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Characterization in the archaeological excavation site of heterotrophic bacteria and fungi of deteriorated wall painting of Herculaneum in Italy

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Abstract

Microbiological characterization of frescos in four different locations (Collegio degli Augustali, Casa del Colonnato Tuscanico, Casa dello Scheletro and Casa del Gran Portale) of excavation sites of Herculaneum was carried out. The use of infrared thermography allowed detecting sample points on frescos with greatest moisture not visible to the naked eye, resulting in structural damage. The microclimatic conditions provided perfect habitat for bacteria and fungi, particularly of spore forming and mould. In fact, heterotrophic bacteria were prevalent in all wall paintings monitored (ranging from 18 ± 2 CFU 100 cm^{-2} to 68 ± 4 CFU 100 cm^{-2}), whereas fungi were also detected but at lower levels (ranging from 9 ± 2 CFU 100 cm^{-2} to 45 ± 3 CFU 100 cm^{-2}). Cultural–based method allow us to identify by 16S and 26S rRNA partial sequence analysis heterotrophic microorganisms belonging to different genera of Bacillus and Aspergillus, Penicillium and Fusarium together with the unusual genera as Microascus and Coprinus. By using this approach, Bacillus-related species (*B. cereus/B. thuringiensis* group, *B. simplex/B.muralis* group, *B. megaterium* and *B. subtilis*) were isolated in all sample points analysed with the exception of the Casa dello Scheletro in which *Micrococcus luteus/Arthrobacter* sp. group and *Streptomyces fragilis* were found. DGGE analysis of PCR amplified V3 region of rDNA from DNA directly recovered from frescos samples, enabled identification of bacterial species not identified using culturable technology as those closest related to Microbacterium group, often associated with Brevibacterium, Streptomyces and Stenotrophomonas. Combination of culture-dependent and independent methods provided better microbiology characterization of heterotrophic microbiota present on the surface of ancient frescos of this important archaeological site.

Key words

Excavation site, Herculaneum, Microbial induced deterioration, DGGE,

Introduction

Herculaneum was badly damaged by an earthquake in 62 and in 79 A.D. It was buried by a mud avalanche following the eruption of Vesuvius, which since hardened into tufa rock and formed a stratum of approximately 12-18 m of rocky lava. It is a prime example of an archaeological site in which the biodeterioration is due to the rapid change in physical conditions when a soil layer is removed from long-buried objects of art. During excavation, the first

damage is caused by man and then by the change in environmental conditions, such as light, moisture and temperature, which encourage the growth of microbial biodeterioration agents. Since, during the biodeterioration process, the organisms involved are usually a selection of those occurring in the soil, results from soil analysis may be relevant to deterioration studies (Gorbushina and Krumbein, 2000; Dornieden *et al*., 2001; Walsh, 2001). The frescos represent a variety of ecological niches where primary colonization is due to photoautotrophic and chemolithoautotrophic microorganisms, followed by secondary colonization due to heterotrophic bacteria that obtain nutrients from various sources (metabolites of autotrophic bacteria, airborne organic contamination, animal faeces and organic compounds in the paint layers themselves). Microbial-induced deterioration processes cause structural as well as aesthetic damage to wall paintings such as the discoloration of materials, the formation of crusts on surfaces and the loss of material (Sarro *et al*., 2006).

Traditionally, the study of microflora which causes this biodeterioration, was based on classical cultivation methods, that were mainly useful to study the physiological potential of microorganisms but do not provide information on all microbial communities to be found in excavated artefacts.

Indeed, many studies using culture-independent techniques, which cultivate microorganisms from different environments, may often represent only minor components of the microbial community as a whole. It is generally accepted that cultivation methods recover less than 1% of the total microorganisms present in environmental samples (Ward *et al*., 1990; Amann *et al*., 1995). Hence microbial investigations based only on cultivation strategies cannot be regarded as reliable in terms of reflecting the microbial diversity present in objects of art. Damage is also known to have been caused by restoration techniques, which in many cases have proved unsuitable. Therefore to ensure proper restoration and conservation it is essential to identify the complete microbial community. Without this information, current restoration efforts can conceivably have the opposite effect, *i.e*., stimulate microbial growth, and thereby accelerate the deterioration process (Bianchi *et al*., 1980; Rolleke *et al*., 1996). Therefore an inventory of the existing microorganisms associated with damage to the paintings is a prerequisite for including biodecay as an integral part of the restoration process. Wagner *et al*. (1994) demonstrated that conventional microbiological efforts can fail to isolate all microorganisms present in natural samples. DNA-based techniques used to identify microorganisms have revealed only a smaller fraction of bacteria isolated from artworks (Giovannoni *et al*., 1990; Ward, *et al*., 1990; Rolleke *et al*., 1996).

Bacterial identification using molecular techniques, especially those including the sequencing of genes coding for ribosomal 16S rRNA, is very important to study bacterial communities found on artefacts. One such method is Denaturing Gradient Gel Electrophoresis (DGGE) of PCR-amplified gene fragments coding for rRNA (Muyzer *et al*., 1993) that allows the separation of partial 16S rDNA amplified fragments of identical length but different sequence due to their different melting behaviour in a gel system containing a gradient of denaturants. As a result, a band pattern is obtained, which reflects the complexity of the microbial community. The use of this method prevents the cultivation of microorganisms before identification when the DNA is extracted directly from the original fresco material and is then amplified by PCR for identification.

The aim of this study was to investigate heterotrophic microbiota of four houses at archaeological excavation site in Italy. For this purpose the molecular approach was adopted, using culture-based and culture-independent techniques, including the amplification of 16S rDNA, analysis of the bacterial community by PCR-DGGE and sequence analyses.

Materials and Methods

Site description and sampling: Investigations were carried out at four different houses in archaeological site of Herculaneum on the Bay of Naples, Italy. Table 1 summarises sampling points (A1, A2, CT4, CT5, CS6, CS7, GP8, GP9) at the four houses and description of damage. For the identification of sampling points, together with the observation of the alterations present on the wall paintings, was employed the infrared thermography (infrared camera IR FlexCam™, TVS- 700). This allowed the detection of the areas with higher moisture, recognised as heat loss by the infrared scanning of wall. Sampling was performed according to Italian legal procedures (DL 3/80) using sterile swabs (10 x 10 cm^2) or, if possible, sterile tweezers by tearing out surface material. The samples were suspended in 9 ml of physiological solution, refrigerated at 4°C and immediately transported in the laboratory for the analysis. This suspension was considered as first dilution and used for microbiological enumerations or direct isolation of microbial DNA.

Culture-dependent Method

Microbiological analysis: Total aerobic heterotrophic bacteria, actinomycetes, ammonia-oxidizing, nitrite-oxidizing, sulphur-oxidizing, ammonifiers and free nitrogen-fixing bacteria were detected at 28°C in liquid and agar media according to Italian law (DL Normal 9/88). Moulds were counted on malt extract agar (MEA) plates (Oxoid) supplemented with chloramphenicol (0.002 gl^{-1}) and DRBC Agar (Oxoid). All tests were carried out in triplicate. Microbiological data were expressed as CFU or MPN 100 cm^{-2}.

Phenotypic characterisation: Isolated colonies of bacteria and mould were randomly isolated from the counting plates on the basis of their colony-morphology (dimension, edge, colour, elevation, consistency), purified by streaking in the same growth medium and differentiated by assessing their micro-morphology (phase-contrast microscopy) and biochemical characteristics (Gram-stains, KOH-lysis and catalase activity) for bacteria.

16S rDNA partial sequence of bacterial isolates: Representative isolates from different groups obtained after phenotypic characterisation were submitted to 16S rDNA partial sequence. DNA isolated by InstaGene™ Matrix (Bio-Rad Laboratories, Hercules, CA) according to the supplier's

recommendations, was stored at -20°C until analysis. 5 ml of DNA (approximately 50 ng) were used as template for PCR assay. Synthetic oligonucleotide primers described by Weisburg *et al.* (1991), fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') were used to amplify the 16S rRNA gene. PCR mixture and conditions were performed as previously reported (Blaiotta *et al.*, 2002). The amplification was carried out in a PTC-100 thermocycler (M J Research Inc.). The presence of PCR products was ascertained by agarose (1.5% w/v) gel electrophoresis, at 100V for 2 hr, purified by using a QIAquick gel extraction kit (Qiagen S.P.A., Milan) and sequenced by using the primer fD1 (Weisburg *et al*., 1991). The DNA sequences were determined by the dideoxy chain termination method by using the DNA sequencing kit (Perkin-Elmer Cetus, Emeryville, CA) according to the manufacturer's instructions. The sequences were analysed by MacDNasis Pro v3.0.7 (Hitachi Software Engineering Europe S. A., Olivet Cedex, F) and compared to the GenBank nucleotide data library using the Blast software at the National Centre of Biotechnology Information (Altschul *et al*., 1997) in order to determine their closest phylogenetic relatives.

Sequencing D1/D2 region of 26S rDNA of fungal isolates: Fungal genomic DNAs were isolated by using DNAZOL Reagent (Invitrogen) according to the supplier's recommendations. Synthetic oligonucleotide primers described by O'Donnel (1993), NL1 (GCATATCAATAAGCGGAGGAAAAG) and NL4 (GGTCCGTGTTTCAAGACGG) were used to amplify the D1/D2 region of 26S rDNA. The 50 µl PCR reactions containing 50 ng template DNA, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.2 µM of each primer, 2.5 U *Taq* polymerase (Invitrogen) and 1X Buffer (Invitrogen), were carried out in a MyCycler thermocycler. The following program was applied: 5 min at 95°C, 30 cycles of 1 min at 95°C, 45 s at 55°C and 1 min at 72°C and a final extension at 72°C for 7 min.

PCR products were purified by using a QIAquick gel extraction kit (Qiagen S.P.A., Milan) and sequenced by using the primer NL1. The DNA sequences were determined, analysed and compared to the GenBank nucleotide data library as above described.

Culture-independent Method

DNA isolation from samples: For DNA isolation from samples directly collected from frescos, 2 ml of surface material suspension were removed and centrifuged at 14000 x g and the pellet was washed with 2 ml of SE buffer (25 mM EDTA, 75 mM NaCl, pH 8.00). The pellet was then resuspended in 500 ml of buffer ET (100 mM EDTA, 10 mM Tris, pH 8.00) and 50 µl of Lysozyme (50 mg ml^{-1}) were added. The mixture was incubated at 37°C for 1 hr. After addition of 3 ml of Pronase E (10 mg ml^{-1}) and 40 µl of SDS solution (25%) incubation was extended for 0.5 hr at 37°C. One vol. of ammonium acetate 5M was then added and the mixture was incubated at –20°C for 30 min. After incubation, the sample was centrifuged at 14000 x g at 4°C for 30 min. The supernatant was captured and one vol. of isopropanol was added. The mixture was then centrifuged at 14000 x g at 4°C for 30 min. Finally, the pellet was air-dried and resuspended in 100 ml of 10 mM Tris-HCl, pH 8.00. The resulting purified DNA was stored at –20°C until use.

Amplification of the16S rRNA V6-V8 region: PCR primers V6F (5'-AACGCGAAGAACCTTAC-3') and V8R (5'-CGGTGTGTACAAGACCC-3') (Nubel *et al*., 1996) were combined to amplify the segment of eubacterial 16S rDNA from nucleotide 968 to nucleotide 1401, respectively (*Escherichia coli* numbering). A GC-clamp was added to the forward primer according to Muyzer *et al*. (1993). A touchdown PCR was performed in which the annealing temperature was decreased from 63 to 54°C at a rate of 1°C every cycle followed by 25 additional annealing cycles at 53°C. A denaturation step of 94°C for 1 min was used, and extension was performed at 72°C for 1 min; a final extension step at 72°C for 5 min ended the amplification cycle. 5 µl DNA (50 ng), 20 picomoles of each primer, 5 nmol of each deoxyribonucleoside triphosphate, 50 nM of $MgCl_2$, 5 µl of 10x buffer and 0.5 U of *Taq* DNA polymerase (Invitrogen) were combined with H_2O to a volume of 50 µl.

DGGE analysis: PCR-amplified segments of 16S rDNAs were analyzed by DGGE according to Muyzer *et al*. (1993) by using a Bio-Rad DCode Universal Mutation System (Bio-Rad Laboratories, Richmond, CA). Samples were loaded in a 0.8-mm polyacrylamide gel to (7% [wt/vol] acrylamide-bisarylamide [37.5:1] by using a denaturant gradient from 25 to 50% and100% denaturant gradient was 7 M urea plus 40% [wt/vol] formamide) increasing in the direction of electrophoresis. Electrophoresis was performed at a constant voltage of 200V and a temperature of 60°C.

Sequencing of DGGE bands: DGGE bands were excised from the gel with sterile scalpel, transferred into 20 µl of sterile water and incubated overnight at 4°C to allow diffusion of the DNA. 2 µl of the eluted DNA was used for re-amplification by using the PCR conditions above described. The PCR products obtained were checked by DGGE; DNA amplified from swabs samples was used as control. The products that migrated as a single band and at the same position with respect to the control were purified and sequenced. The DNA sequences were determined and analysed as above reported.

Microclimatic conditions: The excavation site of Herculaneum is sited in the western part of Campania (Italy) on the bay of Naples. Microclimatic parameters (temperature and relative humidity) were monitored every hour in summer (from 10.00 a.m. to 6.00 p.m.) on sampling days. At each site, ambient temperature (ET, °C) and relative humidity (RH, %) were measured at indoor locations by using a Microclimatic Station BABUC (LSI, Milan, Italy). All data were elaborated by Info GAP software (v 2.0.5.; LSI- LASTEM).

Statistical analysis: Statistical treatment of data by mean and standard deviation (SD) was performed.

Results and Discussion

Sampling, microbiological analysis and microclimatic conditions: The sampling points were chosen on the basis of their historical importance, of the visibility of alteration and of the

Table - 1: Description of sampling places of wall paintings in four different houses in Ercolano's excavations

Houses	Sampling points	Description of damage
Collegio degli Augustali	A1	Discoloration, detachment and green-grey stain on the left wall of the fresco showing Hercules with Juno and Minerva
	A2	Discoloration and detachment on the left wall of fresco showing Hercules with Juno and Minerva
Casa del Colonnato Tuscanico	CT4	Discoloration and detachment on the central wall of the fresco showing Apollo with harp
	CT5	Detachment on the right wall of the fresco showing winged muse
Casa dello Scheletro	CS6	Heavy discoloration and detachment on the central wall of the fresco showing a peacock
	CS7	Brown biofilm and efflorescence from mosaico in the niche of "sacello"
Casa del Gran Portale	GP8	Brown dry crust on the fresco on the central wall showing friezes with armour
	GP9	Discoloration and detachment on the bottom frieze of the left wall

identification of areas with higher moisture. This latter was obtained by using infrared thermography that, through the radiometric video rate infrared camera, allowed us to detect the points of greatest moisture not visible to the naked eye, resulting in structural damage. An example of the thermal infrared image of an internal view of the *sacello* (*Casa dello Scheletro*) is showed in Fig. 1, in which dark blue colour was recognised as dense rising moisture (26.2°C). The description of sampling points on wall paintings in the four different houses in Ercolano's excavation are listed in Table 1. With some exceptions, the most common type of alteration was discoloration, often associated to detachment of the painting layer.

Heterotrophic bacteria were prevalent in all wall paintings monitored, whereas fungi were detected at lower levels. In general the wall paintings sampling showed level of total heterotrophic bacteria ranging from 18 ± 2 CFU 100 cm^{-2}, detected in the sample GP8 of the *Casa del Colonnato Tuscanico,* to 68 ± 4 CFU 100 cm^{-2}, counted in the *Collegio degli Augustali* (sample point A1) *and Casa del Gran Portale* (sample point GP9). The moulds were enumerated from 9 ± 2 CFU 100 cm^{-2}, of the *Collegio degli Augustali* (sample point A2), to 45 ± 3 CFU 100 cm^{-2}, detected in the *Casa dello Scheletro* (sample point CS6) and *Casa del Gran Portale* (sample point GP9). The presence of heterotrophic microorganisms, the first colonizers of moist frescos and building materials (Karpovich-Tate and Rebricova, 1990; Garg *et al*., 1995) could explain, as previously observed (Pepe *et al*., 2010), the diffusion of discolouration detected in all wall paintings analysed. In fact, they excrete organic acids with biocorrosive action thus contributing to the discoloration of the painted surface. Moreover, a large variety of heterotrophic bacteria are commonly found on inorganic substrata containing traces of organic material but do not exhibit pigmented cells (Sorlini *et al*., 1987; Tomaselli, 2003; Tiano and Tomaselli 1989). No functional groups (sulphur-oxidizing, ammonifiers, ammonia-oxidizing, nitrite-oxidizing and free nitrogen fixing) as well as actinomycetes, were detected in the sample points analysed by cultural method.

In the houses examined the daily average temperatures ranged between 17 ± 1.0 (at 6.00 pm) and 25 ± 1.3°C (at 2.00 pm). The *Collegio degli Augustali* showed the highest value of RH (72 ± 2.8%) that decreased (from 49 ± 2.0 to 53 ± 2.1) in all other sites. These microclimatic values demonstrated that the environmental conditions detected provide perfect habitat for potential growth of heterotrophic bacteria and mould spore-forming that can survival for a long time on mural paintings.

Culture-based method: On the basis of the phenotypic characteristics a total of 48 bacteria and 23 mould isolates, were differentiated in eight and nine groups, respectively (data not shown). From one to three representative isolates of each group were chosen for genotypic identification by 16S rRNA or 26S rRNA partial sequence analysis. The results regarding the identification of bacteria and moulds associated with damage of paintings are summarized in Table 2.

Bacillus-related genera were found in all sample points analysed with exception of the sample CS6 (*Casa dello Scheletro*) in which *Micrococcus luteus/Arthrobacter* sp. group coexisted with *Streptomyces fragilis* because its resistance to the exoenzymes excrete by streptomycetes (Karpovich-Tate and Rebrikova, 1990). The genus *Arthrobacter* and *Micrococcus* sp. are commonly detected on frescos (Karpovich-Tate and Rebrikova, 1990; Rölleke *et al*., 1996; Gonzalez *et al*., 1999; Gurtner *et al*., 2000; Heyrman and Swings, 2001; Suihko *et al*., 2007) whereas *Streptomyces fragilis* was isolated, to our knowledge, for the first time from frescos. In particular, *Micrococcus* sp., previously isolated from biofilm in Roman catacombs (Saarela *et al*., 2004) and from wall paintings in the Servilla Tomb in the Carmona necropolis (Heyrman and Swings, 2001), is able to damage the glue and the binder of frescos (Bassi *et al*., 1986). The genus *Arthrobacter* is responsible for the lead oxidation of pigments (Ciferri, 1999).

The type of the *Bacillus* species detected was variable. In the *Collegio degli Augustali* were found *B. cereus* and *B. thuringiensis* (sample point A1) and *B. megaterium* the species *Geobacillus stearothermophilus* (100% identity) (sample point A2). Strains closely related to *B. megaterium* and *B.cereus/B.*

Table - 2: Identification of heterotrophic bacteria and moulds isolated from wall paintings of Ercolano's excavation by culture-dependent analysis

Houses (Sampling points)	No. of isolates*	Partial sequence analysis of 16S or 26S rDNAs	
		Closest relative species (percentage identity)	Accession numbers
Collegio degli Augustali (A1)	3	*Bacillus cereus* (100%)	EU163266
	1	*Bacillus thurigiensis* (100%)	AB363741
	1	*Aspergillus melleus*/ *Aspergillus petrakii*/ *Aspergillus ostianus*/ *Aspergillus ochraceus* (100%)	EF661426/ AF433104/ EF661422/ EF661420
	1	*Fusarium oxysporum* (100%)	FJ614650
Collegio degli Augustali (A2)	1	*Bacillus megaterium* (100%)	AY505510
	1	*Geobacillus stearothermophilus* (100%)	DQ118025
	1	*Aspergillus versicolor* (100%)	AJ937751
Casa del Colonnato Tuscanico (CT4)	2	*Bacillus megaterium* (100%)	DQ105968
	1	*Penicillium vulpinum*/ *Penicillium coprobium*/ *Penicillium paneum*/ *Penicillium dipodomyicola*/ *Pennicillium carneum* /*Penicillium concentricum*/ *Penicillium griseofulvum*/ *Penicillium aethiopicum* (98%)	DQ339572/ DQ339559/ DQ339554/ DQ339570/ DQ339566/ DQ339561/ DQ339557/ U15471
Casa del Colonnato Tuscanico (CT5)	3	*Bacillus megaterium* (100%)	AY505510
	1	*Aspergillus versicolor*/ *Aspergillus puniceus*/ *Aspergillus ivoriensis*/ *Aspergillus protuberus*/ *Aspergillus ustus*/ *Aspergillus multicolor*/ *Aspergillus aeneus*/ *Emericella variecolor*/ *Emericella spectabilis* (98%)	EF652480/ EF652498/ EF652441/ FJ176897/ EF652492/ EF652477/ EF652474/ U29836/ EF652510
Casa dello Scheletro (CS6)	2	*Micrococcus luteus*/ *Arthrobacter* sp. (99%)	DQ659431/ AF235113
	1	*Streptomyces fragilis* (100%)	AB184200
	1	*Microascus cirrosus* (98%)	AF275539
	1	*Coprinus aokii* (99%)	AF041526
Casa dello Scheletro (CS7)	3	*Bacillus simplex*/*Bacillus muralis* (99%)	AJ628747/ AJ628748
	1	*Penicillium chrysogenum*/ *Penicillium paneum*/ *Penicillium aethiopicum*/ *Penicillium griseofulvum* (100%)	U15475/ DQ339554/ U15471/ AF033468
Casa del Gran Portale (GP8)	2	*Bacillus subtilis* (99%)	AY881642
	1	*Microascus cirrosus* (98%)	AF275539
Casa del Gran Portale (GP9)	2	*Bacillus subtilis* (100%)	DQ232747
	1	*Microascus cirrosus*/ *Kernia geniculotricha*/ *Kernia hippocrepida* (97%)	AF275539/ AF275532/ AF275531

*Representative bacteria isolates grouped by morphology of colonies, micro- morphology and physiological characteristics, chosen for the genotypic identification

Table - 3: Identification, based on the Blast comparison in Gene bank, of the bands obtained by PCR-DGGE

Houses (Sampling points)	Band*	PCR-DGGE analysis	
		Closest relative species (percentage identity)	Accession numbers
Collegio degli Augustali (A1)	D	*Brevibacterium* spp. (99%)	AB189329
	B	*Microbacterium laevaniformans/M. kitamiense/M. chocolatum/M. aurantiacum* (99%)	AB004726/AJ717354/AM181503/AM182159
Collegio degli Augustali (A2)	B	*Microbacterium laevaniformans/M. kitamiense/M. chocolatum/M. aurantiacum* (99%)	AB004726/AJ717354/AM181503/AM182159
Casa del Colonnato Tuscanico (CT4)	B	*Microbacterium laevaniformans/M. kitamiense/M. chocolatum/M. aurantiacum* (99%)	AB004726/AJ717354/AM181503/AM182159
	A	*Streptomyces atratus/S. gelaticus/S. sanglieri/S. pulveraceus/S. naraensis/ S. nodosus* subsp. *asukaensis* (100%)	EU158345/EF654097/AB184811/AB184202/ AY094364/AB184808/AB184691/AB184497
Casa del Colonnato Tuscanico (CT5)	B	*Microbacterium laevaniformans/M. kitamiense/M. chocolatum/M. aurantiacum* (99%)	AB004726/AJ717354/AM181503/AM182159
Casa dello Scheletro (CS6)	B	*Microbacterium laevaniformans/M. kitamiense/M. chocolatum/M. aurantiacum* (99%)	AB004726/AJ717354/AM181503/AM182159
Casa dello Scheletro (CS7)	B	*Microbacterium laevaniformans/M. kitamiense/M. chocolatum/M. aurantiacum* (99%)	AB004726/AJ717354/AM181503/AM182159
	C	*Stenotrophomonas maltophilia/Stenotrophomonas* spp. (98%)	DQ109991/EF620453
Casa del Gran Portale (GP8)	B	*Microbacterium laevaniformans/M. kitamiense/M. chocolatum/M. aurantiacum* (99%)	AB004726/AJ717354/AM181503/AM182159
Casa del Gran Portale (GP9)	B	*Microbacterium laevaniformans/M. kitamiense/M. chocolatum/M. aurantiacum* (99%)	AB004726/AJ717354/AM181503/AM182159

*Bands are named as indicated on the DGGE gel shows in Fig. 3.

thuringiensis group were isolated from altered stone-works, frescos and salt efflorescence, demonstrating also their halotolerance (Daffonchio *et al*., 2000; Laiz *et al*., 2000,2003; Gorbushina *et al*., 2004; Pepe *et al*., 2010). *B. simplex*/*B.muralis* group was detected in the *Casa dello Scheletro* (sample point CS7). *Bacillus muralis* is described as a novel species of the genus *Bacillus*, previously detected in samples from wall paintings and monuments (Heyrman *et al*., 2005; Pepe *et al.,* 2010). *B. megaterium* and *B. subtilis* were isolated as sole species in the *Casa del Colonnato Tuscanico* (sample points CT4 and CT5) and *Casa del Gran Portale* (sample points GP8 and GP9). *Bacillus*-related genera, generally isolated from frescos (Sorlini *et al*., 1987; Karpovich-Tate and Rebrikova, 1990; Altenburger *et al*., 1996; Urzi and Realini, 1998; Heyrman and Swings, 2001) and damaged archaeological sites such as Roman catacombs (Saarela *et al*., 2004), are ubiquitous due to their ability to produce spores and hence to grow rapidly on different environments. Moreover, the presence of these microorganisms on the surface of frescos is often associated to micro-fissuring, detachment and discoloration of the paint layer due to production of organic acids that have biocorrosive action, as in the case of Herculaneum frescos analysed. In particular, potential slime-forming *B. subtilis* (Pepe *et al*., 2003), isolated from ochre-to-brown spots, was detected on frescos in the *Casa del Gran Portale*.

It was not found a dominant fungal genus in the different sample points analysed as for bacterial isolates. In fact, some strains closest related to *Aspergillus (A.) melleus/ A. petrakii/ A. ostianus/ A. ochraceus* group, *Fusarium oxysporum* and *A. versicolor* were isolated from sample point A1 and A2 in the *Collegio degli Augustali*. In particular, *Aspergillus versicolor*, is reported as the species generally associated to the deteriorated wall painting and often included in listing of indoor fungi (Garg *et al*., 1995; Berner *et al*., 1997; Gorbushina and Petersen, 2000; Gorbushina *et al*., 2004). *Aspergillus* and *Penicillium* are the major deteriogens of painted surfaces in temperate climes (Shirakawa *et al*., 2002). In fact, they could be considered frequent visitors to the surfaces of the Herculaneum houses located in the bay of Naples, near the sea, since saline deposits may provide a suitable habitat for these genera of fungi. In particular, *Aspergillus versicolor* is more resistant to sodium chloride than other fungal species (Garg *et al*., 1995). Mould strains closest related to different species of *Penicillium* and *Aspergillus*/*Emericella* group were isolated in the sample points CT4 and CT5, respectively, of the *Casa del Colonnato Tuscanico*. in the Herculaneum houses were found on mural paintings of the Casa *dello Scheletro* and *Casa del Gran Portale* (sample points CS6, GP8 and GP9) unusual genera as *Microascus* sp., *Coprinus* sp. and *Kernia* sp. In particular, the species *Microascus cirrosus* and *Coprinus aokii* were not previously found from wall paintings. Many of these chemoorganotrophic fungi have different deteriorating activity includes biocorrosive action by biogenic organic acids production, discoloration of stone surfaces, organic pigments production and mechanical stress to stone structures by hyphal growth (Garg *et al*., 1995; Warscheid and Braams, 2000). The use of molecular methods as sequencing D1/D2 region of 26S rDNA allowed us to obtain the identification of fungal isolates at species level, therefore, as group formed by numerous closest relative species. This situation demonstrate the needing of improved of methods and data banks for the analysis of fungal DNA, since availability of basic information on fungal genes are yet limited (Shirakawa *et al*., 2002).

Culture-independent method: The results regarding the identification of bacteria associated with damage of paintings by using culture-independent method are summarized in Table 3. Generally, the fingerprints obtained by DGGE of all samples were not complex; in fact, for each sample there were just one or two dominant bands (Fig. 2). To each band was assigned a letter (A, B, C and D) corresponding to a position on the DGGE gel. The fingerprints obtained from samples were very similar between them and only the fingerprints of sample points A1, CT4 and CS7 contained extra different bands. The analysis by culture-independent techniques allowed the detection of the genus *Microbacterium* closely related (99% identity) to *Microbacterium laevaniformans*/ *Microbacterium kitamiense*/ *Microbacterium chocolatum*/ *Microbacterium aurantiacum* group (corresponding to the band B in all sample points analysed. To the best of our knowledge, this is the first report concerning the detection of *Microbacterium* sp. in frescos, even if, it was isolated from the deterioration of concrete (Nica *et al*., 2000), in the caves in Spain (Groth *et al*., 1999) as well as in Roman catacomb, with sporadic occurrence (Saarela *et al*., 2004). Moreover, the literature describes the isolation of *Microbacterium* strains from environmental sources and recently from clinical specimens (Funke *et al*., 1995). Since it was detected from the DNA extracted directly from the original fresco material, *Microbacterium* could represent the autochthonous population contributing to the observed biodeterioration phenomena. Taxonomically, the genus *Microbacterium* was redefined by Collins *et al.* (1983), who reclassified ''*Corynebacterium laevaniformans*'' as *Microbacterium laevaniformans* (Funke *et al*., 1995). Recently was proposed to combine the genera *Microbacterium* and *Aureobacterium* in a redefined genus *Microbacterium* (Takeuchi and Hatano, 1998). The genus *Microbacterium* was associated with other bacteria identified as closely related to the genera: *Brevibacterium* sp. (corresponding to the band D) in the *Collegio degli Augustali* (sample point A1); *Streptomyces (S.)* sp. (corresponding to the band A) in the *Casa del Colonnato Tuscanico* (sample point CT4); *Stenotrophomonas* sp. (corresponding to the band C) in the *Casa dello Scheletro* (sample point CS7). Gram-negative bacteria belonging to the species *Stenotrophomonas maltophilia,* was isolated from air and biofilm samples of the Roman catacombs of St. Callixtus and St. Domitilla in Rome (Saarela *et al*., 2004).

Generally, all bacteria were identified at species level with a high sequence identity with database entry (ranged from 99 to 100%), with exception of the *Stenotrophomonas* spp./ *Stenotrophomonas maltophila* group (98% identity).

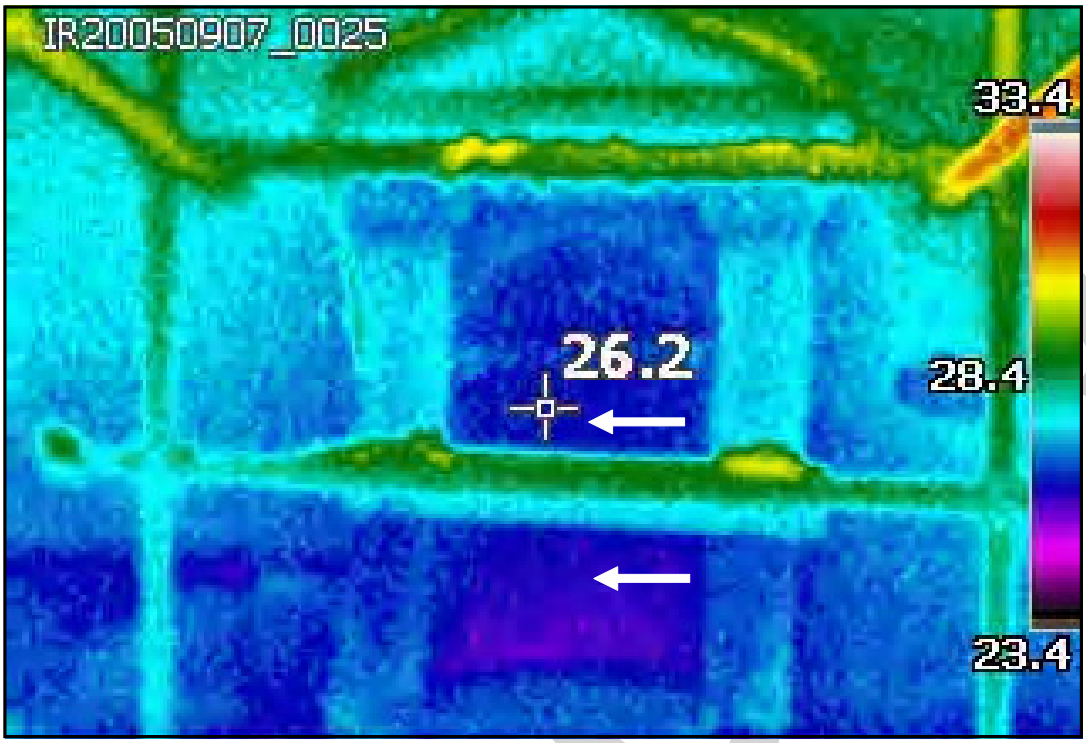

Fig. 1: Thermal infrared image by infrared scanning thermography, of an internal view of the *sacello* (*Casa dello Scheletro*) where dark Blue colour was recognised as dense rising moisture

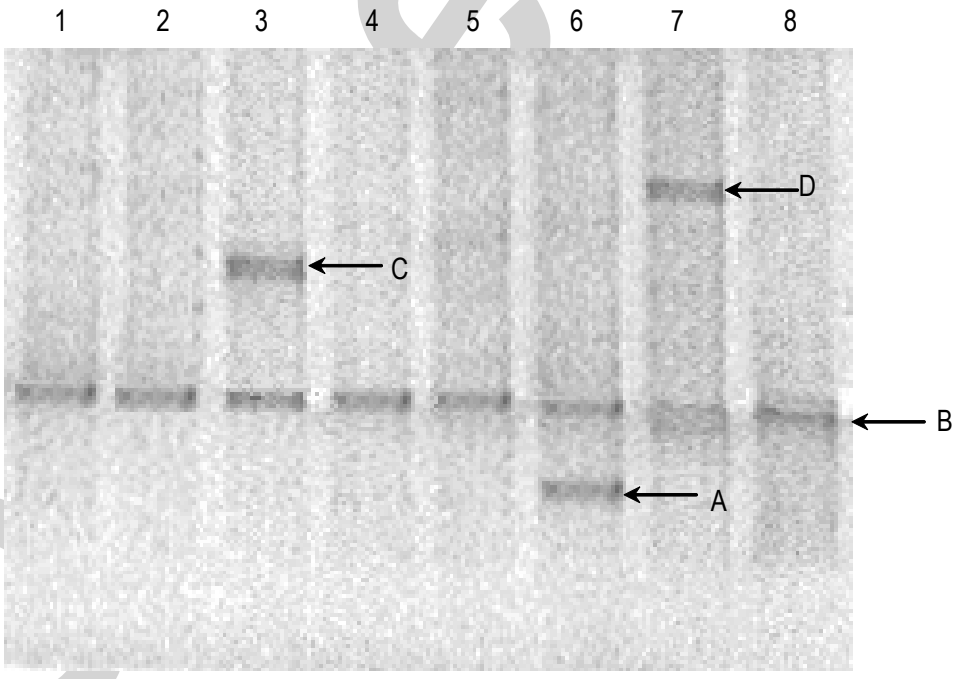

Fig. 2: PCR-DGGE of 16S rRNA V6-V8 region profiles of nucleic acids extracted directly from the wall paintings of the four houses in the excavation site of Herculaneum. Lane 1= sample point CP9; lane 2= sample point CP8; lane 3= sample point CS7; lane 4= sample point CS6; lane 5= sample point CT5; lane 6= sample point CT4; lane 7= sample point A1; lane 8= sample point A2. The letter identified each band

In accordance with previous studies (Gutner *et al*., 2000; Laiz *et al*., 2003), the results obtained by PCR-DGGE analysis differed from those obtained by conventional methods, since no band had the same identification in 16S rDNA sequence analysis and, moreover, the genus *Bacillus* was not found. This finding illustrates the intrinsic limitation of DGGE analysis in visualizing only the predominant species of a microbial community (Muyzer and Smalla, 1998) and the need of combine cultural-dependent and independent methods. According to Scheirlinck *et al.* (2008) and Iacumin *et al*. (2009), as far as the PCR-DGGE outcomes are concerned, low number of bands was visualizing indicating low bacteria species probably due to the length of time spent by the houses under soil.

Microclimatic conditions detected in the four houses analysed were suitable for bacteria and mould growth. Cultural–based method allow us to identify heterotrophic microorganisms belonging to different genera of *Bacillus* and *Aspergillus, Penicillium* and *Fusarium* together with the unusual genera as *Microascus* and *Coprinus.* Sequencing of the 16S ribosomal DNAs, selected on the basis of DGGE profiling, enabled identification of bacterial species not identified using culturable technology as those closest related to *Microbacterium* group. Combination of culture-dependent and independent methods provide better microbiology characterization of heterotrophic microbiota present on the surface of ancient frescos, even if, studies are necessary to better understand the role of the bacteria and fungi on the microbial weathering such as this important archaeological site.

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References

Altenburger, P., P. Kampfer, A. Makristathis, W. Lubitz and H.J. Busse: Classification of bacteria isolated from a medieval wall painting. *J. Biotechnol.*, **47**, 39-52 (1996).

Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman: Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res*., **25**, 3389-3402 (1997).

Amann, R.I., W. Ludwig and K.H. Schleifer: Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev*., **59**, 143-169 (1995).

Bassi, M., A. Ferrari, M. Realini and C. Sorlini: Red stains on the Certosa of Pavia a case of biodeterioration. *Int. Biodeter*., **22**, 201-205 (1986).

Berner, M., G. Wanner and W. Lubitz: A comparative study of the fungal flora present in the medieval wall paintings in the Chapel of the Castle Herberstein and in the Parish Church of St Georgen in Styria Austria. *Int. Biodeter. Biodegr*., **40**, 53-61 (1997).

Bianchi, A., M.A. Favalli, N. Barbieri and M. Bassi: The use of fungicides on mold covered frescos in San Eusebio in Pavia. *Int. Biodeter. Bull.*, **16**, 45-51 (1980).

Blaiotta, G., O. Pepe, G. Mauriello, F. Villani, R. Andolfi and G. Moschetti: 16S–23S rDNA intergenic spacer region polymorphism of *Lactococcus garvieae Lactococcus raffinolactis* and *Lactococcus lactis* as revealed by PCR and nucleotide sequence analysis. *Syst. Appl. Microbiol.*, **25**, 520-527 (2002).

Ciferri, O.: Microbial degradation of painting. *Appl. Environ. Microbiol*., **65**, 879-885 (1999).

Collins, M.D., D. Jones and R.M. Kroppenstedt: Reclassification of *Brevibacterium imperiale* (Steinhaus) and ''*Corynebacterium laevaniformans*'' (Dias and Bhat) in a redefined genus *Microbacterium* (Orla-Jensen) as *Microbacterium imperiale* comb nov and *Microbacterium laevaniformans* nom rev; comb nov. *Syst. Appl. Microbiol.*, **4**, 65-78 (1983).

Daffonchio, D., S. Borin, E. Zanardini, P. Abbruscato, M. Realini, C. Urzì and C. Sorlini: Molecular tools applied to the study of deteriorated artworks. *In*: Of Microbes and Art. The role of microbial communities in the degradation and protection of cultural heritage (*Eds*.: O. Ciferri, P. Tiano and G. Mastromei). Kluwer Academic/Plenum Publishers, New York pp. 21-38 (2000).

Dornieden, T., A.A. Gorbushina and E. Krumbein: Biodecay of cultural heritage as a space/time-related ecological situation – An evolution of a series of studies. *Int. Biodeter. Biodegr*., **46**, 261-270 (2001).

Funke, G., E. Falsen and C. Barreau: Primary Identification of *Microbacterium* spp encountered in clinical specimens as CDC Coryneform Group A-4 and A-5 bacteria. *J. Clin. Microbiol*., **33**, 188-192 (1995).

Garg, K.L., K. Kamal and A.K. Jain Mishra: Role of fungi in the deterioration of wall paintings. *Sci. Total Environ*., **167**, 255-271 (1995).

Giovannoni, S.J., T.B. Britschgi, C.L. Moyer and K.G. Field: Genetic diversity in sargasso sea bacterioplankton. *Nature*, **345**, 60-63 (1990).

Gonzalez, I., L. Laiz, B. Hermosin, B. Caballero, C. Incerti and C. Saiz-Jimenez: Bacteria isolated from rock art painting: The case of Alanterra shelter (south Spain). *J. Microbiol. Methods*, **36**, 123-127 (1999).

Gorbushina, A.A. and W.E. Krumbein: Subaerial microbial mats and their effects on soil and rock. *In*: Microbial sediments (*Eds*.: R. Di Robert and S.M. Awramik). Springer. pp. 161-170 (2000).

Gorbushina, A.A. and K. Petersen: Distribution of micro-organisms on ancient wall paintings as related to associated faunal elements. *Int. Biodeter. Biodegr*., **46**, 277-284 **(**2000).

Gorbushina, A.A., J. Heyrman, T. Dornieden, M. Gonzales-Delvalle, W.E. Krumbein, L. Laiz, K. Petersen, C. Saiz-Jimenez and J. Swings: Bacterial and fungal diversity and biodeterioration problems in mural painting environments of St Martins church (Greene-Kreisen Germany). *Int. Biodeter. Biodegr*., **53**, 13-24 (2004).

Groth, I., B. Vettermann, P. Schumann and C. Saiz-Jimenez: Actinomycetes in karstic caves of northern Spain (Altamira and Tito Bustillo). *J. Microbiol. Methods*, **36**, 115-122 (1999).

Gurtner, C., J. Heyrman, G. Pinar, W. Lubitz, J. Swings and S. Rölleke: Comparative analysis of the bacterial diversity on two different biodeteriorated wall paintings by DGGE and 16S rDNA sequence analysis. *Int. Biodeter. Biodegr*., **46**, 229-239 **(**2000).

Heyrman, J. and J. Swings: 16S rDNA sequence analysis of bacterial isolates from biodeteriorated mural paintings in the servilla tomb (Necropolis of Carmona Seville Spain). *Syst. Appl. Microbiol*., **24**, 417-422 (2001).

Heyrman, J., N.A. Logan, M. Rodríguez-Díaz, P. Scheldeman, L. Lebbe, J. Swings, M. Heyndrickx and P. De Vos: Study of mural paintings isolates leading to the transfer of *Bacillus maroccanus* and *Bacillus carotarum* to *Bacillus simplex* emended description of *Bacillus simplex* re-examination of the strains previously attributed to *Bacillus macroides* and description *of Bacillus muralis* sp nov. *Int. J. Syst. Bacteriol*., **55**, 119-131 (2005).

Iacumin, L., F. Cecchini, M. Manzano, M. Oslualdini, D. Boscolo, S. Orlic, G. Comi: Description of the microflora of sourdoughs by culture-dependent and culture-independent methods. *Food Microbiol.,* **26**, 128-135 (2009).

Karpovich-Tate, N. and N.L. Rebrikova: Microbial communities on damaged frescos and building materials in the Cathedral of the Nativity of the Virgin in the Pafnutii-Borovskii Monastery Russia. *Int. Biodeter. Biodegr.*, **27**, 281-296 (1990).

Laiz, L., D. Recio, B. Hermosinand C. Saiz-Jimenez: Microbial communities in salt efflorescence. *In*: Of microbes and art. The role of microbial communities in the degradation and protection of cultural heritage (*Eds*.: O. Ciferri, P. Tiano and G. Mastromei). Kluwer academic/ Plenum Publishers, New York. pp. 77-88 (2000).

Laiz, L., G. Pinār, W. Lubitz and C. Saiz-Jimenez: Monitoring the colonization of monuments by bacteria: Cultivation versus molecular methods. *Environ. Microbiol.*, **5**, 72-74 (2003).

Muyzer, G., E.C. de Waal and A.G. Uitterlinden: Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.*, **59**, 695-700 (1993).

Muyzer, G. and K. Smalla: Application of deneturating gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek*, **73**, 127-141 (1998).

Nica, D., J.L. Davis, L. Kirby, G. Zuo and D.J. Roberts: Isolation and characterization of microorganisms involved in the biodeterioration of concrete in sewers. *Int. Biodeter. Biodegr.*, **46**, 61-68 (2000).

Normal, D.L 3/80: Stone materials: sampling. CNR-ICR, Cosmas grafica, Rome (1980).

Normal, D.L 9/88: Autotrophic and heterotrophic microflora: isolation in culture. CNR-ICR, Cosmas grafica, Rome (1988).

Nubel, U., B. Engelen, A. Felske, J. Snaidr, A. Wieshuber, R.I. Amann, W. Ludwig and H. Backhaus: Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *J. Bacteriol.*, **178**, 5636-5643 (1996).

O'Donnel, K.: *Fusarium* and its near relatives. *In*: The fungal anamorph: mytotic, meiotic and pleomorphic speciation in fungal systematics (*Eds*.: D.R. Reynolds and J.W. Taylor). CAB international, Wallingford, UK. pp. 225-233 (1993).

Pepe, O., G. Blaiotta, G. Moschetti, T. Greco and F. Villani: Rope-producing strains of *Bacillus* spp. from wheat bread and strategy for their control by lactic acid bacteria. *Appl. Environ. Microbiol.*, **6**, 2321-2329 (2003).

Pepe, O., L. Sannino, S. Palomba, M. Anastasio, G. Blaiotta, F. Villani and G. Moschetti: Heterotrophic microorganisms in deteriorated medieval wall paintings in southern Italian churches. *Microbiol. Res.*, **165**, 21-32 (2010).

Rolleke, S., G. Muyzer, C. Wawer, G. Wanner and W. Lubitz: Identification of bacteria in a biodegraded wall painting by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Appl. Environ. Microbiol.*, **62**, 2059-2065 (1996).

Saarela, M., H.L. Alakomi, M.L. Suihko, L. Maunuksela raaska and T. Mattila-Sandholm: Heterotrophic microorganisms in air and biofilm samples from Roman catacombs with special emphasis on actinobacteria and fungi. *Int. Biodeter. Biodegr.*, **54**, 27-37 (2004).

Sarro, M.I., A.M. Garcia, V.M. Rivalta, D.A. Moreno and I. Arroyo: Biodeterioration of the lions fountain at the Alhambra Palace Granada (Spain). *Build. Environ.*, **41**, 1811-1820 (2006).

Scheirlinck, I., R. Van der Meulen, A. Van Schoor, M. Vancanneyt, L. De Vuyst, P. Vandamme and G. Huys: Taxonomic structure and stability of the bacterial community in belgian sourdough ecosystems as assessed by culture and population fingerprinting. *Appl. Environ. Microbiol.*, **74**, 2414-2423 (2008).

Shirakawa, M.A., C.C. Gaylarde, P.M. Gaylarde, J. Vanderley and W. Gambale: Fungal colonization and succession on newly painted buildings and the effect of biocide. *FEMS Microbiol. Ecol.*, **39**, 165-173 (2002).

Sorlini, C., M. Sacchi and A. Ferrari: Microbiological deterioration of Gambara's frescos exposed to open air in Brescia, Italy. *Int. Biodeter.*, **23**, 167-179 (1987).

Suihko, M.L., H.L. Alakomi, A. Gorbushina, I. Fortune, J. Marquardt and M. Saarela: Characterization of aerobic bacterial and fungal microbiota on surfaces of historic Scottish monuments. *Syst. Appl. Microbiol.*, **30**, 494-508 (2007).

Takeuchi, M. and K. Hatano: Union of the genera *Microbacterium* Orla-Jensen and *Aureobacterium* Collins *et al* in a redefined genus *Microbacterium. Int. J. Syst. Bacteriol.*, **48**, 739-747 (1998).

Tiano, P. and L. Tomaselli: A case of biodeterioration of marble. *Arkos,* **6**, 12-18 (1989).

Tomaselli, L.: Biodeterioration process on inorganic substrata. Coalition: A concerted action from the European Commission (EVK4-CT-1999-2001) on molecular microbiology as an innovative conservation strategy for indoor and outdoor cultural assets. *Newsletters,* **6**, 5-9 (2003).

Urzi, C. and M. Realini: Colour changes of Noto's calcareous sandstone as related to its colonisation by microorganisms. *Int. Biodeter. Biodegr.*, **42**, 45-54 (1998).

Wagner, M., R. Erhart, W. Manz, R. Amann, H. Lemmer, D. Wedi and K.H. Schleifer: Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge. *Appl. Environ. Microbiol.*, **60**, 792-800 (1994).

Walsh, J.H.: Ecological considerations of biodeterioration. *Int. Biodeter. Biodegr.*, **48**, 16-259 (2001).

Ward, D.M., R. Weller and M.M. Bateson: 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature*, **345**, 63-65 (1990).

Warscheid, T. and J. Braams: Biodeterioration of stone: A review. *Int. Biodeter. Biodegr.*, **46**, 343-368 (2000).

Weisburg, W.G., S.M. Barns, D.A. Pelletier and D.J. Lane: 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.*, **173**, 697-703 (1991).