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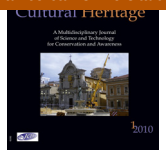

Multiple approaches to identify bacteria in archaeological waterlogged wood

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ABSTRACT

This study was carried out in collaboration with *Soprintendenza del Mare (SM)* that started, since 2004, to plan and realize underwater archaeological parks, such as in the Sicilian islands of Pantelleria (Gadir), Levanzo (Cala Minnola), Ustica (Falconiera), Panarea (Basiluzzo) and Filicudi (Capo Graziano). *In situ* conservation, as well as virtual exhibitions of the same topics, can contribute to ensure the protection and best fruition of underwater cultural heritage. The focus of this study was the identification of bacterial colonies in waterlogged wood samples from the rostrum of a excellent workmanship, that is very likely one of the wrecks attributed to Sextus Pompey fleet (36 BC) and discovered in Acqualadroni, Messina, Sicily, Italy (2008). Samples were analyzed by light and Scanning Electron Microscopy (SEM), *in vitro* culture and molecular technique (DNA base techniques). The results, focused on bacterial consortia, allowed us to reveal the presence of *Pseudomonas* sp., *Sphingomonas* sp., *Xanthomonas* sp. besides *Marinobacter* sp. and *Desulforudis audaxviator*. A prompt and accurate characterization of bacterial colonization represents one of the preliminary step in preservation/restoration projects, especially for waterlogged wood since the metabolic activity of specific bacteria induce and accelerate the deterioration processes. Although it is reported in a case study, this multiple approach is useful for reveal and identify bacterial colonizing both organic and inorganic artifacts.

1. Introduction and research aims

Sicily is one of the most important places in the Mediterranean to study underwater archaeology, mainly due to its central position and its historical role [1].

The state of conservation of submerged archaeological wood depends on the factors closely related to site peculiarity, such as temperature, salinity and oxygen concentration [2]. Bacterial degradation of findings can be manifested in different ways, in relationship with the metabolic activities. The consequence is the loss of some structural components of the wood, such as cellulose, lignin, hemi-cellulose and thus the loss of the original chemical, physical and mechanical properties. Knowledge on microbial deterioration assume great importance for both archaeologists and conservators, and as reported in literature, fungi and bacteria play a critical role in the conservation of almost all cultural assessment, including archaeological wood [3–5]. Recently, fungal colonization has been well described during the drainage of wetlands [6] and two main bacterial species: erosion bacteria (submerged archaeological wood) and tunnelling bacteria (soft rot environments) were pointed out [7,8]. Usually, waterlogged archaeological wood presents a fragile inner structure and it needs special attentions in order to reduce the impact of conservation strategies [9].

In this study, small wood fragments from Mediterranean Sea area (Acqualadroni, Messina, Sicily), were analyzed in order to identify the wood and to evaluate the state of conservation, mainly in relationship to bacterial colonization. Light and Scanning Electron Microscopy (SEM), in combination with *in vitro* culture and molecular analysis demonstrated the presence of bacterial species.

2. Materials and methods

Wood fragments were collected by sterile scalpel and loops from three sites of the rostrum, as shown in Fig. 1 (RA 1, RA 2, RA 3) and utilized for both direct extraction of microbial DNA and inoculation of Nutrient agar plates (NA-*Difco*). After incubation at 30 °C for 18 h, single bacterial colonies have been isolated from the N-agar plates [10].

Total bacterial DNA was extracted from wood fragments by Stool Mini Kit (*Qiagen*), partially modified [11]. DNA from single bacterial colony grew on NA plates, was obtained by cellular lyses at 94 °C for 2 min. Genomic DNA (5 μl aliquots of lyses solution) were utilized as template in PCR reactions.

Specific ribosomal DNA sequences, 16S gene or ITS (Internal Transcribed Spacer) were respectively amplified by specific or universal primers.

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Fig. 1. Wood fragments sampling from different areas (pointed in red) of the wood rostrum structure.

Primers specific for 16S gene were utilized to identify *Cytophaga, Cellvibrio* and *Pseudomonas* bacterial species [12,13].

Universal primers, specific for 16S-23S rDNA – ITS region, were utilized as described in [10,14].

The PCR reaction mixture, up to 50 μl, consisted of: genomic bacterial DNA (5 μl of lyses solution of single colony or 40 ng extracted from wooden fragments), 1× Reaction Buffer, 10 μM forward primer, 10 μM reverse primer, 2 μM dNTP (deossinucleotide-triphosphate) mix, 2 mM $MgCl_2$, 5 U/μl Taq DNA polymerase (*Invitrogen*).

PCR reactions were performed in a Eppendorf Mastercycler gradient by the following amplification profiles: 1 cycle of initial denaturation at 95 °C for 5 min; 30 cycles as follows: denaturation at 94 °C for 1 min, annealing at 50–58 °C for 1 min, extension at

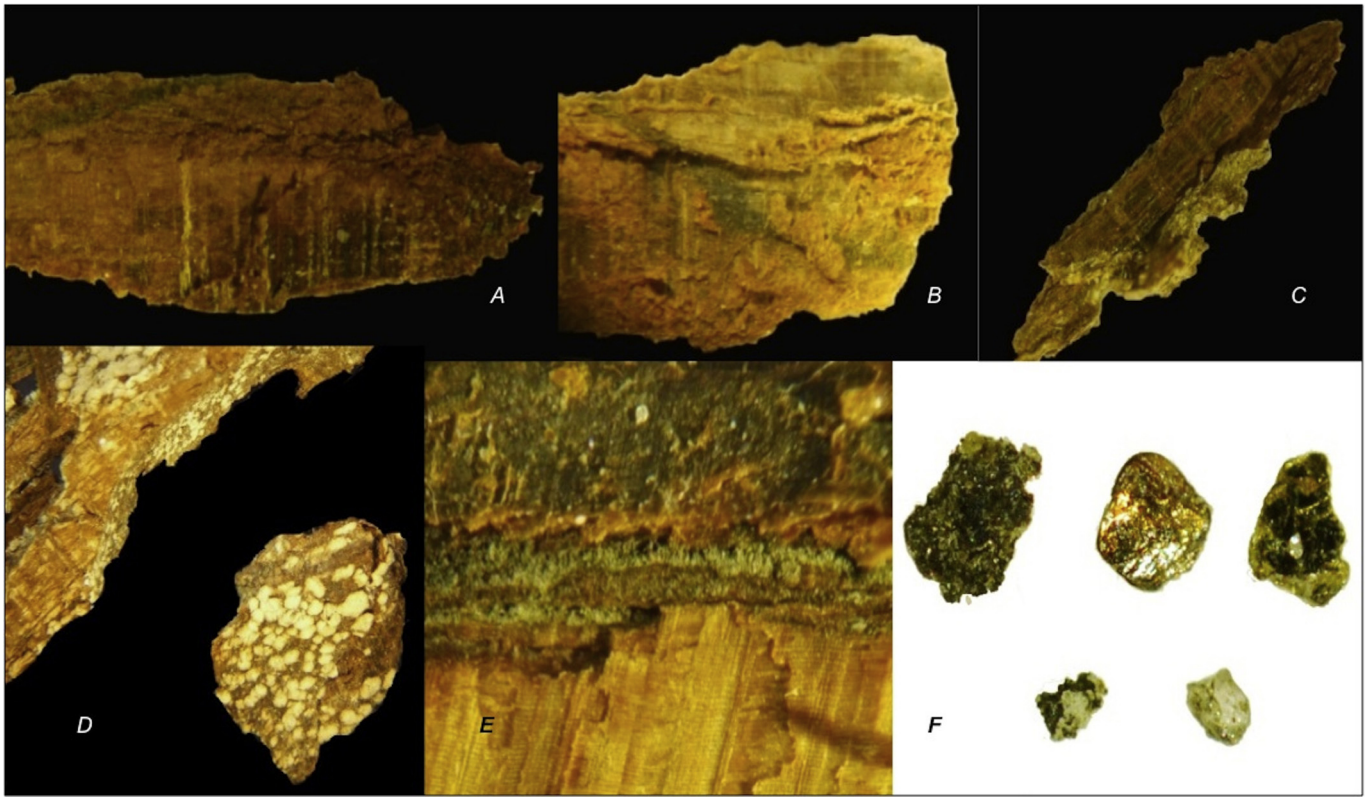

Fig. 2. Stereoscope images (radial and cross sections) of wood fragments. Deposits and chromatic alterations (green-dark spots).

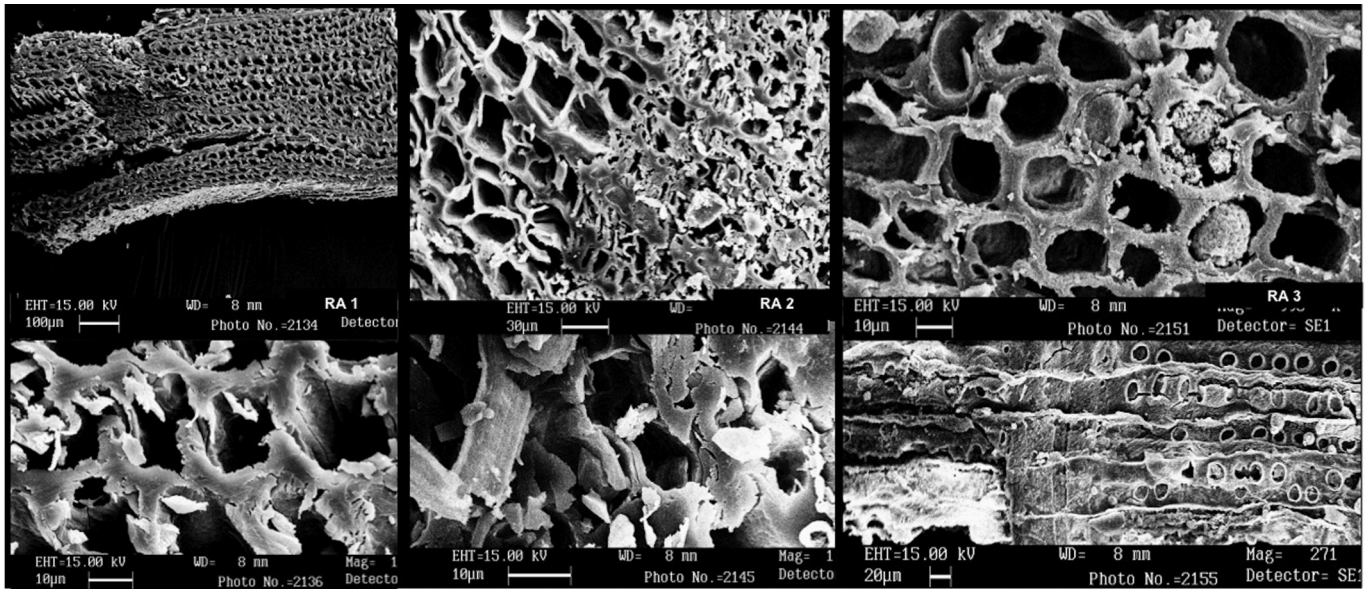

Fig. 3. SEM micrographs of wood structure and sections. RA1) transverse sections, *upper*: deformation of wooden fibres probably due to fast water evaporation; *below*: cellular walls partially degraded; RA2) transverse sections, *upper* and *below*: typical alteration of wood cellular structure related to bacteria colonization; RA3) *upper*, transverse section showing crystalline aggregates inside the wood cell structure; *below*, radial section showing tracheids in the embrittled wooden fragments.

72 °C for 2 min. A final extension step (72 °C for 7 min) was added to ensure that all PCR products were full-length.

DNA fragments corresponding to 16S locus or ITS region amplification were purified by Quick PCR purification kit (QIAGEN) and the sequences determined by Eurofin MWG operon service (http://www.eurofins.com).

The homology research was undertaken on 16S or ITS rDNA sequences, by BLAST analyser (http://blast.ncbi.nlm.gov) [15].

SEM analysis on wood fragments was performed by Leica microscope LEO-400, after have being coated with gold micro-particles (CAR AGAR sputter coater) [9].

3. Results and discussion

It is well known that in degradation of marine waterlogged wood different processes occur, such as accumulation of reduced sulphur compounds, biological deterioration due to bacteria, fungi, marine bacteria and borer [2,5,16]. The aim of this study is to set up a technical protocol for revealing and characterizing bacterial colonization of waterlogged wood, in order to perform a correct diagnosis, indispensable for an adequate conservation strategy.

Wooden structure (radial and cross sections) and relative alterations (RA1, RA2 and RA3 samples) were observed by Optical and Scanning Electron Microscopy. Results in Figs. 2 and 3 showed the altered structure of wood and revealed the presence on the surface of several chromatic spots, green and dark (thiols, iron sulphides), probably due to both bacterial consortia and environmental conditions.

Table 1
Bacterial species identified by molecular analysis.

Samples	DNA from isolated colonies	DNA from wood fragments	
	ITS – PCR	ITS – PCR	16S – PCR
AR1	*Pseudomonas* sp. *Cellulomonas* sp.	*Desulforudis audaxviator*	*Pseudomonas* sp. *Cellulomonas* sp.
AR2	*Pseudomonas* sp. *Cellulomonas* sp.	*Sphingomonas* sp.	*Pseudomonas* sp. *Cellulomonas* sp.
AR3	*Pseudomonas* sp. *Xanthomonas* sp.	*Marinobacter* sp.	*Pseudomonas* sp.

Molecular investigation results, showed in Table 1, confirm the presence of bacteria such as *Cellulomonas* sp., *Sphingomonas* sp., *Xanthomonas* sp, as well as *Marinobacter* sp. (iron-oxidant bacteria) and *Desulforudis* sp. (sulphate-reducing bacteria).

Moreover, cross and radial sections of each wood fragment have been observed by light microscopy, at a magnification range ×10, 5–40 (reflected light) and ×40–200 (transmitted light), identifying the samples as *Pinus* sp. (B. Megna personal communication).

Considering the complexity of degradation microbial of waterlogged wood, the current study contribute to enrich the knowledge on the bacterial consortia colonizing waterlogged wood recovered in Sicilian sea area.

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