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Identification and removal of biodeteriogens on a polychrome wood sculpture

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Riassunto. È stato studiato il biodeterioramento di una statua lignea policroma del XVIII secolo raffigurante l'*Immacolata Concezione con Bambino*, conservata presso l'Università Suor Orsola Benincasa di Napoli. Il legno della statua è risultato appartenente a piante di tiglio (*Tilia* sp.). È stata osservata la presenza di insetti morti, che sono stati identificati, sulla base della morfologia e tipo di danno procurato alla statua, come coleotteri xilofagi della specie *Oligomerus ptilinoides* (Anobiidae). Sono stati identificati come organismi colonizzatori i microfunghi *Alternaria alternaria* e *Fusarium oxysporum* e batteri della specie *Bacillus subtilis*. I trattamenti col biocida Biotin R hanno completamente eliminato i funghi, la categoria microbica risultata maggiormente responsabile dei processi di degrado della scultura. I risultati hanno mostrato che i valori elevati di umidità relativa sono la causa principale del deterioramento della scultura.

Abstract. Biodeterioration active on an eighteenth-century polychrome wood statue representing the *Immaculate Conception with Child* at the University Suor Orsola Benincasa of Naples, Italy, was studied. The wood of the statue was identified as belonging to a species of lime (*Tilia* sp.). Occurrence of dead insects was observed. On the basis of their morphology and type of damage caused to the statue, the insects resulted attributable to xylophagous beetles belonging to the species *Oligomerus ptilinoides* (Anobiidae). Two species of microfungi, *Alternaria alternaria* and *Fusarium oxysporum*, and the bacterium *Bacillus subtilis* were identified as colonizing organisms. Treatments with the biocide Biotin R resulted in the complete elimination of fungi, which were the microbial category most responsible of the degradation processes on the sculpture. Results showed that high values of relative humidity were the main cause of the sculpture's deterioration.

Key words: Bacteria, Biocides, Biodeteriogens, Cultural heritage, Fungi, Insects, Sculpture, Wood

Introduction

Production of wood sculptures of sacred devotional subjects commissioned to adorn churches flourished in Naples, Italy, during sixteenth and eighteenth centuries (Abbate 2009; Borrelli 1970; Fittipaldi 1980).

Between the mid-sixteenth century and the beginning of the seventeenth century, the mystical Neapolitan Sister Orsola Benincasa founded a monastic institute in Naples (Maggio1669), today belonging to the University Suor Orsola Benincasa of Naples. Due to numerous donations and bequests, the institute has been enriched with a considerable number of paintings, sculptures and other kinds of artifacts, including the wood sculpture representing the *Immaculate Conception with Child.*

The sculpture was located in a niche in the church of the ancient monastic institute (Fig. 1). The back of the sculpture was partially cut and removed, probably to adapt the size and depth of the artifact to the available space in the niche. The niche was closed by glass framed in wood (Fig. 1b). In the 1980s, the church was deconsecrated and transformed into a conference hall, known as the "Sala degli Angeli" (Hall of Angels).

Information on the sculpture's provenance and workshop is not available. The *Immaculate Conception with Child* is in the style of classical Neapolitan work from the mid-eighteenth

century (Borrelli 2004). Its dimensions are 180 cm high, 65 cm wide and 50 cm deep. A technical examination of the support has determined that the sculpture is carved in the round by assembling several blocks of wood positioned with the grains parallel to the vertical axis of the statue (Fatigati 2010). The polychromy exhibits a sequence of several layers of gypsum in animal glue coated with a tempera bound gypsum ground. Painted layers include smalt, the pigments azurite, vermilion, minium, and lead white with golden floral decorations (Fatigati 2010). According to the same author, the statue has been re-painted during successive restorations.

Over the years, the sculpture has been subject to a significant biological attack by wood infesting insects, fungi and bacteria leaving the sculpture in very poor condition (Fig. 2a, b). The wooden support is riddled with insect galleries, flight holes, and displays abundant powdered frass. Fungi have caused black stains and encrustations on the sculpture's surface. These types of damage are commonly reported for similar artifacts (Ciferri *et al.* 2000; Warscheid 2000).

In 2011, the sculpture was removed from the niche and transferred to the University Suor Orsola Benincasa's Conservation Laboratory for Wood Artifacts, where the wood-boring pests were eliminated by an anoxic treatment using nitrogen (Castelli & Santacesaria 2012; Tavzes *et al.* 2003). The treatment was not completely effective in preventing growth of fungi and bacteria, since the pests were again visually observable. The microorganisms, while aerobic, are able to survive in the absence of oxygen for a long period of time proving to be resistant to anoxic treatment by entering a quiescent state (Maekawa 2001). In 2012, a treatment based on the injections of Permethrin (CTS 2012) in the flight holes was carried out. Although Permethrin was very effective against insects, once again, it did not address the issue of fungi and bacteria, as shown by optical observations. It was clear

Fig. 1 - **a.** The niche with the sculpture of *Immaculate Conception with Child* on the main altar in the church, actually known as "Room of Angels", at the University Suor Orsola Benincasa of Naples, Italy. **b**. The sculpture in the niche, closed by a glass.

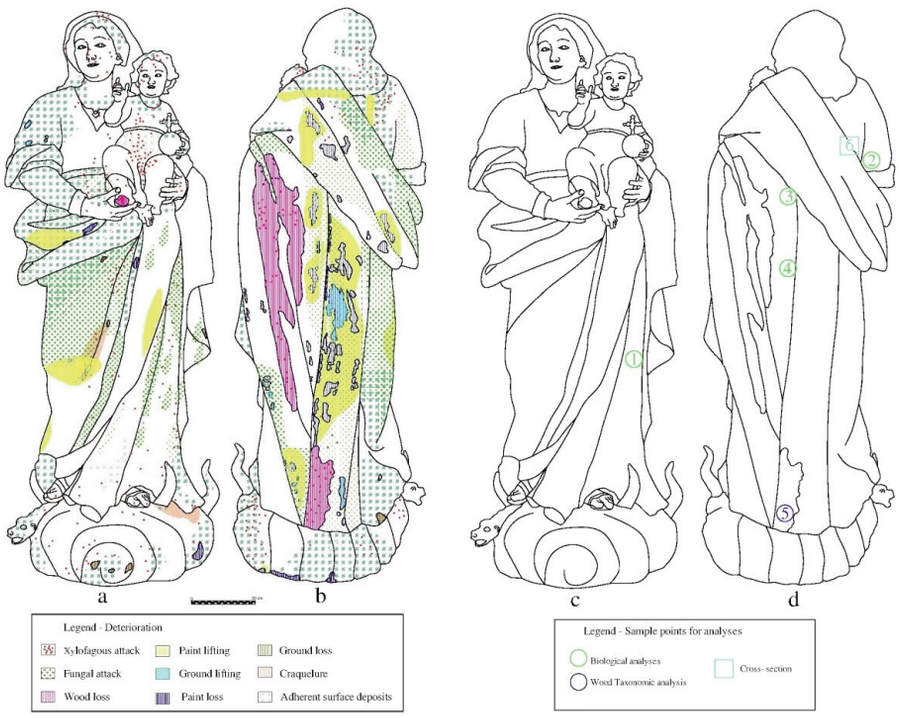

Fig. 2 - Biodeterioration processes occurring on the front (**a)** and on the back (**b**) of the sculpture. The front (**c**) and the back (**d**) of the sculpture with indications of collecting points (numbered from 1 to 6) of samples used for analyses.

that a different treatment was necessary to eliminate the biodeteriogens.

The present study stems from the need to fight the active biological deterioration on the polychrome wood sculpture by finding the appropriate and effective method to remove fungi and bacteria. Preliminary steps were the identification of the sculpture's wood species followed by the identification of encrusting organisms using microscopy and molecular techniques. Concurrently, microclimatic conditions were monitored. With this information at hand, a suitable biocide was selected and applied to the statue.

Experimental

Microclimatic monitoring

Light intensity, air temperature and relative humidity were recorded over a period of six months from December 2012 to June 2013 (Table I) . Light intensity was measured in the centre of Room of Angels, at noon, by using a TESTO 545. Air temperature and relative humidity were recorded at intervals of 15 min by using two data-loggers EL-USB-2RH/Temperature Lascar Electronics. A data-logger was placed in the centre of the Hall, the other one in the niche. Data were processed with the Software Easy log 4.5 (Allegretti *et al.* 2013).

Identification of wood species

Analysis was performed on a micro-sample of wood taken from the sculpture's back (Fig. 2d, sample 5, and Fig. 3a), where wood was exposed for the cut made to adapt the size of the sculpture to the available space in the niche. Observations were made by using an optical microscope in reflected light Nikon Eclipse L150 and photographed with a Nikon Coolpix 990 camera. For interpretation of images (Fig. 3b, c), we referred to analytical keys texts (Berti *et al.* 2002; Schweingruber 1990).

Optical observation of a cross-section

To examine the stratigraphy of the encrustations found on the sculpture and to detect the

Table I - Minimum and maximum values of temperature, light intensity and relative humidity measured in the period of analyses in the centre of Hall of Angels and in the niche with the statue.

	Temperature (°C)	Light Intensity (Lux)	Relative Humidity (%)
Center of Room (Room closed to the public)	17-22	78-82	65-68
Center of Room (Room open to the public)	24-26	78-82	73-75
Niche (Room closed to the public)	15-19	66-70	65-70
Niche (Room open to the public)	19-22	66-70	70-73

status of the biodeterioration, a sample with encrustation formed by biofilm and paint components (Fig. 2d, sample 6) was embedded in epoxy resin; the polished cross sections obtained were observed using a reflected light microscope.

Identification of insects

For the entomological identification, dead insects (Fig. 4c), flight holes (Fig. 4b) and powdered frass (Fig. 4a) found on various areas of the sculpture as remnants of the previous pest treatment (see Introduction) were viewed by an optical microscope in reflected light. The diameter of flight holes was measured by using a caliper.

Sampling and cultivation of encrusting microorganisms

For sampling and cultivation procedures, four samples of encrustations (Fig. 2c, d, samples 1-4) containing colonizing microorganisms were collected by using a sterile scalpel and wiped with a sterile cotton swab. The swab was placed in sterilized capped tubes with 10 mL of mineral medium at pH 6.7. The tubes were transferred to the lab where 1 mL of each sample was diluted in 10 mL sterile water and shaken for 15 min. Resulting suspensions (0.5 mL of each sample) were inoculated in Petri plates (5 cm in diameter) containing a medium specific for fungi (Norris & Ribbons 1969) or bacteria (Cliff *et al.* 2005). All cultures were

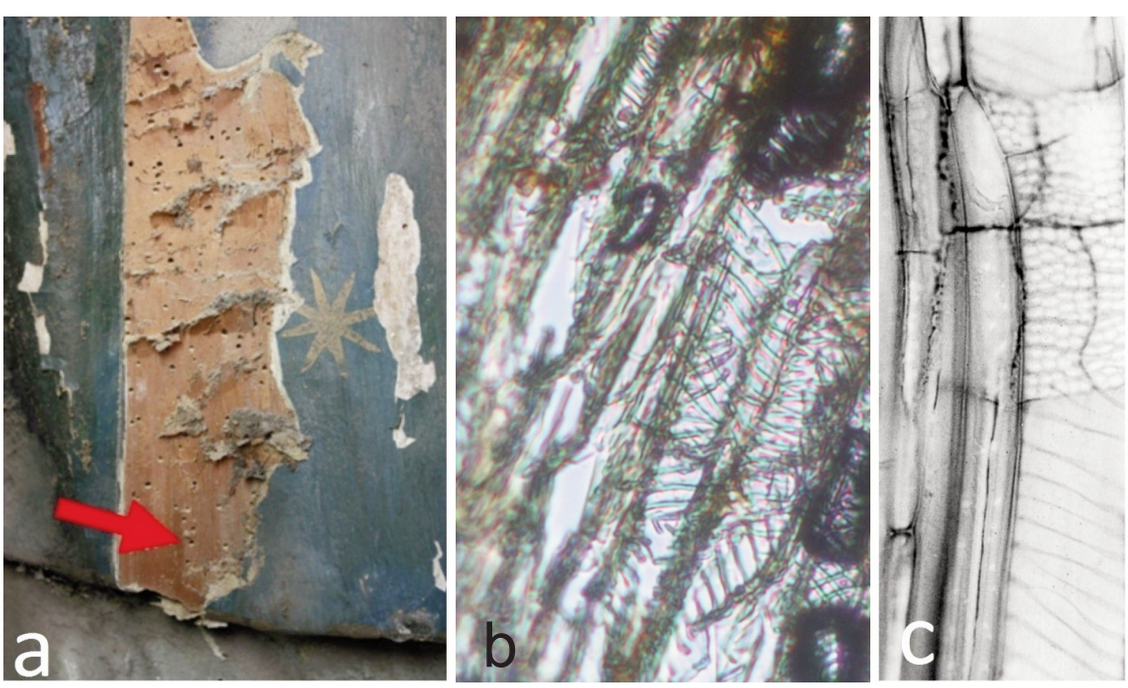

Fig. 3 - Identification of the wood species. **a**. The area of the sculpture (see Fig. 2d, sample 5) where a micro-sample of wood (arrowed) was collected for identification. **b**. Microphotography of the wood radial section (100X). **c**. Comparison with a wood section of lime reported in an analytical keys text (Schweingruber 1990).

incubated for seven days at 28 °C.

Molecular analyses of fungi and bacteria

Molecular analyses become indispensable when classical methods, such as optical and electron microscopy, do not allow a reliable and complete identification of biodeteriogens. Comprehensive and detailed descriptions of procedures and aims of molecular techniques useful in the field of Cultural Heritage are reported by Warscheid (2000) and Dakal & Arora (2012).

For molecular analysis, genomic DNA was isolated from approximately 20 mg of samples directly collected on encrustations (Fig. 2c, d, samples 1-4) following a CTAB (cetyl trimethyl ammonium bromide) procedure (Doyle & Doyle 1990). The samples were ground to a fine powder in liquid nitrogen, transferred to a 2-mL tube containing 0.5 mL CTAB extraction buffer [100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB and 0.2% β-mercaptoethanol] and incubated for 30 min at 60 °C. The homogenate was extracted with an equal volume of chloroform-isoamyl alcohol (24:1) and then centrifuged at 7,000 × *g* for 5 min. The top aqueous layer was recovered and another extraction with chloroform-isoamyl alcohol (24:1) was performed. The top aqueous layer was recovered again, and 70% cold isopropanol was added and mixed gently to precipitate the nucleic acids. After 5 min on ice, the samples were directly centrifuged at 10,000 × *g* for 8 min. The DNA pellet was washed with 70% ethanol, dried and re-suspended in 50 µL of sterile distilled water. The concentration was estimated by comparison with 5 µL of DNA with a DNA standard (Marker II, AppliChem GmbH) on a 0.8% agarose gel containing 0.5 g/mL ethidium bromide.

PCR amplifications were carried out on an estimate of 10 ng of extracted DNA. Ribosomal fragments 16S (800-bp) were amplified by PCR using the universal primers for bacteria SSU1 and SSU2 (Berschick 1997). ITS (600 bp) located between 18S and 5.8S rDNA was amplified by PCR using the universal primers for fungi ITS1 and ITS2 (White *et al.*1990). PCR reactions were carried out in a final volume of 50 µL containing 5 µL of 10X PCR buffer, 100 mM of deoxynucleotide triphosphate, 2.5 mM of magnesium chloride, 0.5 mM of primers, and 1U of Taq polymerase (Quiagen, Hilden, Germany). The PCR program consisted of an initial denaturation at 95 °C for 4 min and 30 cycles including 1 min of denaturation at 94 °C, 45 s of annealing at 56 °C, and 2 min extension at 72 °C. A final extension of 7 min at 72 °C followed by cooling at 4 °C terminated the PCR program. An aliquot of purified PCR product was ligated into the pGEM-T easy Vector system (Promega, Vienna, Austria), following the manufacturer's instructions. The ligation products were then transformed into *Escherichia coli* XL Blue TC, which permitted the identification of recombinants. The recombinants were sequenced following the procedures by Sanger *et al.* (1977) with a 3130 genetic analyzer (Applied

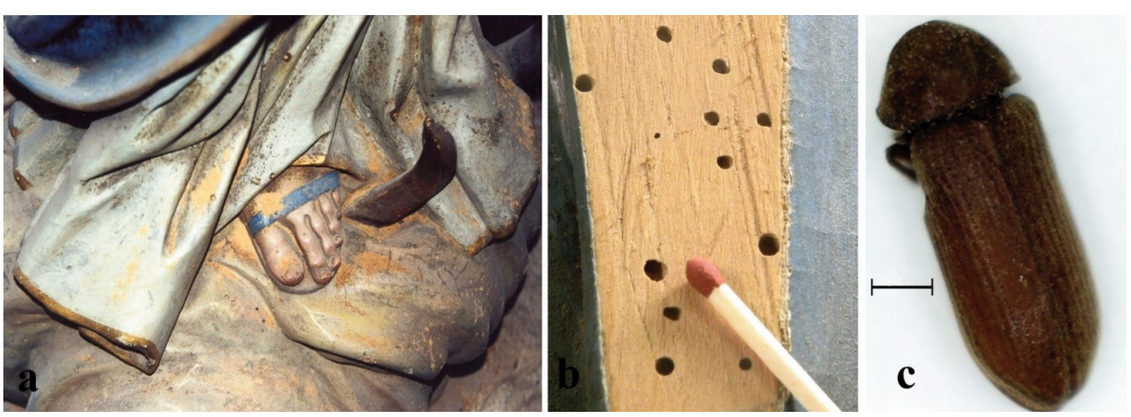

Fig. 4 - Powdered frass (**a**), flight holes (**b**) and the pest beetle identified as *Oligomerus ptilinoides* (**c**). Bar = 1 mm.

Biosystems) and their sequences were edited and aligned using the Bio Edit software (version 7, Hall 1999). Sequences were compared with those in the GenBank sequence database using BLASTN algorithm available at the National Center for Biotechnology (NCBI). Sequences were attributed to taxa only if percentage similarities were > 90%.

Application of the biocide

After an identification of the microorganisms, we chose to utilize Biotin R for several reasons. Application of Biotin R has proved to be very efficacious against biodeteriogens colonizing wood substrata and to be safe for the environment and non-toxic for the operator at least when compared to other analogous biocides (Borgioli *et al.* 2006; CTS 2006). Biotin R active ingredients, 3-iodopropynylbutylcarbamate (IPBC) and 2-Octyl-3(2H) Isothiazolone (OIT) (Williams 2007), have a low solubility in water and are soluble in organic solvents, and are therefore more adequate for use on water sensitive paint such as that of the *Immaculate Conception with Child*. As suggested by Cremonesi & Signorini (2012), we diluted the concentrate product in an organic neutral solvent, namely white spirit, a non-polar hydrocarbon distilled from petroleum. As reported by Cremonesi & Signorini (2012), white spirit has very little if no effect on the sculpture's materials. Additional information on Biotin R is available in Bartolini *et al.* (2007), Borgioli *et al.* (2003) and CTS (2006). About a year and a half after the application, a sample from the treated area was collected using a sterile scalpel and analyzed by *in vitro* testing to verify the microbial viability.

Results and Discussions

Microclimatic monitoring

Table 1 reports minimum and maximum values of temperature, light intensity and relative humidity measured in the centre of Hall of Angels and in the niche with the statue when the Hall was closed to the public and when was open to the public.

Microclimatic conditions monitored in the Room of Angels, with fairly high rates of relative humidity and relatively low values of light intensity, appeared to be the main causes promoting the biodeterioration of the sculpture. Poor ventilation within the niche closed by a glass could also actively contribute to the deterioration.

According to Norma UNI 10829 (1999), ideal thermo-hygrometric values for painted wood and polychrome sculptures are 19-24 °C temperature and 50-60% relative humidity. Another document (Ministero per i Beni e le Attività Culturali 1998) recommends 19-24 °C temperature and 45-65% relative humidity. These last parameters are stated to prevent microbiological attacks on organic materials at 50-60% relative humidity, according to a maximum daily variation ΔRH 2 at the same temperature and maximum daily variation ΔT 1.5 of temperature. Concerning photosensitivity, the painted sculpture is located into the category average no. 2, which supports a maximum illumination of 150 lux (Ministero per i Beni e le Attività Culturali 1998).

In order to prevent further deterioration, a control system of environmental parameters and constant monitoring should be provided in the Room of Angels. Such a control should be specially applied to relative humidity, which is higher than recommended values.

Identification of wood species

Optical examination of wood radial section features, such as rays, ground tissue, libriform fibres, fibre-tracheids, perforation plates, vessels and ray-vessel pits, enabled the wood to be identified as belonging to a species of lime (*Tilia* sp.) (Fig. 3b).

Lime wood has been identified on a number of other wood sculptures in southern Italy (Perusini 2004; Fatigati 2010) from the same time period as the *Immaculate Conception with Child*.

Optical observation of a cross-section

Optical observations of a cross section with black encrustation (Fig. 5) showed three layers: the gypsum ground (Fig. 5, layer 1), the blue coat obtained by using a smalt base and an azurite glaze (Fig. 5, layer 2), and the dark biological deposit on the surface, slightly penetrating the blue layer (Fig. 5, layer 3).

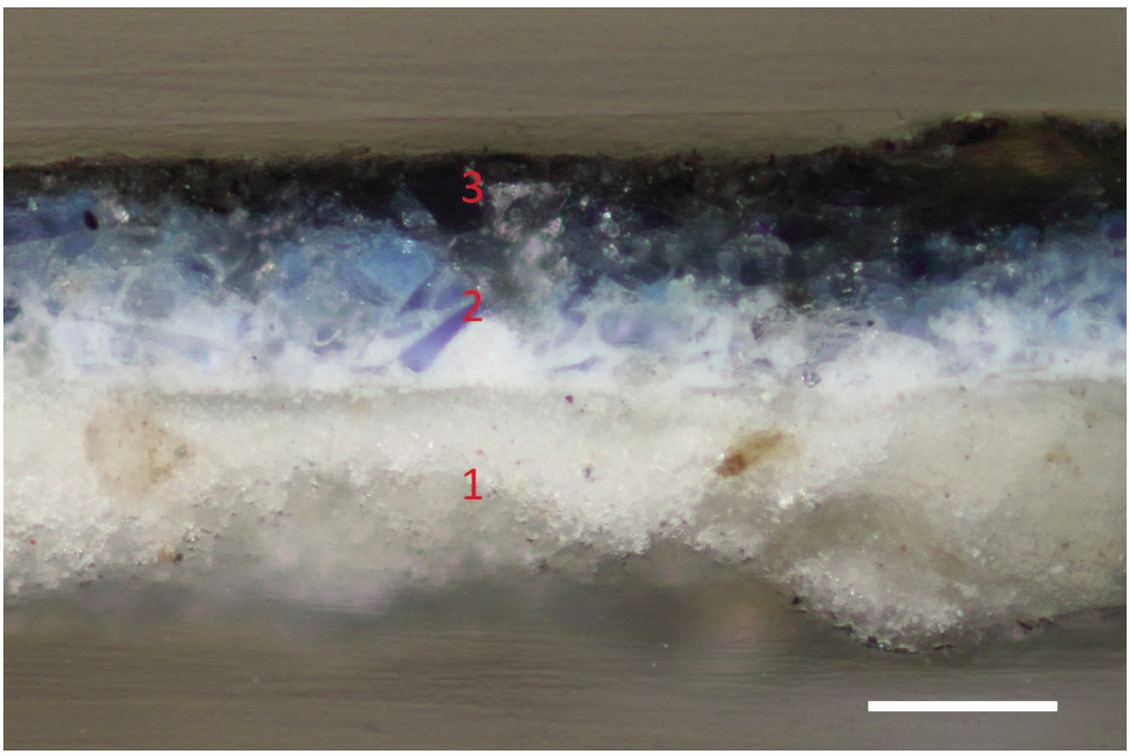

Fig. 5 - Optical observations of stratigraphy of an encrustation from the back of the blue coat (Fig. 2d, sample 6). Three layers formed by gypsum ground (1), blue coat components (2) and a dark biological deposit (3) are indicated. Bar = 250 µm.

Identification of insects

The examined dead insects had body length of 6.5-7 mm, with brown plumage, head recessed into the breastplate, robust mandibles (Fig. 4c); flight holes measured 2-3 mm in diameter (Fig. 4b) and powdered frass had a granular form (Fig. 4a). On the basis of such morphological features, the infesting insect resulted to belong to the beetle species *Oligomerus ptilinoides* (family Anobiidae). This species has been reported as a xylophagous pest on other wooden sculptures (Chiappini *et al.* 2001; Gambetta 2010). Such beetles are common in the region of Naples, and are known to prefer a closed environment (Liotta *et al.* 1989).

Identification of fungi and bacteria

Molecular analyses allowed the identification of fungi and bacteria at species level. Two fungal species, *Alternaria alternaria* and *Fusarium oxysporum*, with the former occurring in all samples and the latter in one sample only (Fig. 2d, sample 2), were identified. The bacterium *Bacillus subtilis*, occurring in all samples, was also identified.

It is important to note that the presence of the beetle *Oligomerus ptilinoides* promotes the proliferation of both fungal and bacterial colonies that easily use organic matter produced by another organism (Liotta *et al.* 1989).

Application of the biocide

Five days after Biotin R treatment, a softening of the encrustations was observed, facilitating their mechanical removal with a scalpel blade. A sample from the treated area was analyzed after about a year and a half by fungal and bacterial specific *in vitro* tests to verify the death of microorganisms and, consequently, the effectiveness of the biocide product. No fungi were detected, whereas bacteria were still present. As restoration works were still in progress at the time of these tests, it cannot be excluded that in this phase the bacteria *Bacillus subtilis*, known for its anti-fungal properties, was colonizing the areas where fungi had already died. This would also explain why the colonies appeared crusty and

had to be removed with a scalpel rather than fluffy, as they would have been if fungi were still actively growing.

Since both percentages of Biotin R resulted effective against fungi, recommendation was made to employ the lower percentage for the treatment of all other affected areas of the sculpture.

In vitro testing repeated one year later, when the restoration of the statue was completed, showed that all microbial categories, included the bacteria, had disappeared.

Conclusions

The biocide Biotin R has proven most effective for the treatment of a wood polychrome sculpture contaminated by microorganisms.

Preliminary procedures, i.e., environmental monitoring and identification of the wood forming the sculpture and of the encrusting organisms, are essential steps to choose an appropriate biocide. The technique based on optical and electron microscopy and DNA analysis proved to be very useful for those identifications. Growth tests were useful to verify the efficacy of the biocide.

Besides suggesting the use of the biocide, it is worth considering that control of microclimatic parameters, such as temperature, light, relative humidity, and ventilation, is always recommended. Works of art like the *Immaculate Conception and Child* are commonly exhibited in closed environment in churches or town halls, where an increase in water content of the air or of the objects' materials place the objects at risk of biodeterioration, especially by microfungi. Biotin R has proven to be particularly effective precisely against microfungi, whereas it did not completely eliminate bacteria. This latter microbial category was observed to be less harmful in the biodegradation process.

Before returning the treated sculpture to its niche, a disinfection of the area surrounding the sculpture was recommended, followed by further periodical disinfection of the closed environment.

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