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EFFICACY OF DEEP EUTECTIC SOLVENTS (DESs) FOR MITIGATING BIODETERIORATION IN CULTURAL HERITAGE: IN SITU EVALUATION

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Abstract

The preservation of stone materials faces significant challenges due to biological deterioration, compromising the visual appearance and structural integrity of artifacts. Current cleaning treatments use toxic chemical biocides, with a high impact on the environment and human health. Research is turning to eco-friendly alternatives, such as Deep Eutectic Solvents (DESs), which offer a promising combination of biocidal properties and eco-friendly, as well as features such as biodegradability, non-flammability and non-volatility. This study defines the efficacy of Choline Chloride-based DESs reported in the literature as biocides in a real case study on an outdoor exposed magmatic effusive rock at the University of Calabria. Several analytical techniques were used to define DESs biocidal efficacy, monitoring their action for 6 months. This study contributes to the evidence of DESs' ability to keep treated surfaces clean, thus promoting the long-term preservation of materials. Macroscopic and microscopic observations, spectrocolorimetry, bioluminometry and FTIR spectroscopy have highlighted the significant potential of DESs as sustainable biocidal solvents for the preservation of stone materials exposed outdoors.

Keywords*: Deep Eutectic Solvents (DESs); Green solvent; Biocide; Cultural Heritage treatment; Stone material*

Introduction

Historic stone architectures, monuments and other artifacts are often located outdoors and are exposed to risks requiring protection due to the combined action of physical, chemical and biological deterioration [1]. The biological deterioration addressed in cultural heritage conservation results from heterogenous ecosystems that find habitat and thrive on artworks' surfaces. These ecosystems are strongly influenced by environmental parameters such as relative humidity, light and temperature [2]. The colonization of various organisms (bacteria, lichens, mosses) forms biological layers known as biofilms, which, together with other factors such as climatic conditions, pollution and the physical-chemical properties of the material itself, contribute to the deterioration of surfaces. These biofilms, in addition to compromising the visual appearance of the artworks, produce substances that cause mechanical damage to the artifact, altering its porosity and its conservation state [3]. This complex process is known as

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biodeterioration, an increasingly problematic threat, especially for artworks and artifacts exposed to the outdoor environment.

Mitigation of biodeterioration on surfaces is typically accomplished through a variety of mechanical, physical, chemical and biological techniques [4]. In general, cleaning procedures involve processes designed to remove or reduce biodegraded layers that accumulate on artworks over time. Currently, the predominant approach to cleaning surfaces subject to biological colonization involves mainly the application of chemical products, which have biocidal action [5]. This is typically followed by a cleaning procedure aimed at mechanically removing any residue from the biodegraded layer. In the context of cultural heritage conservation, specific requirements are requested to the biocides, including the efficacy at low concentrations against target organisms, absence of interference with the artwork's materials and minimal risks to human health and the environment [6-8]. Among used biocides, quaternary ammonium salts, such as PREVENTOL RI50, are commonly used by conservators [9]. However, such biocides pose significant risks to human health, environment and treated materials [10, 11]. This raises concerns about the suitability of their long-term use. Despite their initial efficacy, these biocides manifest disadvantages such as poor durability, lack of specificity against microorganisms and possible promotion of biocide-resistant microbial communities [12, 13]. As a result, research in conservation and restoration is shifting toward more eco-friendly and safe biocide alternatives [14], focusing on plant extracts, essential oils and green solvents [15-17]. Plant extracts and essential oils, although promising, may sometimes lack the efficacy and durability necessary for long-term application to outdoor-exposed artifacts [18].

Recent years have attested Ionic Liquids (ILs) as biocidal solvents aimed at removing and/or preventing the formation of biological patinas on the surface of materials of cultural interest [19]. ILs, belonging to the "green" solvents category, have been employed as an alternative to traditional solvents because of their non-toxic properties, low volatility, antimicrobial and antifungal activity [20]. Numerous research has documented the efficacy of various ILs in counteracting biodeterioration in different contexts [21]. In the context of cultural heritage conservation, ILs have found use as biocidal agents, but now Deep Eutectic Solvents (DESs), known as the "new generation of ILs", are emerging as a potential alternative [22]. Unlike ILs, which consist of anionic and cationic components of synthetic origin, obtained through complex and expensive procedures, DESs are mixtures of Hydrogen Bond Acceptor (HBA) and Hydrogen Bond Donor (HBD), obtained through simpler and cheaper synthetic procedures, are biodegradable, are no volatile and have lower toxicity [23]. DESs have emerged as a class of green solvents with unique chemical and physical characteristics, offering significant potential for various applications [24, 25]. DESs are defined as mixtures of two or more compounds (HBA and HBD) [26]. This specific combination, in a given stoichiometric ratio, results in a substantial decrease in the melting point of the system compared to the individual compounds. The reduction in melting point is mainly attributed to the establishment of hydrogen bonding interactions between the compounds [27, 28]. Typically, existing in a liquid state at room temperature, DESs can be used as versatile solvents for the above-mentioned attractive properties, including 100% atom economy, nonflammability, chemical and thermal stability and water compatibility. The discovery of this new generation of green solvents, DESs, which offers numerous advantages over ILs and demonstrates biocidal properties against bacteria and fungi [29, 30], could represent a new product for the treatment of biodegraded artworks. These advantages stimulate the idea of testing DESs in the cleaning and preservation of monuments and artworks [31-34], promoting the adoption of harmless and eco-

friendly methodologies to counter the biodeterioration of stone artifacts exposed to the outdoor environment.

The focus of the study is to test directly in situ DESs reported in the literature for antibacterial and antifungal properties and to evaluate their long-term efficacy. The study involves the practical application of ChCl-based DESs, namely Choline Chloride/Urea (ChCl/U), ChCl/Glycerol (ChCl/Gly), ChCl/Ethylene Glycol (ChCl/EG), ChCl/Malonic Acid (ChCl/MalAc), ChCl/Oxalic Acid (ChCl/OxAc) and ChCl/Zinc Chloride ($ChCl/ZnCl_2$). The evaluation of efficacy was conducted through macroscopic and microscopic observations, spectrocolorimetric analysis, measurement of cell viability based on detection of ATP and FTIR analyses. DES ChCl/U was tested against bacterial strains such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus cereus* and two yeast strains, *Candida albicans* [35]. ChCl/Gly was investigated for its efficacy against *S. aureus* and *P. aeruginosa* biofilms, which was confirmed by antibiofilm testing [36]. In addition, ChCl/Gly-based ionic dough demonstrated high antibacterial action against *E. coli* and *S. aureus* [37]. ChCl/EG was used as a solvent to extract bioactive compounds and DES-extracts demonstrate antibacterial and antifungal activity against various pathogens (*S. aureus, E. coli, F. solani, A. niger*) [38]. DES ChCl/MalAc and ChCl/OxAc have also shown good antimicrobial action against *Listeria monocytogenes, S. aureus, E. coli* and *Salmonella enteritidis* [39]. Further research has examined the antibacterial activity of ChCl/MalAc against *Enterococcus faelicas, S. aureus, E. coli, P. aeruginosa* and *Candida Albicans* [40]. Other studies evaluated the antibacterial potential of DES ChCl/OxAc against *E. coli, S. aureus, B. subtilis, S. Typhi* and *K. pneumonia* to understand its ability to inhibit bacterial growth in fish samples [41]. Finally, $ChCl/ZnCl_2$ showed a toxic effect on various fungi such as *Phanerochaete chrysosporium, Aspergillus niger, Lentinus tigrinus* and *Candida cylindracea* [42].

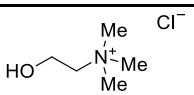
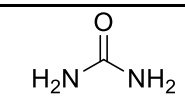
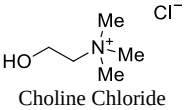
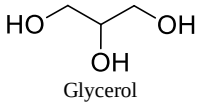
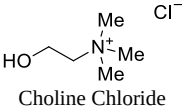
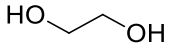
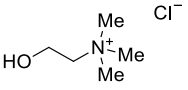
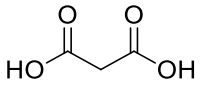
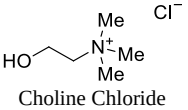
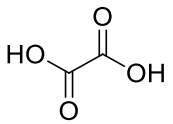
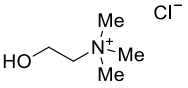
Experimental part

Materials

For the preparation of DESs, the components Choline Chloride (ChCl), Urea, Glycerol, Ethylene Glycol, Malonic Acid, Oxalic Acid and Zinc Chloride were purchased from Sigma Aldrich (Merck KGaA, Darmstadt, Germany). DESs were synthesized using the HBA and HBD components in precise molar ratios. The above components were loaded into a reaction flask, mixed and, heated at temperatures between 80 and 100°C for a period ranging from 1 to 3 hours to obtain homogeneous liquids. DESs were subjected to comparative testing with two kinds of reference biocides: an inorganic biocide, $ZnCl_2$ and an organic biocide, a quaternary ammonium salt. A saturated solution of $ZnCl_2$, a known biocidal agent [43, 44], was prepared. The quaternary ammonium salt selected for comparison was chosen with a medium hydrophobic chain (AUTEAB) and a long hydrophobic chain (PREVENTOL RI50). PREVENTOL RI50 (CTS), a biocide widely used in restoration treatments, was used by dilution with 3% deionized water while the AUTEAB compound, acryloyloxyundecyltriethylammonium bromide [45], provided by UNICAL, was utilized as a 5% solution in deionized water.

Table 1 reported the chemical structures of the DESs. DES 6 ($ChCl/ZnCl_2$), owing to its high viscosity, was added to 7% deionized water for application [46].

Table 1. Composition of DESs tested in this experiment.

DESs name	Composition	Chemical structure	Molar ratio
DES 1	ChCl/U	 Choline Chloride  Urea	1:2
DES 2	ChCl/Gly	 Choline Chloride  Glycerol	1:2
DES 3	ChCl/EG	 Choline Chloride  Ethylene Glycol	1:2
DES 4	ChCl/MalAc	 Choline Chloride  Malonic Acid	1:1
DES 5	ChCl/OxAc	 Choline Chloride  Oxalic Acid	1:1
DES 6	ChCl/$ZnCl_2$	 Choline Chloride $ZnCl_2$	1:2

Methods

All products were applied to an outdoor environment on a magmatic effusive rock exposed in the geological garden of the University of Calabria, exhibiting a homogeneous biological black patina, due principally to the colonization of dematiaceous Hyphomycetes and lichens.

The rock, originating from Lipari in the Aeolian Islands (Messina), consists of andesitic lava flows containing cordierite.

This type of rock was widely used in the past in the cultural heritage field. In particular in 2008 during preliminary excavations preceding the construction of a new pier of the Island at Marina Lunga, near Sottomonastero archaeological remains were casually discovered [47]. Subsequently were discovered a large submerged architectural structure considered a monumental edifice of an old coastal installation [48, 49].

The selected area was divided into ten zones, as reported in Figure 1. DES 1 to 6 were used in the first six zones; subsequently, an untreated section was dedicated (U.A.); then, for comparison purposes, a saturated solution of $ZnCl_2$, AUTEAB and PREVENTOL RI50 were applied.

The experimentation was carried out in December, applying 2 ml of several biocides with a brush in thin layers. During this period, typical microclimatic conditions include temperatures averaging around 8 to 15°C during the day and dropping to approximately 3-10°C at night. They were left to act for 5 days, then removed with a mechanical action by brushing and washing with deionized water. The biocides were reapplied following the same procedure.

Fig. 1. Selected area of the magmatic effusive rock at the University of Calabria.
The zones provided for the treatment with DESs (code DES 1 – 6),
U.A. = untreated area, $ZnCl_2$ = saturated solution of $ZnCl_2$, AUTEAB, PREVENTOL RI50

After application and removal after 5 days, biocidal efficacy was monitored after 3 months and after 6 months to observe the regrowth of the biological patina over time, using the following analytical techniques:

Camera in visible light

Macroscopic observations were collected using an EOS M50 camera in visible light, using colour control to systematize images according to light. Before any biocide treatment, the rock surface had a characteristic uniform biological patina with a predominantly black appearance. In addition, after biocide treatment, the cleaning process was documented to elucidate the effectiveness of the applied biocides in mitigating the formation of the biologic patina in the long term.

Optical microscopy

Portable optical microscopy, dino-lite AM4113T-FVW (50 and 200x magnifications), was employed to monitor the alterations induced on the surface by the tested formulations. The data collected using the microscope facilitated the assessment of microbial contamination levels, changes in microbial consortium morphology and the impact of different treatments over time. Digital microscopy analysis was conducted at 50x magnification.

Spectrocolorimeter

Spectrocolorimetry (3Nh model Y3060), to evaluate the colour of the surface after the biocide cleaning treatment. Through the use of CIE-Lab chromatic space, the L* coordinate, indicating lightness, was evaluated. The a* and b* coordinates were not considered, as we started from a heterogeneous micro- and macro- biological consortium of dark hues and then arrived at the stone exposure, which, although light, maintained heterogeneity in chromaticity. Therefore, the analysis focused exclusively on the alteration of brightness.

Bioluminometer

Bioluminometry conducted with KAIROSafe PD30. The wipe method was used and the tests were repeated three times. The analysis is based on the principle of bioluminescence, which emphasizes the ability of living organisms to emit light through enzymatic reactions that

convert chemical energy into light energy. In particular, this is facilitated by the interaction between the enzyme luciferase, the substrate luciferin and the adenosine triphosphate (ATP) molecule. Therefore, the amount of light emitted is directly proportional to the ATP content in the sample, quantified as relative light units (RLU). A significant decrease in emitted light indicates a reduction in biological activity and, consequently, a greater effectiveness of the biocidal action of the treatment.

FTIR

This analysis was performed to assess the presence of tested biocides 6 months post-application, using Thermo Scientific Summit Pro. This enabled the evaluation of the persistence of biocidal activity on the treated surface over a long period, considering that DESs are nonvolatile formulations. Micro-rock samples were taken from each treated zone and then analyzed by FTIR in ATR mode.

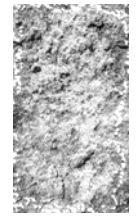
Results and discussion

Macroscopic observations

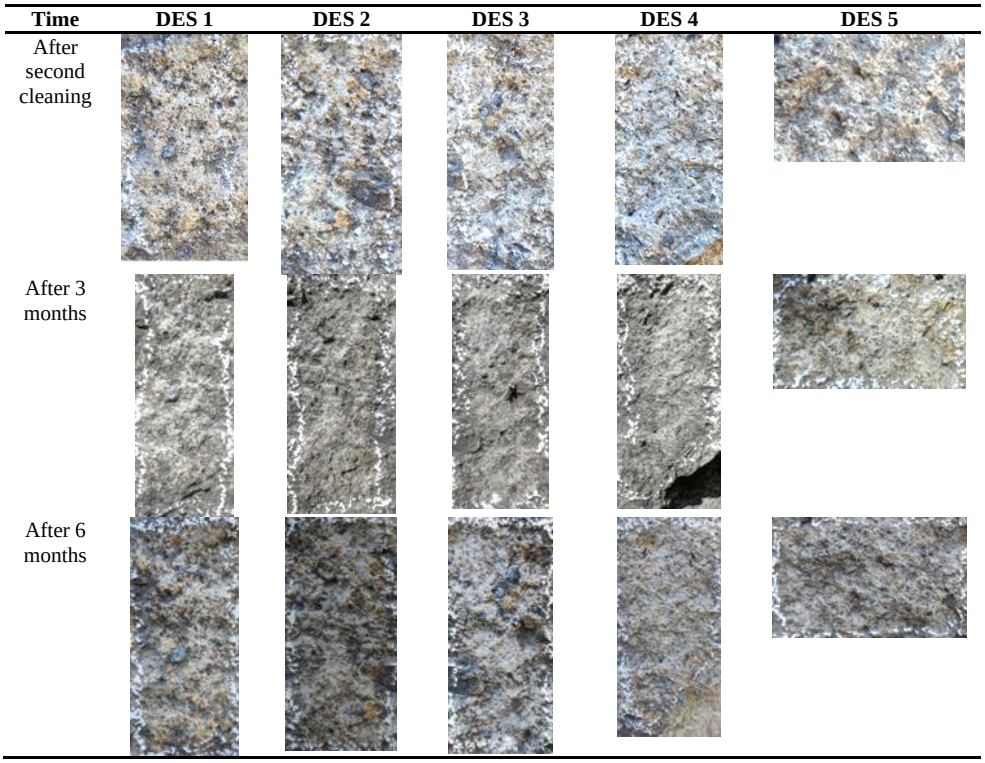
Table 2 reports the images that macroscopically document the visual changes observed on the rock surface during the experimental period. Before biocide treatment, the rock surface exhibited a uniform and dark biological patina. Following the application of DESs (DESs 1 to 6), a saturated solution of $ZnCl_2$, AUTEAB and PREVENTOL RI50, biocidal actions became evident. The first cleaning gave promising results, with macroscopic observations showing a return to the original appearance of the stone surface, without the presence of the biological patina. This success could be attributed to the action of the applied treatments, which proved to be effective in removing the biological patina. After the second cleaning, a similar surface appearance was achieved for all treated zones. During observations, greater biocidal efficacy was noted among the different treated zones, evidenced by a macroscopical cleaner zone. Comparative analysis indicates biocidal action in the treated zones following this descending order: PREVENTOL RI50, DES 5, DES 1, DES 4, AUTEAB, $ZnCl_2$, DES 2, DES3 and DES 6. After 3 months, a slight increase in microbial growth was observed, which became more pronounced after 6 months. This result is attributed to the possible formulations' inability to inhibit the vitality of the spores [4]. From the data collected, it can be seen that in different treated zones, the most promising results at 6 months emerged with the use of DES 5 and DES 4, similar to PREVENTOL RI50 and showed lower activity DES 1, followed by AUTEAB. DES 3 and $ZnCl_2$ showed lower performance, while green-yellow growths were observed in treated zones, especially with DES 2 and DES 6.

Table 2. Macroscopic observations related to DES 1 until DES 6, $ZnCl_2$, AUTEAB, PREVENTOL RI50 collected at different times. The white markings in the images correspond to the chalk used to delineate zones for analysis

Time	DES 1	DES 2	DES 3	DES 4	DES 5
0 time					

Time	DES 1	DES 2	DES 3	DES 4	DES 5
After first cleaning					
After second cleaning					
After 3 months					
After 6 months					

Time	DES 6	U. A.	$ZnCl_2$	AUTEAB	PREVENTOL RI50
0 time					
After first cleaning					



Optical microscopy

Table 3 reported the microscopic observations for each treated zone. With the removal of the biodegraded layer from the rock, the original appearance re-emerged, showing a yellow stone substrate. After the first treatment, biological consortia retain their presence, although they show a noticeable change in appearance, with a clear shift toward brownish tones, suggesting the devitalization of the photosynthetic consortium. After the second treatment, the biological patina was completely removed from the rock substrate. After 3 months, under an optical microscope, a slight increase in microbial growth is observed in the treated zone with DES 1, which becomes significant within 6 months. The treated zone with DES 2 shows a biological consortium with different characteristics than the pre-existing one as early as the 3rd month after treatment (Fig. 2). The treated zone with DES 3 shows particularly interesting biological growth after the 3rd month. DES 4 follows a similar trend to DES 1. After 3 months of treatment, the treated zone with DES 5 shows biological growth. Finally, DES 6 manifests immediate microbial growth that becomes significantly more pronounced after 6 months (Fig. 3), being similar to that observed for DES 2. Whereas the treated zone with $ZnCl_2$ shows the immediate presence of biodeteriogens, with less biocidal action than DES 6. The growth of white-colored biological material is evident. The treated zone with AUTEAB shows a general presence of biological growth after 3 months. Lastly, the treated zone with PREVENTOL RI50 has an obvious biological consortium at 6 months after treatment. In summary, DES 1 and DES 4 have comparable efficacy, similar to PREVENTOL RI50; DES 5 has less pronounced efficacy, followed by AUTEAB, DES 6 and $ZnCl_2$; DES 3 and DES 2 show lower efficacy (DES 1 = DES 4 = PREVENTOL RI50 > DES 5 > AUTEAB > DES 6 > $ZnCl_2$ > DES 3 = DES 2). In general, microbial growth is observed 6 months after treatment.

The observation of the selective regrowth of certain microorganisms in the treated zones with DES 2 and DES 6 (Figs. 2 and 3) can be attributed both to the cleaning process adopted,

which might prefer the proliferation of some organisms over others and to the limited biological patina formation time, set at 6 months.

Table 3. Observation in optical microscopy

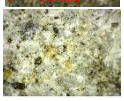
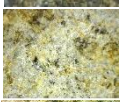
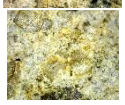
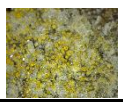
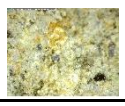
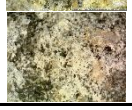
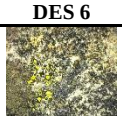
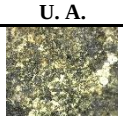
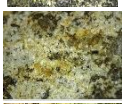
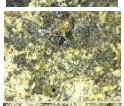
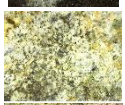
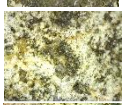
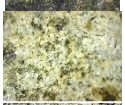
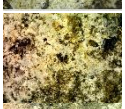
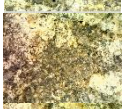
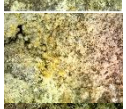
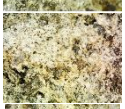
Time	DES 1	DES 2	DES 3	DES 4	DES 5
0 time					
After first cleaning					
After second cleaning					
After 3 months					
After 6 months					
Time	**DES 6**	**U. A.**	**$ZnCl_2$**	**AUTEAB**	**PREVENTOL RI50**
0 time					
After first cleaning					
After second cleaning					
After 3 months					
After 6 months					

Fig. 2. Treated zone with DES 2. Comparison of the biological consortium before treatment and 3 months after treatment

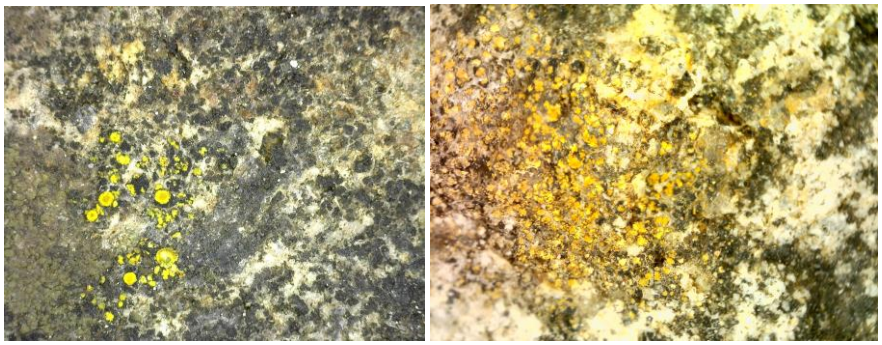


Fig. 3. Treated zone with DES 6. Comparison of the biological consortium before treatment and 6 months after treatment

Microscopically, the observed appearance suggests a possible reappearance of yellow lichens of the genus Xanthoria.

Spectrocolorimetry

Monitoring of biological patina regrowth was conducted by evaluating the L* coordination values of the treated zones at different experimental stages, shown in figure 4. The untreated rock consistently maintained an L* value between 14.10 and 20.90, corresponding to the dark tones of the biodegraded surface.

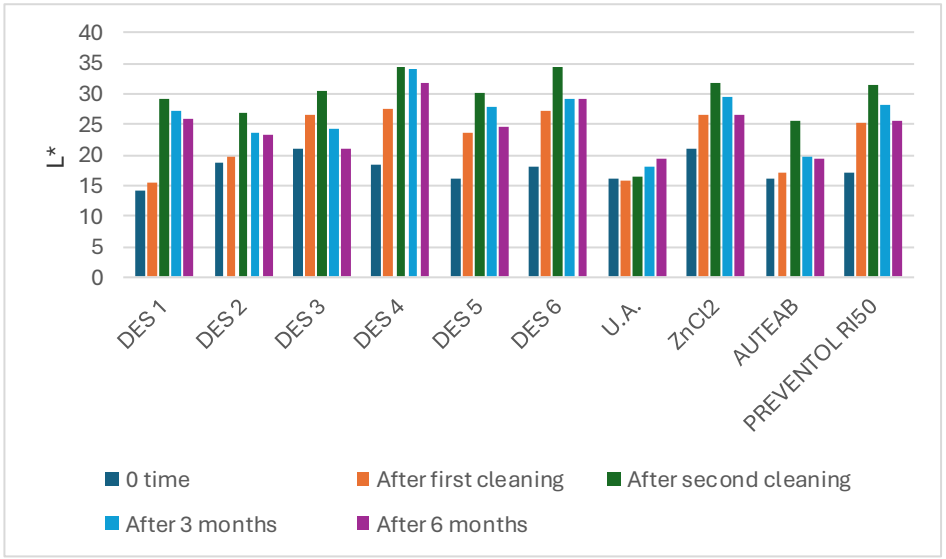


Fig. 4. L* coordinate values of the zones treated first during different phases of the experiment. Standard deviation 0.5 < SD < 3.2

The results show a clear divergence in colour changes between the treated and untreated zones, with the L* value effectively reflecting changes in the appearance of the rock surface. After the first treatment, there is a significant increase in the L* value in the treated zones with DES 4, DES 5, DES 6 and PREVENTOL RI50, indicative of a clear improvement in the colour appearance of the treated surfaces. In contrast, no clear increase in L* value is observed in

treated zones with DES 3, $ZnCl_2$, DES 1, DES 2 and AUTEAB, suggesting the presence of darker tones. This could be attributed to the lack of biocidal efficacy of the products used in removing the biological patina. Whereas, after the second treatment, for treated zones with DES 1, DES 3, DES 4, DES 5, DES 6, PREVENTOL RI50 and $ZnCl_2$, the L* value shows convergence toward that of clean rock (L* = 31.1 ± 2.5), suggesting effective removal of the biological patina and initial restoration of the original surface. After 3 months, there is an initial reappearance of biological consortium on the rock surface, evidenced by decreasing L* values, indicative of darker shades, especially in treated zones with DES 6, DES 3 and AUTEAB. However, for treated zones with DES 4, DES 1, DES 5, $ZnCl_2$, DES 2 and PREVENTOL RI50, the change in L* values after 3 months are minimal. After 6 months, a decrease in L* value was confirmed for some DESs, particularly DES 1, DES 3, DES 4 and DES 5, similar to what was observed for $ZnCl_2$ and PREVENTOL RI50.

Bioluminometry

Table 4 shows the bioluminometric values, which indicate the amount of adenosine triphosphate (ATP) present in each of the experimental zones. Initially, high ATP values in each zone indicate a significant presence of living organisms. After the first cleaning, a significant decrease in ATP values is observed, especially in the treated zones with PREVENTOL RI50 and DES 4, 1 and 5, showing an effective reduction of living microorganisms. The second cleaning leads to a more pronounced biocidal action, as indicated by the lower bioluminometric values. However, in follow-ups at 3 and 6 months after treatment, a gradual increase in bioluminescence values is observed, suggesting regrowth of the microbial consortium and potential recolonization of treated surfaces. After 6 months, treated zones with PREVENTOL RI50 and DES 5 show long-term efficacy and mentioned treated zones with DES 2 and 6 show significant microbiological growth. These results confirm the biocidal efficacy trends observed macroscopically and microscopically. DES 5 emerges as the most effective, followed by DES 4 and 1 compared to PREVENTOL RI50. Furthermore, a reduction in biofilm viability is noted even after a simple cleaning treatment with deionized water and mechanic action, but complete devitalization of the microbial consortium on the stone requires a second cleaning treatment.

Table 4. Bioluminometric values of ATP for each zone. SD < 520

Time	DES 1	DES 2	DES 3	DES 4	DES 5	DES 6	U. A.	$ZnCl_2$	AUTEAB	PREVENTOL RI50
0 time	67209	91289	83698	80732	78341	80924	88115	79249	67312	78225
After first cleaning	3756	4189	4230	3692	3804	4003	33192	4754	4421	2932
After second cleaning	496	646	559	511	413	731	33512	682	481	431
After 3 months	4753	9294	9095	3442	2852	9931	32135	10293	7625	2995
After 6 months	7542	21543	19739	7127	4981	22934	38493	19971	14421	5002

FTIR analysis

The results of the FTIR analysis are presented in figure 5, which displays the super-imposed spectra of rock samples taken from each treated zone, except DES 2, which exhibited no biocidal action. The peaks detected around 997cm^{-1} were attributed to the rock substrate itself. Regarding the DES 1-treated zone, the characteristic vibrational bands of DES 1 ChCl/U

1:2 are observable, which include O-H stretching of a hydroxyl group, N-H stretching of an amine group, NH_2 stretching, within the range of 3250-3500cm^{-1}, C-H bending vibrations of an alkyl group at 1476cm^{-1}, C=O stretching of the amide at 1660cm^{-1} and N-H deformation vibrations at 1621cm^{-1}. The spectrum of the treated zone with DES 3 does not exhibit peaks corresponding to the presence of DES. In the treated zone with DES 4 ChCl/MalAc, vibrational bands are observed around 3250-3500cm^{-1}, corresponding to the O-H stretching of the hydroxyl group, at 1717cm^{-1} corresponding to the C=O stretching of the carbonyl group, 1477cm^{-1} corresponding to the bending vibrations of an alkyl group and 1375cm^{-1} corresponding to CH_3 bending absorption.

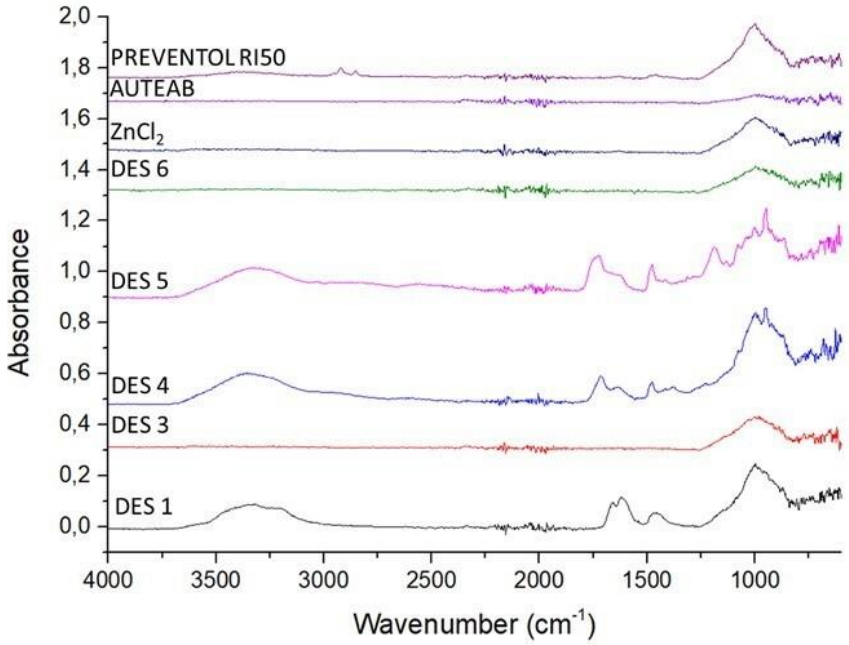


Fig. 5. Overlaying of FTIR spectra of rock samples from each treated zone

The treated zone with DES ChCl/OxAc exhibits a peak around 3250-3500cm^{-1}, corresponding to the vibrational absorption of an O-H group, at 1723cm^{-1} for the C=O of a carbonyl group and at 1477cm^{-1} for C-H bending vibrations of an alkyl group. These findings are consistent with literature reports [50, 51]. Conversely, treated zones with DES 6, $ZnCl_2$ and AUTEAB did not exhibit characteristic peaks confirming their presence. The treated zone with PREVENTOL RI50 exhibits peaks at 2921 and 2852cm^{-1}, typically associated with the asymmetric and symmetric stretching vibrations of alkyl C-H bonds, respectively and a peak at 1466 cm^{-1} attributed to the bending vibrations of alkyl C-H bonds. The presence of DES residues in some treated zones explains the persistence of their biocidal action 6 months after treatment, resulting in reduced microbial growth compared to other treated zones. The analyses conducted indicate that treated zones with DES 1, DES 4, DES 5 and PREVENTOL RI50 remained cleaner, with lower microbial consortia, for an extended duration due to their persistence.

Conclusions

DESs based on Choline Chloride combined with Urea, Glycerol, Ethylene Glycol, Malonic Acid, Oxalic Acid and $ZnCl_2$ were tested as biocides for application in cultural heritage, comparing their efficacy with known biocides such as $ZnCl_2$, AUTEAB and PREVENTOL RI50. Biocidal efficacy was evaluated by applying DESs directly in situ to an effusive magmatic rock with a biodegraded surface.

The results define that some DESs have biocidal efficacy comparable to the conventional biocide PREVENTOL RI50, with persistent action for up to 6 months after treatment. In particular, DES 5 (ChCl/OxAc), DES 4 (ChCl/MalAc) and DES 1 (ChCl/U) have shown significant efficacy in reducing microbial growth in the long term. This efficacy has been evaluated both macroscopically and microscopically and also by spectrocolorimetric, bioluminometric and FTIR spectroscopic analyses. The antimicrobial activity previously documented in the literature for some DESs was confirmed through the tests performed in this study. The results suggest that biocidal efficacy could result from the interaction of the individual constituent components of DESs with the interface of the biodegraded layer, potentially establishing weak bonds with the biofilm. Cations derived from Choline Chloride can interact with functional groups present on the bacterial cell membrane through hydrogen bonds or electrostatic forces. This interaction can cause destabilization of the cell membrane, resulting in structural and functional damage that hinders bacterial growth and survival. DESs consisting of Oxalic Acid (DES 5) and Malonic Acid (DES 4) show enhanced antibacterial activity because they induce an acidic environment within the DES itself. The low pH causes denaturation of the proteins that make up bacterial cell membranes, making the membrane surface less stable and promoting loss of structural integrity. These interactions could contribute to the ability of DESs to counteract microbial growth and preserve materials.

The use of DESs as biocidal agents in the cleaning and conservation of cultural heritage artworks represents an innovative and sustainable strategy for restorers. Their easy and inexpensive synthesis, combined with their inherent properties of safety and low toxicity, make them ideal for application to stone materials, even for extended periods. DESs were applied twice and the biological patina was removed mechanically. The biocide-treated zones were monitored for 6 months, demonstrating how these solutions provide effective protection against biological attack over time. The main result obtained from the study is the development of a sustainable approach to cleaning and preserving stone materials, avoiding the use of potentially harmful chemicals. In addition, the protective and persistent action of DESs effectively counteracts biological growth, thus contributing to the long-term preservation of stone materials. These advantages indicate considerable potential for the use of DESs as "green" biocidal solvents in the restoration of stone materials exposed to the outdoor environment.

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References

[1] C. Alisi, *Biodegradation and biorestoration of stone manufacts*, **Letture Di Georisorse e Ambiente**, **3**, 2011, pp. 94–109.

[2] A. Negi, I.P. Sarethy, *Microbial Biodeterioration of Cultural Heritage: Events, Colonization, and Analyses*, **Microbial Ecology**, **78**, 2019, pp. 1014–1029.

[3] T. Branysova, K. Demnerova, M. Durovic, H. Stiborova, *Microbial biodeterioration of cultural heritage and identification of the active agents over the last two decades*, **Journal of Cultural Heritage**, **55**, 2022, pp. 245–260. https://doi.org/10.1016/j.culher.2022.03.013.

[4] F. Cappitelli, C. Cattò, F. Villa, *The control of cultural heritage microbial deterioration*, **Microorganisms**, **8**, 2020, Artcile Number 1542. https://doi.org/10.3390/microorganisms8101542.

[5] M. A. Kakakhel, F. Wu, J.-D. Gu, H. Feng, K. Shah, W. Wang, *Controlling biodeterioration of cultural heritage objects with biocides: A review*, **International Biodeterioration & Biodegradation**, **143**, 2019, Article Number 104721. https://doi.org/10.1016/j.ibiod.2019.104721.

[6] C. Urzì, F. De Leo, L. Krakova, D. Pangallo, L. Bruno, *Effects of biocide treatments on the biofilm community in Domitilla's catacombs in Rome*, **Science of The Total Environment**, **572**, 2016, pp. 252–262. https://doi.org/10.1016/j.scitotenv.2016.07.195.

[7] D. Pinna, *Can we do without biocides to cope with biofilms and lichens on stone heritage?* **International Biodeterioration & Biodegradation**, **172**, 2022, Article Number 105437. https://doi.org/10.1016/j.ibiod.2022.105437.

[8] A. Unger, *Decontamination and 'deconsolidation' of historical wood preservatives and wood consolidants in cultural heritage*, **Journal of Cultural Heritage**, **13S**, 2012, pp. 196–202. https://doi.org/10.1016/j.culher.2012.01.015.

[9] M. Romani, T. Warscheid, L. Nicole, L. Marcon, P. Di Martino, M. T. Suzuki, P. Lebaron, R. Lami, *Current and future chemical treatments to fight biodeterioration of outdoor building materials and associated biofilms: Moving away from ecotoxic and towards efficient, sustainable solutions*, **Science of the Total Environment**, **802,** 2021, Article Number 149846. https://doi.org/10.1016/j.scitotenv.2021.149846.

[10] C. Zhang, F. Cui, G.M. Zeng, M. Jiang, Z.Z. Yang, Z.G. Yu, M.Y. Zhu, L.Q. Shen, *Quaternary ammonium compounds (QACs): A review on occurrence, fate and toxicity in the environment*, **Science of the Total Environment**, **518-519**, 2015, pp. 352-362. https://doi.org/10.1016/j.scitotenv.2015.03.007.

[11] T.C. Hrubec, R.P. Seguin, L. Xu, G.A. Cortopassi, S. Datta, A.L. Hanlon, A.J. Lozano, V.A. McDonald, C.A. Healy, T.C. Anderson, N.A. Musse, R.T. Williams, *Altered toxicological endpoints in humans from common quaternary ammonium compound disinfectant exposure*, **Toxicology Reports**, **8**, 2021, pp. 646-656. DOI: 10.1016/j.toxrep.2021.03.006.

[12] C. Genova, E. Fuentes, P. Sanmartín, G. Favero, B. Prieto, *Phytochemical compounds as cleaning agents on granite colonized by phototrophic subaerial biofilms*, **Coatings**, **10**, 2020, Article Number 295. https://doi.org/10.3390/coatings10030295.

[13] I. Mulder, J. Siemens, V. Sentek, W. Amelung, K. Smalla, S. Jechalke, *Quaternary ammonium compounds in soil: implications for antibiotic resistance development*, **Reviews in Environmental Science and Biotechnology**, **17**, 2018, pp. 159–185.

[14] F. Di Turo. L. Medeghini, *How green possibilities can help in a future sustainable conservation of cultural heritage in Europe*, **Sustainability**, **13**, 2021, Article Number 3609. https://doi.org/10.3390/su13073609.

[15] R. Russo, F. Palla, *Plant Essential Oils as Biocides in Sustainable Strategies for the Conservation of Cultural Heritage*, **Sustainability**, **15**, 2023, Article Number 8522. https://doi.org/10.3390/su15118522.

[16] D. Isola, F. Bartoli, A. Casanova Municchia, H.J. Lee, S.H. Jeong, Y.J. Chung, G. Caneva, *Green biocides for the conservation of hypogeal mural paintings raised from Western and Eastern traditions: Evaluation of interference on pigments and substrata and multifactor parameters affecting their activity*, **Journal of Cultural Heritage**, **61**, 2023, pp. 116–126. https://doi.org/10.1016/j.culher.2023.03.009.

[17] Q. Li, Y. Hu, B. Zhang, *Phosphonium-based ionic liquids as antifungal agents for conservation of heritage sandstone*, **RSC Advances**, **12**, 2022, pp. 1922–1931.

[18] S. Macedo-Arantes, A. Piçarra, A.T. Caldeira, A.E. Candeias, M.R. Martins, *Essential oils of Portuguese flavouring plants: potential as green biocides in cultural heritage*, **European Physical Journal Plus**, **136**(11), 2021, Article Number: DOI:10.1140/epjp/s13360-021-02018-2.

[19] Lo Schiavo, F. De Leo, C. Urzì, *Present and future perspectives for biocides and antifouling products for stone-built cultural heritage: Ionic liquids as a challenging alternative*, **Applied Sciences**, **10**, 2020, Article Number 6568. https://doi.org/10.3390/app10186568.

[20] R. Sharma, B. Verma, S. Kumar, A. Gupta, P. Kumar Sahu, P. Singh, V. Kumar, *Recent updates on applications of ionic liquids (ILs) for biomedical sciences*, **Journal of the Iranian Chemical Society**, **19**, 2022, pp. 3215–3228.

[21] Q. Li, Y. Hu, B. Zhang, *Polyoxometalate-Ionic Liquids (ILs) and Polyvinyl Alcohol/Chitosan/ILs Hydrogels for Inhibiting Bacteria Colonising Wall Paintings*, **Carbohydrate Polymers**, **256**, 2021, Article Number 117592. https://doi.org/10.1016/j.carbpol.2020.117592.

[22] E.L. Smith, A.P. Abbott, K.S. Ryder, *Deep Eutectic Solvents (DESs) and Their Applications*, **Chemical Reviews Journal**, **114**, 2014, pp. 11060–11082.

[23] J. Płotka-Wasylka, M. De La Guardia, V. Andruch, and M. Vilková, *Deep eutectic solvents vs ionic liquids: Similarities and differences*, **Microchemical Journal**, **159**, 2020, Article Number 105539. https://doi.org/10.1016/j.microc.2020.105539.

[24] T.R. Sekharan, R.M. Chandira, S. Tamilvanan, S.C. Rajesh, B.S. Venkateswarlu, *Deep eutectic solvents as an alternate to other harmful solvents*, **Biointerface Research in Applied Chemistry**, **12**, 2022, pp. 847–860.

[25] K.A. Omar, R. Sadeghi, *Physicochemical properties of deep eutectic solvents: A review*, **Journal of Molecular Liquids**, **360**, 2022, Article Number 119524. DOI:10.1016/j.molliq.2022.119524.

[26] I. Bashir, A. Hussain Dar, K. Kumar Dash, V. Kumar Pandey, U. Fayaz, R. Shams, S. Srivastava, R. Singh, *Deep eutectic solvents for extraction of functional components from plant-based products: A promising approach*, **Sustainable Chemistry and Pharmacy**, **33**, 2023, Article Number 101102. https://doi.org/10.1016/j.scp.2023.101102.

[27] T. El Achkar, H. Greige-Gerges, S. Fourmentin, *Basics and properties of deep eutectic solvents: A review*, **Environmental Chemistry Letters**, **19**, 2021, pp. 3397–3408. DOI:10.1007/s10311-021-01225-8.

[28] Z. Pan, Y. Bo, Y. Liang, B. Lu, J. Zhan, J. Zhang, J. Zhang, *Intermolecular interactions in natural deep eutectic solvents and their effects on the ultrasound-assisted extraction of artemisinin from Artemisia annua*, **Journal of Molecular Liquids**, **326**, 2021, Article Number 115283. https://doi.org/10.1016/j.molliq.2021.115283.

[29] F.S.N. de Oliveira A.R.C. Duarte, *A look on target-specificity of eutectic systems based on natural bioactive compounds*, **Advances in Botanical Research**, **97**, 2021, pp. 271–307.

[30] N. Akbar, N. A. Khan, T. Ibrahim, M. Khamis, A.S. Khan, A.M. Alharbi, H. Alfahemi, R. Siddiqui, *Antimicrobial Activity of Novel Deep Eutectic Solvents*, **Scientia Pharmaceutica**, **91**, 2023, Article Number 9. https://doi.org/10.3390/scipharm91010009.

[31] Y. Jia, G. Sciutto, A. Botteon, C. Conti, M.L. Focarete, C. Gualandi, C. Samorì, S. Prati, R. Mazzeo, *Deep eutectic solvent and agar: A new green gel to remove proteinaceous-based varnishes from paintings*, **Journal of Cultural Heritage**, **51**, 2021, pp. 138-144. https://doi.org/10.1016/j.culher.2021.08.001.

[32] S. Valente, F. Oliveira, I.J. Ferreira, A. Paiva, R.G. Sobral, M.S. Diniz, S.P. Gaudêncio, A.R.C. Duarte, *Hydrophobic DES Based on Menthol and Natural Organic Acids for Use in Antifouling Marine Coatings*, **ACS Sustainable Chemistry & Engineering Journal, 11**(27), 2023, pp. 9989-10000.

[33] C. Biribicchi, A. Macchia, G. Favero, R. Strangis, B. Gabriele, R. Mancuso, M.F.L. Russa, *Sustainable solutions for removing aged wax-based coatings from cultural heritage: exploiting hydrophobic deep eutectic solvents (DESs)*, **New Journal of Chemistry**, **47**, 2023, pp. 5991–6000.

[34] A. Macchia, R. Strangis, S. De Angelis, M. Cersosimo, A. Docci, M. Ricca, B. Gabriele, R. Mancuso, M.F.L. Russa, *Deep Eutectic Solvents (DESs): Preliminary Results for Their Use Such as Biocides in the Building Cultural Heritage*, **Materials**, **15**, 2022, Article Number 4005. https://doi.org/10.3390/ma15114005.

[35] M. Ivanović, D. Grujić, J. Cerar, M. Islamčević Razboršek, L. Topalić-Trivunović, A. Savić, D. Kočar, M. Kolar, *Extraction of Bioactive Metabolites from Achillea millefolium L. with Choline Chloride Based Natural Deep Eutectic Solvents: A Study of the Antioxidant and Antimicrobial Activity*, **Antioxidants**, **11**(4) 2022, Article Number 724. https://doi.org/10.3390/antiox11040724.

[36] Z. Yuan, H. Liu, W. F. Yong, Q. She, J. Esteban, *Status and advances of deep eutectic solvents for metal separation and recovery*, **Green Chemistry, 24**, 2022, pp. 1895-1929.

[37] N. Li, L. Qiu, X. Ji, W. Chen, *Engineering Ionic Dough with a Deep Eutectic Solvent: From a Traditional Dough Figurine to Flexible Electronics*, **Chemistry of Materials Journal, 35**(18), 2023, pp. 7814-7824.

[38] Z. Naseem, M.A. Hanif, M. Zahid, S. Tahir, F. Anjum, H. Bhatti, *Ultrasound-assisted deep eutectic solvent–based extraction of phytochemicals from Mentha arvensis: optimization using Box-Behnken design*, **Biomass Conversion and Biorefinery**, **12**, 2022, pp. 35–45.

[39] B. Zhao, P. Xu, F. Yang, H. Wu, M. Zong, W. Lou, *Biocompatible Deep Eutectic Solvents Based on Choline Chloride: Characterization and Application to the Extraction of Rutin from Sophora japonica*, **ACS Sustainable Chemistry & Engineering Journal, 3**(11), 2015, pp. 2746−2755.

[40] B. Alkhawaja, F. Al-Akayleh, A. Al-Khateeb, J Nasereddin, B.Y. Ghanim, A. Bolhuis, N. Jaber, M. Al-Remawi, N.A. Qinna, *Deep Eutectic Liquids as a Topical Vehicle for Tadalafil: Characterisation and Potential Wound Healing and Antimicrobial Activity*, **Molecules**, **28**(5), 2023, Article Number 2402. DOI: 10.3390/molecules28052402.

[41] R.M. Hanaphi, R. Yusof, S.N.Z. Zakaria, N. M. Saad, *A new application of deep eutectic solvent (DES) in fish preservation*, **IOP Conference Series: Materials Science and Engineering**, **864**, 2020, Article Number: 012048.

[42] M. Marchel, H. Cieśliński, G. Boczkaj, *Deep eutectic solvents microbial toxicity: Current state of art and critical evaluation of testing methods*, **Journal of Hazardous Materials**, **425**, 2022, Article Number 127963. https://doi.org/10.1016/j.jhazmat.2021.127963.

[43] M.M. Almoudi, A.S. Hussein, M.I. Abu Hassan, N. Mohamad Zain, *A systematic review on antibacterial activity of zinc against Streptococcus mutans*, **Saudi Dental Journal**, **30**(4), 2018, pp. 283–291. doi: 10.1016/j.sdentj.2018.06.003.

[44] J. Pasquet, Y. Chevalier, J. Pelletier, E. Couval, D. Bouvier, M.A. Bolzinger, *The contribution of zinc ions to the antimicrobial activity of zinc oxide*, **Colloids and Surfaces A: Physicochemical and Engineering Aspects, 457**, 2014, pp. 263–274. https://doi.org/10.1016/j.colsurfa.2014.05.057.

[45] R. Mancuso, R. Amuso, B. Armentano, G. Grasso, V. Rago, A.R. Cappello, F. Galiano, A. Figoli, G. De Luca, J. Hoinkis, B. Gabriele, *Synthesis and Antibacterial Activity of Polymerizable Acryloyloxyalkyltriethyl Ammonium Salts*, **Chempluschem**, **82**, 2017, pp. 1235–1244. https://doi.org/10.1002/cplu.201700194.

[46] F. Gabriele, M. Chiarini, R. Germani, M. Tiecco, N. Spreti, *Effect of water addition on choline chloride/glycol deep eutectic solvents: Characterization of their structural and physicochemical properties*, **Journal of Molecular Liquids**, **291**, 2019, Article Number 111301. DOI:10.1016/j.molliq.2019.111301.

[47] M. Di Bella, M.A. Mastelloni, F. Italiano, A. Tripodo, S. Quartieri, S. Magazù, G. Sabatino, *Archaeometric constraints on the architectural elements from the submerged installation discovered at the harbor of Lipari (Aeolian Archipelago, Italy)*, **Periodico di Mineralogia**, **89**, 2020, pp. 245-256.

[48] G. De Guidi, E. Nicotra, P. Tisseyre, S. Tusa, *Flood control at Lipari Harbour. Evidences of rapid late-Holocene submergence of the eastern coast of Lipari Island (Italy)*, **DEGUWA/IPR XX, Nuremberg - 16/22 Marz 2015**.

[49] M. Anzidei, A. Bosman, D. Casalbore, S. Tusa, R. La Rocca, *New insights on the subsidence of Lipari Island (Aeolian Islands, southern Italy) from the submerged Roman age pier at Marina Lunga*, **Quaternary International**, **401**, 2016, pp. 162- 173. https://doi.org/10.1016/j.quaint.2015.07.003.

[50] N. Delgado-Mellado, M. Larriba, P. Navarro, V. Rigual, M. Ayuso, J. García, F. Rodríguez, *Thermal stability of choline chloride deep eutectic solvents by TGA/FTIR-ATR analysis*, **Journal of Molecular Liquids**, **260**, 2018, pp. 37-43. https://doi.org/10.1016/j.molliq.2018.03.076.

[51] A.S. Bezerra de Sousa, R. Pereira Lima, M. Cruz Alves da Silva, D. das Neves Moreira, M.M. Estevez Pintado, S. de Melo Silva, *Natural deep eutectic solvent of choline chloride with oxalic or ascorbic acids as efficient starch-based film plasticizers*, **Polymer**, **259**, 2022, Article Number 125314. https://doi.org/10.1016/j.polymer.2022.125314.

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