Diversity of bacterial communities on sunken wood in the Mediterranean Sea

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Sunken woods are very rich and diverse ecosystems supporting large macrofaunal diversity and representing a source of carbon and energy for any heterotrophic organism able to consume plant material. However, relatively little is known about the microbial communities that degrade sunken woods and produce reduced compounds that serve as energy sources for chemosynthetic life. The purpose of this study was to explore the bacterial diversity developing on and within sunken woods in a NW Mediterranean submarine canyon and its adjacent open slope by using 16S rRNA genes survey. We described communities from Pine wood immerged at 1200 m deep in the Blanes Canyon and its adjacent open slope, as well as from material filling wood boring bivalve burrows. We demonstrate that bacterial communities were very different from each other in each of the three wood ecosystems. These highly diverse wood communities contained all the major bacterial phyla, but *Alphaproteobacteria* and *Deltaproteobacteria* were dominant in the open slope and the canyon, respectively. The burrows had more *Gamma-* and *Epsilonbacteria*. In summary, highly diverse bacterial communities with potentially wide metabolic capabilities colonized wood sunken in the Blanes Canyon and its adjacent open slope in the Mediterranean Sea.

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1 Introduction

Marine chemosynthetic ecosystems are driven by microbial communities using energy from chemical compounds to produce biomass. Microbes may use either organic or inorganic materials as carbon sources, but also as electron donor or acceptor for the redox reactions providing them with energy for growth. Those communities become especially significant at depths were sunlight is absent and photosynthesis therefore impossible. The importance of such primary producers found in deep-sea ecosystems was first evidenced from the discovery of high biomass invertebrate assemblages associated to hydrothermal vents [1]. The continuous study of the seafloor has further unveiled a number of other geological features, like cold seeps and gas hydrates, where fluids enriched in reduced chemicals (methane, sulfide, hydrocarbons) are used as electron donors by chemosynthetic microbes. During the last three decades, a continuous effort has been dedicated to these environments, and their novel microbial diversity and pathways involved in energy production and carbon fixation [2-4].

Even though the term chemosynthesis is often used in a restricted sense (i.e. limited to methanotrophy and chemolithoautotrophic CO_2 fixation), microbial communities can also grow from organic carbon arriving from the surface as large sinking organic falls. Rich and abundant communities associated with organic falls on the ocean floor, like wood, vegetable debris or bones, have been described since at least the 19th century and the Challenger expedition. Yet, their relation to chemosynthetic ecosystems is a much more recent knowledge. Whale falls were first shown to sustain invertebrate communities with close relationships to those found at hydrothermal vents and seeps [5]. This was attributed to sulphide enrichment in the surrounding sediment, but new results suggest that methane could be available as an important electron donor as well [6].

Attention on sunken woods as an additional type of substrate sustaining chemosynthetic communities has increased more recently. They were suggested to play an important evolutionary role in the diversification of chemosynthetic ecosystems, representing a stepping stone for the colonization of vent and seep habitats [7]. Wood is not produced in the ocean but is exported from continents and islands and often encountered on the seafloor [8]. Wood can thus represent a pulsed source of energy and carbon allowing the establishment of chemosynthetic communities. Most researches on sunken wood have focused on the faunal diversity colonising that substrate and their symbioses with chemosynthetic microbes [9-11].

Microbial communities directly degrading the wood, and representing the base of the unique sunken wood chemosynthetic ecosystem remain, however, poorly studied.

Early researches based on microscopy and cultures have focused principally on the mechanical process of wood degradation by microbes [12]. But recently, experimental studies based on in situ deployment of wood [13] have provided new insights on the diversity of microbial communities [14, 15], and on the processes of shipwreck degradation [16]. Nevertheless, the identity of wood degrading microbial communities, their ecological function and the metabolic pathways involved are still mostly unknown. The aim of the present research was thus to unveil the diversity of bacterial communities colonizing sunken wood by cloning and sequencing the 16S rRNA gene.

2 Material and methods

2.1 Samples

Triplicate pieces of Pine attached to a mooring line at 1200 m depths, and 20 m above sediments, in the Blanes Canyon (BC) (NW Mediterranean) (41°34'N, 02°50'E) and its adjacent open slope (OS) (41°15' N, 02°48' E) were recovered after 1 year of immersion. Wood chips were cut on board the ship using sterilized tools, flash frozen in liquid nitrogen and placed at -80 ºC. BC samples showed more macroscopic signs of decay, and were highly colonized by wood-boring moluscs belonging to the genus *Xylophaga* (Romano *et al.* 2011, in preparation). The *Xylophaga* burrows contained compacted chimneys formed by digested material excreted by the bivalves as it moves through the wood. This digested wood was also collected and samples were called "burrow".

2.2 DNA extraction

A RETSCH Mixer Mills (Retsch Inc.) was used to grind and homogenize the wood by impact and friction using 25 ml Stainless steel grinding jars and a 20 mm diameter stainless steel ball. One sterile ball was placed inside the sterile grinding jar and dipped into liquid nitrogen. Then, a frozen wood chip of 1-1.5 g was placed into the cold jar, and immersed in liquid nitrogen until it stopped boiling. Tubes were passed through one cycle of bead beating for 1 min at 30 Hz followed by two cycles for 2 min at 30 Hz. The jars were immersed in liquid nitrogen between each bead beating cycle to keep them frozen. One gram of genomic DNA from the wood powder from each sample was extracted using DNeasy PlantMini kit

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(QIAGEN) following the manufacturer's protocol with minor modifications: incubation of buffer AP2 was increased from 5 to 10 min at -20°C, and the eluate was concentrated with 140 µl of Buffer AE.

2.3 PCR, cloning and sequencing the 16S rRNA gene

Bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR) using specific primers 27F [17] and 1492R [18]. PCR amplifications for bacteria were performed in 25 µl containing 1 µl of DNA template, 0.1 µM of each primer, 2 units of SuperTaq polymerase and 1xSuperTaq buffer (HT Biotechnology, Cambridge, UK), 200 µM each dNTP, and 13.5 µl of PCR grade water (Sigma). PCRs were carried out with conditions as follows: 3 min at 95°C, followed by 25 cycles at 95°C for 1.5 min, 50°C for 1 min, 72°C for 2 min and then a final elongation step of 72°C for 10 min.

We constructed one clone library for the wood submerged in the Blanes Canyon (BC), one for wood from the Western open slope adjacent to the canyon (OS) and one for the matter contained in the bivalve burrows (Burrow). Each library was constructed from DNA extracted separately from triplicate pieces of wood before being pooled together for PCR. PCR products were cloned using the TOPO TA cloning kit (Invitrogen) and sequenced with the 27f primer by a commercial laboratory (Macrogen, Seoul, South Korea).

2.4 Phylogenetic analysis and diversity indices

Sequence homology searches were performed using BLASTN on a local Silva100 database [19] and this phylogeny was used for phylum and class level phylogeny. Initial multiple alignments of all sequences were calculated using SINA webaligner [19]. Chimeric sequences were detected using Mallard [20] followed by Pintail [21] comparison with its first BLAST hit. All putative chimeras were excluded from further analysis. Sequences were clustered into Operational Taxonomic Units (OTUs) using the software package Mothur [22] employing the furthest neighbor algorithm. A distance value of 0.01 was determined to be a reasonable cutoff value for OTU definition based upon the number of clusters observed for each sample at each specific distance. One representative sequence of each OTU with 5 sequences or more (≥5) (OTU-rep) was used for further in-depth phylogenetic analyses. This OTU-rep was the sequence that had the minimum distance to the other sequences in the same OTU as calculated in Mothur [22]. A phylogenetic tree of OTU-reps was calculated using the ARB

software package [23] and iTOL [24] was used for tree display together with OTUs abundance data. Richness Chao, abundance-based coverage estimation (ACE), and Shannon alpha diversity indices, as well as rarefaction and abundance-rank plots, were calculated using Mothur [22].

3 Results

3.1 Diversity of bacterial communities

We constructed clone libraries of wood associated bacteria at BC, OS and Burrow, obtaining 76, 78 and 72 sequences respectively . The Burrow community appeared as the most diverse, with the highest Chao index and rarefaction curve (Table 1, Fig. 1). The BC sample was the second most diverse, followed by the OS. None of the rarefaction curves reached a plateau indicating that the *in situ* diversity was greater than the diversity covered by our sampling effort (Fig. 1).

3.2 Composition of bacterial communities

Representatives of most bacterial phyla including *Alpha-, Gamma-, Delta-, Epsilon-proteobacteria, Planctomycetes, Bacteroidetes, Firmicutes, Acidobacteria, Actinobacteria* and *Verrucomicrobia* were present in the wood and burrow samples (Fig. 2). *Proteobacteria* was the major group recovered in all samples (ranging from 39 % to 43 % of the recovered ribotypes), but the subdivisions within this phylum were differentially distributed. *Alphaproteobacteria* formed the dominant bacterial group in all samples, however, their proportion was similar to the *Deltaproteobacteria* in BC (Fig. 2). Burrow samples were characterized by more *Gammaproteobacteria*, *Bacteroidetes* and *Epsilonbacteria* (Fig. 2).

Grouping sequences at a 99 % similarity level resulted in a total of 126 OTUs. At the OTU level, the most abundant phylotypes detected was OTU 56 affiliated to *Deltaproteobacteria* and identified as *Myxococcales* found in OS and BC (Fig. 3). It was followed by OTU 10, affiliated to *Erythrobacter* (*Alphaproteobacteria*) discovered in OS and burrow. Other abundant phylotypes were OTU 23 closely related to *Teredinibacter* (*Gammaproteobacteria*) and observed in BC and burrow. OTU51 closely related to *Alteromonadaceae* (*Gammaproteobacteria*) found more in BC and OS. Interestingly, no *Deltaproteobacteria* or *Planctomycetes* were found inburrow. Most of them were recovered in

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BC and some of them were shared with OS. In contrast, no *Gammaproteobacteria* were observed in OS but they were recovered from BC and burrow. Furthermore, *Epsilonproteobacteria* was found only in burrow (Fig. 2) where it was dominated by OTU 6 represented by *Arcobacter* (Fig. 3). Interestingly, *Firmicutes* and *Verrucomicrobia* derived sequences were also only observed in burrow. Most OTUs belonging to *Bacteroidetes* were affiliated with *Flavobacteriales* and found in OS and BC. Finally, most of the site specific OTUs found within *Alphaproteobacteria* were observed in OS (Fig .3).

The estimated OTUs shared amongst samples are summarized in the Venn diagram (Fig. 4). The numbers of OTUs in burrow, BC, and OS were quite similar, 48, 46, 45, respectively. Surprisingly, no OTUs were shared between all samples. Only 3 OTUs were shared between burrow and BC, these were OTU 22 (*Bacteroidetes*, *Flavobacteria*), OTU 23 (*Gammaproteobacteria*, *Alteromonadales*) and OTU 30 (*Alphaproteobacteria*, *Sphingomonadales*). Three OTUs were shared between burrow and OS: OTU 43 and OTU 37 (*Alphaproteobacteria*, *Rhodobacterales*) and OTU 10 (*Alphaproteobacteria*, *Sphingomonadales*). Seven OTUs were shared between BC and OS: OTU 70 (*Alphaproteobacteria*, *Rhizobiales*), OTU 51 (*Gammaproteobacteria*, *Alteromonadales*), OTU 84 (*Bacteroidetes*, *Flavobacteria*), OTU 56 and OTU 83 (*Deltaproteobacteria*, *Myxococcales*), and OTU 90 and OTU 74 (*Planctomycetes*).

4 Discussion

The goal of this study was to determine the bacterial diversity in Pinewood samples experimentally submerged in the Blanes Canyon and its adjacent open slope. The qualitative distribution of bacterial 16S rRNA sequences indicated a significant difference in community composition between sunken wood environments. Bacterial phylotypes were most often unique and characteristic to each habitat, with very little overlap between samples. The results support the idea of highly diverse and specific microbial communities on sunken woods in the Mediterranean Sea.

Proteobacteria largely dominated bacterial communities overall and the majority of sequences were assigned to the subclass *Alphaproteobacteria*. It seems probable that microorganisms belonging to that class could play an important ecological role on all sunken wood ecosystems. Unfortunately, the metabolism of *Alphaproteobacteria* that were detected

on our samples is not known as the sequences were related to uncultured organisms. However, some possible metabolisms present on sunken woods could be extrapolated. For instance, some *Gammaproteobacteria*, which were more abundant in Blanes Canyon burrows, belonged to *Teredinibacter*. *Teredinibacter* is a bacterial symbiont of Teredinidae (i.e., the so called shipworm molluscs), other species of marine wood-boring bivalves more common in shallower environments. Because shipworms are unable to metabolize wood cellulose directly, they developed a symbiosis with cellulolytic nitrogen-fixing bacteria that provide the host with the necessary enzymes for survival on a diet of wood cellulose [25]. These symbiotic bacteria reside in distinct structures lining the interlamellar junctions of the gill [25, 26]. Our results show that these bacteria may be shared between different groups of wood boring bivalves such us the shallow *Teredo* spp. and the deep-sea *Xylophaga* spp.

On the other hand, the presence of *Bacteroidetes*, with the majority belonging to *Flavobacteriaceae* (predominantly chemoorganoheterotrophs) recovered in both wood samples could be explained by especial capability for degrading proteinaceous, saccharidic, pectinic or cellulosic substrates [27]. These bacteria are ubiquitous and abundant in organic-rich habitats and probably play a major role in the turn-over of organic matter [27]. Some *Flavobacteriaceae* have also been shown to be abundant in whale fall-associated sediments [6] and could therefore represent ubiquitous components of microbial communities associated to large organic falls in the sea.

Interestingly, we found *Epsilonbacteria* exclusively in burrow samples with sequences closely related to the *Arcobacter* genus that have been previously detected on the bone surfaces of whale falls [28]. Above all, it is recognized that *Epsilonbacteria* are the prevalent bacterial phylotype in deep-sea hydrothermal vent ecosystems [29-31]. *Epsilonbacteria* are a heterogeneous group for which microaerophily and sulphur metabolism is a very common features [32]. Furthermore, the genus *Arcobacter* was recently described in deep-sea hydrothermal sulfur mat [33]. Although one can only speculate on the metabolism of sunken wood *Arcobacter*, they might be sulfur-oxidizing bacteria and could be involved in the formation of sulfur filaments.

Firmicutes and *Verrucomicrobia* were also observed only in burrow samples. It is interesting to note that those bacteria were reported for a first time in organic rich mucous secretions of the hydrothermal vent species polychaete *Paralvinella palmiformis* and some of them have remarkable feature to use preferentially sugars as substrates for growth [32].

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Verrucomicrobia were known to exist in many aquatic habitats, not only at moderate temperatures but also at cold temperatures in the deep sea and in Antarctica. Moreover in the review of Schlesner et al. (2006), several reports identified members of *Verrucomicrobia* in extreme environments such as sulfide-rich water and sediments, a soda lake and hot spring. Interestingly, a member of the *Verrucomicrobia* was identified in the chitin tubes of the giant vent worm *Riftia pachyptila*, illustrating another connection between this group and thermal environments [34]. Like *Flavobacteriaceae* discussed above, *Verrucomicrobia* groups are known for their ability to degrade biomacromolecules [32]. This could explain that these organisms colonize sunken woods.

In summary, highly diverse microorganisms with potentially wide metabolic capabilities colonized sunken wood from the Blanes Canyon and its adjacent open slope. The Blanes canyon seemed to have higher bacterial diversity than outer slope, and some of the bacterial sequences were related to bivalve symbionts.

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Table 1. Diversity of bacterial 16S rRNA clone libraries from pine samples submerged around the Blanes Canyon. Wood samples were recovered after 1 year at 1200 m deep. Phylotypes and diversity were based on OTUs defined by > 99 % similarity between sequences.

Samples	No. of clones	No. of phylotypes	Singletons	Shannon	Chao1
Blanes Canyon Wood	76	46	34	3.550178	126
Open Slope Wood	78	45	27	3.640197	80
Burrow matter	72	48	37	3.654075	143

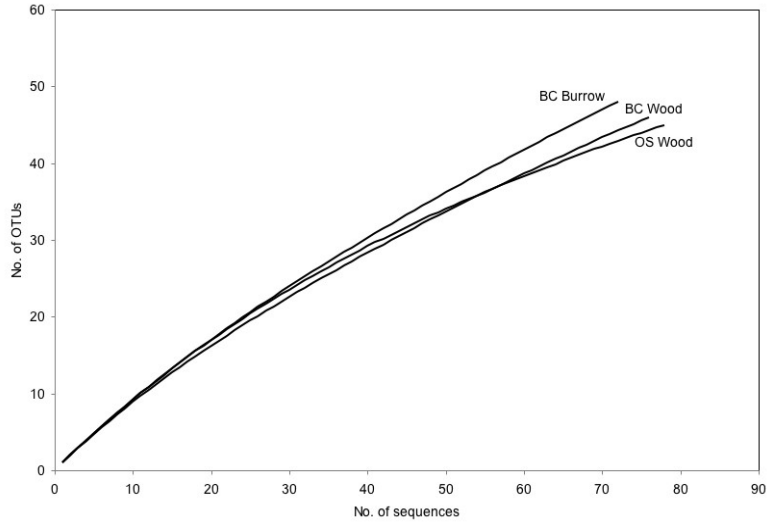

Fig. 1. Rarefaction curves calculated for 16S rRNA clone libraries of Bacteria from sunken wood: BC (Blanes Canyon), OS (open slope) and Burrow (matter inside the bivalve burrows). Rarefaction was calculated with Mothur at the 1% level.

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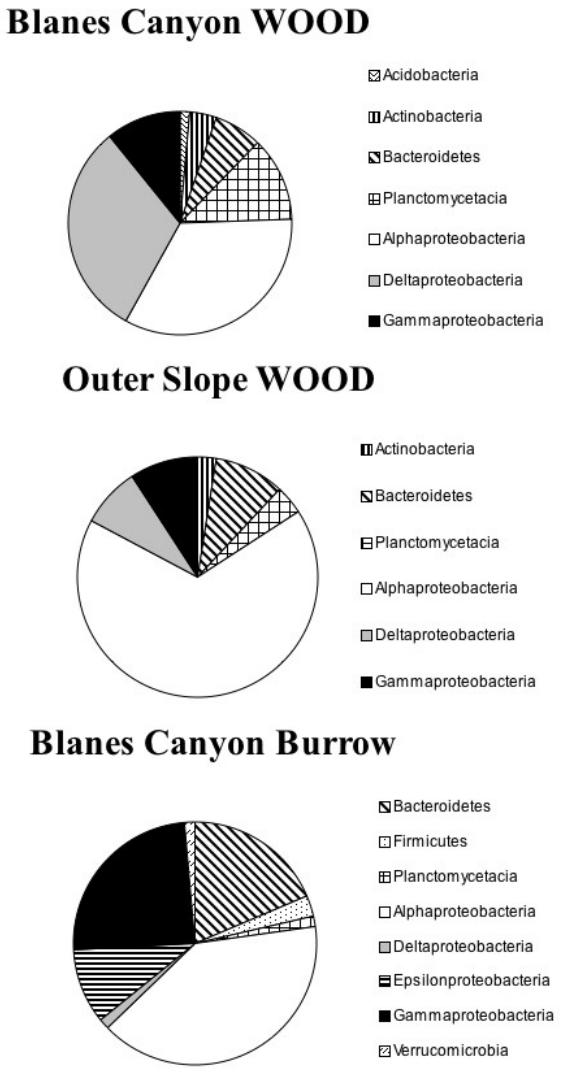

Fig. 2. Bacterial community structure at the phylum or class level found in the sunken wood samples collected around the Blanes Canyon.

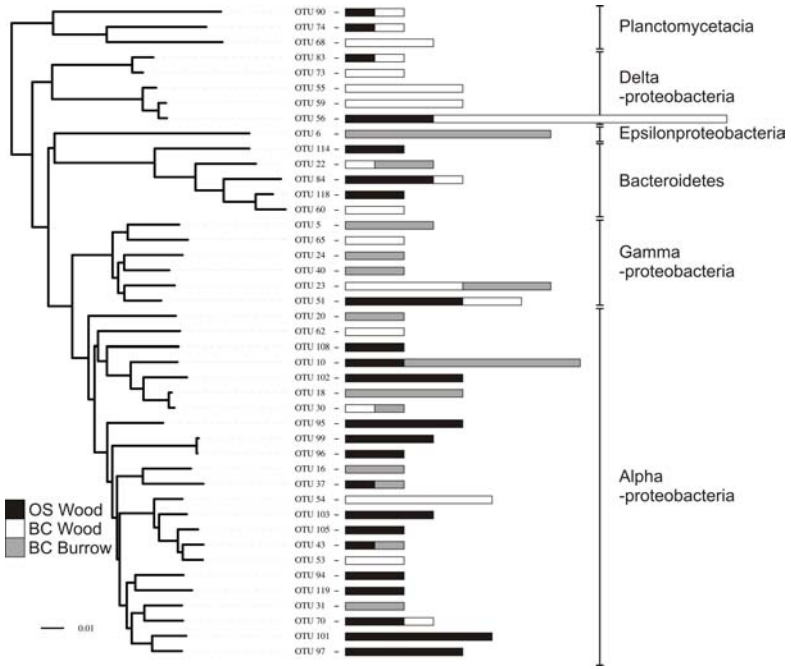

Fig. 3. Neighbor-joining distance phylogenetic tree (Jukes-Cantor correction) of all OTUs recovered in sunken wood samples. Bars represent the number of sequences contained in each OTU. BC: wood from the Blanes Canyon; OS: wood from the open slope; Burrow: material from burrows of Blanes Canyon bivalves.

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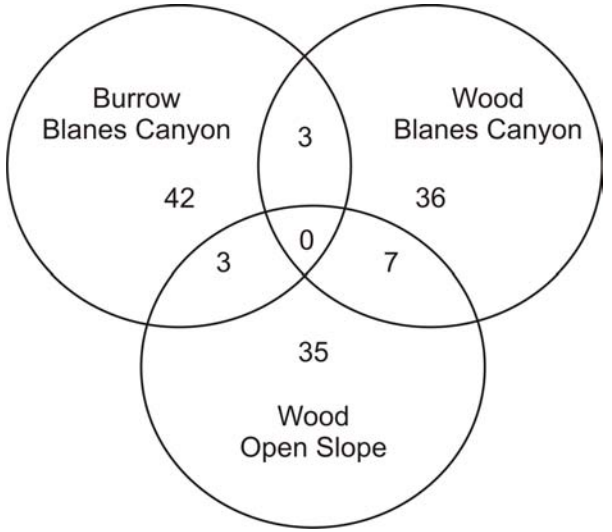

Fig. 4. Venn diagram showing OTUs, defined at a 99% cutoff, shared between the sunken wood samples collected around the Blanes Canyon.