Sampling methods for outdoor sculptures: Comparison of swabs and cryogels by flow cytometry as novel alternatives for assessment and quantification of microbial contamination

Nádia C. Silva[a], Manuela Pintado[a], Patrícia R. Moreira[a,b,*]

[a] *Universidade Católica Portuguesa, CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal*

[b] *Universidade Católica Portuguesa, CITAR – Centro de Investigação em Ciência e Tecnologia das Artes, Escola das Artes, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal*

* Corresponding author

Abstract

Flow cytometry was evaluated for the first time for the microbiological characterisation of samples collected from the surfaces of stone and mortar sculptures. The study was carried out on outdoor sculptures located in the district of Porto (Portugal), from which samples were collected using swabs and poly(2-hydroxyethylmethacrylate) (poly(HEMA)) cryogels as non-invasive sampling methods. The use of poly(HEMA) cryogels was tested as an alternative to swabbing due to the difficulties often encountered in sampling stone surfaces and retrieving microorganisms from swab fibres. The quantification of the microorganisms collected by the two sampling methods and the determination of their cell viability were performed using a combination of the fluorochromes thiazole orange (TO) and propidium iodide (PI). Both methods were effective to collect samples for analysis by flow cytometry, which proved to be a technique of interest and potential use for biodeterioration studies of cultural heritage (CH). Data visualisation of viable and non-viable microbial cells was clearer with cryogels, although higher concentrations of total microorganisms were achieved with swabs. Our results show that swab sampling is a more adequate method, although poly(HEMA) cryogels can be an option to consider for specific microbial viability analyses for biodeterioration evaluation of stone artworks.

Keywords: preventive conservation, biodeterioration, sculpture, stone, flow cytometry

1. Introduction

As the importance of the biodeterioration phenomena becomes more recognised, the need to establish the quantification and characterisation of the microbiological contamination of stone art objects and monuments, as part of conservation and restoration plans, has become more evident [1–6]. On the one hand, it is fundamental to acquire information about the composition and diversity of the microbial communities to establish the potential for biodeterioration to which the object or monument may be subjected. On the other hand, it is

important not to overlook the activity and viability of the microorganisms and gather data on their active, non-active or dormant state [7,8]. Until recently, most studies on microbial viability in CH involved measuring the concentration of fluorescein after fluorescein diacetate (FDA) hydrolysis by the enzymatic action of microorganisms or, more commonly, the detection of adenosine triphosphate (ATP) on the surfaces under investigation [7–14]. These assays, particularly those involving the detection of ATP by bioluminescence using commercial kits, are a quick, relatively cost-effective, and easy way to evaluate the microbiological contamination of various types of CH objects [7]. However, ATP measurements must be performed with caution, as the assay is more suitable for monitoring the overall surface contamination or testing the efficacy of a biocide treatment than to be used as the only method for evaluating microbial viability or for precise quantitative determinations [7,13]. This is partly because ATP assays also detect extracellular ATP, organic residues and contaminants (some of which originated from previous treatments applied to the artworks) and should not, therefore, be a direct and sole reflection of microbial viability and metabolic activity [7,15,16].

Therefore, the search for other techniques that complement the microbiological studies should be encouraged to allow for a broad view of the microbiomes in CH. The use of rapid and well-established techniques, such as flow cytometry, can be a valuable and effective tool not only to quantify the microbial communities present in the artworks but also to infer their viability and metabolic state using appropriate fluorochromes. Flow cytometry has been successfully used over the years in several fields of research, for the analysis of environmental, pharmaceutical, medical and food samples [17,18,27,28,19–26]. In the context of CH, however, its application is still limited, although some studies have used flow cytometry as a complement to other analytical tools, either in the study of the microorganisms involved in the biodeterioration processes of artworks or the evaluation of the efficiency of potential biocide treatments [29–34].

Moreover, sampling art objects for biodeterioration studies can be a challenging task, with restores, conservators and scientists seeking to extract as much information as possible while causing the least damage to the artworks [35]. This challenge is emphasised when dealing with delicate and highly valuable artworks where there are limitations to the amount or number of samples that can be collected [35]. Also, the variety of analytical techniques and methodologies that can be applied, as well as the lack of standardised procedures, add extra difficulties to the choice of the most appropriate sampling method [36].

When studying the microbiological contamination of stone sculptures, buildings and monument façades, sampling frequently involves the collection of stone fragments and flakes [37]. However, this approach is not always possible, and there are cases where it is necessary or desirable to use non-invasive techniques. The most common non-invasive or micro-invasive sampling approaches in CH include the application of scalpels, swabs, membrane filters and adhesive tapes, which have been explored in several works over the years [38–45]. Nevertheless, new sampling techniques continue to be developed to increase the range of options available for the various types of case studies. For example, de Carvalho and co-authors (2019) [46] developed a method for sampling a canvas painting, by using sterilised filter tips connected to a

pipe and smoothly vacuuming several sampling points with a small vacuum pump. Later, Piñar and co-workers (2020) [35] adapted this approach and used micro-aspiration to collect dust particles, small fibres and microorganisms from the surfaces of some of the most emblematic and famous drawings by Leonardo da Vinci.

Sampling with swabs can be a simple, low-cost and practical choice. However, depending on the type of surface to be sampled, swabbing can be difficult (particularly in rough surfaces such as stone), as is the recovery of microbial cells from swab fibres for downstream analyses. It has been reported that the use of swabs has some disadvantages, particularly due to the difficulties associated with the recovery of bacterial cells for quantification purposes and poor correlations with the microbial quantification of surfaces, possibly related to the entrapment of microbial cells in the swabs' fibres [47–49]. Some known challenges concerning surface sampling with swabs, that affect the reproducibility and repeatability of the technique, include (i) the difficulties to standardise the procedures for sample collection, such as the angle and pressure applied during sampling, (ii) the use of swab fibres made of different materials (cotton, polyester, rayon, etc.) and (iii) the multitude of wetting agents/diluents that can be used to moisten the swabs or to recover microorganisms from their fibres [48].

Alternatives to swab sampling of artworks have been investigated in the past. For example, Pinãr and co-authors (2015) [38] have demonstrated that the use of membranes, particularly those made of nylon, was a more accurate sampling method for fingerprinting and phylogenetic analyses of the microorganisms involved in the formation of foxing spots on Leonardo da Vinci´s self-portrait. It has been suggested that nylon membranes offer more accurate results than cotton swabs since swabs can overestimate the microbiome of the surfaces and do not account for the endolithic microorganisms [38]. Particularly concerning stone artwork, the epilithic microbiota, colonising the surfaces of artworks, may not always be directly involved or have a significant role in the biodeterioration of a particular historical object, since microorganisms can penetrate the pores of the stone and become endolithic to find protection from UV radiation and temperature and moisture fluctuations [50,51]. In such cases, swab sampling is unable to identify the endolithic microbiota and can bias the conclusions of the study if this issue is ignored. On the other hand, adhesive tapes can minimise these constraints and be an interesting technique for the study of the stratified microbiome of art objects, by successively peeling off the biofilm in the same sampling area to analyse the microbiome from the surface level to the deepest layers [40,52,53].

In the continuous search for non-invasive and non-destructive sampling methods for CH objects, hydrogels and cryogels could represent an alternative for sampling stone surfaces and avoiding some of the drawbacks associated with swabbing. Polymeric hydrogels are already used in CH, usually containing variable concentrations of organic solvents, for cleaning easel paintings, sculptures and monuments [54–57]. As for cryogels, they usually have large pores, are made of inexpensive materials and can be produced in any desirable shape (blocks, cylinders, discs, etc.) [58,59], and may, therefore, be adapted to the surface chosen for sampling.

2. Research Aim

This study aims to evaluate the use of flow cytometry to quantify and determine the viability state of microorganisms collected from outdoor stone and mortar sculptures. Additionally, this study compares the effectiveness of swabs and poly(HEMA) cryogels as non-invasive methods for sampling outdoor sculptures for microbiological contamination studies. The poly(HEMA) cryogels were chosen as a model for the use of gels for surface sampling and tested as a possible alternative to overcome the difficulties associated with swabbing uneven surfaces. The comparison of the sampling methods was performed by flow cytometry using the fluorochromes TO and PI.

3. Materials and Methods

3.1. Materials

HEMA, N,N´-methylene-bis(acrylamide), ammonium persulfate and N,N,N´,N´-tetramethylethylnediamine for the preparation of the poly(HEMA) cryogels were obtained from Sigma-Aldrich (USA). Tween 80, peptone, Na_2HPO_4 and KH_2PO_4 were also obtained from Sigma-Aldrich (USA). KCl was from Merck (Germany) and NaCl was acquired from Panreac (Spain). Yeast extract and the culture media Nutrient Agar (NA) and Potato Dextrose Agar (PDA) were acquired from BIOKAR Diagnostics (France), while Czapek Dox Liquid Medium was obtained from Oxoid (United Kingdom). Sterile 30 µm-pore-sized Celltrics® filters were acquired from Sysmex (Germany) and the BD™ Cell Viability Kit for flow cytometry was purchased from BD Biosciences (USA).

3.2. Sculptures and sampling sites

The samples were obtained from five outdoor stone and mortar sculptures, which are located in parks and public squares in urban regions of the district of Porto, Portugal (Figure 1). The sculptures were chosen in order to include artworks made of different materials, at different stages of degradation and visible biocontamination. In each sculpture, five areas were selected for sampling using swabs and cryogels. The selected sculptures are described below:

- *Sol, Lua e Vento* (1997), a grey granite sculpture by Satoru Sato, located in Santo Tirso and part of the collection of the International Museum of Contemporary Sculpture (approximate coordinates: 41°20'35.3"N 8°28'15.6"W).

- *Movimento* (1994), a marble sculpture by Augusto Jorge Ulisses, located in the gardens of the Faculty of Fine Arts of the University of Porto (approximate coordinates: 41°08'43.0"N 8°36'02.7"W).

- *Repouso* (1953), a mortar sculpture by Gustavo Bastos, located in the gardens of the Faculty of Fine Arts of the University of Porto (approximate coordinates: 41°08'44.1"N 8°36'01.8"W).

- *Rosalía de Castro* (1951), a pink granite sculpture by Salvador Barata Feyo, located in Praça da Galiza, Massarelos (approximate coordinates: 41°09'08.3"N 8°37'36.1"W).

- *Afonso de Albuquerque* (1930), an Ançã limestone sculpture by Diogo de Macedo, located in Largo de D. João III, Lordelo do Ouro (approximate coordinates: 41°09'27.2"N 8°39'39.0"W).

3.3. Sampling methodology

3.3.1. Preparation of poly(HEMA) cryogels

Poly(HEMA) cryogels were prepared according to Keleş and co-authors (2015) [58] with slight modifications. Briefly, 21.43 mmol HEMA was mixed with N,N´-methylene-bis(acrylamide) solution (3.67 mmol in 20 mL of deionised water). The cryogels were synthesised by free radical polymerisation by adding 0.175 mmol ammonium persulfate to the solution and cooling it in an ice bath for 3 min, followed by the addition of 0.335 mmol N,N,N´,N´-tetramethylethylnediamin and stirring of the reaction mixture for 1 min. The reaction mixture was poured into Petri dishes and frozen at -20 °C for 24 h. The cryogels were thawed at room temperature, washed with deionised water to remove unreacted monomers and cut into circular discs (1 cm^2). Finally, the cryogel discs were sterilised by immersion in ethanol, washed with sterile deionised water and put under UV-C lights for 15 min. The cryogel discs were stored in sterile deionised water at room temperature until use.

3.3.2. Sample collection

Sterile cotton swabs were pre-moistened in a working solution (1% Tween 80 in 1 g L^{-1} peptone water) and used for swabbing the surfaces of the sculptures methodically, in several directions, while rolling the swabs to expose unused sides. The samples from each sampling area were obtained by swabbing two adjacent zones of 1 cm^2. The swabs were placed inside sterile tubes containing 10 mL of working solution by breaking off the swabs' heads. Sampling with poly(HEMA) cryogels was performed in a similar way, by applying sterile circular discs (1 cm^2) to the sampling areas of each sculpture and gently pressing them against the surfaces. The cryogel discs were placed in tubes containing 10 mL of working solution. All samples were immediately transported to the laboratory at room temperature and promptly processed for flow cytometry analysis.

Figure 1 – Identification of the sculptures, in the district of Porto, Portugal. A – *Sol, Lua e Vento*, made of grey granite. B – marble sculpture *Movimento*. C – *Repouso*, made of mortar. D – pink granite sculpture *Rosalía de Castro*. E - *Afonso de Albuquerque*, made of Ançã limestone.

3.3.3. Sample preparation

The preparation of the samples for flow cytometry was performed following a protocol adapted from Anvarian and co-authors (2016) [60]. The tubes containing the samples collected with swabs and cryogels were vortexed for 2 min and put in an ultrasonic bath for 15 min to release the microbial cells to the working solutions. In a sterile environment, the swabs and the cryogels were removed and discarded. To eliminate large dust particles and debris, the suspensions were firstly centrifuged at 1,600 rpm (292 ×g) for 5 min at 4 °C. The obtained pellets (Pellet 1) were dispersed in 0.5 mL of phosphate-buffered saline (PBS) (8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.44 g L^{-1} Na_2HPO_4, 0.24 g L^{-1} KH_2PO_4) and kept in an incubator at 4 °C, whereas the supernatants were transferred to clean sterile tubes and centrifuged at 4,000 rpm (1,825 ×g) for 20 min at 4 °C to harvest the microbial cells. The pellets collected from this centrifugation (Pellet 2) were resuspended in 5 mL of PBS and kept at 4 °C, and the supernatants were centrifuged at 4,000 rpm (1,825 ×g) for 45 min at 4 °C to collect remaining cells. Lastly, the supernatants from this final centrifugation were discarded and the pellets (Pellet 3) were dispersed in 0.5 mL of PBS. Under sterile conditions, the resuspended pellets were sequentially filtered (Pellet 2, 3 and 1) through sterile 30 µm-pore-sized filters and the filtrates were stored at 4 °C until flow cytometry analysis.

3.4. Flow cytometry

Flow cytometry analysis was performed using a BD Accuri™ C6 flow cytometer (BD Biosciences, USA) equipped with two lasers (488 nm and 640 nm) and four optical filters (FL1 533/30 nm, FL2 585/40 nm, FL3 >670 nm, and FL4 675/25 nm). FSC and SSC detectors were also used for observation of forward and side light scatter parameters, respectively. The samples were firstly analysed without the addition of fluorochromes and were appropriately diluted when necessary. Data acquisition per sample was limited to 10,000 events and 25 µL at a flow rate of 14 µL min^{-1}. To detect cells of interest and evaluate cell viability, the samples were stained with TO and PI according to the manufacturer's instructions (BD™ Cell Viability Kit, BD Biosciences, USA) and data were acquired under the same conditions. The final concentrations of the fluorochromes were 420 nmol L^{-1} for TO and 43 µmol L^{-1} for PI.

Flow cytometry data were observed and analysed with BD Accuri™ C6 v1.0.264.21 software (BD Biosciences, USA). The identification of the microorganisms (TO-positive) was done by analysing the fluorescence peaks on the FL1 channel. Once the areas corresponding to TO-positive populations were determined, the discrimination of viable and non-viable populations on FL2 (PI) vs FL1 (TO) plots was performed and gates corresponding to these populations were drawn. Negative controls were carried out with the working solution. Isolates of *Bacillus cereus, Chlorella vulgaris* and *Penicillium chrysogenum* obtained from our laboratory collection were used to prepare single suspensions of cells (*B. cereus* and *C. vulgaris*) and spores and mycelia (*P. chrysogenum*); these suspensions were stained with TO and PI and used as controls to distinguish and gate the regions of labelled microorganisms from background noise. To prepare these controls, *B. cereus* colonies grown in NA plates at 30 °C for 24 h were suspended in the

working solution. *C. vulgaris* was grown in BG-11 liquid media [61] at 25 °C for 14 d with constant light and then transferred to the working solution. The *P. chrysogenum* spore suspension was prepared by growing the strain in PDA plates at 25 °C for 6 d, followed by the addition of working solution to the plates to release and collect the spores. The suspensions were diluted as necessary to obtain final concentrations of 1-2 x 10^7 CFU mL^{-1}, as calculated by Neubauer chamber counting. For the preparation of the *P. chrysogenum* mycelia suspension, the strain was grown in Czapek Dox Liquid Medium supplemented with 5 g L^{-1} of yeast extract, at 25 °C and 80 rpm for 6 d, and then filtered in a sterile environment; the filtered mycelia were transferred to a sterile flask, diluted in deionised water to reach a final concentration of 1 g mL^{-1} and then fragmented using a T 25 digital ULTRA-TURRAX® (IKA, Germany) at 25,000 rpm for 30 s.

3.5. Statistical analysis

Statistical analysis was performed using IBM SPSS® Statistics v27 software (IBM, USA) to check for statistically significant differences in the concentrations of microorganisms and viable/ non-viable cells obtained from swab and cryogel samples, for each sculpture. The Shapiro-Wilk test was used to confirm that the values were normally distributed, followed by the t-test for independent samples to compare the samples collected with swabs and cryogels.

4. Results

Figure 2 shows some examples of the density plots of FL2 (PI) vs FL1 (TO) fluorescence obtained from swab and cryogel samples, where the regions comprising the particles of interest (TO-positive) are shown separated by vertical markers to isolate them from the regions containing debris, acellular particulates and background noise. The same figure shows the populations corresponding to viable (TO-positive/PI-negative) and non-viable (TO-positive/PI-positive) cells, which were defined based on the fluorescence properties of the particles stained with PI.

The flow cytometry data that resulted from the analyses of the samples obtained from the surfaces of the five sculptures using the two sampling methods are summarised in Figure 3. All samples yielded high concentrations of total microorganisms, ranging from 5.51 ± 0.22 and 6.18 ± 0.17 $\log_{10}$ cells mL^{-1} for the swab samples and from 4.84 ± 0.15 to 5.43 ± 0.30 $\log_{10}$ cells mL^{-1} for the cryogel samples, and regardless of the material from which the sculptures are made. Regarding the analyses of cell viability, most of the cells appear to be non-viable or to have compromised membranes. The higher concentrations of non-viable cells compared to viable cells could be related to either the low availability of nutrients in the stone/mortar substrates or to the methodology adopted for processing the samples to reduce the amount of debris, which may have, at some point, contributed to the disruption of the cell membranes. Further studies may be required to better elucidate the high number of non-viable cells. Additionally, it was observed, for both sampling methods, that a large part of the events detected by flow cytometry seems to be debris and particulate matter collected from the surfaces of the sculptures. As an example, over 87% of the recorded events in the swab samples from *Afonso de Albuquerque* were not stained by TO (data not shown), even after sample processing prior to the analyses.

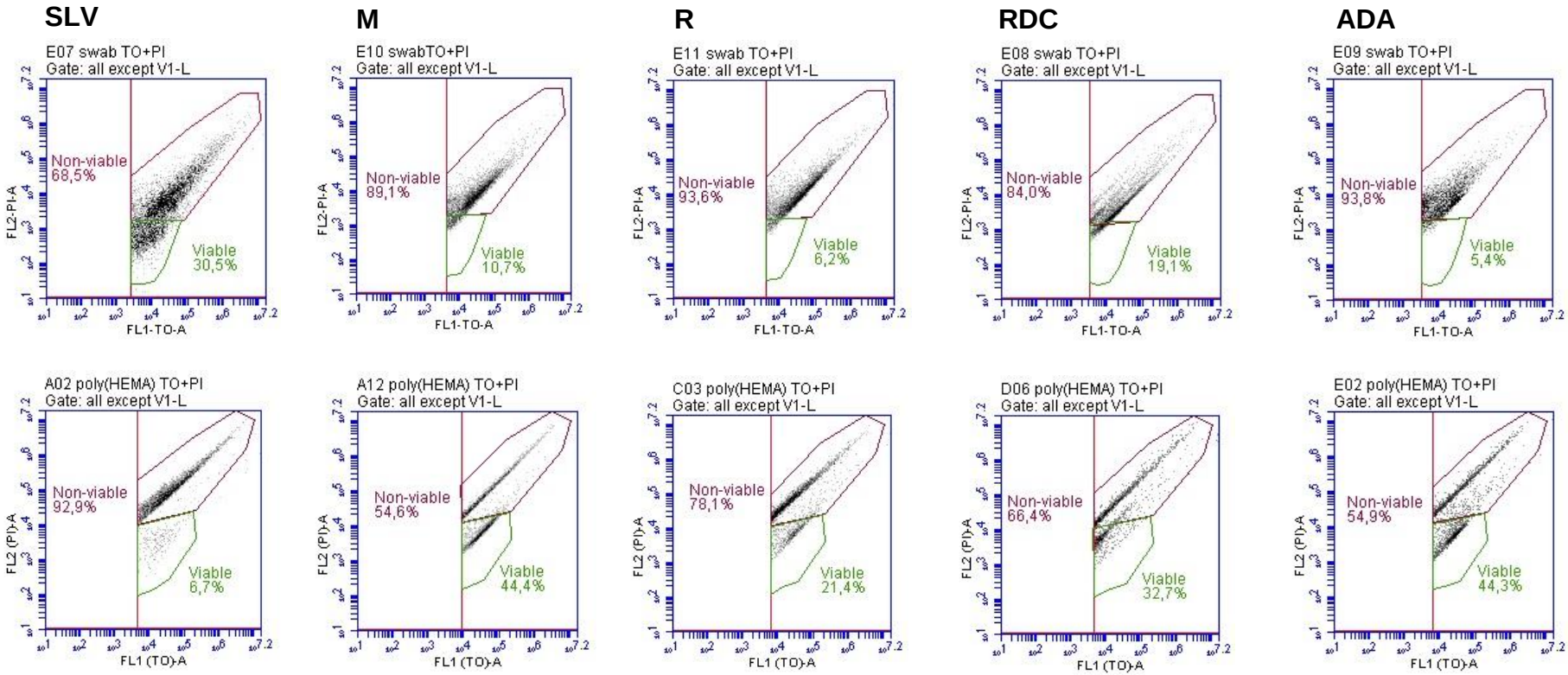

Figure 2 – Density plots obtained by flow cytometry analysis of some of the samples collected with swabs (top) and poly(HEMA) cryogels (bottom) from the sculptures *Sol, Lua e Vento* (SLV), *Movimento* (M), *Repouso* (R), *Rosalía de Castro* (RDC) and *Afonso de Albuquerque* (ADA). The samples were stained with thiazole orange (TO) and propidium iodide (PI). The vertical markers separate the TO-labelled events from the regions containing debris, acellular particulates and background noise, while the regions comprising viable (PI-negative) and non-viable (PI-positive) cells are outlined in the TO-positive regions.

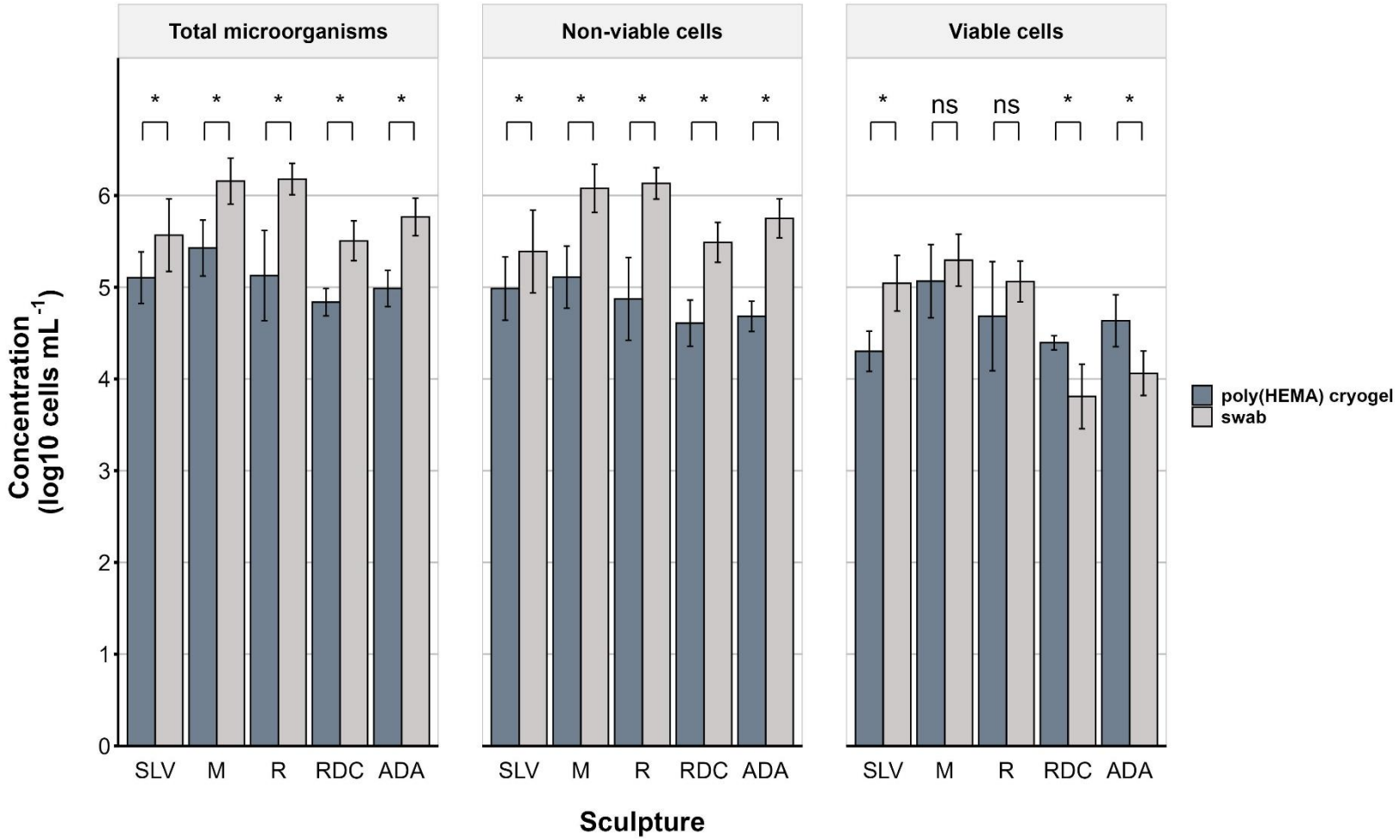

Figure 3 – Quantification of microbial cells by flow cytometry of samples collected with swabs and poly(HEMA) cryogels from the sculptures *Sol, Lua e Vento* (SLV), *Movimento* (M), *Repouso* (R), *Rosalía de Castro* (RDC) and *Afonso de Albuquerque* (ADA). The values shown are the mean ± SD (n = 10). Within each sculpture, the symbol ***** indicates statistically significant differences between poly(HEMA) cryogels and swabs and the letters **ns** indicate non-significant differences (t-test for independent samples; α = 0.05).

When comparing the two sampling methods concerning the concentrations of total microorganisms, viable cells and non-viable cells (Figure 3), statistically significant differences ($p < 0.05$) were found, for each sculpture, between the capacity of swabs and cryogels to collect and retrieve microbial cells, despite the very high standard deviation values obtained for some of the samples. When considering the concentrations of total microorganisms, a greater number of cells were identified in the samples collected with swabs than with cryogels. The same trend was observed when considering the recovery and identification of non-viable cells, but only in the sculpture *Sol, Lua e Vento* the concentrations of viable cells were significantly higher ($p < 0.05$) in the swab samples than in those obtained with cryogels.

Lastly, it is noteworthy that even though the overall number of events recorded by the flow cytometer was higher in the swab samples, a clearer and more defined separation of cell populations based on viability was observed in the samples collected with cryogels (Figure 2).

5. Discussion

Sampling with cryogels allowed the recovery of microorganisms and the discrimination of viable and non-viable populations by flow cytometry, but cell concentrations were significantly lower than those obtained from swabs. It seems that even though swabbing environmental samples has some known disadvantages, particularly for quantification purposes, in some cases swab sampling may still be the most adequate method available [62]. In our study, the use of swabs proved to be a more fitting approach to ensure a greater recovery of microorganisms from the surfaces of the sculptures, despite all the drawbacks that may exist associated with swab sampling. However, sampling with cryogels still allowed the recovery of a substantial number of microorganisms, and the separation of microbial cell populations based on their membrane integrity was clearer than with swabs, which facilitated the evaluation of the viability state of the microorganisms. It is not clear why this happened, though the porous nature of the cryogels [59] might have contributed to the better preservation of the cells as opposed to the swabs, which may have contributed to the rupture of cell membranes by mechanical action during sampling and to the lower concentrations of viable cells obtained from some of the sculptures [48]. If the objective of the research is a clear separation of these populations or a flexible sampling method due to the possibility of preparing the cryogels in any desirable shape adaptable to the surfaces (blocks, discs, etc.), then the use of a gel, such as the one tested in our study, might be a choice to consider.

Furthermore, flow cytometry was adopted to quantify the microorganisms collected by both swabs and cryogels and to infer their viability by evaluating the integrity of their cell membranes. Flow cytometry has been largely used in various fields of expertise, not only to characterise human cells but also to study microorganisms and biofilms collected from various sources. The applications of flow cytometry include, among others, the study of natural and agricultural soils, sediments and sludge samples, the characterisation of bacterial cells in marine and freshwater, the study of the effects of antibiotics on cell viability, the quantification of viable

bacterial cells in milk powders and the characterisation of yeasts in the beer and wine industries [63,64,73,65–72]. Flow cytometry has also become a promising analytical tool in the context of CH, even though its use for these types of samples is still not common practice. In fact, to the best of our knowledge, flow cytometry has not yet been established for the quantification of microorganisms directly obtained from CH samples and our study is the first attempt to use flow cytometry for cell quantification and assessment of cell viability in studies concerning the biocontamination of sculptures. Nonetheless, some studies have been published where flow cytometry was applied in the context of CH. Mesquita and co-authors (2013) [29] used flow cytometry to evaluate the growth, viability and metabolic activity of *P. chrysogenum*, *Aspergillus nidulans* and *A. niger* spores, as common contaminants of CH objects, after a disinfection treatment with increasing levels of gamma radiation. The differences in the size and complexity of the spores were evaluated by FSC and SSC detectors, as well as their viability and metabolic activity through staining with PI and dihydroethidium (DHE) [29]. In the work of Silva (2017) [30], the physiology of *Bacillus* sp. cells was investigated by flow cytometry using a multi-parameter analysis with Annexin V and 7-aminoactinomycin D (7-AAD) to monitor the response of the bacterial cells to nutrient starvation and supplementation and to evaluate the production of bioactive compounds with potential to be applied as green biocides for CH applications. Gheorghe and co-authors (2021) [31] reported the efficiency of MgB_2 materials against several strains of filamentous fungi isolated from wooden and stone CH objects. The authors used flow cytometry to evaluate the toxicity effects of MgB_2 nanoparticles against Hep2 cells after staining with PI, for the assessment of MgB_2 as an environmentally safe antifungal [31]. The identification of the microorganisms involved in the occurrence of a rosy-red discolouration on frescoes in the St. Brizio Chapel (Orvieto Cathedral, Italy) was performed by Cappitelli and co-authors (2009) [32]. The authors cultivated microbial cells extracted from samples of the frescoes and implemented a flow cytometry protocol to confirm cyanobacteria as the principal cause of the discolouration of the frescoes, by observing the green fluorescence given by the SYBR-Green I staining of non-pigmented microorganisms and the red-orange autofluorescence of the natural pigments of phototrophic microorganisms [32]. Likewise, studies have been published in which fluorescent probes and hybridisation techniques were used in combination with flow cytometry to characterise CH samples. González-Pérez and co-authors (2017) [33] developed an RNA-fluorescence in situ hybridisation (RNA-FISH) protocol for the analysis of bacteria and yeasts and used flow cytometry to analyse isolates of *Escherichia coli* and *Saccharomyces cerevisiae* labelled with Cy3 and 6-FAM fluorophores. Flow cytometry was used to analyse the impact of cell growth phase and [probe]/[cell] ratios on the fluorescence intensity of probe-labelled cells, and the results pointed out the possible use of RNA-FISH in combination with flow cytometry (Flow-FISH) for the analysis of microorganisms present in CH samples [33]. Similarly, Vieira and colleagues (2016) [34] developed a FISH protocol for the detection of bacteria and yeasts present in mortars. Flow cytometry was applied to observe the fluorescence decay of *E. coli* cells after long exposure to UV-C light, thus helping the authors in the choice of appropriate filters to achieve long-lasting and stable signals for the study of the samples by epifluorescence microscopy [34]. The average

fluorescence intensity and the percentage of fluorescent cells after the application of the FISH protocol to bacteria and yeast cells suspensions was also evaluated by flow cytometry [34].

The protocol implemented for the analysis of the swab and cryogel samples obtained from the five sculptures involved a gating strategy using a combination of TO and PI. On the one hand, the ability of TO to bind to nucleic acids allows the labelling of all cells, regardless of their physiological state, which facilitates the distinction between microbial cells and noise signals or debris present in the cell suspensions [60,74]. On the other hand, staining with PI was used to infer cell viability, as cells with intact membranes are impermeable to PI (suggesting viability), while damaged membranes (occurring in non-viable cells) allow PI to enter the cells and compete with TO for DNA binding sites [60,75]. As with all analytical techniques, some challenges were encountered, which are mostly related to the microbiological diversity of the samples and to impurities that remained in the suspensions after the cleaning protocol. The great diversity of taxonomic groups found in these types of samples, as well as the presence of microorganisms with different cell membrane integrity and at different metabolic states, can influence the identification and quantification of microorganisms by flow cytometry. Additionally, membrane permeability and binding efficiency to nucleic acids vary significantly among fluorochromes and groups of organisms [63,76]. Moreover, even though the samples were processed before the analyses, a large number of inorganic particles or cell debris were detected (over 50% of all events detected were not stained by TO; data not shown), which were not eliminated during the cleaning process due to their small sizes and may have hampered the penetration of the fluorochromes into the cells. The presence of inorganic or dead organic particles in the samples, particularly when attached to bacteria and extracellular DNA, can reduce the accuracy of the technique and bias the cell counts due to the difficulty to distinguish particles of interest from interfering compounds [63,64]. These difficulties may have contributed to the high standard deviation values obtained for some of the samples, although flow cytometry is still an interesting and promising analytical tool for this research field.

To further clean the samples before the analyses and remove extra interfering particles, one could consider using filters with smaller pore sizes, although this may result in the loss of cells of interest, such as filamentous fungi and microalgae of larger sizes. A compromise must be found between the elimination of contaminating particles that may contribute to non-specific staining and the excessive removal of cells that could significantly bias the quantification of microorganisms [64,77]. Furthermore, the addition of ethylenediamine tetraacetic acid (EDTA) to the working solutions used for sample collection or to the staining buffers could be useful to chelate metal ions and reduce their possible interference with flow cytometry analyses. Also, EDTA facilitates the detachment of bacterial cells from biofilms; however, its use must be carefully considered when performing cell viability assays due to the chelation of cations necessary for membrane stability and its effect on cell lysis [48,78,79], which may skew the results. Moreover, a multiparametric analysis involving other fluorescent probes for staining specific structures of microbial cells, such as Nile Red, fluorescein isothiocyanate (FITC) and calcofluor white for labelling of membrane lipids, proteins and carbohydrates [28,76], could be suitable not only to

distinguish particles of interest from interfering compounds but also for screening particular groups of microorganisms. The addition of fluorochromes to measure the metabolic activity of microbial cells, such as DHE for the detection of reactive oxygen species (ROS) [29,76], may also be of interest to help quantify the microorganisms with real risk to contribute to the biodeterioration processes of stone, particularly when combined with the analysis of the membrane integrity of the cells.

6. Conclusions

The use of swabs is a more adequate sampling method for the quantification of microbiological communities colonising stone and mortar sculptures when compared to poly(HEMA) cryogels. Although data visualisation of the viable and non-viable cells populations was clearer when sampling with cryogels, swab sampling allowed a greater recovery of total microorganisms from the surfaces of the sculptures, which may be relevant for quantification studies of microbiological contamination. Moreover, flow cytometry proved to be an efficient and useful technique for biocontamination and biodeterioration assessments of outdoor sculptures, both for microbial quantification and cell viability purposes.

Acknowledgements

The authors would like to thank the International Museum of Contemporary Sculpture, the Faculty of Fine Arts of the University of Porto and the Porto City Hall for granting access to the sculptures to collect samples for this study.

Funding

This work was supported by national funds from FCT – Fundação para a Ciência e a Tecnologia through project BIONANOSCULP [grant number PTDC/EPH-PAT/6281/2014]. Nádia C. Silva was supported by FCT and Fundo Social Europeu - FSE through Programa Operacional Regional Norte [grant number SFRH/BD/138935/2018]. The authors would also like to thank the scientific collaboration under the FCT projects FCT project UID/Multi/50016/2013 and UID/Multi/50016/2019.

Declarations of Competing Interest: none

References

[1] J.D. Rodrigues, Defining, mapping and assessing deterioration patterns in stone conservation projects, J. Cult. Herit. 16 (2015) 267–275. https://doi.org/10.1016/j.culher.2014.06.007.

[2] A. Mihajlovski, D. Seyer, H. Benamara, F. Bousta, P. Di Martino, An overview of techniques for the characterization and quantification of microbial colonization on stone

monuments, Ann. Microbiol. 65 (2015) 1243–1255. https://doi.org/10.1007/s13213-014-0956-2.

[3] O. Salvadori, A.C. Municchia, The role of fungi and lichens n the biodeterioration of stone monuments, Open Conf. Proc. J. 7 (2016) 39–54. https://doi.org/10.2174/2210289201607020039.

[4] P. Tiano, Biodeterioration of stone monuments a worldwide issue, Open Conf. Proc. J. 7 (1:M3) (2016) 29–38. https://doi.org/10.2174/2210289201607020029.

[5] A.C. Pinheiro, N. Mesquita, J. Trovão, F. Soares, I. Tiago, C. Coelho, H.P. de Carvalho, F. Gil, L. Catarino, G. Piñar, A. Portugal, Limestone biodeterioration: A review on the Portuguese cultural heritage scenario, J. Cult. Herit. 36 (2019) 275–285. https://doi.org/doi.org/10.1016/j.culher.2018.07.008.

[6] Y. Wang, H. Zhang, X. Liu, X. Liu, W. Song, Fungal communities in the biofilms colonizing the basalt sculptures of the Leizhou Stone Dogs and assessment of a conservation measure, Herit. Sci. 9 (2021) 36. https://doi.org/10.1186/s40494-021-00508-1.

[7] N. Unković, M.L. Grbić, M. Stupar, J. Vukojević, G. Subakov-Simić, A. Jelikić, D. Stanojević, ATP bioluminescence method: Tool for rapid screening of organic and microbial contaminants on deteriorated mural paintings, Nat. Prod. Res. 33 (2019) 1061–1069. https://doi.org/10.1080/14786419.2015.1108975.

[8] P. Sanmartín, A. DeAraujo, A. Vasanthakumar, Melding the old with the new: Trends in methods used to identify, monitor, and control microorganisms on cultural heritage materials, Microb. Ecol. 76 (2018) 64–80. https://doi.org/10.1007/s00248-016-0770-4.

[9] E.S. Çıplak, K.G. Akoğlu, Enzymatic activity as a measure of total microbial activity on historical stone, Heritage. 3 (2020) 671–681. https://doi.org/10.3390/heritage3030038.

[10] F. Troiano, A. Polo, F. Villa, F. Cappitelli, Assessing the microbiological risk to stored sixteenth century parchment manuscripts: A holistic approach based on molecular and environmental studies, Biofouling. 30 (2014) 299–311. https://doi.org/10.1080/08927014.2013.871539.

[11] P. Tiano, L. Tomaselli, C. Orlando, The ATP-bioluminescence method for a rapid evaluation of the microbial activity in the stone materials of monuments, J. Biolumin. Chemilumin. 3 (1989) 213–216. https://doi.org/10.1002/bio.1170030409.

[12] A.A. Gorbushina, N.N. Lyalikova, D.Y. Vlasov, T. V. Khizhnyak, Microbial communities on the monuments of Moscow and St. Petersburg: Biodiversity and trophic relations, Microbiology. 71 (2002) 350–356. https://doi.org/doi.org/10.1023/A:1015823232025.

[13] M.L. Grbić, N. Unković, M. Stupar, J. Vukojević, T. Nedeljković, Implementation of ATP biolumenescence method in the study of the fungal deterioration of textile artefacts, FIBRES Text. East. Eur. 22 (2014) 132–136.

[14] J. Leplat, A. Francois, F. Bousta, White fungal covering on the wall paintings of the Saint-Savin-sur-Gartempe Abbey church crypt: A case study, Int. Biodeterior. Biodegradation. 122 (2017) 29–37. https://doi.org/10.1016/j.ibiod.2017.04.007.

[15] G.Y. Lomakina, Y.A. Modestova, N.N. Ugarova, Bioluminescence assay for cell viability,

Biochem. 80 (2015) 701–713. https://doi.org/10.1134/S0006297915060061.

[16] V. Keasler, B. Bennett, C. Keller, P. Whalen, J. Cairns, R.M. De Paula, Expanding the microbial monitoring toolkit: Evaluation of traditional and molecular monitoring methods, Int. Biodeterior. Biodegrad. 81 (2013) 51–56. https://doi.org/10.1016/j.ibiod.2012.07.002.

[17] C. Alba, A.C. Marin, A.G. McNicholl, A. Montalban-Arques, I. Mora-Gutierrez, A.J. Sánchez-Arroyo, T. Soler, D. García-Fresnadillo, J.P. Gisbert, T. Alarcón, D. Bernardo, A quick flow cytometry protocol to assess Helicobacter pylori viability, J. Microbiol. Methods. 177 (2020) 106043. https://doi.org/10.1016/j.mimet.2020.106043.

[18] M. Genovese, E. Poulain, F. Doppler, R. Toussaint, M. Boyer, Bacillus spore enumeration using flow cytometry: A proof of concept for probiotic application, J. Microbiol. Methods. 190 (2021) 106336. https://doi.org/10.1016/j.mimet.2021.106336.

[19] C. Longin, C. Petitgonnet, M. Guilloux-Benatier, S. Rousseaux, H. Alexandre, Application of flow cytometry to wine microorganisms, Food Microbiol. 62 (2017) 221–231. https://doi.org/10.1016/j.fm.2016.10.023.

[20] M. Vitelli, H. Budman, M. Pritzker, M. Tamer, Applications of flow cytometry sorting in the pharmaceutical industry: A review, Biotechnol. Prog. 37 (2021) e3146. https://doi.org/10.1002/btpr.3146.

[21] M. van der Meulen, J.E.C. Bromberg, K.H. Lam, R. Dammers, A.W. Langerak, J.K. Doorduijn, J.M. Kros, M.J. van den Bent, V.H.J. van der Velden, Flow cytometry shows added value in diagnosing lymphoma in brain biopsies, Cytom. Part B (Clinical Cytom. 94 (2018) 928–934. https://doi.org/10.1002/cyto.b.21641.

[22] J.M.M. Gomes, I. Charlie-Silva, A.K. Santos, R.R. Resende, J.A.S. Gomes, A.T. de Carvalho, J.D.C. Junior, Flow cytometry in the analysis of hematological parameters of tilapias: Applications in environmental aquatic toxicology, Environ. Sci. Pollut. Res. 28 (2021) 6242–6248. https://doi.org/https://doi.org/10.1007/s11356-020-12119-7.

[23] J. Comas-Riu, N. Rius, Flow cytometry applications in the food industry, J. Ind. Microbiol. Biotechnol. 36 (2009) 999–1011. https://doi.org/10.1007/s10295-009-0608-x.

[24] D. Moratto, M. Chiarini, V. Giustini, F. Serana, P. Magro, A.M. Roccaro, L. Imberti, F. Castelli, L.D. Notarangelo, E. Quiros-Roldan, Flow cytometry identifies risk factors and dynamic changes in patients with COVID-19, J. Clin. Immunol. 40 (2020) 970–973. https://doi.org/10.1007/s10875-020-00806-6.

[25] M.E. Heinrichs, D. De Corte, B. Engelen, D. Pan, An advanced protocol for the quantification of marine sediment viruses via flow cytometry, Viruses. 13 (2021) 102. https://doi.org/10.3390/v13010102.

[26] M. Díaz, M. Herrero, L.A. García, C. Quirós, Application of flow cytometry to industrial microbial bioprocesses, Biochem. Eng. J. 48 (2010) 385–407. https://doi.org/10.1016/j.bej.2009.07.013.

[27] I. Vermes, C. Haanen, C. Reutelingsperger, Flow cytometry of apoptotic cell death, J. Immunol. Methods. 243 (2000) 167–190. https://doi.org/10.1016/S0022-1759(00)00233-7.

[28] A. Álvarez-Barrientos, J. Arroyo, R. Cantón, C. Nombela, M. Sánchez-Pérez, Applications of flow cytometry to clinical microbiology, Clin. Microbiol. Rev. 13 (2000) 167–195. https://doi.org/10.1128/CMR.13.2.167-195.2000.

[29] N. Mesquita, A. Portugal, G. Piñar, J. Loureiro, A.P. Coutinho, J. Trovão, I. Nunes, M.L. Botelho, H. Freitas, Flow cytometry as a tool to assess the effects of gamma radiation on the viability, growth and metabolic activity of fungal spores, Int. Biodeterior. Biodegrad. 84 (2013) 250–257. https://doi.org/10.1016/j.ibiod.2012.05.008.

[30] M.T.C. da Silva, Novel biocides for cultural heritage, University of Évora, Portugal, 2017. http://dspace.uevora.pt/rdpc/handle/10174/21001.

[31] I. Gheorghe, I. Avram, V.M. Corbu, L. Măruţescu, M. Popa, I. Balotescu, I. Blăjan, V. Mateescu, D. Zaharia, A.Ş. Dumbravă, O.E. Zetu, I. Pecete, V.C. Cristea, D. Batalu, M.A. Grigoroscuta, M. Burdusel, G.V. Aldica, P. Badica, A.D. Datcu, N. Ianovici, C. Bleotu, V. Lazar, L.M. Diţu, M.C. Chifiriuc, In vitro evaluation of MgB2 powders as novel tools to fight fungal biodeterioration of heritage buildings and objects, Front. Mater. 7 (2021) 601059. https://doi.org/10.3389/fmats.2020.601059.

[32] F. Cappitelli, P. Abbruscato, P. Foladori, E. Zanardini, G. Ranalli, P. Principi, F. Villa, A. Polo, C. Sorlini, Detection and elimination of cyanobacteria from frescoes: The case of the St. Brizio Chapel (Orvieto Cathedral, Italy), Microb. Ecol. 57 (2009) 633–639. https://doi.org/10.1007/s00248-008-9441-4.

[33] M. González-Pérez, C. Brinco, R. Vieira, T. Rosado, G. Mauran, A. Pereira, A. Candeias, A.T. Caldeira, Dual phylogenetic staining protocol for simultaneous analysis of yeast and bacteria in artworks, Appl. Phys. A Mater. Sci. Process. 123 (2017) 142. https://doi.org/10.1007/s00339-016-0725-0.

[34] R. Vieira, M. González-Pérez, A. Pereira, A. Candeias, A.T. Caldeira, Novos desenvolvimentos para a deteção de leveduras e bactérias presentes em argamassas, Conserv. Património. 23 (2016) 71–77. https://doi.org/10.14568/c2015047.

[35] G. Piñar, M.C. Sclocchi, F. Pinzari, P. Colaizzi, A. Graf, M.L. Sebastiani, K. Sterflinger, The microbiome of Leonardo da Vinci's drawings: A bio-archive of their history, Front. Microbiol. 11 (2020) 593401. https://doi.org/10.3389/fmicb.2020.593401.

[36] G. Piñar, K. Sterflinger, Natural sciences at the service of art and cultural heritage: An interdisciplinary area in development and important challenges, Microb. Biotechnol. 14 (2021) 806–809. https://doi.org/10.1111/1751-7915.13766.

[37] N.A. Cutler, A.E. Oliver, H.A. Viles, A.S. Whiteley, Non-destructive sampling of rock-dwelling microbial communities using sterile adhesive tape, J. Microbiol. Methods. 91 (2012) 391–398. https://doi.org/10.1016/j.mimet.2012.09.022.

[38] G. Piñar, H. Tafer, K. Sterflinger, F. Pinzari, Amid the possible causes of a very famous foxing: Molecular and microscopic insight into Leonardo da Vinci's self-portrait, Environ. Microbiol. Rep. 7 (2015) 849–859. https://doi.org/10.1111/1758-2229.12313.

[39] A. Pyzik, K. Ciuchcinski, M. Dziurzynski, L. Dziewit, The bad and the good - Microorganisms in cultural heritage environments - An update on biodeterioration and

biotreatment approaches, Materials (Basel). 14 (2021) 177. https://doi.org/10.3390/ma14010177.

[40] X. Ding, W. Lan, J.-D. Gu, A review on sampling techniques and analytical methods for microbiota of cultural properties and historical architecture, Appl. Sci. 10 (2020) 8099. https://doi.org/10.3390/app10228099.

[41] C. Urzì, F. De Leo, Sampling with adhesive tape strips: An easy and rapid method to monitor microbial colonization on monument surfaces, J. Microbiol. Methods. 44 (2001) 1–11. https://doi.org/10.1016/S0167-7012(00)00227-X.

[42] A. Grottoli, M. Beccaccioli, E. Zoppis, R.S. Fratini, E. Schifano, M.L. Santarelli, D. Uccelletti, M. Reverberi, Nanopore sequencing and bioinformatics for rapidly identifying cultural heritage spoilage microorganisms, Front. Mater. 7 (2020) 14. https://doi.org/10.3389/fmats.2020.00014.

[43] T. Rosado, L. Dias, M. Lança, C. Nogueira, R. Santos, M.R. Martins, A. Candeias, J. Mirão, A.T. Caldeira, Assessment of microbiota present on a Portuguese historical stone convent using high-throughput sequencing approaches, Microbiologyopen. 9 (2020) 1067–1084. https://doi.org/10.1002/mbo3.1030.

[44] P.J.A. Skipper, L.K. Skipper, A survey of bacterial colonisation of historic limestone buildings: Lincoln Cathedral and St. Peter at Gowts, United Kingdom, Rehab 2014 – Proc. Int. Conf. Preserv. Maint. Rehabil. Hist. Build. Struct. (2014) 1003–1012.

[45] M.-L. Suihko, H.-L. Alakomi, A. Gorbushina, I. Fortune, J. Marquardt, M. Saarela, Characterization of aerobic bacterial and fungal microbiota on surfaces of historic Scottish monuments, Syst. Appl. Microbiol. 30 (2007) 494–508. https://doi.org/10.1016/j.syapm.2007.05.001.

[46] H.P. de Carvalho, S.O. Sequeira, D. Pinho, J. Trovão, R.M.F. da Costa, C. Egas, M.F. Macedo, A. Portugal, Combining an innovative non-invasive sampling method and high-throughput sequencing to characterize fungal communities on a canvas painting, Int. Biodeterior. Biodegrad. 145 (2019) 104816. https://doi.org/10.1016/j.ibiod.2019.104816.

[47] M.E.A. Eissa, A.M. Mahmoud, A novel improved bioburden recovery method using swabbing technique, Int. J. Microbiol. Res. 3 (2012) 208–215. https://doi.org/10.5829/idosi.ijmr.2012.3.3.64231.

[48] G. Moore, C. Griffith, Problems associated with traditional hygiene swabbing: The need for in-house standardization, J. Appl. Microbiol. 103 (2007) 1090–1103. https://doi.org/10.1111/j.1365-2672.2007.03330.x.

[49] G. Moore, C. Griffith, A comparison of surface sampling methods for detecting coliforms on food contact surfaces, Food Microbiol. 19 (2002) 65–73. https://doi.org/10.1006/fmic.2001.0464.

[50] X. Liu, R.J. Koestler, T. Warscheid, Y. Katayama, J.-D. Gu, Microbial deterioration and sustainable conservation of stone monuments and buildings, Nat. Sustain. 3 (2020) 991–1004. https://doi.org/10.1038/s41893-020-00602-5.

[51] G.M. Gadd, Geomicrobiology of the built environment, Nat. Microbiol. 2 (2017) 16275.

https://doi.org/10.1038/nmicrobiol.2016.275.

[52] X. Zhang, Q. Ge, Z. Zhu, Y. Deng, J.-D. Gu, Microbiological community of the Royal Palace in Angkor Thom and Beng Mealea of Cambodia by Illumina sequencing based on 16S rRNA gene, Int. Biodeterior. Biodegrad. 134 (2018) 127–135. https://doi.org/https://doi.org/10.1016/j.ibiod.2018.06.018.

[53] A. Kusumi, X. Li, Y. Osuga, A. Kawashima, J.-D. Gu, M. Nasu, Y. Katayama, Bacterial communities in pigmented biofilms formed on the sandstone bas-relief walls of the Bayon temple, Angkor Thom, Cambodia, Microbes Environ. 28 (2013) 422–431. https://doi.org/10.1264/jsme2.ME13033.

[54] E. Carretti, S. Grassi, M. Cossalter, I. Natali, G. Caminati, R.G. Weiss, P. Baglioni, L. Dei, Poly(vinyl alcohol)-borate hydro/cosolvent gels: Viscoelastic properties, solubilizing power, and application to art conservation, Langmuir. 25 (2009) 8656–8662. https://doi.org/10.1021/la804306w.

[55] E. Carretti, I. Natali, C. Matarrese, P. Bracco, R.G. Weiss, P. Baglioni, A. Salvini, L. Dei, A new family of high viscosity polymeric dispersions for cleaning easel paintings, J. Cult. Herit. 11 (2010) 373–380. https://doi.org/10.1016/j.culher.2010.04.002.

[56] M. Sun, J. Zou, H. Zhang, B. Zhang, Measurement of reversible rate of conservation materials based on gel cleaning approach, J. Cult. Herit. 16 (2015) 719–727. https://doi.org/https://doi.org/10.1016/j.culher.2014.01.010.

[57] F. Gabriele, M. Tortora, L. Bruno, C. Casieri, M. Chiarini, R. Germani, N. Spreti, Alginate-biocide hydrogel for the removal of biofilms from calcareous stone artworks, J. Cult. Herit. 49 (2021) 106–114. https://doi.org/10.1016/j.culher.2021.02.009.

[58] B. Keleş, T. Inanan, N. Tüzmen, A. Denizli, Cadmium removal performances of different dye ligands attached cryogel disks, Croat. Chem. Acta. 88 (2015) 139–149. https://doi.org/10.5562/cca2549.

[59] V.I. Lozinsky, I.Y. Galaev, F.M. Plieva, I.N. Savina, H. Jungvid, B. Mattiasson, Polymeric cryogels as promising materials of biotechnological interest, Trends Biotechnol. 21 (2003) 445–451. https://doi.org/10.1016/j.tibtech.2003.08.002.

[60] A.H.P. Anvarian, Y. Cao, S. Srikumar, S. Fanning, K. Jordan, Flow cytometric and 16S sequencing methodologies for monitoring the physiological status of the microbiome in powdered infant formula production, Front. Microbiol. 7 (2016) 968. https://doi.org/10.3389/fmicb.2016.00968.

[61] R. Rippka, J. Deruelles, J.B. Waterbury, M. Herdman, R.Y. Stanier, Generic assignments, strain histories and properties of pure cultures of cyanobacteria, J. Gen. MicrobioZogy. 110 (1979) 1–61. https://doi.org/10.1099/00221287-111-1-1.

[62] L. Rose, B. Jensen, A. Peterson, S.N. Banerjee, M.J. Arduino, Swab materials and Bacillus anthracis spore recovery from nonporous surfaces, Emerg. Infect. Dis. 10 (2004) 1023–1029. https://doi.org/10.3201/eid1006.030716.

[63] A. Frossard, F. Hammes, M.O. Gessner, Flow cytometric assessment of bacterial abundance in soils, sediments and sludge, Front. Microbiol. 7 (2016) 903.

https://doi.org/10.3389/fmicb.2016.00903.

[64] M. Bressan, I.T. Gattin, S. Desaire, L. Castel, C. Gangneux, K. Laval, A rapid flow cytometry method to assess bacterial abundance in agricultural soil, Appl. Soil Ecol. 88 (2015) 60–68. https://doi.org/10.1016/j.apsoil.2014.12.007.

[65] P.C. Braga, C. Bovio, M. Culici, M. Dal Sasso, Flow cytometric assessment of susceptibilities of Streptococcus pyogenes to erythromycin and rokitamycin, Antimicrob. Agents Chemother. 47 (2003) 408–412. https://doi.org/10.1128/AAC.47.1.408-412.2003.

[66] S. Flint, K. Walker, B. Waters, R. Crawford, Description and validation of a rapid (1 h) flow cytometry test for enumerating thermophilic bacteria in milk powders, J. Appl. Microbiol. 102 (2007) 909–915. https://doi.org/10.1111/j.1365-2672.2006.03167.x.

[67] S. Müller, A. Lösche, Population profiles of a commercial yeast strain in the course of brewing, J. Food Eng. 63 (2004) 375–381. https://doi.org/https://doi.org/10.1016/j.jfoodeng.2003.08.010.

[68] A.R. Boyd, T.S. Gunasekera, P. V. Attfield, K. Simic, S.F. Vincent, D.A. Veal, A flow-cytometric method for determination of yeast viability and cell number in a brewery, FEMS Yeast Res. 3 (2003) 11–16. https://doi.org/10.1016/S1567-1356(02)00125-3.

[69] P. Malacrinò, G. Zapparoli, S. Torriani, F. Dellaglio, Rapid detection of viable yeasts and bacteria in wine by flow cytometry, J. Microbiol. Methods. 45 (2001) 127–134. https://doi.org/10.1016/S0167-7012(01)00243-3.

[70] G. Grégori, S. Citterio, A. Ghiani, M. Labra, S. Sgorbati, S. Brown, M. Denis, Resolution of viable and membrane-compromised bacteria in freshwater and marine waters based on analytical flow cytometry and nucleic acid double staining, Appl. Environ. Microbiol. 67 (2001) 4662–4670. https://doi.org/10.1128/AEM.67.10.4662-4670.2001.

[71] A. Pianetti, T. Falcioni, F. Bruscolini, L. Sabatini, E. Sisti, S. Papa, Determination of the viability of Aeromonas hydrophila in different types of water by flow cytometry, and comparison with classical methods, Appl. Environ. Microbiol. 71 (2005) 7948–7954. https://doi.org/10.1128/AEM.71.12.7948-7954.2005.

[72] J. Wojciech, M. Kamila, B. Wojciech, Investigation of the population dynamics within a Pseudomonas aeruginosa biofilm using a flow based biofilm model system and flow cytometric evaluation of cellular physiology, Biofouling. 34 (2018) 835–850. https://doi.org/10.1080/08927014.2018.1508569.

[73] M. Kerstens, G. Boulet, M. Van kerckhoven, S. Clais, E. Lanckacker, P. Delputte, L. Maes, P. Cos, A flow cytometric approach to quantify biofilms, Folia Microbiol. (Praha). 60 (2015) 335–342. https://doi.org/10.1007/s12223-015-0400-4.

[74] I.O.L. McHugh, A.L. Tucker, Flow cytometry for the rapid detection of bacteria in cell culture production medium, Cytom. Part A. 71A (2007) 1019–1026. https://doi.org/10.1002/cyto.a.20488.

[75] F. Joux, P. Lebaron, Use of fluorescent probes to assess physiological functions of bacteria at single-cell level, Microbes Infect. 2 (2000) 1523–1535. https://doi.org/10.1016/S1286-4579(00)01307-1.

[76] B.P. Tracy, S.M. Gaida, E.T. Papoutsakis, Flow cytometry for bacteria: Enabling metabolic engineering, synthetic biology and the elucidation of complex phenotypes, Curr. Opin. Biotechnol. 21 (2010) 85–99. https://doi.org/10.1016/j.copbio.2010.02.006.

[77] P. Klauth, R. Wilhelm, E. Klumpp, L. Poschen, J. Groeneweg, Enumeration of soil bacteria with the green fluorescent nucleic acid dye Sytox green in the presence of soil particles, J. Microbiol. Methods. 59 (2004) 189–198. https://doi.org/10.1016/j.mimet.2004.07.004.

[78] R. Cavaliere, J.L. Ball, L. Turnbull, C.B. Whitchurch, The biofilm matrix destabilizers, EDTA and DNaseI, enhance the susceptibility of nontypeable Hemophilus influenzae biofilms to treatment with ampicillin and ciprofloxacin, Microbiologyopen. 3 (2014) 557–567. https://doi.org/10.1002/mbo3.187.

[79] E. Banin, K.M. Brady, E.P. Greenberg, Chelator-induced dispersal and killing of Pseudomonas aeruginosa cells in a biofilm, Appl. Environ. Microbiol. 72 (2006) 2064–2069. https://doi.org/10.1128/AEM.72.3.2064-2069.2006.