Special Issue: Biodeterioration and Biotechnology of Cultural Heritage

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THE SPANISH BIODETERIORATION AND BIODEGRADATION GROUP

Diego A. Moreno

The Biodeterioration and Biodegradation Group (http://www.semicro.es/biodet/presentacion.htm) was founded on 1989 within the Spanish Society for Microbiology (SEM) (http://www.semicro.es/).

Among Group’s activities are organization of Biodeterioration specialized meetings, monographic round tables on up-to-date, specific subjects, doctorate and postgraduated courses on “Materials Biodeterioration”, in which students receive a wide and in-deep training on the biodeterioration processes that materials suffer in different environments.


Coinciding with the XXI SEM Meeting, held in Seville in September 2007, a round table on “Biodeterioration and Biotechnology of Cultural Heritage” was organized. The four communications discussed are published in this current issue of Coalition.

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BACTERIAL DIVERSITY IN THE CAVE OF ALTAMIRA

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The study of microorganisms involved in deterioration of prehistoric paintings is of great importance to preserve these singular samples of cultural heritage. This study presents, as an example, results from a research carried out in Altamira Cave (Cantabria, Spain). Altamira Cave contains numerous and well known paintings from the Upper Palaeolithic, dated back around 15,000 years. This work focused in understanding the microorganisms inhabiting this singular system and the potential consequences of its microbial diversity for conservation of these valuable paintings.

Introduction

The importance of microorganisms in cultural heritage have been generally overlooked because either the methods used were not appropriated for the proposed objectives or their potential implications in cave conservation was not considered. In environmental microbiology, using the appropriate methodology is of critical importance. Classical microbiological methods involve the use of culturing techniques for the detection of microorganisms. Culturing implies a need to grow up a microorganism in the laboratory to be detected. Since the growth conditions of most microorganisms in our planet are still unknown, we can only culture up to 1% of the microorganisms present in any given sample (Ward et al. 1990, Gonzalez and Saiz-Jimenez 2004). Microscopy observation, for instance, is unable to provide microbial identification due to the relatively
low morphological diversity with respect to the known specific diversity of microorganisms.

In recent years, a novel methodology is being developed to approach the detection of environmental microorganisms independently of a need to culture them. The use of molecular techniques based on the analysis of nucleic acids is transforming the way in which environmental microbiology, and the general microbiology, is being looked up. Today, it is known that the microbial diversity in our planet is huge, well beyond what can be experimentally determined (Curtis et al. 2002). These molecular methods allow to detect microorganisms without a need for culture them and so the researchers can get a vision of the actual microbial community in situ, without modifications due to microbial growth in the laboratory.

Studies available so far report on the isolation and analysis of a very reduced number of microbial groups. For instance, some heterotrophic bacteria and fungi are often cultured and are generally relatively easy to grow up in the laboratory. However, most physiological groups of microorganisms have never, or rarely, being grown up from cultural heritage samples. Examples, shown in Table 1, include most anaerobic bacteria such as the sulfate-reducing bacteria, denitrifying bacteria, or methanogenic archaea, lithotrophic microorganisms (both aerobic and anaerobic ones), and many others. An example of frequently cultured microorganisms are the phototrophs. Microalgae and Cyanobacteria have been reported to grow on historic sites, generally leading to green-colored films able to cover large surfaces of murals, paintings, or walls of a variety of monuments. Some phototrophs able to grow up at reduced light intensities have been often reported to develop in caves with permanent illumination installed (Albertano and Urzi 1999).

Table 1. Some physiological groups of microorganisms classified by their expected relative abundance in caves with prehistoric paintings, the risk involved in attempting to culture them, the frequency of reports for these cultures, and some common examples

<table>
<thead>
<tr>
<th>Type of metabolism</th>
<th>Oxygen tolerance</th>
<th>Relative abundance</th>
<th>Frequency of cultivation</th>
<th>Basic examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterotrophic</td>
<td>Aerobic</td>
<td>High</td>
<td>High</td>
<td>Many Bacteria, Fungi</td>
</tr>
<tr>
<td>Heterotrophic</td>
<td>Anaerobic</td>
<td>High</td>
<td>Low</td>
<td>Clostridium</td>
</tr>
<tr>
<td>Lithotrophic</td>
<td>Anaerobic</td>
<td>Low/Medium</td>
<td>Very low</td>
<td>Thiobacillus</td>
</tr>
<tr>
<td>Lithotrophic</td>
<td>Aerobic</td>
<td>Low</td>
<td>Very low</td>
<td>Thiobacillus</td>
</tr>
<tr>
<td>Sulfate-reducers</td>
<td>Anaerobic</td>
<td>Medium</td>
<td>Very low</td>
<td>Desulfovibrio</td>
</tr>
<tr>
<td>Methanogens</td>
<td>Anaerobic</td>
<td>Low</td>
<td>Very low</td>
<td>Methanosarcina</td>
</tr>
<tr>
<td>Denitrifiers</td>
<td>Anaerobic or Aerobic</td>
<td>Low/Medium</td>
<td>Very low</td>
<td>Pseudomonas denitrificans</td>
</tr>
<tr>
<td>Acetogens</td>
<td>Anaerobic or Aerobic</td>
<td>Low/Medium</td>
<td>Very low</td>
<td>Acetobacterium</td>
</tr>
<tr>
<td>Fe-reducers</td>
<td>Anaerobic</td>
<td>Low</td>
<td>Very low</td>
<td>Shewanella</td>
</tr>
<tr>
<td>Phototrophic</td>
<td>Aerobic</td>
<td>Low to High</td>
<td>High</td>
<td>Cyanobacteria, microalgae</td>
</tr>
<tr>
<td>Unknown</td>
<td>Unknown</td>
<td>Low</td>
<td>Never</td>
<td>Candidate Divisions</td>
</tr>
</tbody>
</table>

Methodology
A case study carried out in Altamira Cave (Cantabria, Spain) is presented here. The methodology used during this study has been previously described (Gonzalez et al. 2003, Gonzalez and Saiz-Jimenez 2004, Portillo et al. 2008). Briefly, DNA and RNA were extracted from minute samples. Analysis based on DNA allow to detect the microorganisms present in a sample, while those performed based on RNA provide with information on those showing significant metabolical activity within their community. Bacterial and archaeal 16S rRNA gene fragments were amplified by PCR (Polymerase Chain Reaction) and processed for DNA library construction with the aim of sequencing and microbial community fingerprints by DGGE.
(Denaturing Gradient Gel Electrophoresis). Bioinformatic analyses were performed in order to obtain the taxonomic affiliation of the retrieved sequences which was compared with molecular profiles of these communities obtained by DGGE.

**Results and Discussion**

Reports from the culture of microorganisms from samples collected in Altamira Cave showed that three major groups represented practically the total microbial community in this cave (Laiz et al. 2003). These groups were the Actinobacteria (mostly *Streptomyces*), Proteobacteria (mostly Gammaproteobacteria), and Firmicutes (mostly *Bacillus* spp.). Recently, molecular analysis have shown the presence of a large number of different microorganisms involved completely different physiologies (Schabereiter-Gurtner et al. 2002, Gonzalez and Saiz-Jimenez 2005, Portillo et al. 2008). A preliminary study on this topic was presented by Schabereiter-Gurtner et al. (2002) showing the presence of several microbial groups, never detected before in Altamira Cave; these groups were Acidobacteria, Bacteroidetes, Planctomycetes, and Chloroflexi. These groups represented about 45% of the detected microorganisms in that study. In this study of Schabereiter-Gurtner et al. (2002), Proteobacteria represented 50% of the total studied community. Actinobacteria appeared to represent just about 5% of total in contrast to the great abundance found by culturing procedures. Firmicutes, relatively abundant by culturing methods, was not even present in this preliminary study (Schabereiter-Gurtner et al. 2002). The differences generated by the different methods used in the analyses were obvious and highly significant, leading to a change of the perspective related to the conservation of the cave.

More recent and intense surveys of the microbial communities in Altamira Cave have shown the presence of a much large complexity in these communities (Gonzalez et al. 2008). For instance, the group Proteobacteria has been the most frequently detected although it includes representatives from the Alpha, Beta, Delta, and Gamma Proteobacteria. The Alpha and Gammaproteobacteria were the most commonly found. The major components of the microbial communities in Altamira Cave had been identified and were represented by bacteria related to the genus *Sphingomonas* (Alphaproteobacteria), Acidobacteria, Gammaproteobacteria (Enterobacteriales and Pseudomonadales), Deltaproteobacteria (Desulfovibrionales), Betaproteobacteria (Burkholderiales and Rhodocyclales), and Actinobacteria (Actinomycetales). The Actinobacteria represent a low fraction of the total, generally around 5% of total sequences which is in agreement to the results of Schabereiter-Gurtner et al. (2002). From our results, the Firmicutes were also detected using molecular methods although they constitute a low percentage of the bacterial community.

Table 2. Some of the microbial groups rarely or never reported before as important components of the microbial communities in Altamira Cave. Estimate of their diversity, current knowledge of their status, and the level of knowledge on their metabolism are reported.

<table>
<thead>
<tr>
<th>Microbial group</th>
<th>Status of detection</th>
<th>Abundance</th>
<th>Physiology and role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfate-reducing bacteria</td>
<td>Active/ mostly uncultured</td>
<td>Very high</td>
<td>Known</td>
</tr>
<tr>
<td>Low-temperature Crenarchaeota</td>
<td>Active/uncultured</td>
<td>Very high</td>
<td>Mostly unknown</td>
</tr>
<tr>
<td>Acidobacteria</td>
<td>Active/uncultured</td>
<td>Very high</td>
<td>Barely known and highly diverse</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Active/uncultured</td>
<td>Medium/high</td>
<td>Mostly unknown and highly diverse</td>
</tr>
<tr>
<td>Planctomycetes</td>
<td>Active/uncultured</td>
<td>Medium/high</td>
<td>Mostly unknown and highly diverse</td>
</tr>
<tr>
<td>Chloroflexi</td>
<td>Uncultured</td>
<td>Medium</td>
<td>Mostly unknown</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>Active/uncultured</td>
<td>Medium</td>
<td>Mostly unknown</td>
</tr>
<tr>
<td>Nitrospira</td>
<td>Uncultured</td>
<td>Medium</td>
<td>Mostly known</td>
</tr>
</tbody>
</table>
Interestingly, besides the microorganisms frequently reported, a number of microbial groups have never, or sporadically, been reported in this cave (Table 2), even if they constitute a highly significant fraction of the total community. For instance, within the Proteobacteria, the sulfate-reducing bacteria belonging to the Deltaproteobacteria, have never been reported previously and we have detected their presence as metabolically active microorganisms (by RNA analysis). Previous finding on sulfate reducing bacteria have been rarely mentioned. Sulfate reducing bacteria could have important consequences for pigment conservation as previously reported (Portillo et al. 2006). The Acidobacteria have been extensively studied by Zimmermann et al. (2005) although their metabolism and role in the studied cave remains to be investigated.

The Archaea have been never reported in Altamira Cave until the study by Gonzalez et al. (2006). Specifically, the low-temperature Crenarchaeota represent a highly diverse and ubiquitous microbial group in Altamira Cave although their role, threaten to the paintings and metabolism is still unknown due to the difficulty in culturing these microorganisms. Other microbial groups barely known and present in Altamira Cave as metabolically active members of the communities and in a significant proportion of these microbial communities are reported in Table 2. The metabolism of most of these groups is unknown and so, their potential risk for the conservation of the cave is pending of further developments in microbiology.

These last studies that we are carrying out suggest that the actual diversity in Altamira Cave is much higher than shown in the results obtained so far. Figure 1 shows cumulative curves of sequencing results obtained in Altamira Cave during the campaigns carried out in the last years. We have been able to identify the bacterial groups (i.e., at the Division level) present in the cave as determined from molecular analysis based on DNA. When this same analysis is performed on the different microorganisms present in Altamira Cave, we detect that the cumulative curve barely experiments a downwards curvature. This result clearly indicates that the actual number of different microorganisms in Altamira Cave is huge, and much higher than previously imagined, even if those microorganisms belong to already detected bacterial divisions. When the analysis is performed using molecular techniques based on RNA, that is, on those microorganisms showing considerable metabolic activity within the microbial community, then we observe that the number of bacterial divisions detected in the studied cave is similar to that determined from DNA; this suggests that most of the bacterial divisions detected in this cave somehow have representatives with considerable activity. However, when
analyzing the results of the different microorganisms detected in Altamira Cave using RNA-based techniques, we observed that the cumulative curve based on different metabolically active microorganisms levels off significantly suggesting that the number of metabolically active microorganisms from Altamira Cave are not as abundant as those present. Consequently, Altamira Cave presents a huge number of different microorganisms although only a fraction of them are actively participating in microbial development or growth in the cave.

While microbiologists know well that environmental conditions directly influence the development of specific microorganisms, and that a high microbial diversity is present in this cave, unpredictable changes in these microbial communities could potentially occur if the environmental conditions of the cave change. Thus, one of the preliminary objectives in the conservation of caves with prehistoric paintings should be to maintain them in the closest conditions to those that have maintained these caves for thousands of years (Allemand and Bahn, 2005). Mass tourism, illumination set ups, and other modifications to the cave environmental conditions could result in unpredicted changes in the microbial communities, likely to enhance the growth of microorganisms that never had any significance in this cave.

The study of microbial communities in Altamira Cave have revealed the presence of metabolically active microorganisms rarely mentioned in previous studies. Examples are the Crenarchaeota (Archaea), the Acidobacteria and several other bacterial groups (Table 2). These groups represent a significant fraction of the microbial communities. This and the fact that the role of these microbial communities is poorly understood would represent a risk for the conservation of this UNESCO World Heritage site.

Acknowledgements
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References

CHARACTERIZATION OF PHOTOSYNTHETIC BIOFILMS CAUSING BIODETERIORATION

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Introduction
The vital activities of epilithic microorganisms contribute to undesirable changes in works of art. The organisms responsible for biodeterioration build complex structured communities, called biofilms, detracting from aesthetics and eventually inducing chemical and physical damage (Albertano et al. 2003).
Qualitative and quantitative characterization of biodeterioration demands an understanding of the substratum, organisms and abiotic factors implicated (Ariño et al. 1997). Such information may help administrators to choose the appropriate preventive and eradication methods for cultural heritage at risk (Roldán et al. 2003). Currently the study of biofilms has emerged as a powerful new approach for research on microorganisms. Both traditional approaches and molecular information have a strong potential application in the characterization and taxonomy of microorganisms building biofilms. At the same time sound taxonomy will need redescription on the basis of characteristics other than simple morphological traits.

The microscopy techniques most frequently used to determine the diversity of microorganisms in terms of morphology, ecology and physiological adaptations as well as the structure of the biofilms and relationship with the substrata include scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM), apart from transmission electron microscopy and the classical light microscopy. We illustrate a few practical examples, among the great variety of these techniques, to survey photosynthetic specimens and to determine their intrinsic features. Protocols may require modifications according to organism characteristics. All the techniques were performed withdrawing only micro samples, which is important when works of art are involved. In addition several of these techniques can be applied sequentially to the same sample.

In the following sections, details will be given about the different techniques used to obtain all the information available about samples taken from dim light caves and Roman catacombs.

**Microscopy methods: tools and techniques**

**Scanning electron microscopy**

Scanning electron microscopy (SEM) images have great depth of field yielding a characteristic three-dimensional appearance, useful to examine surface topology and distribution of specimens as well as for monitoring the interactions between biofilms and substrates (Figures 1-3).

Clean samples or calcareous investments work well and the procedure is especially helpful for calcite crystals deposited on sheaths or any surface structure (Figure 2).

Figure 1. SEM-micrograph of epilithic and chasmoendolithic rod-shaped microalga, cyanobacteria and diatoms on a strongly rough calcite substratum. (Zuheros cave, Cordoba, Spain).

Figure 2. SEM-micrograph of a biofilm formed by filaments of *Scytonema julianum*, *Leptolyngbya* sp and filamentous actinobacteria. Note the triradiate calcita needles on the sheaths of *S. julianum* (Collbato cave, Barcelona, Spain).

However conventional SEM requires samples to be imaged under vacuum and biological material tend to be susceptible to dehydration. Sheaths and mucilaginous outer layers may be condensed or blur the surface of the specimens. Other types of SEM overcome some of these processing inconveniences. For example, Environmental Scanning Electron Microscope (ESEM) allows the observation of hydrated samples without coating or further process. Scanning microscopes can be coupled to Backscattered Electron imaging (BSE) (Figure 3) or Energy Dispersive Spectroscopy (EDS), both analytical techniques used predominantly chemical characterization (inorganic vs. biological) or for the elemental analysis of a sample (Figure 4).
Figure 3. SEM-BSE images of a calcite stone showing subsurface heavily colonized by microalgae and cyanobacteria (Nerja cave, Malaga, Spain).

Figure 4. SEM-EDS image of a stalactite with clay impurities from decalcification. A. The biofilm consisted primarily of cyanobacteria and microalgae living on the surface but also in fissures and between crystals (chasmoendoliths). B. Spectroscopic data portrayed as a graph plotting x-ray energy vs. count rate. The peaks correspond to characteristic elemental emissions (Collbato cave, Barcelona, Spain).

Confocal Scanning Laser Microscopy

CLSM is a tool for 3-D localization of fluorescent organisms or items dyed with fluorescent labels externally or inside the substrata. The technique provides an efficient way to determine the presence, the viability and the spatial organization of specific organisms. It offers the possibility for non-invasive optical sectioning by subtracting out-of-focus planes of the image. It allows the in situ observations, that is, to examine the surface and the in depth structure of the sample with minimal preparation and without disturbing the structure (Hernández-Maríné et al. 2003, Neu and Lawrence 1997).

In the observation of biofilms Confocal Scanning Laser Microscopy (CSLM) was used for imaging in fluorescence mode. Autofluorescence from photosynthetic pigments was viewed in the red channel using the 543 and 633 nm lines of an Ar/HeNe laser in the emission range of 590 to 800 nm. Extracellular polysaccharides (EPS), or mucilage, were labeled with the broad-spectrum carbohydrate-recognizing lectin concanavalinA-Alexa Fluor 488 (Molecular Probes, Inc., Eugene, OR, USA) (0.8 mM final concentration). Con-A was observed in the green channel using the 488 nm line of an Ar laser in the emission range of 490 to 530 nm. Controls (i.e. without Con-A labeling) were also run to correct for autofluorescence. DNA-selective dye Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA) was viewed in the blue channel using the 351 and 364 nm lines of a UV laser in the emission range of 400 to 480 nm.

We acquired optical sections in both x-y planes obtained at different intervals (z step) along the optical axis. The images were sequentially acquired using the range of excitation and emission wavelengths described above. The image size was 512 x 512 pixels with an 8-bit grayscale. Images are presented as multichannel, extended focus and 3D projections using Imaris software (Bitplane, Zürich, Switzerland). Optical sections were processed with the maximum intensity projection algorithm in which the maximum fluorescence intensities of each x,y point of all the optical series along the z-axis are integrated in a two-dimensional compound image. The projections are used to describe the spatial distribution of organisms within the biofilms as well as differences in biofilm thickness and architecture (Figures 5-7).
Figure 5. Compound images of CLSM (red=pigment fluorescence, green=Con-A labelled EPS, blue=Hoechst 33258 stained nucleic acids) from Zuheros cave. Left. *Chlorella*-like biofilms presented Chlorococcales at the bottom and occasionally scarce diatoms at the top. Three-dimensional extended focus projections in *x*-y, *x*-z and *y*-z views of 42 (step=0.4 µm) sections in the *z*-direction of the biofilm. 16.40 µm total thickness. Right. Biofilm formed by *Chlorophyta*, filamentous and coccal cyanobacteria. Some of the cells presented natural blue fluorescence (arrow). Three-channel SFP: pigments, EPS and DNA of 38 *x*-y optical sections. (*z* step=0.4 µm). Thickness=14.80 µm. Scale bar is 10 µm.

Figure 6. Confocal double-fluorescence ( autofluorescence and EPS) images of filamentous cyanobacteria forming biofilms inside Roman hypogea. 3D extended focus images. Steady state conditions. Porous biofilm of *Leptolyngbya* sp. (73.5 µm total thickness).

Figure 7. Confocal double-fluorescence (autofluorescence and EPS) images of filamentous cyanobacteria forming biofilms inside Roman hypogea. Senescent conditions. Porous biofilm of *Leptolyngbya* sp. (8 µm total thickness).

Subcellular identification of photosynthetic pigments
Confocal microscopy with the lambda-scan function was used to determine the optimal detection and separation of emission spectra for pigments (Roldán et al. 2004 b,c). Wavelength scans were performed using the 488 nm line of an Argon laser. Each image sequence (wavelength scan) was obtained by scanning the same *x*-y optical section using a bandwidth of 20 nm for the emission (the *λ* coordinate of *x*-y-*λ* data sets). The *x*-y-*λ* data set was acquired at the *z* position having maximum fluorescence. Gains and offsets were the same for each field and remained constant throughout the scanning process. The variation in intensity of a particular spectral component was represented on the screen using a false-color scale. Warm colors such as white and red represent maximum intensities, whereas cool colors, like blue, represent low intensities (the intensity of each pixel was set to 256 levels of grey). The image size was 512 x 512 pixels. Mean Fluorescence Intensity (MFI) of the *x*-y-*λ* data sets was measured using Leica Confocal Software, version 2.0. The region of interest (ROI) function of the software was used to determine the spectral signature of a selected area from the scanned image. For the fluorescence analysis, ROIs of 1 µm² taken from the thylakoid region inside the cell were set in each *x*-y-*λ* stack of images. Lambda
scans (n = 20 ROIs) were obtained in at least three independent experiments. The mean and standard error for all the ROIs were calculated (Figure 8).

Figure 8. CSLM images and Lambda-scans of *in vivo* aerophytic biofilm. Optical sections and spectral profiles derived from λexc of 488 nm. A. 3D maximum intensity projections in x-y and orthogonal views in z-direction of the biofilm. The image represents the maximum auto-fluorescence emitted in the range of 590-775 nm (shady area) when excited at 543-nm. The pseudocolor scale is shown at the bottom left. T= Surface of the sample. Scale bar = 10 µm. 98 x-y optical sections of a stratified biofilm with a continuous upper layer of *Chlorella*-like (head arrow) and a discontinuous bottom layer of *Cyanosarcina parthenonensis* (arrow). The volume under observation is 146.77 x 146.77 x 200 µm³. Z step = 0.2 µm. Zoom factor: 2. Thickness: 19.4 µm. B. *In vivo* mean emission spectra of the different species present in the biofilm. The difference emission profiles obtained indicate the presence of different groups of algae and cyanobacteria.

The above microscopy techniques complemented each other, providing an efficient way for determining the presence and viability of biofilms and for the design and monitoring of control strategies for conservation of cultural monuments.

**Diversity and biofilm structure**

**Organisms**

Not all aerophytic endolithic and hypogean environments are identical, but in most of the ones that we have studied specially adapted groups such as bacteria, coccal and filamentous sheathed cyanobacteria and mosses develop, sometimes including diatoms, fungi hyphae and actinobacteria. They build up into biofilms that form highly structured communities and that, in addition to different types of cells, also contain extracellular polymeric substances (EPS), particulate matter and water (Costerton et al. 1999). In the special case of hypogean monuments, biofilms are aerophytic growing at the surface/air interface when light is available (Albertano et al. 1994, Hoffmann 2001, Hernández-Maríné et al. 2003, De los Ríos and Ascaso 2005).

A general pattern in relation to environmental conditions in subterranean monuments could not be determined. However, when substrata are illuminated and the microclimate was strongly influenced by the outdoors, fluctuating throughout the year, microflora colonies were quite rich, with special abundance of mucilaginous and dark coloured biofilms typical from terrestrial aerophytic or atmospheric habitats. The community, sometimes dominated by *Scytonema julianum* in areas protected from rain, was composed of algae and cyanobacteria such as *Gloeothecae* sp. and *Nostoc* sp., among others. *Trentepohlia* sp. and many crustose lichens, which had this alga as a photobiont, were also abundant. Differences in the availability of liquid water and substratum coherence could also explain variations in species composition and patchiness. With moderate abiotic oscillations and low light but not yet a stress factor cyanobacteria were the most visible constituents, occasionally mixed with green algae and diatoms which abundance diminished with decreasing irradiance. With stable abiotic factors in dim light areas only a few cyanobacteria were able to survive, among them the coccial *Gloeocapsopsis magma*, the filamentous *Leptolyngbya* spp. and *Geitleria calcearea* and *Loriella osteophila*, both characterized by the presence of carbonate precipitates on the polysaccharide uncoloured sheaths that surrounded the trichomes (Hernández-Maríné et al. 2001, Roldán et al. 2004b).

The influence of microclimatic conditions, especially light and relative humidity was reflected on the aspect of the biofilms. In fluctuating areas the biofilms were formed by
mucilaginous and dark coloured coccal cyanobacteria, that displayed those protective strategies against desiccation and irradiation. On the other hand, the persistence of humidity at the dew point and dim light in hypogean monuments made the excretion of colored mucilaginous material unnecessary in the more sheltered and dark areas.

**Biofilms**

The biofilms were generally porous, stratified and very heterogeneous in thickness (from 80 to 550 µm), as determined by the three dimensional projections from autofluorescence and EPS (Figure 2). Interestingly, the reaction of morphologically similar phototrophic cells to Con-A varies with their relative position inside the biofilm. For the majority of biofilms, the EPS were most abundant at the upper layer (Figures 5-7).

Living phototrophic and the heterotrophic microorganisms were distinguished by coupling the results from Hoechst 33258 DNA staining with those from pigment autofluorescence studies. The nucleic acid stain does not show an extended heterotrophic bacterial community in the biofilms that were fluorescent and well developed. However, in several brown biofilms, predominantly on hard substrata, some senescent and weakly fluorescent cyanobacterial cells were observed. Moreover, heterotrophic bacterial communities were highly developed in zones having weak photosynthetic pigment fluorescence.

**Pigments**

Species belonging to one phylogenetic group, such as Cyanobacteria, Bacillariophyta or Chlorophyta, showed spectrophotometric profiles distinguishable in shape and pigment absorption maxima from other phylogenetic groups. Chlorophyll α has been identified in all photosynthetic organisms. In addition, most cyanobacteria use phycobiliproteins (phycoerythrin, phycocyanin and allophycocyanin) to capture light energy and then pass it on to the chlorophylls. Among cyanobacteria, phycoerythrin- and non-phycoerythrin containing species were easily discriminated. In general, when present, green algae and diatoms were on top of the biofilms whereas coccal cyanobacteria such as *Nostoc punctiforme* or *Gloeocapsopsis magma* were able to develop under the canopy of filamentous or inside the cracks and fissures. Green algae only developed in fissures or inside the rocks near lamps or in highly illuminated areas.

**Conclusion**

The biological growth on stone, although not necessarily harmful, is thought undesirable and generally referred as unwanted. The mucus-like biofilms strongly adhere to substrates, immediately detracting from aesthetics, and eventually inducing chemical and physical damage (Albertano et al. 1995, Ariño et al. 1997, Hernández-Mariné et al. 2001, Roldán et al. 2004a). The process depends on environmental conditions, nutrients and mineralogical type and stone surface topography.

The long-term preservation of cultural heritage requires a holistic approach (Kumar and Kumar 1999, Warscheid and Braams 2000). Most of the treatments to remove phototrophic biofilms consist of cleaning and biocide applications which often have undesirable lateral effects. Hence there is a pressing need to further develop techniques to monitor the natural conditions in which phototrophic biofilms grow, and to evaluate the qualitative and quantitative changes that preventive or eradicative treatments have on biofilms.

SEM and CSLM are advantageous for use in protecting cultural patrimony sites because they allow the *in vivo* exploration of very small samples and provide information in real time and space. Moreover, the laser penetration allows epilithic and endolithic microorganisms, as well as nearby minerals to be simultaneously recognized on the valuable surfaces. In addition, when coupled to wavelength scans the main features of the technique are: (i) analysis for both global and single fluorescent pixels, providing their three dimensional localization *in vivo*; (ii) direct analysis of fluorescent pigments from a single *cell in situ* in thick samples without isolation; (iii) establishing a simultaneous relationship between fluorescence properties, morphology and position inside complex microbial assemblies; and (iv) discrimination of cells with particular fluorescence signatures within the colony, and correlation with individual cell states.

The information obtained about the three-dimensional structure of the biofilms, the microorganisms forming each of its layers and
their respective reaction to mechanic and chemical treatments and their role in the biodeterioration processes can only be understood as a whole and needs to be done with very little manipulation of the precious works of art. ESEM and CSLM allows for each of these conditions and has the additional advantage of avoiding pre-treatments of the samples that could give false impressions of the whole living community.

References

BACTERIAL BIOMINERALIZATION APPLIED TO THE PROTECTION-CONSOLIDATION OF ORNAMENTAL STONE: CURRENT DEVELOPMENT AND PERSPECTIVES

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Introduction

The deterioration of ornamental stone, as well as other materials used both in sculpture as well as in the construction of historical buildings or in new works, has in recent times accentuated due to environmental pollution and acid rain, especially in urban and/or industrialized areas. In the last few decades, awareness has grown concerning the importance of these types of problems because, in a great number of cases, key architectonic monuments have been degraded and even lost.

During this time, conventional treatments, both inorganic was well as organic, used for the consolidation and/or impermeabilization of these deteriorated materials have yielded poor results because of the incompatibility between the stone or material treated and the nature of the material applied in the treatment. In many cases, treatments have in fact proved harmful by accelerating the alteration (Clifton 1980, Torraca 1976), (Figure1).

An attempt to use treatments more in line with the nature of these materials has, in the last few decades, directed attention to biomaterials, generally carbonates, produced

In the case of bacterial biomineralization, the most common process is induced biomineralization (Lowenstam and Weiner 1989), with certain exceptions (such as the production of magnetite by magnetotactic bacteria) (Bäuerlein 2003), and this process is commonly extracellular, occurring in the area surrounding the cell, often leading to the mineralization of the bacterial cells (i.e., Ehrlich 1999, Rodriguez-Navarro et al. 2007).

Bacteria can change the chemistry in their surroundings by the release of metabolites. This, together with the contribution of structures (i.e., cells, cell membranes or cell debris; González-Muñoz et al. 1996, Schultze-Lam et al. 1996) that would act as nuclei for heterogeneous crystallization, lead to mineral precipitation. Therefore, the precipitation of minerals induced by bacteria can take place under conditions in which precipitation would not occur in their absence. In addition, the types of minerals that the bacteria can produce are very diverse (i.e., calcium oxalate, silicates, calcite, apatite; Ben Omar et al. 1997) and consequently so are the materials that can be cemented (consolidated) by the deposition of a broad range of compatible minerals. Nevertheless, applied research on cementing agents has to date focussed mainly on consolidation by calcite deposition, partly because bacterial carbonatogenesis is a widespread phenomenon among bacteria, occurring through many metabolic pathways. However, for the problem at hand, it is generally accepted that bacteria suited for stone-consolidation treatments are aerobic heterotrophs or microaerophiles (Castanier et al. 2000). In general, metabolic pathways able to increase the environmental pH toward alkalinity can, in the presence of calcium ions, foster calcium carbonate precipitation.

Some authors consider that these processes represent a method to produce just a sacrificial layer, being the part most exposed to the deteriorating action of physical, chemical, and biological agents. However, other authors think that these biomineralization processes also create a calcium-carbonate cement that consolidates and protects the material being treated.

Consequently, the newly formed carbonate: 1) reduces the water permeability of the stone while maintaining its transpiration to permit gas exchange; and 2) promotes a greater cementing of the mineral particles that make up the stone, conferring it greater resistance. These processes should occur, moreover, without altering the colour of the stone and without plugging its natural pores. In this sense, noteworthy results have been achieved by Rodriguez-Navarro et al. (2003) treating calcarenite with cultures of Myxococcus xanthus, in which case the original porosity of the stone was 28% and that of the treated stone 26%, with almost no alteration of the mean pore-size distribution. With regard to stone consolidation, after the use of different culture media, treated and untreated samples exposed to five 5-min cycles of ultrasonic treatment showed that the untreated stone lost somewhat more than 0.9% of its weight while the samples treated with M. xanthus in one of the media did not reach 0.4% loss, and another remained below 0.6%. Figure 2 shows two samples of Macael marble (Almería, Spain), one untreated and the other treated, following the protocol described for calcarenite in the work of Rodriguez-Navarro et al. (2003). The newly formed calcite crystals are visibly very compact, and grow epitaxially on the substrate.
Despite of the many investigation carried out on this problem and the goodness of the results obtained, some researchers call for attention to the drawbacks that these types of treatments can have. For example, the supply of nutrient media to the stone or to the construction materials with the end of encouraging the bacterial growth of those able to produce the precipitation of CaCO$_3$ could promote the development of undesirable microbiota that could later harm the treated material (Perito et al. 2000).

Researchers and European projects

A great number of researchers, from one standpoint or another, work on these types of processes some of them working within the framework of a series of European projects. As representatives of different lines under development, the following ones have been chosen, from which a brief commentary will be provided:

- **Research team of Adolphe, Castanier, Loubière, Le Métayer-Levrel and collaborators (French group).**
- **Research team of González-Muñoz and Rodríguez-Gallego (group of the Universidad de Granada, Spain).**
- **Group of Verstraete (Ghent University, Belgium).**
- **Group of Bang (South Dakota School of Mines and Technology, USA).**
- **Project BIOBRUSH (http://www.heritage.xtd.pl/pdf/Bio_3_May.pdf), coordinator Eric May (University of Portsmouth, UK).** Within the framework of this project, the group of Claudia Sorlini (Università degli Studi di Milano, Italy).
- **Project BIOREINFORCE (http://www.ub.es/rpat/bioreinforce/bioreinforce.htm), coordinator Piero Tirano (Istituto por la Conservazione e la Valorizzazione dei Beni Culturali, Sesto Fiorentino, Italy).** Within the framework of this project, the group of Brunella Perito and Giorgio Mastromei (Università degli Studi di Firenze, Italy).

**French group.** The large group of French researchers, including, among others, Adolphe, Castanier, Loubière, Le Métayer-Levrel, have been working on applying bacterial biomineralization to the consolidation of stone since the 1980s. These works have led them to develop two patents (European Patent n° 90400G97.0, Adolphe et al. 1990; French Patent n° 9505861, Castanier et al. 1995) and, based on these, have developed the process CALCITE. With these patents, they support the use of any microorganism for the restoration of stone materials, though in the CALCITE process the bacterium used is *Bacillus cereus*. The data offered by these authors indicate that the treatments made (e.g. in the tower of the church Saint Mèdard and in the castle walls of Champs-sur-Marne, both structures from the 12th century) have the following effects: do not notably alter colour; slightly reduce the original roughness of the stone; provide a calcite film for a few micrometers; reduce water permeability; the general biotope is not changed; the bacteria provided by the treatment are well calcified; the undesirable microbial populations decreased; the number of fungal spores are augmented (data taken from the Spanish version of the CALCITE procedure).

The works of the research group of the **Universidad de Granada** present treatments that improve the results shown by the French group. In this sense, a coherent carbonate cement of 10–50 µm coated the treated stones and this cement was rooted down to a depth of 1 mm while, at the same time, stone porosity remained unaltered (Rodriguez-Navarro et al. 2003) as commented above. In addition, this group has investigated the role of microbiota in stone when the treatment is applied. The culture media used by these authors activate, among the microbiota inhabiting the stone, those bacteria able to induce the precipitation of calcium carbonate. Such a precipitation notably contributes to the consolidation of the stone (Jimenez-Lopez et al. 2007). In some of these treatments, the new calcium carbonate produced was rooted down to a depth of 5 mm (unpublished data).

**Group of Verstraete** proposes: (a) the use of diverse species of *Bacillus* selected on the basis of their capacity to produce urease, of the bacterial membrane potential $\zeta$ at pH 9.0, and of the production of a biofilm (Dick et al. 2006); and (b) a treatment method that proceeds sequentially and in alternating cycles (1) production of the biofilm and (2) CaCO$_3$ precipitation. The application of the treatment was made on Euville limestone (from quarries of the “Département de la Meuse” in France). These researchers conclude that “*B. sphaericus* strains with a very negative $\zeta$ -
potential, a high initial urea degradation, and a continuous formation of dense calcium carbonate crystals are most suitable for coherent calcite production on degraded limestone. These strains also significantly decreased capillary water absorption of the treated limestone.”

Along another line, the Group of Bang has investigated the microbiological remediation of concrete cracks and demonstrated the improvement of compressive strengths of cement mortar cubes in the presence of microorganisms and in particular reported that the “physicochemically versatile Poly Urethane has shown to be an effective enhancement tool in microbiologically induced calcite precipitation in concrete cracks” (Bang et al. 2001). In this work, they utilized B. pasteurii and suggest that the role of urease in CaCO3 precipitation is paramount.

European project BIOBRUSH (2002-2005).
Some of the objectives proposed were:
1: Collection, cataloguing and analysis of stone samples from historic buildings and monuments across Europe, showing evidence of salt deposits and incrustations.
2: Selection, screening and identification of bacterial cultures for use in bioremediation.
3: Evaluation of delivery systems to carry biological agents onto the stone.
4: Assessment of mineral changes in stones during bioremediation treatment in laboratory studies.
5: Assessment of the effect of the bioremediation process on bulk-stone properties in laboratory studies with stone cubes and blocks.
6: Field trials of bioremediation on buildings and monuments in Europe.”

European project BIOREINFORCE (2001-2004).
“The objective of the project is to develop and validate a new methodology for monumental stones conservation based on biomineralization processes. This could satisfy the request for more durable and safer products in order to reduce the costs, delay the maintenance interventions and pose no risk both for the personnel and the environment, conciliating the end-users and stakeholders with the application of innovative treatments. The molecular biology and the bacterial genetic engineering are the innovative technologies chosen to improve the bio-mediated calcite precipitation method. These tools will be applied for finding the genetic expression of crystal formation in bacteria. This will be cloned and the bio-inducing proteins will be overproduced by an appropriate expression vector (host cell). With
these bio-derived low cost renewable macromolecules, a Bio-Mediated calcite Treatment (BMT) will be developed for the stone reinforcement, due to new calcite precipitation inside its pores. The BMT will be finally validated, by end-users, in monumental test-sites applications.”

As stated above, some authors such as Perito et al. (2000) suggest that negative processes may occur as a consequence of the application of bacterially-inoculated culture media. Therefore, in the execution of this European project, in accord with its objectives, alternative methods have been proposed and developed in what could be considered two research lines. One of these is the one followed by the group of Piero Tiano. These researchers have investigated, in particular, the growth of new calcite crystals inside stone pore space with a biomineralization process induced by the “Organic Matrix Macromolecules (OMMs)” extracted from marine shells and by Polyaspartic acid (Poly A) (Tiano et al. 2006), in the absence of living cells. In their study, these authors present excellent results when the treatments are applied under laboratory conditions, but monumental test-sites have not rendered the results expected. Also, the drawbacks of applying organic matter do not appear to be avoided, since the treatments used (OMMs or Poly A) could foment the development of heterotrophic microorganisms that may use these macromolecules as a carbon, nitrogen, and energy source.

The other line, developed by the group of Brunella Perito, has sought to identify the genes involved in bacterial carbonatogenesis with the aim of large-scale production of the corresponding macromolecules for use in free applications of bacterial cultures. These authors, in a recent publication (Barabesi et al. 2007), show that Bacillus subtilis has an operon, icfA, which is involved in calcite precipitation, and that the gene etfa is essential for this precipitation. On the other hand, as a consequence of the research in this work, they suggest the possibility of a link between calcite precipitation and fatty-acid metabolism. The extraordinary genetic work done by these authors does not, however, allow the identification of the macromolecules implied in the carbonatogenesis process.

Consequently, the development of a large-scale method based on low-cost renewable macromolecules for the reinforcement of the stone is not possible at the present.

**Conclusions and new perspectives**

In consideration of the above in relation to the use of microorganisms for bioremediation, it can be stated, in agreement with Alison Webster and Eric May (2006), that “although the technology is still in its infancy and, therefore, not readily available, the results so far indicate that it promises to offer a viable alternative to those working to preserve our cultural heritage”. In addition, the research performed to date will indicate paths that are useful to follow and those that are not. The data indicate that bacterial carbonatogenesis processes are appropriate and promising for treating and reinforcing altered stone and it is advisable, in order to proceed in a suitable manner and to avoid undesirable secondary effects, to investigate in depth the following aspects: 1) nutritive solutions used as bacterial culture media, so as to help activate carbonatogenic microbiota while inhibiting the development of microbiota harmful to the stone; 2) the role of the formation of biofilm, to study the cases in which its presence might be positive and in which cases it could provoke the inappropriate loss of porosity and/or provoke undesired supply organic matter.

In addition, a consensus should be sought among researchers working in this field in order to establish both the type of characteristics or parameters that should be evaluated as well as the techniques to undertake such an evaluation. This would enable the effective comparison of the treatments applied by the different researchers. I suggest that evaluations should be made on measurement of the porosity and water uptake, surface hardness, stone cohesion, and chromatic coordinates of the stone surface. In terms of the techniques to be used, a distinction should be made between those exclusively for laboratory treatment (testing laboratory samples) and those that could be used also in the field (validation of treatments on monumental test-sites). To date, the most adequate appear to be those used by Rodriguez-Navarro et al. (2003) and Tiano et al. (2006):
Finally, I would also like to mention the works that in recent years have questioned the paradigm of controlled biomineralization: i.e., DiMasi et al. (2003), Nassif et al. (2005), Yu et al. (2005). In this regard, the question of DiMasi et al. (2003): “When is template directed mineralization really template directed?” is truly provocative. I think that it is interesting to go further in this research, since it could provide new insights regarding consolidation treatments based on biomineralization processes.

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References


The application of viable bacteria for the biocleaning of cultural heritage surfaces

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Microorganisms generally are considered biodeterioration agents. Indeed they are often responsible of alteration of works of art, but they can be also used as means of conservation. In fact during the last ten years a new technology, based on the use of microorganisms to clean the surfaces of altered works of art, has been set up.

The application of this new technology to stone monuments and frescoes gave very interesting results.

The procedure used for this application consists on: (1) the selection of appropriate bacterial strains that must be effective in the alteration materials removal and safe for the works of art, operators and environment; (2) the selection of the delivery system effective to immobilise the bacteria and adapt for an easy application also to vertical surfaces and vaults; (3) to set up the procedure of delivery system application and to define the duration of the treatment; monitoring of environmental parameters during the application; (4) removal of the bacteria after the treatment and long-term monitoring of the treatment effects.

Selection of the bacteria
The bacteria used in these cases are, respectively: (a) sulfate-reducers, able to convert sulfates to hydrogen sulfide; initially, Desulfovibrio desulfuricans has been used, but subsequently it has been substituted with Desulfovibrio vulgaris subsp. vulgaris, because the latter showed a better tolerance to the oxygen; (b) nitrate-reducers, able to convert nitrates to molecular nitrogen; among the bacteria of this functional group a Pseudomonas stutzeri has been selected among the several bacteria tested; (d) aerobic heterotrophic bacteria able to attack and degrade synthetic undesired polymers. In this last case the applications are not yet performed on the real works of art,

Until now viable cells of bacteria can be applied to remove the following alterations and undesired materials from the surfaces: (i) “sulfatation” of calcareous stones, resulting in black, brown and grey crusts; (ii) “nitratation” resulting in disgregation and pulverization; both alterations are caused by atmospheric pollutants on the stones and, in the case of “nitratation”, also by the capillary rise of water rich in nitrates in the walls; (iii) layers of organic patinas used in particular techniques of frescoes detachment from the walls; (iii) residues of synthetic polymers during conservation treatments in order to protect the works of art. (Figure 1).

Figure 1. Mechanism of action of bacteria.
but only on samples in laboratory. The products of their transformations are gaseous molecules that evolve in the atmosphere without leaving residues on the treated surfaces.

**Delivery system**

Regarding the materials for delivery systems, several matrices have been tested and among all in a first time an inorganic material, namely sepiolite powder, had been selected. Subsequently this material has been substituted with an organic support matrix, the carbogel, a powder that, after addition of a bacterial suspension, originates a gel that entraps the bacteria.

**Conditions of applications**

During the application, temperature and humidity were monitored; in fact environmental conditions affect the activity of microorganisms and, as a consequence, the time necessary for the treatment.

The duration varies according to the chemical composition of the material and the thickness of the layer to remove. To remove organic glue from the surface of frescoes 12 hours were enough, while to remove, for example, sulfates from the stones, 15-36 hours were necessary; when the thickness was particularly high a second and – sometimes – a third treatment was required.

**Final monitoring**

At the end of the treatment bacteria must be removed and the surface polished with care in order guarantee the absence of bacteria. This is a precaution; in fact bacteria are applied under nutritional conditions that do not allow their growth. In some cases after one or more month new monitoring is performed in order to verify the absence of bacterial cells used in the treatment.

Several biocleaning intervention have been carried out in situ mainly to remove black crusts from: the base of the Pietà Ronadanini by Michelangelo Buonarroti (Castello Sforzesco, Milan, It); some areas of the church façade of S. Maria delle Grazie (Milan, It); statues of the Castle of Buon Consiglio at Trento (It); some areas of the façade of the cathedral of Milan (It); removal of layers of nitrates has been carried out from the external walls of the cathedral of Matera (It); organic glue layer have been removed from 20 square meters of the frescoes of Spinello Aretino in the Monumental Cemetery of Pisa (It) (Ranalli et al. 2005). Sulfates were removed up to 98% and the black crusts were eliminated (Figure 2); also nitrates were drastically reduced (up to 80%).

All the biocleaning interventions carried out in situ have been performed in agreement and under the control of the local authorities of the Cultural Heritage Ministry, that shows an interest to experiment new techniques for conservation.

![Figure 2. Result of the bioremoval of a black crust rich in sulfates.](image-url)

In order to verify the performances of the biocleaning compared with a traditional chemical cleaning method, a in situ experiment was carried out on a lunette of Candoglia marble of the cathedral of Milan. Biological treatment was based on the use of *D. vulgaris* suspended in a pH 7 phosphate buffer embedded in carbogel; chemical treatment on the use of an ammonium carbonate-EDTA mixture. The duration of the treatment, environmental conditions and the alteration (a black crust) were the same.

After the application, the results of the biocleaning were evaluated on the basis of the criteria proposed by Vergès-Belmin: conservation of the *patina noble* (mainly made of oxalates which have a preservative function); absence of physical and chemical harm; homogeneity of removal of the surface deposits; persistence of the cleaned state; efficiency of cleaning; colour; aesthetics. The results showed that the chemical cleaning 1) caused a non-homogeneous removal of the black crusts from the surface; 2) attacked the oxalates patina; 3) eliminated gypsum among...
the marble grains resulting in decohesion phenomena; 4) formed undesired sodium sulfate crystals.

On the contrary, the biological cleaning showed: 1) a better and more homogeneous removal of black crusts; 2) no attack to the oxalates patina; 3) no attack to the sound stone.

After the treatment, the presence of calcite was evidenced on both biologically and chemically cleaned surfaces. However, it is claimed that biocalcite is better than calcite chemically formed. The bioprecipitation was considered a significant advantage of the microbiological activity, making the biological treatment not only a cleaning procedure but also a consolidation treatment (Cappitelli et al. 2007).

Granted that the bacteria for biocleaning can be used only in the cases above listed, on the basis of the experiences acquired, it is possible to claim that: in comparison to traditional methods (chemical and mechanical treatment), the biological treatment is non-invasive, extremely selective, and friendly for the environment; in addition, it does not require equipment. Moreover, viable cells of bacteria are able to produce inducible enzymes when they get in contact with the molecules to be broken down; these enzymes are not available on the market (Antonioli et al. 2005).

Companies, with competence in the field of the biotechnologies and in the microbial biomass production, have shown a great interest in the production and marketing of these bioformulates. We think that this biotechnology could offer new employment opportunities for the young microbiologists working in the field of the cultural heritage conservation.

References

Congress Announcement

Taormina-Naxos (ME, Italy), October 6th to 11th, 2008.

The IBBS-14 Symposium is dedicated to different topics dealing with biodeterioration and biodegradation of organic and inorganic materials including cultural heritage objects, medical devices, corrosion of metals and other applied aspects such as hydrocarbons and pollutants biodegradation and techniques applied to the study of microorganisms involved in these processes.

It takes place every three years, assembling scientists from different parts of the World. It is the first time this Symposium has been organized in Italy: and Sicily is a wonderful place to stay combining the scientific aspect of the Symposium with the unique landscape, typical Sicilian food and works of art of different époques (from prehistorique to modern time). Attendants of this Symposium will enjoy all these aspects.

The IBBS-14 scientific presentations, the poster discussion and workshops are located close to each other in a resort village and will allow close contact among the participants to discuss their own experience in addition to the scientific sessions.