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Tolerance of *Pseudomonas oleovorans* biofilms to disinfectants commonly used in endoscope reprocessing?

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ABSTRACT

Reprocessing failure of endoscopes may result in outbreaks of serious infections in vulnerable patients caused by Gram-negative bacteria. *P. oleovorans* (PSOL) was detected in 6 automated endoscope washer-disinfectors (AEWDs) in two reprocessing units during routine check and probing for quality control. Ten endoscopes were probed yielding the growth of PSOL. Two different PSOL strains were identified by genotyping. Biofilms and planktonic cells of both PSOL (N = 2) and of *Pseudomonas aeruginosa* PAO1 as reference were incubated with increased disinfectant concentrations modelling the disinfection process in the AEWD. PSOL in planktonic form was eradicated by GLUT1% (1 g/100 g) at 55 °C. GLUT at a higher concentration of 3 % resulted in the eradication of PSOL biofilms at 25 °C. The persistent growth of PSOL in quality controls indicates inadequate disinfection. Increase of the concentration of GLUT would be an option to eradicate PSOL. However, increasing the concentration of GLUT may lead to corrosion of the sensible instruments and toxic side-effects in patients. Further investigation on disinfectant type and concentration, the reservoir of contamination and defining additional disinfection steps are warranted.

1. Introduction

Endoscopy is a very frequent intervention for diagnostic, preventive and therapeutic purposes. Reprocessing and disinfection in particular of these sensible instruments with several channels with small diameters are prerequisite for safe patient care [1]. Infections associated with contaminated endoscopes have been described as the most common infections linked to medical devices [2] caused by pathogens such as *Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella enteritidis* [3,4].

As endoscopes are thermolabile devices, high-level disinfection (HLD) is performed in automated endoscope washer-disinfectors (AEWDs) using chemo-thermal disinfection [5,6]. Pre-cleaning before automated reprocessing is pivotal to ensure no residues compromise the performance of AEWD [7,8]. Microbial contamination despite high-level disinfection may impair patients' safety and has to be dealt with immediately, usually by taking the affected AEWD machine out of service and thus impairing clinical service [9]. To ensure the quality of endoscopy and patients' safety reprocessing of endoscopes has to be accompanied by periodical checks according to the European standard EN ISO 15883-4: before initial use, annual revalidation, and after any repair or change of disinfection protocol or the disinfectant. In the summer of 2021, routine testing of 27 AEWDs at the University Hospital revealed an increased occurrences of samples with the growth of *P. oleovorans* (PSOL) in one AEWD in the operation tract (OT) and in five AEWDs in the endoscopy unit of the gastroenterology department. There are few reports on PSOL in AEWD machines and endoscopic surfaces. The Tyrolean Endoscopy Surveillance Study, in which 29-endoscopy centres tested endoscopes and final rinse water for contamination, identified *Pseudomonas oleovorans* as the most common microorganism [10,11]. However, there has been no detailed study of the bacteria and its possible origin. Persistent PSOL in AEWD machines may be a potential source of contamination of the endoscopes and consequently of infection in vulnerable patients.

In the current study, we investigate the characteristics of 2 clinical PSOL strains including their ability to form biofilms, as well as the

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effects of the commonly used disinfectant GLUT in different concentrations. Additionally, to identify the reservoir of PSOL within the AEWD, all water inlets points, openings and channel adapters, were investigated for the presence of PSOL.

2. Material and methods

2.1. Setting

The University Hospital is an 1800 bed academic hospital offering services from primary to tertiary care including hemato-oncology, organs and bone marrow transplantation, advanced surgery and huge intensive care service, paediatrics and neonatology. Endoscopy (gastrointestinal, respiratory tract, urology) is performed at different locations including the operation theatre tracts, the intensive care unit tracts, several outpatients' clinics etc. All of these locations have a least two AEWD machines to supply immediate cleaning and disinfection of the endoscopes.

The endoscopes included in this report are reprocessed in AEWDs manufactured by Olympus GmbH, Hamburg, Germany. The washing and disinfection process is performed at 55 °C according to the manufacturer's instructions. The quality of the disinfection of the endoscopes and the final rinse water is checked according to national guideline ÖGSV-L04a (https://oegsv.com/wp/guidelines/) based on the European standard EN ISO 15883-4. Briefly, to sample ready-to-use endoscopes, 20 mL of sterile 0.9 % NaCl solution is flushed through the biopsy/aspiration channel from the proximal inlet to the distal end and collected in a 50 mL aseptic microbiological container without adjuvants. To verify the reprocessing quality of AEWDs after a completed cleaning and high-level disinfection cycle, 500 mL of final rinse water is collected, centrifuged, and tested for bacterial growth on agar plates after filtration. Bacterial enumeration and identification is performed according to standard microbiology protocols as a standard procedure.

2.2. PSOL isolates

RAPD-PCR was carried out on these isolates for genotyping. The antimicrobial susceptibility of all PSOL isolates, was determined by using the Kirby-Bauer disk diffusion method on MHE agar plates (Biomerieux, France). Susceptibility testing to piperacillin, piperacillin/tazobactam, meropenem, imipenem, ceftazidime, cefepime, tobramycin, amikacin, ciprofloxacin, and aztreonam were performed according to EUCAST protocols (The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 12.0, 2022). As a PSOL reference strain was not available, the *Pseudomonas aeruginosa* reference strain PA01 was used as reference.

2.2.1. Effect of GLUT on PSOL planktonic state

Neodisher Septo DN2 is a commonly used disinfectant containing 20 g of glutaraldehyde per 100 g (GLUT20 %). We tested different concentrations of disinfectant, starting from 0.2 % (0.2 g/100 g), which is equal to 1 % of GLUT20 % as recommended by the manufacturer (Dr. Weigert, Hamburg; Germany), to GLUT1.5 % (1.5 g/100 g). These tests were conducted at 55 °C for 5 min. Tests were performed using EN 1040 reference method, which is designed to test basic bactericidal activity; EN 13727, to simulate customer-specific contaminants; and EN 14561, to mimic the disinfection of medical instruments. All of the reference methods required that the number of bacteria be reduced by 5 $\log_{10}$ in order to confirm the sensitivity to the disinfectant. In addition, the minimum inhibitory concentration (MIC) of GLUT on planktonic PSOL at 25 °C was assessed according to EUCAST guidelines using 96-well-plates. In brief, bacteria (1.5×10^5 CFU/mL) were exposed to increasing concentrations of GLUT (ranging from 0.25 % to 3 %) for 5 min. Disinfection was stopped using 5 % sodium thiosulfate (150 μL for 15 min), and then 100 μL of each well was plated on Columbia agar plates (BD, Heidelberg, Germany) to quantify the surviving bacteria. In accordance with the requirements of the reference methods used, the neutralizing effect of 5 % sodium thiosulfate on the disinfectant was confirmed with bacterial growth after application of the disinfectant.

2.2.2. Effect of GLUT on PSOL biofilms

Based on the ASTM E2799-17 reference method, we tested biofilms using the Calgary device. Biofilms of PSOL (2.5×10^6 CFU/mL) were grown on pegs attached to the lids of 96-well plates (Nunc, Thermo Scientific, Germany) in a medium consisting of 150 μL of tryptic soy broth (TSB, DifcoTM Germany) containing 0.2 % glucose. The biofilms were cultured at 37 °C with shaking at 110 rpm. After 24 h, the pegs, with biofilms, were rinsed with 300 μL of PBS and transferred to wells containing freshly prepared disinfectant at varying final concentrations of GLUT0.5 %, 1 %, 2 %, 3 %, and 4 % at 55 °C. Disinfectant activity was stopped by 5 % sodium thiosulfate.

Crystal violet assay (CV): For the evaluation of the biofilm biomass, biofilms were fixed with 200 μL of 2.5 % glutaraldehyde, and stained using 0.4 % CV for 10 min. The optical density (OD) of CV dissolved in ethanol, which corresponds to the mass of the biofilm, was measured at 590 nm using a microplate reader (FLUOstar Omega-BMG Labtech, Germany).

Viable colony count (VCC), was used to record the quantity of surviving bacteria after disinfectant treatment. Treated biofilms were dissolved using 200 μL of Sputasol (Thermo Fisher Scientific™, Remel™Suisse) and an orbital shaker [12]. Subsequently, 100 μL of each solution was subjected to serial dilution and then pipetted onto Columbia agar plates and incubated for 48 h.

The XTT cell proliferation assay, to show the activity of bacteria in biofilms after treatment with disinfectants, was performed using 50 μL of the labelled XTT reagent (AppliChem, Darmstadt, Germany) with a final concentration of 0.3 mg/mL. The absorbance of the tetrazoline salts was measured using a microplate reader at a wavelength of 490 nm.

Confocal laser scanning microscopy (CLSM), biofilm imaging, before and after treatment with GLUT1%, was performed using an Axiolan confocal upright microscope (Carl Zeiss, Oberkochen, Germany, argon laser, 488 nm and 561 nm) and 6-well ibidi plates. The protective biofilm EPS within the biofilms, was visualized following the incubation of the sections with 50 μg/mL concanavalin A (ConA-FITC C7642; Sigma-Aldrich Inc, St. Louis, Mo). Surviving cells were visualized using live/dead staining (Invitrogen™ LIVE/DEAD™ BacLight™ Bacterial Viability Kit Thermo Fisher Scientific, Germany).

2.3. AEWDs, water inlets points, openings and channel adapters

To identify the source of PSOL contact cultures were taken from the predilection sites in the AEWD machines. The sampled sites A = Albarran pump, B = openings endoscope connection, C = hot air opening, D = rinsing water/circulation pump, E = detergent/disinfectant inlets, F = water inlet into the machine, are shown in Fig. 1.

All sampling points (Fig. 1: A, B, C, D, E, F) were examined with swabs (e-SWAB, Hain Lifescience GmbH., Germany) subsequently incubated in Tryptic Soy Broth (TSB) (BD, Heidelberg, Germany). After two weeks, TSB was examined for the bacterial growth using Columbia agar plates and incubation for more than 72 h. In addition, the channel adapters (Fig. 2) that connect the endoscopes channels ports to the water and air supply lines in the AEWDs were replaced and tested for the presence of PSOL.

Each channel adapter (Olympus GmbH, Hamburg, Germany) was brushed and rinsed. The brushes were rolled onto Columbia agar plates (BD, Heidelberg, Germany) then placed in Falcon tubes containing 20 mL of TSB and incubated for 3 weeks. Similarly, the rinse solutions (20 mL of TSB) were also incubated for 3 weeks. The adapters were then opened up and their inside carefully scraped under sterile conditions for analysis by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). In addition, next-generation sequencing

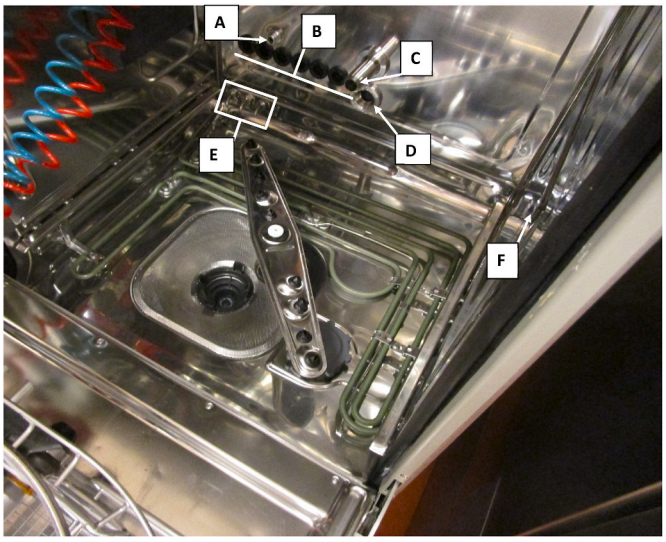

Fig. 1. The inside of an automated endoscope washer-disinfector and the sampling points A-F.

(NGS) was used to further assess the scraped material for the presence of bacterial DNA. By combining these techniques, we were able comprehensively analyze the microbial communities inhabiting the adapters interiors and gain insight into their spatial distribution, morphology and genetic composition. This multifaceted approach provides a deeper understanding of the microbial ecology within the tubes and can inform further investigations into their roles and interactions within the environment.

Confocal laser scanning microscopy (CLSM): The scraped samples were mounted on a microscope slide. A drop containing 10 μL of a live/dead dye mixture of propidium iodide and Syto9 was then dropped onto the samples for 5 min (Invitrogen™ LIVE/DEAD™ BacLight™ Bacterial Viability Kit, Thermo Fisher Scientific, Germany). An LSM780 (Zeiss, Oberkochen, Germany) confocal microscope system (argon laser, 488 nm and 561 nm) was used to capture the images.

Scanning electron microscopy (SEM): The scraped samples for SEM analysis were fixed with glutaraldehyde for 20 min, dried in an alcohol dilution series. Then coated with gold and then examined in a scanning electron microscope (JSM 6310; Jeol Ltd., Tokyo, Japan) at 15 kV acceleration voltage.

Next generation sequencing (NGS): To detect bacterial DNA on the inner surfaces, the channel adapters were externally cleaned with RNA cleaner (1.5 % hypochlorite) and cut in half lengthwise. The inner

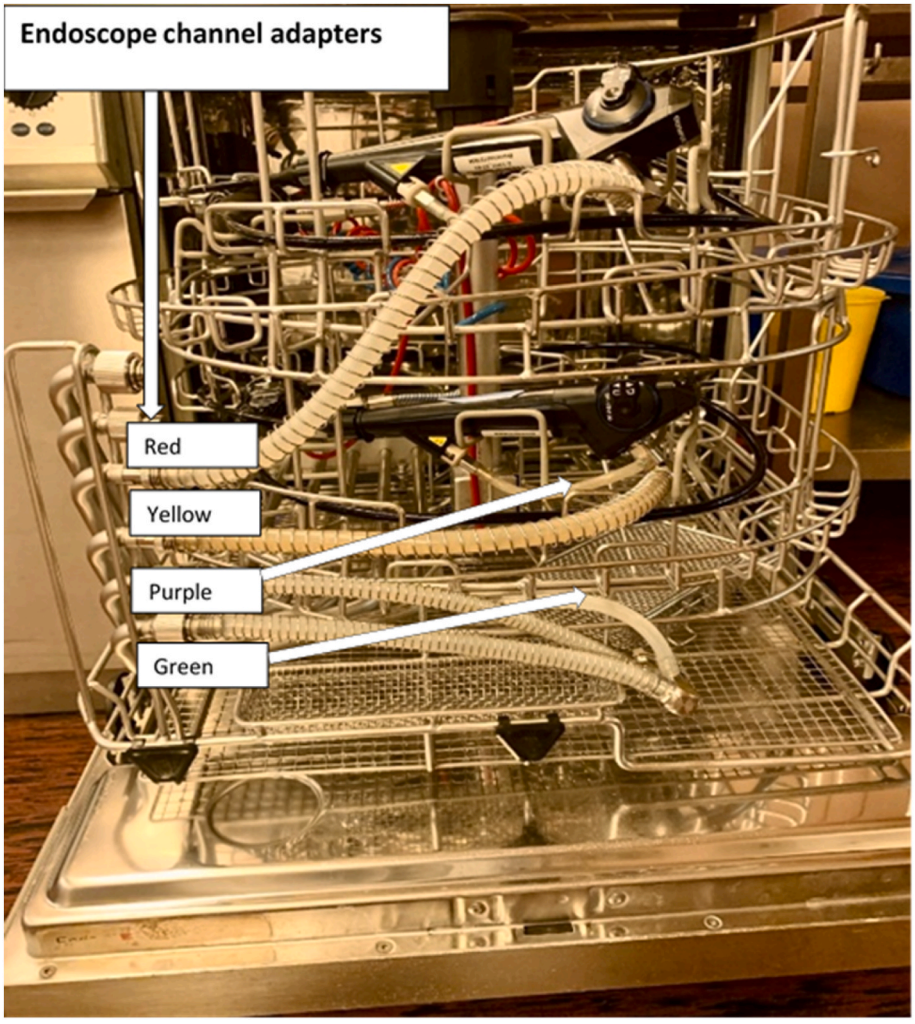

Fig. 2. The interior of an automated endoscope washer-disinfector and the tested channel adapters to the endoscope. The adapters with largest diameter, marked red and yellow, are usually connected to the suction channel and instrument/biopsy channel of the endoscope. The green and purple adapters connect adapters connect to the ports of air/water channels depending on the reprocessed endoscope model. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

surface of the adapters was scraped using a metal spatula and 100 μL of PBS. The scraped material was collected with a pipette, transferred to an epi-vial, centrifuged and stored in a freezer at −80 °C for further analysis. After DNA extraction from the scraped material using a modified CTAB-SDS-chloroform method [13], the overall composition of bacterial DNA in the samples was assessed by microbiome analysis using the MiSeq® platform (Illumina, San Diego, California). Primers 27F (5'-CCT ACG GGN GGC WGC AG-3') and 1391R (5'-GACTACHVGGTATC-TAATCC-3') were selected for bacterial 16S rRNA amplification and phylogenetic classification. In addition, bioinformatic analysis and NGS quality were assessed using FASTQC 0.11.4 software [14].

A microbiome analysis was conducted using the Illumina MiSeq platform (Illumina, USA) by paired-end sequencing of the 16S rRNA V3–V4 region with the 600-cycle MiSeq Reagent Kit v3 (Illumina, USA). Library preparation followed the 16S Metagenomic Sequencing Library Preparation protocol by Illumina, utilizing the primer sequences recommended by Klindworth et al. (2013) [15]. Bioinformatic processing involved primer trimming with Cutadapt. Filtering, denoising, merging of paired-end reads, and chimera removal were carried out using the DADA2 R package in RStudio 2022.12.0 + 353. Taxonomy was assigned to the resulting amplicon sequence variants (ASVs) with the SILVA v138.1 Ref NR 99 SSU database. Data visualization was performed using the ggplot2 R package in RStudio and Microsoft Excel 2016.

2.4. Data analysis

Statistical analysis after treatment with disinfectant was performed using ANOVA, two-sided unpaired and paired (for percentage activity reduction) *t*-test (IBM SPSS software, version 24). *P* values < 0.05 were considered as significant.

3. Results

3.1. PSOL isolates

The final rinse water of the affected AEWDs (7 of 27 tested) showed 2–44 CFU/mL of PSOL. In the endoscopes (10 of 80 tested), PSOL was most frequently found in the air-water channel (2–100 CFU/mL), followed by the instrument/biopsy channel (1–74 CFU/mL) and the jet port (2–100 CFU/mL). The albarran lever was unaffected in all cases.

RAPD-PCR classified the 15 PSOL isolates into two distinct strains. Eleven isolates of one strain were ciprofloxacin resistant and mostly found in AEWDs of the OT area. The other 4 isolates of the second strain were susceptible to all antibiotics tested and was found in AEWDs of the gastroenterology. For further testing, we randomly selected one isolate from each strain (PSOL942 resistant and PSOL966 sensitive).

3.1. 1Effect of GLUT on PSOL planktonic state

All reference methods (EN 1040, EN 13727, and EN 14561) used for testing on PSOL942, PSOL966 and the PA01 confirmed the efficacy of GLUT1% (1 g/100 mL) at 55 °C on planktonic cells, mostly. However, single colonies of ciprofloxacin-resistant PSOL 942 were found 48 h after exposition to GLUT1% (Table 1). The subsequently measured 1.5 % concentration had a bactericidal effect on all isolates tested. At lower temperatures (25 °C), the disinfectant failed to eliminate planktonic forms of both PSOL strains. In contrast, the reference strain PA01 was sensitive to all (0.25 %, 0.50 % and 0.75 % data not shown) tested disinfectant concentrations (Table 2).

3.1.2. Effect of GLUT on PSOL biofilms

PSOL-biofilms as measured by CV assay, were smaller than those of PA01 ($p < 0.05$) (Fig. 3).

The CLSM visualization yielded similar results. The biofilm thickness reached approximately 35 μm for both PSOL strains, while PA01 had an average thickness of 70 μm. Interestingly, the behavior of the visualized biofilms EPS appears to be different. The compact EPS of PSOL942

Table 1
The impact of GLUT1% and GLUT1.5 % on planktonic cells of PSOL and PA01 was assessed at 55 °C using three reference methods. The mean $\log_{10}$ CFUs of six replicates were measured (0.25 %, 0.5 %, 0.75 % data not shown). NG = no growth.

		Growth $\log_{10}$ CFU/mL at 55 °C				
			after 24 h		after 48 h	
		control	1 %	1.5 %	1 %	1.5 %
EN1040_basic suspension assay	*P. oleovorans* 942	2×10^7	NG	NG	NG	NG
55 °C	*P. oleovorans* 966	1.6×10^7	NG	NG	NG	NG
	P. aeruginosa 01	3×10^6	NG	NG	NG	NG
EN13727_low protein loading (0.3 g/l) assay	*P. oleovorans* 942	2.8×10^9	NG	NG	0.5	NG
55 °C	*P. oleovorans* 966	1.8×10^9	NG	NG	NG	NG
	P. aeruginosa 01	10×10^7	NG	NG	NG	NG
EN14561_carrier test	*P. oleovorans* 942	4×10^9	NG	NG	NG	NG
55 °C	*P. oleovorans* 966	7×10^9	NG	NG	NG	NG
	P. aeruginosa 01	4×10^9	NG	NG	NG	NG

Table 2
The effect of different concentrations of GLUT on planktonic PSOL and PA01 was examined at 25 °C. Results represent the average CFU growth on agar plates from six replicates. NG confirmed the $5\log_{10}$ reduction. NG = no growth.

	control	Growth CFUs/mL at 25 °C				
		1.0 %	1.5 %	2.0 %	2.5 %	3.0 %
P. oleovorans 942	10^7	>200	>200	>200	1.16	NG
P. oleovorans 966	10^8	88	1.14	NG	NG	NG
P. aeruginosa 01	107	NG	NG	NG	NG	NG

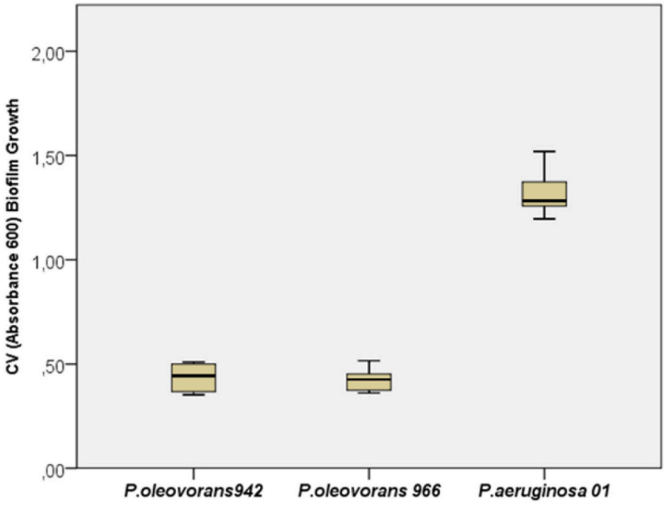

Fig. 3. The average biofilm biomass measured using the optical density of PSOL and Pa01 isolates (nx3) is shown in the crystal violet assay. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

appears to protect the biofilm surface from the disinfectant and cover the underlying bacteria in biofilms. In contrast, the EPS of PA01 degraded without a clear function, resulting in a greater reduction of biofilm mass compared to PSOL (Fig. 4). However, with the CLSM we could not detect and visualize any significant differences in the amount of surviving bacteria after treatment with 1 % disinfectant.

As expected, the effect of 55 °C temperature alone was not sufficient to eliminate bacteria in biofilms. Only the combination of GLUT1% and 55 °C temperature was able to reduce the growth of PSOL by more than $5\log_{10}$. Here, the use of 0.5 % disinfectant (GLUT0.5 %) in combination with 55 °C reduced the growth of PA01 biofilms by $5\log_{10}$, but not that of the two PSOL strains (Fig. 5a). It is worth noting that, the turbidity (absorbance) of the culture medium after treatment with GLUT1% showed similar values to those of the controls at 37 °C and 55 °C (Fig. 5b). This could be an indication of residual biofilm, which could serve as a hiding place for the surviving bacteria, and probably only additional mechanical action on the biofilms could reduce them in the long term. As shown in Fig. 6a and b, the values measured after 24 h of disinfectant exposure at 55 °C were generally elevated compared to those measured after 4 h ($p = 0.003$).

3.2. AEWDs, water inlets points, openings and channel adapters

Closer sampling of the deeper areas of the point F, the water inlet into the machine (Fig. 7), revealed colonization with PSOL, along with *P. aeruginosa* and *Sphingomonas* sp. Other non-fermenting bacteria, but no PSOL, were found at all other sampling points A-E.

Except for common contaminants, no growth of PSOL was detected in the samples obtained by flushing and brushing the channel adapters. Single CFUs of environmental microorganisms such as *Micrococcus luteus*, *Staphylococcus* sp., *Paenibacillus glucanolyticus*, and *Bacillus cereus* were found and considered to be contaminants only. In contrast, SEM and CLSM visualization revealed biofilm formation inside the channel adapters. (Figs. 8 and 9).

The following NGS analysis of scraped material from the inside of the adapters showed mainly DNA of *Bacillus species* and *Enterobacterales* such as *Klebsiella oxytoca* or *Serratia marcescens* (Fig. 10). Bacterial load was highest in the adapters of air-water channels (marked purple), while other adapters appeared to be less affected by adhesive bacteria. Notable: NGS analysis of channels adapters' colonization revealed no source of PSOL.

4. Discussion

Pseudomonas species biofilms resist eradication, particularly when present in medical devices harboring channels with narrow lumina. These biofilms may disrupt and spread to patients. PSOL was first isolated in water-oil emulsions used as lubricants and coolants in metal cutting and industrial wastewater, but was detected also in the last rinse water from AEWDs [10,16]. PSOL has been found in wound samples, sepsis, peritonitis, meningitis and one case of infective endocarditis [17,18]. Hypersensitivity pneumonitis has also been reported in a worker with cystic fibrosis [19]. Like other *Pseudomonas species,* it is able to grow and move actively in the environment at 4–42 °C, with optimum growth at 35 °C [20]. Due to its environmental ubiquity, *Pseudomonas* sp. may harbor various resistance genes including integrin blaVIM-2 associated with human infection [21]. PSOL has been described to carry resistance genes, eg. the multidrug resistance proteins MdtA and MdtB [22]. PSOL isolated from industrial wastewater were found to be susceptible to most antibiotics [22]. In our study, eleven out of fifteen PSOL isolates collected were resistant to ciprofloxacin. Additionally a decreased susceptibility to glutaraldehyde, the active ingredient in disinfectants used in our AEWDs, was found in two of our investigated PSOL strains. Additionally a decreased susceptibility to glutaraldehyde, the active ingredient in disinfectants used in our AEWDs, was found in two of our investigated PSOL strains. Reduced susceptibility to disinfectants has been described for enterobacteria, and may be a factor for emerging resistance to antibiotics used to treat infections in humans [23,24]. Adkin and colleagues reported that the mechanism of action of ciprofloxacin resistance is similar to that of glutaraldehyde, causing an eightfold increase in the MIC of ciprofloxacin in *P. aeruginosa* primed with glutaraldehyde. Even in the absence of apparent increased biocidal tolerance [24].

We suspect that the decreased susceptibility to GLUT observed in our isolates may be a consequence of repeated exposure to non-lethal concentrations of glutaraldehyde during repeated reprocessing. This might

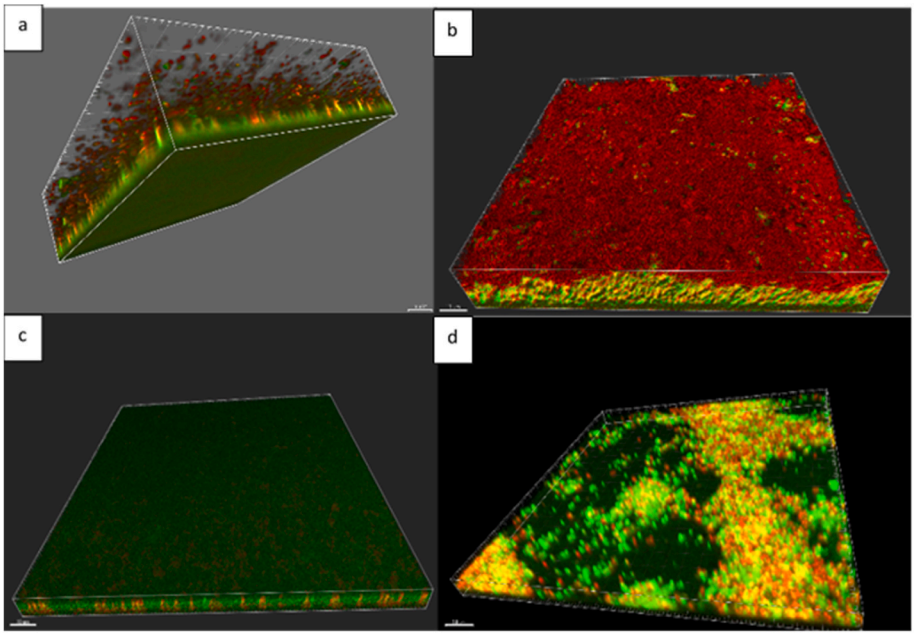

Fig. 4. Confocal laser scanning microscopy images of the biofilm matrix. Extracellular polymeric substance (EPS) was visualized using ConA/PI staining, where red represents bacteria and green represents EPS. This visualization was performed on PSOL942 and PA01 before (a, b) and after (c, d) treatment with GLUT1% at 55 °C (magnification 630x). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

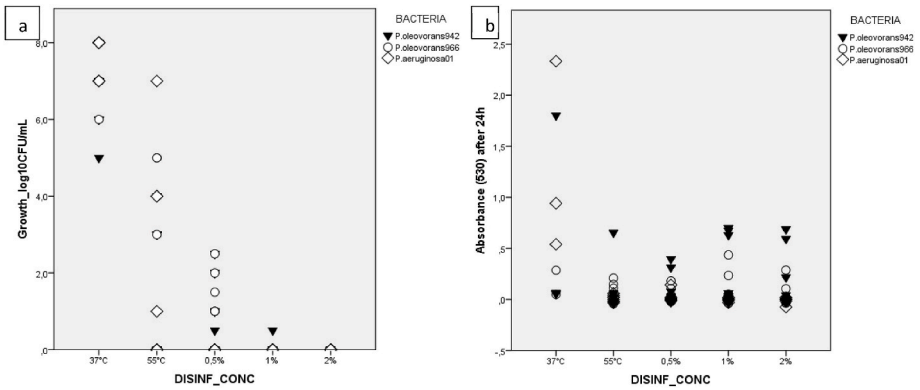

Fig. 5. Measuring bacterial growth 24 h after exposing biofilms to different concentrations of GLUT and temperatures: (a) viable colony count assay shows the number of surviving bacterial colonies (CFUs) and (b) the turbidity (absorbance) of the growth medium. Each symbol represents a measurement performed in an absorbance assay in fourfold repetition. Temperatures 37 °C and 55 °C served as controls without disinfectant. Notably, there were no significant differences in biofilm growth between the ciprofloxacin-resistant (PSOL942) and ciprofloxacin-sensitive (PSOL966) isolates.

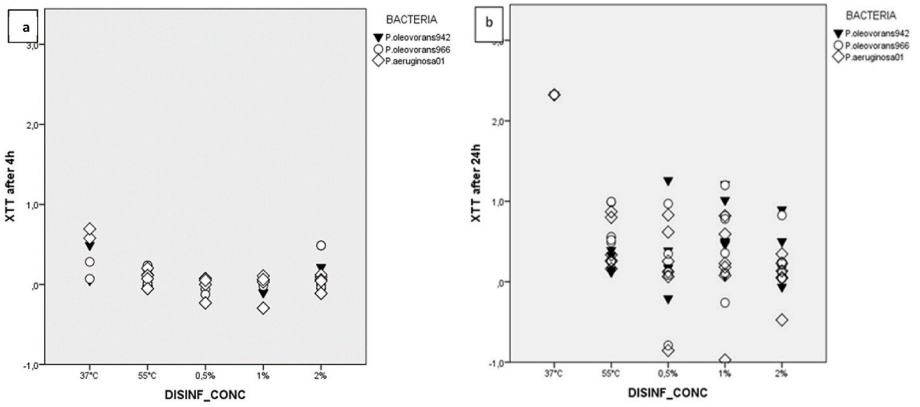

Fig. 6. Metabolic activity investigation using XTT assay. Measurements were taken at 37 °C and 55 °C and after exposure to disinfectant (a) 4 h, and (b) 24 h after treatment.

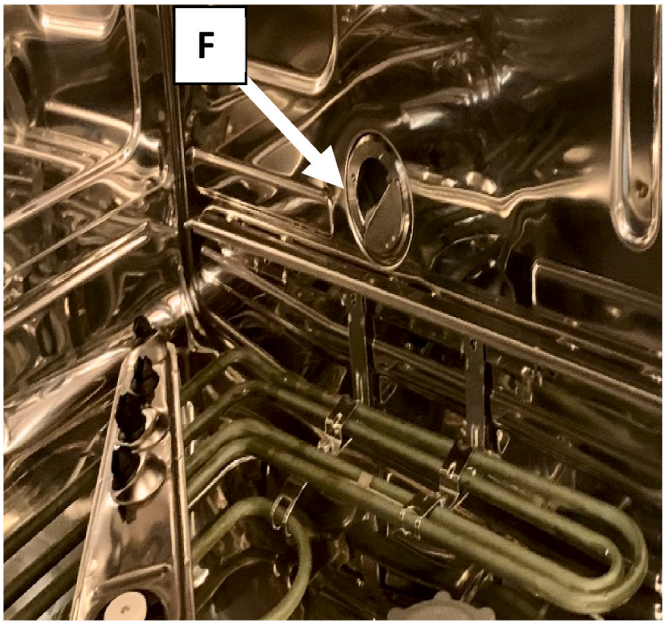

Fig. 7. The inside of the automated endoscope washer-disinfector and point F viewed close up. The affected "water inlet into the machine". Bacteria were found after a deep sampling of the canal behind the opening.

influence the subsequent ciprofloxacin resistance.

In our in vitro tests, *P. aeruginosa* was eradicated using GLUT 0.2 % at the following temperatures of 55 °C and at 25 °C as recommended by the manufacturer. Even biofilms of *P. aeruginosa* were destroyed when the recommended disinfectant concentrations were used at the above temperature settings. In contrast, PSOL planktonic cells and biofilms were killed only at the higher concentrations of GLUT1%. It is known that the extrapolymeric substances (EPS) produced by bacteria in biofilms serve as a source of carbon for energy production and, above all, help to overcome harsh environmental conditions [25]. We observed that, *Pseudomonas aeruginosa* EPS was adversely affected by the disinfectant, losing its functionality. The visualized EPS of PSOL on the other hand, was not disrupted by the disinfectant and remained on the surface of the bacteria in the biofilm. This observed protective behavior of PSOL biofilm EPS could be the reason for the increased biofilm vitality 24 h after disinfectant treatment as shown in Fig. 6. Wang et al. did not describe any such behavior in their extensive literature review on biofilm EPS [26,27]. As our results are based on the examination of only two representative strains of *P. oleovorans,* further studies on this topic are needed.

The first step in identifying the source or reservoir of PSOL was to sample the endoscope channel adapters used during reprocessing. These adapters were not single-use but were intended to be shared among multiple AEWDs. We hypothesized that PSOL was hidden in the channel adapters and transmitted due to their repeated use across all AEWDs. NGS detected bacterial species in the biofilms inside the channel adapters, such as *Bacillus* sp. and *Enterobacterales*. As reported by

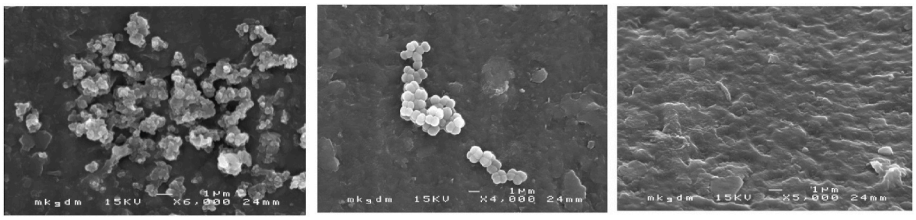

Fig. 8. Using scanning electron microscopy, the inner wall of the channel adapters consistently showed biofilm-like structures on the surface. In addition, individual bacteria were visible on the surface, as in the SEM (5000x magnification).

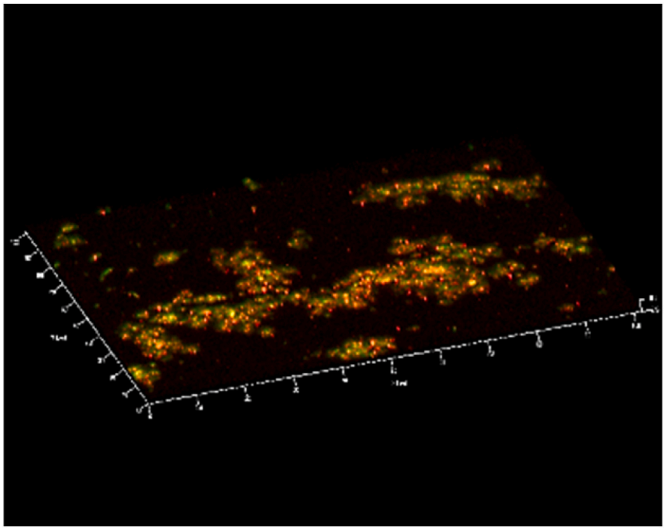

Fig. 9. Confocal laser scanning microscopy of scraped material from inside the channel adapters included live/dead staining (green for live cells/red for dead cells). Notably, there were visible red dots indicating bacteria with damaged membranes and stained DNA. There were likely microorganisms with reduced metabolism appearing in light green or yellow (magnification 630x). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Dergham et al., *Bacillus species* often colonize AEWDs and protect biofilm bacteria, such as *Enterobacterales*, from the action of disinfectants [28]. Bacteria were mostly detected in adapters with small diameters, which is consistent with the literature [29]. Despite this potential risk, PSOL was not detected in the channel adapters, not even in minute quantities. A plausible explanation for the absence of PSOL could be the frequent replacement of these channel adapters, preventing any significant buildup or contamination.

With this in mind, we carried out further tests on the machine's water inlets and water circuit.

Water for our AEWDs comes from the general water supply system, which is fed with clean Viennese drinking water (controlled yearly according to Austrian legislation). The water then circulates through the machine, where it is first deionized using an ion exchanger and subsequently disinfected by a UV unit. Since PSOL was found in the last rinse water and on the surfaces of the endoscope after the disinfection process, we assumed that PSOL could be washed in during reprocessing. At the time of sampling, there was no evidence of PSOL in the general water supply. PSOL was detected in the water inlet into the machine, located downstream of the ion exchanger and the UV disinfection unit. It seems that despite the mandatory UV disinfection of the tap water in the machine after deionization, it was not possible to prevent the spread of bacteria, which were probably present in biofilms. We suspect that the original source of the bacteria was a potential biofilm in the ion exchange pump. Unfortunately, accessing the ion exchange pump is not possible without disassembling the machine to confirm our suspicions.

We also find it interesting that none of the microorganisms found with PSOL in the water inlets, such as *Pseudomonas aeruginosa* and *Sphingomonas* spp., were detected on the endoscope surface or in the final rinse water during control sampling. This aligns with our in vitro susceptibility test results, indicating that disinfectant residues in the rinse water effectively kill *Pseudomonas aeruginosa* and other non-fermenters but do not affect PSOL. According to these results, increasing the GLUT concentration above 1 % could eliminate PSOL, but at this concentration its damaging effect on the sensitive endoscope surface is detrimental, as evidenced by Bradley et al. [30]. In addition, GLUT on the endoscope surfaces can be toxic causing inflammation of the intestinal mucosa in patients as described by Dolce et al. and Delabrousse et al. [31,32].

So far, there are no known PSOL infections attributable to or associated with the use of endoscopes. However, we should consider Rutala et al.'s comment that endemic transmission of infections related to gastrointestinal endoscopes may go unnoticed due to inadequate or nonexistent monitoring of ambulatory procedures and the extended time between colonization and infection [5]. Therefore, the presence of PSOL in AEWD machines and in endoscopes may continue to contribute to the spread of PSOL and antimicrobial resistance.

5. Conclusions and the future work

Studying the resistance mechanisms of PSOL's exopolysaccharides (EPS) and their role in protecting bacteria within biofilms could provide important insights into improving disinfection protocols and addressing bacterial resistance. The presence of PSOL in the AEWDs raises the question if standardized testing of new disinfectants using only *P. aeruginosa* as a reference strain may overlook the survivability of other non-fermenters. To find the source or reservoir as well as the mechanisms why PSOL can survive in the AEWD machine, further studies on the metabolic potential of PSOL for survival in the AEWD environment and development of resistance are needed.

Furthermore, are PSOLs only associated with AEWDs that use disinfectants containing glutaraldehyde? Are PSOLs the adapted survivors due to "disinfection pressure" in the niche of AEWDs? A variety of disinfectants are available, but controlled laboratory and clinical studies have shown that they can vary widely in their efficacy and suitability for use [33].

PSOL could serve as a valuable marker for maintenance of the AEWDs. Its presence may indicate inadequate maintenance of the machine itself or issues with the supply water. Therefore, detecting PSOL could be a useful marker for the effectiveness of the disinfection process and its appearance signal the need for a technical inspection. Regular monitoring of PSOL levels could help ensure that the AEWDs are functioning properly and that maintenance protocols are being followed. In addition, any redesign of AEWDs should aim to enhance the accuracy of cleaning and disinfection processes. The redesign should also improve the accessibility of all components of the AEWD for thorough examination and maintenance. This would help reducing the risk of contamination and improving overall device performance.

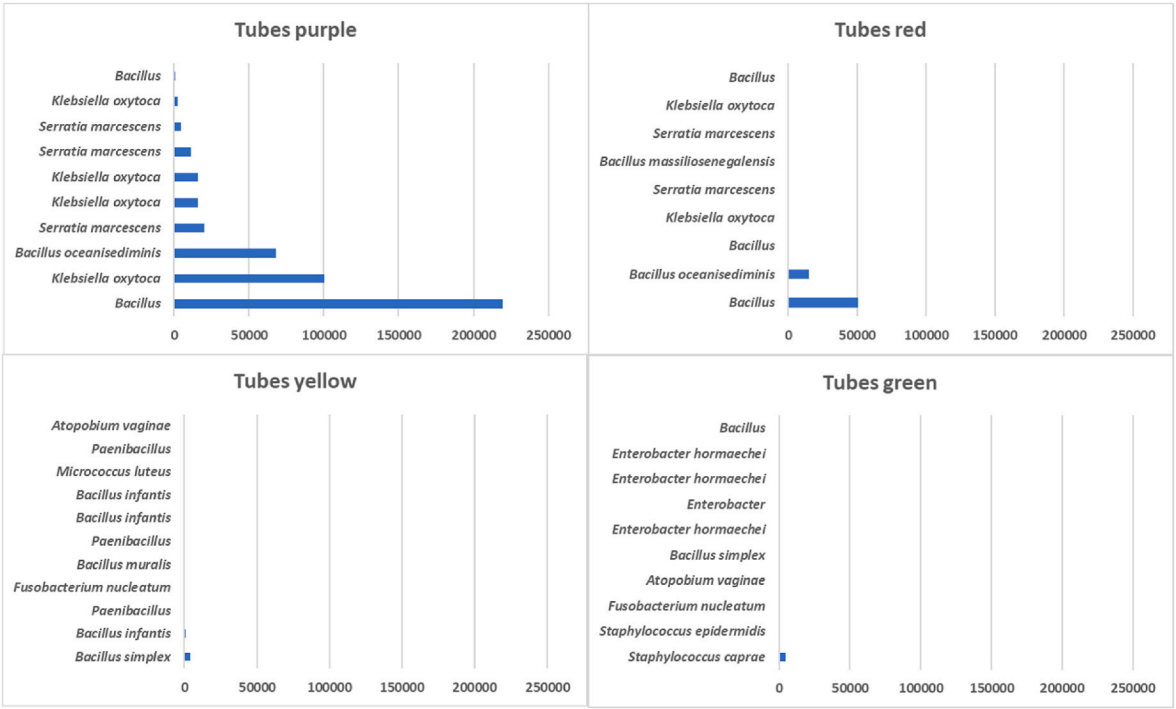

Fig. 10. Bacterial DNA identified by Next Generation Sequencing inside the channel adapters. The ten most frequent reads and the concentration of bacterial DNA found for each species. PSOL was not found.

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CRediT authorship contribution statement

Beata Leeb-Zatorska: Writing – original draft, Methodology, Data curation, Conceptualization. **Miriam Van den Nest:** Writing – review & editing, Methodology, Investigation. **Julia Ebner:** Writing – review & editing, Methodology, Conceptualization. **Doris Moser:** Visualization, Investigation, Data curation. **Kathrin Spettel:** Validation, Investigation, Data curation. **Lukas Bovier-Azula:** Writing – review & editing, Software, Formal analysis. **Magda Diab-El Schahawi:** Writing – review & editing, Validation. **Elisabeth Presterl:** Writing – review & editing, Supervision.

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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