

Antimicrobial properties of silver nanoparticles against biofilm formation by *Pseudomonas aeruginosa* on archaeological textiles

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Abstract: The aims of this work were to: (i) microscopically analyse the pre- and post-Columbian archaeological textiles using Scanning Electron Microscopy with Energy Dispersive X-Ray Analysis (SEM-EDX); (ii) microbiologically analyse the archaeological textiles (from the Southern Andean Area, La Plata Museum); (iii) determine the ability of *Pseudomonas* sp. isolates from archaeological textiles to biofilm formation by SEM; (iv) assess the anti-biofilm properties of AgNPs protecting cotton against *Pseudomonas* sp. Results showed the presence of bacteria with proteolytic and lipolytic activities on archaeological textiles, including *Clostridium* sp. and *Pseudomonas aeruginosa*. Two nucleotide sequences of 16S ribosomal RNA gene of *P. aeruginosa* strains were deposited in GeneBank NCBI database with accession numbers: KP842564 (strain 1) and KP842565 (strain 2). Those strains exhibited different morphological and growth characteristics: strain 1 with ability to form biofilms on archaeological textiles was rod-shaped, produced bluish-green pigment, and smaller than strain 2; and strain 2 was pleomorphic and produced brown pigment. The use of silver nanoparticles (90 ppm, ϕ 10–80 nm) allowed to protecting textiles against *P. aeruginosa* growth by 63%–97%, depending on the strain and exposition time.

Keywords: archaeological textiles, *Pseudomonas* sp., protection, silver nanoparticles, biofilm, SEM-EDX

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1. Introduction

For thousands of years, textiles have played a key role in the social, economic, and ideological life of the Andean world populations.

“Every nation that inhabited this universe dressed according to their symbols, expressed with its own iconography, with a particular manufacturing, colour, and style that set her apart from the others and indicating their cultural identity and landscape from where

they belonged”^[1]. The textiles dating from the pre- and post-Columbian times were made mainly from wool and cotton. The designs included geometrical patterns, representations of gods, men, plants, animals, and supernatural beings.

Archaeological textiles in suitable conditions (high humidity, high temperature, and presence of organic substances) may be overgrown with microorganisms, which cause the weakening or loosening of structure, odour emission, aesthetic changes, the presence of staining and discolorations on the surface, slimy substances, and finally complete deterioration^[2–6]. The biodeterioration of archaeological textiles may have important economic and cultural consequences. The colonisation and development of biofilms formed by fungi and bacteria drastically modify the material. Biofilms are aggregates of microorganisms encased in their matrix of extracellular polymeric substances (EPS). These EPS consist of a mixture of polysaccharides, proteins, nucleic acids, and other highly hydrated biopolymers. Microorganisms such as fungi (i.e., *Cladosporium* sp.) and bacteria (*Pseudomonas* sp.), which form biofilm on textiles also cause the degradation of cellulose or proteins — the main component of natural fibres such as cotton and wool^[7]. Proper storage of textiles in special containers, under suitable and stable conditions of light (<50 lux), temperature (18–20°C), and relative humidity (<55%) is effective to avoid undesirable results^[8–10]. The most effective method to inhibit the biodeterioration is disinfection (the destruction of pathogenic and saprophytic microorganisms and their spores by physical or chemical agents). Chemical agents used in disinfection include quaternary ammonium salts, ethylene oxide, alcohols, aldehydes such as formaldehyde, glutaraldehyde, phenolic compounds, biguanides, halogen compounds, dyes, peroxides, surfactants, heavy metals (e.g., Ag, Cu, Au)^[11]. The most common method for disinfection of historical objects is ethylene oxide; its high effectiveness is incomparable with any other fumigant. Unfortunately it is a carcinogenic, mutagenic gas, causing allergic reactions and having an irritating action on the respiratory tract^[12,13].

In the past few years, there has been increasing interest in silver nanoparticles (AgNPs), due to its high antimicrobial properties^[14,15]. Previous studies performed by Gutarowska *et al.* show that silver nanoparticles in the form of mist are effective in disinfection of historical materials such as textiles, paper, leather and wood^[16]. AgNPs misting reduced bacterial

number by 87%–99% and fungal by 32%–54% on cotton fabrics. Moreover, AgNPs misting protection method does not significantly influence the optical and mechanical properties of cotton fabrics, even after accelerated light ageing^[17].

So far, there are no studies concerning the use of silver nanoparticles for inhibition of biofilm formation on archaeological textiles. The objectives of this study were to: (i) analyse the pre- and post-Columbian archaeological textiles microscopically; (ii) analyse the archaeological textiles microbiologically; (iii) determine the ability of *Pseudomonas* sp. isolates from textiles for its biofilm formation; (iv) assess the anti-biofilm properties of cotton protected-AgNPs from *Pseudomonas* sp.

2. Materials and Methods

2.1 Characterisation of the Samples

The tested samples came from the pre-Columbian archaeological textiles (1250–1450 AD) from the Southern Andean area. The samples came from excavations in the Puna Argentina, Santa Rosa de Tastil, performed between 1967 and 1969, and guided by Diana Rolandi^[18]. They are sheltered in Deposit 25 of Archaeological Division at La Plata Museum. Sample 1 was made of cotton, while samples 2 and 3 were made of wool.

2.2 Microscopic Analysis of Archaeological Textiles

The microscopic analysis of surface modified fabrics were carried out by means of scanning electron microscope (SEM) Nova NanoSEM 230 from FEI company (SE Detector, HV: 15kV, Low Vacuum) with X-ray microanalyser EDX (Energy-dispersive X-ray Spectroscopy) (EDAX, USA). The quantitative chemical analysis of studied materials was carried out by the use of ViP Quant software with ZAF and low vacuum correction procedures. The samples were prepared by fixing the fabric to holder with the aid of conductive carbon adhesive tape.

2.3 Microbial Contamination of Archaeological Textiles

Sampling was done from 1 cm² surface of the textiles using sterile cotton swabs^[19]. Proteolytic, amylolytic, and lipolytic bacteria were counted in differential culture media^[20,21]. Proteolytic activity was determined on Frazier Gelatin Agar (Merck®, Germany/Britania®, Argentina). Amylolytic activity was determined on

Starch Agar (Difco™, USA/Britania®, Argentina) by addition of Lugol's reagent. Lipolytic activity was determined on Tributiryn Agar (Fluka/Britania®, Argentina). All samples were incubated at 28°C ± 2 for 3 days. The colonies were counted and expressed as CFU/cm².

The presence of sulphite-reducing bacteria from genus *Clostridium* sp. was checked by inoculation of aliquots into differential-reinforced *Clostridium* broth (DRCM Merck®, Germany) and incubated at 28°C ± 2 for 15 days in anaerobic conditions^[22]. All samples were done in duplicates.

The *Pseudomonas* sp. strains were grown on blood agar (Oxoid®, UK) and Cetrimide agar (Merck®, Germany). The phenotypic method for detecting metallo-β-lactamases was done using a commercially available screen kit (RCS) (Rosco Diagnostica A/S, Taastrup, Denmark)^[23] and incubated at 36°C for 24 h.

Bacteria were typified according to the Gram staining and biochemical tests described in *Bergey's Manual of Systematic Bacteriology*^[24]. One of the strains of *Pseudomonas* sp. was typified as *Pseudomonas aeruginosa* (strain 1) while the other one as *Pseudomonas* sp. (strain 2). For molecular identification, two *Pseudomonas* sp. strains were chosen due to its ability to form biofilm. For further studies, two *Pseudomonas* strains were selected for laboratory studies due to its bioadhesion ability, pigment production, and viability and growth on textile fibres.

2.4 Molecular Identification of *Pseudomonas* sp. Isolates

Molecular identification of two *Pseudomonas* sp. strains isolated from archaeological textiles was performed according to methodology presented by Kręgiel *et al.*^[25]. The nucleotide sequences of 16S ribosomal RNA gene were deposited in GeneBank NCBI database with accession numbers: KP842564 (strain no. 1) and KP842565 (strain no. 2). Both strains were also deposited in Pure Culture Collection at the Institute of Fermentation Technology and Microbiology at Lodz University of Technology (ŁOCK) with accession numbers: ŁOCK 0998 (strain no. 1) and ŁOCK 0999 (strain no. 2).

2.5 Bioadhesion and Biofilm Formation Assay

Bacterial strains were inoculated on solid Mineral Medium^[5] on agar slant with samples of archaeological textiles as a sole carbon source. The bioadhesion and biofilm formation were assessed after 72 h of bacterial

incubation using scanning electron microscope (SEM) (FEI Quanta200). Samples were kept in a closed chamber with ethanol (100%)^[26] for 24 h and metalised with Au/Pd prior to microscopic observation.

2.6 Preparation of Silver Nanoparticles

Colloidal silver nanoparticles (produced by Mint Metale Szlachetne S.A., Poland) were prepared at a concentration of 90 ppm; particle sizes of 10–15 nm (60%–70%) and 50–80 nm (30%–40%). The process was undertaken in a chamber (1.73 m³), temperature T=25°C, RH=90%, air flow 1 m/s. The process lasted 8 h (8 disinfection cycles). The chamber and disinfection procedure are patent protected (Polish Patent no. P-399 507)^[27]. The disinfection chamber, the silver nanoparticle colloid preparation and its application are described in detail in previous study^[28]. The amount of silver nanoparticles deposited on the cotton fabric during one disinfection process, established using the FAAS (Flame Atomic Absorption Spectroscopy) method, was 4.5 ppm/g. The chosen disinfection parameters ensure the safeness for the treated textile samples^[16].

2.7 Antibacterial Properties of AgNPs against *Pseudomonas aeruginosa* on Textile Samples

Model of the studies were performed on cotton to assess the ability of AgNPs to inhibit *Pseudomonas* sp. growth. Cotton fabrics were supplied by Andropol S.A. (Poland). The characteristics of this fabric are given in the previous article^[17]. Fabric were also analysed in terms of colour changes due to bacterial growth.

The cotton samples were antimicrobially protected by misting with silver nanoparticles preparation (90 ppm, 8 misting cycles)^[29]. They were placed on the Petri dishes with Mineral Medium^[5]. The inoculum concentrations of *Pseudomonas aeruginosa* were established using culture method, with equalled 10⁸ CFU/mL for both strains. The inocula (100 μL) were applied on cotton samples with AgNPs and incubated for 5 days in a climatic chamber, KBF720 (Binder GmbH, Germany), at T = 28°C and RH = 80%. After 0, 3 and 5 days, the number of microorganisms was determined by AATCC 100:2012 test method^[30]. Each sample were placed in 50 mL of sterile saline (0.85% NaCl) and shaken for 20 min (200 cpm; ELPIN+ S.C. 358A, Poland). Then, using serial dilutions, the number of microorganisms in CFU/cm² was determined on TSA (Tryptic Soy Agar, Merck, Germany). Each strain had its own control (without AgNPs). Samples were

done in triplicate. The arithmetic mean and standard deviation of the number of microorganisms on cotton with and without silver nanoparticles were calculated. The reduction, R (%) in the number of microorganisms on cotton with AgNPs was determined using the following equation:

$$R = \frac{N_0 - N}{N_0} \times 100\%$$

where:

N is the number of microorganisms on the sample with AgNPs (CFU per sample);

N_0 is the number of microorganisms on the sample without AgNPs (CFU per sample).

An ANOVA statistical analysis was performed to assess the statistically significant difference between the number of microorganisms on cotton with and without AgNPs.

3. Results and Discussion

The SEM-EDX technique can be used for fibre identification of archaeological textiles even when the fabric is almost completely mineralised^[31]. The analysis of pre- and post-Columbian textiles (samples 1, 2, and 3) showed high content of carbon (1: 40.81%; 2: 46.19%; 3: 55.01%, w/w), which proves that natural

fibres are good carbon source for microorganisms. Samples made of wool (2 and 3) were also rich in sulphur (2: 2.17%; 3: 2.39%, w/w) and sample 3 was also rich in nitrogen (9.62%, w/w). The presence of other compounds showed that the tested fabrics were mineralised and highly contaminated with sand and salts. Moreover, iron was detected on sample 1 (1.81%, w/w) (Figure 1), which may be related to respiratory activity of anaerobic bacteria. It might be the causes of reddish spots (foxing type) on textiles, as a result of oxidation processes.

The determined number of lipolytic bacteria ($3 \times 10^4 - 3 \times 10^6$ CFU/cm²) was high, while there was low concentration of proteolytic bacteria (1–4 CFU/cm²) (Figure 2). Amylolytic bacteria were not detected at any of the tested samples.

The mechanism of microbial degradation of textiles depends on their chemical composition. Cotton (cellulose-based fabrics) is damaged by enzymatic hydrolysis (1,4-endo- β -D-glucan cellobiohydrolase, endo-1-4- β -D-glucan glucanohydrolase, glucohydrolase of β -D-glucosides^[32–34]). The microorganisms responsible for cellulose-based fibres degradation are mostly fungi (*Alternaria* sp., *Aspergillus* sp., *Aureobasidium* sp., *Chaetomium* sp., *Mucor* sp., *Penicillium* sp., *Rhizopus* sp., *Trichoderma* sp.). However, bacteria also

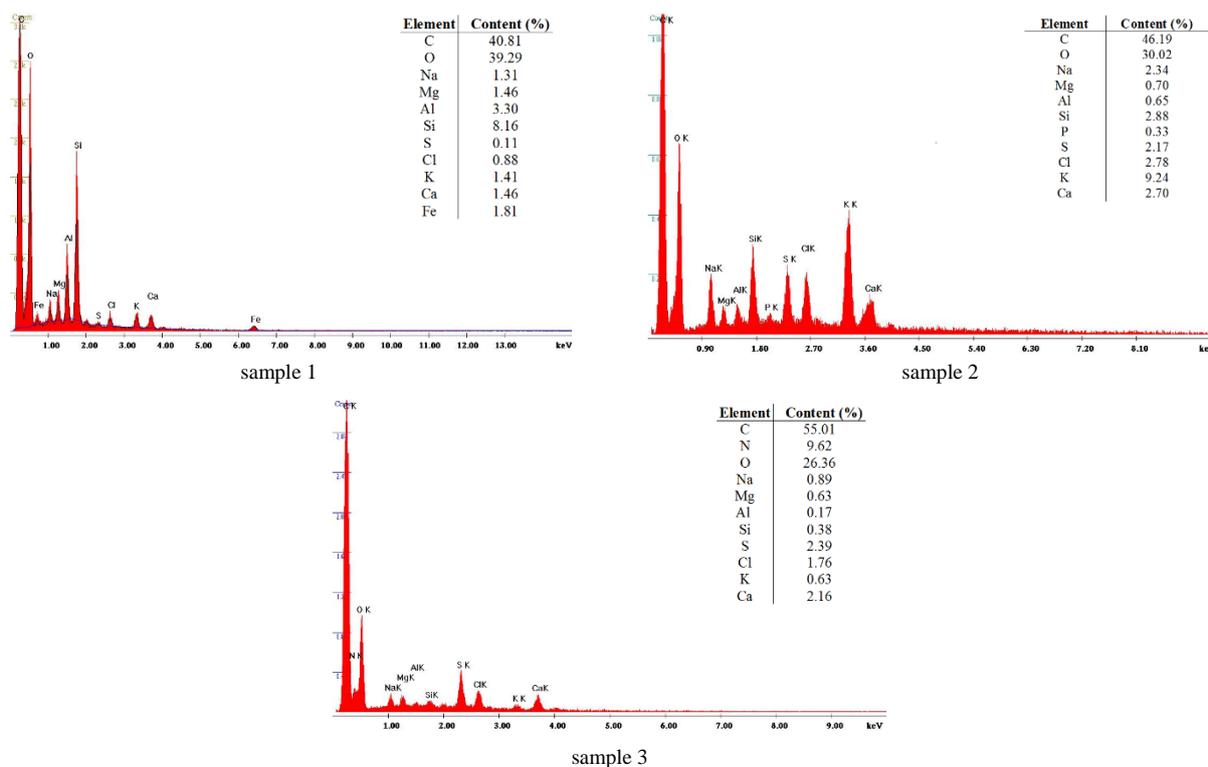


Figure 1. Element content on archaeological textiles (SEM-EDX).

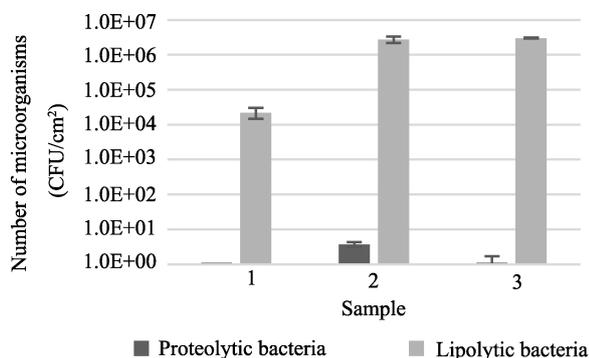


Figure 2. Bacterial contamination of archaeological samples.

play a significant role (*Bacillus* sp., *Cellulomonas* sp., *Cellvibrio* sp., *Clostridium* sp., *Pseudomonas* sp., *Streptomyces* sp.)^[35,36].

The wool is characterised by high content of various amino acids. The keratinolysis process is the result of three reactions: sulfitolysis, proteolysis and deamination^[34,37]. Microorganisms known from literature for wool biodeterioration activity are bacteria such as *Alkaligenes bookeri*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Bacillus* sp. (*B. agri*, *B. mycoides*, *B. mesentericus*, *B. megatherium*, *B. subtilis*) and fungi, namely *Aspergillus* sp., *Alternaria* sp., *Cephalothecium* sp., *Dematium* sp., *Fusarium* sp., *Oospora* sp., *Penicillium* sp. and *Trichoderma* sp.^[36].

Among the microorganisms inhabiting tested archaeological textiles were *Clostridium* sp. and *Pseudomonas* sp., which are known for archaeological textiles colonisation^[37]. The literature data show that *Clostridium* genus can produce textile degrading enzymes such as lipases^[21,38].

Isolated *Pseudomonas* strains grow as bright yellow slimy colonies and produce pigments changing the colour of cotton fibres (Figure 3). *Pseudomonas* sp. strain no. 1 produces a bluish-green pigment, a typical for *Pseudomonas aeruginosa*, while *Pseudomonas* sp. strain no. 2 produces brown pigment and colonies glow in UV light (Figure 4). Using the molecular methods, both strains of *Pseudomonas* sp. were identified as *Pseudomonas aeruginosa*.

Pseudomonas aeruginosa strain 1 show the adherence to the archaeological textiles and SEM analysis proved its bioadhesion ability and biofilm formation (Figure 5). They also exhibited proteolytic and lipolytic properties in culture media, which confirms with previous studies^[5]. Microorganisms inhabit the material due to its natural composition and sufficient moisture content, adhering to the textile that develops biofilms. The microbial growth and its metabolites cause structural damages, which are a hazard for the fibres^[7].

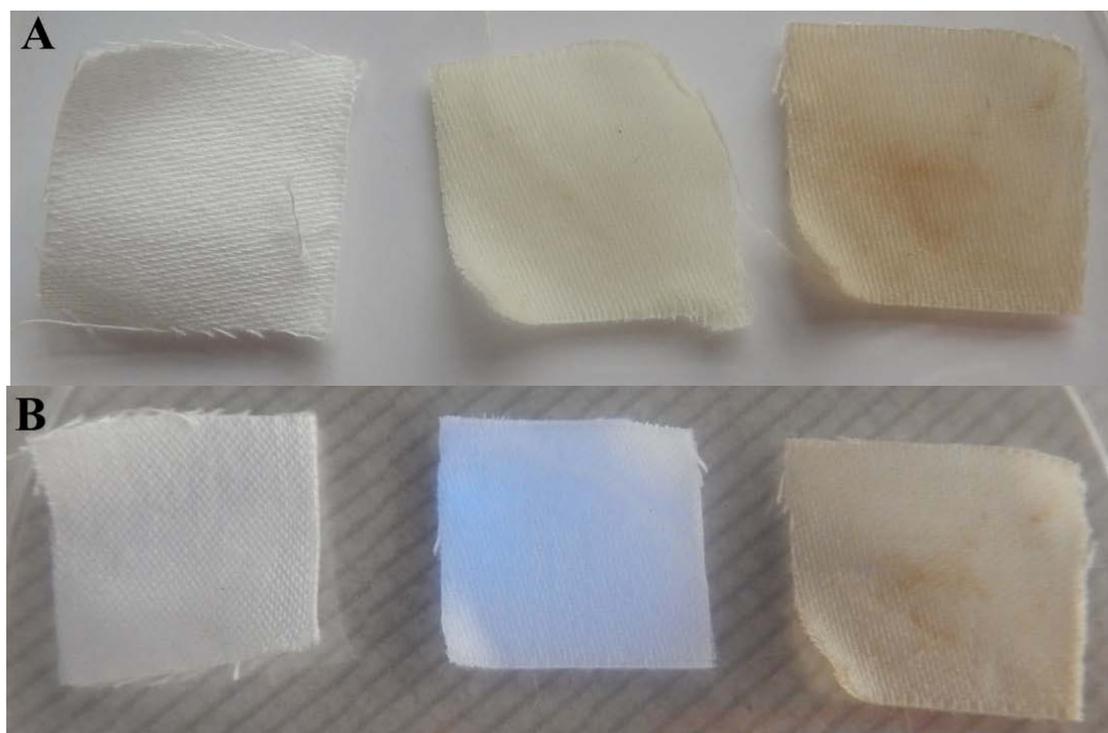


Figure 3. *Pseudomonas aeruginosa* growing on cotton samples. (A) Daylight; (B) UV light; Left: control; Centre: strain no. 1; Right: strain no. 2.

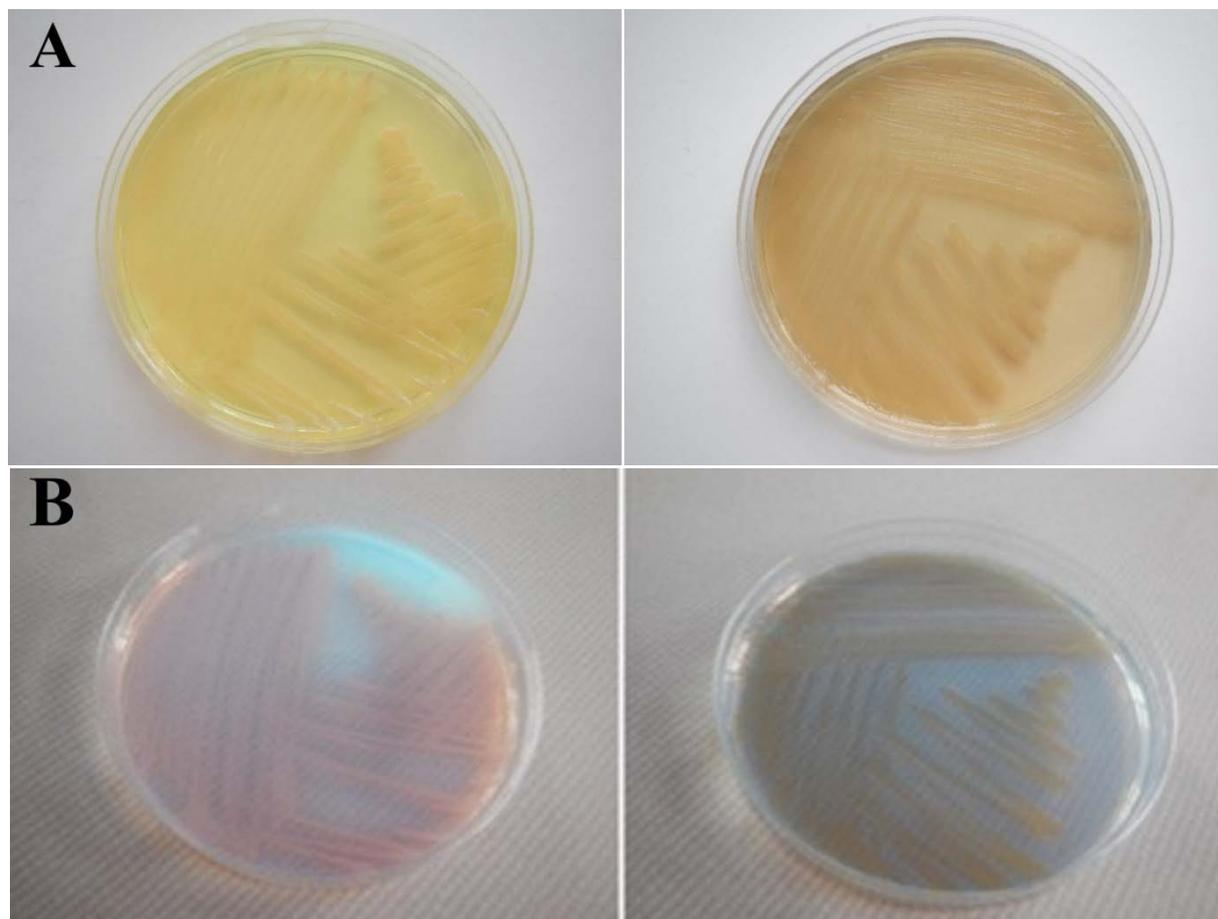


Figure 4. *Pseudomonas aeruginosa* on tryptic soy agar. (A) Daylight; (B) UV light; Left: strain no. 1; Right: strain no. 2.

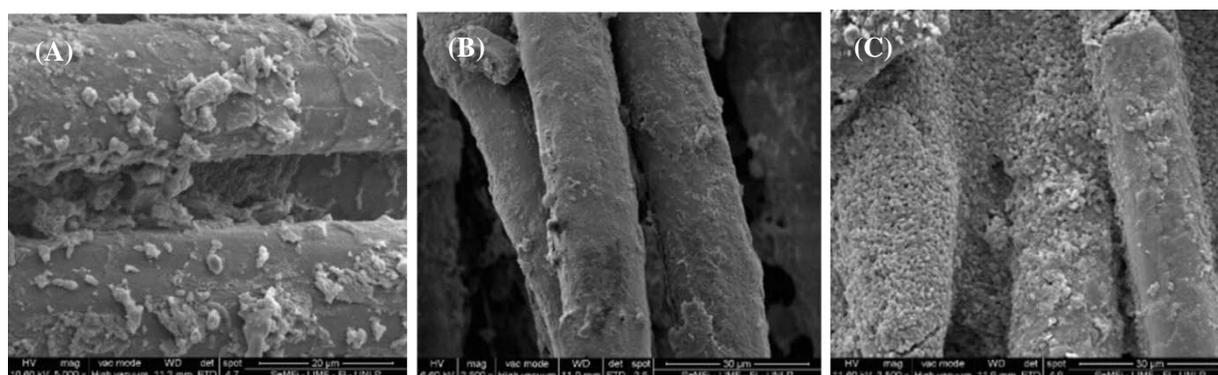


Figure 5. SEM images of: (A) Archaeological textile sample with adhered bacteria and material dirt (5000X, Bar 20 μm); (B) Adhered *Pseudomonas aeruginosa* (strain 1) and biofilm formed on archaeological textile fibre (72 h); (C) Adhered *Pseudomonas aeruginosa* (strain 2) on archaeological textile fibre (72 h).

Microbiological analyses proved various abilities for microbial growth on cotton for both tested strains. Number of *Pseudomonas aeruginosa* strain 1 increased during the incubation on control cotton sample from 1.5×10^6 CFU/cm² to 3.6×10^7 CFU/cm² on day 5 of incubation time. On the other hand, the

amount of *Pseudomonas aeruginosa* strain 2 decreased during the incubation from 4.3×10^5 CFU/cm² to 4.3×10^4 CFU/cm² (Table 1, control samples). Strain 1 has a greater ability to form biofilms on cotton than strain 2, which proves the observations from SEM analysis.

Table 1. *Pseudomonas aeruginosa* growth on cotton samples with/without AgNPs

Day	Number of microorganisms (CFU/cm ²)					
	<i>Pseudomonas aeruginosa</i> strain 1			<i>Pseudomonas aeruginosa</i> strain 2		
	Control without AgNPs	AgNPs	R (%)	Control without AgNPs	AgNPs	R (%)
0	M: 1.5×10 ⁶	M: 1.2×10 ⁶	21.5	M: 4.3×10 ⁵	M: 6.4×10 ⁵	–
	SD: 7.0×10 ⁵	SD: 3.1×10 ⁵		SD: 1.6×10 ⁵	SD: 2.6×10 ⁴	
3	M: 1.1×10 ⁷	M: 6.3×10 ^{6*}	43.0	M: 5.0×10 ⁴	M: 5.0×10 ⁴	–
	SD: 4.6×10 ⁶	SD: 2.5×10 ⁶		SD: 2.0×10 ⁴	SD: 2.0×10 ⁴	
5	M: 3.6×10 ⁷	M: 1.3×10 ^{7**}	62.9	M: 4.3×10 ⁴	M: 6.3×10 ^{2*}	97.1
	SD: 2.5×10 ⁷	SD: 1.7×10 ⁷		SD: 1.6×10 ⁴	SD: 3.2×10 ³	

M: arithmetic mean; SD: standard deviation; -: no reduction

*: significantly different to the sample without AgNPs, ANOVA with significance level $p < 0.05$

The silver nanoparticles inhibited the growth of both *Pseudomonas* strains. For strain 1, the maximum decrement in the microorganisms number was from 3.6×10^7 CFU/cm² to 1.3×10^7 CFU/cm², which constituted up to 63% (day 5). For strain 2, the maximum decrement in the microorganisms number was higher and reached 97% (day 5) — from 4.3×10^4 to 6.3×10^2 CFU/cm². The differences between number of microorganisms with and without nanosilver are significantly different after 5 days of incubation for both strains and after 3 days for strain 1, respectively (Table 1, AgNPs samples).

Pseudomonas aeruginosa is known for its resistance to antibiotics and several antimicrobial agents. The literature shows that AgNPs can be very efficient in limiting *P. aeruginosa* biofilm formation, however, it happens only when there is a direct contact of nanoparticles with the bacterial cells (biofilm and planktonic) in liquid culture. A number of studies show very high reduction of *P. aeruginosa* biofilm, up to 100%, due to AgNPs action^[39–41]. The data obtained in this study for microbial number reduction is lower (up to 2 log units for strain 2). However, on the textiles there is no direct contact of microorganisms with the silver nanoparticles; thus, it is impossible to achieve very high disinfection effectiveness without material destruction. Literature shows no studies presenting the limitation of *Pseudomonas aeruginosa* biofilm on archaeological textiles. On the other hand, studies considering the influence of biocides on new textiles for *P. aeruginosa* are unfortunately not promising. Jakimiak et al.^[42] tested cotton finished with quaternary ammonium compounds, while Takai et al.^[43] tested textiles (cotton, polyester/cotton) with different antibacterial finishing substances (Ag.Zn.Cu – Zeolite; Ag.Zn.ammonium Zeolite; aliphatic imide;

quaternary ammonium derivative; chitosan). Both research teams observed absence of antimicrobial effect against *P. aeruginosa* (pure culture collection and wild strains). Such results suggested that the data presented in the study of silver nanoparticles misting can be very promising in the antimicrobial protection of textiles, especially those related to archaeological textiles.

Conflict of Interest and Funding

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