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**Sunken woods on the ocean floor provide diverse specialized  
habitats for microbes**

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## ABSTRACT

Marine waterlogged woods on the ocean floor provide the foundation for an ecosystem resulting in high biomass and potentially high macrofaunal diversity, similarly to other large organic falls. However, the microorganisms forming the base of wood fall ecosystems remain poorly known. To study the microbial diversity and community structure of sunken woods, we analyzed over 2500 cloned archaeal and bacterial 16S rRNA gene sequences from samples with different geographic locations, depths and immersion times. The microbial communities from different wood falls were diverse, suggesting that sunken woods provide wide-ranging niches for microbes. Microorganisms dwelling at sunken woods change with time of immersion most likely due to a change in chemistry of the wood. We demonstrate, for the first time in sunken woods, the co-occurrence of free-living sulfate reducing Bacteria (SRB) and methanogens and the presence of sulfide oxidizers. These microorganisms were similar to those of other anaerobic chemoautotrophic environments suggesting that large organic

falls can provide similar reduced habitats. Furthermore, quantification of phylogenetic patterns of microbial community assembly indicated that environmental forces (habitat filtering) determined sunken wood microbial community structure at all degradation phases of marine woodfalls. We also include a detailed discussion on novel archaeal and bacterial phylotypes in this newly explored biohabitat.

## INTRODUCTION

Terrigenous organic material such as wood can be exported to the marine environment by river waters and storm events. Once in marine waters, wood logs will sink and eventually end up on the ocean floors (Wolff, 1979). Patterns and processes of the ecology and evolution of sunken woods and the transformation of this organic matter in marine environments are not yet fully understood. Sunken wood harbors a high diversity of macrofauna (Gareth Jones *et al.*, 1976; Wolff, 1979) and comparative studies between organic falls and hydrothermal vent fauna suggest that wood falls could play a major role in the adaptation, dispersal, and evolution of chemosynthesis-based communities on the sea floor (Bennett *et al.*, 1994; Distel *et al.*, 2000; Smith & Baco, 2003).

Once on the ocean floor, wood falls may become anaerobic due to sustained decay of its major components: hemicellulose, cellulose and lignin. Hemicellulose is more readily degradable than cellulose while lignin is very difficult to degrade and can maintain the structure of the wood even when the other components are degraded (Clausen, 1996). The degradation of sunken woods is expected to be successional. First, soft rot fungi and hydrolytic bacteria will yield cellobiose and glucose. Bacteria and Archaea may then ferment these compounds to organic acids, CO<sub>2</sub> and H<sub>2</sub>. In marine environments, due to high sulfate concentration, sulfate-reducing bacteria could out-compete methanogens for the use of H<sub>2</sub> (Leschine, 1995). This will lead to an enrichment of H<sub>2</sub>S at the surface of the wood, as observed by

Laurent *et al.* (2009) in sunken wood from a mangrove ecosystem. Chemoautotrophic microorganisms could in turn use the H<sub>2</sub>S and/or methane byproducts of wood degradation as energy source, as observed in marine mud volcanoes, hydrothermal vents or cold seeps (Jorgensen & Boetius, 2007), whale falls (Deming *et al.*, 1997; Goffredi & Orphan, 2010; Goffredi *et al.*, 2008), and bivalve symbiotic bacteria inhabiting sunken woods (Duperron *et al.*, 2008; Lorion *et al.*, 2009). Microbial communities on waterlogged wood could thus have a potential for high diversity since it entails two components, a wood degrading heterotrophic community and a chemosynthetic community.

In spite of their importance for marine ecosystems evolution, sunken woods harbor an as yet little explored free-living microbial community. Early studies characterized bacteria according to their physical action on wood, i.e. tunneling versus eroding bacteria (Jurgens *et al.*, 2003; Kohlmeyer, 1978; Mouzouras *et al.*, 1988). Recently, a free-living SRB was isolated from a wood fall (Khelaifia *et al.*, 2011) and Landy *et al.*, (2008) showed the presence of Bacteroidetes and Pseudomonas in archeological waterlogged wood. Palacios *et al.* (2009) found significantly different bacterial fingerprinting profiles in samples with low vs. high signs of decay, independent of the biogeographic origin or depth of the sample, suggesting temporal succession in communities populating waterlogged woods. Archaea, important chemoautotrophes in other marine anaerobic environments, were present only in woods with notable decay and surrounding sediments (Palacios *et al.*, 2009).

Ecosystems differ in the way coexisting species are distributed (Horner-Devine *et al.*, 2007). One way to explore the principal ecological processes underlying the microbial community distribution is to estimate if the bacterial assemblage shows evidence of a significant phylogenetic structure. To study tree topologies, two indices, net related index (NRI) and the nearest taxa index (NTI) have been proposed (Webb *et al.*, 2002) and recently also utilized for microbial ecology (Barberán & Casamayor, 2010; Horner-Devine & Bohannan, 2006). The analysis is based on the hypothesis that when

communities are structured by processes such as competitive exclusion, species will be more phylogenetically distant than expected by chance, leading to a pattern of “phylogenetic overdispersion”. Inversely, “phenotypic attraction” will be observed through a pattern of “phylogenetic clustering” if environmental forces (habitat filtering) are the main processes structuring the community. We examined the phylogenetic structure of sunken wood microbial communities to determine whether abiotic filtering is the dominating ecological process driving the sunken woods microbial community composition. The importance of this process is becoming increasingly evident in microbial ecosystems (Amaral-Zettler *et al.*, 2011; Barberán & Casamayor, 2010; Herrera *et al.*, 2010; Horner-Devine & Bohannan, 2006; Ojeda *et al.*, 2010). We hypothesize that abiotic filtering will be more prevalent in long-time compared to short-time immersed woodfalls because a larger number of available micro-niches will decrease competition among community members. Furthermore, we wanted to study the phylogenetic and ecological diversity of Bacteria and Archaea associated with wood falls. We expected that sunken woods would harbor a great diversity, possibly forming specialized microbial communities compared to other marine habitats. We also envisaged that an in-depth phylogenetic analysis would give clues on the potential wood degradation pathways in the marine environment.

## **MATERIAL AND METHODS**

### *Sample collection*

Sunken wood samples were collected from the Pacific Ocean and the Mediterranean Sea (Fig. 1). Pine, coconut and mangrove wood pieces (1x1x3 cm) were experimentally implanted on the seafloor in the South Pacific (New Caledonia 22°31'S, 166°15'E, 1100m depth) and collected by trawling after 20 months of immersion. Douglas fir wood pieces (8.75x11.25x60 cm) were implanted in the North Pacific (Santa Cruz Basin, California, 33°30' N, 119°22 W, 1624m depth) and collected using an ROV. The

Douglas fir piece was subdivided into outer (<1cm from surface) and inner (>1cm) parts, which had different signs of decay. Ficus and oak samples were driftwood that had been naturally sunken and found during dredging in the West Pacific (Vanuatu, 15°3'7" -5'5" S, 166°55'5" -23" W, 1000m depth) and the Mediterranean (42°29'447" -732" N, 3°10'201" -9'937" E, 57m depth), respectively. They had advanced signs of decay compared to artificially immersed wood pieces. Wood chips were cut, frozen and kept at -80°C until DNA extraction, except for the Douglas fir samples (FirCal) which were subsampled while frozen. For an in-depth description of sampling locations, handling and DNA extraction see Palacios *et al.* (2009). Briefly, samples were mechanically powdered in a TissueLyser (Qiagen, Courtaboeuf, France) with 25 mL grinding jars (Retsch GmbH, Haan, Germany) and 2-cm diameter steel beads that had been immersed in liquid nitrogen. DNA was extracted with the DNeasy Plant Mini kit (Qiagen) with minor modifications (Palacios *et al.* 2009).

*Polymerase chain reaction (PCR) amplification, clone libraries construction, and sequencing*

Genomic DNA from wood samples was PCR amplified using universal bacterial 16S rRNA gene primers 9bf (5'-GRGTTTGATCCTGGCTCAG-3') (Burggraf *et al.*, 1992; Eder *et al.*, 2001) and 1492R (5'-CGGCTACCTTGTTACGACTT-3') (Teske *et al.*, 2002). PCR amplifications for bacteria were performed with 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 molecular grade water (Sigma). PCRs were carried out using a Robocycler (Stratagene, Amsterdam, The Netherlands) for 3 min at 95°C, 25 cycles at 95°C for 1.5 min, 50°C for 1 min, 72°C for 2 min; and a final elongation step of 72°C for 10 min. Archaeal 16S rRNA gene amplifications were performed using primers A109F (5'-ACKGCTCAGTAACACGT-3') (Reysenbach & Pace, 1995) and A1492R (5'-GGCTACCTTGTTACGACTT-3') (Palacios *et al.*, 2009). Fifteen replicate PCR amplifications were pooled to reduce bias. To minimize the presence of heteroduplex molecules, we employed a reconditioning PCR of 4-5 cycles with 1µl of mixed

original PCR reactions (Thompson *et al.*, 2002). PCR products were purified using Qiaquick (Qiagen) and cloned with the TOPO TA kit with pCR2.1 vector (Invitrogen) and sent for sequencing to Macrogen Inc. (Seoul, Korea). The sequencing reaction was performed using the reverse primer both for Bacteria and Archaea.

### *Sequence processing*

Sequences were processed through an automated perl pipeline that used PHRED (Ewing *et al.*, 1998) for base-calling and LUCY (Chou & Holmes, 2001) for quality trimming and vector removal. Sequences shorter than 500 bp were excluded from analysis. Sequence homology searches were performed using BLASTN on a local Silva100 database (Pruesse *et al.*, 2007). Sequences were assigned to the phylum or class level based upon their BLAST hits to previously described sequences in GenBank. Initial multiple alignments of all sequences were calculated using SINA webaligner (Pruesse *et al.*, 2007). Chimeric sequences were detected using Mallard (Ashelford *et al.*, 2006) followed by Pintail (Ashelford *et al.*, 2005) comparison with its first BLAST hit. All putative chimeras were excluded from analysis and this resulted in 2385 bacterial and 512 archaeal sequences used for further analysis. Sequences were clustered into Operational Taxonomic Units (OTUs) using the software package mothur (Schloss *et al.*, 2009) employing the furthest neighbor algorithm. A distance value of 0.01 was the best cutoff value for OTU definition based upon the number of clusters observed for each sample at each specific distance (Fig. S1A). The curves flatten out at this value, where most microdiversity concentrates and where we can assume that OTUs form cohesively neutral units of diversity ((Palacios *et al.*, 2008), and references therein). This cutoff was used for all the community analyses, however we provide the Shannon index at 0.03 distance level in Table 1 for comparison. One representative sequence of each OTU with 5 sequences or more (OTU-rep) was used for in-depth phylogenetic analyses. Sequences obtained have been submitted to the GenBank database under accession numbers JN106724-JN107532.



### *Phylogenetic analyses*

Bacterial sequence phylogenetic analyses were performed using the ARB software package (Ludwig *et al.*, 2004) with sequences aligned using the SINA webaligner (see above). Pairwise distance matrices among sequences were generated using the ARB neighbor-joining (NJ) algorithm with Jukes-Cantor corrected distance values. Bootstrap analysis (100 replicates) was performed using the PHYLIP package (Felsenstein, 1993). A manual filter was generated by employing the "filter by base frequency" (frequency = 50) tool in ARB, and manually masking out highly variable regions. A phylogenetic tree of OTU-reps was calculated and iTOL (Letunic & Bork, 2007) was used for tree display together with OTUs relative abundance data. Phylogenetic trees with the OTU-reps together with the nearest neighbors were made using backbone NJ trees containing near full-length sequences as described above and adding shorter sequences representing the OTUs *a posteriori* by parsimony in ARB. Furthermore, one NJ tree of all sequences and one tree containing all OTUs (679 OTUs) was generated in ARB for downstream phylogenetic community structure analyses.

### *Ecological diversity analyses*

Richness Chao and Shannon alpha diversity indices, as well as rarefaction and abundance-rank plots, were calculated using mothur. To compare microbial communities between samples (beta diversity), we calculated the Bray-Curtis index, as well as the phylogenetically based parsimony test (Martin, 2002) using mothur. Output pairwise distance matrices among samples from these beta metrics were clustered using the UPGMA algorithm in mothur (Schloss *et al.*, 2009). For assessing phylogenetic structure of community assemblages, a phylogenetic tree containing all OTUs was used as input for Phylocom 4.1 (Webb *et al.*, 2008). Within this program, we used the COMSTRUCT function to calculate Net Related Index (NRI) and Nearest Taxon Index (NTI) which measure alpha diversity pairwise distances

among OTUs in each sample (Webb *et al.*, 2002). As metrics of inter-community phylogenetic dissimilarity (phylogenetic beta diversity) we use function NTCOMDIST from Phylocom, which use nearest taxon (MNTD) phylogenetic distances between all pairs of OTUs in each of every pair of samples. Output pairwise distance matrices were clustered using UPGMA.

## RESULTS

### *Microbial species diversity in sunken woods*

To investigate the microbial diversity associated to sunken woods we chose a subset of mainly deep-sea samples from a broader fingerprinting study on sunken woods (Palacios *et al.* 2009) with representative samples from short and longtime immersed woods (Fig. 1). Both bacterial and archaeal 16S rRNA gene primers were used, and the alpha diversity measures for Bacteria are presented in Table 1. FicPac had the highest diversity followed by OakMed, as shown by alpha diversity indices (see Table 1, also for sample names) and rarefaction curves (Fig. S1B). MangPac was overall the less diverse sample. Rank abundance curves (Fig. S1C) showed that less diverse communities were composed of a few OTUs that dominate the assemblage and the most diverse communities had a more even phylotype distribution (shallower slopes of OakMed and FicPac, Fig. S1C). To determine the phylogenetic structure of the microbial communities in our samples, we calculated the Net Related Index (NRI) and Nearest Taxon Index (NTI). These indices measure the degree of phylogenetic clustering of taxa across a phylogenetic tree in one particular sample and are based on the mean pairwise phylogenetic distance (MPD) and mean phylogenetic distance to closest relative (MNTD). The significance of these metrics is then compared to null distributions ( $10^5$  randomized runs) (Table 1). NRI and NTI values significantly higher than zero indicates that a community is clustered, while values significantly lower than zero

indicates “phylogenetic overdispersion”, for more details, see Horner-Devine & Bohannan (2006).

Indeed, we observed an NTI value above zero for all samples ( $NTI > 0$ ,  $P < 0.001$ ) and longer immersed samples, FicPac and OakMed, also had relatively higher NRI values (Table 1). The phylogenetic overdispersion pattern was rejected for all samples ( $NTI$  and  $NRI < 0$ ,  $P < 0.001$ ).

Archaeal 16S rRNA genes amplified and cloned from FicPac, OakMed, and FirCalOut resulted in only 213, 210 and 67 archaeal sequences, respectively. The other samples yielded no archaeal PCR products or very few archaeal sequences. FicPac and OakMed were the most diverse samples containing 101 OTUs (Chao = 235) and 35 OTUs (Chao = 57), respectively. FirCalOUT had only 7 OTUs, MangPac contained 2 OTUs belonging to DHVE6 group and CocoPac 1 OTU belonging to Methanosarcina.

All beta diversity measures separated sunken wood samples in two clusters, one containing OakMed and FicPac and the other grouping the rest of the samples. Samples had different bacterial communities as per the long branches and close-to-one distances within clusters on the UPGMA dendrogram (Fig. 1, left). Parsimony test, which uses a tree containing all sequences and do not take into account sequences relative abundances, confirmed that sunken wood samples were phylogenetically different ( $P < 0.001$ ). COMDISTNT “phyloordinations” from Phylocom corroborates the relationship among samples obtained using previous beta diversity methods (Fig. 1, right dendrogram), however, within-cluster branches in the UPGMA tree were much shorter. Phylocom uses a tree of all OTUs and weights distances by taxa relative abundances.

#### *Bacterial and archaeal community composition in sunken woods*

A broad taxonomic classification showed that sunken woods differed at Bacteria phylum and class levels (Fig. 2), but OakMed and FicPac appeared more similar to each other than the others. A finer phylogenetic analysis was performed using the most relatively abundant bacterial OTUs populating sunken woods. This included OTUs with 5 or more sequences (OTU-rep) that are presented in Fig. 3 and

table S1. A detailed description of the OTUs is presented in Supplemental Text 1 (ST1). Also included in ST1 is the distribution of rare OTUs, which were overrepresented in the more diverse samples, OakMed and FicPac (Table 1).

Proteobacteria was by far the most frequent phylum in sunken woods. All samples contained Alpha-, Delta- and Gammaproteobacteria, but their relative abundance varied considerably between samples (Fig. 2A). For example, Alphaproteobacteria dominated the PinPac sample, but was a minor constituent of other woods. Furthermore, Deltaproteobacteria was present in all samples in substantial amounts (>14%) and it was the most dominant phylum in OakMed, FicPac and FirCalOUT. Beta- and Epsilonproteobacteria were not present in all samples but they were found to be relatively abundant in the MangPac sample. Also, Bacteroidetes was especially frequent in MangPac and FirCalIN (Fig. 2A).

Indeed, the most predominant OTU in the whole study was found within the class Bacteroidia. This OTU, namely SWB 1 (Sunken Wood Bacteria), contained 91 sequences mostly from MangPac, CocoPac and FirCalIN (Fig 3). SWB 6, with 61 sequences, was very closely related to SWB 1. These OTUs fall within the Marinilabiaceae (Fig. S2). Firmicutes, Planctomycetes and Spirochaetes were present in all samples in varying amounts but never as dominant phyla (<16% of the total clones in any sample). The group "Others" also varied greatly between samples (see ST1 for further description). Furthermore, FicPac and OakMed contained a relatively large amount of "Unclassified" clones. Many of the unclassified sequences from these two samples had a top BLAST hit to clones from a hypersaline microbial mat (EU245176) (Isenbarger *et al.*, 2008), but with only 95% identity. The other "Unclassified" were phylogenetically more spread out.

The phylogenetic classification of Archaea sequences showed that FicPac and OakMed were more similar to each other than to FirCalOUT in terms of community composition (Fig. 2B). FicPac and OakMed contained around 40% of Crenarchaeota, including Miscellaneous Crenarchaeotic Group (MCG)

(Inagaki *et al.*, 2003) and Marine Benthic Group B (MBGB) (Vetriani *et al.*, 1999) (Fig. 2B). We also found OTUs within the Crenarchaeota Group Ib, originally from soils but also detected in freshwater samples and deep subsurface (Schleper *et al.*, 2005). The most relatively abundant OTU among the Archaea, SWA 117, was found within the MCG (Inagaki *et al.*, 2003) (Fig. 4). This group is usually found in deep marine subsurface sediments (Teske & Sorensen, 2008) but SWA 117 were closely related to sequences found in salt marsh sediments (Fig. S5 and Table S1).

Euryarchaeota were present in all sunken woods but especially in FirCalOUT (Fig. 2B).

Methanosarcinales dominated the FirCalOUT sample, but was also present in FicPac and OakMed.

Numerous euryarchaeal OTUs fell within the DHVE6 and DHVE5 clades (Fig. 4), originally detected in hydrothermal vents (Takai & Horikoshi, 1999). Finally, we delineated a new clade of Euryarchaeota, named “Wood Group”, positioned between Methanogens and DHVE5 and DHVE6 clusters with <93% similarity to database sequences (Fig. 4 and S5).

## DISCUSSION

### *Sunken woods harbor diverse microbial communities at all degradation stages*

Our results showed a high microbial species diversity in all sunken woods ( $H^{197} = 3.39-4.74$ ;  $H^{99}=3.59-4.95$ ) (Table 1). This is comparable to organic rich sediments such as those found associated with whalefalls ( $H^{97}=3.93-4.59$ ) (Goffredi & Orphan, 2010) or the more diverse deep-Sea Mud Volcano sites ( $H^{98}=1.47-4.03$ ) (Pachiadaki *et al.*, 2011; Pachiadaki *et al.*, 2010). Indeed, many of the first BLAST hits were from marine sediments and carbon rich habitats such as whale falls, mud volcanoes and hydrothermal vents (Table S1). Furthermore, parsimony test show that all communities are significantly different, suggesting that sunken woods can provide diverse habitats for microorganisms.

Alpha diversity measures show that there was a lower microbial diversity in short-time compared to long-time immersed wood samples (Table 1, Fig S1), this difference was supported by clustering analysis using beta diversity measures (Fig. 1). Microorganisms in the younger samples are probably more specialized towards a limited range of substrates such as the breakdown of cellulose, cellobiose and fermentation of sugars. Then, as the breakdown of the wood matrix proceeds, more electron and carbon sources become available and micro-niches will be created. As degradation continues the wood becomes anaerobic which can in some cases promote diversity (Bowman & McCuaig, 2003), this is likely the case for the older samples FicPac and OakMed. Furthermore, wood-boring fauna are expected to promote micro-niches within the wood matrix.

We have been able to distinguish between the processes (biotic vs. abiotic) responsible for community assembly in wood falls by observing patterns of phylogenetic relatedness compared to patterns in randomly generated null phylogenies (Horner-Devine & Bohannan, 2006; Webb *et al.*, 2008). We demonstrate “phylogenetic clustering” of OTUs at terminal nodes for all samples (pairwise phylogenetic alpha diversity NTI  $\gg$  0) (Table 1). Phylogenetic clustering, also called “phenotypic attraction” (Webb *et al.*, 2008), implies that species composition in sunken wood ecosystems is not managed by biological processes such as competition but is driven by the chemical conditions in the wood. Furthermore, an NTI value significantly above zero means that OTUs cluster together at terminal tree nodes, while a high NRI value indicates phylogenetic clustering at deeper phylogenetic levels (Webb *et al.*, 2002). Samples immersed for a longer time (OakMed and FicPac) had also relatively higher NRI values (Table 1), suggesting that environmental forces become more relevant with more extreme (anaerobic) chemical conditions. These results are in agreement with a direct observation of the distribution of bacterial and archaeal taxa in wood fall samples both at deep (Fig. 2) and shallow (Fig. 3 and 4) phylogenetic levels. For instance, archaeal communities gets established only in wood with advanced signs of decay (also see Palacios *et al.* (2009)). Furthermore, Deltaproteobacteria became

more frequent with immersion time at the expense of Alpha-, Beta-, Gamma and Epsilonproteobacteria (Fig. 3). This community succession was confirmed by comparing the community composition of inner (FirCalIN) and outer (FirCalOUT) wood parts. The attack of the wood starting from the exterior to the interior (Jurgens *et al.*, 2003; Palacios *et al.*, 2009), the outside sample had developed to a community more similar to that of older samples contrarily to FirCalIN, which is more similar to younger samples (see for instance Fig. 2). However, note that FirCalOUT rank abundance plot does not yet reflect the evenness of a mature community (Whittaker, 1972) as FicPac and OakMed plots do (Fig. S1 Panel C).

#### *Marine wood falls as niches for organic matter hydrolyzing bacteria*

The most common OTU SWB 1, together with the closely related SWB 6, were found within Bacteroidetes. These OTUs, present in all the short time immersed samples, were phylogenetically close to sequences found in sediment underneath whale falls (Goffredi & Orphan, 2010) and associated with *Osedax* sp. (Goffredi *et al.*, 2005). These OTUs could represent one of the first stages of organic matter degradation in the marine environment. The closest cultured relative to SWB 1 and 6, *Marinifilum fragile*, is a facultative anaerobe that ferments a variety of sugars (Na *et al.*, 2009). Indeed the Bacteroidetes "*Cytophaga-Flavobacterium*" cluster members are known to break down complex organic compounds (Cottrell & Kirchman, 2000; Kirchman, 2002) in marine waters. Bacteroidetes were also found to be the dominant microbiota in archeological wood samples (Landy *et al.*, 2008). Furthermore, we found several OTUs within the Flavobacteriales order (Table S1 and Fig. 3 and S2), a group involved in cellulose hydrolyzation (Barbeyron *et al.*, 2001; Bowman, 2006). These OTUs were primarily found in the 30 month samples and to some extent in 20 month samples, but not in the older samples, suggesting their participation in the intermediary stage of cellulose breakdown in sunken wood. Also in the younger samples, two very relatively abundant OTUs were found within the Myxococcales (Deltaproteobacteria), SWB 50 and 406. These OTUs belonged to the family Sorangiineae and grouped

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together with clones derived from soil. Myxococcales are indeed commonly found in terrestrial soils degrading organic material (Shimkets *et al.*, 2006) but have also been found in the marine environment (Iizuka *et al.*, 1998; Pham *et al.*, 2008). Furthermore, because SWB 50 and 406 group separately from the known cellulose degrading genera *Sorangium* and *Byssovorax* (Reichenbach *et al.*, 2006) (Fig. S4), we speculate that this is a new marine wood-degrading group within the Myxococcales.

Other possible aerobic cellulolytic bacteria found in younger samples are Actinomycetales in PinPac (ST1) and SWB 4 belonging to the genus *Erwinia* (Gammaproteobacteria), found in PinPac and FirCallN (Fig. 3 and Fig. S3). Furthermore, we retrieved two OTUs from younger samples that grouped together with *Teridinibacter* species (Fig. S3), a symbiotic heterotrophic bacterium of wood-boring bivalves, that hydrolyses cellulose and can fix nitrogen under microaerophilic conditions (Distel *et al.*, 2002). Finally SWB 37 and SWB 5, within the Alteromonadales (Gammaproteobacteria), grouped together with *Psychromonas kaikoa* an obligate piezophile and facultative anaerobe that can grow on cellobiose, originally isolated from the cold-seep sediments in the Japan Trench (Nogi *et al.*, 2002).

We observed putative anaerobic cellulolytic bacteria in longer immersed samples FicPac and OakMed, as well as FirCalOUT, for example SWB 203 and 276 belonging the genera *Clostridia* and *Ruminococcus* respectively (Fig. S2). This suggests a shift from aerobic to anaerobic cellulose degradation. Moreover, members of the Fibrobacteres are known cellulose-hydrolyzing bacteria and these were solely found in FicPac and OakMed (Fig. 3 and S2). However, the OTUs found in sunken woods formed a separate group within the Fibrobacteres compared to known cellulose degraders from the same group, so a statement concerning the function should be made with caution. The Archaeal MCG group was also characteristic to older samples (Fig. 2 and 4). This group has no cultured representative but it has been hypothesized that its members are heterotrophic anaerobes that use complex organic substrates (Teske & Sorensen, 2008).



## *The role of sulfur cycling and methanogenesis in wood degradation*

Organic falls are truly special in the marine environment because with time of immersion the chemistry of the matrix changes towards anaerobiosis due to the sustained decay that these ecosystems can afford (Clausen, 1996). As sulfate reduction is favored over methanogenesis in organic matter degradation in the marine environment (Reeburgh & Heggie, 1977; Winfrey & Zeikus, 1977), we expected sulfur cycling to be an important mechanism in the wood matrix degradation. In this line, shorter time immersed samples, PinPac, CocoPac and FirCalIN, contained relatively less sulfate reducing (SR) Deltaproteobacteria sequences than the rest (Fig. 3). Within these samples, the OTUs fell mainly within the Desulfuromonadales which only reduces elemental sulfur and the Desulfobulbaceae which is known to carry out disproportionation of inorganic sulfur compounds (reviewed in Finster (2008)) (Fig. 3 and S4). On the other hand, longer immersed samples, OakMed, FicPac, and FirCalOUT, were dominated by SR Deltaproteobacteria (Fig. 3) belonging mainly to the family Desulfobacteraceae and the SEEP-SRB1 Group (Knittel *et al.*, 2003). This is a group of metabolically versatile sulfate reducing bacteria (SRB) known to be partners of Archaea groups ANME 1 and 2 in the ANaerobic oxidation of MEthane (Knittel *et al.*, 2003; Orphan *et al.*, 2001). Concomitantly, we detected the occurrence of methanogens in OakMed and FicPac only (Fig. 2b), mostly from the order Methanosarcinales, but also some Methanomicrobiales (Fig. 4 and Fig S5). Sulfate reducers and methanogens were thought to be spatially or temporally separated (Reeburgh & Heggie, 1977) due to competition for common substrates, such as H<sub>2</sub> and acetate (Abram & Nedwell, 1978; Lovley *et al.*, 1982; Winfrey & Zeikus, 1977). However, in high carbon environments, both processes occur (Oremland & Taylor, 1978; Senior *et al.*, 1982). Cellulose degradation will primarily yield acetate, but methanol may be the product of bacterial (Donnelly & Dagley, 1980) and fungal (Ander & Eriksson, 1985) degradation of lignin. Methanogens use methanol more readily than SRB (Oremland *et al.*, 1982). Thus, SRB and methanogens may not directly compete for the same substrate in sunken woods, explaining why we find more methylotrophic methanogens,

represented by Methanosarcinales, in our samples as compared to sediments underneath whale falls, where the trend was towards the presence of more hydrogenotrophic methanogens with time (Goffredi *et al.*, 2008) giving support to our hypothesis that competition is less important than habitat filtering at structuring communities. Interestingly, sulfur oxidizers, other important players in the sulfur cycling, were detected only on short time immersed sunken logs. OTU SWB 170, belonging to the Gammaproteobacterial order Thiotrichales, was found in CocoPac, PinPac and MangPac. Indeed, SWB 170 was 98% identical to a bacterial sequence retrieved from sulfide oxidizing mats at cold seeps (Grunke *et al.* 2011), suggesting that this OTU might have sulfide oxidizing potential. SWB 17, a relatively abundant Spirochete, might also be involved in sulfide oxidation as it is close to a newly discovered species *Spirochaeta perfilievii* sp. nov. (Dubinina *et al.*, 2011) capable of sulfur oxidation. We also found several Epsilonproteobacteria OTUs close to "Candidatus *Arcobacter sulfidicus*", a known sulfide oxidizer (Taylor *et al.*, 1999; Wirsén *et al.*, 2002). The ecological significance of these groups in the younger samples is uncertain but they surely have a specialized function in the intermediate phase of wood degradation.

## CONCLUSIONS

Through robust phylogenetic analysis 16S rRNA gene sequences we demonstrate for the first time in marine waterlogged woods the presence of microorganisms related to methane and sulfur cycles and the close relationship of marine sunken woods to other anaerobic chemoautotrophic environments. Our samples were very diverse, suggesting that marine wood falls provide numerous micro-niches to the ocean floor. They also contained a high number of hits with very low similarity to sequences archived in the databases indicating that wood falls harbor a truly unique ecosystem particularly before advanced decay. These poorly studied communities need to be further explored to better comprehend the

physiology and functions of its components. Quantification of phylogenetic patterns in community assembly supported the hypothesis that environmental forces largely determine the underlying patterns of diversity in long and short-time immersed wood fall microbial communities because the large number of available habitats decrease competition among community members at all degradation phases. Future work including continual monitoring of chemical parameters and the concomitant study of phylogenetic patterns will help to verify this hypothesis. Our results leave an open door to speculations on the past and future evolutionary ecology of ocean floor chemosynthetic ecosystems given the increase of terrigenous organic material being exported to the sea with global climate changes.

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## FIGURE And TABLE LEGENDS

**Table 1.** Measures of alpha diversity and related data for sunken wood samples from this study. N. Seq = number of sequences after removing presumed chimeras and sequences shorter than 500 bp, OTU = number of OTUs, Singl.=number of singletons, <5% = percentage of sequences that belong to OTUs with less than 5 sequences, Chao = Chao1 estimated richness index,  $H^{99}$  and  $H^{97}$  = Shannon index for community diversity when clustering at 99% and 97% identity level, respectively, NRI = Net Related Index and NTI = Nearest Taxon Index.

**Fig. 1.** Sunken wood samples from this study and community comparison based upon beta diversity measures. Overview of sunken woods samples and community comparison from UPGMA dendrograms using the Bray Curtis index (left) and the mean nearest taxon phylogenetic pairwise distance metric (MNTD) from COMDISTNT in Phylocom (right) after clustering OTUs at 0.01 distance. The distance from the tip of the dendrogram to the root is 1 for both trees.

**Fig. 2.** Bacterial and archaeal community structure at the phylum or class level for sunken wood samples. A) Bacteria. "Others" represents less frequent phyla non-generalized over samples, see Results and Supplemental Text 1 for details. "Unclassified" represent OTUs that could not be affiliated with known phyla. B) Archaeal community structure of major phyla in sunken wood samples FicPac, OakMed and FirCalOUT. Note that the other samples contained no or very few archaeal sequences. Other Crenarchaeota: includes Group I and BBA6. Other Euryarchaeota: includes GN-4n1.

**Fig. 3.** Phylogenetic tree of major bacterial OTUs. Neighbor-joining distance phylogenetic tree (Jukes-Cantor correction) of all OTUs containing 5 or more sequences (OTU rep) recovered in sunken wood samples. A total number of 560 positions were used to make the tree. *Deinococcus aquaticus* (AM940971) was used as outgroup (not shown). Bootstrap values are shown by closed circles (90% or more) and open circles (60-90%). Nodes with no circle are supported by less than 60%. Scale on bottom relates to number of sequences in each OTU.

**Fig. 4.** Phylogenetic tree of major archaeal OTUs. Neighbor-joining distance tree (Jukes-Cantor correction) of all archaeal OTUs containing 2 or more sequences (OTU-rep) recovered in sunken wood samples. A total number of 526 positions were used to make the tree. *Bacillus subtilis* (X60646) was used as outgroup (not shown). Bootstrap values are shown by closed and open circles, representing values of 90% or more and 60-90%, respectively. Nodes that have no circle are supported by less than 60%.

#### SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

**Table 1.** Measures of alpha diversity and related data for sunken wood samples from this study. N. Seq = number of sequences after removing presumed chimeras and sequences shorter than 500 bp, OTU = number of OTUs, Singl.=number of singletons, <5% = percentage of sequences that belong to OTUs with less than 5 sequences, Chao = Chao1 estimated richness index,  $H^{99}$  and  $H^{97}$ = Shannon index for community diversity when clustering at 99% and 97% identity level, respectively, NRI = Net Related Index and NTI = Nearest Taxon Index.

	N. Seq	OTUs	Singl.	<5%	Chao	$H^{99}$	$H^{97}$	NRI	NTI
PinPac	353	134	88	37	325	4.19	4.07	0.91	2.92**
MangPac	317	74	37	19	135	3.59	3.51	-0.42	3.03**
CocoPac	352	95	46	18	144	3.83	3.67	0.83	5.00**
FirCalIN	343	88	43	19	152	3.77	3.39	-0.43	4.32**
FirCalOUT	340	94	48	25	164	3.85	3.68	0.32	4.12**
OakMed	340	158	108	63	409	4.56	4.33	1.44*	5.50**
FicPac	339	202	158	66	718	4.95	4.74	1.74*	2.83**

\* = Samples showing significantly positive phylogenetic clustering at  $P < 0.06$ ,

\*\* = Samples showing significantly positive phylogenetic clustering at  $P < 0.01$





