

# Anti-biofilm activity of antimicrobial hypochlorous wound irrigation solutions compared to common wound antiseptics and bacterial resilience in an innovative in-vitro human plasma biofilm model (hpBIOM)

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## Research

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# Abstract

Background Biofilms pose a relevant factor for wound healing impairment in chronic wounds. With 78% of all chronic wounds being affected by biofilms, research in this area is of high priority, especially since data for evidence-based selection of appropriate antimicrobials and antiseptics is scarce. Therefore, the objective of this study was to evaluate the anti-biofilm efficacy of commercially available hypochlorous wound irrigation solutions compared to established antiseptics.

Methods Using an innovative complex in-vitro human plasma biofilm model (hpBIOM), quantitative reduction of *P. aeruginosa*, *S. aureus* and MRSA biofilms by three hypochlorous irrigation solutions (two <0.08% and one 0.2% NaClO) were compared to 0.1% octenidine-dihydrochloride/phenoxyethanol (OCT/PE) and 0.04% polyhexanide (PHMB). Efficacy was compared to a non-challenged planktonic approach as well as with increased substance volume over the prolonged treatment course (up to 72h). Qualitative visualisation of biofilms was performed by scanning electron microscopy (SEM).

Results Both tested antiseptics (OCT/PE and PHMB) induced significant biofilm reductions within 72h, whereby OCT/PE in an increased volume even managed complete eradication of *P. aeruginosa* and MRSA biofilms after 72h. The tested hypochlorous wound irrigation solutions however achieved no relevant penetration and eradication of biofilms, despite increased volume and exposure. Only 0.2% NaClO managed a low reduction over prolonged treatment time.

Conclusion The results in the here used complex human plasma biofilm model closely mimic the clinical situation of a high challenging environment for antimicrobials to perform in. Under these conditions, the low-dosed hypochlorous wound irrigation solutions are significantly less effective than antiseptics and thus unsuitable for biofilm eradication.

## Background

In wound management, controlling microbial bioburden is a key factor of prophylactic and therapeutic regimes. While wound contamination and colonization can mostly be handled with vigilance and mechanical cleansing, local infection with the potential threat of systemic spread requires antimicrobial intervention (1). Possible interventions range from preserved antimicrobials-containing wound irrigation solutions to antiseptics, debridement and systemic antibiotics in case of systemic spread. Biofilm formation thereby represents a specifically difficult to diagnose and manage complication in wound therapy. According to recent evaluations over 78% of all chronic wounds are challenged by biofilm formation (2). Biofilms are structured communities of microorganisms attached to a surface (e.g. wound bed) encased within an extracellular matrix (ECM) referred to as the extracellular polymeric substance (EPS) (3). Microorganisms embedded in the EPS demonstrate a significant tolerance and resilience to antimicrobial substances, biocides and the host immunity due to a variety of factors such as polymicrobial heterogeneity, genetic diversity and resistance transfer, dormant metabolism and the EPS itself functioning as a high-protein mechanical and diffusion barrier for antimicrobials (3–6). As a result,

wound antimicrobials and antiseptics need to be thoroughly investigated regarding their anti-biofilm efficacy (7).

Several *in-vitro* models have been developed for this purpose, growing biofilms on plastic or metal surfaces mostly without additional organic, let alone human, material (7–9). Such approaches are feasible for evaluating biocides and disinfectants for (surface) decontamination, but in case of wound antimicrobials the more complex composition of the wound microenvironment (cytokines, proteases, fibrin, cells, etc.) with high-protein, organic challenge as well as the human immune system need to be considered. Therefore, our research group developed an innovative human plasma biofilm model (hpBIOM) for quantifiable testing of anti-biofilm activity of antimicrobial and antiseptic agents in a challenging wound biofilm environment, closely resembling the *in-vivo* situation (10, 11).

Due to the recent renaissance of hypochlorous acid based antimicrobial wound irrigation solutions and antiseptic products (12), and the contrasting evidence regarding the anti-biofilm efficacy of such agents this study focused on the evaluation of such agents in direct comparison to established antimicrobial and antiseptic agents. Prolonged exposure times for remanence detection, increased substance volumes and varying agents (with differing substance concentrations) were investigated in the innovative biofilm model and compared to a planktonic test setup to provide a comprehensive analysis.

## Material And Methods

### Microbial Strains

*Staphylococcus aureus* (DSM-799) and *Pseudomonas aeruginosa* (DSM-939; both DSMZ, Braunschweig, Germany) as well as a clinical MRSA strain (provided by the Institute for Medical Laboratory Diagnostics, Helios University Hospital Wuppertal, Germany) were selected. All strains were previously tested for biofilm formation on plastic surfaces (data not shown). All strains were cultivated on casein/soy peptone agar plates (CSA) according to standard protocols and the second subculture was used for experiments.

### Antiseptics and antimicrobials

Two categories of antimicrobials were chosen for evaluation, five solutions in total (Table 1). Three chlorine-based and -releasing agents, used as preserved wound irrigation solutions with additional antimicrobial effect: ActiMaris® forte (0.2% sodium hypochlorite (NaClO)/ 3% sal maris; AMF), Lavanox® (< 0.08% sodium hypochlorite (NaClO); LVX) and Kerrasol® (< 0.08% sodium hypochlorite (NaClO); KSL).

Table 1

Overview of the tested antiseptics and antimicrobial wound irrigation solutions, product specifications, manufacturer and composition as specified by the manufacturer.

Test solution	Product	Manufacture	Composition	Category	
OCT/PE	Octenisept®	Schülke & Mayr GmbH	0.1 g Octenidin-dihydrochlorid, 2.0 g 2-Phenoxyethanol (per 100 mL)	Antiseptic	
PHMB	Lavisorb®	Fresenius Kabi AG	0.4 g polyhexanide, 0.02 g macrogolum 4000 (per 1000 mL)	Antiseptic	
NaClO	AMF	ActiMaris®forte	ActiMaris AG	0.2% NaClO, 3% sea-salt, H <sub>2</sub> O	Antimicrobial irrigation solution
	LVX	Lavanox®	Serag Wiessner GmbH & Co KG	H <sub>2</sub> O, < 0.08% NaClO	Antimicrobial irrigation solution
	KSL	KerraSol™	Crawford Healthcare GmbH	H <sub>2</sub> O, < 0.08% NaClO	Antimicrobial irrigation solution

The second category includes two of the most established antiseptics in wound management:

Octenidine-dihydrochloride/phenoxyethanol (Octenisept®; 0.1% OCT/PE) and polyhexanide (Lavisorb®; 0.04% PHMB). All products were handled in a sterile manner and according to manufacturer instructions.

## Neutralizing agent and dissolving solution

To terminate antimicrobial activity after specific designated exposure times, a neutralizing solution was used comprising of 40 g tween 80, 30 g saponin, 4 g lecithin, 3 g sodium thiosulfate (all Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and 10 g sodium dodecyl sulfate (SDS) (Caesar & Loretz GmbH, Hilden, Germany) ad 500 mL aqua bidest. The neutralizing solution was intentionally double concentrated to fit the limited capacity in the experimental setup (keeping a ratio of 1:8 active agent to neutralizing solution). Concentration as well as volume of the neutralizing solution have been preliminarily validated and demonstrated a sufficient neutralizing efficacy for all tested antiseptics and antimicrobials, non-toxicity towards microbial strains used in a planktonic as well as a biofilm setup and also did not interfere with the integrity of the newly developed biofilm model.

For dissolving the stable model after successful conduction of experiments to recover and quantify surviving microorganisms, a bromelain solution was used. Therefore, ten tablets of Bromelain-POS® (URSAPHARM Arzneimittel GmbH, Saarbrücken, Germany) were dissolved in 100 mL phosphate buffered saline (PBS) and the solution stored at 4 °C until further use. Before usage, bromelain was warmed to 37 °C for improved enzyme performance. The bromelain solution has been preliminarily validated as well and showed no antimicrobial effect of its own.

# Human Plasma Biofilm Model (hpBIOM) preparation

The development and use of the hpBIOM has been described in detail elsewhere (10, 11) and was adapted to fit the specific agents and purpose pursued here. In brief, human plasma preserves (citrate buffered) and buffy coat from anonymous donors were obtained from the DRK-Blutspendedienst West (Hagen, Germany). To remove residual erythrocytes, the buffy coat was centrifuged at 3000 rpm at room temperature (RT) for 30 min. Subsequently, plasma and buffy coat were merged, gently mixed in a sterile glass bottle and continuously agitated at 22 °C. A microbial test suspension was prepared by colony picking and adjusted to a 0.5 McFarland standard (approx.  $1.5 \times 10^8$  cfu/mL) using a spectrophotometer (EON™; BioTek Germany, Bad Friedrichshall, Germany). Finally, a 'master mix' was prepared by adding the appropriate amount of microbial test solution to the plasma and buffy coat mixture, resulting in a final bacterial concentration of  $1.5\text{-}3 \times 10^6$  cfu per individual hpBIOM. Next, 18.26  $\mu\text{L}$   $\text{CaCl}_2$  (500 mM) per mL plasma was added to the master mix, to induce coagulation, gently mixed and immediately transferred into 12-well plates (1.5 mL per model/well containing plasma, buffy coat and pathogenic bacteria). The plates were incubated for 12 h on a rotation shaker at 50 rpm and 37.0 °C for the plasma solution to polymerize with pathogens rearranging and forming an extracellular matrix, finally yielding a stable biofilm disc/clot with integrated bacteria (approx. concentration of  $1.5 \times 10^7$  cfu/mL), EPS as well as human plasma and immune cells.

## Antimicrobial treatment of the hpBIOM and quantification of bacterial survival

After hpBIOM preparation, each clot was treated with 300  $\mu\text{L}$  of antiseptic/antimicrobial test substance for 0.5, 2, 6 and 24 hours. To additionally investigate the effect of prolonged exposure (in terms of remanence effect without reapplication) and/or increased substance volume (1 mL), experiments were additionally extended to 48 and 72 hours with 300  $\mu\text{L}$  or 1 mL of active substance against the clinically isolated MRSA strain and *P. aeruginosa*. After the specified treatment periods, antimicrobial activity was terminated by adding 1.2 mL of the specified neutralizing solution to each well, detaching the model from the well walls with a pipette tip (in order to fully distribute the neutralizing solution around the model) and placing the plates on a rotation shaker at RT for 5 min  $\pm$  10 s. Subsequently, each hpBIOM was carefully transferred into a 15 mL falcon tube with 1 mL bromelain solution for dissolving the model. An additional 0.5 mL of bromelain solution was used to wash out the well to detach remaining microorganisms and added to the falcon tube.

In case of the 1 mL test setup, models were already detached and transferred into 15 mL falcon tubes for neutralization, due to the higher necessary amount of neutralizing solution (4 mL to keep a 1:8 ratio) and bromelain subsequently added to the tube after neutralization. After 2–3 h, the hpBIOM was completely dissolved. For quantification, the resulting solution was thoroughly vortexed, serially tenfold diluted, 50  $\mu\text{L}$  of each dilution spread on agar plates and incubated overnight at 37 °C under aerobic conditions. Surviving microorganisms (in cfu/mL) were determined using a Colony Counter Pen (eCount™, VWR Leicestershire, UK).

## Quantitative suspension method (QSM)

To compare the anti-biofilm effect of the tested antiseptics and antimicrobials to their effect on planktonic bacteria, all tested substances were also evaluated in a quantitative suspension method (QSM). The QSM used is based on the DIN EN 13727 (13) yet has been modified for direct comparison to the hpBIOM. Thereby, a bacterial test suspension was prepared by colony picking and adjusted to  $1.5 \times 10^7$  cfu/mL initial concentration (as for hpBIOM). 1.5 mL of the prepared bacterial test suspension was treated with 300  $\mu$ L of the respective antiseptic or antimicrobial for 0.5, 2, 6 or 24 hours. After treatment, 1.2 mL neutralizing solution was added and incubated for 5 min  $\pm$  10 s on a rotation shaker at RT to terminate antimicrobial activity. Subsequently, surviving microorganisms (in cfu/mL) were quantified in the same manner on agar plates as described for the hpBIOM.

## Visualization of biofilm using scanning electron microscopy (SEM)

To visualise the morphology and structure of the bacterial biofilm with and without antiseptic/antimicrobial treatment, scanning electron microscopy (SEM) was performed for selected experimental setups (see Table 2). After neutralization of the antiseptic/antimicrobial agents, the models were fixed with a glutaraldehyde/PVP-solution containing 2.5% glutaraldehyde, 2% polyvinylpyrrolidone (PVP) and 0.5% NaNO<sub>2</sub> in 0.1 M cacodylate buffer for 1 h at 4 °C. Samples were washed in 0.1 M cacodylate buffer and stored at 4 °C until preparation of freeze fracture fragments with liquid nitrogen. For glycocalyx staining, the samples were subsequently incubated in a solution containing 2% arginine-HCL, glycine, sucrose and sodium glutamate for 18 h at RT. The samples were rinsed with aqua dest. and 0.1 M cacodylate buffer followed by immersion in a mixture of 2% tannic acid and guanidine-HCL for 5.5 h at RT. After another rinsing step with aqua dest. and 0.1 M cacodylate buffer samples were incubated over night at 4 °C. For staining, the samples were placed in a 1% OsO<sub>4</sub> solution for 30 min at RT. After three rinsing steps with 0.1 M cacodylate buffer the samples were again stored over night at 4 °C. Finally, samples were dehydrated using isopropyl alcohol and acetone and dried in liquid CO<sub>2</sub> using a critical point dryer (BAL-TEC AG, Balzers, Liechtenstein). Via the sputter coater (BAL-TEC AG, Balzers, Liechtenstein), samples were sputtered with gold palladium and finally examined with a Zeiss Sigma SEM (Zeiss, Oberkochen, Germany) using 2 kV acceleration voltage and an inlens detector.

Table 2

Specification and overview of the exemplary experimental setups used for visualisation of biofilm formation in the hpBIOM using scanning electron microscopy (SEM). Images (a) - (c) in Fig. 4 demonstrate an exemplary bacterial biofilm development over time. Images (a) – (d) in Fig. 5 show the biofilm surface structure under treatment with antiseptic (OCT/PE; b & c) and antimicrobial wound irrigation solution (< 0.08% NaOCl; d) over time compared to the initial structure.

Image	Setup/Substance	Pathogen	Maturation time*	Treatment period
4 (a)	CTRL	<i>P. aeruginosa</i>	12 h	-
4 (b)	CTRL	<i>P. aeruginosa</i>	18 h	-
4 (c)	CTRL	<i>P. aeruginosa</i>	36 h	-
5 (a)	CTRL	<i>P. aeruginosa</i>	12 h	0 h
5 (b)	OCT/PE	<i>P. aeruginosa</i>	12 h	6 h
5 (c)	OCT/PE	<i>P. aeruginosa</i>	12 h	24 h
5 (d)	NaOCl (< 0.08%)	<i>P. aeruginosa</i>	12 h	24 h
* before application of test substance, if any (not in case of CTRL)				

## Statistical Analyses

All experiments were performed in triplicates with three different anonymous donors. Microbial reduction rates (in  $\Delta\log_{10}$  cfu/mL) were calculated for all tested antiseptic/antimicrobial solutions. Data is expressed as means  $\pm$  standard error of the mean (SEM) and was analysed using the statistics program GraphPad PRISM (Version 8.2.1; GraphPad Software Inc., La Jolla, USA). Statistical analysis was performed using two-way ANOVA, followed by Holm-Sidak posthoc test for evaluation of multiple comparisons. A p-value of  $p \leq 0.05$  was considered statistically significant. (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ; \*\*\*\* $p \leq 0.0001$ ).

## Results

### Antimicrobial efficacy on planktonic bacteria (QSM) within 24 h

On bacteria in a planktonic state, both antiseptics, OCT/PE and PHMB, achieved a highly significant reduction of *P. aeruginosa* ( $5.77 \pm 1.41$  and  $6.25 \pm 0.93 \log_{10}$  steps, respectively;  $p < 0.0001$ ), *S. aureus* ( $7.18 \pm 0.00$  and  $5.82 \pm 1.36 \log_{10}$  steps;  $p < 0.0001$ ) and MRSA (both  $7.18 \pm 0.00 \log_{10}$  steps;  $p < 0.0001$ )

within 30 min of exposure (Figs. 1a-c). After 2 h of exposure, OCT/PE and PHMB both fully eradicated all three tested pathogens, except PHMB against *S. aureus*, needing 6 h for complete eradication (Fig. 1c).

The three tested antimicrobial hypochlorous wound irrigation solutions containing NaClO/HClO demonstrated no bacterial reduction against any tested pathogen within 24 h in the here conducted planktonic QSM (Fig. 2).

All reduction rates of the QSM are summarised in Supplementary table 1.

## **Anti-biofilm efficacy (hpBIOM) within 24 h**

In a complex biofilm model such as the hpBIOM, the tested antiseptics showed a distinctly reduced effect within 24 hours: against a methicillin-resistant (MRSA) as well as a methicillin-susceptible (MSSA) *S. aureus* biofilm, PHMB showed no statistically significant reduction within 24 h compared to an untreated control (Fig. 1b & c;  $p = 0.90/0.93$ ), while OCT/PE at least managed a statistically significant low reduction of  $0.83 \pm 0.23$  (Fig. 1b;  $p = 0.014$ ) and  $1.28 \pm 0.32 \log_{10}$  steps (Fig. 1c;  $p = 0.0002$ ).

A higher efficacy could be observed against *P. aeruginosa* biofilms. Both antiseptics induced a statistically significant reduction within 24 h of treatment compared to the control, whereby OCT/PE achieved  $2.68 \pm 0.46 \log_{10}$  steps ( $p = 0.0008$ ) and PHMB  $2.97 \pm 0.59 \log_{10}$  steps (Fig. 1a;  $p = 0.0002$ ).

As in the QSM, the tested hypochlorous wound irrigation solutions showed no antimicrobial/-biofilm activity in the here performed experiments. No bacterial reduction could be observed against any tested pathogen within 24 h (Fig. 2).

## **Anti-biofilm efficacy (hpBIOM) under prolonged exposure (up to 72 h) and/or increased substance volume (1 mL)**

Prolonged exposure times of up to 72 h for 0.3 mL OCT/PE and PHMB increased bacterial reduction of all three pathogens with a continuous decrease in bacterial counts yielding highest reductions after 72 h of exposure compared to the untreated control (Fig. 1a-c). For MRSA and MSSA, OCT/PE reached higher overall reduction rates than PHMB after 72 h (MRSA –  $5.45 \pm 1.73$  vs.  $1.96 \pm 0.79 \log_{10}$ ; MSSA –  $3.19 \pm 0.29$  vs.  $1.97 \pm 0.79 \log_{10}$ ; Fig. 1b & c). Against *P. aeruginosa* biofilms, PHMB achieved higher reduction rates than OCT/PE after 72 h ( $5.23 \pm 1.95$  vs.  $4.54 \pm 1.34 \log_{10}$ ; Fig. 1a). In case of antimicrobial hypochlorous solutions, a prolonged exposure with 0.3 mL showed no improved impact (Fig. 2)

When increasing the substance volume per treatment to 1.0 mL, both OCT/PE and PHMB demonstrated a significant increase of bacterial reduction within 24 h compared to 0.3 mL (Fig. 3). OCT/PE achieved a nearly complete eradication of MRSA and *P. aeruginosa* after 24 h and especially against MRSA biofilms it showed a significantly higher reduction than PHMB ( $6.64 \pm 0.53$  vs.  $2.63 \pm 0.63 \log_{10}$ ;  $p < 0.0001$ ; Fig. 3b). For hypochlorous solutions, an increase in volume did not result in an increase in antimicrobial efficacy within 24 h (Suppl. table 2).

The highest anti-biofilm efficacy was observed under the combination of prolonged exposure and increased substance volume of 1 mL (Fig. 3a & b). OCT/PE even managed to completely eradicate both MRSA and *P. aeruginosa* biofilms after 72 h. In case of *P. aeruginosa* biofilms, PHMB achieved full eradication before OCT/PE, after only 48 h (Fig. 3a), while against MRSA it proved significantly less effective (2.73 log<sub>10</sub> reduction steps less than OCT/PE after 72 h;  $p = 0.0004$ ), not achieving complete eradication and even demonstrating a certain regrowth between 48 and 72 h (Fig. 3b).

In terms of the antimicrobial hypochlorous solutions, the highest concentrated product (AMF; 0.2% sodium hypochlorite) demonstrated a low bacterial reduction against MRSA biofilm of  $2.35 \pm 0.58$  log<sub>10</sub> steps ( $p = 0.0016$ ) after 48 h. However, between 48 and 72 h a beginning regrowth could be observed (Fig. 3b) as well.

## Discussion

Biofilms represent a major challenge, especially in chronic wound care and one of the main factors for wound chronicity and impaired healing (1, 7, 14–16). Adequately addressing this challenge by advancing understanding and developing precise, comprehensive and new therapeutic strategies is one of the most important research goals in modern wound management (7, 17, 18).

Due to the extensive resilience of microorganisms residing within such polymicrobial communities embedded in extracellular polymeric substance (EPS), rigorous and highly efficient antiseptic regimen are necessary. While a form of repetitive, effective debridement represents the fundamental base of every anti-biofilm strategy (1, 7, 19), debridement alone is insufficient for complete biofilm eradication (7, 20), needing a combination with highly efficient antimicrobial and antiseptic agents (7, 21, 22). However, reported efficacy of available antimicrobial and antiseptic agents varies greatly depending on various factors and to date no agents can be recommended over another as being best suited for the treatment of chronic wound biofilms (7, 17). Foremost, heterogeneity in study design/experimental setup and lacking translation from *in-vitro* to *in-vivo* studies have been identified as main limitations in current research (17, 18). Another major concern is the liberal extrapolation of results in certain test scenarios to supposedly similar situations. This is the case for experimental setups as demonstrated by the significant differences in efficacy between a standard planktonic (DIN EN 13727 (13)) and biofilm *in-vitro* assay in this study (Figs. 1a-c) as well as previous works comparing different biofilm models, calling for more comprehensive testing. The results emphasize the direct dependency of an agent's efficacy on the environment it acts in.

Most static as well as liquid-flow-based *in-vitro* models are limited to factors such as growth on plastic surfaces and lack of adequate chronic wound reflecting organic conditions, let alone the heterogenous, individual conditions in human chronic wound biofilms (9). The development of our human plasma biofilm model (hpBIOM; Fig. 4a-c) (10, 11), aims to narrow the gap between *in-vitro* and *in-vivo* biofilm research and provide a translational approach. The use of a complex biofilm model based on human material, including plasma and active immune cells, addresses the interactions of microbial biofilms with

the human wound environment (3, 14) as well as the relevant efficacy loss of antimicrobials under challenge (23, 24), providing a 'close-to-reality' test scenario.

The presented results on commercially available hypochlorous wound irrigation solutions compared to high-potency antiseptics highlight the necessity for such models and the careful distinction within agent classes. In a previous publication, we demonstrated and discussed the differences between commercially available hypochlorous wound irrigation solutions depending on agent concentration, pH-value and test setup against planktonic bacteria (12): while LVX, KSL and especially AMF, showed a high short-term efficacy on planktonic bacteria, no efficacy of either agent could be detected in the complex human biofilm model over the course of 72 h (Fig. 2). OCT/PE and PHMB, on the contrary, achieved significant reductions of microorganisms with increasing exposure time (Fig. 1a-c) and visual breaking open of biofilm structures (Fig. 5a-c). Compared to other approaches (11), no re-application of test-substances within 72 h was performed, demonstrating a certain remanence effect of OCT/PE and PHMB. In contrast to our earlier publication (12), hypochlorous agents not only failed to reduce microbial counts in biofilms but also in the here used planktonic method (QSM; Fig. 2). This is most likely attributed to the experimental setup with a deliberately chosen lower 'agent to microbial test suspension'-ratio in the planktonic QSM (1:5 compared to 8:1 in standards), to unify the ratio in both models (QSM and hpBIOM). OCT/PE and PHMB however, still achieved complete eradication of all tested planktonic microorganisms within 6 hours (Fig. 1a-c) even in low ratio setups. In a planktonic setup, higher ratios for hypochlorous irrigation solutions also resulted in higher reduction rates ((12) and unpublished data).

To investigate whether an increase in substance volume would yield anti-biofilm effects of hypochlorous solutions, the volume capabilities of the hpBIOM were exhausted to administer as much agent as possible (1.0 instead of 0.3 mL): while OCT/PE and PHMB showed a significant increase in anti-biofilm efficacy, even partially achieving complete eradication, the only hypochlorous solution, demonstrating a (small) increase in efficacy compared to the lower volume was AMF (Fig. 3a & b). LVX and KSL showed no reduction (Suppl. Tbl. 2). This underlines the dose-dependency of antiseptics and antimicrobials as well as the relevance of additional aspects such as dilution, mechanical detachment/debridement and reduction in surface tension to the overall antimicrobial and cleansing effects of especially wound irrigation solutions reported in other studies(25, 26). The discrepancies in studies evaluating different forms of chlorine-releasing solutions, reporting higher efficacies, than reported in this study, mainly derive from the vast heterogeneity of study designs. Many *in-vitro* studies used stationary biofilms on plastic surfaces without organic challenge (27–29), even though the relevance of such challenge has been widely described (9, 30). Also, the exact composition of solutions needs to be considered, differentiating between solutions containing mainly sodium-hypochlorite, hypochlorous acid or both, as well as the concentration and pH of solution and environment, whereby more acidic and alkaline conditions and solutions can be more effective, as described before (12, 27, 29, 31, 32) and shown in this work, as the only hypochlorous irrigation solution showing any anti-biofilm effect was the more alkaline, higher-concentrated (0.2% NaClO) AMF (Fig. 3b).

## Conclusion

The results clearly underline fundamental principles in chronic wound biofilm treatment in a complex biofilm model closely mimicking the situation in a human wound biofilm setting. They highlight the necessity of highly potent antiseptics applied for a sufficient amount of time, to achieve biofilm penetration and effectively reduce bacterial counts (if used alone). State of the art treatment remains the combination with debridement to mechanically break open dense biofilm structures. Low-dosed, (near-)neutral pH hypochlorous wound irrigation solutions on the contrary seem unsuitable for first-line anti-biofilm treatment, as supported by the inability to reduce bacterial counts in this complex human plasma biofilm model. Naturally, these results should be translated to clinical practice with caution, since certain limitations such as the use of mono-species biofilms and relatively short maturation times (12h) still apply. However, more mature and multi-species biofilms would exhibit even higher bacterial resilience, as partly already demonstrated in another work of our research group (11) and therefore pose an even greater challenge for hypochlorous irrigation solutions. The anti-biofilm capacities of such wound irrigation solutions are therefore quite limited making them rather suited for decontamination and decolonisation of acute and chronic wounds and prevention of re-contamination/-infection, rather than primary treatment of mature biofilms compared to high potency antiseptics.

## Abbreviations

AMF  
Actimaris® forte  
CaCl<sub>2</sub>  
calcium chloride  
CFU  
colony forming units  
CSA  
casein/soy peptone agar  
DIN  
german institute for standardization  
DRK  
german red cross  
ECM  
extracellular matrix  
EPS  
extracellular polymeric substance  
HClO  
hypochlorous acid  
hpBIOM  
human plasma biofilm model

KSL  
Kerrasol®  
LVX  
Lavanox®  
MRSA  
methicillin-resistant *S. aureus*  
NaClO  
sodium hypochlorite  
NaNO<sub>2</sub>  
sodium nitrite  
OCT/PE  
octenidine dihydrochloride / phenoxyethanol  
OsO<sub>4</sub>  
osmium tetroxide  
PHMB  
Polyhexamethylene -biguanide  
PVP  
polyvinylpyrrolidone  
QSM  
quantitative suspension method  
SEM  
scanning electron microscopy

## **Declarations**

### **Ethics approval and consent to participate**

Not applicable.

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request or are available as supplementary data files.

### **Competing interests**

The authors declare that they have no competing interests.

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### **Authors' contributions**

JDR, LH and ES designed the study. JDR and LH performed experiments, data analysis and drafted the figures. MB helped with data analysis, interpretation and experimental setup. JDR, LH and ES drafted and finalized the manuscript. All authors read and approved the final manuscript.

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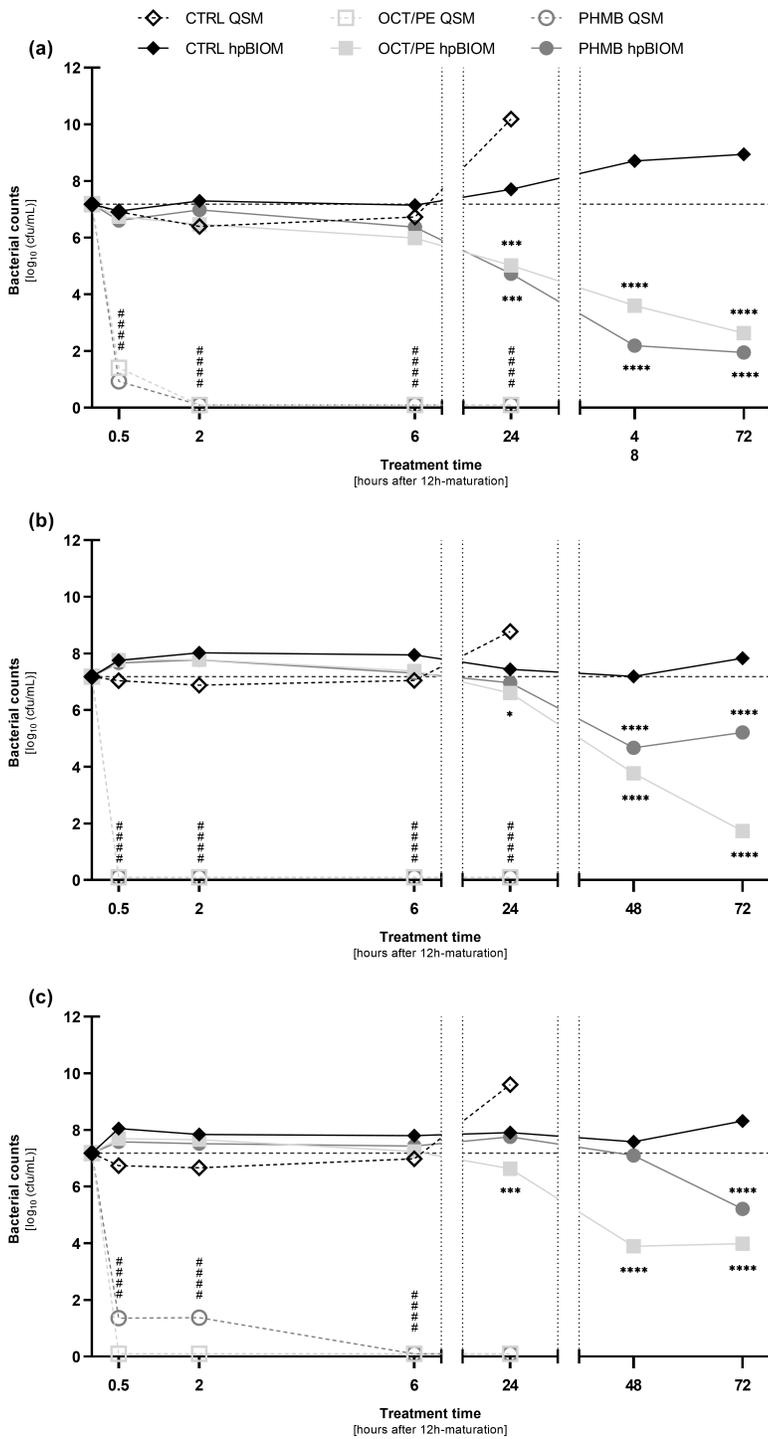
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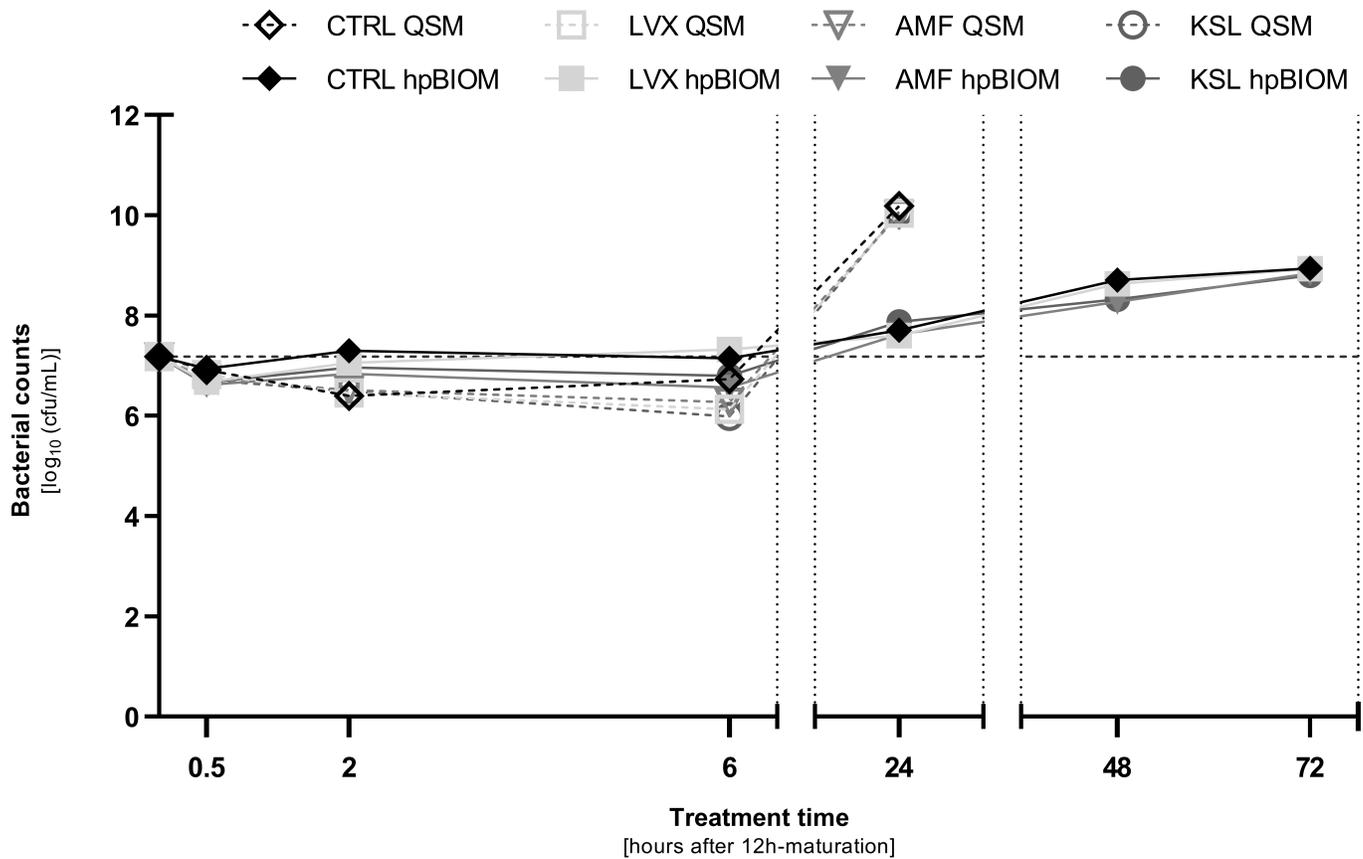
## Figures



**Figure 1**

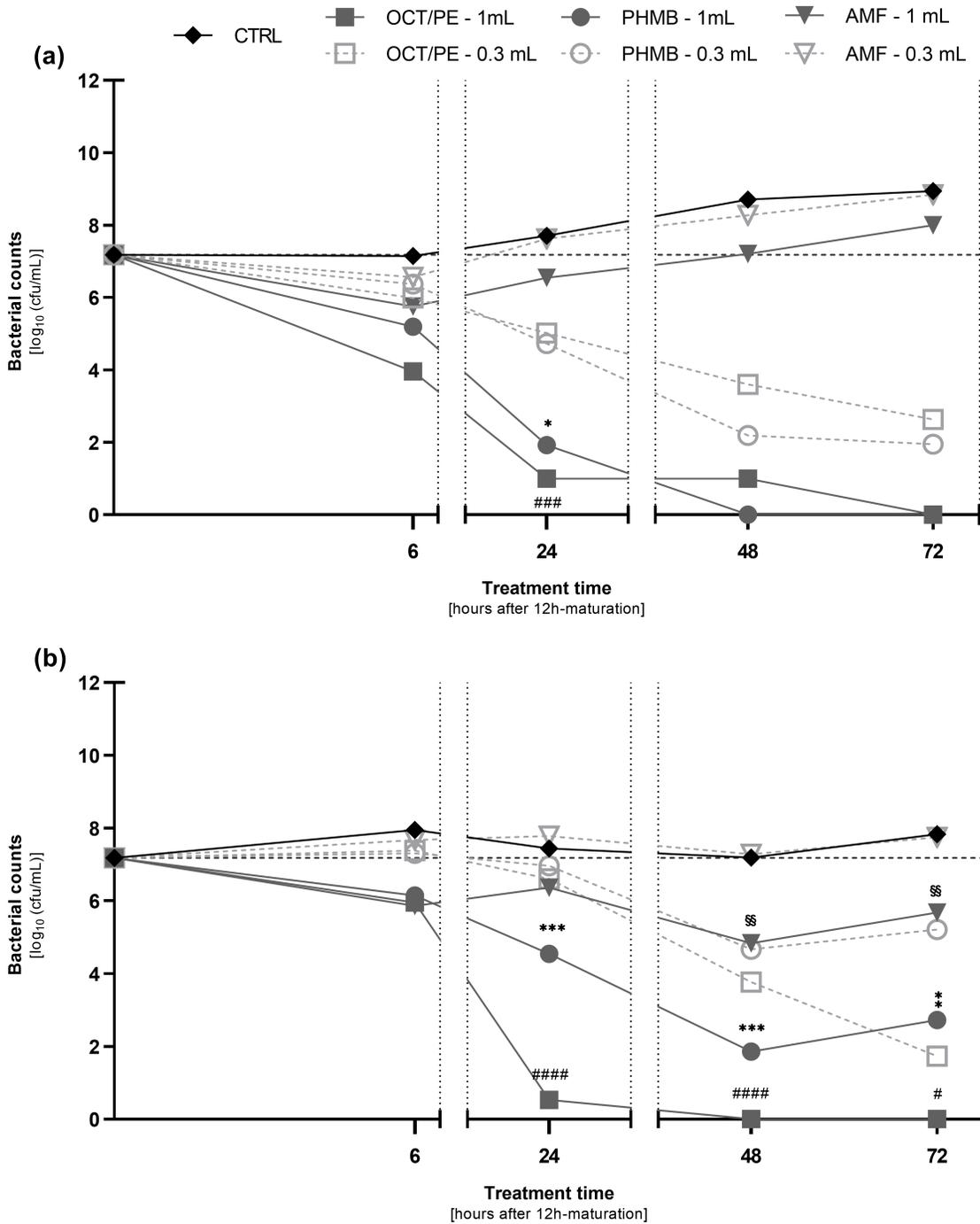
a-c. Reduction of *P. aeruginosa* (a), MRSA (b) and *S. aureus* (c) in planktonic (QSM) vs. biofilm (hpBIOM) form by 0.3 mL of tested antiseptics octenidine-dihydrochloride/phenoxylethanol (OCT/PE) and polyhexanide (PHMB) compared to an untreated control (CTRL). Remaining bacterial counts (in log<sub>10</sub> cfu/mL) are outlined over the course of 72h of treatment after an initial biofilm maturation period of 12h. Values are expressed as means, significant reductions compared to the untreated control are expressed

as \*P ≤ 0.05, \*\*P ≤ 0.01, \*\*\*\*P ≤ 0.001 or \*\*\*\*P ≤ 0.0001 (# in case of QSM). (Time-kill-curves of bacteria in planktonic state (QSM) are only depicted for 24h).



**Figure 2**

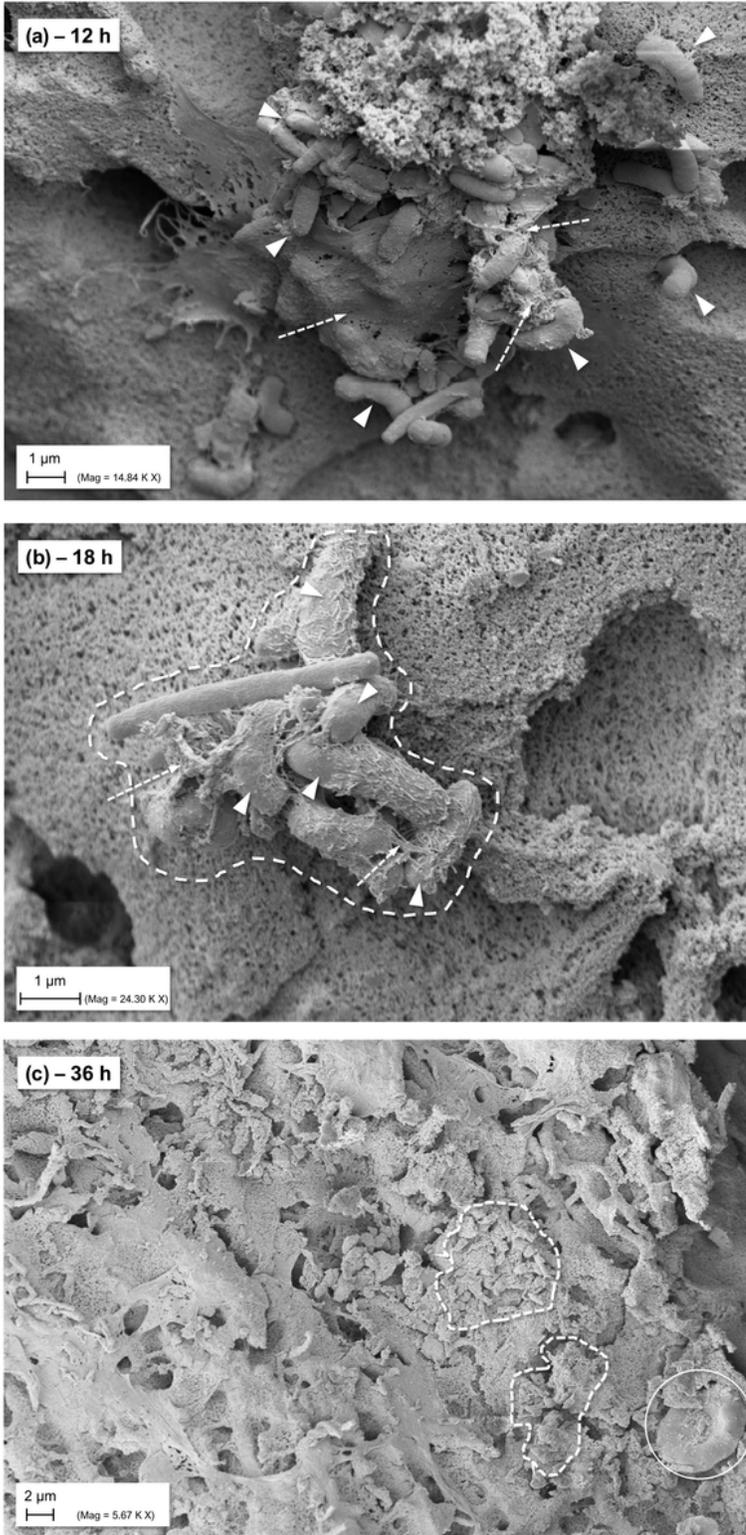
Reduction rates of 0.3 mL of tested antimicrobial irrigation solutions Lavanox® (LVX; <0.08% NaClO), Kerrasol® (KSL; <0.08% NaClO) and Actimaris® forte (AMF; 0.2% NaClO, 3% sal maris) compared to an untreated control (CTRL) in planktonic (QSM) and biofilm (hpBIOM) form (here exemplary against *P. aeruginosa*). Bacterial counts (in log<sub>10</sub> cfu/mL) are outlined over the course of 72h of treatment after an initial biofilm maturation period of 12h. Values are expressed as means and time-kill-curves of bacteria in planktonic state (QSM) are only depicted for 24h.



**Figure 3**

a & b. Comparison of the reduction of *P. aeruginosa* (a) and MRSA (b) in the biofilm model (hpBIOM) between 0.3mL and 1.0mL of tested antimicrobial substances in a prolonged exposure of up to 72h. Graphs depict bacterial counts (in log<sub>10</sub> cfu/mL) after treatment with octenidine-dihydrochloride/phenoxyethanol (OCT/PE), polyhexanide (PHMB) or 0.2% sodium hypochlorite (AMF) compared to an untreated control (CTRL). Values are expressed as means and a significant increase in

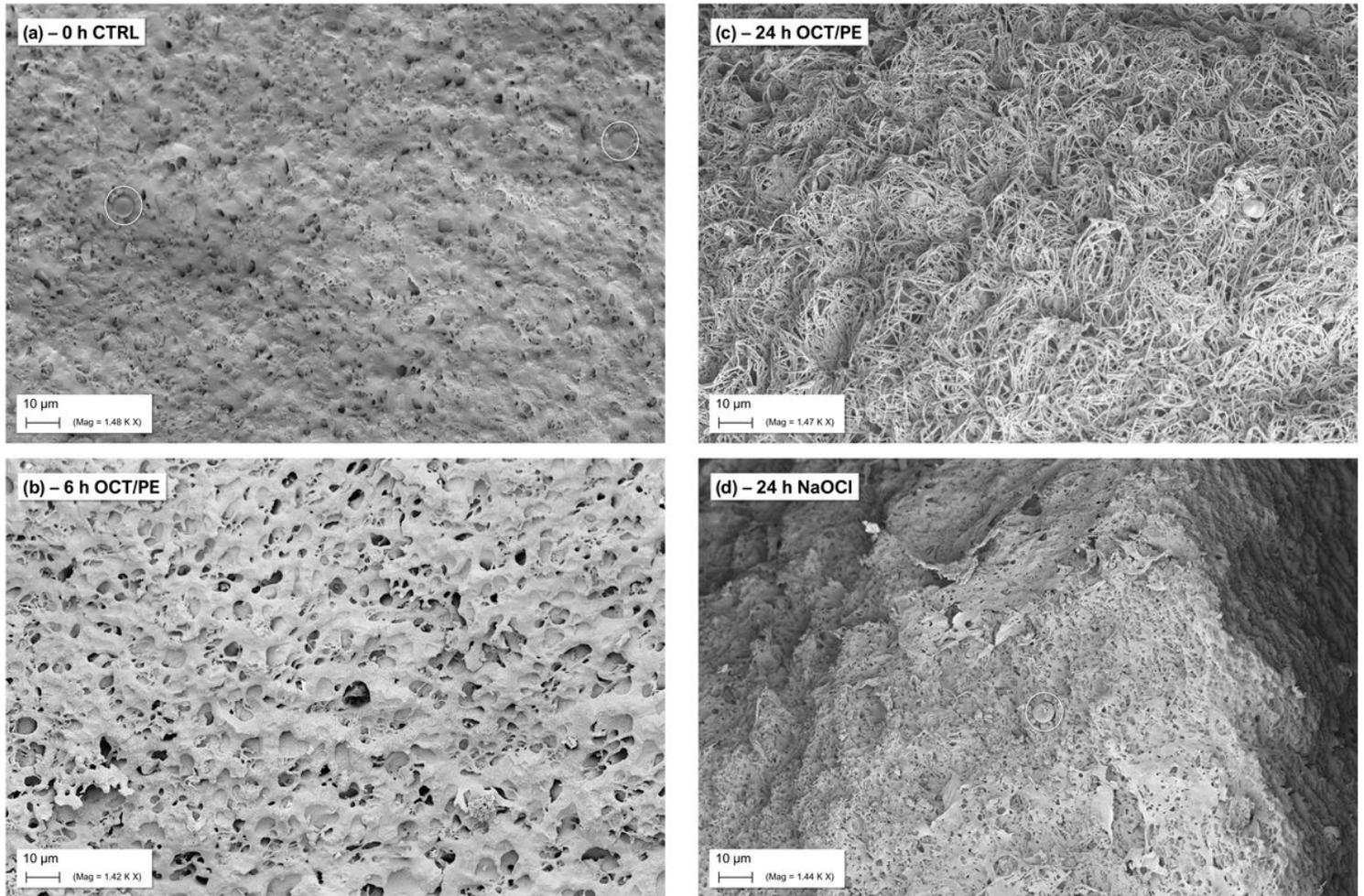
reduction under 1.0mL substance volume is expressed as \*P ≤ 0.05, \*\*P ≤ 0.01, \*\*\*\*P ≤ 0.001 or \*\*\*\*P ≤ 0.0001 (\* for PHMB; # for OCT/PE and § for AMF).



**Figure 4**

a-c. Scanning electron microscopy (SEM) images of biofilm maturation and development in the hpBIOM (here *P. aeruginosa*). (a) Formed microcolony in a 12h matured biofilm with bacterial attachment and initially formed EPS/glycocalyx (arrows with dashed lines); arrowheads indicate bacteria. (b) 18h

matured biofilm: dashed framing circles bacterial microcolony with single bacteria (arrowheads) connected by EPS/glycocalyx (arrows with dashed lines). (c) Surface view of 36h matured biofilm with densely integrated and glycocalyx-surrounded bacteria (dashed framing) and human cells (white circle; erythrocyte).



**Figure 5**

a-d. Scanning electron microscopy (SEM) visualisation of biofilm surface alteration in the hpBIOM (here *P. aeruginosa*) under treatment with antiseptics and antimicrobial wound irrigation solutions. (a) Densely connected surface structure of a 12h-matured biofilm before substance application, white circles depict human erythrocytes. (b) After 6h of treatment with OCT/PE: surface structure appears less compact with several holes as potential entry points. (c) After 24h of treatment with OCT/PE: surface is deranged and 'broken-open' into a loosened structure with fine filaments, readily penetrable. (d) After 24h of treatment with <math>< 0.08\%</math> NaOCl: remaining densely connected surface structure, no visible penetration, additional build-up superficial EPS structures (white circle depicts human erythrocyte).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [FINALSupplementaryDataTablesRembe.docx](#)