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Dimitri Kalenitchenko

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# Université Pierre et Marie Curie

Ecole doctorale 129

*Laboratoire d'Ecogéochimie des Environnements Benthiques (LECOB)*

## **Dynamique et rôle des microorganismes dans l'écosystème bois coulé en milieu profond**

Dimitri Kalenitchenko

Thèse de doctorat d'écologie microbienne

Dirigée par Pierre E. Galand

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Devant un jury composé de :

Pr. Nadine Le Bris (Professeur UPMC)	Présidente
Dr. Josep Maria Gasol (Directeur de Recherche, Institut de Ciències del mar)	Rapporteur
Dr. Thierry Bouvier (Directeur de Recherche, Université de Montpellier)	Rapporteur
Dr. Jennifer Biddle (Assistant Professor, Université du Delaware)	Examineur
Dr. Eric Chauvet (Directeur de Recherche, Université de Toulouse)	Examineur
Dr. Pierre E. Galand (Directeur de Recherche, UPMC)	Examineur



À ma grand-mère,

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## Introduction

Chaque année, environ 4000 milliards de tonnes de carbone (Pan *et al.*, 2011) sont fixés par les forêts. Une fraction de ce carbone, encore inconnue à ce jour, est transférée à l'océan sous la forme de bois. Ce transfert est souvent le résultat d'événements météorologiques violents induisant la montée du niveau des cours d'eau (West *et al.*, 2011; Wohl and Ogden, 2013). L'eau est capable, grâce à la force d'Archimède, de transporter sur des kilomètres une branche, un tronc voire un arbre entier. Des chalutages dans l'océan Indo-Pacifique ont confirmé, dans 53 % des stations étudiées, la présence de bois sur le fond de l'océan (Samadi *et al.*, 2010). Grâce à cet apport non négligeable de matières organiques, certaines espèces ont pu au cours de l'évolution se spécialiser dans l'utilisation de cette ressource. La plus emblématique de ces espèces est un bivalve marin nommé *Teredo navalis* (Linné, 1758). Cette espèce, à l'origine adaptée aux débris naturels de bois, a trouvé dans les constructions maritimes d'origine anthropique une nouvelle niche écologique. L'impact de ces bivalves étaient tel que au XVIII<sup>e</sup> siècle, la marine britannique recouvrait l'intégralité de la coque de ses navires de cuivre afin de se protéger de ce bivalve foreur.

La pose des premiers câbles télégraphiques sous marins au cours du XIX<sup>e</sup> siècle a permis de mettre en évidence une autre espèce de bivalves foreurs de bois, mieux adaptée aux grandes profondeurs *Xylophaga sp* (Purchon, 1941). Cette espèce colonise et se nourrit de la gaine en chanvre des câbles sous marins jusqu'à atteindre l'isolant fait en « gutta percha » (une gomme obtenue à partir de feuille d'arbre), entraînant des courts-circuits et nécessitant la remontée du câble pour réparation {Electricians:1975cs}. L'intérêt pour le bois et les espèces associées était donc à l'origine motivé essentiellement par des intérêts économiques.

Vers la fin du XX<sup>e</sup> siècle, la découverte de la capacité du bois à produire de l'hydrogène sulfuré a provoqué une nouvelle vague d'intérêt pour cet écosystème (Ljungdahl and Eriksson, 1985; Peck, 1984) jusqu'à la découverte dans les années 2000, d'un lien phylogénétique entre des moules colonisant les bois coulés et celles présentes au niveau des sources hydrothermales. Cette découverte aboutit à l'hypothèse selon laquelle les bois auraient servit d'étape évolutive lors de la colonisation des abysses par les bivalves côtiers (Distel *et al.*, 2000; Samadi *et al.*, 2007). Toutefois, l'écologie des microorganismes qui sont à la base de la transformation de ce substrat d'origine terrigène en un système pouvant soutenir la chimiosynthèse, reste peu connue.

## Un substrat d'origine terrestre : le bois

Dans cette étude, nous utiliserons deux essences communes en méditerranée, la première est un pinophyte *Pinus pinea* et le second, un angiosperme *Quercus suber*. Afin de comprendre la dégradation du bois en milieu marin, il est essentiel de développer les éléments qui caractérisent ce substrat.

### Structure

Le bois se compose de deux structures principales, les cellules du xylème et du phloème. Les cellules du xylème assurent le transport de la sève brute contenant l'eau et les nutriments présents dans le sol alors que les cellules du phloème assurent le transport de la sève élaborée produite par les feuilles. Ces deux types de cellules sont produits par un méristème secondaire appelé cambium : ce tissu produit les cellules du xylème vers l'intérieur du tronc et les cellules du phloème vers l'extérieur du tronc. Le méristème produit continuellement les deux types de cellules, toutefois, la production du xylème est beaucoup plus rapide que celle du phloème. L'essentiel de ce qu'on nomme bois est donc constitué de cellules du xylème. Les couches successives de xylèmes accumulés au cours des années constituent l'aubier et les couches successives de phloème, le liber (Figure 1).

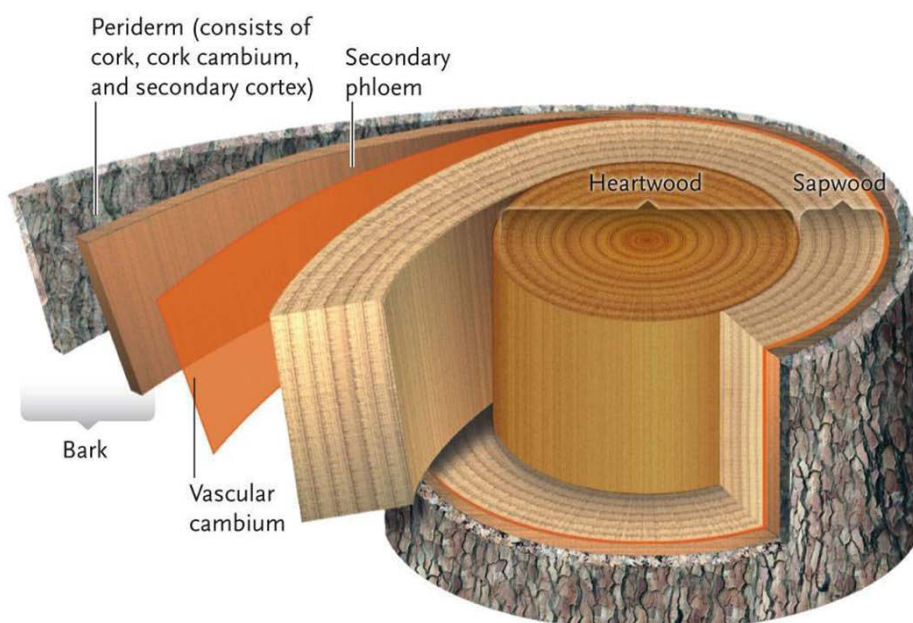


Figure 1 : Anatomie d'un bois duramenisé représentant les principales structures : le duramen (Heartwood), l'aubier (Sapwood), le cambium, le liber (Secondary phloem) et l'écorce (Bark). © Thomson Higher Education

Chez certains arbres, les cellules du xylème les plus anciennes cessent d'être utilisées par la plante et forment un tissu mort appelé duramen dont le seul rôle est le maintien de l'arbre. Cette structure est facilement observable car elle s'accompagne la plupart du temps d'un changement de couleur.

Une des différences majeures entre les deux essences étudiées est la composition du xylème. Le xylème des pinophytes est composé de trachéides (90-95%) et de cellules du parenchyme (5-10%) (Sjostrom, 1993). Les trachéides sont des cellules allongées d'une longueur de 1 cm en moyenne. La sève brute circule d'un trachéide à l'autre via des ouvertures appelées ponctuations (Figure 2). Les ponctuations présentent la particularité d'être fermée par une membrane semi-perméable présentant au centre un système de protection contre l'embolie, appelé torus (Figure 2). Les cellules du parenchyme ont essentiellement un rôle de stockage et de production de résine qui est conduite au travers de canaux résinifères formés par les cellules du parenchyme (Rowell *et al.*, 2012).

Chez les angiospermes, la conduction de sève brute se fait en majeure partie via un autre type de cellule appelée élément de vaisseau. Ces cellules se différencient des trachéides par une structure appelée plaque perforée présente à chacune des extrémités de la cellule. Contrairement aux ponctuations, les plaques perforées ne possèdent pas de membrane, ce qui facilite le transport de la sève brute. Les éléments de vaisseaux mis bout à bout peuvent former des tubes de plus de 1 m et peuvent représenter 25% du volume du bois (Sjostrom, 1993). Le diamètre des éléments de vaisseaux est de *ca.* 130  $\mu\text{m}$  (Pereira *et al.*, 2009), ce qui est 10 fois plus large que le diamètre d'un trachéide. Le reste du bois est composé de trachéides (qui ont essentiellement un rôle de soutien) et de cellule du parenchyme.

## **Composition chimique**

Le bois est essentiellement composé de cellulose (*ca.* 40%), d'hémicellulose (*ca.* 25%), de lignine (*ca.* 30%) et de composés extractibles (< 5%) (Sjostrom, 1993).

### *La cellulose*

La cellulose, découverte il y a à peine 150 ans par Anselm Payen, est le polysaccharide le plus abondant sur terre (Preston, 1975; Ekman *et al.*, 1986). C'est un polymère linéaire constitué de molécules de glucose reliées entre elles par des liaisons  $\beta$ 1 - 4 glycosidique (Figure 3). Ce sont ces liaisons qui confèrent à la cellulose sa relative rigidité (Fengel, 1971). Le degré de polymérisation moyen, c'est-à-dire le nombre de monomères liés, a été évalué à 10 000

(Goring and Timell, 1962) ce qui représente une longueur moyenne de 5 $\mu$ m. La synthèse des molécules de cellulose n'étant pas orientée spatialement, les microfibrilles de cellulose synthétisées tendent à se lier entre elles afin de former un cristal dont la cohésion est assurée par des liaisons hydrogènes. Dans sa forme cristalline, seule la surface externe du cristal de cellulose est biodisponible.

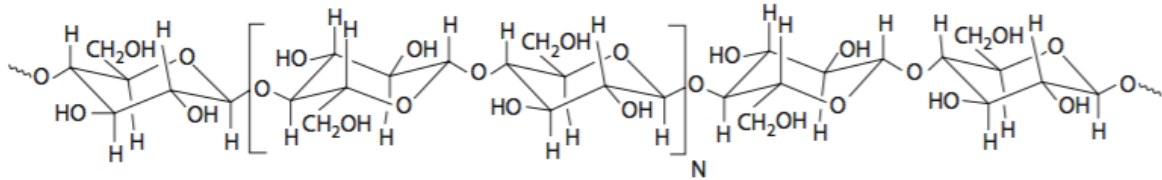


Figure 3 : Structure partielle de cellulose. D'après Rowell *et al.*, 2012

### *Les hémicelluloses*

Les hémicelluloses servent à stabiliser les fibres de cellulose au sein de la paroi. Les hémicelluloses regroupent une grande variété de molécules avec un degré de polymérisation moins élevé que la cellulose (100-200) (Rowell *et al.*, 2012). Les hémicelluloses les plus abondantes chez les angiospermes sont les glucuronoxylane (15-30%) et les glucomannane (2-5%) (Sjostrom, 1993). Chez les pinophytes, c'est le galactoglucomannane (15-23%) et l'arabinoglucuronoxylan (7-10%) qui dominent (Sjostrom, 1993).

### *La Lignine*

La lignine est un composé encroûtant qui vient recouvrir et protéger le maillage formé par la cellulose et les hémicelluloses. Elle est formée de composés aromatiques qui sont des polymères du phénylpropane. Ces molécules sont par la suite liées entre elles à l'aide de liaisons carbone-oxygène-carbone ou carbone-carbone. (Rowell *et al.*, 2012)

## Les composés extractibles

Les composés extractibles incluent tous les composés qui ne sont pas inclus dans les parois. Parmi les composés extractibles, on retrouve des monosaccharides regroupés sous le terme de sucres non structuraux (Hoch *et al.*, 2003) (Figure 4) qui représentent la fraction la plus labile des sucres du bois.

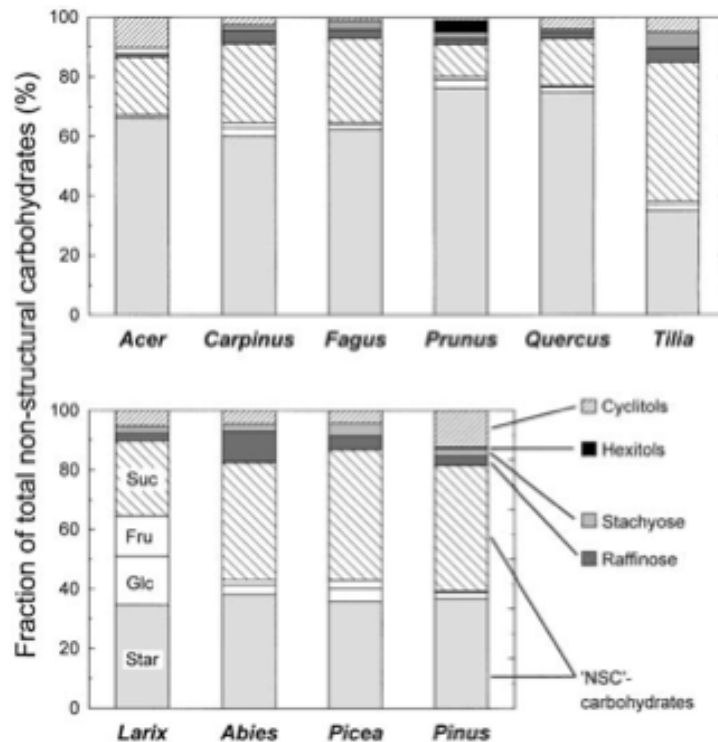


Figure 4: Fraction des sucres non structuraux (NSC) de branches d'arbres matures (>10 ans) récoltées en 2002. Sucres non structuraux : Star (Amidon), Glc (Glucose), Fru (Fructose) et Suc (Sucrose). D'après Hoch *Et Al.*, 2003.

Parmi ces composés extractibles, des acides gras jouent le rôle de réserve dont l'abondance est plus importante chez les pinophytes que chez les angiospermes (Fengel and Wegener, 1984). Chez les pinophytes, les composés extractibles incluent également les terpènes qui sont les principaux constituant de la résine.

## Structure cellulaire

Les cellules qui constituent le bois sont formées de plusieurs couches : la lamelle moyenne, la paroi primaire et la paroi secondaire constituée des couches S<sub>1</sub>, S<sub>2</sub> et S<sub>3</sub> (Figure 5). Ces différentes parois sont caractérisées par leur composition ainsi que par l'arrangement tridimensionnel des microfibrilles de cellulose.

La lamelle moyenne a un rôle de liaison entre les cellules et est principalement composée de pectine lorsque la cellule est vivante (Sjostrom, 1993). Les pectines sont des polysaccharides riches en acides galacturoniques servant de matrice lors de la mise en place des microfibrilles de cellulose (Willats *et al.*, 2001). Lorsque les cellules du xylème sont matures et prêtes à assurer leur rôle de cellule conductrice de sève, un processus appelé la lignification, provoque le remplacement des pectines par de la lignine afin d'assurer la cohésion intercellulaire (Ros Barceló, 1997). Après la lignification, la lamelle moyenne est composée de 38 à 88 % de lignine (Ros Barceló, 1997).

La paroi primaire correspond à la paroi de la cellule avant le processus de lignification conduisant au dépôt de la paroi secondaire. Elle est fine (0.2-1µm) et formée après lignification de cellulose, d'hémicellulose et d'une proportion importante de lignine. Dans cette structure, les fibres de cellulose sont orientées perpendiculairement à l'axe de la cellule (Sjostrom, 1993).

La paroi secondaire est faite de trois couches. Ces couches sont formées par un dépôt de lamelles constituées de fibres de cellulose parallèles entre elles et maintenues par des hémicelluloses et de la lignine. La couche extérieure S<sub>1</sub> est la première à être déposée lors de la lignification, elle mesure 0.2 à 0.3 µm d'épaisseur et est constituée de 3 à 4 lamelles. L'angle formé par ces lamelles avec l'axe de la cellule est de 50 à 70°. La couche S<sub>2</sub> mesure entre 1 et 5 µm selon les espèces et peut contenir jusqu'à 150 lamelles formant un angle de 5 à 30°. Enfin, la couche S<sub>3</sub> plus fine (0.1 µm) constituée de quelques lamelles, forme un angle de 50 à 90° avec l'axe de la cellule et délimite la zone conductrice de sève appelée lumen.

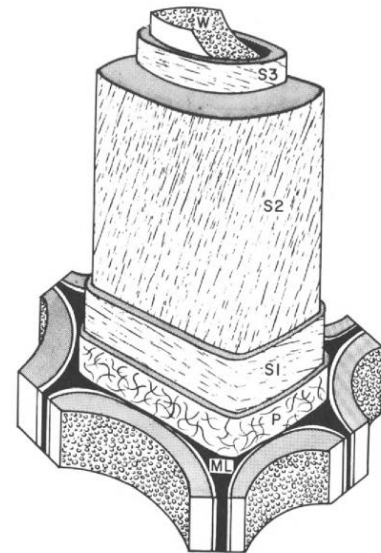


Figure 5 : Schéma simplifié montrant les différentes structures d'une cellule de bois lignifiée, la lamelle moyenne (ML), la paroi primaire (P) et les trois couches formant la paroi secondaire (S<sub>1</sub>, S<sub>2</sub> et S<sub>3</sub>). D'après Sjostrom, 1993

## Dégradation

Le bois représente une source de carbone importante pour les microorganismes. Dans le milieu terrestre, un certain nombre de microorganismes libres ont su s'adapter à ce substrat réfractaire (Ljungdahl and Eriksson, 1985).

Les bois émergés sont principalement dégradés par les champignons. Ils ont été classés en trois catégories en se basant sur les observations macroscopiques liées à la dégradation.

*Les moisissures blanches* sont des *Basidiomycetes* capables de dégrader l'intégralité de la paroi cellulaire incluant la lignine. Au sein de ce groupe, certains vont préférentiellement dégrader la lignine provoquant la délignification de la paroi et son blanchissement (Leonowicz *et al.*, 1999), alors que d'autres, vont attaquer l'ensemble des parois cellulaires incluant la lamelle moyenne (Blanchette, 1991).

*Les moisissures brunes* sont des *Basidiomycetes* qui dégradent rapidement les carbohydrates présents dans la paroi (Blanchette, 2000). Leur attaque se caractérise par une perte en masse rapide et invisible en lien avec la dépolymérisation de la cellulose (Wilcox, 1968). Cette dégradation sélective aboutit à un enrichissement relatif de la paroi cellulaire en lignine (Blanchette, 2000).

*Les moisissures molles* appartiennent au phyla des *Ascomycetes* et des *Fungi imperfecti* et sont essentiellement impliquées dans la dégradation des carbohydrates composants la paroi cellulaire. Deux types d'attaques peuvent être observées. Les attaques de type I forment des cavités à l'intérieur de la paroi secondaire alors que les attaques de type II provoquent la dégradation de l'intégralité de la paroi secondaire (Greaves, 1968).

En parallèle de la dégradation par les champignons, un certain nombre de bactéries ont su s'adapter à l'utilisation de ce substrat (Figure 6) :



*Bactéries érosives.* Ces bactéries se développent dans le lumen des cellules et commencent par attaquer la paroi S<sub>3</sub>. Une fois la paroi S<sub>3</sub> détruite, elles s'attaquent à la paroi S<sub>2</sub> et peuvent aller jusqu'à atteindre la lamelle moyenne, toutefois, celle-ci n'est jamais dégradée par ce type de bactéries (Blanchette *et al.*, 1989) (Blanchette *et al.*, 1989). La dégradation de la paroi secondaire en milieu anoxique est caractérisée par la présence d'une substance amorphe constituée de reste de parois et de bactéries à l'emplacement où se trouvait la paroi secondaire (Blanchette *et al.*, 1989; Singh and Butcher, 1991). Cette substance amorphe forme des poches, voire remplace l'intégralité de la paroi secondaire.

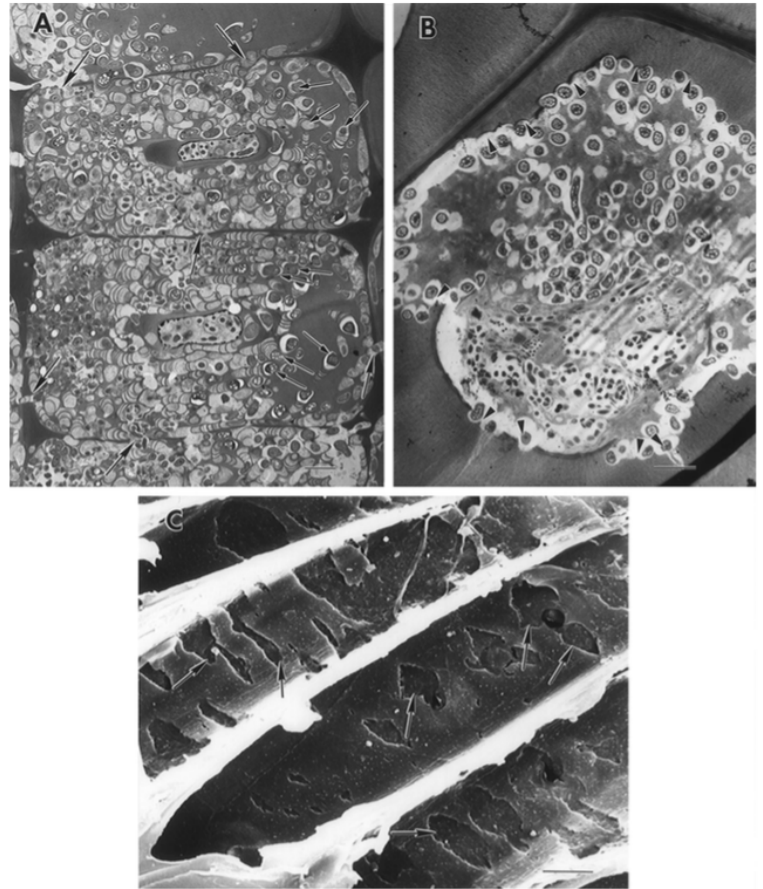


Figure 6: Dégradation de la paroi cellulaire du xylème par des bactéries de tunnel (A), érodantes (B) et de cavitation (C). D'après Blanchette, 2000

*Bactéries formant des tunnels.* Comme leur nom l'indique, ces bactéries ont la propriété de créer des tunnels. Elles commencent par pénétrer la paroi S<sub>3</sub> puis chaque bactérie va creuser un tunnel qu'elle remplira de résidus de dégradations (Singh, 1997). Un bois dégradé par ce type de bactéries présentera une portion de paroi S<sub>2</sub> très dégradée, de laquelle rayonnera des tunnels bactériens (Nilsson and Singh, 1984; Singh and Butcher, 1991) qui peuvent aller jusqu'à dégrader la lamelle moyenne (Agassiz).

*Bactéries formant des cavités.* Ces bactéries attaquent également la couche S<sub>2</sub> et se développent à proximité des ponctuations. La cavité est orientée dans le sens perpendiculaire aux microfibrilles et présente des bord anguleux (Lemaitre, 1995).

# L'écosystème bois coulé

## Découverte

La première expédition scientifique à discuter de l'intérêt du bois coulé d'un point de vue scientifique, date du XIX<sup>e</sup> siècle (Agassiz) et fait suite à la découverte de l'existence et de la persistance de bois au fond des océans par l'expédition Challenger (1872-1876). Au cours de cette expédition, plusieurs espèces inféodées au bois ont été décrites, notamment un nouveau genre de crustacé, *Xylopagurus* (Milne Edward, 1880) qui vit caché dans des débris de bois (Bruun, 1959) (Figure 7).

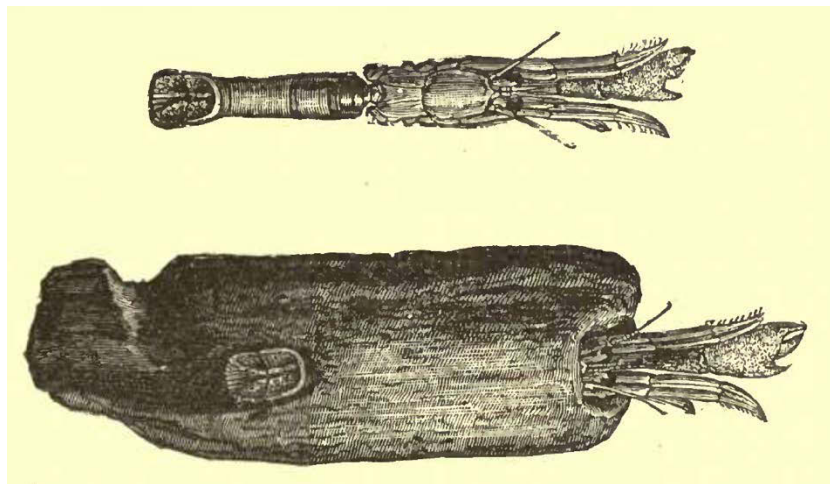


Figure 7 : *Xylopagurus rectus*. D'après Bruun, 1957

L'expédition Galatea qui s'est déroulée entre 1950 et 1952 a procédé à des chalutages benthiques dans plusieurs régions du globe mettant en évidence l'abondance du bois en milieu marin (Purchon, 1941). Le chalutage de la fosse des Philippines démontre que le bois est suffisamment réfractaire pour traverser, sans être entièrement décomposé, 7 000 m de colonne d'eau. Cette expédition a conduit à la formulation de la première hypothèse quant à l'utilisation du bois en milieu marin. En 1957, l'hypothèse était que des bactéries coloniseraient le bois, le rendant plus labile, permettant alors à la macrofaune d'avaler l'ensemble "bois pré-dégradé et bactéries" ainsi formé.(Distel *et al.*, 2011).

## Espèces ingénieurs

Ces expéditions ont permis d'accroître les connaissances sur les bivalves foreurs inféodés à cet écosystème particulier décrit quelques années auparavant, notamment les plus inaccessibles d'entre eux appartenant à la famille des *Xylophagidae* (Turner, 1966). En effet, contrairement aux *Tereneidae* qui ont colonisé les côtes, les membres des *Xylophagidae* ont



divergé à une époque encore inconnue pour former une famille adaptée aux grandes profondeurs (Hill *et al.*, 1927). Les *Tereneidae* sont une famille de bivalves dont le corps est extrêmement bien adapté à la colonisation du bois en milieu côtier. Du fait de cette spécialisation, ce sont à l'heure actuel les bivalves marins dont le pouvoir de destruction sur les constructions humaines est le plus important (Turner, 1966), comme en témoignent les photographies des constructions sur pilotis prises dans la baie de San Francisco (Figure 8).

Figure 8 : Photographies anciennes dans la baie de San Francisco, de constructions détruites par *Terredo Navalis*. D'après Hill *et al.*, 1927

En effet, cette famille de bivalves a su, au cours de l'évolution, développer des mécanismes lui permettant de survivre au stress biotique auquel la vie associée au bois côtier l'expose. La colonisation de bois flottant ou proche des côtes impose au bivalve des phases d'émersions induisant deux risques majeurs. Tout d'abord du fait de l'évaporation, l'eau de mer environnante va se vaporiser, entraînant une augmentation de la salinité de l'eau présente dans le morceau de bois. Si cette émergence se poursuit, elle expose le bivalve à la déshydratation. Afin de lutter contre ces risques, les *Tereneidae* tapissent l'intégralité de leur terrier de calcaire (Voight, 2015) et développent des palettes leur permettant de fermer hermétiquement leur terrier. Grâce à la réserve d'eau emmagasinée dans celui-ci, les *Tereneidae* peuvent

survivre à des salinités environnantes deux fois supérieures à l'eau de mer (Voight, 2015) et résister à des émergences complètes de plus de 72h (Voight, 2005).

La famille des *Xylophagidae* a pris une toute autre direction en colonisant les milieux profonds. Le risque d'émergence étant très faible, l'évolution a sélectionné des organismes ne recouvrant pas leur tube de calcaire (Herring, 2002) économisant ainsi de l'énergie. Bien que les bivalves foreurs aient quelques prédateurs connus comme l'oursin *Xyloplax* (Voight, 2005), les vers tubicoles appelés polyclad (Herring, 2002) ou la moule carnivore *Idas argenteus* (Ockelmann and Dinesen, 2011), ce sont eux qui, en forant le bois, vont provoquer la destruction de leur habitat et ainsi leur propre mort (Voight, 2015).

La capacité qu'ont les bivalves foreurs à se nourrir de bois date d'avant la séparation des deux familles citées précédemment. Les caractères liés à ce mode de vie sont donc partagés entre les deux familles (Distel *et al.*, 2011). Afin de forer le bois, la larve puis l'adulte, utilisent des dents sculptées sur la face antérieure des valves aussi bien chez les *Tereneidae* {miller1924boring} que chez les *Xylophagidae* (Voight, 2007). Ces dents vont user le bois et permettre à l'animal de former la cavité dans laquelle il va vivre et produire la sciure de bois dont il se nourrit. Récemment, une étude par tomographie a permis de montrer qu'un *Xylophagidae*, *Xylophaga depalmai* forait  $0.4380 \pm 0.1440 \text{ cm}^3$  de bois par an, ce qui, à l'échelle de la population étudiée (1489 individus), provoque la disparition de 51.88 % d'un échantillon de  $196 \text{ cm}^3$  (14 cm x 14 cm x 1 cm) en seulement 6 mois (Amon *et al.*, 2015).

Les bivalves foreurs ont donc un rôle essentiel lors de la dégradation du bois en milieu marin car ce sont eux qui vont avoir l'impact le plus important sur la structure du bois et donc sur la base de l'écosystème bois coulé.

## **Un nouvel hotspot de biodiversité chimiosynthétique profond ?**

### *Caractéristiques des systèmes chimiosynthétiques profonds*

La découverte du premier système chimiosynthétique profond intervient lors d'une campagne sur la dorsale des Galapagos à 2 500 m de profondeur en mai 1976 (Lonsdale, 1977). L'objectif de cette campagne était d'étudier le fonctionnement des dorsales et les caractéristiques physico-chimiques du panache hydrothermal. La première description de l'écosystème « ... fractures in the basalt are frosted with white and bright yellow chemical precipitates, and that within 15 m of the fissure there is a remarkable community of large and abundant benthic organisms. » (Lonsdale, 1977) révèle que l'hypothèse de l'époque selon laquelle la diversité

diminuait avec la profondeur (Hessler and Sanders, 1967) n'était pas applicable à l'échelle globale et que des écosystèmes entiers très diversifiés pouvaient se développer sans lien avec l'énergie solaire (Lonsdale, 1977) (Corliss *et al.*, 1979). La seule source d'énergie disponible était la chimioautotrophie, c'est-à-dire la fixation de carbone inorganique à partir d'une réaction d'oxydoréduction entre un donneur d'électrons inorganiques et un accepteur d'électrons inorganiques. Au niveau des sources hydrothermales, le donneur d'électrons provient du fluide. La composition du fluide hydrothermal est liée aux roches traversées (Damm, 1995). Le fluide se forme lorsque l'eau de mer percole au travers de la croûte océanique où elle va se réchauffer au contact du magma et atteindre une température pouvant aller jusqu'à 350°C (Tivey, 1995). À ce moment là, l'action conjointe de la température associée à une pression importante va d'une part réduire une partie des sulfates présents dans l'eau de mer et provoquer, d'autre part, le lessivage du basalte formant la croûte océanique (Ono *et al.*, 2007). Le lessivage du basalte va enrichir le fluide en composés inorganiques réduits tels que l'hydrogène sulfuré, l'hydrogène et le méthane (German and Seyfried, 2014). La présence de ce fluide a permis à des communautés de microorganismes symbiotiques et non symbiotiques de se développer. Ces microorganismes chimiosynthétiques utilisent les composés réduits du fluide hydrothermal comme donneur d'électrons et l'oxygène ou les nitrates de l'eau de mer comme accepteur d'électrons (Jannasch and Mottl, 1985) (Lam *et al.*, 2004) (de Angelis *et al.*, 1993; Anantharaman *et al.*, 2013; 2015). La majeure partie de ces microorganismes non symbiotiques est liée à la classe des *Epsilonproteobacteria* (Campbell *et al.*, 2006) et utilise le cycle de Krebs inversé pour fixer le carbone inorganique dissous (Hügler *et al.*, 2005). Toutefois, la productivité bactérienne non symbiotique ne suffisait pas à expliquer l'étonnante biodiversité décrite dans ces environnements. C'est l'observation d'un vers *Siboglinidae* appelé *Riftia pachyptila* (Jones, 1981), dépourvu de bouche et d'anus, qui va révéler l'existence du phénomène appelé symbiose chimiosynthétique (Distel *et al.*, 1988). Ce terme de symbiose vient de l'interdépendance qu'ont les *Riftia* avec leurs organismes symbiotiques qui sont des bactéries sulfo-oxydantes. Ces bactéries sont situées dans un organe particulier appelé trophosome (Cavanaugh *et al.*, 1981; Felbeck, 1981) qui est irrigué par le sang particulier de ce vers contenant deux types d'hémoglobines capables de transporter à la fois les sulfures et l'oxygène (Powell and Somero, 1983). Les bactéries symbiotiques de *Riftia* sont des gammaprotéobactéries appelées *Candidatus Endoriftia persephone*, qui oxydent les sulfures et incorporent le carbone via le cycle de Calvin Benson ou le cycle de Krebs inversé (Robidart *et al.*, 2008). Par la suite, d'autres associations de ce type ont été mises en évidence notamment chez les Bathymodiolinae (Duperron *et al.*, 2008; 2009) où les

bactéries sont localisées dans un organe particulier des branchies appelé bacteriocyte (Fiala-Médioni *et al.*, 1986). Une des particularités des symbiotes des Bathymodiolinae est la cooccurrence de bactéries sulfo-oxydantes et de microorganismes methanotrophes capables d'utiliser le méthane du fluide comme source de carbone et d'énergie (Fiala-Médioni *et al.*, 2002).

Ce type de symbiose s'est également développé sur les zones dites de suintements froids. Le terme froid fait référence à la température du fluide réduit qui percole au travers des sédiments et dont la température est proche de celle de l'eau de mer. Ce fluide réduit est lié à la présence de débris organiques fossiles qui se retrouvent enfouis, au cours des temps géologiques, sous des couches de plusieurs mètres de sédiment. Là, ils se transforment lentement en hydrocarbures et provoquent des dégazements de méthane (Levin, 2010). Lors de la remontée du méthane dans les sédiments, celui-ci va se trouver au contact d'un consortium entre des bactéries sulfato-réductrices et des archaea (Boetius *et al.*, 2000; Hoehler *et al.*, 1994; Hansen *et al.*, 1998). Ce consortium dérive l'énergie produite par la réduction des sulfates afin de permettre aux archaea d'oxyder le méthane (Boetius *et al.*, 2000). L'oxydation anaérobie du méthane provoque l'enrichissement du fluide en hydrogène sulfuré et permet le développement à la surface du sédiment, d'organismes chimiosynthétiques non symbiotiques tels que les bactéries *Beggiatoa* (Sassen *et al.*, 1993). Ces sites de suintements froids ont également été associés à des concentrations importantes de macrofaune telle que les vers *Lamellibrachia luymesii*. Ces vers tubicoles utilisent leur tube enfoncé dans le sédiment afin d'accéder à l'hydrogène sulfuré et le transportent jusqu'à leur symbiote sulfo-oxydant (Freytag *et al.*, 2001; Julian *et al.*, 1999).

Un troisième système profond est capable de soutenir la chimiosynthèse, il s'agit des cadavres d'animaux marins de grande taille. L'exemple le plus étudié est celui de la baleine. Ce système présente la particularité, par rapport à ceux cités précédemment, de pouvoir être recréé artificiellement en coulant volontairement un cadavre au niveau d'un site facile d'accès. Le déploiement artificiel de carcasses de baleine a permis notamment de révéler la succession écologique associée à la décomposition d'une carcasse de baleine (Smith and Baco, 2003). Tout d'abord, un premier stade où des charognards appartenant à la macrofaune, se nourrissent de la partie la plus labile de la baleine, à savoir la chair et les viscères (Smith, 1985; Isaacs and Schwartzlose, 1975; Dayton and Hessler, 1972), à une vitesse de 40 à 60 kg de matière organique retirée par jour (Smith and Baco, 2003). Puis, un second stade où les

polychètes et les crustacées utilisent la matière organique accumulée dans les sédiments sous jacents (Turner, 1977; Pearson and Rosenberg, 1978). Ces deux premiers stades ont une durée estimée de quelques mois à quelques années. Une fois le squelette nettoyé de la matière organique labile, une phase dite sulfurique intervient (Smith and Baco, 2003). La dégradation de l'importante quantité de lipides présents dans les os de la baleine (3 tonnes pour une baleine de 40 tonnes (Robineau and Buffrénil, 1993)) par des communautés sulfato-réductrices aboutit à la mise en place d'un dégagement d'hydrogène sulfuré au niveau des os (Treude *et al.*, 2009; Shapiro and Spangler, 2009). Ce dégagement de composés réduits dans l'eau de mer oxygénée aboutit à la création d'une niche favorable au développement de mats chimiosynthétique sulfo-oxydantes (Deming *et al.*, 1997; Smith and Baco, 2003). Les os de baleine ne sont pas colonisés par de la macrofaune symbiotique mais par un vers appelé *Osedax* ayant formé une symbiose avec des microorganismes capables de dégrader les lipides contenus dans l'os (Verna *et al.*, 2010). L'apport de carbone que représente la carcasse de baleine au sédiment, provoque un enrichissement de ce dernier en hydrogène sulfuré et en méthane, du fait de l'activité de bactéries sulfato-réductrices et d'archaea méthanogènes dans le sédiment (Treude *et al.*, 2009; Goffredi *et al.*, 2008). À l'endroit où le sédiment est le plus riche en composés réduits (c'est-à-dire proche de l'os), des bivalves symbiotiques, tels qu'*Idas washingtonia* et des palourdes de la famille des Vesicomidae, se développent (Smith and Baco, 2003; Smith *et al.*, 2014; Lorion, Duperron, Gros, Cruaud and Samadi, 2009b; Génio *et al.*, 2014).

#### *Le bois, un 4<sup>ème</sup> hotspot de biodiversité chimiosynthétique*

Les récentes campagnes BOA dans l'océan Indo-Pacifique ont permis de révéler une diversité importante d'organismes inféodés à l'arrivée d'un fragment de bois au fond de l'océan. (Samadi *et al.*, 2010; Schwabe *et al.*, 2015; Pailleret *et al.*, 2007) (Figure 9)



Figure 9: Diversité des organismes découverts lors des campagnes BOA .  
D'après Samadi et al. 2010

Une récente revue de la littérature liée aux bivalves xylophages (Voight, 2015) émet l'hypothèse que dans l'environnement profond, les foreurs (et surtout leurs symbiontes) joueraient le rôle de producteur primaire en transformant le bois réfractaire en biomasse disponible, notamment pour leurs principaux prédateurs les oursins *Xyloplax* (Voight, 2005) et les polyplacophores. Selon cette revue, le fait de retirer les bivalves foreurs du système induirait l'effondrement de l'ensemble du système unique et diversifié observé par les campagnes océanographiques. Parallèlement au processus de dégradation du bois, les scientifiques ont remarqué qu'un morceau de bois plongé dans l'eau de mer provoquait le dégagement d'hydrogène sulfuré (Leschine, 1995; Fors *et al.*, 2008; Yücel *et al.*, 2013). Précédemment, nous avons décrit différents systèmes chimiosynthétiques dans lesquels la cooccurrence d'un composé réduit, ici l'hydrogène sulfuré avec un oxydant, rend possible la mise en place de réactions d'oxydo-réduction produisant suffisamment d'énergie pour fixer le carbone inorganique. La désignation de l'écosystème bois coulé comme système chimiosynthétique profond n'est pas uniquement basé sur la cooccurrence d'un oxydant et d'un réducteur mais aussi sur la diversité de la faune chimiosynthétique associée à cet écosystème. Cette faune est caractérisée par des animaux phylogénétiquement proches de la faune associée aux carcasses de baleine, suintements froids et sources hydrothermales (Distel *et al.*, 2000). Les organismes chimiosynthétiques comprennent notamment les vers vestimentifères (Dando *et al.*, 1992; Hughes and Crawford, 2009) et les mollusques des genres *Idas* et *Adipicola* (Duperron *et al.*, 2008) (Gros and Gaill, 2007b) (Gros and Gaill, 2007a) possédant des symbiontes sulfo-oxydants.



# Microorganismes associés au bois coulé

## Microorganismes Symbiotiques associés à la dégradation du bois

Les bivalves foreurs participent activement à la dégradation du substrat bois, néanmoins, cette dégradation ne peut avoir lieu sans l'aide de symbiotes bactériens. Le bois réduit en sciure par l'action mécanique des bivalves foreurs, doit encore être dégradé afin d'être assimilable par le bivalve hôte. Nous avons vu que le bois était constitué en majeure partie de cellulose et que son utilisation nécessitait d'avoir recourt à des endoglucanases capables de briser les liaisons  $\beta$ 1 - 4 glycosidique liant les molécules de glucoses entre elles (Ljungdahl and Eriksson, 1985). L'utilisation de la cellulose par les bivalves foreurs est rendue possible grâce à l'association de ces bivalves avec des bactéries endosymbiotiques produisant une endoglucanase (Waterbury *et al.*, 1983) (Greene *et al.*, 1989) (détectée dans une vacuole à l'intérieur des cellules) présente dans une invagination des branchies appelée glande de Deshayes (Deshayes, 1848). Toutefois, la digestion du bois n'a pas lieu dans cette structure mais dans un organe spécial appelé caecum (Purchon, 1941; Turner, 1973; Distel and Roberts, 1997). Chez les *Tereneidae*, la preuve a été apportée que l'enzyme (endoglucanase) produite par les bactéries dans la glande de Deshayes est purifiée et transportée jusqu'au caecum afin de digérer le bois (O'Connor *et al.*, 2014). En revanche, chez les *Xylophagidae*, seule la présence de bactéries dans les branchies a été démontrée mais leur fonction dans la digestion du bois n'a été que supposée (Distel and Roberts, 1997). La bactérie responsable de la production d'endoglucanase chez les *Tereneidae* est isolée en 2002 (Distel *et al.*, 2002) et nommée *Teredinibacter turnerae*. Cette bactérie, en plus de la fabrication d'endoglucanase, possède la capacité en condition anoxique de fixer l'azote moléculaire (Distel *et al.*, 2002). Ce dernier est fixé et transféré au bivalve foreur qui compense ainsi le manque l'absence d'azote dans les carbohydrates issus du bois (Leschine, 1995; Lechene *et al.*, 2007).

## Microorganismes Symbiotiques associés à l'oxydation de l'hydrogène sulfuré

Les microorganismes associés à une moule couramment retrouvée en association avec les bois coulés, *Idas sp.* (Distel *et al.*, 2000; Pailleret *et al.*, 2007) sont localisés dans ses branchies (Gros and Gaill, 2007a). Cette association est extracellulaire, c'est-à-dire que les bactéries sont situées à la surface de cellules branchiales modifiées appelées bacteriocytes (Gros and

Gaill, 2007a) avec lesquelles les microorganismes pourraient échanger des composés carbonés (Duperron *et al.*, 2008). Une étude comparant les bactéries associées aux branchies de bivalves retrouvés à la fois sur les os de baleines et les bois coulés, a montré que l'espèce *Adipicola crypta* et une espèce non identifiée d'*Idas sp.* peuvent coloniser à la fois les os de baleine et les bois coulés (Lorion, Duperron, Gros, Cruaud and Samadi, 2009a). Les symbiontes de l'espèce inconnue d'*Idas sp.* sont extracellulaires et possèdent des symbiontes sulfo-oxydants (Duperron *et al.*, 2008). Dans le cas d'*Adipicola crypta*, les symbiontes bactériens sont proches de ceux rencontrés chez *Idas sp.* (99.5%) mais sont situés à l'intérieur de la vacuole des bacteriocytes (on parle alors d'endosymbiose). Récemment, le séquençage de l'ADN 16S de symbiontes d'*Idas iwaotakii* (une autre espèce d'*Idas* associée au bois coulé) (Lorion *et al.*, 2010) a conclu que 4 des phylotypes bactériens rencontrés avaient une similarité supérieure à 97% avec des gammaprotéobactéries symbiotiques sulfo-oxydantes potentiellement chimioautotrophes isolées dans des branchies d'autres Bathymodionlinae (Thubaut *et al.*, 2013). En résumé, l'ensemble des études portant sur les bactéries associées aux branchies de bathymodionlinae colonisant les bois coulés, a classé ces bactéries dans la classe des gammaprotéobactéries (Duperron *et al.*, 2008; Lorion, Duperron, Gros, Cruaud and Samadi, 2009b). De plus, les études portant sur les gènes fonctionnels ont montré le potentiel génétique de ces bactéries à oxyder le sulfure d'hydrogène afin de fournir l'énergie nécessaire à la fixation du carbone inorganique (Duperron *et al.*, 2008; Lorion, Duperron, Gros, Cruaud and Samadi, 2009a).

## **Microorganismes non symbiotiques associés à la dégradation du bois**

Comme nous venons de le voir, de nombreuses études se sont intéressées aux organismes macroscopiques associés au bois coulé mais la base de ce système, représentée par les microorganismes libres, reste relativement peu connue. On sait, d'après les études sur les bois archéologiques préservés de l'attaque des foreurs (Sandström *et al.*, 2002; Björdal *et al.*, 2000), que des bactéries marines sont capables de dégrader la paroi lignocellulosique des cellules du bois (Blanchette, 2000). Toutefois, comme en témoignent les épaves d'apparence intacte immergées pendant plus de 300 ans (Sandström *et al.*, 2002), cette dégradation est un processus très lent (Björdal *et al.*, 1999). Les microorganismes responsables de la dégradation du bois en milieu marin sont essentiellement des bactéries érosives (Björdal *et al.*, 1999; Björdal and Nilsson, 2008; Kim *et al.*, 1996) (Singh *et al.*, 1989; Kim and Singh, 2000), des bactéries de tunnels (2003) et des champignons *Ascomycetes*, provoquant des dégradations de

type moisissures molles (Singh and Butcher, 1991; Jones, 2000; Mouzouras *et al.*, 1988; Jones, 2011). La dépolymérisation extracellulaire de la paroi secondaire des cellules du xylème par les endoglucanases produites par les microorganismes (Palacios *et al.*, 2006) conduit à la libération de cellobiose (polymère formé de 2 glucoses), de cellodextrine (polymère formé de n glucoses) et de glucose dans le milieu. Ces composés sont par la suite fermentés par les bactéries cellulolytiques et par des bactéries détritivores non cellulolytiques profitant de la libération de ces composés labiles dans le milieu extracellulaire (Leschine, 1995). Le rôle de ces bactéries détritivores est essentiel car bien qu'elles ne soient pas capables de dégrader directement la cellulose, elles favorisent sa dégradation en limitant l'accumulation des produits de la dépolymérisation qui sont des inhibiteurs des endoglucanases (Ljungdahl and Eriksson, 1985). La fermentation des carbohydrates produits va conduire à la production d'acetate, de formate, d'hydrogène et de CO<sub>2</sub>. En milieu marin, ces composés seront essentiellement utilisés par les bactéries sulfato-réductrices utilisant les sulfates comme accepteur terminal d'électrons (Leschine, 1995; Khelaifia *et al.*, 2011). Cette activité sulfato-réductrice conduit à la production d'hydrogène sulfuré, un composé réduit essentiel pour la communauté chimiosynthétique du bois coulé. Une partie de cet hydrogène sulfuré va diffuser vers la surface du bois (Laurent *et al.*, 2013; Yücel *et al.*, 2013) tandis qu'une autre fraction va se lier à la lignine de la lamelle moyenne (Fors *et al.*, 2008).

La dynamique temporelle de la communauté associée au bois en milieu profond reste peu connue. Des études basées sur des cultures ont montré que la communauté semblait évoluer au cours du temps (Austin *et al.*, 1979; Cundell and Mitchell, 1977) et qu'elle contenait des bactéries appartenant au phylum des protéobactéria (Christine Helms *et al.*, 2004). Par la suite, l'utilisation de l'électrophorèse sur gel dénaturant (DGGE) a permis de montrer que parmi les bactéries formant la communauté des bois archéologiques, ce sont probablement des bactéries appartenant au phylum des *Sphingobacteria* qui seraient responsables de l'érosion des parois (Landy *et al.*, 2008). L'étude des facteurs biotiques influençant cette communauté a permis de montrer que la composition de la communauté bactérienne semblait être variable en fonction du temps d'immersion et du type de bois colonisé (angiosperme ou pinophyte) (Palacios *et al.*, 2009). L'étude en profondeur de cette communauté montre que dans certains cas, il pouvait y avoir dans le bois, cooccurrence de bactéries sulfato-réductrices et d'*Archaea* méthanogènes (Fagervold *et al.*, 2012). Cette cooccurrence est assez surprenante du fait que les méthanogènes et les sulfato-réducteurs sont en compétition pour l'utilisation de l'acétate et de l'hydrogène. Néanmoins, lorsque la quantité de matière organique est suffisamment

importante, ces deux processus peuvent avoir lieu simultanément (Oremland and Barrie F Taylor, 1978).

Nous avons vu précédemment que la paroi lignocellulosique du bois pouvait également être dégradée par les bivalves foreurs (Amon *et al.*, 2015) avec l'aide de leurs bactéries associées (Distel *et al.*, 2002). L'impact de la présence de ces foreurs sur la communauté microbienne a pu être approché grâce à des expériences de déploiement de bois dans un canyon et sur le talus continental adjacent (Fagervold *et al.*, 2013). Les canyons possèdent la particularité de concentrer les macro débris et les larves de bivalves foreurs (Romano *et al.*, 2013) et par conséquent, la colonisation par les bivalves est plus importante et plus rapide dans le canyon qu'à l'extérieur du canyon (Romano *et al.*, 2013). La présence des bivalves foreurs (dans cette étude *Xylophaga sp.*) a pour effet d'augmenter la diversité bactérienne (Fagervold *et al.*, 2013) via la création de nouvelles niches écologiques par les foreurs, notamment des niches adaptées à la présence de Epsilonproteobactérie sulfoxydantes (Bessette *et al.*, 2014).

La présence de bois sur le fond provoque, comme dans le cas des os de baleine, une modification du sédiment sous jacent (Bienhold *et al.*, 2013). Le sédiment enrichi en matières organiques dérivées de la dégradation du bois provoque une anoxie du sédiment. Cette anoxie favorise le développement de niches écologiques adaptées aux bactéries sulfato-réductrices. L'activité sulfato-réductrice de ces bactéries aboutit à une augmentation de la concentration d'hydrogène sulfuré dans le sédiment à proximité du bois (Bienhold *et al.*, 2013).

## **Bilan des connaissances actuelles**

Cela ferait 300 millions d'années que des bois sont transportés vers l'océan (Distel *et al.*, 2000). Nous avons vu que la présence de bois en dégradation dans le milieu marin était capable de soutenir une communauté chimiosynthétique (Bienhold *et al.*, 2013). Des études de phylogénies ont montré que les bivalves côtiers auraient commencé par coloniser le bois (Fujiwara *et al.*, 2010) et auraient évolué vers la chimiosynthèse à la fin du crétacé (Kiel and Goedert, 2006). Le développement de la chimiosynthèse aurait permis à la macrofaune de coloniser les carcasses de reptiles marins tels que les plésiosaures, en association avec des ancêtres du vers *Osedax* dont le rôle moderne est la dégradation des lipides contenus dans les os (Kaim *et al.*, 2008; Fujiwara *et al.*, 2010; Danise and Higgs, 2015). Après l'extinction massive à la fin du crétacé, ces communautés auraient survécu en utilisant les cadavres de poissons téléostéens et de tortues de mer (Danise and Higgs, 2015). Au cours de l'éocène et de l'oligocène (54-24 millions d'année), la faune chimiosynthétique aurait colonisé

simultanément les carcasses de baleines (apparues au début de l'oligocène) ainsi que les sources hydrothermales et les suintements froids (Fujiwara *et al.*, 2010; Lorion *et al.*, 2013). Le substrat bois représenterait donc la genèse de l'ensemble des systèmes chimiosynthétiques profonds connus (Distel *et al.*, 2000; Samadi *et al.*, 2007). Toutefois, l'intérêt pour le bois coulé a d'abord été motivé par la protection des installations humaines contre la macrofaune inféodée à ce substrat. C'est seulement récemment que la communauté bactérienne à la base de la mise en place du système chimiosynthétique commence à être explorée. Cependant, les études d'écologies microbiennes précédemment citées ne font état que d'échantillons dont la durée d'incubation est supérieure à 9 mois (Fagervold *et al.*, 2013) ou inférieure à 1 jour (Bienhold *et al.*, 2013). Cette absence de données sur la mise en place de l'écosystème chimiosynthétique bois coulé donne lieu à des hypothèses, notamment sur le rôle joué par les bivalves foreurs lors de l'établissement de cet écosystème. Les publications les plus récentes (Bienhold *et al.*, 2013) (Voight, 2015) proposent que ce serait le forage du bois par les bivalves foreurs qui provoquerait l'apparition de niches anoxiques et donc la production de sulfure d'hydrogène. Néanmoins, des mesures chimiques effectuées sur des bois en aquarium ont montré que, après seulement 1 mois et en l'absence de foreurs, le bois dégageait une quantité importante de sulfure d'hydrogène (*ca.* 1 mM) (Yücel *et al.*, 2013). Enfin, il a été montré dans l'ensemble des systèmes chimiosynthétiques connus, la présence de mat bactérienne sulfo-oxydante (Girnth *et al.*, 2011) (Grünke *et al.*, 2011; Gentz and Schlüter, 2012; Alain *et al.*, 2004; CD Taylor *et al.*, 1999).

Dans le cas des bois, seules de rares observations (Palacios *et al.*, 2009; Laurent *et al.*, 2013; Yücel *et al.*, 2013) ont été faites et aucune donnée sur la composition de ces biofilms n'existe au début de cette thèse. Toutefois, le fait qu'un biofilm de couleur blanche (Palacios *et al.*, 2009) semble se développer à l'interface entre le bois produisant des sulfures (Yücel *et al.*, 2013) et l'eau, laisse penser que ce biofilm pourrait être sulfo-oxydant.

## Objectifs de la thèse

Cette thèse a pour objectif principal la compréhension du rôle joué par les microorganismes lors de la mise en place de l'écosystème chimiosynthétique associé à la dégradation du bois coulé en environnement marin profond. Afin d'atteindre cet objectif, 3 axes valorisés par quatre articles, seront développés :

- L'axe de travail décrit dans l'article I vise à déterminer les conditions environnementales influençant la communauté microbienne au cours de la mise en place de l'écosystème chimiosynthétique.
- L'axe de travail décrit dans les articles II et IV permettra de comprendre l'impact des bivalves foreurs sur la communauté microbienne et la production d'hydrogène sulfuré à la base de la communauté chimiosynthétique.
- Enfin, l'axe de travail décrit dans l'article III permettra de comprendre la dynamique et les fonctions associées à la communauté microbienne lors de la mise en place d'un hypothétique biofilm sulfo-oxydant à la surface du bois.

# Sites d'expérimentation et méthodes

## Sites d'études

Les canyons sous-marins sont des structures géologiques correspondantes à des vallées sous-marines entaillant le plateau continental (Harris and Whiteway, 2011). L'hiver, lorsque le mistral et la tramontane (deux vents froids en provenance du nord) soufflent, une masse d'eau froide (donc dense) se forme en surface. Passé un certain seuil de densité ( $\geq 29.126 \text{ kg.m}^{-3}$  (de Madron *et al.*, 2013)), cette masse d'eau va plonger jusqu'à atteindre une profondeur où l'eau environnante possède une densité similaire à celle de la masse d'eau plongeante (Canals *et al.*, 2006). Cette masse d'eau plongeante utilise les canyons sous-marins qui représentent le chemin le plus court pour rejoindre les couches d'eau profonde avec lesquelles elles seront en équilibre (de Madron *et al.*, 2013). Le volume d'eau associé à ce phénomène en méditerranée nord ouest est estimé entre 14 000 et 7 600  $\text{km}^3$ . Ce volume d'eau entraîne lors de sa plongée la matière organique terrigène, incluant les bois, vers les grands fonds via les canyons sous-marins (Canals *et al.*, 2013). Ce système concentre la matière organique terrigène, favorisant ainsi la faune associée à ces substrats et notamment les bivalves foreurs associés au bois (Romano *et al.*, 2013). Ces systèmes sont donc idéals pour tester l'axe de travail associé aux articles II et IV car la colonisation par les bivalves *Xylophaga sp.* sera rapide et certaine. De plus, ce système concentrant naturellement la matière organique terrigène, représente le lieu idéal pour déployer des bois afin d'étudier la dynamique de mise en place du système chimiosynthétique développé dans l'article I. Dans cette étude, nous utiliserons deux canyons sous-marins pour nos expérimentations, le canyon de Blanes pour l'article IV et le canyon de Lacaze-Duthiers pour l'article I et II (Figure 10).

En raison des contraintes liées à l'expérimentation en milieu naturel profond (accessibilité et coût), certains travaux de cette thèse (décrits dans les articles I et IV) ont été effectués dans un

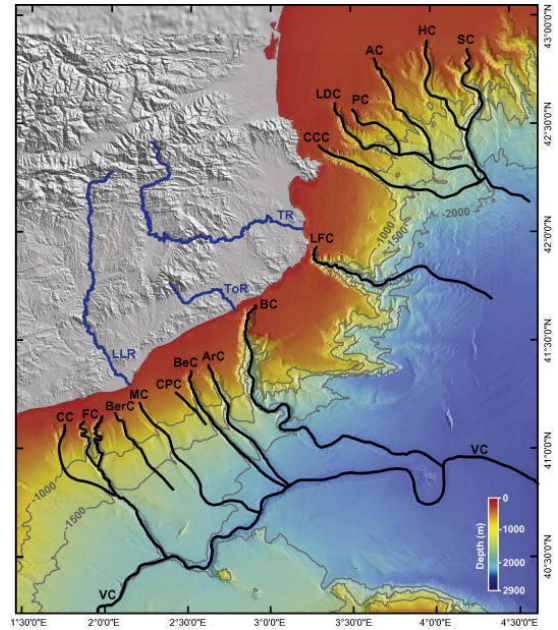


Figure 10: Carte des canyons sous marins de la côte catalane incluant nos deux sites d'études, le canyon de Blanes (BC) et le canyon de Lacaze-Duthiers (LDC). D'après Canals, 2013

mésocosme reproduisant certaines conditions abiotiques de l'eau méditerranéenne profonde (Zavatarielli and Mellor, 1995) comme la température ( $13^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ) et l'obscurité.

## **Méthodes**

Les méthodes utilisées dans cette thèse sont décrites en détails dans les articles scientifiques et résumé dans la table 1.



Table 1 : Résumé des méthodes utilisées dans cette thèse

Analyse	Méthode	Utilisées et décrites dans l'article	Référence
Extraction de l'ADN du bois	Kit d'extraction d'ADN de plante	I, II, III et IV	MOBIO Laboratories, Carlsbad, CA
PCR	Primer 16S rRNA bactérien	I, II, III et IV	Vergin et al. 1998; Teske et al. 1994
	Primer 16S rRNA archaea	I	Ovreas et al., 1997; Yu et al., 2005
	Primer ITS champignons	I	Gardes and Bruns, 1993; White et al., 1990
Séquencage d'amplicons	454	I et IV	Brandford, CT, USA
	Illumina Miseq	II et III	Illumina, San Diego, CA, USA
Nétoyage et analyse des amplicons	Ampliconnoise	I	Quince et al., 2011
	Mothur	I, II, III	Schloss et al. 2009
	Pyrotagger	IV	Kunin V e tHugenholtz P, 2010
Séquencage de métagénomés	Illumina Miseq	II et III	Illumina, San Diego, CA, USA
	Illumina Hiseq	III	Illumina, San Diego, CA, USA
Nétoyage et analyse des métagénomés	MG-RAST	II et III	Meyer et al., 2008
Mesure des lipides totaux	Sulphosphovanillin	I	Barnes and Blackstock, 1973
Mesure de la concentration en hydrogène sulfuré	Electrode Voltamétrique Au/Hg	I	Luther et al., 2001
	Electrode potentiométrique Ag/Ag2S	II	NKE, SPHT, Hennebont, Fr
Tomographie à rayon X	Tomographe de 39 W	II	RX solutions, Chavanod, France
CARD-FISH	Sonde Eubactérie	III	Amann et al., 1990
	Sonde Epsilonprotéobactéries	III	Lin et al., 2006
	Sonde Gammaprotéobactéries	III	Manz et al., 1992
Analyse de la diversité	Shannon	I,II,IV	Shannon et Weaver, 1976
	NTI/NRI	I et IV	Webb, 2000
	Bray-Curtis	I,II,IV	Bray et Curtis 1957
	MIC	I et IV	Reshef et al., 2011
Analyse multivariées	NMDS	III	Torgerson, 1958
	ADONIS	I	Anderson, 2001
Analyses Statistiques	ANOVA	I,III	Fisher, 1925
	Welch t test	II	Welch, 1947
	Mann-Whitney	I,II	Mann et Whitney 1947
	Kruskal-Wallis	II	Kruskal et Wallis 1952



**Article I: Temporal and spatial constraints on community  
assembly during microbial colonization of wood in  
seawater**

## ORIGINAL ARTICLE

# Temporal and spatial constraints on community assembly during microbial colonization of wood in seawater

Dimitri Kalenitchenko<sup>1</sup>, Sonja K Fagervold<sup>1</sup>, Audrey M Pruski<sup>1</sup>, Gilles Vétion<sup>1</sup>, Mustafa Yücel<sup>1,2</sup>, Nadine Le Bris<sup>1</sup> and Pierre E Galand<sup>1</sup>

<sup>1</sup>Sorbonne Universités, UPMC Univ Paris 06, CNRS, Laboratoire d'Ecogéochimie des Environnements Benthiques (LECOB), Observatoire Oceanologique, Banyuls sur Mer, France and <sup>2</sup>Middle East Technical University (METU), Institute of Marine Sciences, Erdemli, Turkey

**Wood falls on the ocean floor form chemosynthetic ecosystems that remain poorly studied compared with features such as hydrothermal vents or whale falls. In particular, the microbes forming the base of this unique ecosystem are not well characterized and the ecology of communities is not known. Here we use wood as a model to study microorganisms that establish and maintain a chemosynthetic ecosystem. We conducted both aquaria and *in situ* deep-sea experiments to test how different environmental constraints structure the assembly of bacterial, archaeal and fungal communities. We also measured changes in wood lipid concentrations and monitored sulfide production as a way to detect potential microbial activity. We show that wood falls are dynamic ecosystems with high spatial and temporal community turnover, and that the patterns of microbial colonization change depending on the scale of observation. The most illustrative example was the difference observed between pine and oak wood community dynamics. In pine, communities changed spatially, with strong differences in community composition between wood microhabitats, whereas in oak, communities changed more significantly with time of incubation. Changes in community assembly were reflected by changes in phylogenetic diversity that could be interpreted as shifts between assemblies ruled by species sorting to assemblies structured by competitive exclusion. These ecological interactions followed the dynamics of the potential microbial metabolisms accompanying wood degradation in the sea. Our work showed that wood is a good model for creating and manipulating chemosynthetic ecosystems in the laboratory, and attracting not only typical chemosynthetic microbes but also emblematic macrofaunal species.**

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## Introduction

Deep-sea chemosynthetic ecosystems build upon the use of reduced chemicals by microorganisms and develop on and around benthic features such as hydrothermal vents, cold seeps, whale falls or woods falls (Jorgensen and Boetius, 2007). Woods transported from land to sea sink once waterlogged and create unique ecosystems on the ocean floor. The refractory nature of wood, which becomes quickly anaerobic in seawater (Yücel *et al.*, 2013), leads to a slow degradation of its organic matter and the production of sulfide (Laurent *et al.*, 2013) typical for chemosynthetic ecosystems. Wood falls ecosystems have been the focus of a number of

research projects as they are thought to have a key role in the dispersal and evolution of deep-sea chemosynthetic organisms (Distel *et al.*, 2000; Kiel *et al.*, 2009), and because they are home for an emblematic fauna harboring microbial symbionts (Dubilier *et al.*, 2008). The free-living microorganisms that digest wood or produce sulfide remain, however, poorly studied.

Little is currently known about microbial communities that colonize the wood itself. Wood fall bacterial communities that were early characterized according to their physical action on the wood matrix (Mouzouras *et al.*, 1988; Jurgens *et al.*, 2003), and first described through the isolation of cultivable representatives (Cundell and Mitchell, 1977; Austin *et al.*, 1979), were precisely identified only recently by molecular tools. A large cloning and sequencing effort gave the first insights into the diversity of natural wood fall bacterial and archaeal assemblages (Fagervold *et al.*, 2012). It demonstrated the presence of sequences associated to possible fermenters and sulfide-oxidizing bacteria, as well as

Correspondence: D Kalenitchenko, Sorbonne Universités, UPMC Universités Paris 06, CNRS, Laboratoire d'Ecogéochimie des Environnements Benthiques (LECOB), Observatoire Oceanologique, Banyuls sur Mer 66650, France.

E-mail: dimitri.kalenitchenko@obs-banyuls.fr

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the co-occurrence of free-living sulfate-reducing bacteria. *Archaea* have also been detected on wood falls, especially on samples with substantial signs of decay (Palacios *et al.*, 2009). They are potentially involved in the production of methane (Fagervold *et al.*, 2012), but the observation of the association of a giant *Thaumarchaeota* with sulfur-oxidizing bacteria (Muller *et al.*, 2010) suggests the presence of diverse archaeal metabolisms on wood falls. The first molecular descriptions of wood microbes gave clues to potential metabolisms associated with the degradation of wood in the marine environment. Cellulose could be hydrolyzed into simple sugars by bacteria or fungi. Marine fungi have the potential to degrade cellulose and lignin (Raghukumar *et al.*, 1994; Pointing *et al.*, 1998), but their presence on marine wood falls has rarely been documented (Raghukumar, 2012; Rämä *et al.*, 2014). Simple sugars will in turn be fermented to small carbon molecules that could then be used by sulfate reducers and methanogens, which represent the last step of the anaerobic degradation of organic matter. Finally, the sulfide produced by sulfate-reducing bacteria can be re-oxidized aerobically or with nitrate as electron acceptor, thus setting the basis for the wood fall chemosynthetic ecosystems.

The first insight into the ecology of wood falls bacteria communities was published recently through experimental deployments of woods in the sea (Palacios *et al.*, 2009; Bienhold *et al.*, 2013; Fagervold *et al.*, 2013). The results showed how local environmental features could influence bacterial community assembly, but contrasting results have been reported. In one case, similar communities were present in woods situated at geographically distant sites and at different depths of immersion (Palacios *et al.*, 2009). While in other cases, the location of the wood inside a submarine canyon (Fagervold *et al.*, 2013; Bessette *et al.*, 2014), in the vicinity of carbonate crust (Bienhold *et al.*, 2013), or its colonization by wood-boring *Xylophaga* bivalves (Fagervold *et al.*, 2014) strongly structured the microbial communities. The type of wood may also be important in structuring sunken wood bacterial communities (Palacios *et al.*, 2009; Fagervold *et al.*, 2013). Data on community ecology remain, however, rare, as they require experimental approaches that are particularly challenging in the deep sea. Evidences of possible differences in community composition between the different parts of the wood are, for instance, lacking, and there are no direct evidences of temporal succession in the microbial communities. Previous studies relied on samples collected solely after several month of immersion (Palacios *et al.*, 2009; Bienhold *et al.*, 2013; Fagervold *et al.*, 2013), which made it difficult to study the temporal dynamics of microbial wood colonization and community assembly.

Microbial community assembly can theoretically be dictated by neutral processes, such as genetic drift or immigration of species with similar traits and fitness, but assembly can also be ruled by species

sorting, which is the selection of species by environmental conditions (Langenheder and Székely, 2011). We hypothesize that in wood fall ecosystems, the local environmental conditions are key factors for structuring communities with time, as the degradation of organic matter by bacterial consortia, and the production of reduced chemicals will continuously transform the habitat.

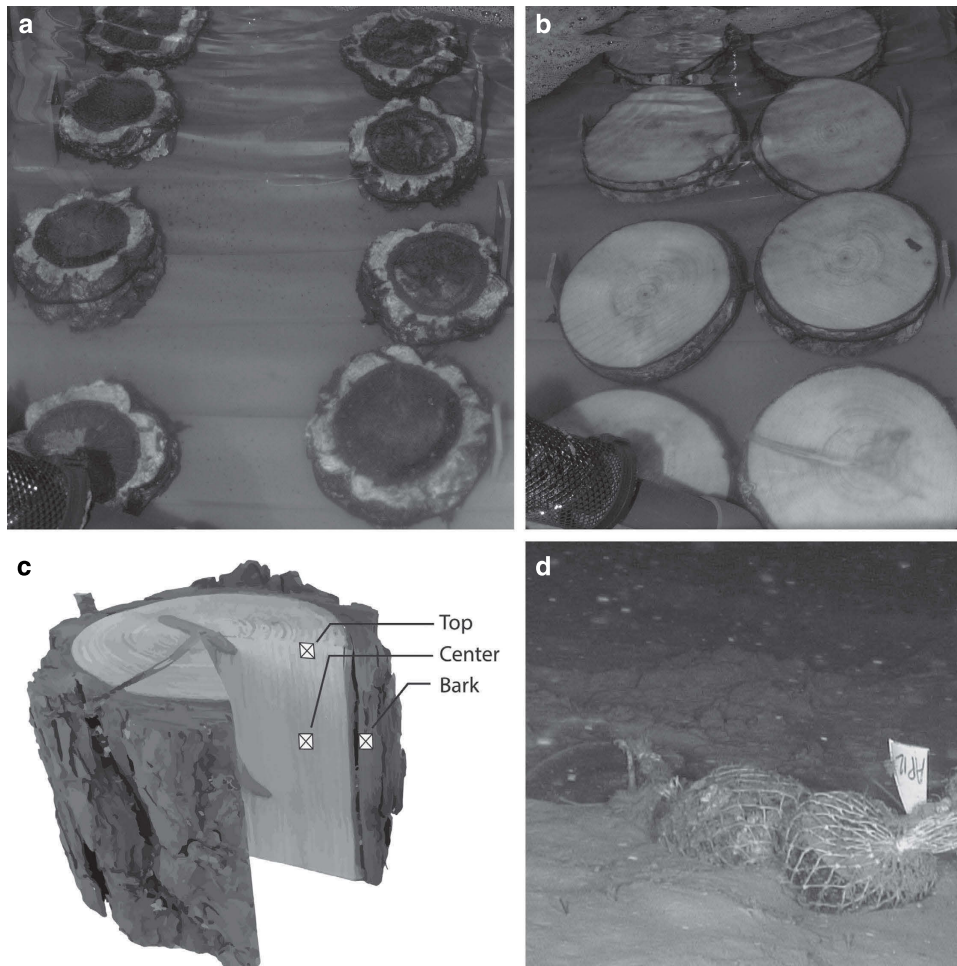
The major aim of this study was to determine how environmental conditions dictate community assembly during the colonization and establishment of a deep-sea chemosynthetic ecosystem. We use wood as a model ecosystem that can easily be manipulated and monitored in aquaria, and compared the results to *in situ* experiments from deep-sea deployments. In order to encompass diverse environmental constraints on community assembly, we focus on both a spatial scale, represented by wood microhabitats (wood bark, inside the wood and surface of the wood), and temporal scale that is the immersion time and the effect of wood species (pine vs oak). We also compared woods from a natural environment (submarine canyon) with woods from an artificial environment (aquarium). We used pyrosequencing for a precise description of the bacterial, archaeal and fungal community structure and diversity, and measured changes in wood lipid content and sulfide concentrations to assess the potential activity of microorganisms.

## Material and methods

### Experimental design

**Aquaria experiment.** Wood pieces used for the experiments originated from pine and oak trees planted in 1980s at the Banyuls sur Mer botanical garden. Eight pieces of oak (*Quercus suber*) and eight pieces of pine (*Pinus pinea*) woods (10 × 16 cm; Figures 1a and b) cut from a tree branch were incubated in tanks filled with natural seawater at 13 ± 1 °C in the dark, simulating the temperature and light conditions of the deep Mediterranean Sea (Zavatorielli and Mellor, 1995). These tanks were filled continuously with oxygenated water pumped directly 30 m away from the coast at 4 m depth.

Pieces of wood were collected five times during a 307-day incubation period after 30, 78, 121, 240 and 307 days. Wood was sampled with a saw washed with ethanol and rinsed with sterile water. Samples were collected from the following three locations on each wood piece: center, surface and bark (Figure 1c). These three types of samples were chosen to represent different potential microbial microhabitats. The wood center was not in direct contact with seawater, the surface constituted an interface between seawater and wood, and finally the bark, also at the interface of seawater, which had a different chemical composition than wood. Samples were frozen immediately after collection in liquid nitrogen and stored at -80 °C.



**Figure 1** Experimental design with eight pieces of oak (a) and eight pieces of pine (b) wood incubated in the dark in aquaria filled with natural seawater thermostated at 13 °C. (c) Position of the different microhabitat wood samples: the bark sample is at the interface between bark and seawater, the top sample is at the interface between sapwood and seawater and the center sample has not been in direct contact with seawater. (d) Pine and oak woods deployed in situ at 520 m depth in the Lacaze-Duthiers canyon in the NW Mediterranean Sea.

*In situ experiment.* Twelve pieces of pine and oak wood cut from the same tree as the one used for the aquaria experiment were immersed in the Lacaze-Duthiers canyon in the Gulf of Lions (NW Mediterranean Sea), 25 km away from shore (42° 32' 26" N, 03° 25' 9" E) at 520 m depth (Figure 1d) in November 2010. Wood immersion and recovery were conducted with the ROV 'Super Achille' deployed from the research vessel Minibex. Three pieces of pine and three pieces of oak were recovered after 210 days and again after 365 days of immersion. The wood pieces were strongly degraded by wood-boring bivalves, thus we could not sample the three different wood microhabitats. Therefore, only the center was sampled following the same protocol as for the aquarium experiment and was stored immediately at -80 °C.

#### DNA extraction and pyrosequencing

Wood samples were powdered with a TissueLyzer (RETSCH Mixer Mill, Retsch Inc., MM301; Palacios *et al.*, 2009). Stainless steel grinding jars (25 ml,

Retsch Inc., Newtown, PA, USA; MM 400), containing one stainless steel ball, and the sample were kept in liquid nitrogen for 5 min, then powdered for 1:30 min at a frequency of 25 Hz. This step was repeated three times. The powder was recovered and placed inside 2 ml Eppendorf tubes. One tube was used for DNA extraction and the other was reserved for biochemical analysis. DNA extraction was performed with the Power plant MoBio kit (MOBIO Laboratories, Carlsbad, CA, USA) following an adapted version of the MoBio protocol (Fagervold *et al.*, 2013). The extraction was followed by a cleanup step by isopropanol precipitation before resuspending DNA in TE buffer.

A portion of the 16 S rRNA gene was amplified using the *Bacteria*-specific primers 28 F (5'-TTTGA TCNTGGCTCAG-3') and 519 R (5'-GTNTTACNG CGGCKGCTG-3'), and the *Archaea* primers 340 f (5'-CCTACGGGGYGCASCAG-3'; Ovreas *et al.*, 1997) and 806r (5'-GGACTACNNGGTATCTAAT-3'; Yu *et al.*, 2005). A portion of the fungal internal transcribed spacer (ITS) was amplified with primers ITSF1F (5'-CTTGGTCATTTAGAGGAAGTAA-3';

Gardes and Bruns, 1993) and ITS4R (5'-TCCTCCGTTATTGATATGC-3'; White *et al.*, 1990). We sequenced 42 samples for *Bacteria* (30 from the aquarium and 12 from the canyon). For *Archaea*, all samples were first screened by PCR and only 12 samples gave positive results and were sequenced (nine from the aquarium and three from the canyon). The same 12 samples were sequenced for *Fungi* (Supplementary Table 1). Pyrosequencing was conducted by a commercial laboratory (Research and Testing Laboratory, Lubbock, TX, USA) on a Roche 454 FLX (Brandford, CT, USA) using commercially prepared Titanium reagents. Sequences have been deposited to the GenBank Sequence Read archive under number SRR1564096 for bacterial sequences and SRR1958899 for archaeal and fungal sequences.

#### Sequence data analysis

Sequences were processed following the standard operating procedure in Mothur (Schloss *et al.*, 2011). Briefly, sequences were denoised using Mothur's implementation of PyroNoise (Quince *et al.*, 2011) with default parameters, and chimeras were removed using uchime (Edgar *et al.*, 2011). Sequences were grouped into operational taxonomic units (OTUs) at a 97% similarity cutoff, and taxonomy was assigned using the Silva database (v. 108; Pruesse *et al.*, 2007) for *Bacteria* and *Archaea* and the UNITE database (v6; Kõljalg *et al.*, 2013) for *Fungi*, based on a representative sequence of each OTU, chosen as the most abundant sequence of an OTU. Finally, data sets were resampled down to an equal number of 1663 sequences per sample for *Bacteria*, 2000 sequences for *Archaea* and 750 sequences for *Fungi*. Three archaeal samples were excluded from the analysis because they had too few sequences (Supplementary Table 1).

#### Diversity metrics and statistics

We calculated rarefaction curves and the Shannon diversity index ( $H'$ ) in Qiime (Caporaso *et al.*, 2010b). We also estimated phylogenetic diversity by computing the Net Relatedness Index (NRI) (Webb, 2000) using the picante package in R (Kembel *et al.*, 2010). NRI calculations are based on the mean pairwise distance that measures branch length between each OTU in a phylogenetic tree. The tree was built using representative sequence of each OTU aligned with Pynast (Caporaso *et al.*, 2010a). The phylogenetic tree was built using the FastTree algorithm (Price *et al.*, 2010). NRI significance was tested against a null model in which OTUs are randomly drawn from the sequence pool.

An unweighted pair group method with arithmetic mean cluster analysis was conducted based on the Bray–Curtis dissimilarity index. The strength of the cluster's nodes was assessed by jackknife analysis and the significant difference between larger clusters was tested by running a SIMPROF analysis (Clarke *et al.*, 2008;

Yoshioka, 2008). To test the effect of microhabitat and time on the bacterial communities, we used a permutational multivariate analysis of variance (ADONIS) using a Bray–Curtis distance matrices (Anderson, 2001). The significance of the test was verified with a Monte Carlo permutation (9999 times).

Analysis of variance, the Wilcoxon and the Mann–Whitney statistical test were computed to test whether lipid concentrations varied with time. Differences were considered significant when  $P < 0.05$ . All statistics were computed with the R software (V 2.15.13, Vienna, Austria).

#### Association network

Associations between bacterial OTUs were characterized by computing the maximal information coefficient between each pair of abundant OTUs (Reshef *et al.*, 2011). Maximal information coefficient provides a score that represents the strength of a relationship between data pairs. The matrix of maximal information coefficient values  $> 0.4$ , which reduces the false discovery rate ( $P = 5 \times 10^{-5}$ ), and corresponding to positive linear correlations, was used in Cytoscape 3.1.0 to visualize the network of associations (Smoot *et al.*, 2011) using the spring-embedded algorithm (Kamada and Kawai, 1989). Subnetworks of OTUs were defined with the hierarchical clustering algorithm HC-PIN (Wang *et al.*, 2011) and the modularity of each subnetwork was calculated. Modularity is the sum of the differences between the number of edge within the subnetwork and the square number of edges that linked another subnetwork (Clauset *et al.*, 2004).

#### Lipid concentrations

We quantified lipid concentrations to compare pine and oak wood characteristics. Lipids used as reserves characterize pine as a fat tree compared with oak that uses starch, a glucose polymer, for storage (Fengel and Wegener, 1984). Powdered woods were freeze dried before analysis and total lipids were determined for each sample on triplicates using the sulfophosphovanillin assay (Barnes and Blackstock, 1973). Briefly, lipids were extracted from 25 mg of dried material with 1.5 ml of chloroform/methanol (2v:1v) for 20 min. An aliquot of the lipid extract was evaporated to dryness and hydrolyzed with concentrated sulfuric acid. The phosphoric acid–vanillin reagent was added to 100  $\mu$ l of hydrolysate and the optical density at 520 nm was read on a PerkinElmer Lambda 25 spectrophotometer (Waltham, MA, USA). Concentrations were estimated using cholesterol as a standard (Barnes and Blackstock, 1973). Variability within triplicates was on average 6.5% (ranging from 0.6 to 16%).

#### Sulfide measurements

Discrete measurements of sulfide were performed on wood pieces using a voltammetric three-electrode

cell with Au/Hg electrode as working electrode (Luther *et al.*, 2001). The electrode was placed in contact with the wood surface during these measurements. Each measurement consisted of five scans following a 10-s cleaning step, and five such measurements were performed on a chosen point on the experimental substrate (Yucel *et al.*, 2013).

## Results

### Wood lipid and sulfide concentrations

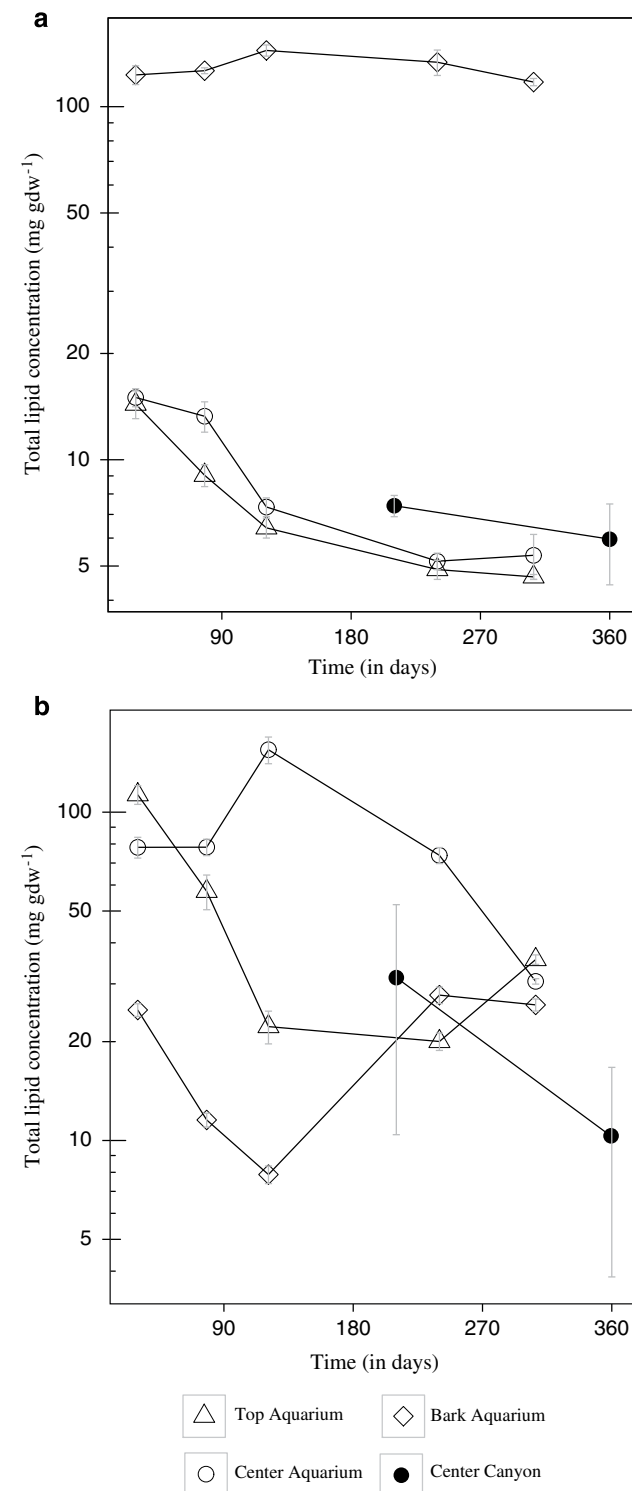
In the aquaria experiment, three pine and oak wood microhabitats (the bark, the center and the top of the wood) were monitored to test whether the different microhabitats changed with time. Oak bark lipid concentrations, which were 10 times higher than in the top and center of the wood (Figure 2a), did not vary significantly along the course of the experiment. In contrast, center and top lipid concentrations decreased significantly (top: analysis of variance,  $F=406.83$ ,  $P<0.01$ ,  $n=15$ ; center: analysis of variance,  $F=90.031$ ,  $P<0.01$ ,  $n=15$ ), especially during the first 121 days of incubation, and were reduced by more than half after 307 days. Pine samples (Figure 2b) contained significantly more lipids than oak in all three microhabitats (Wilcoxon,  $W=1291$ ,  $P<0.01$ ,  $n=90$ ). On the first sampling point, lipid concentrations were higher in the top and center than in the bark (Figure 2b). As in the oak, lipid concentrations mostly decreased with time in the wood center, but no significant trends were detected in the other microhabitats.

In the canyon experiment, we monitored the center of pine and oak wood after 210 and 365 days of immersion. We also observed a decrease of lipid concentrations with time in both pine (Mann-Whitney test,  $W=63$ ,  $P=0.050$ ,  $n=18$ ) and oak samples (analysis of variance,  $F=4.15$ ,  $P=0.058$ ,  $n=18$ ).

In the aquaria experiment, discrete measurements of sulfide concentrations were done on the wood during the first four months of the experiment when a white biofilm developed on the wood surface (Supplementary Figure 1). Sulfide was never detected on the bare wood surface itself but significant amounts were measured on the wood biofilms (Supplementary Table 2). Sulfide was always detected on the oak biofilm with values ranging from 2 to 46  $\mu\text{M}$  after 78 days. On pine biofilms, sulfide was detected frequently after 21 days of incubation but less often later in the experiment (Supplementary Table 2). After 122 days, the biofilm disappeared and no more sulfide measurements were done.

### Community and phylogenetic diversity

Pyrosequencing produced a total of 410 923 raw bacterial sequences from 42 samples gathered from the aquaria and *in situ* experiments. A total of



**Figure 2** Total lipid concentrations and standard errors in oak (a) and pine (b) woods over time in different microhabitats (bark, top and center) in aquaria experiments and *in situ* canyon deployments. The y axis is in log scale.

164 386 sequences remained after denoising and 154 604 after chimera checking (Supplementary Table 1). Sequences clustered at a similarity threshold of 97% grouped into 7326 OTUs. *Bacteria*



rarefaction curves for pine and oak samples (Supplementary Figure 2) showed that our sequencing effort did not cover the full community diversity. In pine samples from the aquarium, the bark was the most diverse microhabitat with a Shannon index that varied between 7 and 8 (Figure 3a). The lowest value occurred after 121 days. In the top microhabitats, diversity decreased with time from 6.5 to <5. The center microhabitat showed large variations in diversity: days 30, 121 and 307 had a lower diversity than days 78 and 240. Oak center microhabitat had the same patterns of diversity as pine. However, the oak bark was less diverse, and the oak top microhabitat diversity increased strongly between days 30 and 78 to a value of 8, exceeding what was observed in pine (Figure 3b). Shannon diversity indexes were always higher in the canyon than in the aquarium (Supplementary Table 1).

Phylogenetic diversity (Figures 3c and d) was calculated with the NRI on aquaria samples. Positive NRI values indicate a clustered phylogeny, whereas a negative index indicates overdispersion with OTUs less related to each other. The patterns of phylogenetic diversity were different than the patterns observed for community diversity. In pine, the three microhabitats showed different patterns. The largest variation was observed in the top with lowest values after 121 days (Figure 3c). In oak, the NRI decreased with incubation time in the three microhabitats but showed significant values for the bark only (Figure 3d). In top and middle, the NRI decreased during the first 240 days before increasing again after 307 days.

For *Archaea*, a total of 11 609 sequences remained for the diversity analysis and grouped into 50 OTUs. The diversity was lower than for *Bacteria* with a Shannon index ranging from 1.43 to 1.73 (Supplementary Table 1).

For fungal communities, 12 735 amplified sequences remained in total. Fungal diversity was also lower than *Bacteria* with a Shannon index ranging from 0.62 to 2.92 (Supplementary Table 1).

#### Similarity between communities

For *Bacteria*, the cluster analysis based on Bray–Curtis similarity split the communities in four significant groups (SIMPROF,  $P > 0.01$ ): two from the canyon and two from the aquarium experiment (Figure 4). Within the canyon and the aquarium, the two subgroups clustered according to the type of wood (pine vs oak).

Within the aquarium, oak samples were significantly separated in the following three main groups: one containing samples incubated for 30 days, one with samples incubated for 78 and 121 days, and one with samples incubated for 240 and 307 days. For the pine samples, two main clusters were distinguished. All bark microhabitat samples were grouped in one of the cluster, and all but one top

samples were grouped in the other cluster. In comparison with bark and top, center samples were more spread through the clusters (Figure 4). The nonparametric multivariate ADONIS test confirmed the significant effect of time on oak bacterial communities ( $F = 2.8$ ,  $P = 0.0046$ ,  $R^2 = 18\%$ ) and the significant effect of microhabitat on pine bacterial communities ( $F = 2.0$ ,  $P = 0.0106$ ,  $R^2 = 25\%$ ).

For the canyon, samples could not be separated into different microhabitats owing to the advanced degradation state of the wood. For both pine and oak, immersion time appeared to separate the communities as most replicated samples taken after 210 days grouped away from 365 days samples (Figure 4). It should be noted that the entire canyon cluster was composed of long branches indicative of large differences between community compositions.

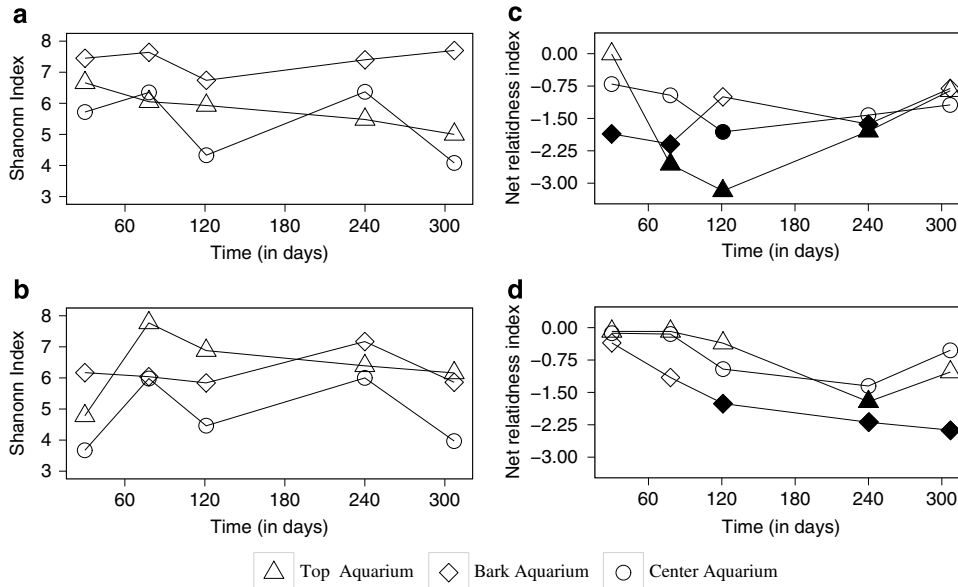
For *Archaea*, we detected sequences in oak only. Samples were grouped in two clusters that separated canyon from aquarium samples (Supplementary Figure 3). In the aquarium, the bark microhabitat samples grouped together away from the top microhabitats. After 240 days, *Archaea* were detected in the bark only.

For *Fungi*, the aquarium samples after the first month grouped away from communities sampled after 78 and 240 days (Supplementary Figure 4), and samples from 78 days center and top grouped separately from samples from the bark after 78 and 240 days.

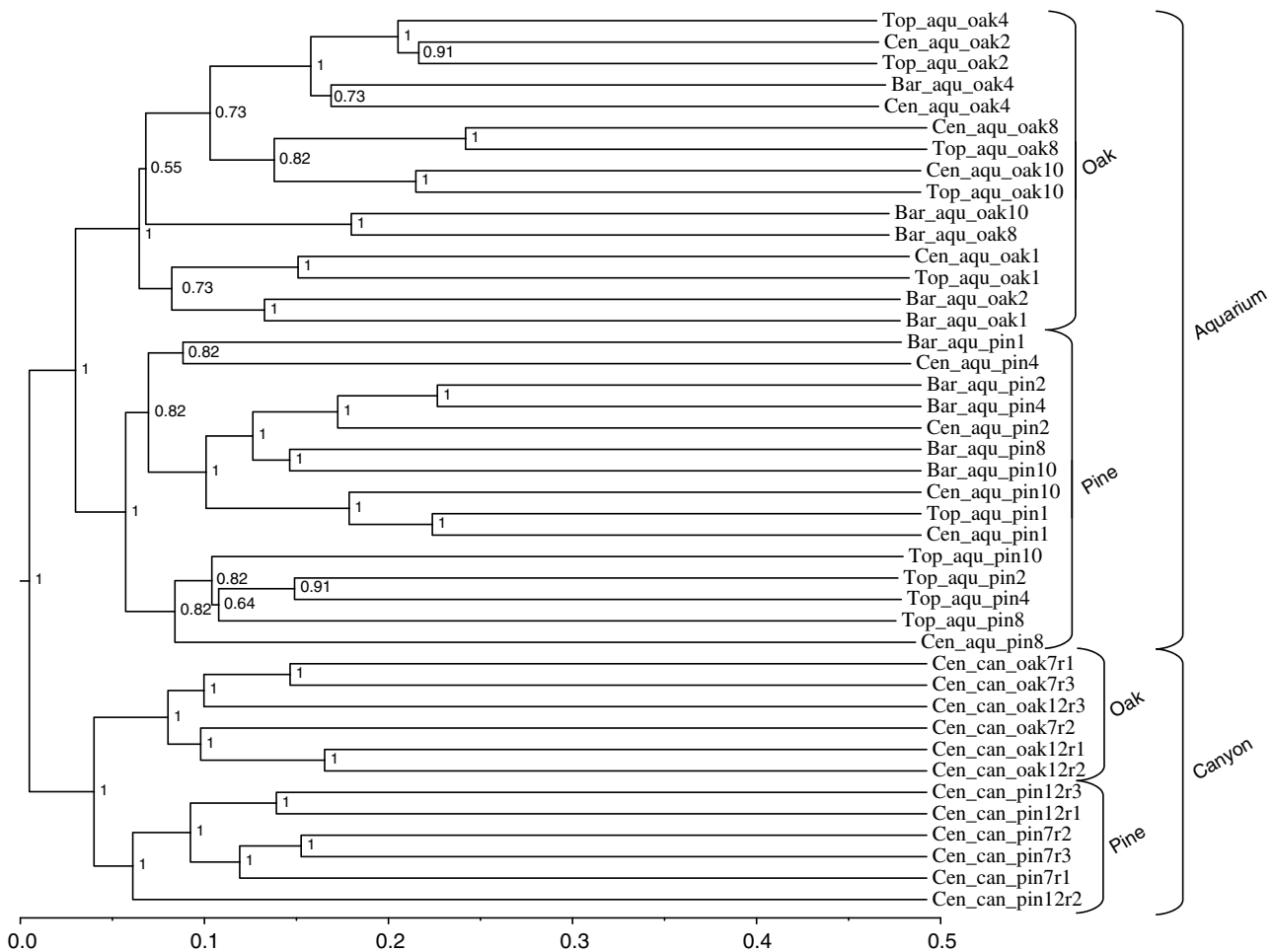
#### Community composition

**Bacteria communities.** Among the abundant OTUs found in aquarium oak samples incubated for 30 days, a large number of bacterial sequences were affiliated to *Reinekea marinisedimentorum* (98% similarity). *Reinekea marinisedimentorum* produce acids from glucose under aerobic and anaerobic conditions, and are able to reduce nitrate (Romanenko *et al.*, 2004; Figure 5). A deep-branching *Gammaproteobacteria* was also abundant as well as *Spirocheta* sequences only distantly related to cultivated strains. The relative number of *Spirochaeta* sequences increased after 78 and 240 days in all microhabitats (Supplementary Figures 5 and 6). These sequences were represented by one single OTU distantly related to any cultivated strain (Otu10983). *Desulfobacteraceae* sequences appeared after 78 days, as well as few sequences affiliated to the *Maribacter* genus. After 240 and 307 days, an uncultured *Gammaproteobacteria* OTU was detected that increased in abundance concomitant with a decrease of *Desulfobacteraceae* sequences after 307 days. The proportion of *Bacteroidetes* sequences increased from 240 to 307 days.

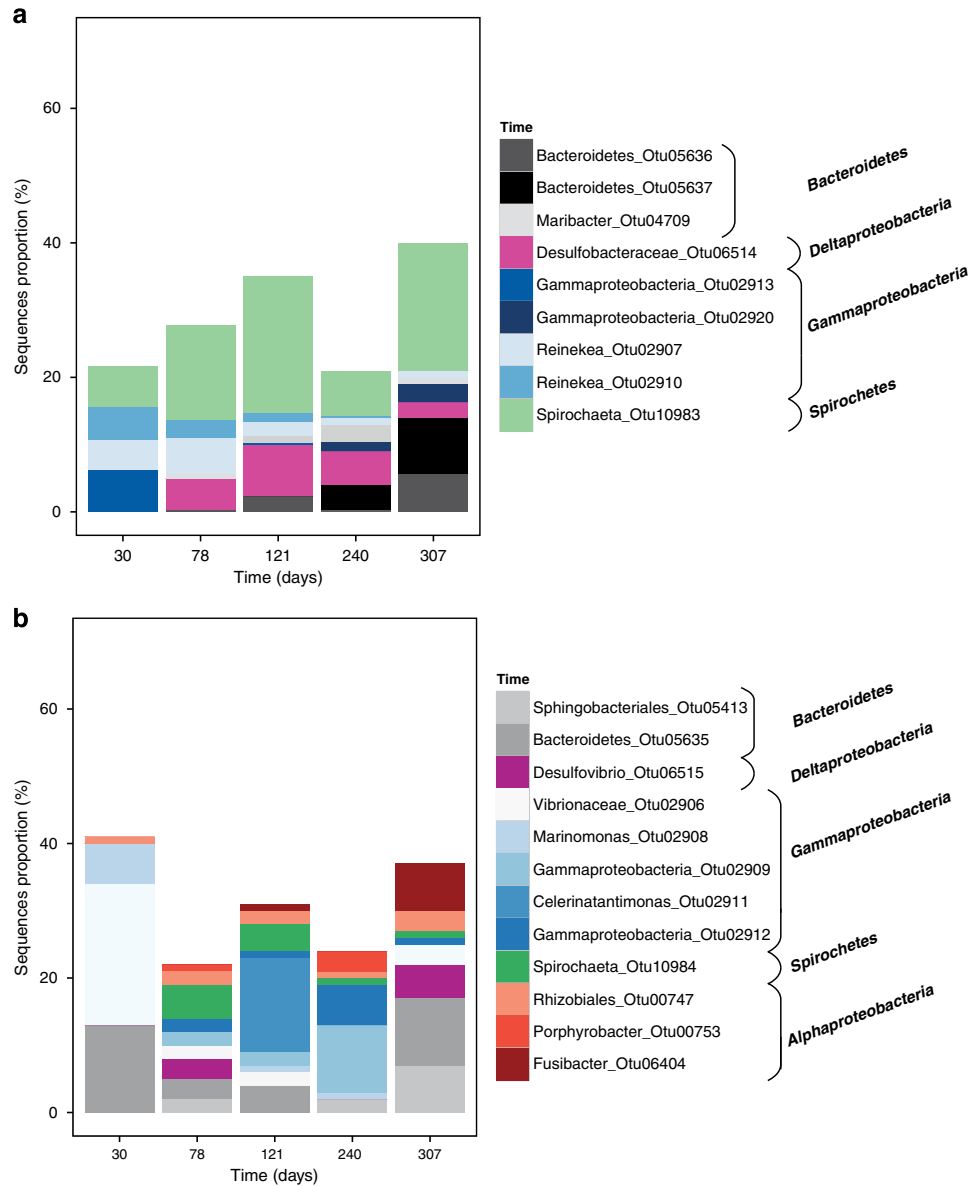
Pine samples from the aquarium (Supplementary Figure 7) did not present any clear temporal trend of OTU dynamics when pooling the three microhabitats together (Figure 5). A total of four OTUs were present in all pine samples (Figure 5): an unknown



**Figure 3** Shannon index (a, b) and NRI (c, d) for bacterial communities in the bark (◇), top (Δ) and center (○) microhabitats in pine (a, c) and oak (b, d) woods. Filled symbols indicate significant NRI values.



**Figure 4** Cluster analysis based on the Bray–Curtis index showing similarity between bacterial community assemblies in top (Top), center (Cen) and bark (Bar) microhabitats in pine and oak woods from the aquarium experiment (aqu) and *in situ* canyon deployments (can). Numbers indicate the time of incubation in month, and r1, r2 and r3 indicate replicate samples in the canyon.



**Figure 5** Taxonomic affiliation and proportion of the five most abundant OTUs in each sample for oak (a) and pine (b) woods from the aquarium. The three wood microhabitats are grouped together. Details are shown in Supplementary Figure 5.

*Rhizobiales* (Otu747), an unknown *Bacteroidetes* (Otu5635), a *Vibrio*, which was closed to *Vibrio aestuarinus* with 95% similarity (Otu2906), and an OTU similar (99%) to *Desulfovibrio piezophilus* (Otu6515), a sulfate reducer originally isolated from a deep-sea wood fall in the Mediterranean Sea (Khelaihia *et al.*, 2011). Among other pine OTUs, the few that had a close match to database sequences were *Alteromonas stellipolaris* (99% similarity; Otu2918), which is a chemoheterotrophic aerobic bacterium that uses cellulose degradation downstream products as a carbon source (Van Trappen *et al.*, 2004). *Marinomonas mediterranea* (99% similarity; Otu2908) was found especially in the bark (Supplementary Figure 5), which is aerobic and motile sugar oxidizer (Solano and Sanchez-Amat, 1999). Interestingly, OTUs found in the center

microhabitat were often distantly related to known strains (ca. 90%). Pine and oak wood shared two abundant OTUs, one belonging to *Spirochaetes* (Otu10984) and the other belonging to *Bacteroidetes* (Otu5635).

In the canyon samples (Supplementary Figure 8), the largest difference in community composition was observed between the type of wood and then between incubation times (Figure 3). Regarding the type of wood, *Flavobacteriales*, *Methylophiliales* and *Alteromonadales* were typical for pine samples, whereas the *Rhodobacteriales* and *Spirochaetales* class characterized the oak.

*Archaea and fungi communities.* Most of the archaeal sequences were only distantly related to known sequences. Among the ones that we could

identify more precisely, otu35 (Supplementary Figure 9) was 99% similar to *Methanococcoides alaskensis*, a methanogen that uses trimethylamine as catabolic substrate with methane as end-product (Singh *et al.*, 2005). Its sequence was more abundant after 78 days in the top microhabitats in the aquaria experiment (Supplementary Figure 9). The other abundant known sequence in the top was a marine group I *Thaumarchaeota* (otu1) belonging to the *Nitrospumilus* genus that is commonly found in seawater (Könneke *et al.*, 2005). After 240 days in the aquarium, *Archaea* were present only in the bark microhabitat with a dominance of *Thaumarchaeota* similar to sequences previously found in marine sponges and sediments.

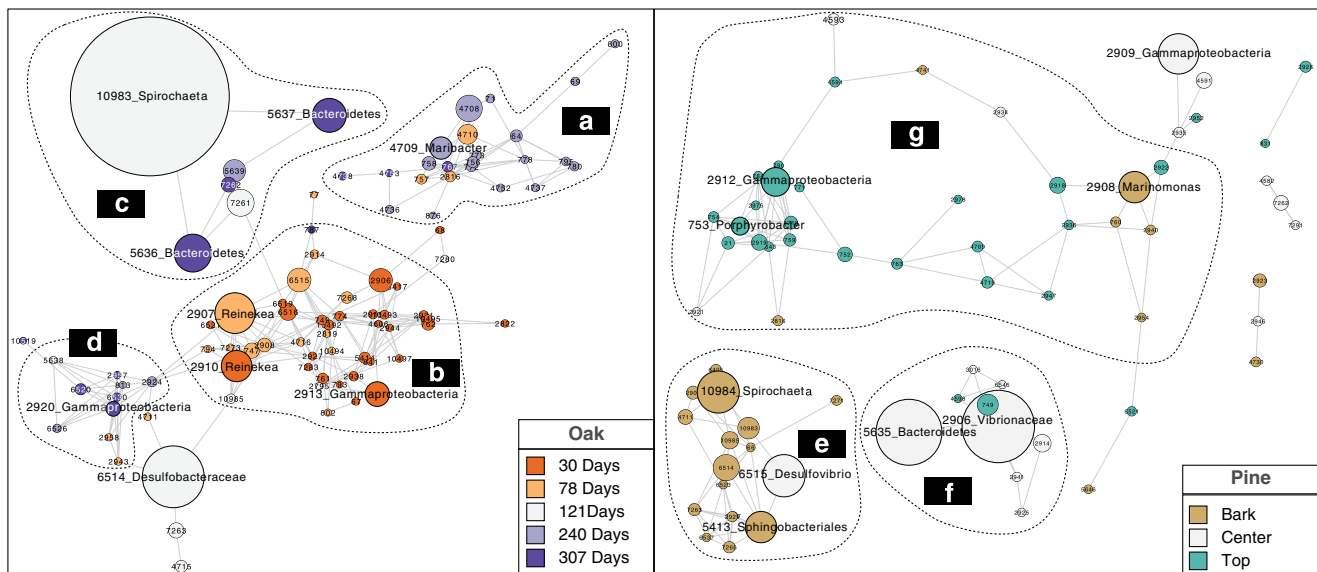
Canyon *Archaea* were different from the ones detected in the aquarium (Supplementary Figure 9). Sequences belonged to the lake dagow sediment and rice cluster V cluster of *Euryarchaeota* (Barberán *et al.*, 2011) and were distantly related ( $\leq 95\%$ ) to sequences found in marine sediments.

The most abundant fungal sequences (otu001; Supplementary Figure 10) detected after 30 days of incubation in the aquaria was identified as a *Phialemonium* (100% similarity), a genus that has been previously found in marine surface waters (Oliveira *et al.*, 2013). After 78 days in the aquaria, the bark community was characterized by two *Ascomyta* (otu2 and otu9; Supplementary Figure 10). They had low similarity ( $\leq 95\%$ ) to sequences from the database. The otu2 was identified as a *Leotiomyces*, a class containing marine fungi found in cold seeps (Nagahama *et al.*, 2011) and hydrothermal vents (Burgaud *et al.*, 2009). Other OTUs (otu3, 7 and 11) belonging to the *Ascomyta*

phylum were also present in the center and top, but only otu11 could be precisely assigned (97% similarity) to sequences from spring water (Oliveira *et al.*, 2013). In the Canyon after 210 days, the most abundant fungal OTU was an *Ascomyta* that was similar to sequences found in the sea and mangrove sediments with 100% similarity, and another OTU that was 99% similar to a sequence from methane hydrate-bearing deep-sea marine sediments (Lai *et al.*, 2007).

### Association network

A network was constructed to identify OTUs that were closely associated to each other in the aquaria experiments. In oak, four subnetworks could be distinguished by the hierarchical clustering algorithm HC-PIN (Wang *et al.*, 2011), which mathematically dissociates groups of OTU into subnetworks. The subnetwork (Figure 6a) with the highest modularity ( $\infty$ , that is, densest connections between nodes) was composed of 23 OTUs, and 71% of these OTUs were more abundant after 240 days of incubation (Figure 6a). Most of them belonged to *Alphaproteobacteria* (48%) and *Flavobacteria* (38%). Forty other OTUs formed a second subnetwork with a modularity of 12 (Figure 6b). This group was composed of a majority of OTUs that were abundant after 30 (63%) or 78 days (30%) in the aquarium. This subnetwork was more diverse than the previous one and was dominated by *Gammaproteobacteria* (28%) and *Alphaproteobacteria* (23%). The third subnetwork (Figure 6c) revealed by the algorithm was smaller with only seven nodes and a modularity of 8. Three OTUs (5636, 5637 and 10 983)



**Figure 6** Network showing associations between OTUs identified by maximal information coefficient values. Nodes represent abundant OTUs. Numbers indicate OTU names. Taxonomic assignment is given for the largest OTUs. Node size is proportional to the number of sequences in each OTU. The left panel presents the oak sample network from the aquarium and the right panel represents the pine samples from the aquarium. Dashed lines delineate subclusters defined by the HC-PIN algorithm. Colors indicate OTUs typical for the different incubation times or microhabitats. **a-g** indicates subnetworks discriminated by the HC - PIN analysis.

reach their maximum abundance between 121 and 307 days. The last subnetwork was made of 10 nodes composed of 50% of *Gammaproteobacteria* and 30% of *Deltaproteobacteria*, among which *D. piezophilus* (6520; Figure 6d). In summary, we identified one large subnetwork made of OTUs that reached their highest sequence abundance per sample at the beginning of the experiment (b), then a subnetwork with maximum OTU sequence abundance after 240 days of experiment (a) and, finally, two subnetworks that were not clearly associated to incubation time. It should be noted that many abundant OTUs did not appear in any subnetwork because they did not show specific associations to other OTUs, probably because they were ubiquitous across the different samples.

Looking at the same projection for pine, we observed a first subnetwork (modularity =  $\infty$ ) made of 17 nodes, among which 94% reached their highest sequence number in the bark microhabitat (Figure 6e). These OTUs were mostly composed of *Deltaproteobacteria* and one of them (6515) was 99% similar to *D. piezophilus* as mentioned earlier. There were also three OTUs related to *Spirochaetales* (10983, 10984 and 10985), which are known to ferment simple sugars such as glucose in acetate, CO<sub>2</sub> and H<sub>2</sub>, and *Oceanospiralles* (2908). Those two orders contained OTUs that were similar to those found in the oak (*Spirochaeta littoralis* and *M. mediteranea*). Subnetwork 2 (modularity =  $\infty$ ) contained nine OTUs and seven of them reached maximum sequence abundance in the center microhabitat (Figure 6f). Five OTUs belonged to the *Gammaproteobacteria* and one OTU belonged to the *Vibrionales* order (2906). The third and last subnetwork was the largest with 31 OTUs and a modularity of 32.5. Close to 71% of the OTUs from this cluster reached a maximum sequence number in the top microhabitat. A total of 13 OTUs belonged to *Gammaproteobacteria* and 9 belonged to *Alphaproteobacteria*. A noteworthy OTU (4593) in this subnetwork was related to an *Arcobacter* able to oxidize sulfide.

In summary, the networks showed that most of the abundant OTU in oak varied together based on incubation time, while in pine, microhabitat was the strongest structuring factor for microbial communities. However, some interactions between microhabitats were detected. Microbes from the bark had few associations to OTUs from the center microhabitat, and most of the abundant center microhabitat OTUs had some connections to OTUs abundant in the top. The top microhabitat, as seen in the subnetwork g, was connected to OTUs abundant in the bark and center.

## Discussion

Our deployment of wood substrates in marine waters demonstrated that the patterns of microbial

colonization of a same object, wood, changed markedly depending on the environmental context. The location, which encompasses factors such as the biological, chemical and physical properties of water, and the vicinity of sediments were the strongest structuring factors as illustrated by the difference observed between woods incubated in deep-sea water in the canyon and wood incubated in coastal water in the aquarium. Moreover, our results also showed that within a given environment, the factors controlling microbial communities differed. First, the type of wood was the strongest controlling factor as illustrated by the difference between pine and oak wood communities in both canyon and aquarium, and as shown earlier (Palacios *et al.*, 2009; Fagervold *et al.*, 2014). Then, communities changed between wood microhabitats, within the same wood, as seen in pine in the aquarium. The temporal scale appeared, however, to have a stronger impact on community assembly in oak wood, which had communities changing with time of incubation rather than between microhabitats. It shows that under similar environmental conditions, in this case, wood incubated over time in the same aquarium, the pattern of microbial colonization is influenced by different constraints (time vs microhabitat) depending on the substrate (pine vs oak).

The comparison of phylogenetic diversity between communities in the different microhabitats of the wood also highlighted a scale-dependent control of community structure. In oak, communities' phylogenetic diversity changed temporally. Communities were always more phylogenetically clustered after 30 days of incubation, indicating that they were more related to each other in the beginning of the experiment. This pattern suggests that microbial community assembly was shaped by species sorting (that is, regulated by the abiotic environment; Webb *et al.*, 2002). In this case, only one specific fraction of the environmental bacterial pool is able to colonize and persist in the wood matrix when colonization starts. This abiotic selection process is called habitat filtering (Webb *et al.*, 2002) and was shown earlier to structure wood fall communities on the sea floor (Fagervold *et al.*, 2012). Interestingly, the level of habitat filtering decreased with time. It reached a minimum after 240 days, which was indicative of an overdispersed phylogeny. Dispersion suggests that new microhabitats became available selecting for more distantly related bacterial species that would be more ecologically different. This scenario is interpreted as an increased importance of competitive exclusion for community structuring. Only species that do not compete with each other, by occupying distinct microhabitats, are able to develop in the environment. We can hypothesize that at this stage the full cascade of organic matter degradation is taking place from aerobic processes on the wood surface to anaerobic metabolisms in the wood center. After 240 days, values became more variable, suggesting that the wood environment underwent

perturbations. The colonization by wood-boring species of the family *Terenididae*, which we observed in the wood after 240 days of incubation, transformed the wood matrix creating new microhabitats for microbial colonization (Bessette *et al.*, 2014; Fagervold *et al.*, 2014). In pine wood, the different microhabitats showed different dynamics of phylogenetic diversity, illustrating that the local spatial scale had an important role in structuring the phylogenetic structure of communities. The top microhabitats had the strongest variation of phylogenetic diversity with time, whereas the middle microhabitat changed less. Overall our data showed that the importance of habitat filtering changed with both time and microhabitats, illustrating the complexity of the ecological interactions shaping the wood fall chemosynthetic ecosystem.

The presence of different communities, in different microhabitats and/or at different times, formed the basis of microecosystems with distinct potential microbial metabolisms. Even though caution should be taken when inferring potential metabolisms from 16 S rRNA-based studies, the close similarity of some of our sequences to cultivated strains indicated the potential for sugar degradation, fermentation, sulfate reduction, methanogenesis and sulfide oxidation in the wood. In contact with seawater, aerobic bacteria such as *Marinomonas* or other heterotrophs may be degrading the sugars originating from the cellulose. Cellulose breakdown is likely of bacterial origin as illustrated by the presence of cellulose degrading *Clostridiales* (Ljungdahl and Eriksson, 1985; Shiratori *et al.*, 2006), but fungi, the major wood degrader in terrestrial environment (Ljungdahl and Eriksson, 1985; Raghukumar, 2008), could also participate in the process. We were able to directly detect fungi and to assign them to fungal groups without using long incubations (Azevedo *et al.*, 2010; Pang *et al.*, 2011) or culture methods (Rämä *et al.*, 2014). Whether these were able to degrade cellulose remains to be shown, as none of our ITS sequences had perfect match to cultivated strains. Nevertheless, our data give a rare indication of fungal sunken wood colonization in the sea and add to recent reports of high fungal diversity on sea floor logs (Rämä *et al.*, 2014). Deeper in the wood, where oxygen disappears, anaerobic fermenters belonging to *Spirochaeta*, *Bacterioidales* and *Vibrionales* probably take advantage of the sugar released from the cellulose. After 78 days, a sulfidic stage was observed with the presence of *Desulfobacterales* and *Desulfovibrionales* in the center. *Desulfovibrionales* are sulfate-reducing bacteria that probably take advantage of fermentation end-products. The production of sulfide inside the wood has been documented earlier (Yucel *et al.*, 2013), and our discrete measurements of sulfide concentrations on the wood surface indicated that sulfate reduction also took place in our experiments. Methanogenesis is the other main end process of the anaerobic degradation of organic matter (Canfield, 1993).

The presence of 16 S rRNA gene sequences from methanogens in the top of the wood after 78 days suggests that methane could also be produced when wood is degraded in marine water. The potential for methanogenesis was not detected later in the incubation as only archaeal sequences belonging to possible ammonia oxidizing *Thaumarchaeota*, common on the surface of marine sediments, were found on the bark at the end of the experiment. The metabolism of the canyon wood *Archaea* belonging to the lake dagow sediment and rice cluster V clusters of the *Euryarchaeota* phylum (Barberán *et al.*, 2011) is not known.

The difference between canyon and aquarium bacterial communities could be linked to several factors such as the source of the microbial colonizers, the influence of the sample location on the wood matrix itself and different wood degradation kinetics between canyon and aquarium. Our results confirm previous reports showing that woods incubated in different environments had different bacterial communities (Bienhold *et al.*, 2013; Fagervold *et al.*, 2013) They contrast, however, with a fingerprinting-based study that showed high similarity between woods incubated at different geographical locations or depths (Palacios *et al.*, 2009).

Interestingly, despite the large difference observed between canyon and aquarium, some sequences, such as the one identified as belonging to *D. piezophilus*, were common to both the natural and the artificial environments. The fact that *D. piezophilus*, originally found in deep-sea habitats, is able to colonize poorly connected environments, highlights that even rare bacterial species have an extraordinary dispersal capability in the sea. The presence of abundant sequences of *D. piezophilus* in our aquarium woods also indicates that our experiments in cold water in the dark were able to attract such emblematic species. It also demonstrates that *D. piezophilus*, thought to be a typical piezophile (Khelaifia *et al.*, 2011; Pradel *et al.*, 2013), may also thrive under atmospheric pressure conditions.

The clear differences in term of community composition between oak and pine in both the aquarium and the canyon could be due to different wood chemical composition. Our finding confirms earlier results indicating that different types of wood may harbor different microbial communities (Palacios *et al.*, 2009; Fagervold *et al.*, 2014). Pine wood contained higher concentrations of lipids than oak wood. These lipids used as reserves characterize pine as a fat tree compared with oak that uses starch, a glucose polymer, for storage (Fengel and Wegener, 1984). The kind of substrate available for bacterial community thus differed between wood species. We hypothesize that the significant initial decrease in the oak wood lipid content was due to bacterial or fungal activity. We could not, however, identify by 16 S rRNA gene analysis the specific bacterial group harboring lipase, the enzyme allowing lipid

hydrolysis, as it is common across bacterial lineages (Arpigny and Jaeger, 1999).

## Conclusion

We provide a direct demonstration that wood falls are dynamic microecosystems with high spatial and temporal community turnover. Community assembly was dictated by different constraints that depended on the scale of observation. The most illustrative example was the difference between pine communities, apparently structured spatially according to the wood microhabitat, and oak communities, which changed more significantly with time. Changes in community assembly were reflected by changes in phylogenetic diversity that could be interpreted as shifts between assemblies ruled by species sorting to assemblies structured by competitive exclusion. These ecological interactions mirrored the dynamics of the potential microbial metabolisms accompanying wood degradation in the sea. Finally, we showed that wood is a good model for creating and manipulating potential chemosynthetic ecosystem in the laboratory, and attracting not only typical chemosynthetic microbes but also emblematic macrofaunal species.

## Conflict of Interest

The authors declare no conflict of interest

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Supplementary Information accompanies this paper on The ISME Journal website (<http://www.nature.com/ismej>)

## Supplemental material

**Supplementary Table 1** Origin of the samples used in this study, number of sequences obtained after cleaning and Shannon index. Samples that were discarded because they had too few sequences are shown in red.

Sample name	Origin	Wood	Microhabitat	Month	Number of sequences	Shannon
Bar_aqu_oak1	Aquarium	Oak	Bark	1	5693	7.45
Cen_aqu_oak1	Aquarium	Oak	Center	1	3092	5.72
Top_aqu_oak1	Aquarium	Oak	Top	1	3858	6.66
Bar_aqu_pin1	Aquarium	Pine	Bark	1	3261	6.17
Cen_aqu_pin1	Aquarium	Pine	Center	1	2322	3.67
Top_aqu_pin1	Aquarium	Pine	Top	1	4944	4.78
Bar_aqu_oak2	Aquarium	Oak	Bark	2	4605	7.64
Cen_aqu_oak2	Aquarium	Oak	Center	2	4182	6.35
Top_aqu_oak2	Aquarium	Oak	Top	2	3676	6.05
Bar_aqu_pin2	Aquarium	Pine	Bark	2	5315	6.04
Cen_aqu_pin2	Aquarium	Pine	Center	2	3084	5.98
Top_aqu_pin2	Aquarium	Pine	Top	2	3222	7.77
Bar_aqu_oak4	Aquarium	Oak	Bark	4	4993	6.74
Cen_aqu_oak4	Aquarium	Oak	Center	4	4013	4.33
Top_aqu_oak4	Aquarium	Oak	Top	4	3751	5.93
Bar_aqu_pin4	Aquarium	Pine	Bark	4	4483	5.84
Cen_aqu_pin4	Aquarium	Pine	Center	4	3235	4.46
Top_aqu_pin4	Aquarium	Pine	Top	4	2965	6.88
Cen_can_oak7r1	Canyon	Oak	Center	7	3549	7.47
Cen_can_oak7r2	Canyon	Oak	Center	7	3453	6.80
Cen_can_oak7r3	Canyon	Oak	Center	7	3673	6.42
Cen_can_pin7r1	Canyon	Pine	Center	7	3631	6.76
Cen_can_pin7r2	Canyon	Pine	Center	7	3187	6.94
Cen_can_pin7r3	Canyon	Pine	Center	7	2505	6.30
Bar_aqu_oak8	Aquarium	Oak	Bark	8	4194	7.40
Cen_aqu_oak8	Aquarium	Oak	Center	8	2331	6.37
Top_aqu_oak8	Aquarium	Oak	Top	8	1764	5.48
Bar_aqu_pin8	Aquarium	Pine	Bark	8	4460	7.17
Cen_aqu_pin8	Aquarium	Pine	Center	8	3594	6.00
Top_aqu_pin8	Aquarium	Pine	Top	8	4144	6.39
Bar_aqu_oak10	Aquarium	Oak	Bark	10	4493	7.70
Cen_aqu_oak10	Aquarium	Oak	Center	10	1663	4.08
Top_aqu_oak10	Aquarium	Oak	Top	10	3243	5.00
Bar_aqu_pin10	Aquarium	Pine	Bark	10	4819	5.86
Cen_aqu_pin10	Aquarium	Pine	Center	10	3048	3.97
Top_aqu_pin10	Aquarium	Pine	Top	10	4426	6.16
Cen_can_oak12r1	Canyon	Oak	Center	12	3062	7.27
Cen_can_oak12r2	Canyon	Oak	Center	12	3845	7.53
Cen_can_oak12r3	Canyon	Oak	Center	12	2587	6.88
Cen_can_pin12r1	Canyon	Pine	Center	12	3978	7.13
Cen_can_pin12r2	Canyon	Pine	Center	12	3673	8.16
Cen_can_pin12r3	Canyon	Pine	Center	12	4588	8.03
Cen_aqu1_ITS	Aquarium	Oak	Center	1	1632	0.62
Top_aqu1_ITS	Aquarium	Oak	Top	1	2788	1.14
Bar_aqu1_ITS	Aquarium	Oak	Bark	1	1692	2.08
Cen_aqu2_ITS	Aquarium	Oak	Center	2	1683	1.43
Top_aqu2_ITS	Aquarium	Oak	Top	2	1159	1.84
Bar_aqu2_ITS	Aquarium	Oak	Bark	2	2939	1.42
Cen_can7r1_ITS	Canyon	Oak	Center	7	362	-
Cen_can7r2_ITS	Canyon	Oak	Center	7	526	-

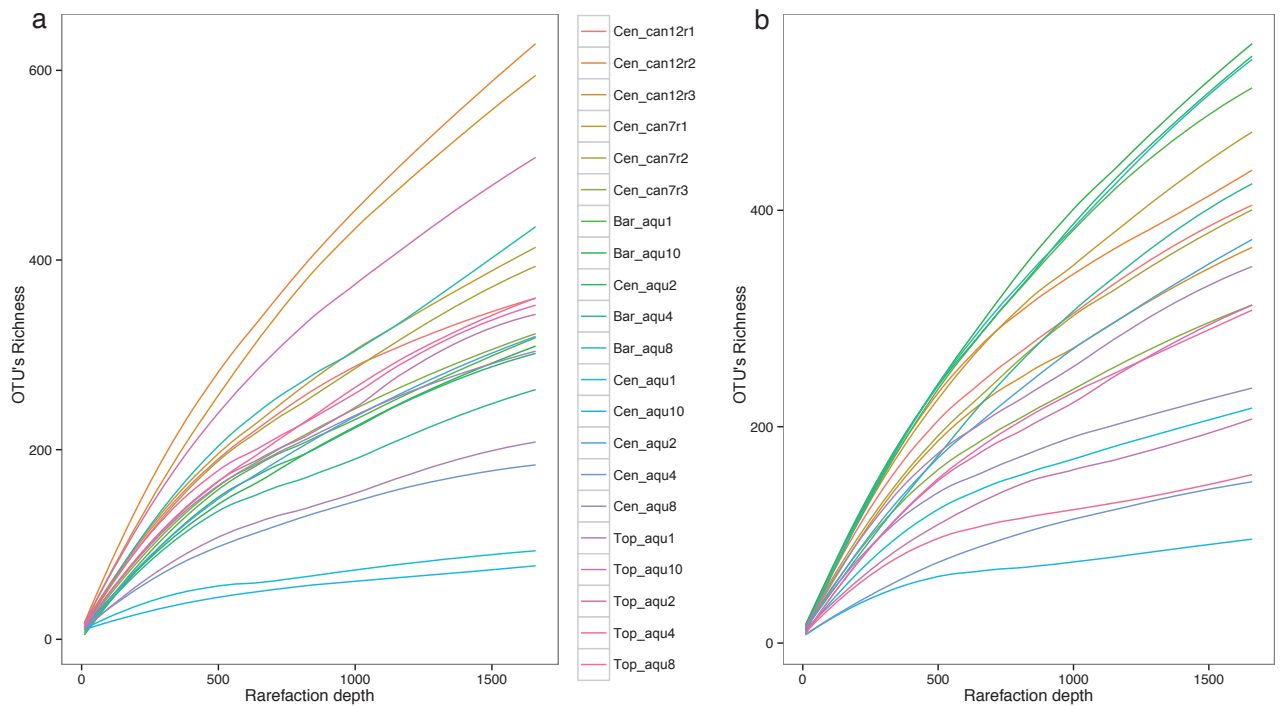
Cen_can7r3_ITS	Canyon	Oak	Center	7	415	-
Cen_aqu8_ITS	Aquarium	Oak	Center	8	0	-
Top_aqu8_ITS	Aquarium	Oak	Top	8	93	-
Bar_aqu8_ITS	Aquarium	Oak	Bark	8	842	2.91
Cen_aqu1_ARC	Aquarium	Oak	Center	1	1	-
Top_aqu1_ARC	Aquarium	Oak	Top	1	979	1.55
Bar_aqu1_ARC	Aquarium	Oak	Bark	1	215	-
Cen_aqu2_ARC	Aquarium	Oak	Center	2	336	-
Top_aqu2_ARC	Aquarium	Oak	Top	2	1762	1.45
Bar_aqu2_ARC	Aquarium	Oak	Bark	2	1451	1.73
Cen_can7r1_ARC	Canyon	Oak	Center	7	2580	1.57
Cen_can7r2_ARC	Canyon	Oak	Center	7	1809	1.64
Cen_can7r3_ARC	Canyon	Oak	Center	7	581	-
Cen_aqu8_ARC	Aquarium	Oak	Center	8	0	-
Top_aqu8_ARC	Aquarium	Oak	Top	8	0	-
Bar_aqu8_ARC	Aquarium	Oak	Bark	8	3028	1.57

**Supplementary Table 2** Discrete measurement of sulfide concentrations in the aquaria at the interface between wood and seawater. All measurements were made on the biofilm that develop at the interface.

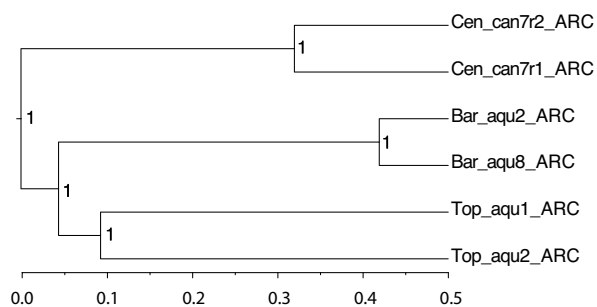
	Time in Days	Sulfide ( $\mu\text{M}$ )
Oak	21	4.2 +- 0.6
	30	3.9 +- 2.3
	30	4.4 +- 2
	32	20.7 +- 2.6
	32	14.1 +- 1.3
	35	7.8 +- 1.4
	35	2.6 +- 1
	51	2.0 +- 0.4
	78	29.1 +- 7.7
	78	46.4 +- 6.2
78	12.4 +- 3.8	
Pine	21	12.6+-2.5
	30	12.8+- 3.6
	30	18.6+- 5
	30	0
	32	0
	32	28.6+- 15
	32	0
	35	0
	35	17.4+- 7.4
	38	0
	38	0.7+- 0.3
	122	0
122	0	



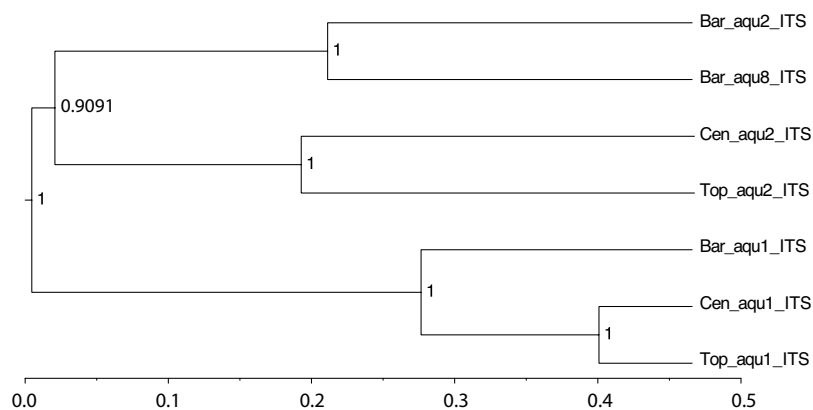
**Supplementary Figure 1** White microbial mat developing on oak wood surface in an aquarium incubation. Wood powder in the centre originates from *Teredinae* bivalves boring activity.



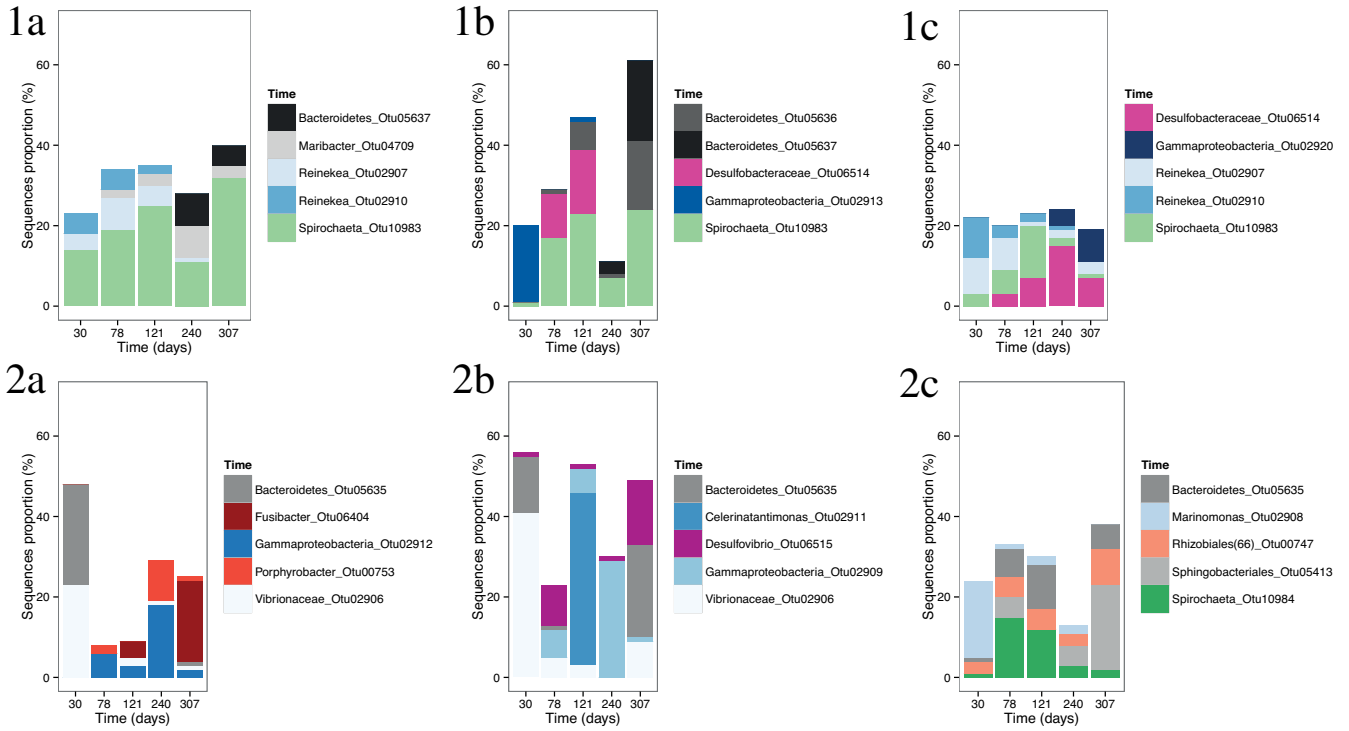
**Supplementary Figure 2** Rarefaction curves for bacterial OTUs clustered at 97% similarity in pine (a) and oak (b) wood samples.



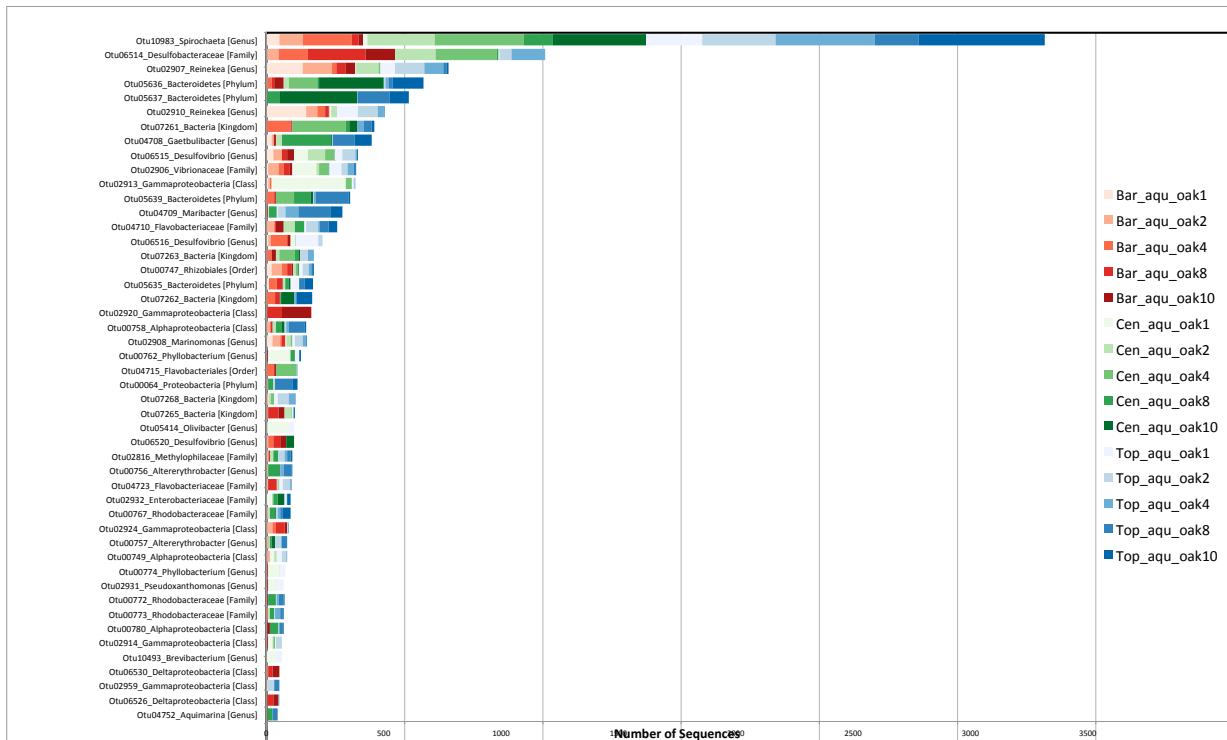
**Supplementary Figure 3** Cluster analysis based on a Bray Curtis dissimilarity matrix of archaeal OTUs in oak samples from the aquarium experiment (aqu) and from the in situ canyon deployment (can). Numbers after the names indicate the time of incubation in months. r1 and r2 are replicate samples. Bootstrap values are shown on the branches.



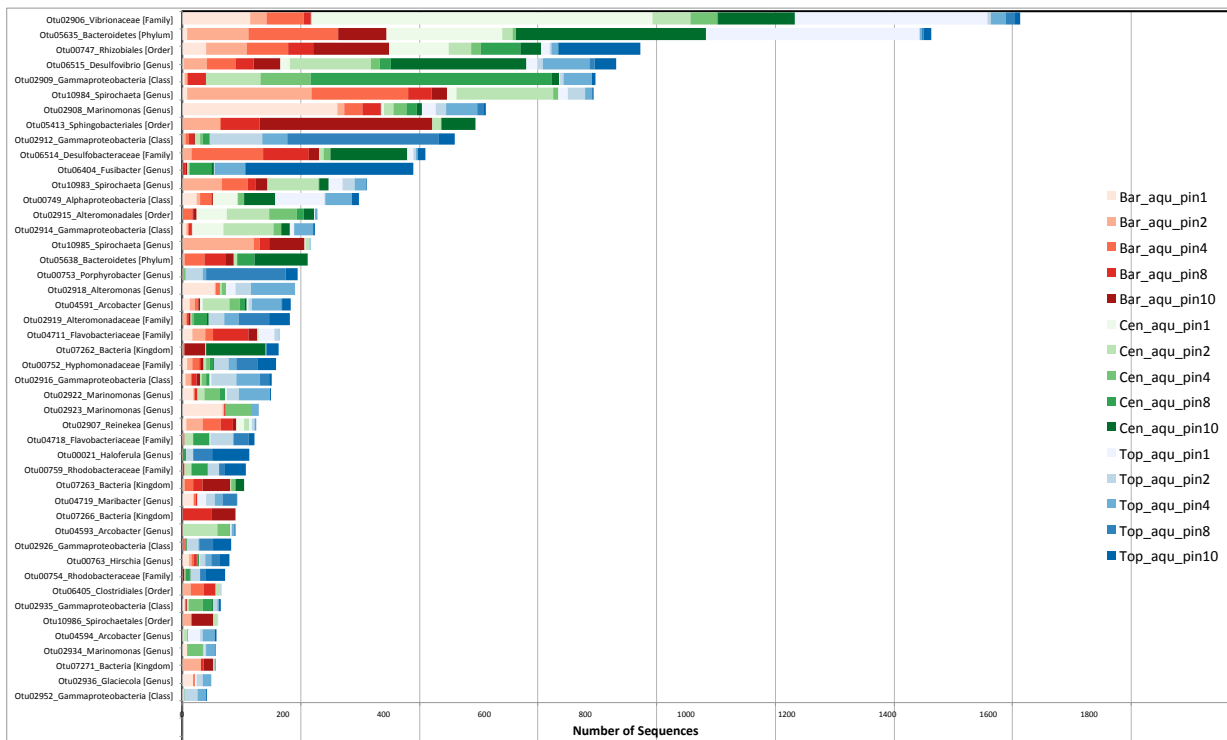
**Supplementary Figure 4** Cluster analysis based on a Bray Curtis dissimilarity matrix of fungal OTUs in oak samples from the aquarium experiment (aqu). Numbers after the names indicate the time of incubation in months. Bootstrap values are shown on the branches.



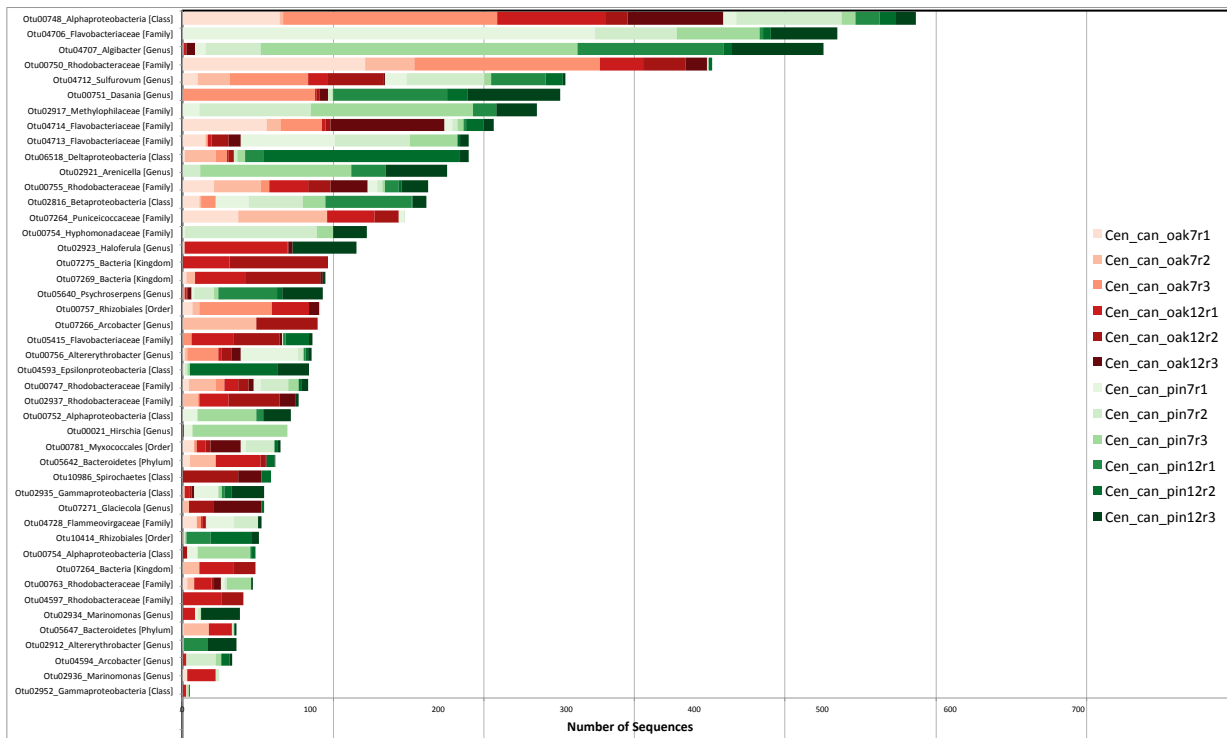
**Supplementary Figure 5** Sequence abundance of the five most abundant bacterial OTUs in each sample for oak (1) and pine (2) wood and in the top (a), centre (b) and bark (c) microhabitats. All samples are from the aquaria experiment.



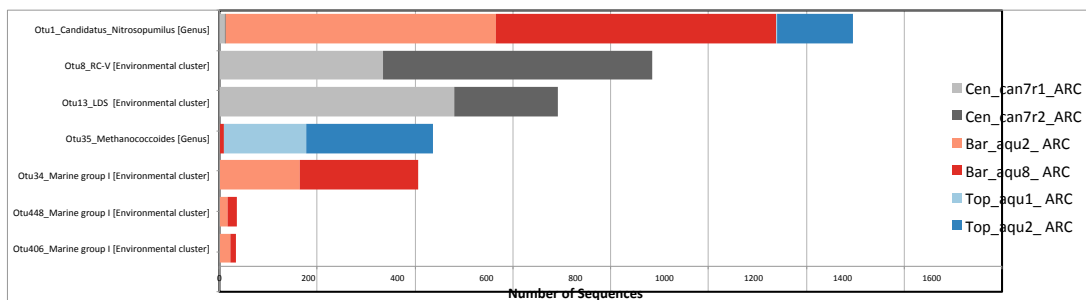
**Supplementary Figure 6** Proportion of the most abundant bacterial OTUs found in oak during the aquaria experiment. Abundant OTU are defined as OTUs with > 1% sequence abundance in at least 2 samples. The taxonomic assignment is based on the lowest annotated taxon. Sample labels show the microhabitat (bark, top or center) and the time of incubation in months (1,2,4,8 or 10).



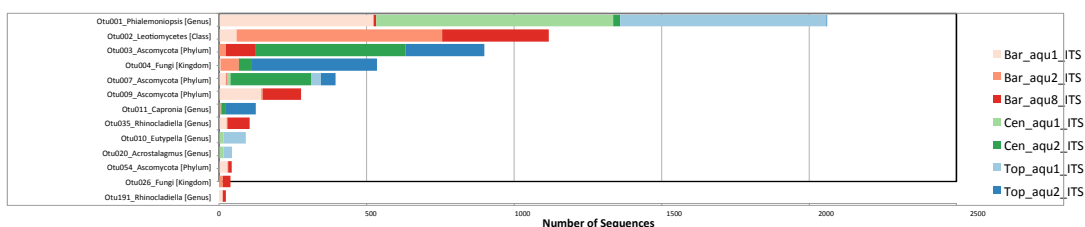
**Supplementary Figure 7** Proportion of the most abundant bacterial OTUs found in pine during the aquaria experiment. Abundant OTU are defined as OTUs with >1% sequence abundance in at least 2 samples. The taxonomic assignment is based on the lowest annotated taxon. Sample labels show the microhabitat (bark, top or center) and the time of incubation in months (1,2,4,8 or 10).



**Supplementary Figure 8** Proportion of the most abundant bacterial OTUs found in the canyon experiment. Abundant OTU are defined as OTUs with > 1% sequences abundance in at least 2 samples. The taxonomic assignment is based on the lowest annotated taxon. Sample labels show the type of wood (oak or pine), the time of incubation in months (7 or 12), replicate samples (r) and replicate number (1, 2, 3).



**Supplementary Fig. 9** Proportion of the most abundant archaeal OTUs found in oak during the canyon and aquarium experiment. Abundant OTU are defined as OTUs with > 1% sequences abundance in at least 2 samples. The taxonomic assignment is based on the lowest annotated taxon. Sample labels show the type of wood (oak or pine), the time of incubation in months (7 or 12), replicate samples (r) and replicate number (1, 2, 3).



**Supplementary Figure 10** Proportion of the most abundant fungal OTUs found in oak during the canyon and aquarium experiment. Abundant OTU are defined as OTUs with > 1% sequences abundance in at least 2 samples. The taxonomic assignment is based on the lowest annotated taxon. Sample labels show the microhabitat (bark, top or center), the sample site (canyon or aquarium) and the time of incubation in months (1, 2, or 8).



## **Article II: Bacteria alone establish the chemical basis of the wood-fall chemosynthetic ecosystem in the deep sea**

Dimitri Kalenitchenko<sup>1</sup>, Nadine Le Bris<sup>1</sup>, Laetitia Dadaglio<sup>1</sup>, Erwan Peru<sup>1</sup>, Arnaud Besserer<sup>2</sup>, Pierre E. Galand<sup>1</sup>

<sup>1</sup> Sorbonne Universités, UPMC Univ Paris 06, CNRS, Laboratoire d'Ecogéochimie des Environnements Benthiques (LECOB), Observatoire Océanologique, Banyuls sur Mer, France

<sup>2</sup> Université de Lorraine, Laboratoire d'étude du matériau bois (LERMAB), Campus bois, Epinal, France

### **Abstract**

Wood-fall ecosystems host chemosynthetic bacteria that use hydrogen sulfide as an electron donor. The production of hydrogen sulfide from decaying wood in the deep sea has long been suspected to rely on the activity of wood-boring bivalves, *Xylophaga sp.* However, recent mesocosm experiments have shown hydrogen sulfide production in the absence of wood borers. Here, we combined in situ chemical measurements, amplicon sequencing and metagenomics to test whether the presence of *Xylophaga sp.* affected hydrogen sulfide production and wood microbial community assemblages. During a short-term experiment conducted in a deep-sea canyon, we found that wood-fall microbial communities could produce hydrogen sulfide in the absence of *Xylophaga sp.* The presence of wood borers had a strong impact on the microbial community composition on the wood surface but not in the wood centre, where communities were observed to be homogeneous among different samples. The wood centre community did not have the genetic potential to degrade cellulose or hemicellulose but could use shorter carbohydrates such as sucrose. We conclude that wood centre communities produce fermentation products that can be used by the sulfate-reducing bacteria detected near the wood surface. We thus demonstrate that microorganisms alone could establish the chemical basis essential for the recruitment of chemolithotrophic organisms in deep-sea wood falls.

## Introduction

Wood falls are described as one of the four chemosynthetic deep-sea ecosystems that harbour diverse and specialized macrofauna (Bernardino *et al.*, 2010; Kiel *et al.*, 2009), meiofauna (Cuvelier *et al.*, 2014) and microorganism communities (Bienhold *et al.*, 2013; Fagervold *et al.*, 2013; Bessette *et al.*, 2014). The inclusion of wood falls among chemosynthetic ecosystems refers to the presence of animal species, which are phylogenetically related to vent and seep lineages (Dando *et al.*, 1992; Distel *et al.*, 2000; Lorion *et al.*, 2009) and host chemoautotrophic symbiotic bacteria. These bacterial symbionts are sulfur oxidizers that may use the hydrogen sulfide released during wood degradation (Duperron *et al.*, 2008). The origin of this reduced compound is different for wood than for hydrothermal vents, where sulfides originate from abiotic reactions at high temperatures and pressures (German and Damm, 2006), or for cold seeps, where it is a breakdown product of the anaerobic oxidation of methane formed from the degradation of fossil organic matter trapped in sediments (Levin, 2010). By contrast, in organic falls, reduced compounds directly originate from the biological degradation of the substrate itself. In the well-studied whale fall system, the chemoautotrophic community relies on the heterotrophic activity of microorganisms using the lipid content of bone (Deming *et al.*, 1997; Smith and Baco, 2003). These microorganisms were identified as sulfate-reducing bacteria that used sulfate as a terminal electron acceptor and produced hydrogen sulfide while using small organic compounds, such as acetate as a carbon source (Treude *et al.*, 2009; Shapiro and Spangler, 2009; Nedwell, 1984). Small organic compounds are breakdown products of the anaerobic breakdown of whale bone lipids (Smith and Baco, 2003). By contrast, wood is mostly made of cellulose and lignin, two highly refractory compounds (Gessner, 2005). Their degradation in anaerobic environments, such as sunken woods, is performed by complex communities of microorganisms, including fungi and bacteria (Ljungdahl and Eriksson, 1984; Leschine, 1995).

Woods on the seafloor harbour highly diverse bacterial communities that include potential sugar degrading, fermenting and sulfate-reducing bacteria but very few potential cellulose degraders (Bienhold *et al.*, 2013; Palacios *et al.*, 2009; Fagervold *et al.*, 2013). In aquaria, these diverse communities showed clear ecological succession during a one-year incubation, and the presence of 16S rRNA gene sequences from sulfate reducers was related to hydrogen sulfide accumulations first detected after 1 month of immersion (Kalenitchenko *et al.*, 2015) (Yücel *et al.*, 2013). Over this short time-scale, wood sulfate reducers could use small organic compounds derived from polysaccharide fermentation. The major

polysaccharide present in wood is cellulose but plants also use sucrose to transport the carbon fixed on leaves or needles through the phloem (Büttner and Sauer, 2000; Lemoine *et al.*, 2013). Sucrose is used by many heterotrophic bacteria as a carbon source (Reid and Abratt, 2005). Even if present as only a small part of the carbohydrate pool, sucrose may represent a less refractory carbon source than cellulose for opportunistic bacteria.

Cellulose degradation in sunken wood is hypothesized to be mostly conducted by macrofaunal borers such as *Xylophaga sp.* (Voight, 2015). Furthermore, it is thought that without the presence of wood borers, which hydrolyze cellulose in association with their bacterial symbionts (Distel and Roberts, 1997), hydrogen sulfide production may not be sufficient to sustain the development of a chemosynthetic community (Bienhold *et al.*, 2013). *Xylophaga sp.* are hypothesized to create niches for bacteria through the development of anoxic zones, which originate from the accumulation of faeces rich in cellulose degradation breakdown products and the production of mucus (Purchon, 1941; Bienhold *et al.*, 2013). It was recently shown that *Xylophaga sp.* faeces host specific microbial communities (Fagervold *et al.*, 2014), which supports the idea that *Xylophaga sp.* play an important role in the sunken wood ecosystem. However, recent results obtained from aquaria experiments suggest a different picture for the early stages of wood colonization. Sulfate-reducing bacteria quickly occupied the wood matrix, and hydrogen sulfide reached millimolar concentrations ( $> 1 \text{ mM}$  [ $\text{H}_2\text{S}$ ]) before any wood borers were detected (Yücel *et al.*, 2013; Kalenitchenko *et al.*, 2015).

In this study, we first tested whether the presence of wood microbes alone could lead to the production of hydrogen sulfide and provide support for a chemosynthetic ecosystem in the natural conditions of a deep-sea environment. Second, we tested whether the presence of borers modifies microbial community assembly and if it could favour sulfide availability for wood chemoautotrophs. We designed an exclusion experiment that was deployed for 2 months in a submarine canyon known to harbour *Xylophaga sp.* We used pine wood to compare our results to previous studies (Kalenitchenko *et al.*, 2015) (Fagervold *et al.*, 2013; Yücel *et al.*, 2013) and protected half of our replicate samples with a mesh to prevent *Xylophaga sp.* larval colonization. We characterized the microbial community by sequencing a portion of the bacterial 16S rRNA gene on an Illumina MiSeq system and by constructing a shotgun metagenome to reveal the genetic potential of the community. Hydrogen sulfide concentrations were measured continuously in situ and used as a marker for sulfate-reducing bacterial activity.

## Materials and methods

### *Experimental design*

Wood samples were cut from one branch of a 30-year old pine tree (*Pinus pinea*) from the Banyuls-sur-Mer “Biodiversarium” botanical garden. Six pieces of wood logs (10 cm in length and 14 cm in diameter) were placed in plastic cages (15x18 cm). Cages were made of a rigid plastic mesh with a 13 mm by 13 mm grid. Three cages were randomly selected and covered with a 50 µm mesh to prevent colonization by wood borer larvae (Culliney and Turner, 1976). Three additional wood-containing cages were left unprotected. Sulfide potentiometric electrodes were inserted 25 mm into the wood through 2 mm diameter holes drilled into one mesh-covered and one unprotected piece of wood. The hole diameter was minimized to avoid trapping water and sealed with Teflon tape. The pieces of wood were attached to an experimental platform and deployed at a depth of 520 m in the Lacaze-Duthiers submarine canyon in the Gulf of Lion (NW Mediterranean Sea), 25 km away from the shore (42°32'26''N, 03°25'9''E) on the 27<sup>th</sup> of April 2013 (Supplementary Figure 1). The platform (with cages) was recovered 85 days later (Supplementary Figure 2). Both operations were performed with a ROV Super-Achille operated from the R/V Minibex (COMEX SA) as part of a larger integrated programme dedicated to the submarine canyon ecosystem.

### *Wood sampling and DNA extraction*

Wood samples were obtained by extracting wood cores with a 4.35 mm-diameter increment borer from logs recovered after 85 days of immersion. Each wood core was divided in two sub-samples: one surface sub-sample (S) covering the first centimeter of the wood, and one centre sample (C) taken from 4 to 5 cm deep inside the wood (Figure 1). Two wood cores were extracted from each of the three wood replicates: one sample was drilled 1 cm away from the bark in the sapwood (Sa) and another 6 cm away from the bark in the heartwood (He) (Figure 1). For the two wood logs equipped with a hydrogen sulfide sensor, an additional core was drilled at the electrode position (sample name ends with a \*).

Wood core samples (1 cm long and 4.35 mm in diameter) were powdered using a TissueLyser (RETSCH Mixer Mill, Retsch Inc., MM301, Düsseldorf, Germany) (Palacios *et al.*, 2009). Samples were placed in stainless steel grinding jars (25 ml, Retsch Inc., MM400, Düsseldorf, Germany), kept in liquid nitrogen for 5 min, and then powdered for 3 X 1.5 min at 25 Hz. DNA was extracted and purified from the wood powder with the Power Plant pro

Mobio Kit (MOBIO Laboratories, Carlsbad, CA, USA) following an adapted version of the MoBio Protocol (Fagervold *et al.*, 2013). An extra clean-up step using isopropanol was performed to remove remaining DNA contaminants.

### *16S rRNA gene and metagenome sequencing*

A portion of the 16S rRNA gene was amplified using bacteria-specific primers 28F (5'-TTTGATCNTGGCTCAG-3') and 519R (5'-GTNTTACNGCGGCKGCTG-3') and then sequenced by a commercial laboratory (Research and Testing Laboratory, Lubbock, TX, USA) on an Illumina MiSeq sequencer to produce 2x300 base pair (bp)-long paired end sequences.

For a functional description of the wood centre community, one sample was selected for a 4 Gb shotgun metagenome sequencing. The library was prepared with 50 ng of DNA using the Nextera DNA sample preparation Kit (Illumina, San Diego, CA, USA) producing a library with an average insert size of 500 bp. The sequencing was performed on a MiSeq 2x300 bp Illumina sequencer by a commercial laboratory (Research and Testing Laboratory, Lubbock, TX).

### *Sequence data analysis*

The 16S rRNA sequences were analyzed using the MiSeq standard operating procedure in Mothur (Kozich *et al.*, 2013). Briefly, sequences were paired, giving *ca.* 500 bp long fragments. Sequences were then trimmed for quality, and chimaeras were removed using UCHIME (Edgar *et al.*, 2011). Sequences were finally grouped using average linkage into Operational Taxonomic Units (OTUs) at a 97 % sequence similarity threshold, and taxonomy was assigned using the Silva database (v.108) (Pruesse *et al.*, 2007). Datasets were resampled down to 2,425 sequences per sample. Sequences were deposited in the NCBI database under SRA accession number SRP058532

For metagenome data, adapter and primers were first removed from the sequences, and then paired-end reads were joined with a minimum overlap setting of 8 bp and a maximum difference of 10 %. Both paired and unpaired reads were retained for further analysis. Low-quality regions (phred score  $\leq$  15) were trimmed using SolexaQA (Cox *et al.*, 2010). Reads that passed the quality trimming were dereplicated using a k-mer approach that identifies sequences with identical twenty character prefix, and then, artificial duplicate sequences were removed (Gomez-Alvarez *et al.*, 2009). A machine learning approach known as

FragGeneScan (Rho *et al.*, 2010) identified open reading frames (ORF) that were annotated using BLASTX (Camacho *et al.*, 2009) against the M5NR (Wilke *et al.*, 2012), KEGG (Kanehisa and Goto, 2000) and Silva (Pruesse *et al.*, 2007) databases. Analyses were conducted with the MG-RAST pipeline (Meyer *et al.*, 2008), and sequences are available under the accession number 4612269.3.

### *Diversity and statistical analysis*

The Shannon and Simpson diversity indices were calculated on a resampled OTU table. Comparisons of alpha diversity indices were achieved in R (2.15.13; Vienna, Austria) using non-parametric Mann–Whitney or the Kruskal-Wallis tests, followed by a multiple comparison test depending of the number of categories compared.

Beta diversity between samples was estimated with the Bray-Curtis dissimilarity index and then represented using an unweighted pair group method with arithmetic mean (UPGMA) clustering. Significant differences between subclusters were tested using a SIMPROOF analysis (Yoshioka, 2008). We constructed heatmaps that presented the community composition at the family level, including only families that contained > 1 % of the total sequence pool. Based on these heatmaps, we performed a Welch t-test to compare microbial community composition between samples. Analyses were conducted using the STAMP software (Parks *et al.*, 2014), and the relationship between OTUs and samples was displayed using the CIRCOS software (Krzywinski *et al.*, 2009).

For the metagenome, we selected gene sequences belonging to the most abundant bacterial family based on the M5 nonredundant protein database (Wilke *et al.*, 2012) with an e-value cut-off of  $1e^{-5}$ , a minimum alignment coverage of 80 % and a minimum length of 15 amino acids (aa). The selected sequences were then compared to the KEGG ontology database (Kanehisa and Goto, 2000) with an e-value cut-off of  $1e^{-5}$ , a minimum alignment coverage of 60 % and a minimum length of 15 aa. Pathway hierarchies were represented using Cytoscape (Smoot *et al.*, 2011).

### *Tomography data treatment and hydrogen sulfide measurement*

An unprotected wood piece was scanned on a 39 Watt RX Solution Tomograph with a high tension of 0 to 130 kW and an anode current of 0 to 300  $\mu$ A. The wood was scanned after freezing the sample at  $-80^{\circ}\text{C}$  to kill and fix the macrofaunal taxa and drying to 12 % of relative humidity to allow an optimum visualization of the wood structures. The presence of

*Xylophaga sp.* individuals in the wood was confirmed by the presence of previously identified *Xylophaga sp.* shells (Romano *et al.*, 2014) at the bottom of the burrows.

Hydrogen sulfide was monitored inside the wood using Ag/Ag<sub>2</sub>S electrodes connected to an autonomous potentiometric sensor (NKE, SPHT), similar to the one used in a shallow water mangrove wood experiment (Laurent *et al.*, 2013; 2009) and in other deep-sea sulfidic environments (Mullineaux *et al.*, 2012). Calibration was performed in the laboratory, before and after deployment, to check the stability of the electrodes and allow semi-quantitative estimates. Because the associated glass electrode placed inside the wood failed, no pH measurement is available, preventing the quantification of sulfide concentration. Nevertheless, the signal of the Ag/Ag<sub>2</sub>S electrodes can be used to identify an increase in sulfide concentration above the detection threshold (<1 μM, 2) and to compare the sulfide ranges between the two conditions. Here, we present raw potential data to document the variation of sulfide over time, and particularly the steep decrease in potential matching the increase of sulfide measured above the detection threshold. We also calculated raw estimates of sulfide concentrations assuming a pH of 6.5 (i.e., corresponding to an equimolar concentration of H<sub>2</sub>S and HS<sup>-</sup> in seawater), reflecting a median value in the pH range measured in immersed wood logs with borers, following transfer to the aquarium.

## Results

### *Degradation of wood samples*

All wood samples were recovered 85 days after being immersed at a depth of 520 m. Samples protected with mesh (Figure 1a) did not have *Xylophaga sp.* holes on their surface but were covered by the remains of a white biofilm. In comparison, the wood samples without mesh had, on average, 40 % of their surface covered by *Xylophaga sp.* holes. The hole bored artificially before the experiment for insertion of the sulfide electrode was close (0.9 mm) to a *Xylophaga sp.* hole. The potential impact of *Xylophaga* inside the wood pieces was estimated on a longitudinal scan obtained using X-ray tomography (Figure 1c). The black areas show the *Xylophaga sp.* burrows, and the white circular parts at the end of the burrows are the shells of the animals. The burrows on the top of the wood were longer than the burrows at the bottom (with mean lengths of 2.6 cm and 1.2 cm, respectively). After 85 days of immersion, *ca.* 30 % of the wood volume had been bored by *Xylophaga sp.*

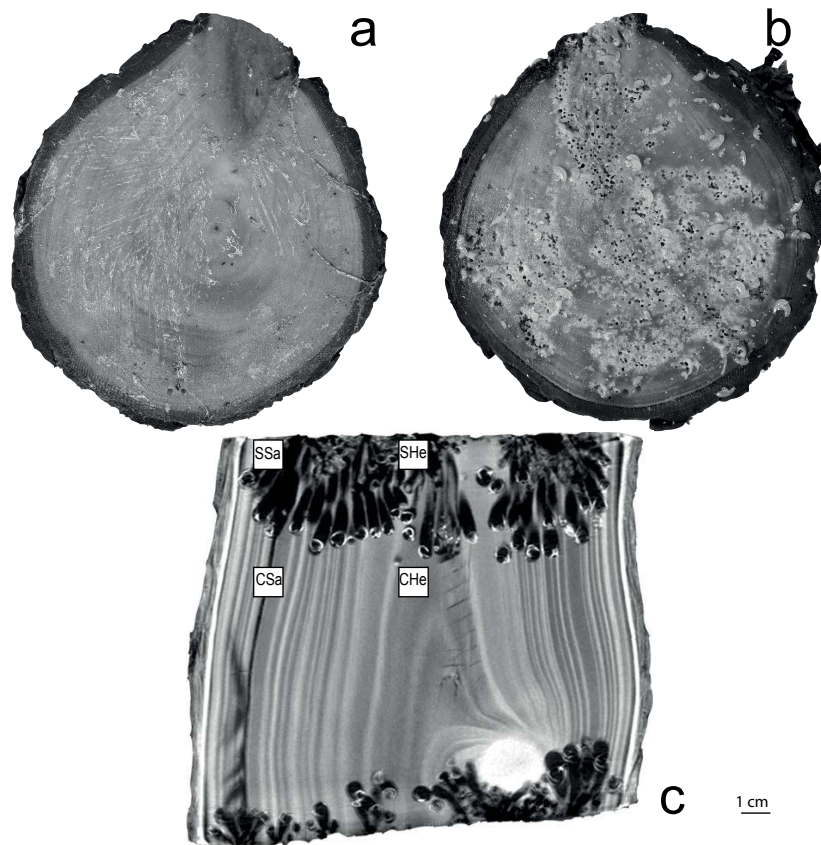


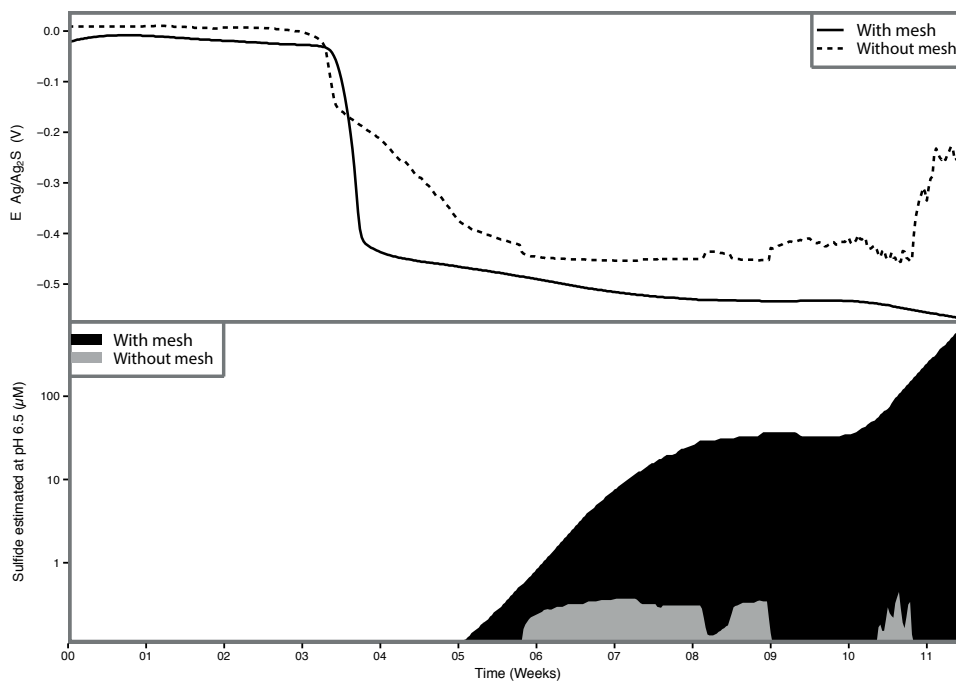
Figure 1: Picture of the intact surface of a wood sample protected by a 50 µm mesh (a) and Xylophaga sp. holes on the surface of a wood sample that was not protected (b). Woods were immersed for 85 days in a deep-sea canyon. Longitudinal tomographic cross section of an unprotected wood showing Xylophaga sp. burrows as dark regions inside the wood (c). The different sampling points are indicated as surface sapwood (SSa), surface heartwood (SHe), centre sapwood (CSa) and centre heartwood (CHe).

### *Continuous measurement of sulfide concentration*

Potential values measured for both mesh-protected and unprotected wood samples remained stable during the first 21 days of immersion, indicative of the absence of sulfide. After this plateau, a steep potential decrease was measured (Figure 2a), indicating that dissolved hydrogen sulfide was starting to accumulate in the pore water surrounding the probe, at a depth of 25 mm inside the wood (Figure 2a). After 23-24 days of incubation, a more progressive potential decrease was observed, reaching a minimum of  $-0.47$  V after 36 days in the mesh-protected wood and  $-0.42$  V after 40 days in the unprotected wood, which suggests a more progressive accumulation of sulfide over time in the unprotected wood. In the unprotected wood, the electrode potential continued to decrease to reach a minimum of  $-0.57$  V at the end of the experiment. Unprotected wood presented a fluctuating curve with potential slight re-increases after 57 and 63 days, followed by a large re-increase after 75 days, to result in a final potential of  $-0.25$  V.



Sulfide concentration estimates calculated for a pH of 6.5, which provide semiquantitative information concerning the sulfide ranges achieved in both wood samples, indicate markedly different patterns for the two sets of woods (Figure 2b). The estimated concentration exceeded a value of 0.1  $\mu\text{M}$  after 35 days in the protected wood and 5 days later in the unprotected wood. Subsequent hydrogen sulfide accumulation was, however, more pronounced when *Xylophaga sp.* were excluded. After 42 days, the estimated sulfide concentration exceeded 1  $\mu\text{M}$  in the protected wood and continued to increase up to several tens of micromoles in the following month, whereas in the presence of *Xylophaga sp.*, the estimated concentration remained below 1  $\mu\text{M}$ . Hydrogen sulfide estimates indicated that a concentration of several hundred micromolar may be reached after 85 days at the end of the deployment period. Wood colonized by *Xylophaga sp.* showed only limited and ephemeral sulfide accumulation after 42 days and between 70 and 77 days during the deployment (Figure 2b).



**Figure 2** Raw and converted data from sulfide probe continuous measurements. The upper graph shows the probes' electrical potential signal over time 25 mm inside protected (continuous line) and unprotected (dashed line) wood samples. The lower graph represents the sulfide concentrations calculated for a pH=6.5. The grey and black area frontier represents the sulfide concentration in the unprotected sample, and the upper limit of the black area represents the sulfide concentration in the protected samples.

## Community alpha and beta diversity

Alpha diversity measured as the Simpson and Shannon index showed a significant difference between the high community diversity measured in surface samples compared to the low diversity measured in wood centre samples (Supplementary Figure 3) (Shannon, Mann–Whitney test,  $W = 23$ ,  $p\text{-value} = 2.2e^{-3}$ ; Simpson,  $W = 19$ ,  $p\text{-value} = 8.7e^{-4}$ ). There were no significant differences in community diversity between protected and unprotected samples (Supplementary Figure 3).

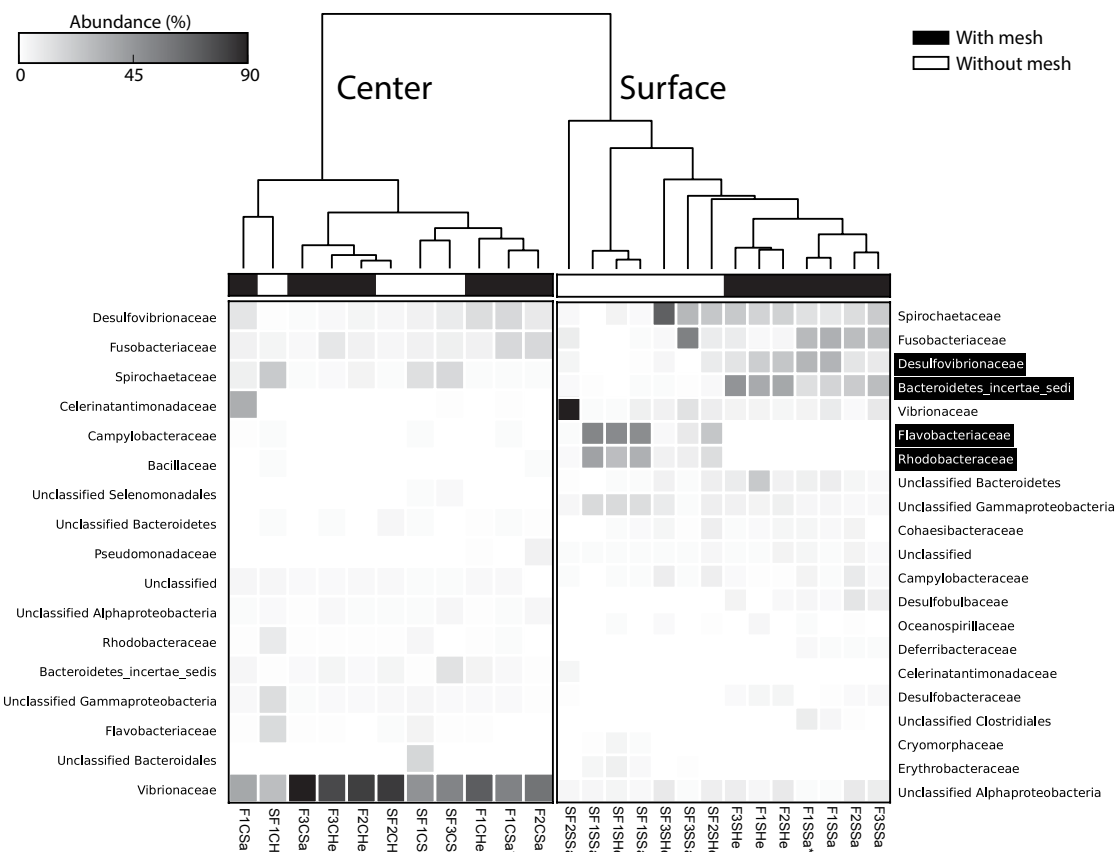


Figure 3: Bacterial community composition similarity between wood samples represented as a UPGMA cluster analysis based on the Bray–Curtis similarity index. Black cluster tips indicate protected samples, and white tips show unprotected samples. The heatmap represents the proportion of the most abundant bacterial families ( $> 1\%$  of the total number of sequences present in a cluster). Families marked in black showed a significant difference in relative sequence abundance (Welch t-test,  $\alpha = 0.05$ ) between protected and unprotected samples.

The UPGMA cluster analysis separated the bacterial communities into two large clusters (SIMPROF,  $p > 0.01$ ) (Figure 3). One group contained all the samples taken from 5 cm-deep inside the wood, whereas the other group contained samples from the wood surface. Within the surface cluster, all communities from the mesh-protected wood grouped together and were separated from the unprotected wood communities (Figure 3). Unprotected wood communities were more heterogeneous in their composition as shown by a loose clustering

and longer branches in the dendrogram (Figure 3). Within the centre communities, there was no significant difference between the protected and unprotected samples.

The heatmap represented the distribution of 21 bacterial families from the wood surface and 17 from the wood centre (Figure 3). Sequences affiliated with the *Vibrionaceae* family dominated all of the wood centre samples (60 % of the sequences  $\pm$  20 %). For surface samples, there were differences in the sequence abundance for the *Bacteroidetes incertae sedi* ( $p = 9.07e-4$ ), *Desulfovibrionaceae* ( $p = 3.16e-3$ ), *Flavobacteriaceae* ( $p = 0.024$ ) and *Rhodobacteraceae* ( $p = 0.022$ ). *Desulfovibrionaceae* and *Bacteroidetes incertae sedi* families were more abundant in the absence of *Xylophaga sp.*, whereas *Flavobacteriaceae* and *Rhodobacteraceae* were more abundant in the presence of *Xylophaga sp.* The *Bacteroidetes incertae sedi* members had low similarity to NCBI database sequences (<97 %). The *Desulfovibrionaceae* were all assigned to the *Desulfovibrio* genera. One-third of the *Flavobacteriaceae* were assigned to the *Cellulophaga* and 17 % to the *Psychroserpens* genera (>97 % similarity). One-third of the *Rhodobacteraceae* belonged to the *Litoreibacter* genera and 12 % to the *Sulfitobacter* genera (>97 % similarity) (Supplementary Figure 4).

#### *Wood surface bacterial community composition*

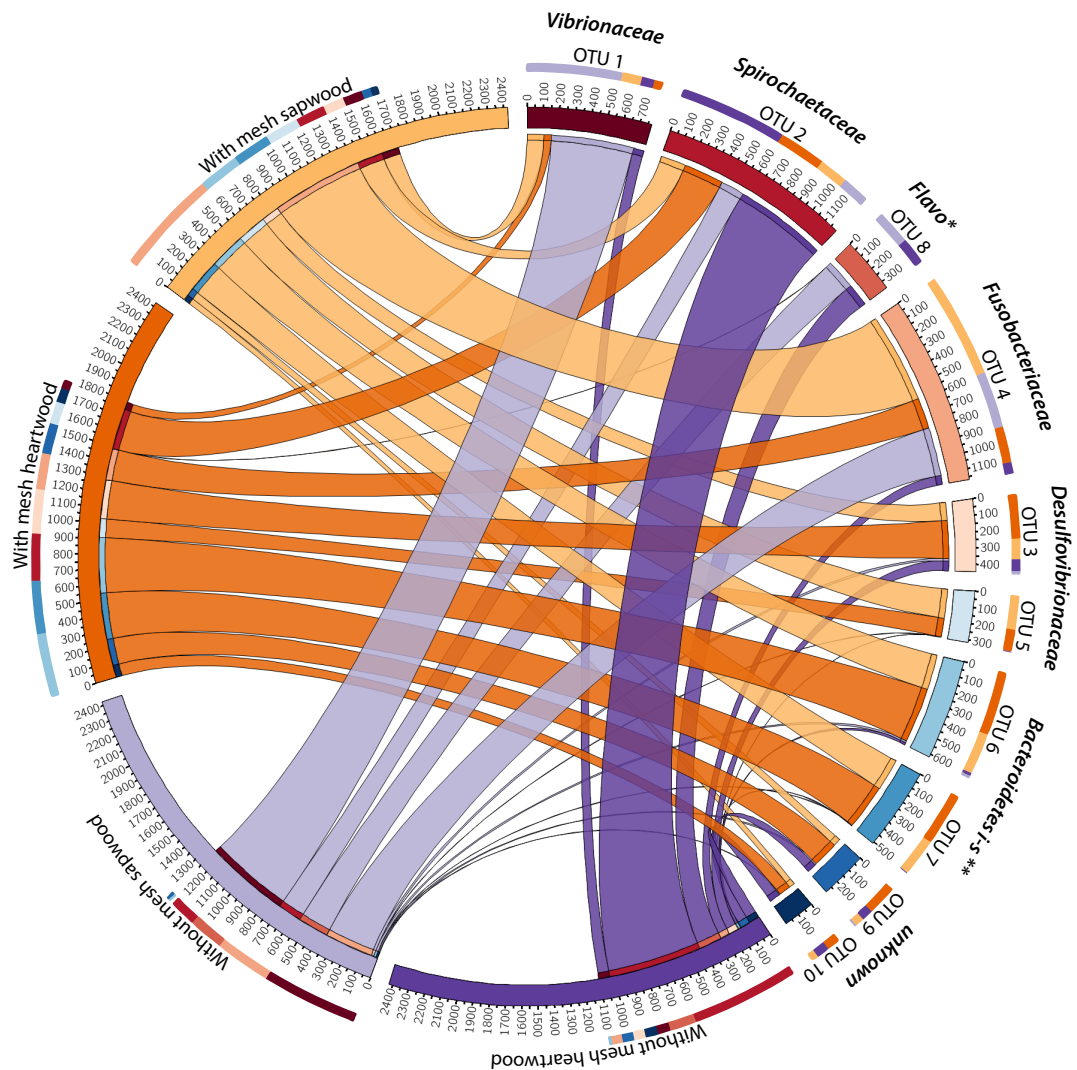
For a more precise description of the wood surface community composition, we analyzed the communities at the OTU level. A circular diagram shows the relationship between the ten most abundant OTUs and the different wood surface samples (Figure 4). The abundant OTUs (>1 % of the sequences) represented 50 % of the sequences in the wood without protection whereas they represented 70 % of the sequences in the protected wood (Figure 4).

OTU 1 and 8 sequences were found mostly in the surface sapwood samples without mesh. OTU 1 was identified as belonging to the *Vibrionaceae* family but was distantly related to any known bacterium sequences (94 %). OTU 8 was related to an uncultured *Cellulophaga* clone (97 % sequence similarity) found in northwest Spanish coastal sediments.

OTU 3 and 5 sequences were found mostly in wood surface that had been protected by the mesh. Both OTU sequences belonged to the *Desulfovibrio* genera (95 and 99 % similarity, respectively). This genus is known to contain bacteria that are able to produce sulfide from sulfate. OTU 3 was closely related to a well-characterized *Desulfovibrio* that was isolated from deep-sea wood fall (Khelaifia *et al.*, 2011). OTU 6, 7 and 8 were also typical for mesh samples and belonged to the *Bacteroidetes* family, which is linked with high molecular

weight polymer degradation rather than monomers (Fernández-Gómez *et al.*, 2013; Cottrell and Kirchman, 2000).

OTU 2 and 4 were found in both protected and unprotected woods, OTU 2 was found more frequently in protected heartwood, and OTU 4 was found more frequently in sapwood with mesh. OTUs 2 and 4 presented low similarity to *Spirochaetaceae* (93 %) and *Fusobacteriaceae* sequences (92 %), respectively.



Flavo\*: *Flavobacteriaceae*  
 Bacteroidetes i-s \*\*: *Bacteroidetes\_incertae\_sedis*

Figure 4: Circular diagram showing the sequence proportion of the ten most abundant OTUs in the different wood surface samples. The ribbon width represents the number of sequences from an OTU that belong to a sample pool. Purple ribbons indicate an unprotected sample category whereas orange ribbons show protected samples categories. Darker ribbons connect OTUs to heartwood samples whereas lighter ribbons connect OTUs to sapwood samples. The bars below the OTU names indicate the contribution of each sample pool to these OTUs.

### *Functional genes in the wood centre*

The wood centre samples were dominated by 16S rRNA sequences belonging to the *Vibrionaceae* family (Figure 3). To explore the potential metabolism of the wood centre bacteria, we constructed a metagenome from the sample F3CSa (Figure 3). This sample was chosen because it contained 98 % of *Vibrionaceae* 16S rRNA gene sequences grouped into a single OTU, which had a low similarity with database sequences (93 %). MiSeq Illumina sequencing produced 15,743,663 sequences (3,458,774,336 bp). A total of 83 % passed quality checking, 0.5 % were assigned to ribosomal RNA genes and 70 % contained ORFs. Using these ORFs, 321,058 functions were predicted based on the KEGG ontology database.

The metagenome and amplicon sequencing taxonomy (Supplemental Figure 3) showed similar patterns with a strong dominance of the *Gammaproteobacteria*, which contains the family *Vibrionaceae*. Based on the M5NR assigned taxonomy, we selected 179,277 *Vibrionaceae* sequences from the metagenome. Genes were assigned to pathways grouped hierarchically into levels and functions, and these hierarchical data were plotted as a network (Figure 5). Genes were distributed across five KEGG categories: metabolism (90,812 sequences), environmental information processing (35,136 sequences), genetic information processing (32,117 sequences), cellular processes (16,317 sequences) and human disease (4,040 sequences).

*Metabolism.* Among carbohydrate metabolisms, we detected genes coding for enzymes involved in the common glycolysis pathway that converts glucose to pyruvate. Genes that code for fermentative enzymes were also assigned to this pathway (Supplementary Table 1). We found 451 copies of the alcohol dehydrogenase gene (*adhE*), 61 copies of acetyl-CoA synthase (*acs*) and 10 copies of the D-lactate dehydrogenase gene (*ldhA*). We detected the need for sucrose assimilation and hydrolysis in all of the genes (Supplementary Table 2).

Regarding sulfur metabolism, we only detected genes involved in assimilatory sulfur reduction (Supplementary Table 3), and no genes coding for dissimilatory sulfur reduction were detected among the wood centre *Vibrionaceae* sequences.

*Cellular processes.* We detected all of the genes that code for flagellar assembly (Supplementary Table 4). Furthermore, we detected genes involved in the type III secretion system 1 (TTSS1) flagellar export apparatus within the pathway ko02040. However, no genes coding for the TTSS1 core apparatus were detected. Genes for chemotaxis (Supplementary Table 5) were mostly involved in flagellum regulation. A total of 15 different genes were detected including the *mcp* gene, which codes for the methyl accepting chemotaxis protein.

*Environmental information processing.* We found the *Vibrionaceae* OTU to be abundant, with 40 genes involved in two-component systems (Supplementary Table 6). The *arcA* and *arcB* genes indicate a cell's potential to detect the absence of oxygen and then switch metabolism from aerobic to anaerobic respiration (Alvarez and Georgellis, 2010). In this metagenome, the two-component system pathway included *Lux* genes known for their implication in the Lux operon regulation (Engebrecht *et al.*, 1983). However, the *LuxC*, *D*, *A*, *B*, and *E* genes were not detected.

Bacterial secretion system genes (Supplementary Table 7) code for secretion system II and VI. The bacterial system could be mediated here through the twin arginine translocation pathway (144 sequences) and the *sec*-translocase system (2291 sequences), which are specialized in the translocation of folded and unfolded proteins, respectively, through the secretion system II (Natale *et al.*, 2008).

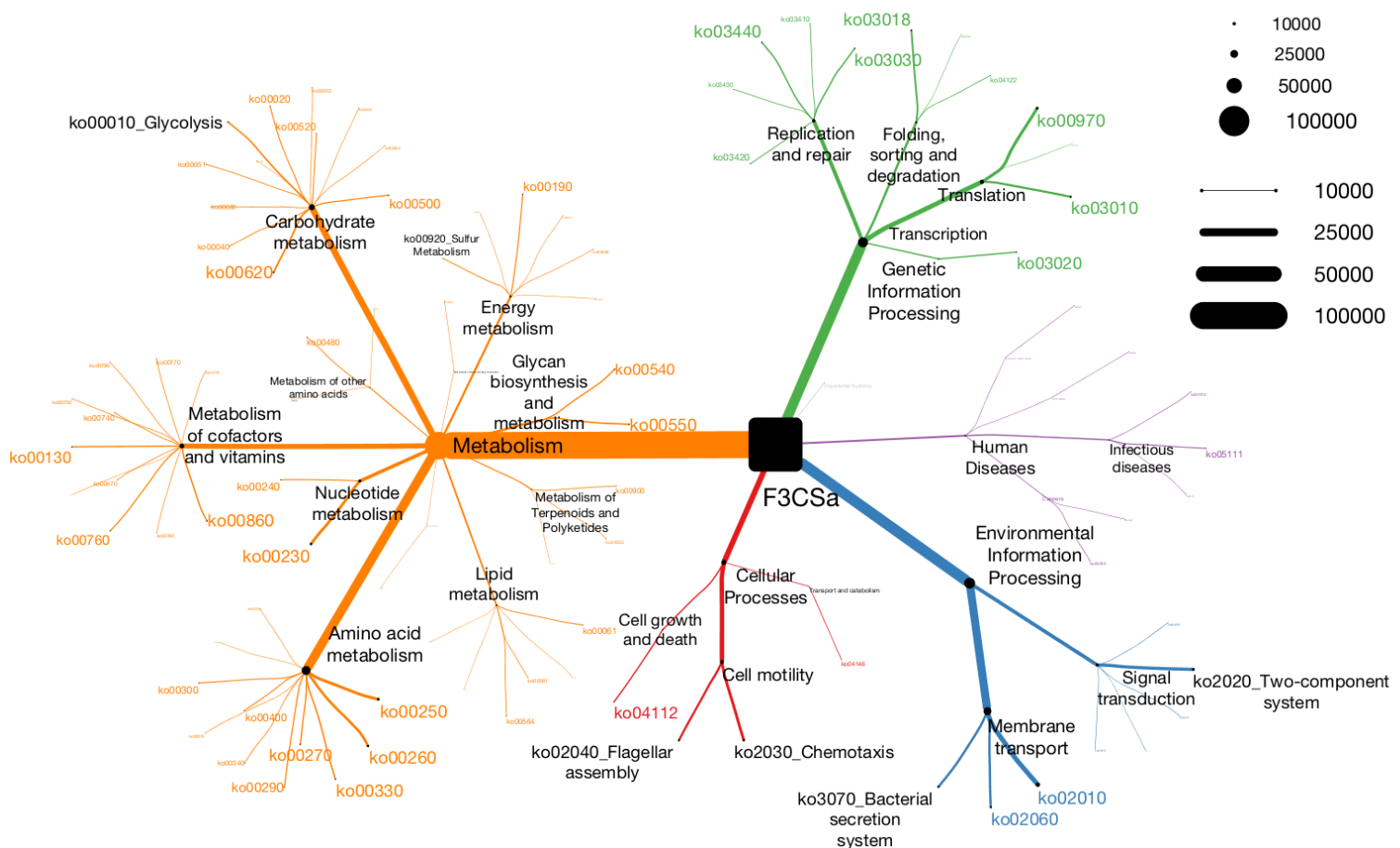


Figure 5: Hierarchical network showing the KEGG hierarchical classification of the most abundant bacterial pathways found in the wood centre metagenome (F3CSa). Names (ko) in black indicate pathways that are detailed in the Results section. The list of genes belonging to these pathways is presented in Supplementary Tables 1, 3 to 7. Edge and node widths are proportional to the number of sequences belonging to each sub-category.

## Discussion

Our 520 m depth experiment revealed the ability of sunken wood microorganisms to produce hydrogen sulfide in the absence of *Xylophaga sp.* under natural deep-sea conditions. We thus demonstrate that microorganisms alone could produce a significant amount of reduced compounds essential for the chemolithotrophic growth of symbiotic or free-living microorganisms. This short-term exclusion experiment in a submarine canyon validates that microbial organic substrate degradation in seawater can lead to a high level of sulfide production in the absence of wood borers, consistent with the result of earlier experiments based on aquaria incubations with the same range of hydrogen sulfide concentrations (millimolar) (Yücel *et al.*, 2013). This finding contradicts the hypothesis that wood-boring bivalve colonization is the primary driver of the creation of anoxic niches rich in cellulolytic material available for bacteria to produce sulfide (Bienhold *et al.*, 2013; Voight, 2015; Turner, 1973). In the early stage of immersion, bacteria appear to be the primary colonizers that may favour the subsequent settlement of macrofaunal species such as the wood-boring bivalves or chemosynthetic mussels.

The impact of *Xylophaga sp.* on the wood matrix was nevertheless very important. The bivalves had degraded *ca.* 30 % of the wood after only 2 months of immersion, and no such degradation of the wood matrix was observed in the protected wood. The disruptive effect of *Xylophaga* has long been observed in the deep sea. The first observations made from the *Alvin* in 1972 described sunken wood after 104 days of immersion as “weakened and falling apart while touched by the submarine arm” (Turner, 1973). Experiments showed more recently that *Xylophaga sp.* are fast growing deep-sea organisms (Tyler *et al.*, 2007). *Xylophaga sp.* also grew quickly in our Western Mediterranean experiment, but, more interestingly, they significantly impacted the wood microorganism communities.

Surface microbial communities from *Xylophaga sp.*-colonized woods were significantly different from communities of protected woods. In addition, sulfide accumulation in the wood was lower in the presence of *Xylophaga sp.*, suggesting that bivalve colonization impacted microbial activity. *Xylophaga sp.* containing woods had more bacteria belonging to the *Flavobacteriaceae* and *Rhodobacteriaceae* families. Because members of the *Rhodobacteriaceae* family are aerobic, we hypothesize that the *Xylophaga sp.* favour the introduction of oxygen into the wood. Seawater is filling the burrows with the *Xylophaga sp.* siphon movements (Voight, 2015). Burrows may also introduce other seawater electron acceptors (sulfate, nitrate), creating new redox gradients that structure the microbial

communities. Within the *Rhodobacteriaceae*, the *Litoreibacter* (OTU17) and *Sulfitobacter* (OTU32) genera are both strictly aerobic (Romanenko *et al.*, 2011; Sorokin, 1995), and *Sulfitobacter* is a potential chemolithotroph (5 *sulfitobacter* genera out of the 9 known to date) that is able to oxidize sulfide (Sorokin, 1995). The hydrogen sulfide consumption by OTU32 could explain the lower sulfide accumulations in *Xylophaga*-colonized woods. However, the lower concentrations may also be due to lower production rate, as only a few potential sulfate reducers were detected, and other electron acceptors are expected to be more abundant in this environment. The *Flavobacteriaceae* family also contains aerobic bacteria (Bernardet and Nakagawa, 2006) that are capable of degrading a large variety of macromolecules (Reichenbach, 2006), from cellulose to fucoidan (Sakai *et al.*, 2002). Among them, members of the *Cellulophaga* genera (OTU 8) may be strictly aerobic and able to use disaccharides such as cellulobiose but not cellulose (Pati *et al.*, 2011).

In the absence of *Xylophaga sp.*, the wood remained anoxic as suggested by the dominance of sequences from anaerobic or facultative anaerobic bacteria from the *Bacteroidetes incertae sedi* and *Desulfovibrionaceae* families. This corroborates previous aquaria experiments that showed wood became suboxic after only 2 days in seawater (Yücel *et al.*, 2013). These earlier aquaria measurements also indicate that the possible absence of oxygen on the surface of the mesh-protected *in situ* woods was not due to a mesh-effect. Anoxia certainly favours fermentative pathways inside the wood as indicated by the presence of OTUs that belong to the *Marinifilum* genus, which has been previously found in sunken wood (Fagervold *et al.*, 2012) and comprises facultative anaerobes that ferment sugars (Na *et al.*, 2009). Fermentation products such as acetate could then be used as a carbon source by organisms from the *Desulfovibrionaceae* family, which are able to reduce sulfate to hydrogen sulfide. The *Desulfovibrionaceae* OTU3 was very close to *Desulfovibrio piezophilus* previously found on sunken wood (Kalenitchenko *et al.*, 2015; Khelaiifia *et al.*, 2011).

There were also significant differences in composition and diversity between communities from the surface and the centre of the wood, which is in accordance with results obtained earlier in an aquaria experiment (Kalenitchenko *et al.*, 2015). In both protected and unprotected woods, the wood centre was not impacted by the *Xylophaga sp.* burrows. All centre samples were dominated by one single OTU identified as belonging to the *Vibrionaceae* family but distantly related to database sequences (94 % similarity). *Vibrionaceae* have been detected in sunken wood after only 1 day of immersion (Bienhold *et al.*, 2013) and are massively present in all of our samples collected after 2 months. We thus



believe that they are one of the first wood colonizers, and have constructed a metagenome to identify possible wood centre *Vibrionaceae* metabolic pathways.

Our data showed the presence of genes coding for alcohol dehydrogenase, which is a key enzyme in the fermentation of pyruvate to ethanol in the glycolysis pathway. We were, however, not able to detect any genes related to the degradation of polysaccharides such as cellulose or cellulobiose, which may indicate that our wood *Vibrionaceae* is only able to degrade monosaccharides such as glucose, or disaccharides such as sucrose as a carbon source. This corroborates the idea that cellulose degradation is mostly conducted by symbiotic bacteria hosted by *Xylophaga sp.* (Distel and Roberts, 1997). Regarding sulfur metabolism, the wood centre *Vibrionaceae* OTU had only assimilatory (and not dissimilatory) sulfate reduction genes, and thus probably did not produce the high sulfide concentrations that we measured *in situ* close to the wood surface.

The presence of flagella-related genes and chemotaxis genes in the wood centre *Vibrionaceae* metagenome indicates that cells had the potential to move along chemical gradients sensed through the protein coded by the *mcp* gene (Miller *et al.*, 2009). Chemotaxis and motility are important features of plant-colonizing bacteria, which use their flagella to move toward their optimum growth niche and to spread within the plant tissue (Hardoim *et al.*, 2008). We also detected *Lux* genes that could be involved in *HapR* regulation (Zhu *et al.*, 2002). One of the *HapR* regulation features is to control biofilm formation (Waters *et al.*, 2008). Slower biofilm production may reduce physical barriers and allow for better circulation of bacteria within the wood. This was shown in the well-described *Vibrio cholera* strains for which an increase in cell density also negatively regulates vibrio virulence toward host cells. We also detected genes coding for the secretion system VI, an important feature of inter-bacterial interactions that can be used for killing invader bacteria or phage infected bacteria and for intra community non antagonist bacterial signalling (Russell *et al.*, 2014). However, we did not detect any toxin coding genes, which indicates that our wood centre *Vibrionaceae* probably does not produce any known toxins.

## **Conclusion**

We demonstrated the capacity of the sunken wood microbial community to produce hydrogen sulfide, which is the base of the wood chemosynthetic ecosystem, in the absence of *Xylophaga sp.* in a natural deep-sea environment. The accumulation of hydrogen sulfide in the wood was negatively correlated with the presence of *Xylophaga sp.* The bivalves' burrows

may allow seawater circulation, which creates aerobic niches suitable for sulfide oxidizing bacteria. Deeper in the wood, away from the area affected by *Xylophaga sp.*, our metagenome data draw the picture of a dominant *Vibrionaceae* strain capable of defending its niche against other bacteria and fermenting the sucrose naturally present in the wood matrices. This vibrio could thus produce the substrate needed by the sulfate-reducing bacteria situated further up in the wood in contact with sulfate containing seawater.

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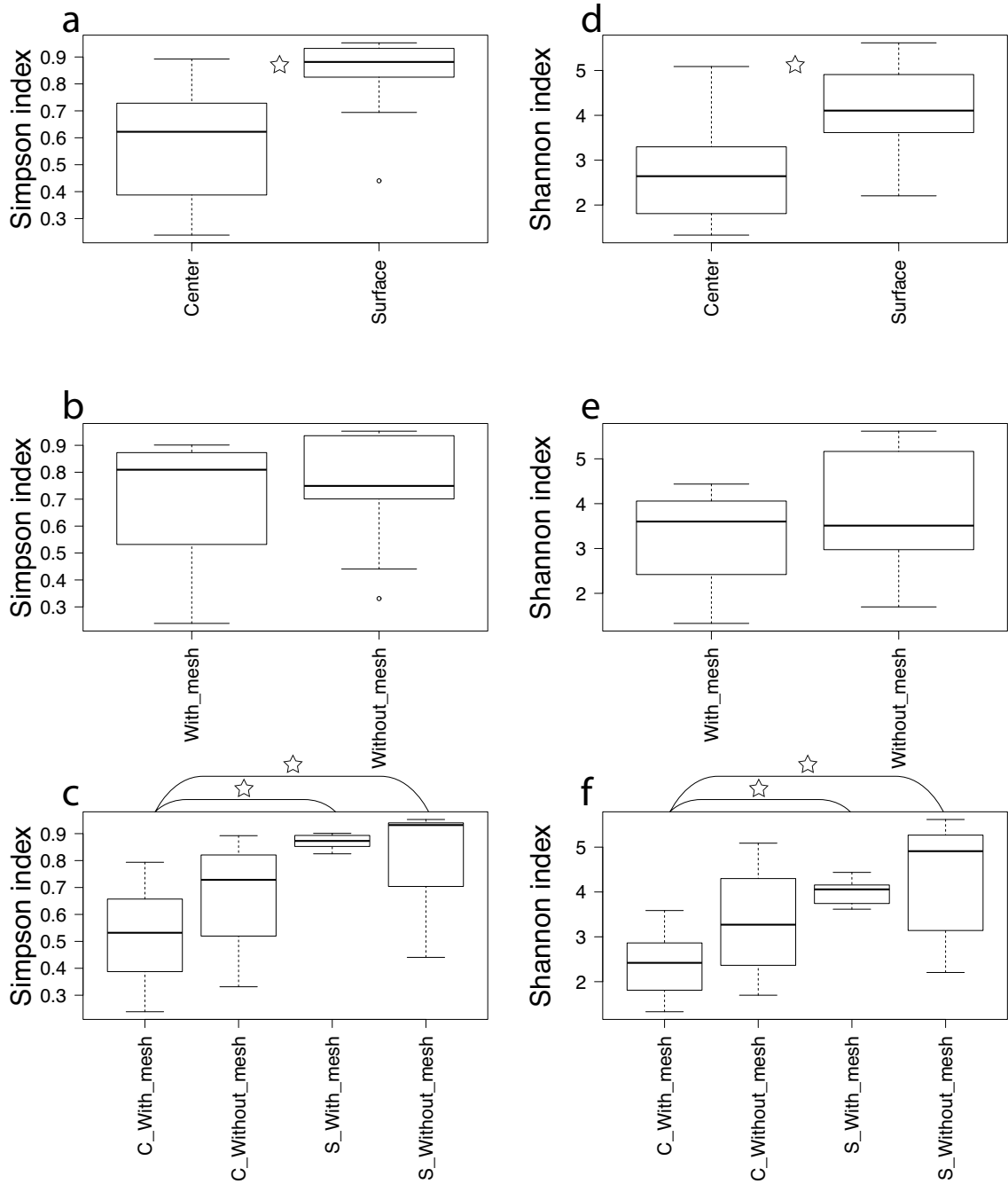
## Supplemental material



**Supplementary figure 1** An unprotected cage (left) and a mesh protected cage (right) equipped with hydrogen sulphide sensors. The picture was taken by the R.O.V. Super Achille at 520m depth in the Lacaze-Duthiers submarine canyon at the beginning of the experiment.

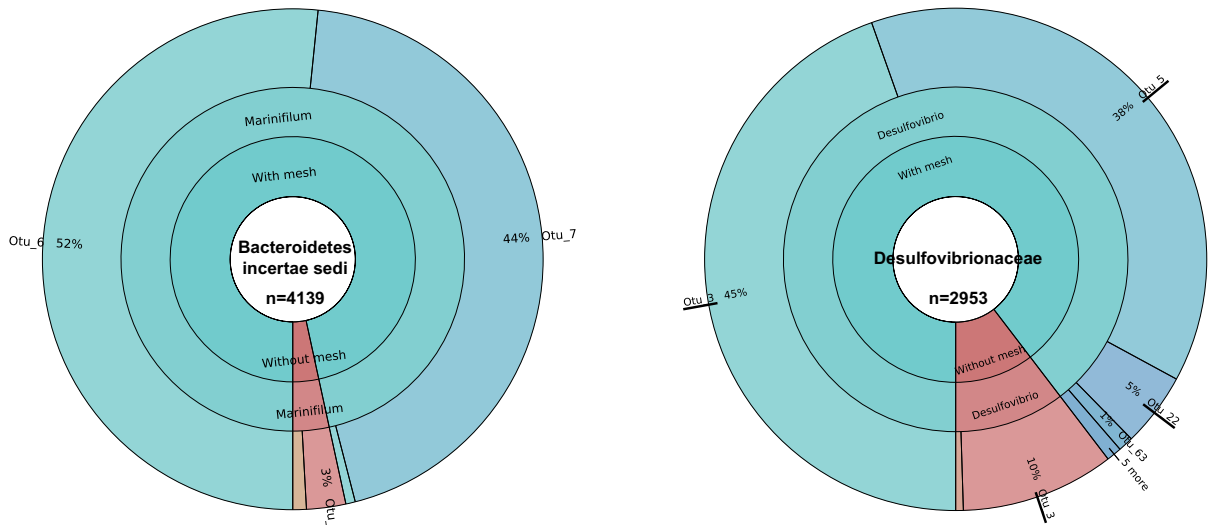


**Supplementary figure 2** An unprotected cage (left) and a mesh protected cage (right) equipped with hydrogen sulphide sensors after 85 days of immersion in the Lacaze-Duthiers submarine canyon.

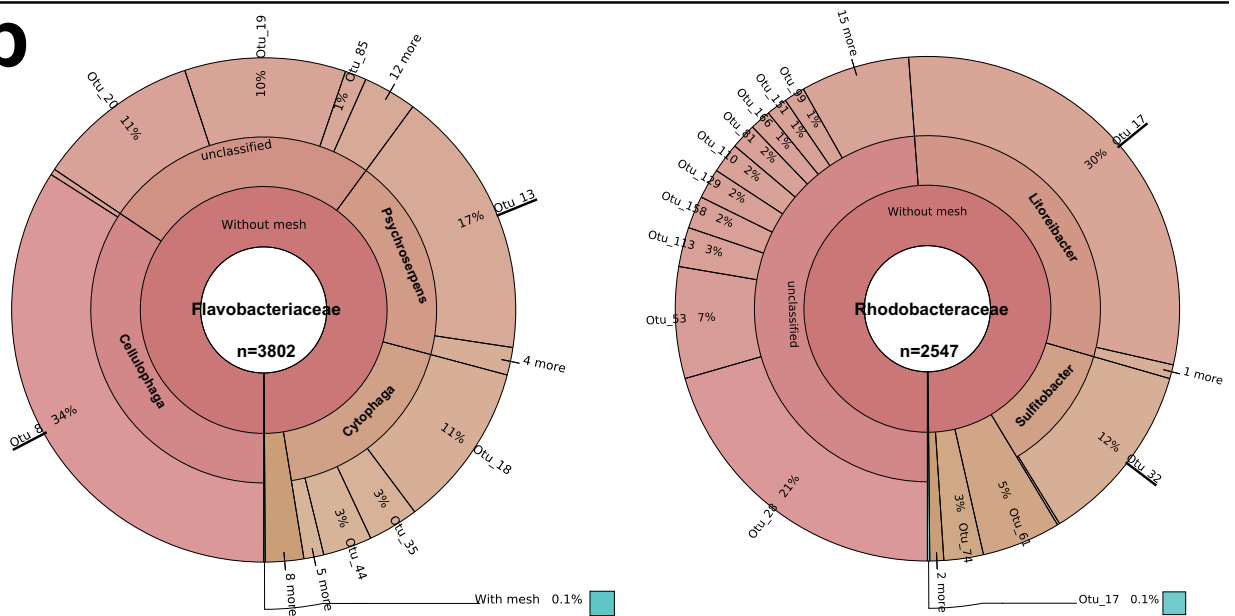


**Supplementary figure 3** Simpson (a,b,c) and Shannon (d,e,f) diversity index for different groups of samples. Groups are center, surface, with mesh, without mesh, center with mesh (C\_with\_mesh), center without mesh (C\_without\_mesh), surface with mesh (S\_with\_mesh) and surface without mesh (S\_without\_mesh). Significant differences between groups are indicated by a star.

**a**



**b**



**Supplementary figure 4** Taxonomic composition of the four families that showed a significant difference in sequence abundance between mesh protected (blue) and unprotected (red) wood surface samples. The upper graphs (a) show families that were more abundant in mesh protected samples and the lower graphs (b) show families that were more abundant in the unprotected samples.



**Supplementary table 1** Glycolysis or gluconeogenesis genes sequence abundance in the putative *Vibrionaceae* OTU from the F3CSa wood center metagenome. Sub-pathways were assigned according to the KEGG gene mapper.

<b>Glycolysis / Gluconeogenesis: ko00010</b>				
<b>Function</b>	<b>Abundance</b>	<b>e-value</b>	<b>avg % ident</b>	<b>Sub-pathway</b>
<i>pyk_K00873</i>	663	-43.31	82.14	
<i>aceE_K00163</i>	602	-56.3	90.7	
<i>lpd_K00382</i>	494	-51.69	91.67	
<i>pckA_K01610</i>	361	-54.7	87.14	
<i>gapA_K00134</i>	344	-43.01	86.38	
<i>fbp_K03841</i>	308	-55.2	89.99	
<i>glpX_K02446</i>	304	-44.41	87.15	
<i>tpiA_K01803</i>	280	-44.02	81.26	
<i>pgm_K01835</i>	272	-52.15	84.39	
<i>aceF, pdhC_K00627</i>	264	-38.02	88.89	Glycolysis
<i>pfkA_K00850</i>	257	-47.2	89.21	
<i>pgk_K00927</i>	244	-47.08	90.82	
<i>pgi_K01810</i>	234	-51.28	80.58	
<i>fbaA_K01624</i>	209	-56.05	90.45	
<i>galM_K01785</i>	161	-15.33	69.1	
<i>eno_K01689</i>	133	-53.98	92.51	
<i>gpmA_K01834</i>	92	-44.08	78.9	
<i>celF_K01222</i>	28	-42.37	81.11	
<i>bigA_K01223</i>	1	-28.64	80.18	
<i>adhE_K04072</i>	451	-47.43	82.61	Fermentation to Ethanol
<i>acs_K01895</i>	61	-41.06	77.29	Fermentation to Acetate
<i>ldhA_K03778</i>	10	-37.54	73.72	Fermentation to Lactate

**Supplementary table 2** Sucrose metabolism genes sequence abundance in the in the putative *Vibrionaceae* OTU , from the F3CSa sample metagenome. Sub-pathways were assigned according to KEGG gene mapper.

**Sucrose metabolism**

Function	Abundance	e-value	avg % ident	Sub-pathway
<i>scrA_K02809</i>	137	-47.81	82.67	Assimilation
<i>sacA_K01193</i>	35	-19.04	67.34	Hydrolysis
<i>scrK_K00847</i>	154	-28.48	69.88	
<i>pgi_K01810</i>	234	-51.28	80.58	fructose to glucose
<i>pgm_K01835</i>	272	-52.15	84.39	

**Supplementary table 3** Sulfur metabolism genes sequence abundance in the putative *Vibrionaceae* OTU from the F3CSa sample metagenome. Sub-pathways were assigned according to KEGG gene mapper.

**Sulfur metabolism: ko00920**

Function	Abundance	e-value	avg % ident	Sub-pathway
<i>cysJ_K00380</i>	417	-40.4	79.68	
<i>cysN_K00956</i>	321	-42.83	78.86	
<i>cysI_K00381</i>	256	-46.6	84.74	
<i>cysD_K00957</i>	207	-58.8	90.54	Assimilatory sulfate reduction
<i>cysH_K00390</i>	144	-47.12	79.23	
<i>cysC_K00860</i>	134	-37.76	79.02	

**Supplementary table 4** Flagellar assembly genes sequence abundance in the putative Vibrionaceae OTU from the F3CSa sample metagenome. Sub-pathways were assigned according to KEGG gene mapper.

<b>Flagellar assembly: ko02040</b>				
<b>Function</b>	<b>Abundance</b>	<b>e-value</b>	<b>avg % ident</b>	<b>Sub-pathway</b>
<i>fliD_K02407</i>	435	-32.6	73.72	Filament cap
<i>fliC_K02406</i>	980	-41.74	82.3	Filament
<i>flgK_K02396</i>	289	-42.11	78.18	Hook-Filament junction
<i>flgL_K02397</i>	257	-44.33	77.33	
<i>flgE_K02390</i>	231	-50	84.12	Hook
<i>flgD_K02389</i>	183	-46.01	83.39	
<i>flgG_K02392</i>	158	-42.71	89.81	Distal rod
<i>flgH_K02393</i>	158	-32.51	78.8	L ring
<i>flgF_K02391</i>	154	-36.76	81.44	Proximal rod
<i>fliE_K02408</i>	101	-31.02	74.18	
<i>flgB_K02387</i>	119	-41	80.7	
<i>flgC_K02388</i>	78	-43.38	88.54	P ring
<i>flgL_K02394</i>	263	-43.53	84.62	
<i>fliF_K02409</i>	275	-47.4	81.42	MS ring
<i>fliG_K02410</i>	247	-47.25	89.33	C ring
<i>fliM_K02416</i>	237	-52.85	89.5	
<i>fliN_K02417</i>	170	-40.94	94.66	
<i>flhA_K02400</i>	346	-51.33	91.68	Motor/switch
<i>flhB_K02401</i>	162	-39.27	79.38	
<i>fliI_K02412</i>	209	-47.92	89.76	Type III secretion system
<i>fliR_K02421</i>	162	-46.58	78.82	
<i>fliH_K02411</i>	140	-26.26	71.67	
<i>fliP_K02419</i>	121	-38.39	85.53	
<i>fliQ_K02420</i>	60	-32.91	80.58	
<i>fliO_K02418</i>	8	-8.33	68.49	Early gene product
<i>flgA_K02386</i>	162	-27.55	64.04	
<i>flgN_K02399</i>	117	-21.09	65.96	
<i>fliS_K02422</i>	79	-32.46	87.54	Late gene product
<i>fliJ_K02413</i>	36	-29.14	66.58	
<i>flgM_K02398</i>	89	-18.99	75.01	

**Supplementary table 5** Chemotaxis genes sequence abundance in the putative Vibrioceae OTU from the F3CSa sample metagenome. Sub-pathways were assigned according to KEGG gene mapper.

<b>Chemotaxis: ko02030</b>				
<b>Function</b>	<b>Abundance</b>	<b>e-value</b>	<b>avg % ident</b>	<b>Sub-pathway</b>
<i>mcp_K03406</i>	3303	-22.6	69.07	
<i>cheV_K03415</i>	933	-46.33	85.26	
<i>cheA_K03407</i>	450	-40.69	88.69	
<i>motA_K02556</i>	263	-49.39	90.29	
<i>cheB_K03412</i>	263	-38.56	87.39	
<i>fliG_K02410</i>	247	-47.25	89.33	
<i>fliM_K02416</i>	237	-52.85	89.5	
<i>cheW_K03408</i>	225	-43.46	86.74	Flagellum regulation
<i>cheR_K00575</i>	193	-43.75	85.42	
<i>motB_K02557</i>	192	-37.74	83.28	
<i>fliN_K02417</i>	170	-40.94	94.66	
<i>cheC_K03410</i>	161	-25.73	86.93	
<i>cheX_K03409</i>	135	-45.52	93.46	
<i>cheY_K03413</i>	86	-36.14	95.59	
<i>cheZ_K03414</i>	31	-27.58	80.4	
<i>rbsB_K10439</i>	150	-46.85	85.11	D-Ribose chemosensor

**Supplementary table 6** Two-component system genes sequence abundance in the putative Vibrionaceae OTU from the F3CSa sample metagenome. Sub-pathways were assigned according to KEGG gene mapper.

<b>Two-component system: ko02020</b>				
<b>Function</b>	<b>Abundance</b>	<b>e-value</b>	<b>avg % ident</b>	<b>Sub-pathway</b>
<i>phoB_K07657</i>	135	-50.1	93.72	Phosphate assimilation
<i>envZ_K07638</i>	291	-31.24	77.54	Osmotic regulation
<i>ompR_K07659</i>	157	-44.63	90.39	
<i>HtrA_K04771</i>	226	-49.63	79.73	
<i>cpxA_K07640</i>	212	-36.61	72.65	Misfolded protein degradation
<i>cpxR_K07662</i>	167	-25.8	84.17	
<i>glnD_K00990</i>	609	-40.07	78.71	Nitrogen Assimilation
<i>cusB_K07798</i>	2	-18.1	69.58	Copper efflux
<i>fliA_K02405</i>	147	-48.11	81.48	Flagella regulon
<i>arcB_K07648</i>	350	-37.91	81.32	Anaerobic respiration
<i>arcA_K07773</i>	228	-53.92	91.25	
<i>citG_K05966</i>	34	-18.39	63.62	Citrate fermentation
<i>citF_K01643</i>	14	-42.44	73.22	
<i>dcuB_K07792</i>	173	-43.65	83.23	Anaerobic fumarate resp.
<i>MaeA_K00027</i>	270	-56.03	87.34	Malate utilization
<i>frdA_K00244</i>	648	-52.61	88.85	Fumarate reductase
<i>frdB_K00245</i>	329	-49.11	88.12	
<i>frdD_K00247</i>	166	-36.1	79.46	
<i>frdC_K00246</i>	6	-35.33	78.06	
<i>barA_K07678</i>	417	-34.63	81.31	Carbon storage regulation
<i>uvrY_K07689</i>	119	-42.67	93.91	
<i>csrA_K03563</i>	86	-20.96	95.41	
<i>glnG_K07712</i>	359	-42.57	89.22	Nitrogen Assimilation
<i>glnA_K01915</i>	285	-50.63	86.36	
<i>glnB_K04751</i>	103	-32.54	90.63	
<i>glnL_K07708</i>	91	-46.74	87.88	
<i>atoB_K00626</i>	125	-40.02	72.68	Short chain fatty acid metabolism
<i>rpoN_K03092</i>	251	-36.06	86.37	Pilus synthesis
<i>pilA_K02650</i>	49	-9.86	65.04	
<i>luxO_K10912</i>	294	-39.41	86.42	Lux family
<i>luxP_K10910</i>	217	-30.12	69.95	
<i>luxQ_K10909</i>	207	-14.93	65.85	
<i>luxU_K10911</i>	98	-14.35	64.82	
<i>fliR_K10941</i>	301	-30.43	77.7	Class III flagellar system

<i>fliC_K10943</i>	258	-41.97	85.41	
<i>fliB_K10942</i>	191	-45.8	80.95	
<i>clp_K10914</i>	148	-50.42	98.92	Biofilm formation
<i>petB_K00412</i>	70	-36.75	66.9	OXPHOS
<i>cydA_K00425</i>	380	-56.18	87.21	
<i>cydB_K00426</i>	139	-36.75	78.33	Aerobic respiration

**Supplementary table 7** Bacterial secretion system genes sequence abundance in the putative Vibrioceae OTU from the F3CSa sample metagenome. Sub-pathways were assigned according to KEGG gene mapper.

**Bacterial secretion system: ko3070**

Function	Abundance	e-value	avg % ident	Sub-pathway
<i>hcp_K11903</i>	670	-49.31	86.16	
<i>vgrG_K11904</i>	433	-31.09	65.13	
<i>impL, vasK, icmF_K11891</i>	396	-36.11	75.13	Type VI
<i>clpV_K11907</i>	195	-49.4	83.57	
<i>dotU_K11892</i>	99	-29.68	75.98	
<i>lip_K11906</i>	93	-36.26	75.76	
<i>gspD_K02453</i>	594	-42.02	77.76	
<i>gspK_K02460</i>	234	-15.32	62.82	Type II
<i>gspG_K02456</i>	152	-38.77	80.87	
<i>gspF_K02455</i>	146	-25.89	69.11	
<i>gspE_K02454</i>	137	-37.18	73.35	
<i>gspL_K02461</i>	82	-6.97	69.31	
<i>gspI_K02458</i>	80	-14.52	77.81	
<i>gspC_K02452</i>	40	-8.31	60.94	
<i>tatC_K03118</i>	142	-33.6	74.27	Tat
<i>tatB_K03117</i>	1	-8.75	86.03	
<i>tatA_K03116</i>	1	-8.55	67.18	
<i>secA_K03070</i>	636	-50.95	84.47	Sec-SRP
<i>secD_K03072</i>	344	-43.7	84.42	
<i>yidC, spoIIJ_K03217</i>	320	-40.36	78.93	
<i>secY_K03076</i>	260	-49.76	92.93	
<i>ftsY_K03110</i>	170	-53.52	88.78	
<i>secF_K03074</i>	169	-42.29	84.94	
<i>secB_K03071</i>	140	-51.14	89.38	
<i>secG_K03075</i>	87	-27.22	84.08	
<i>secE_K03073</i>	86	-24.57	85.61	
<i>yajC_K03210</i>	79	-37.06	82.15	
<i>ffh_K03106</i>	237	-48.32	91.38	-

# **Article III: Ecological succession leads to chemosynthesis in mats colonizing wood in sea water**

Dimitri Kalenitchenko<sup>1</sup>, Marlène Dupraz<sup>1</sup>, Erwan Peru<sup>1</sup>, Carole Petetin<sup>2</sup>, Nyree J. West<sup>2</sup>, Pierre E. Galand<sup>1</sup>

<sup>1</sup> Sorbonne Universités, UPMC Université Paris 06, CNRS, Laboratoire d'Ecogéochimie des Environnements Benthiques (LECOB), Observatoire Océanologique, Banyuls sur Mer, France

<sup>2</sup> Sorbonne Universités, UPMC Univ Paris 06, CNRS, Observatoire Océanologique de Banyuls (OOB), F-66650 Banyuls/mer, France

## **Abstract**

Chemosynthetic mats involved in cycling sulfur compounds are often found in hydrothermal vents, cold seeps and whale falls. However, there are only few records of wood fall mats, even though the presence of hydrogen sulfide at the wood surface should create a perfect niche for sulfide oxidizing bacteria. Here we report the growth of microbial mats on wood incubated under conditions that simulate the Mediterranean deep-sea temperature and darkness. We used amplicon and metagenomic sequencing combined with fluorescence in situ hybridization (FISH) to test whether a microbial succession occurs during mat formation and whether the wood fall mats present chemosynthetic features. We show that the wood surface was first colonized by sulfide oxidizing bacteria belonging to the *Arcobacter* genus after only 30 days of immersion. Subsequently, the number of sulfate reducers increased and the dominant *Arcobacter* phylotype changed. The ecological succession was reflected by a change in the metabolic potential of the community from chemolithoheterotrophs to potential chemolithoautotrophs. Our work provides clear evidence for the chemosynthetic nature of wood fall ecosystems and demonstrates the utility to develop experimental incubation in the laboratory to study deep-sea chemosynthetic mats.



## Introduction

Wood falls represent an important carbon load for the seabed (Blair and Aller, 2012). They form the foundation for unique deep sea ecosystems, but, intriguingly, some wood colonizing organisms do not use directly organic wood carbon. They prefer instead to consume inorganic carbon and use as an energy source, inorganic compounds such as hydrogen sulfide (Duperron *et al.*, 2008). High hydrogen sulfide concentrations have been measured on wood falls in situ (Laurent *et al.*, 2013) and in aquaria (Yücel *et al.*, 2013) with values similar to the ones measured in hydrothermal vent fluids (Le Bris *et al.*, 2006). The production of hydrogen sulfide has been related to the presence of bacteria from the *Desulfovibrio* genera, detected after only one month of wood immersion (Kalenitchenko *et al.*, 2015), which respire sulfate and use fermentation products like acetate as a carbon source. However, after several months of immersion, when all the labile organic matter present in the wood was consumed, it was hypothesized that the microbial community needs a macrofaunal partner (*Xylophaga sp.*) to convert the refractory wood carbon into labile organic matter (Voight, 2015). New fermentative niches can be created within macrofaunal feces (Fagervold *et al.*, 2014; Purchon, 1941) that could result in high hydrogen sulfide concentration after one year (Bienhold *et al.*, 2013). These environmental chemical conditions are similar to the ones found in other deep-sea sulfidic habitats where microbial mats colonize exposed surfaces. Similarly, chemosynthetic microbial mats should be expected to develop on wood surfaces.

Epixylic mats (mats that develop on the surface of the wood) are well studied in fresh water environments and consist of microbial communities dominated by fungi able to degrade lignin and cellulose (Golladay and Sinsabaugh, 1991) (Tank and Webster, 1998) (TANK and WINTERBOURN, 1995). In contrast, little is known about epixylic mats in the marine environment. Their presence has been reported only recently in studies from shallow waters or mesocosm experiments (Laurent *et al.*, 2013; Yücel *et al.*, 2013; Kalenitchenko *et al.*, 2015) but the identity and function of the mat forming bacteria are unknown. For deep sea woods, sampling is difficult because the long distances travelled when moving up through the water column washes the wood leaving no trace of the fragile biofilm structure (Samadi *et al.*, 2010).

Deep sea mats have, however, been studied in other chemosynthetic ecosystems such as mud volcanos (Girnth *et al.*, 2011), cold seeps (Grünke *et al.*, 2011; Gentz and Schlüter, 2012) or hydrothermal vents (Alain *et al.*, 2004; Taylor *et al.*, 1999). These studies highlighted the key role played in sulfidic habitats by *Epsilonproteobacteria* (Campbell *et al.*,

2006) during the establishment of deep sea chemosynthetic ecosystems. For carbon acquisition some members of this class use the reverse TCA cycle (rTCA) to fix inorganic carbon (López-García *et al.*, 2003) and chemosynthetic fauna inhabiting hydrothermal vents probably rely on bacterial carbon (Van Dover and Fry, 1989) and more precisely on carbon fixed using the rTCA cycle (López-García *et al.*, 2003). *Epsilonproteobacteria* were also described as the first colonizer of new surfaces exposed to the mixing zone surrounding vent chimneys (Alain *et al.*, 2004) (Heijs *et al.*, 2005) validating their key role in deep sea sulfidic environments. However, other bacterial groups are important and three main kinds of sulfidic chemosynthetic mats have been described. *Thiomargarita* (*Gammaproteobacteria*) mats develop within highly dynamic habitats where hydrogen sulfide and oxygen (or nitrate) gradients are unstable (Girnth *et al.*, 2011). *Arcobacter* (*Epsilonproteobacteria*) mats develop in niches where hydrogen sulfide and oxygen co-occur (Grünke *et al.*, 2011). Finally, *Beggiatoa* (*Gammaproteobacteria*) mats reduce the narrow overlap region between hydrogen sulfide and oxygen (or nitrate) and promote a steep gradient within the mat (Gentz and Schlüter, 2012). Due to the difficulty of accessing deep-sea samples, these mat categories present only a snapshot of the mat communities at a given time and the possible ecological succession of the microbial community composition remains unexplored.

During the formation of microbial mats, bacteria change their lifestyle from a free living stage to a multicellular community by secreting extracellular polymeric substances (EPS) that can represent 90% of a mat volume (Flemming and Wingender, 2010). Mat establishment strongly affect chemical gradients between the water and the substrate (Santegoeds *et al.*, 1998) and consequently can create new ecological niches. In wood falls, we suspect that changing chemical conditions measured on the wood surface (Yücel *et al.*, 2013) may result in a surface community adapted to changes in sulfide concentrations, similar to community changes observed in deep-sea chemosynthetic ecosystems (Alain *et al.*, 2004). We hypothesize that mat development creates with time new niches that might be colonized by specific bacterial groups. Furthermore, wood is composed mostly of polysaccharides (Sjostrom, 1993) so the microbial mat may use polysaccharides directly from the wood surface as observed in freshwater fungal biofilms (Golladay and Sinsabaugh, 1991).

In the present study we aim to fill the knowledge gaps in the least studied of the four deep-sea chemosynthetic ecosystems, wood falls. The overarching goal of this study is to provide a first description of a wood fall microbial mat and to verify if the mat bacterial community changes with time. We used a mesocosm experiment, which we developed to mimic deep-sea conditions (Kalenitchenko *et al.*, 2015), to overcome the difficulty of

repeatedly recovering fragile wood fall mats from the deep ocean. We combined illumina amplicon sequencing, metagenomics, fluorescent in situ hybridization (FISH) and image monitoring to investigate the diversity and function of the microbial community. This experiment allowed us to test (i) whether a microbial succession occurs during mat formation and (ii) whether the wood fall biofilms present chemosynthetic features.

## **Material and method**

### *Experimental set up*

Five wood logs (15 cm in diameter and 10 cm in length) were placed in aquaria filled with 5  $\mu\text{m}$ -filtered seawater pumped (flow: 8 L h<sup>-1</sup>) from the Mediterranean Sea at 4 m depth, 30 m away from shore. Seawater temperature was maintained at 13  $\pm$  1°C and oxygen concentrations were maintained at 100% saturation by bubbling. Wood logs originated from a same pine tree (*Pinus pinea*) cut the day before the start of the experiment in the Banyuls-sur-Mer “Biodiversarium” botanical garden. Pictures of each of the five wood logs were taken every day to monitor the color, the thickness and the area covered by the mats developing on the wood surface.

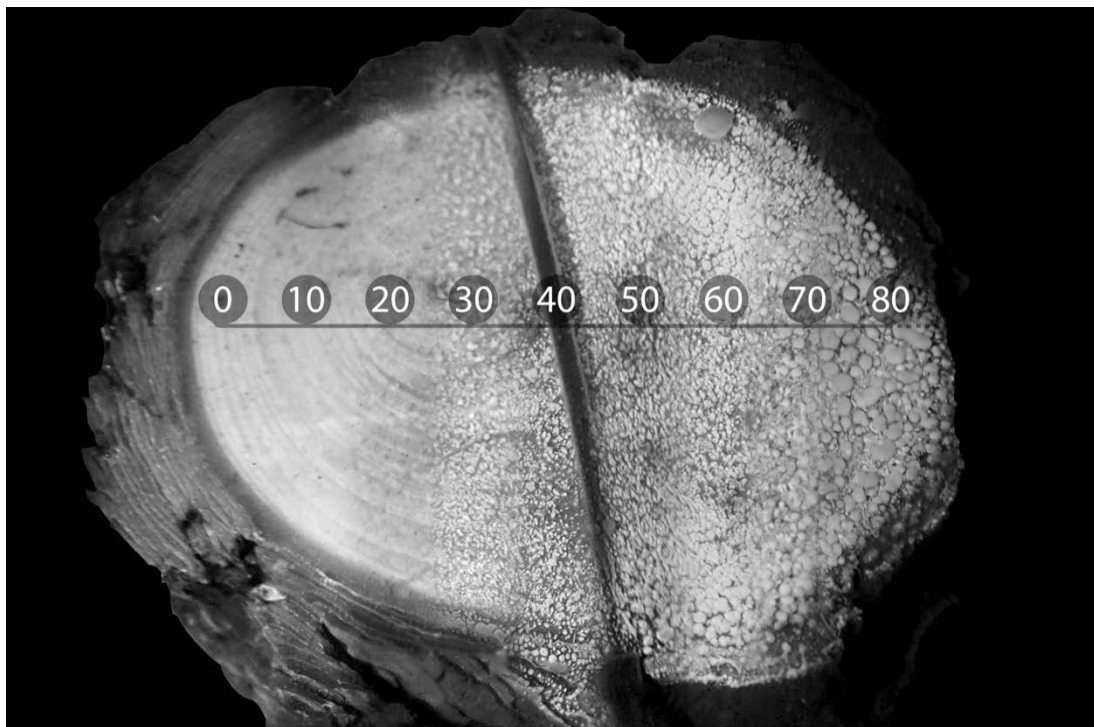


Figure 1: Composite picture showing the growth of a bacterial mat on the surface of a piece of wood immersed in seawater. Numbers indicate days of immersion. The data presented in this paper are from samples taken between 30 and 50 days of immersion.

### *Sampling, fixation and filtration*

Wood mats were sampled after 32, 35, 38, 43, 45 and 49 days of wood immersion. For each sampling time, mats were sampled with a punch (4.35 mm in diameter) from wood logs chosen randomly. Samples were always taken more than 50 mm away from an earlier sampling site to avoid any “earlier sampling site” effect. One CARD-FISH sample and two DNA samples were collected at each time point. For DNA analysis, samples were stored in 2 mL cryotubes at -80°C. For CARD-FISH, samples were suspended and fixed with 1 ml of paraformaldehyde (PFA) solution diluted to 4% with phosphate buffered saline (PBS) for 6 hours at 4°C. Then 50 µl of this solution was filtered onto a 25 mm diameter filter (Whatman, Nuclepore 0.2µm), the filters rinsed three times with a 1X PBS solution and then stored at -20°C.

### *Catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH)*

CARD-FISH was performed according to a previously published protocol (Pernthaler *et al.*, 2004). Nuclepore filters with mat cells were embedded in 0.1% low gelling point agar. Cells on the embedded filters were permeabilised with lysozyme buffer (10 mg/ml of Lysozyme (105 000 U mg<sup>-1</sup>, SIGMA, Saint-Louis, MO,USA) in 0.05 M EDTA, 0.1 M Tris-HCl (pH 8.0) and endogenous peroxidases inactivated by treatment with 0.01 M HCl. Filters were cut in 6 equal parts and 4 parts were incubated with the following probes; a universal *Bacteria* probe (EUB338-I) (Amann *et al.* 1990), a negative control probe (NON338) which is the reverse complement of the universal probe, an *Epsilonproteobacteria* class probe (EPSY549, (Lin *et al.*, 2006)), and a *Gammaproteobacteria* class probe (GAM42a, (Manz *et al.*, 1992)). Because GAM42a may also target some *Betaproteobacteria* we used the unlabelled BET42a probe (Manz *et al.*, 1992) as a competitor probe. Filter fragments were covered with hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH8.0), 10% dextran sulfate, 0.02% sodium dodecyl sulfate (SDS), 55% formamide (Sigma) and 1% Blocking Reagent (Roche)) containing the probe at 2.5 ng µl<sup>-1</sup>, and incubated in a hybridization chamber prepared with 55% formamide solution during 3 hours at 35°C. Filters were washed in washing buffer (5 mM EDTA (pH 8), 20 mM Tris-HCl (pH 8), 0.01% (w/v) SDS and 13 mM NaCl) for 10 min at 37°C and the equilibrated in 1X PBS for 15 min at room temperature. Filters were covered with the CARD substrate mix and incubated for 30 min at 37°C in the dark. The CARD substrate was prepared by mixing the amplification buffer (10% dextran sulfate, 2 M NaCl, 0.1% (w/v) blocking reagent, in 1X PBS (pH 7.6) and H<sub>2</sub>O<sub>2</sub> solution (0.15% in PBS) at a

1:100 ratio) with the substrate fluorescein tyramide (Perkin Elmer) at a ratio of 1 part tyramide to 200 parts amplification buffer. Filters were mounted on slides using a mounting mix (Citifluor and Vectashield at a 4:1 ratio and DAPI at 0.5 µg/ml). Observations and counts were achieved with a BX61 microscope (Olympus) equipped with FITC (U-N41001 HQIF) and DAPI (U-MNU2) filter sets. For each sampling time and each probe, 12 fields of view were counted for the CARD-FISH labeled bacteria and DAPI labeled bacteria and the relative abundance of labeled cells expressed as a % of the DAPI-labeled cells.

#### *DNA extraction, illumina amplicon and metagenome sequencing*

Frozen samples were thawed on ice, centrifuged 30 s at 6000 rpm and supernatants were discarded. DNA extraction was performed using the “PowerPlant DNA isolation kit” (MOBIO Laboratories, Carlsbad, CA, USA). Purified DNA was suspended in 50µl of Tris-EDTA (TE) buffer.

For amplicon sequencing, a portion of the 16S gene was amplified using the *Bacteria* specific primers 28 F (5'-TTTGATCNTGGCTCAG-3') and 519 R (5'-GTNTTACNGCGGCKGCTG-3'). DNA samples were sequenced on a Miseq Illumina sequencer (Illumina, San Diego, CA, USA) using Miseq reagent kit V3 (Illumina, San Diego, CA, USA) producing 2x300bp long reads in a commercial laboratory (MR DNA, Lubbock, TX, USA).

For metagenomes, a double sequencing strategy was adopted to get a better coverage. Samples were sequenced first on a Miseq Illumina sequencer (Illumina, San Diego, CA, USA) using the Miseq reagent kit V3 (Illumina, San Diego, CA, USA) to get longer reads (2x300 bp) and then on a Hiseq Illumina sequencer (Illumina, San Diego, CA, USA) (2x150 bp) to get a larger number of reads. Metagenome sequencing was conducted by a commercial laboratory (MR DNA, Lubbock, TX, USA) using commercially prepared reagents.

#### *Sequence data analysis*

16S rDNA sequences were processed following the standard operating procedure in Mothur (Kozich *et al.*, 2013). Briefly, sequences were paired, producing ca. 500 bp fragments. Sequences were then quality trimmed and chimera were removed using UCHIIME (Edgar *et al.*, 2011). Sequences were grouped in operational taxonomic units (OTU) if they were more than 97% similar. The taxonomy of the most abundant sequence of each OTU was assigned using the SILVA SSU 119 database (Pruesse *et al.*, 2007). All samples were resampled down

to 5131 sequences per sample. Three sample duplicates (d32, d45 and d49) were removed from further analysis because the amplification was not successful.

Metagenome sequences from Miseq and Hiseq sequencing were concatenated into two files, one forward (containing Miseq and Hiseq forward reads) and one reverse (containing Miseq and Hiseq reverse reads). Miseq sequences are longer, which improves gene assignment, whereas Hiseq sequences are short but in high abundance, which increases the coverage of the community gene diversity. The combination of these two sequencing methods maximized our chance to get a precise and deep coverage of the communities. Forward and reverse reads were joined with a minimum overlap of 8 bp and a maximum difference of 10%. Both paired and unpaired reads were retained for further analysis. Low-quality region trimming (phred score  $\leq 15$ ) was done with SolexaQA (Cox *et al.*, 2010). Sequences that passed the quality trimming were dereplicated using a k-mer approach that identified sequences that contain identical twenty-character prefix. Artificial duplicated sequences were removed (Gomez-Alvarez *et al.*, 2009) and then a machine learning based approach, FragGeneScan (Rho *et al.*, 2010) identified open reading frames (ORF), which were annotated by BLASTX (Camacho *et al.*, 2009) against the KEGG (Kanehisa, 2002) and SILVA (Pruesse *et al.*, 2007) databases. Analyses were conducted with the MG-RAST pipeline (Meyer *et al.*, 2008) and sequences were deposited under the accession numbers 4623131.3, 4623132.3 and 4623133.3.

To compare the metabolic potential between communities we counted the sequence abundance of functional genes linked with aerobic respiration, carbon cycle (aerobic and anaerobic carbon fixation, carbon monoxide oxidation, fermentation, sulfur cycle (sulfur mineralization, sulfur oxidation and dissimilatory sulfate reduction), nitrate cycle (Nitrate reduction) and methane cycle (methane production and consumption). We also looked at polysaccharide degradation genes that include cellulosome and starch sequestration systems. For proteins encoded by two or more subunits, we used the mean of the sequence abundance in the subunits.

#### *Computation of microbial diversity and function*

Comparison of bacterial community composition between different sampling times was achieved using a non-metric multidimensional scaling (NMDS) based on a Bray Curtis dissimilarity matrix. The matrix was calculated on a resampled dataset that contained only

abundant OTUs. An abundant OTU within a sample was defined as an OTU that encompassed more than 1‰ of the sequences.

The resampled dataset was also used to plot the relative bacterial class abundance over time for comparison with the CARD-FISH counts. An analysis of variance (ANOVA) was conducted on the replicate CARD-FISH counts to test if the abundance of the different bacterial class varied significantly with time. The ANOVA prerequisite (normality, independence and homoscedasticity) were verified.

We constructed ternary diagrams with ggtern (v1.0.3.1) to compare functional gene and OTU abundance between samples.

## Results

### *Mat growth*

During the first 20 days of immersion the surface of the 5 pieces of wood (Figure 1) were exempt of mat. The first traces of mats appeared after 20 days and colonized the entire wood surface within 10 days after their appearance. After 30 days of immersion the mat surface was not homogeneous and showed both white dots and transparent areas (Figure 1). Our study focused on the first stage of mat formation (32 to 49 days). During this period the mat thickness increased from ca. 1 mm to more than 4 mm. The pattern of mat development was similar on the five replicate pieces of wood (Supplementary figure 1).

### *Bacterial community dynamics*

Miseq illumina sequencing produced 161201 raw 16S rRNA paired end reads. After pairing, quality filtering and chimera checking 100426 sequences remained which clustered into 40 OTUs at 97% similarity.

Non-metric multidimensional scaling (NMDS) based on a Bray-Curtis distance matrix separated the samples according to time of immersion. The NMDS stress value was 0.057 indicating a good ordination of the data. Samples from the beginning of the experiment (d32 to d43) were associated to a group of seventeen OTUs (Figure 2). These OTUs belonged to the order *Gammaproteobacteria* (Otu10-11-12-21-22-23-24-26-30-31-38), *Epsilonproteobacteria* (Otu1-2-6-8-42) and *Flavobacteria* (Otu13). Communities originating from samples incubated for a longer time (d45 and d49) were highly discriminated by axis 1

and were associated to OTUs from the class *Deltaproteobacteria* (Otu3-9-19-32) and *Deferribacteres* (Otu16).

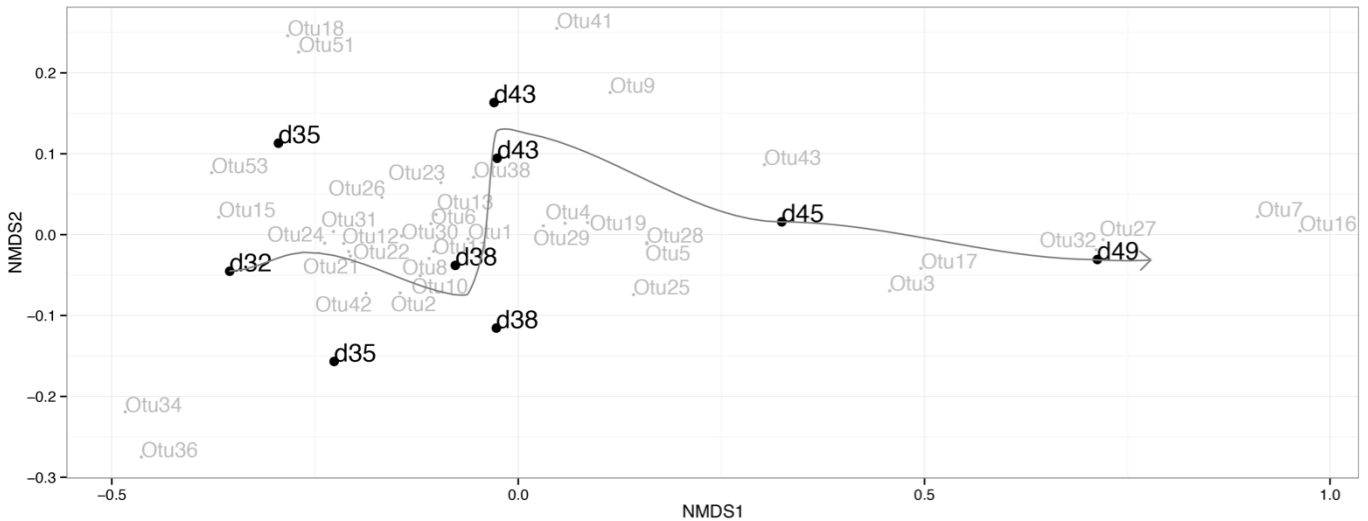


Figure 2: Non-metric multidimensional scaling plot showing changes in bacterial community composition with time. Black dots represent sample names and grey points show OTUs plotted within the NMDS space. Stress value=0.057.

We then observed the dynamics of each of these bacterial classes along time (Figure 3a). *Gammaproteobacteria* sequence abundance did not vary with time ( $11\% \pm 3\%$ ), however, the proportion of *Epsilonproteobacteria* sequences decreased after 38 days (66%) and reached a minimum after 49 days (30%). The *Deltaproteobacteria* sequence abundance was a mirror image of that of the *Epsilonproteobacteria* and remained below 11% between day 32 and day 38, and became the dominant class after 49 days (50%). Some bacterial classes were detected only at the end of the experiment like the *Deferribacteres* that were not detected before 38 days and became increased in abundance ( $> 1\%$  of the total number of sequences) after 49 days (3%). Replicate samples (d35, d38 and d43) showed similar patterns reflecting a homogenous community composition in the mats.

The dynamics based on high throughput sequencing results could be biased by the PCR amplification step. Based on the sequencing results we targeted two bacterial classes to validate the temporal dynamics with CARD-FISH. We targeted the *Epsilonproteobacteria*, as a class that showed variations in abundance, and the *Gammaproteobacteria* was selected as a stable class. Cell counts were expressed as percentage of labeled cells relative to DAPI stained cells. The CARD-FISH results showed patterns similar to the sequencing results (Figure 3b). *Epsilonproteobacteria* abundance decreased significantly with time (ANOVA,  $N=54$ ,  $F= 69.885$ ,  $p\text{-value}= 3.453e\text{-}11$ ) from 82% of the DAPI stained cells ( $\pm 12\%$ ) after 32 days, to 67% ( $\pm 13\%$ ) after 38 days and 40% ( $\pm 15\%$ ) at the end of the experiment. The



abundance of *Gammaproteobacteria* remained close to 8% ( $\pm 5\%$ ) of the DAPI stained cells and did not present any significant variations.

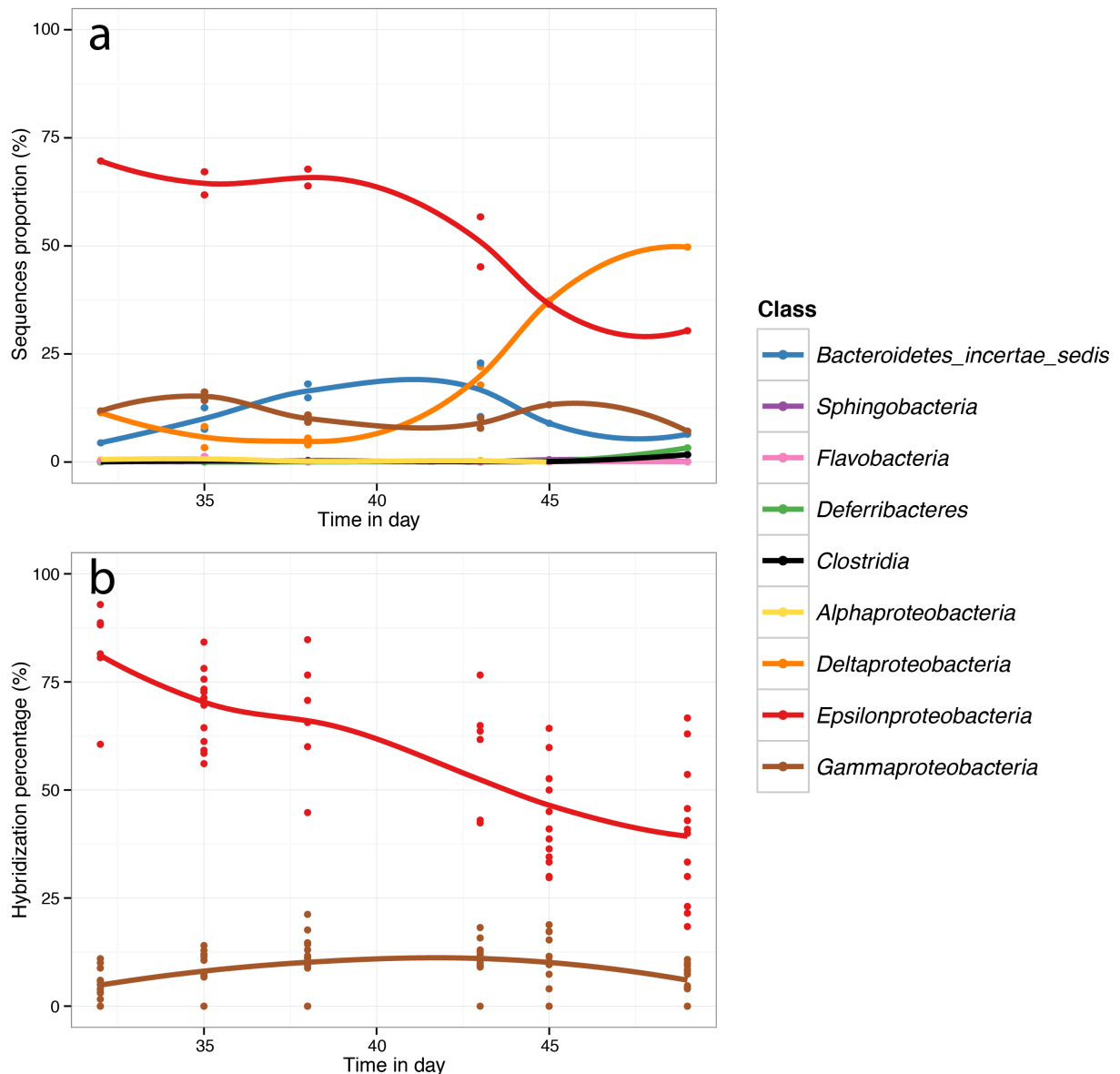


Figure 3: Proportion of bacterial classes in the wood fall mats at different times of immersion. Bacteria were quantified based on the number of 16S rRNA sequences (a) and by CARD-FISH counts (b). The curve was fitted to the mean values when replicates were present.

### *Bacterial community composition*

From the temporal dynamics results we defined 3 key time points in the development of the mat: d32 (abundant *Epsilonproteobacteria*), d45 (equally abundant *Epsilonproteobacteria* and *Deltaproteobacteria*) and d49 (abundant *Deltaproteobacteria*), and observed at the OTU level how relative abundances changed between these three time points (Figure 4a).

At the class level, *Gammaproteobacteria* abundance did not change with time but at the OTU level there were variations. After 32 days, *Gammaproteobacteria* contained many different equally abundant OTUs but as time passed only a few abundant OTUs dominated the class (Figure 4a, Supplementary Figure 2). At d32, the main *Gammaproteobacteria* OTUs were Otu 10, Otu 11 and Otu 12, together accounting for 57% of the *Gammaproteobacteria* sequences. After 45 days, a single OTU (Otu5) dominated the *Gammaproteobacteria* (84% of these sequences) and remained the most abundant OTU at 49 days (91% of these sequences). Otu 10, 11 and 12 were similar to NCBI deposited sequences NR\_042965.1 (100% similarity), NR\_029031.1 (100% similarity) and KC476293.1 (99% similarity) respectively. These three OTUs belonging to the *Oceanospirillaceae* family were attributed to the *Marinomonas* genus for Otu 10 and 11 and to the *Thalassolituus* genus for Otu12. Otu5 was distantly related to any known strains (< 94% similarity) but a similar sequence was previously found (100% similarity to GQ455175.1) in mats from coral black band disease (Ben-Dov *et al.*, 2011).

For the *Epsilonproteobacteria*, there was a clear succession of the dominant OTUs with time, beginning with Otu2, Otu1 and Otu8 at d32, Otu1 persisting and dominating at d45, being replaced by Otu7 at d49. Otu1 and Otu2 were not related to any known strains ( $\leq 94\%$  or  $\leq 95\%$  respectively) whereas Otu7 was 95% similar to *candidatus Arcobacter sulfidicus* and Otu8 was 99% similar to *Arcobacter nitrofigilis* strain (EU106661.1).

*Deltaproteobacteria* OTUs were most abundant at the later time points d45 and d49 and were dominated by Otu3 (> 96 % of the *Deltaproteobacteria*). This OTU sequence was 100 % similar to a sequence from cold seeps (AM404369.1), and 99% similar to a sequence identified as a *Desulfovibrio frigidus* strain (NR\_043580.1) that uses sulfate and sulfite as electron acceptor and thus produces hydrogen sulfide as down product.

Other bacterial classes were represented by one Otu only: *Bacteroidetes incertae sedis* (Otu4), *Sphingobacteria* (Otu25), *Flavobacteria* (Otu13), *Deferribacteres* (Otu 16) and *Clostridia* (Otu17). Otu13 was typical for the beginning of the experiment (d32) and was 98% similar to a sequence identified as *Winogradskyella sp.* (JX174421.1). Otu25 showed an increased abundance at 45 days and was only distantly related to database sequences (< 94%). Otu16 and Otu17 were more abundant at the end of the experiment (d49) and were only 92 and 95 % similar to NCBI database sequences. Otu4 showed only small variations in abundance at the different time points and was also distantly related to any deposited sequences (<95%).

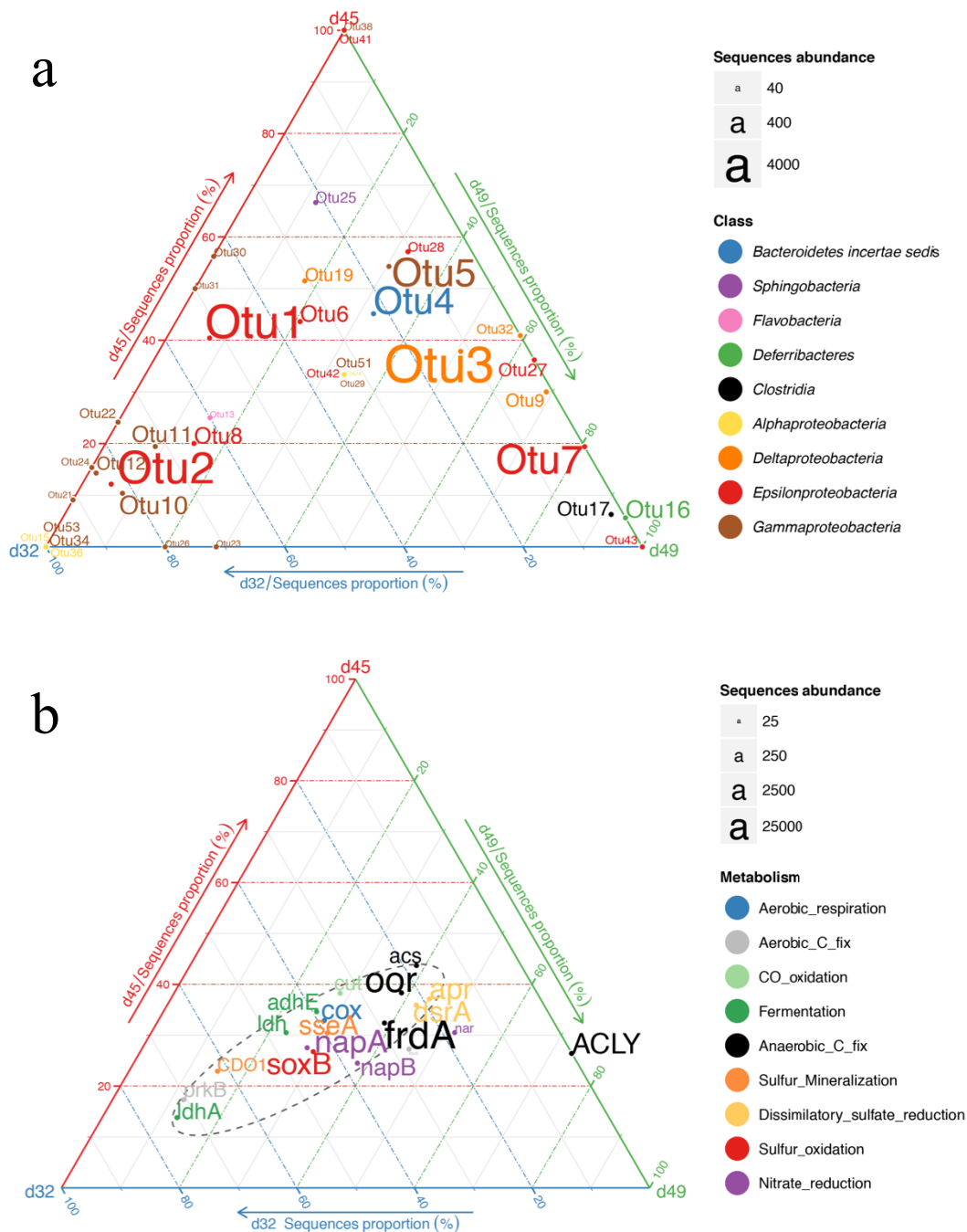


Figure 4: Triplots representing OTU (a) and functional gene (b) proportions within three samples. The blue axis represents the proportion of sequences after 32 days of immersion (d32), the red axis the proportion after 45 days (d45) and the green axis after 49 days (d49). OTU colors represent the different bacterial classes and gene colours show the different metabolic pathways. OTU and gene name sizes are proportional to the relative abundance of each gene or OTU out of the entire dataset.

### Functional genes diversity

We constructed a metagenome for each of these time points (d32, d45 and d49), which contained 20341628, 21322937 and 20555592 sequences respectively. A total of  $64.5\% \pm 4\%$

of the sequences passed the quality checking step and  $49\% \pm 3,4\%$  were assigned to known proteins.

Overall, functional genes were less dispersed among samples than OTUs (Figure 4b). All the microbial communities that formed the wood fall mats had the potential for aerobic respiration, aerobic carbon fixation, carbon monoxide oxidation, fermentation, sulfur mineralization, dissimilatory sulfate reduction, sulfur oxidation and nitrate reduction based on the detection of marker genes for these metabolisms in the three metagenomes (Figure 4b). We did not detect any genes linked with methane production or oxidation (*mcr* or methane monooxygenase). Even though many functional genes were present in all metagenomes, some gene abundances changed with time. Most of the genes were included within a stretched ellipse that went from the sample d32 corner to a middle point situated between sample d45 and d49 (Figure 3b). This shape reflects three main groups of genes: first, genes that were detected in the three samples (close to the triplot centroid), second, genes that were detected mostly in sample d32 (close to the d32 side of the ellipse), and third, genes that were detected in both d45 and d49 in nearly equal proportions (close to the 50% value of the d49 axis).

Fermentation genes, especially the D-lactate dehydrogenase gene (*ldhA*) were essentially detected within sample d32, which contained 74% of the *ldhA* genes detected. Other fermentation genes (*ldh* and *adhE*) were less influenced by time (Figure 3b). Phosphoribulokinase gene (*prkB*), a key enzyme of the Calvin cycle, was more abundant (310 sequences) in the d32 sample compared with the d45 and d49 samples (76 and 53 sequences respectively). Another gene marker for aerobic carbon fixation, the ribulose-bisphosphate carboxylase small chain gene (*rbcS*), was rarely detected in our mats (3 to 5 sequences depending on the sample). Sulfur mineralization marker genes, cysteine dioxygenase (CDO1) and thiosulfate/3-mercaptopyruvate sulfurtransferase (*sseA*), were more abundant at d32 (62% of the CDO1 and 40% of the *sseA* genes) than at d45 and d49. The sulfur oxidation protein gene (*soxB*) was also more present in the d32 sample (44% of the *SoxB*) than after 45 and 49 days (27 and 29 % of the *SoxB* respectively). The marker genes for nitrate reduction, cytochrome c-type protein (*napB*), periplasmic nitrate reductase (*napA*) and nitrate reductase (*nar*), were not clearly associated to any sample, reflecting a conserved function during the mat development. The same pattern was observed for the cytochrome c-oxidase (*cox*) and carbon monoxide dehydrogenase (*cut*) genes that did not show any temporal dynamics. Anaerobic carbon fixation marker genes were more abundant in the d45 and d49 samples. The ATP citrate lyase gene (*ACLY*), involved in the reverse TCA cycle, fell outside the ellipse and was only detected after 45 (26% of the *ACLY* genes) and 49 days (74% of the *ACLY* genes).

Other anaerobic carbon fixation genes, the fumarate reductase flavoprotein subunit (*frdA*), the 2-oxoglutarate ferredoxin oxidoreductase (*oor*) and the acetyl-CoA decarbonylase/synthase (*acs*), were closer to the triplot centroid than the *ACLY* gene, but closer to the d45 and the d49 samples than to the d32 sample. The two dissimilatory sulfate reduction genes, sulfite reductase (*dsrA*) and adenylylsulfate reductase (*apr*), were affiliated to the *Deltaproteobacteria* class in the KEGG taxonomy database, validating their affiliation to the dissimilatory sulfate reduction metabolisms. They were both detected more often in the d49 (42% of the *dsrA* and 44% of the *apr* genes) and d45 sample (36% of the *dsrA* and 37% of the *apr*) than in the d32 sample.

Polysaccharide degradation gene screening (Supplementary figure 3) did not reveal cellulosome related genes such as the *sca* cluster genes (encoding the cellulosomal structural proteins, scaffoldins) as observed in the rumen gut (Flint *et al.*, 2008). However, we did detect *Sus* cluster genes coding for the starch sequestration system found in *Bacteroidetes* (Flint *et al.*, 2008).

## Discussion

Our experiment revealed the identity and function of the microbes constituting wood fall associated mats and gave the first opportunity to study the dynamics of this unknown ecosystem. We could thus demonstrate an ecological succession in the bacterial community assembly and potential metabolisms, and establish the chemosynthetic nature of wood colonizing mats.

### *Ecological succession during epixylic mat formation*

The first bacterial community that reproducibly colonized wood falls after ca. 1 month of immersion was mainly composed of *Epsilonproteobacteria*. These pioneer communities were dominated by an unknown OTU distantly related to the *Arcobacter* genus (Otu2, Figure 5). Another dominant *Epsilonproteobacteria* (Otu8) was closely related to *Arcobacter nitrofigilis* (Pati *et al.*, 2010), which is a chemoorganotrophic bacteria that use nitrate as electron acceptor. The white appearance of the biofilm may indicate the capacity of Otu8 to produce elemental sulfur from hydrogen sulfide oxidation as observed for *Arcobacter sulfidicus* (Sievert *et al.*, 2007). Hydrogen sulfide or organic compounds (used by the *Arcobacter nitrofigilis*-related Otu8) were all shown to be breakdown products of wood degradation (Yücel *et al.*, 2013; Leschine, 1995; Kalenitchenko *et al.*, 2015). During this first stage of mat

formation, the *Epsilonproteobacteria* shared their habitat with seven *Gammaproteobacteria* OTUs related to the aerobic genera *Thalassolituus* and *Marinomonas* (Prabakaran *et al.*, 2005; Yakimov *et al.*, 2004). Our results, together with observations made on a sulfide oxidizing community in the black sea (Jørgensen *et al.*, 1991), suggest that this first, potentially sulfide oxidizing mat community, plays an important role in protecting the wood fall surrounding environment against hydrogen sulfide produced during the wood decay.

After ca. 45 days, the bacterial communities started to change with an increase of the relative proportion of *Deltaproteobacteria* sequences that was inversely correlated with a significant decrease of *Epsilonproteobacteria* sequences. *Deltaproteobacteria* were dominated by sequences related to the *Desulfovibrio* genera (OTU3) previously found associated to wood falls (Khelaifia *et al.*, 2011) (Kalenitchenko *et al.*, 2015). This genera of sulfate reducing bacteria (SRB) is strictly anaerobic, uses fermentation end products as a carbon source, respire sulfate and thus produces hydrogen sulfide (Pradel *et al.*, 2013). The mat thickness increased with time, which probably reduced the penetration of potential electron acceptors like oxygen and nitrate, thus promoting the development of anaerobic and nitrate depleted niches (Okabe *et al.*, 2005; van Houten *et al.*, 1994) on the wood side of the mat. These new ecological niches presented ideal conditions for the development of SRB but were probably not favorable for chemoorganotrophic bacteria, like *Arcobacter nitrofigilis* (Otu8), which disappeared as the mat grew.

Interestingly, the mat showed a unique feature at this stage since the community was composed half of SRB and half of an *Arcobacter* related OTU. This is unusual because SRB and nitrate reducing bacteria, like *Arcobacter nitrofigilis*, usually compete for hydrogen as electron donor (Tang *et al.*, 2013). In our experiment, hydrogen production is derived from the wood fermentation (Leschine, 1995), which is a slow process, but must have been sufficient to provide the needs of the different bacteria. The presence of SRBs maintaining the production of hydrogen sulfide could provide an ideal niche for potential sulfur oxidizing *Arcobacter* (like unknown Otu1 or Otu2), probably located on the biofilm upper layers, where oxygen or nitrate from the seawater and hydrogen sulfide from the wood co-occur. Such a co-occurrence of sulfate reducers and sulfur oxidizers within the same microbial mat has not been previously documented in other chemosynthetic ecosystems (whale falls, cold seeps or hydrothermal vents) but such consortia are known to occur in symbiosis with oligochaete worms (Blazejak *et al.* 2005).

At the end of our experiment, the biofilm was up to 4 mm thick. As the mat grew, the penetration of electron donors probably did not increase (Santegoeds *et al.*, 1998) and as a

consequence, the niche suitable for bacteria dependent on electron acceptors like oxygen and nitrate did not increase (Figure 5). However, sulfate ions are abundant in seawater (Sverdrup *et al.*, 1942) and could still penetrate within the mat and also within the wood where it could be reduced (Yücel *et al.*, 2013). The penetration of sulfate within the mat may maintained the sulfate reducing bacteria ecological niche, which probably occupied all the space between the wood surface and the hydrogen sulfide oxidizing layer, resulting in an increase in the proportion of sequences related to the *Desulfovibrio* genera (Figure 5). The increasing distance between the mat-water interface and the wood surface probably resulted in a selection of bacteria that did not use the organic carbon from the wood. These bacteria, like the chemolithoautotroph *Arcobacter sulfidicus* related OTU (Otu7), would prefer to fix the inorganic carbon present in seawater. *Arcobacter* related OTUs may be responsible for the white coloration observed on our mat because they do an incomplete oxidation of sulfide to elemental sulfur, which produces white sulfur filaments visible to the naked eye (Sievert *et al.*, 2007). In addition to the visual observation of this elemental sulfur, we detected *Deferribacteres* OTUs that scavenge this elemental sulfur and use it as terminal electron acceptor (Garrity *et al.*, 2001) giving another clue about the sulfur filaments formation. These community changes also impacted the *Gammaproteobacteria* class that evolved from seven aerobic OTUs after 32 days to only one unknown OTU probably more adapted to anoxia, at the end of the experiment.

#### *Metabolic potential of the epixylic mat*

Using a metagenomic approach we were able to detect the presence of genes coding for key enzymes involved in the potential metabolisms of bacterial groups cited above. Our results indicate that from the first stage of colonization to the end, the microbial community had all the potential functional genes needed for the growth and maintenance of the epixylic mat. The presence of *napA*, *napB* and *nar* gene validated the finding that the bacterial community had the potential to produce the nitrate reductase protein (Smith *et al.*, 2007) and thus confirmed the capacity of the microbial pool to use nitrate as electron acceptor.

Our 16S rRNA data indicated that the upper mat layer moved from a chemoorganotrophic stage to a chemolithoautotrophic stage. This was confirmed by the slight increase of the relative sequence abundance of reductive TCA (rTCA) cycle genes, including the fumarate reductase (*frdA*), the 2-oxoglutarate ferredoxin oxidoreductase (*oor*) and the acetyl-CoA decarboxylase (*acs*), involved in the CO<sub>2</sub> fixation pathway. However, the ATP

citrate marker gene (*ACLY*), the key enzyme of the rTCA cycle, was not detected in the beginning of the colonization indicating the microbial community inability to run the rTCA cycle during the first development stage. The rTCA pathway is widely used by *Epsilonproteobacteria* (Hügler *et al.*, 2010; Campbell and Cary, 2004) to fix carbon dioxide in anaerobic or microanaerobic conditions, which confirms that our community acquired the potential to fix inorganic carbon between 32 and 45 days of immersion.

The detection of *Desulfovibrio* related sulfate reductase (*dsr*) and adenylylsulfate reductase (*apr*) genes evidenced the community's potential for sulfate reduction. The proportion of *dsr* and *apr* genes increased at the end of the experiment supporting the hypothesis about the metabolism of *Desulfovibrio* related bacteria (Otu3), which quickly dominated the wood community. The common presence of *Desulfovibrio* in wood falls (Kalenitchenko *et al.*, 2015; Fagervold *et al.*, 2013; Bienhold *et al.*, 2013; Pradel *et al.*, 2013) and measures of hydrogen sulfide pulses on wood surfaces after 1 month (Yücel *et al.*, 2013; Kalenitchenko *et al.*, 2015), suggests the importance of this genera for the production of hydrogen sulfide from wood falls. In addition, the bacterial community's potential to use hydrogen sulfide as electron donor was confirmed by the detection of the sulfur oxidation protein coding gene *soxB*. This finding supports the theory that the unknown Otu2, most closely related to *Arcobacter*, detected at the beginning of the experiment was able to use hydrogen sulfide as electron donor and may be responsible for the white elemental sulfur accumulation in the mat. However, there is no sign that the early colonizers can fix inorganic carbon through the rTCA cycle. We therefore hypothesize that the first dominant OTU used chemolithoheterotrophy which may give a competitive advantage over chemolithoautotrophy in high organic carbon environments (Sorokin; Moran *et al.*, 2004). After 45 days, the bacterial mat conditions probably allowed the establishment of bacteria with the potential for chemolithoautotrophy, which use carbon dioxide as carbon source and inorganic compound (hydrogen sulfur) as an electron donor. We conclude that after the first stages of colonization, the biofilm presented all the features of a chemosynthetic ecosystem.



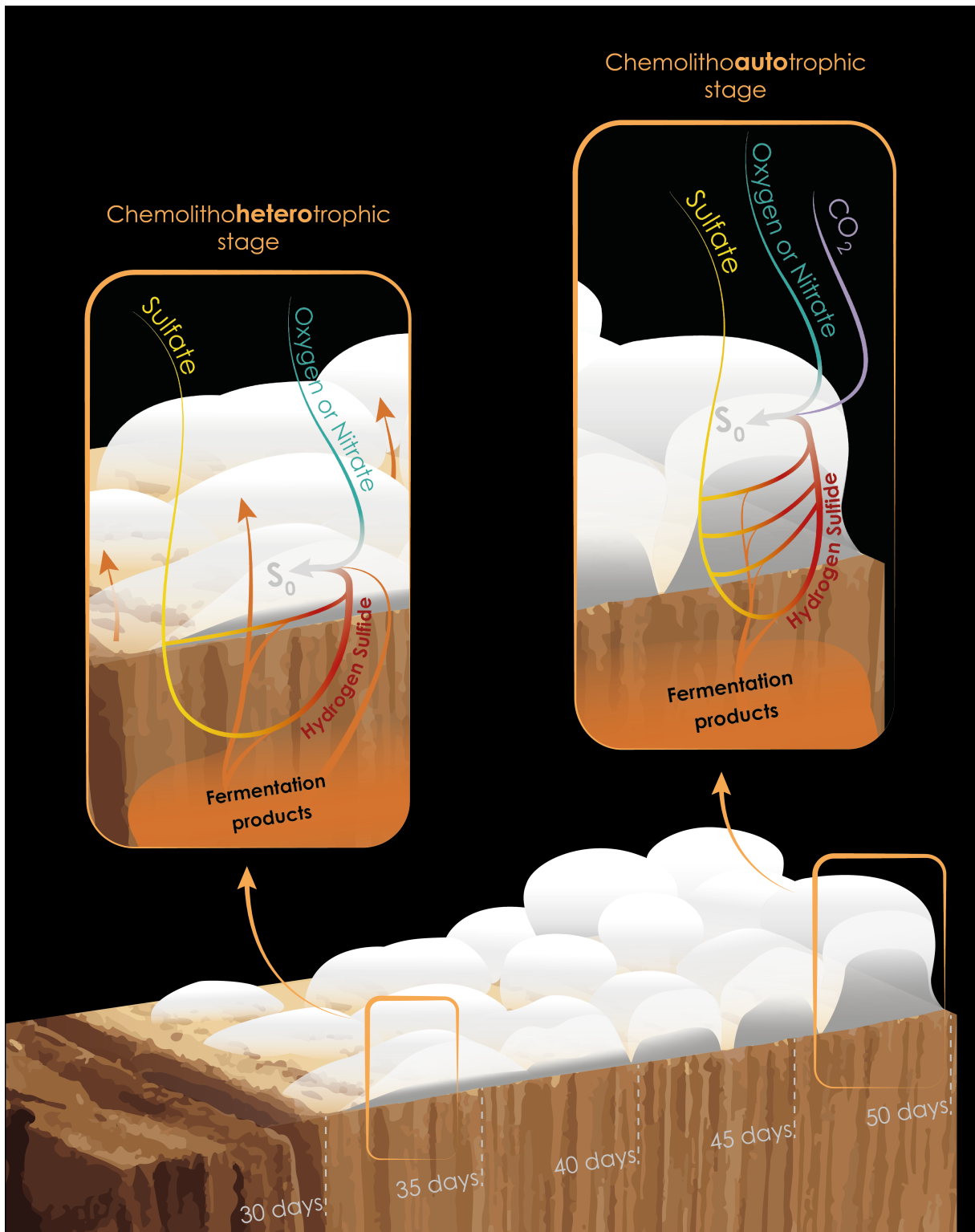


Figure 5: Schematic illustration of the ecological succession within the epixylic mat. The two zoomed areas portray the potential metabolisms detected in the mat. The size of the area representing sulfate reducing bacteria (grey area) and sulfide oxidizing bacteria (white area) is proportional to the number of 16S rRNA sequences detected at the different time points.

## Conclusion

Here we present a first description of the diversity and potential metabolisms of the bacterial communities forming mats on wood falls in the sea. First, an *Arcobacter*-related community dominated chemoorganotrophic mats developed on hydrogen sulfide and organic carbon from wood fermentation (Figure 5). Second, the thickening of the mat created new ecological niches for the co-occurrence of sulfide oxidizing and sulfate reducing bacteria. Finally, at a later stage, we hypothesize the selection of chemolithoautotrophic *Arcobacter*, instead of chemolithoheterotrophic *Arcobacter*, caused by the preferential utilization of organic carbon from fermentation products by the growing population of sulfate reducing bacteria. Our work also demonstrates the utility to develop experimental incubation in the laboratory to study deep-sea chemosynthetic mats. The easy access to samples allows short-term temporal studies for a precise description of mat formation.

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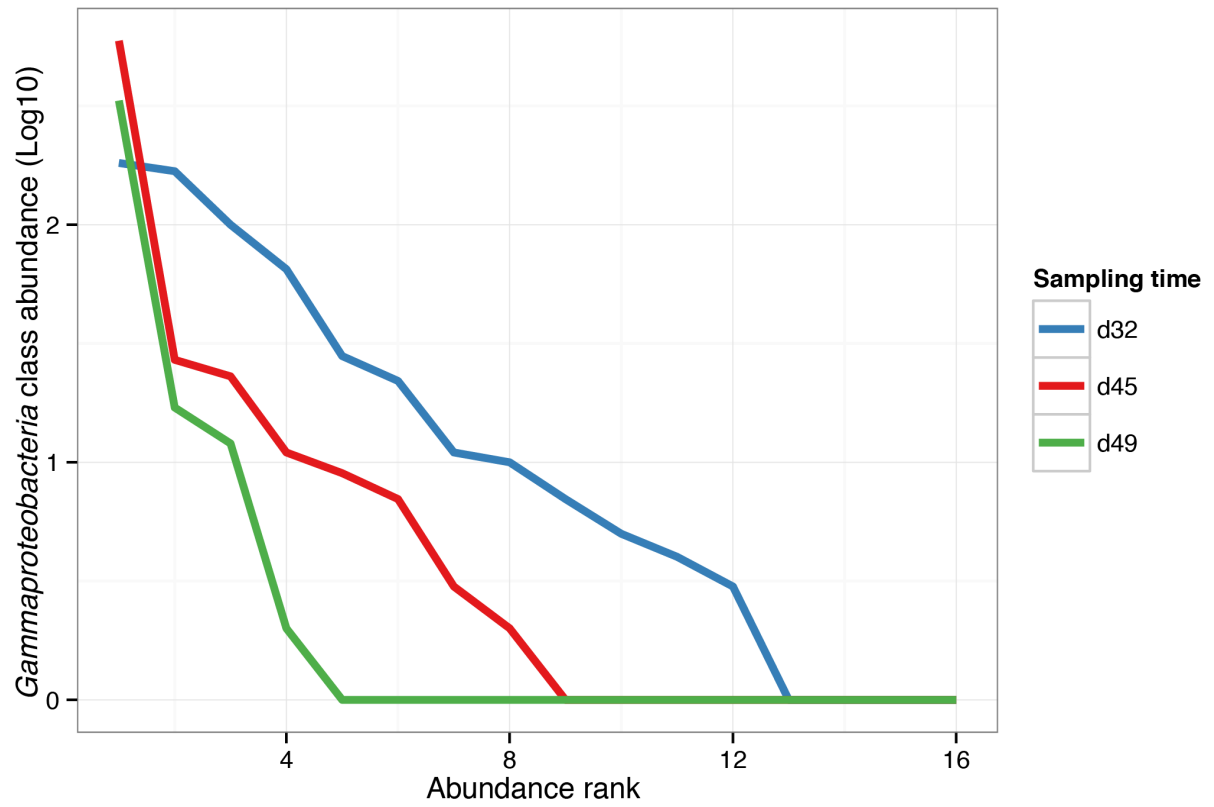
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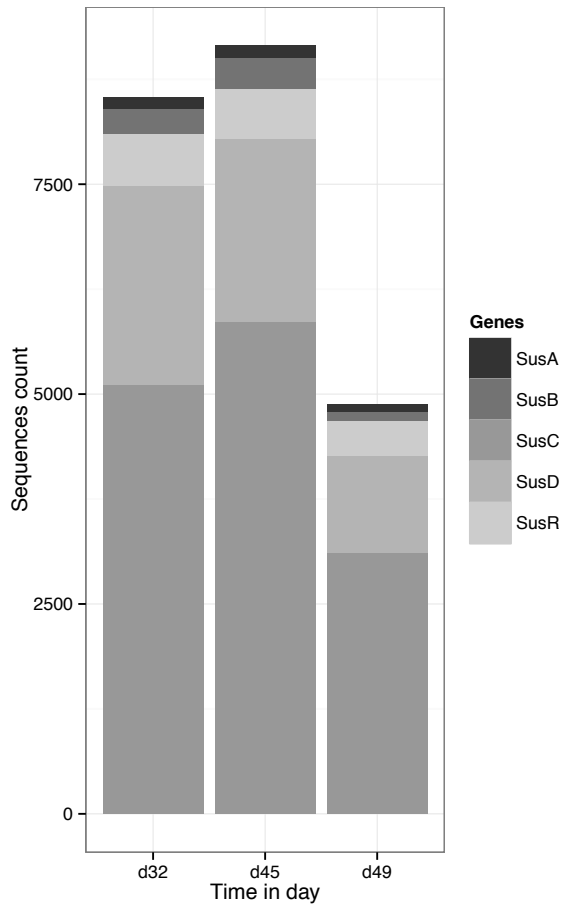
Supplementary material



Supplementary figure 1: Photographs of the five incubated woods taken after 21, 32 and 49 days of immersion showing the same patterns of mat colonization dynamics.



Supplementary figure 2: Whittaker plot of the *Gammaproteobacteria* class affiliated OTU highlighting a decrease in diversity with time.



Supplementary figure 3: Abundance of sequences belonging to the *Bacteroidetes* starch sequestration system within the three wood fall mat metagenomes



**Article IV: Microbial Communities in Sunken Wood Are  
Structured by Wood-Boring Bivalves and Location in a  
Submarine Canyon**



# Microbial Communities in Sunken Wood Are Structured by Wood-Boring Bivalves and Location in a Submarine Canyon

Sonja K. Fagervold<sup>1,2</sup>, Chiara Romano<sup>3</sup>, Dimitri Kalenitchenko<sup>1,2</sup>, Christian Borowski<sup>4</sup>, Amandine Nunes-Jorge<sup>4</sup>, Daniel Martin<sup>3</sup>, Pierre E. Galand<sup>1,2\*</sup>

**1** Sorbonne Universités, UPMC, Univ Paris 06, UMR8222, LECOB, Observatoire Océanologique, Banyuls-sur-Mer, France, **2** CNRS, UMR 8222, Laboratoire d'Écogéochimie des Environnements Benthiques (LECOB), Banyuls-sur-Mer, France, **3** Centre d'Estudis Avançats de Blanes (CEAB-CSIC), Blanes (Girona), Catalunya, Spain, **4** Max Planck Institute for Marine Microbiology, Bremen, Germany

## Abstract

The cornerstones of sunken wood ecosystems are microorganisms involved in cellulose degradation. These can either be free-living microorganisms in the wood matrix or symbiotic bacteria associated with wood-boring bivalves such as emblematic species of *Xylophaga*, the most common deep-sea woodborer. Here we use experimentally submerged pine wood, placed in and outside the Mediterranean submarine Blanes Canyon, to compare the microbial communities on the wood, in fecal pellets of *Xylophaga* spp. and associated with the gills of these animals. Analyses based on tag pyrosequencing of the 16S rRNA bacterial gene showed that sunken wood contained three distinct microbial communities. Wood and pellet communities were different from each other suggesting that *Xylophaga* spp. create new microbial niches by excreting fecal pellets into their burrows. In turn, gills of *Xylophaga* spp. contain potential bacterial symbionts, as illustrated by the presence of sequences closely related to symbiotic bacteria found in other wood eating marine invertebrates. Finally, we found that sunken wood communities inside the canyon were different and more diverse than the ones outside the canyon. This finding extends to the microbial world the view that submarine canyons are sites of diverse marine life.

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\* E-mail: pierre.galand@obs-banyuls.fr

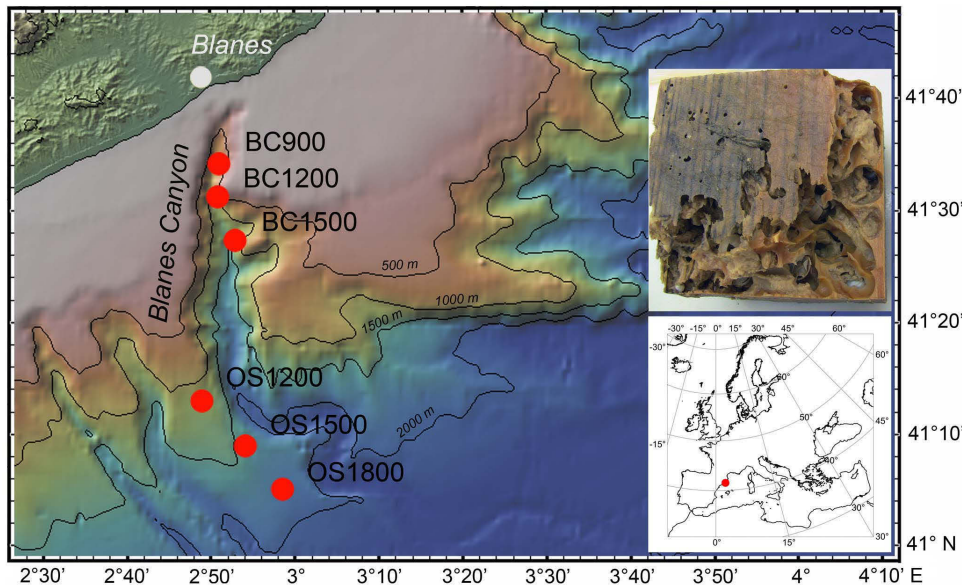
## Introduction

Debris of terrestrial plants can be exported from land to sea by rivers and streams, especially during flooding events [1]. Once saturated with water, the debris sinks and brings a discrete load of organic carbon to the ocean floor. The importance of plant debris for the oceans organic carbon cycle has recently been acknowledged [2]. However, sunken wood has long been in the center of scientific interest because it can harbor distinct and specialized faunal communities [3] and because sunken wood is hypothesized to play a key role in the maintenance and dispersion of chemosynthetic species in the deep sea [4,5]. Among the emblematic species found on sunken wood are wood boring marine invertebrates, which use the wood matrix as shelter and food. A significant amount of energy is stored in the wood as cellulose which is degraded by cellulolytic organisms, and their degradation products can be used by animals that host microbial symbiotic communities [6–8].

*Xylophaga* Turton, 1822 from the family Pholadidae, is the most common genus of xylotrophic (wood eating) bivalves found in sunken wood in the deep sea [9,10]. These animals bore into wood

using their shell and ingest the wood particles produced. They use wood as an energy source, but the origin of the cellulase used to hydrolyze the cellulose is still uncertain because studies on the physiology of *Xylophaga* do not exist. Shallow-water wood-boring bivalves from the family Teredinidae (known as shipworms) have been more extensively studied. Shipworms have strongly modified and enlarged gills that harbor cellulolytic and nitrogen-fixing *Gammaproteobacteria* [11,12]. Cellulolytic enzymes produced by the gill endosymbionts are assumed to contribute to lignocellulose breakdown in the digestive system, however, this has not been demonstrated and the transfer mechanism of cellulases from the gills to the digestive tract remains unclear. The gills of *Xylophaga* are much smaller and less modified [13]. They also contain dense populations of endobacteria, but it is not known if these help digesting cellulose [14]. Further, one of the characteristics of *Xylophaga* species is that they fill their burrows with pellets of feces mixed with mucus while shipworms expel their fecal pellets outside the burrow. Thus, it is possible that *Xylophaga* create new niches for microbial colonization within the wood.

Microorganisms are also present in the wood itself where they degrade wood cells. As submerged woods become anaerobic after



**Figure 1. Map of sampling sites and wood with *Xylophaga* burrows.** Map showing the position of the experimental moorings in relation to the Blanes Canyon (BC) and its outer slope (OS), drawn using GeoMapApp (<http://www.geomapapp.org>). Insert, cube of pine wood (8 X 8 X 8 cm) colonized by *Xylophaga* after 12 months of immersion in Blanes Canyon. doi:10.1371/journal.pone.0096248.g001

only a few days [15], it is expected that pathways associated to fermentation, with electron acceptors other than oxygen, play a significant role. Indeed, studies on sunken wood in the deep Mediterranean revealed that this substrate can harbor rich bacterial communities [16–18] including fermenting bacteria, microorganisms involved in sulfur cycling and methane production, and new clades of Bacteria and Archaea with unknown physiologies [18]. As the application of molecular techniques has revealed a large diversity of microbes associated to sunken woods, they have also allowed a first understanding of the ecology of sunken wood microbial communities, showing that wood type, immersion time and the environmental conditions surrounding submerged wood may promote contrasted bacterial communities [19,16–18]. For instance, bacterial communities associated with oak wood that had been artificially submerged in the Blanes Canyon (western Mediterranean) were dominated by *Alphaproteobacteria* of the family *Rhodobacteriales*, *Gammaproteobacteria* and *Bacteroidetes* [17]. In contrast, bacterial communities recovered with wood deployments from the Eastern Mediterranean differed on higher taxon levels and they were characterized by the presence of *Flavobacteria* [16]. Moreover, factors controlling community assembly remain poorly understood and the possible effect of wood-boring bivalves on bacterial community composition has never been explored.

This study aims at investigating the composition and diversity of microbial communities in wood, and in particular if external factors exhibit structuring influence. A major focus is on the effects of wood-boring bivalves on the wood associated microbial communities, in particular with respect to the abundant fecal pellets deposited by the bivalves in their burrows. We hypothesize that fecal pellets increase habitat diversity and that pellet communities are distinct from those in the wood matrix. Our second goal was to test if the special environmental conditions inside submarine canyons provide structuring influence on the microbial wood communities, as canyons are known to be hotspots for benthic biomass and productivity [20–22]. We used pine wood that was experimentally deployed in the deep Blanes Canyon off

the Mediterranean Spanish coast and in similar water depths on an adjacent open slope area. We characterized the microbial communities in the wood matrix and fecal pellets by analyzing the bacterial 16S rRNA gene targeted by 454 pyrosequencing. In addition, we analyzed bacteria in *Xylophaga* gills to test if they relate to wood communities.

## Materials and Methods

### Experimental set up

Traps with triplicate cubes (8×8×8 cm) of pine and triplicate cubes of oak wood were deployed along the axis of Blanes Canyon at 900, 1200 and 1500 m water depth. Additional traps of pine and oak cubes were deployed next to Blanes Canyon on the western outer slope at 1200, 1500 and 1800 m depth (Figure 1 and Table 1). The oak wood samples have been presented previously [17] but are included in Table 1 for a comprehensive overview. The traps were suspended 20 m above the seafloor. Cubes from 1200 m depth were collected in November 2009 after 9 months of immersion and samples from 900, 1500 and 1800 m depths were collected after 12 months of immersion. Pine cubes from Blanes Canyon and the outer slope were used for the analysis of microbial communities in the wood and in the fecal pellets of wood boring *Xylophaga* bivalves. Microbial wood communities in the oak cubes from Blanes Canyon have been analyzed previously but they were used here for comparative analyses of microbial fecal pellet communities in pine and oak, as the pellets in these oak tubes were not previously analyzed.

Immediately upon recovery of the cubes, wood chips to be used for microbial analysis were cut using sterilized tools, flash frozen in liquid nitrogen and kept at  $-20^{\circ}\text{C}$  until further processing. Fecal pellets were collected from *Xylophaga* burrows in pine and oak cubes. Fecal pellets from individual wood triplicates were separately frozen at  $-20^{\circ}\text{C}$ . Further, to estimate the % wood consumed, the rest of wood the cubes were carefully dissected by hand and all wood-boring bivalves were extracted and stored in 70% ethanol for taxonomic identification. To ensure that recently

**Table 1.** Overview of the different samples in the whole experiment.

Trap	Depth (m)	Duration (Months)	Location	Samples	Wood type	Matrix
BC 900	894	12	Canyon	BC Pine 900 m	Pine	Wood
				BC Pine 900 m	Pine	Pellet
				BC Oak 900 m*	Oak	Wood
				BC Oak 900 m	Oak	Pellet
BC 1200	1195	9	Canyon	BC Pine 1200 m	Pine	Wood
				BC Pine 1200 m	Pine	Pellet
				BC Oak 1200 m*	Oak	Wood
				BC Oak 1200 m	Oak	Pellet
				Bla1.2	Pine	Gills
				Bla10	Pine	Gills
BC 1500	1468	12	Canyon	BC Pine 1500 m	Pine	Wood
				PC Pine 1500 m	Pine	Pellet
				BC Oak 1500 m*	Oak	Wood
				Bla1.1	Oak	Gills
OS 1200	1184	9	Slope	OS Pine 1200 m	Pine	Wood
				OS Oak 1200 m*	Oak	Wood
OS 1500	1497	12	Slope	OS Pine 1500 m	Pine	Wood
				OS Oak 1500 m*	Oak	Wood
OS 1800	1806	12	Slope	OS Pine 1800 m	Pine	Wood
				OS Oak 1800 m*	Oak	Wood

Details on the locations of the traps used for pine and oak (Fagervold et al 2013) wood immersion experiments, as well as the samples taken from each trap. Abbreviations Bla 1.2, Bla 10, Bla 11 and Bla 1.1 refer to gills extractions from four *Xylophaga* sp. A individuals.

\*= Samples from Fagervold et al (2013) that were also used in this study.

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settled individuals were included, extraction was performed with the aid of a magnifier (2X) or a dissecting microscope. Shell length (SL) of each specimen of *Xylophaga* spp. was measured to the nearest 0.1 mm with digital calipers. The volume of each *Xylophaga* was calculated as the volume of a sphere with a radius equal to SL/2. Considering that each specimen created a burrow in the wood, the volume consumed by each *Xylophaga* was estimated to three times its volume. The % of wood consumed was estimated as the ratio between the volume occupied by *Xylophaga* spp. and the total volume of the wood cube.

No specific permissions were required for deploying submerged moorings in Blanes Canyon as it is not a protected area, moreover this study did not involve endangered or protected species.

### Wood and pellet DNA extraction, PCR and pyrosequencing

Procedures for DNA extraction, PCR and pyrosequencing were performed as described earlier [17]. Briefly, representative pieces from each wood cube used for analyses of microbial wood communities (Table 1) were powdered by bead beating (RETSCH Mixer Mill. Retsch, Inc. MM 301) using 25-ml grinder jars (Retsch, Inc. MM 400 Stainless steel) and 20-mm diameter stainless steel balls. The grinder jars were dipped into liquid nitrogen to keep the wood brittle. The fecal pellet material was already in powder form after drying. Approximately 100 mg of the powders from woods or fecal pellets were used to extract genomic

DNA with the Mobio PowerPlant kit (Ozyme, Saint-Quentin-en-Yvelines, France).

Initially, DNA extracts from individual triplicates of two selected treatments were amplified and sequenced: 1200 m pine wood chips and 1200 m pine pellet. Bacterial communities were then compared in a cluster analysis (see below), together with communities from oak wood obtained from a previous study from the Blanes Canyon [17]. The results revealed that community composition of individual triplicates were always more similar within than across treatments (Figure S1). We therefore pooled the triplicate DNA extracts from each treatment for further processing. This resulted in 6 pooled DNA extracts for wood (3 pine from the Blanes Canyon and 3 from the outer slope) and 5 pooled DNA extracts from fecal pellets (3 from pine and 2 from oak inside the Blanes Canyon) (Table 1).

A portion of the 16S rRNA gene was amplified by PCR using modified versions of universal bacterial 16S rRNA primers 27F (5'-AGRGTGTTGATCMTGGCTCAG-3') [23] and 519R (5'-GTVTTACCGCGGCTGCTG-3') [24] as described in previously [17]. Amplicons obtained with the 27F primer were modified at the 5' end by addition of the Roche 454 A-adaptor sequence and a 10-nucleotide identifier barcode (multiplex identifier, MID). Emulsion PCR and Roche 454 pyrosequencing (Genome Sequencer, FLX Titanium chemistry) were performed at the Genotoul platform of INRA, Toulouse (France).

## Dissection, DNA extraction, PCR and pyrosequencing of *Xylophaga* gills

Bacterial 16S rRNA gene sequences from *Xylophaga* gill bacteria originated from a separate study using a different sequencing approach. Four individuals of *Xylophaga* sp. A, which was the most abundant wood-boring bivalve in the Blanes Canyon pine and oak deployments [25] were dissected. Three individuals originated from pine (Bla1.2, Bla10, Bla11), one from oak (Bla1.1; Table 1). DNA was extracted from their symbiont-containing gills following the protocol of Zhou et al. [26]. Amplification and 454 pyrosequencing of ~480 bp long DNA fragments covering the V3 region of bacterial 16S rRNA genes was performed using primers bac339F (5'-CTCCTACGGGAGGCAGCAG-3') and bac815R (5'-TTGTGCGGGCCCCCGTCAATT-3') in a commercial laboratory (MR DNA, Shallowater, TX, USA). Bacterial DNA was amplified in a single-step PCR in which adaptors and barcodes were linked to the 5' region of the amplicons using the HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA). Amplification conditions were as follows: 94°C for 3 min, 28 cycles at 94°C for 30 s, 53°C for 40 s, 72°C for 1 min, and a final elongation step at 72°C. Equal concentrations of all PCR products were combined and purified with Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Combined samples were sequenced with a Roche 454 FLX titanium instrument and reagents, following the manufacturer's procedures.

Attempts to amplify bacterial 16S rRNA genes from dissected gut tissue with the general bacterial primers 8F and 1492R [27] were unsuccessful and analysis of microbial gut communities was not further followed.

## Sequence data analyses

All reads that had mismatches to the 16S rRNA primers, contained ambiguous nucleotides (N) or were shorter than 270 nucleotides (excl. the forward primer) were removed. The remaining sequences were subjected to stringent quality trimming to remove reads containing  $\geq 3\%$  bases with Phred values  $< 27$  (0.2% per-base error probability). This minimizes the influence of erroneous reads when clustering at 97% for OTU definition [28,29]. Sequences were then de-replicated and clustered at a 97% threshold using Uclust [30]. Sequences from each OTU were classified by comparison to the Greengenes database [31]. Read quality filtering and length trimming, dereplication, clustering at 97% sequence identity, taxonomic classification and dataset partitioning based on barcodes were conducted with Pyrotagger [32]. The taxonomic affiliations of the most abundant OTUs ( $> 1\%$  of the sequences) were further verified against sequences from the NCBI databases using BLAST [33]. To compare bacterial communities for diversity analysis, all samples were randomly resampled to the size of the sample containing the fewest sequences ( $n = 798$ ) using Daisy Chopper [34]. Calculation of the Shannon diversity index ( $H'$ ) and cluster analysis were performed using the software PAST [35]. A similarity percentage analysis, SIMPER [36], was conducted to identify the phylotypes contributing the most to the dissimilarity between different samples. Sequences have been submitted to MG-RAST (<http://metagenomics.anl.gov/linkin.cgi?project=5773>) for public availability.

Because the 16S rRNA genes of free-living bacteria and gill bacteria from *Xylophaga* were amplified with different primer pairs, the obtained sequences did not cover identical gene fragments. However, the overlap included the entire hypervariable V3 region (*E. coli* positions 433–497) that is widely used in phylogenetic studies using next generation sequencing methods, and this region yields sufficient information for a direct comparison between

wood, pellet and gill communities of *Xylophaga*. All sequences were therefore realigned and an OTU table based upon 100% sequence identity built from the common overlapping region of 80 bp. The analysis was done in mothur [37] using the Silva SEED database provided as a reference alignment.

## Network association

A network analysis was conducted to characterize the relationships among bacterial OTUs as described earlier [38]. Maximal information-based nonparametric (MINE) statistics were applied by computing the maximal information coefficient (MIC) between each pair of OTUs [39]. MIC captures associations between data and provides a score that represents the strength of a relationship between data pairs. A matrix of MIC values  $> 0.5$  and corresponding to positive linear correlations was used with Cytoscape 2.8.3 to visualize the network of associations [40]. In these visualizations, bacterial OTUs are represented as nodes and are connected by lines that are proportional in length to the MIC value. The force-directed layout based on the Fruchterman-Rheingold Algorithm [41] was edge-weighted by the MIC value.

## Results

### Wood degradation and bacterial diversity

Pine wood showed different levels of degradation depending of the location, wood loss being higher in BC than in OS (18.3 vs 2.8% in average, respectively) (Figure S2). *Xylophaga* spp. had degraded large inner parts of the cubes, leaving their burrows filled with wood pellets (Figure 1, insert). Sequencing of the bacterial 16S rRNA gene originating from wood and pellet yielded a total of 55 874 quality checked sequences. The amount of sequences varied among samples but rarefaction analysis (Figure S3) shows that the sequencing effort was not exhaustive. Bacterial community diversity was significantly higher in the wood immersed in Blanes Canyon compared to wood deployed on the open slope (Figure S3), as estimated with the number of OTUs and the Shannon index (t-test,  $p = 0.01$ , Table 2). On the other hand, the bacterial diversity found in the pellets filling the burrows of *Xylophaga* spp. from the canyon did not differ significantly from that in the wood matrix ( $p = 0.09$ ).

### Bacterial community composition in the wood

Cluster analysis at the OTU level based on a Bray Curtis distance matrix showed that bacterial communities could be separated in two main groups: pellet and wood communities (Figure 2). Within the wood samples, communities grouped according to location. All samples from Blanes Canyon grouped together and were separate from the open slope samples. Further, a comparison of sequences from this study with oak wood sequences from Fagervold et al. [17] showed that pine wood communities were different from oak wood communities (Figure S1). This was also true when comparing the pellet samples only, in that they grouped according to wood type. Depth was not a structuring factor for the community composition in pellets (Figure 2).

The microbial composition differed at high taxonomic level (phylum/class level) (Figure 3). Pine wood samples contained more *Alphaproteobacteria*, *Planctomycetes* and *Deltaproteobacteria* while pine pellet communities contained more *Gammaaproteobacteria* and *Bacteroidetes* (Figure 3). Regarding wood samples, canyon communities harbored less *Alphaproteobacteria* and more *Deltaproteobacteria* than the open slope. Concerning pellet samples, oak contained more *Alphaproteobacteria* than pine. In turn, gill communities were dominated by *Gammaaproteobacteria* sequences with the exception of

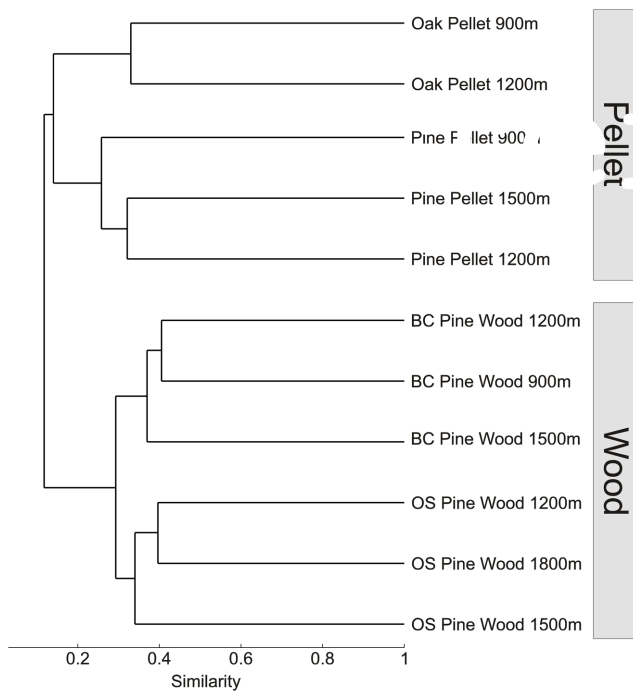
**Table 2.** Alpha diversity.

Sample	Subsampled (n = 798)				
	OTUs	H'	Chao-1	Cov	
Wood	OS Pine 1200 m	169	4.37	259	91
	OS Pine 1500 m	166	4.06	284	90
	OS Pine 1800 m	174	4.46	263	91
	BC Pine 1200 m	229	4.81	399	85
	BC Pine 1500 m	251	4.93	434	83
	BC Pine 900 m	244	4.85	456	83
Pellet	Pine 1200 m	202	4.45	303	89
	Pine 1500 m	142	3.84	192	93
	Pine 900 m	235	4.65	491	83
	Oak 900 m	194	4.51	286	89
	Oak 1200 m	127	4.13	156	95
Gills	Bla1.1	131	3.81	313	91
	Bla10	66	1.69	86	97
	Bla11	39	2.58	41	99
	Bla1.2*	7	0.57	7	99

Number of sequences and diversity of wood, pellet and gill samples from Blanes Canyon (BC) and its open slope (OS). H': Shannon index, Chao-1: Chao true diversity estimator, Cov: coverage.

\*not subsampled (n = 263).

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**Figure 2. Sample clustering.** Dendrogram based on Bray-Curtis distance representing the similarity between bacterial communities sequenced from the wood matrix and from burrow pellets obtained from wood immersed at various depths in Blanes Canyon (BC) and its adjacent open slope (OS).

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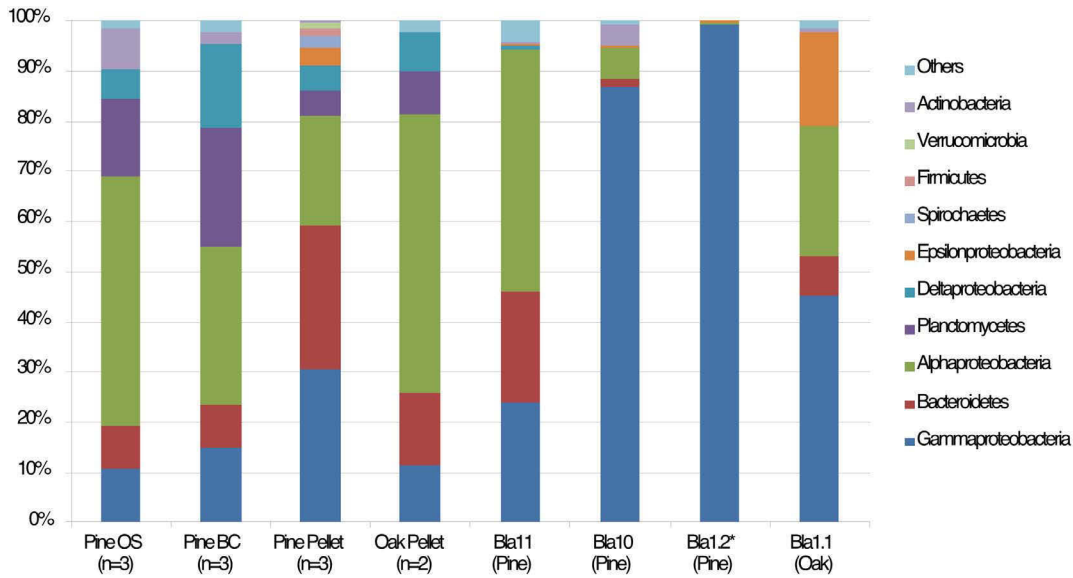
the sample collected from oak, Bla11, which contained more *Alphaproteobacteria* (Figure 3).

**OTU co-occurrence and taxonomy in the wood**

Microbial communities in pine pellets from Blanes Canyon shared very few OTUs with wood samples from the same wood cubes, and the few OTUs in common were not abundant (Figure 4). Among the typical pine pellet OTUs, the most abundant were OTU 31, a *Gammaproteobacterium* distantly related to unpulshed sequences from a marine biofilm, followed by OTU 13, a *Bacteroidetes* distantly related (90%) to algae associated communities, a *Gammaproteobacterium* (OTU 12) distantly related (93%) to sequence from sunken wood [42], and an *Epsilonproteobacterium* (OTU 15) previously detected as abundant in sequences cloned from pine pellets (OTU6, [19]). Among less abundant pellet OTUs, one *Gammaproteobacterium* (OTU 83) exhibited 99% similarity to a symbiotic bacterium from the Teredinidae *Lyrodus pedicellatus* [43].

Pine pellet communities had more in common with those from oak pellets than from the pine wood. Nevertheless, the oak pellets contained more *Alphaproteobacteria* closely related to coral tissue clones [44,45] (OTU 21) (97%). Among the OTU shared between pellet samples, many belonged to *Bacteroidetes* often distantly related to sponge bacteria (OTU 62 and 77, 94–95% similarity, [46] or deep sediments (OTU 45, 95% similarity, [47]).

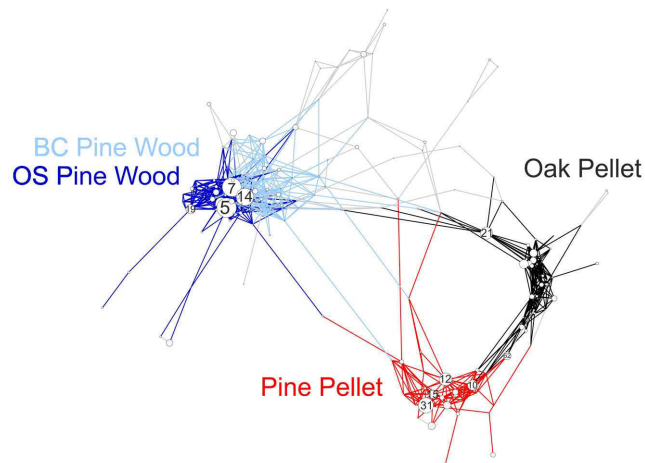
On the other hand, communities from wood in the canyon shared many OTUs with open slope communities, as illustrated by the short distance separating these samples in the network (Figure 4). In pine wood from the canyon, an abundant *Planctomycetes* OTU (OTU 14) was 100% similar to sequences found in the digestive tract of a chiton, a wood associated mollusk [42], and it was 99% identical to digestive tract bacteria found in a sea urchin from a wood fall [48]. Further, an abundant



**Figure 3. Distribution of bacterial taxa.** Relative proportion of the most abundant bacterial phylum or class (>1% of the sequences) found in wood, pellet and gill (Bla) samples from Blanes Canyon (BC) and its adjacent open slope (OS). \* = contained significantly less sequences than the rest of the samples (n = 263). doi:10.1371/journal.pone.0096248.g003

*Deltaproteobacterium* OTU (OTU 51) was closely related (99%) to clones found in sunken wood [42]. A less abundant *Gammaproteobacterium* was distantly related to *Teredinibacter turnerae*, an intracellular endosymbiont of shallow water marine wood-boring Teredinidae [49]. In wood from the open slope, the most abundant OTU (OTU 5) belonged to the *Rhodobacterales* order of *Alphaproteobacteria* and was 100% similar to a sequence found

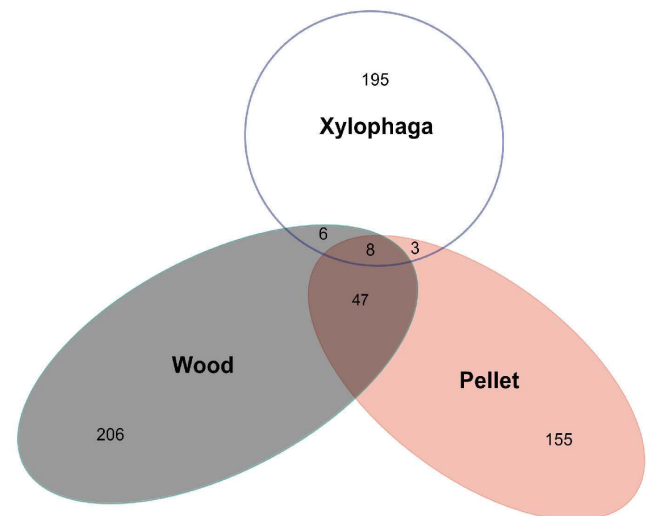
earlier in oak wood from Blanes Canyon (Blanes 1043, [17]) and distantly related (91%) to bacteria found in guts of the marine wood-feeding gastropod *Pectinodonta* sp. (Patellogastropoda, Mollusca) [50] and the chiton *Nierstraszella lineata* [42]. Another abundant OTU (OTU 7) was identified as a *Gammaproteobacterium* related to clones from gorgon or seafloor lava (96%). This OTU was not detected in previous oak wood samples from Blanes Canyon.



**Figure 4. OTU network.** Network showing associations between OTUs found in wood and pellets from wood immersed at various depths in Blanes Canyon (BC) and its adjacent open slope (OS). OTUs are represented as nodes (white circles) in the network and the size of each node is proportional to the number of sequences contained in each OTU. Lines connect the nodes that are the most correlated (MIC values > 0.5). The identifying numbers of the most abundant OTUs are written in the nodes. Colors highlight associations between the OTUs that most explain the differences between groups of samples (Oak pellet, Pine pellet, BC Pine wood and OS Pine wood). OTUs that best explain differences between samples were identified by a SIMPER analysis. doi:10.1371/journal.pone.0096248.g004

**Xylophaga gill community**

A total of 8260 sequences were obtained after quality check from the four individuals of *Xylophaga* (Table 1). The number of sequences varied between samples as did the diversity, but the gill



**Figure 5. Venn diagram representing bacterial OTUs unique and common to the wood samples, the pellet and the *Xylophaga* gills.** doi:10.1371/journal.pone.0096248.g005

community from *Xylophaga* was always less diverse than wood or pellet communities (Table 1). Bacteria from gills of *Xylophaga* were different from those found in wood and pellets (Figure 5). Only 17 *Xylophaga* OTUs (8%) were found in the pellets or in wood while pellets and wood shared 55 of their OTUs (21 to 26%) (Figure 5).

Many sequences from the gills of *Xylophaga* were closely related to those of symbiotic bacteria from shipworms. In specimen Bla1.2, at least 96% of the sequences were from possible symbionts. The most abundant OTU, which represented 84% of the sequences, was 99% similar to a sequence of a *Gammaproteobacterium* from the teredinid shipworm *L. pedicellatus* (Clone RT14, [43]). In Bla10, at least 75% of the sequences were from possible symbionts. The most abundant OTU (73%) was 95% similar to the 16S rRNA gene of the T7901 strain of the *Gammaproteobacterium* *T. turnerae* [49]. In Bla11, more than 45% of the sequences were possible symbionts. The most abundant OTUs were less dominant than in the other *Xylophaga* specimens with only 24% of the sequences distantly affiliated to a symbiont of *L. pedicellatus* (Clone RT20, [43]). For specimen Bla1.1, we probably failed to amplify specific gill symbionts as the most abundant OTU related to symbionts from Teredinidae represented only 5% of the sequences.

## Discussion

The incubation of wood pieces in Blanes Canyon and its adjacent open slope showed that the boring activity of *Xylophaga* sp. A transformed the wood environment by creating distinct niches for bacterial communities. The communities associated with the gills of *Xylophaga* were different and probably composed of symbiotic bacteria, while the niche created by the pellets promoted the development of bacterial communities that were very different from those in the wood matrix. The large differences observed at phylum and OTU levels suggest that the pellet populations represent a distinct community rather than a subset of the wood communities.

The accumulation of pellets consolidated with mucus is typical for *Xylophaga* [13]. While Teredinidae expulse the products of their wood boring activity from their burrows, *Xylophaga* species most often form chimneys of compacted fecal pellets around their siphons, thus lining the bored tunnels. These pellets have a very different chemical composition compared to wood, as up to 80% of the wood cellulose may be lost during digestion by *Xylophaga* spp. [51]. Pellets may thus form a new substrate for microorganisms that is depleted in cellulose but enriched in mucus, in comparison to wood. Alternatively, the community found in pellets might be similar to that found inside the gut of *Xylophaga*. These animals ingest the wood shavings produced by their shell, which they use as rasp to dig burrows. Wood particles are then stored in a large caecum before passing through the stomach and intestine. However, previous studies found few microbes in the caecum of *Xylophaga* [14] and Teredinidae [52] while large numbers of bacteria occurred in the fecal pellets in the intestines of Teredinidae [52]. It is not known if bacterial communities follow the same spatial distribution in the digestive tracts of *Xylophaga*, but similar life strategies and common evolutionary history of the two wood-boring bivalve groups [53] may suggest so. Bacteria colonizing the pellets may thus exit the digestive tract with the excreted feces and survive in the burrows. However this hypothesis could not be verified in this study, as the gut microbiota was not analyzed. Further, communities from pine pellets were different from those in oak pellets. This difference could be due to the presence of a second species, *Xylophaga* sp. B, that was predominantly colonizing pine while it was rare in oak [25]. It is possible

that this species has different gut microflora than the ubiquitously abundant *Xylophaga* sp. A [25] and this could influence the composition of pellet communities in pine and oak. However, it is also possible that the composition of the wood itself is controlling the composition of pellet communities. Remarkably, we could not identify known cellulolytic strains among the bacteria detected in the fecal pellets. However, since cellulolytic pathways are widespread over many bacterial phyla, it cannot be excluded that the uncultured bacteria we detected may be able to degrade cellulose. One metabolic pathway that might be inferred from the identified OTUs is fermentation. OTU 17 shares 98% sequence identity to the fermentative Alphaproteobacterium *Polymorphum gilvum* [54], and this close relationship may be an indication that fermentative bacteria can take advantage of the wood remains.

We detected several abundant OTUs in the gills of *Xylophaga* sp. A closely related to sequences from bacteria associated to wood-boring Teredinidae. The very close similarity (99%) of these OTUs to symbionts from the shipworm *Lyrodus pedicellatus* is a strong indication that we were able to detect gill symbionts from *Xylophaga* sp. A. Bacteria had been observed earlier in gills of *Xylophaga* [14] but they have never been isolated or taxonomically characterized. Further, the most abundant OTU in *Xylophaga* sp. A matched the symbionts of the most abundant phylotype (clade P3) in the Teredinidae *L. pedicellatus* [43]. We also detected sequences related to the cultivated shipworm endosymbiont *Teredinibacter tumerae*, a cellulolytic *Gammaproteobacterium* that has been isolated from many teredinid host species [12]. The presence of similar sequences in deep-sea and shallow environments indicates that wood-boring endosymbionts may be adapted to a wide range of environments and hosts.

The location of the sunken wood, a factor that is linked to different levels of wood degradation, clearly shaped the community composition in the wood matrix. Wood deposited in the canyon, which was largely and more rapidly degraded and colonized by the wood-boring bivalves, harbored different and more diverse communities than wood in the open slope. Canyons channel the transport of organic matter from the continental shelf to the deep sea, which is specially enhanced during dense shelf water cascading events [55]. This process in combination with frequent storms from easterly directions in the western Mediterranean area and offshore convection appear to be the main drivers for the transfer of organic matter to the deep Mediterranean Sea [56,57] and they may in particular contribute to high benthic biomass and productivity that make the hot spot character of submarine canyon ecosystems [21]. Our results thus extend to the microbial world the view that submarine canyons are sites of enhanced marine diversity. The difference in bacterial community composition between woods immersed inside and outside the canyon may also be due to different sources of microbes colonizing that wood. Allochthonous bacteria could be transported to the canyon as a result of ecosystem forcing events, which may lead to the establishment of different communities in the canyon water column or sediments compared to the open slope. However, we cannot discard the possibility that the difference in bacterial communities between locations are linked to the different phases of the wood decomposition process. The pine wood communities where characterized by a large proportion of *Alphaproteobacteria* followed by *Planctomycetes* and *Gammaproteobacteria*. These classes of bacteria were also observed previously in oak wood [17], emphasizing their role in wood degradation. However, there were differences between pine and oak at the OTU level. The most abundant pine OTUs were absent in oak, like the *Gammaproteobacterium* OTU 7, or less abundant, as in the case of the



*Alphaproteobacterium* OTU 5. Some pine OTUs were also 100% similar to sequences found on sunken wood, inside a chiton gut [42], and in a sea urchin collected around the Vanuatu Island [48]. This suggests that some bacteria associated with sunken wood are distributed worldwide. The presence of these bacteria in the guts of different wood-ingesting animal species suggests that they may either be associated to a large variety of macroorganisms or that they may represent globally distributed sunken-wood microorganisms that are ingested by the animals together with the wood.

## Supporting Information

**Figure S1 Clustering of all samples.** Hierarchical clustering using the Bray Curtis index showing the similarity of the microbial communities between the different samples.

(PDF)

**Figure S2 Wood consumption.** Percentage of consumed pine wood after immersion at various depths in Blanes Canyon (BC)

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# Supplementary material

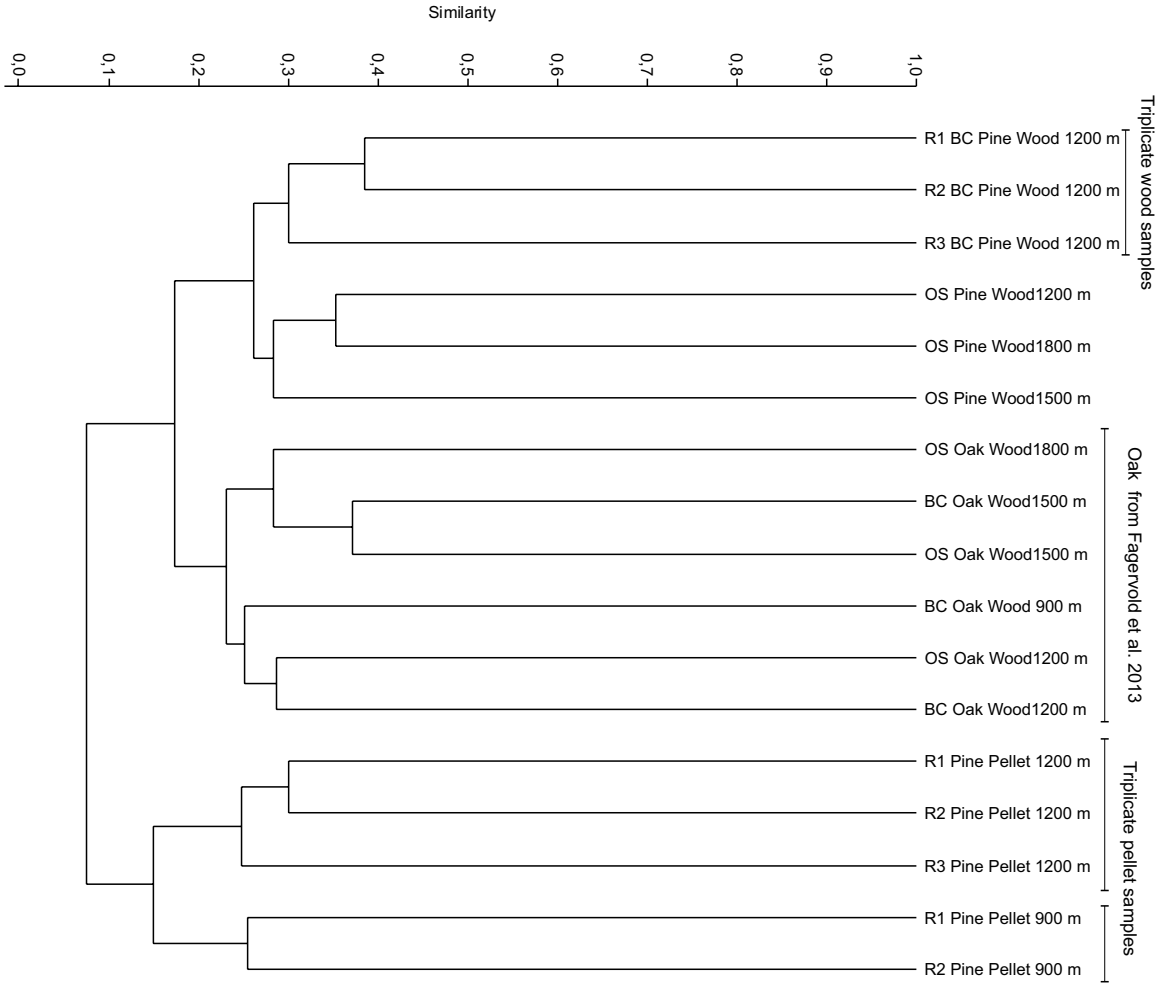


Figure S1 :Hierarchical clustering using the Bray Curtis index showing the similarity of the microbial communities between the different samples.

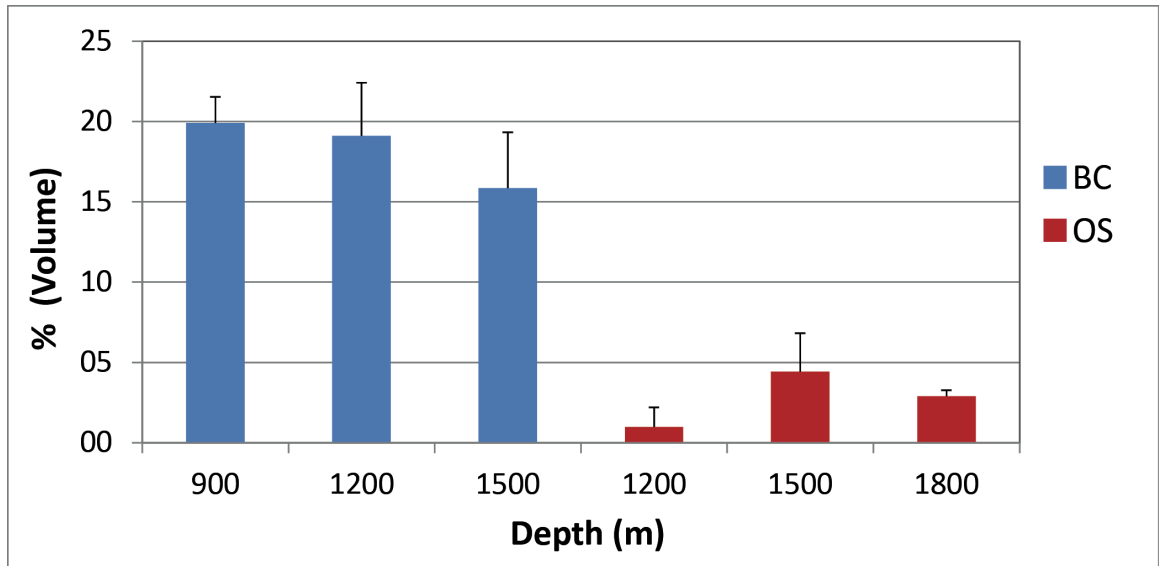


Figure S2 :Percentage of consumed pine wood after immersion at various depths in Blanes Canyon (BC) and its adjacent open slope (OS), expressed as percentage of the initial volume of the respective wood cubes.

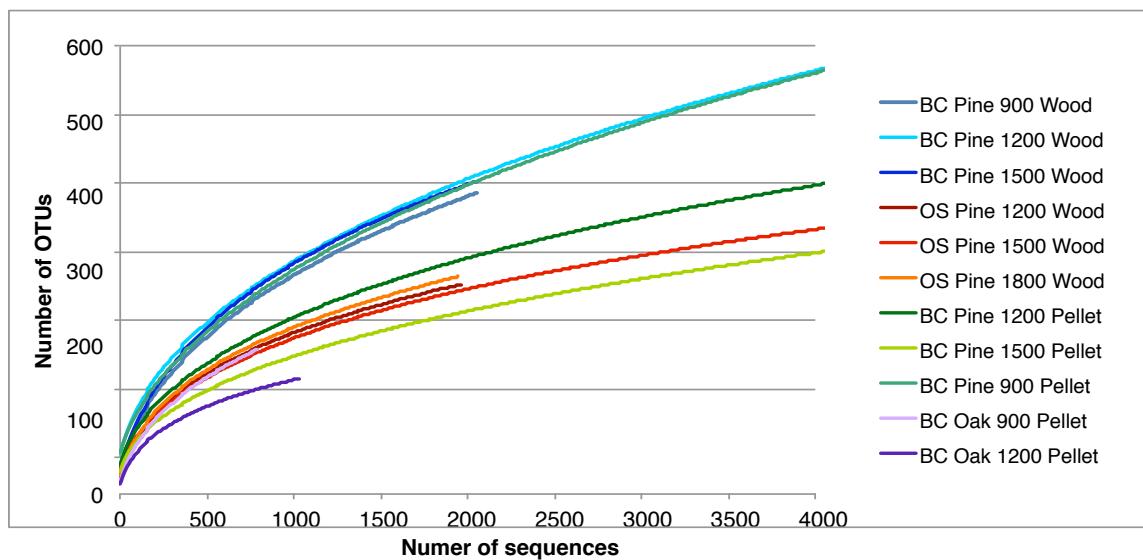


Figure S3 :Number of sequences versus OTUs formed for all sequenced samples, except the gill samples. Broadly, blue lines represents BC Pine Wood, redish lines OS Pine Wood, greenish lines Pine Pellet sampels and purple lines oak pellets.

## Discussion et perspectives

### La communauté microbienne associée au bois coulé

#### (Article I)

Avant ce travail, l'étude de la communauté microbienne associée au bois coulé s'intéressait principalement à l'observation des microorganismes après 9 mois au minimum d'incubation. Ces travaux préliminaires ont mis en évidence grâce à l'utilisation de la microscopie et du séquençage, que la dégradation des cellules du bois variait en fonction de l'essence et du temps d'immersion (Fagervold *et al.*, 2013, Palacios *et al.*, 2009). Cette variation temporelle peut être liée à une succession écologique comme celle observée lors de la dégradation des carcasses de baleine (Smith *et al.*, 2003). Du fait, nous émettons l'hypothèse qu'une succession écologique a lieu lors de la dégradation du bois.

Afin de pouvoir étudier la dynamique de dégradation du bois, nous avons utilisé une expérimentation en aquarium permettant un accès facile aux échantillons. En se basant sur la composition des communautés, nous avons vu que celles présentes dans l'aquarium étaient différentes de celles présentes dans le canyon. Les raisons de ces différences sont multiples et peuvent notamment être liées à l'absence de sédiment dans les aquariums ou encore au type de faune colonisant les bois (*Xylophaga sp.* dans le canyon et *Tereneidae* dans l'aquarium). Une telle variabilité de communauté avait déjà été observée entre des bois incubés à l'intérieur et à l'extérieur d'un canyon et avait été liée à l'activité des bivalves foreurs, plus importante dans le canyon (Fagervold *et al.*, 2013).

Grâce à la comparaison entre un angiosperme (*Quercus suber*) et un pinophyte (*Pinus pinea*), nous avons confirmé qu'il existait une communauté microbienne associée au bois coulé (Palacios *et al.*, 2009; Fagervold *et al.*, 2012; Rämä *et al.*, 2014) et que celle-ci était influencée par la composition et la structure (Fengel and Wegener, 1984) (Sjostrom, 1993) de chaque essence d'arbre .

Malgré ces différences entre essences, tous les bois incubés en aquarium indépendamment de leur essence sont colonisés par une communauté capable de produire, à la surface du bois, du sulfure d'hydrogène mesuré après seulement 21 jours. Le sulfure d'hydrogène est essentiel à la mise en place du système chimiosynthétique. En effet, ce composé réduit permet aux bactéries associées aux bivalves présents sur les bois coulés (Duperron *et al.*, 2008), de produire l'énergie nécessaire à la fixation du dioxyde de carbone. Le dioxyde de carbone sera

alors transféré à l'animal hôte permettant à ce dernier de vivre sans apport de carbone fixé avec l'énergie solaire.

Nos données ont permis d'associer la présence de ce sulfure d'hydrogène à une population de bactéries appartenant à la famille des *Desulfovibrionaceae* dans les échantillons de pin, et des *Desulfobacteraceae* dans les échantillons de chêne. Les membres de ces deux familles possèdent la capacité de réduire les sulfates en sulfure d'hydrogène (Kuever, 2014b) (Kuever, 2014a) démontrant que dans ce cas, la composition de la communauté change mais que la fonction dans l'écosystème reste la même. Le sulfure d'hydrogène est un produit de l'utilisation du sulfate (présent dans l'eau de mer) comme accepteur terminal d'électrons par les bactéries sulfato-réductrices (Sørensen *et al.*, 1981; Lutz Ehrlich and Newman, 2008) au centre du morceau de bois. Le donneur d'électrons, dans le cas des bactéries sulfato-réductrices, est le plus souvent un composé organique dérivé de la fermentation comme l'acétate (Khelaifia *et al.*, 2011; Sørensen *et al.*, 1981). Toutefois, les résultats obtenus lors de cette première expérience ne nous ont pas permis de savoir si les composés organiques simples utilisés par les bactéries sulfato-réductrices provenaient de la dégradation de la cellulose ou de composés plus simples appartenant à la fraction libre (non pariétale) du bois. Les bactéries abondantes susceptibles d'être à l'origine de la fermentation de la cellulose et des hémicelluloses sont les *Bacteroidetes* comme pressenti dans des études précédentes (Fagervold *et al.*, 2012; Landy *et al.*, 2008; Kirchman, 2002). Néanmoins, afin de confirmer le potentiel de ces unités taxonomiques opérationnelles, il faudrait pouvoir être capable de séquencer leur metaprotéome afin de savoir si les enzymes liées au cellulosome sont présentes. Le cellulosome est un complexe protéique permettant aux bactéries (notamment des membres des *Bacteroides*) de dégrader la cellulose dans un milieu anaérobie (Flint *et al.*, 2008) comme le bois (Yücel *et al.*, 2013). Sans ce complexe extracellulaire, les bactéries ne seraient pas capables de fixer la cellulose et de la dégrader. Lors de la dégradation de la cellulose et des hémicelluloses par le cellulosome, des polymères de glucose sont importés dans la paroi pour être fermentés (Flint *et al.*, 2008). Toutefois, des bactéries ne possédant pas ce complexe peuvent utiliser les polymères de glucose avant que ceux-ci ne soient incorporés par la cellule cellulolytique (Singh and Butcher, 1991; Björdal *et al.*, 2000). Ces bactéries opportunistes pourraient correspondre notamment au *Spirochaetes* et au *Vibrionaceae*, détectés lors de notre expérience en aquarium.

La détection d'une première phase sulfurique après seulement 1 mois contredit la thèse actuelle selon laquelle la première phase sulfurique apparaîtrait après 6 mois - 1 an d'incubation et serait dépendante de la présence de bivalves foreurs (Bienhold *et al.*, 2013).

La raison de cette contradiction est que l'étude de Bienhold et al. a inférée le fonctionnement global du système à partir d'observations faites après 1 an d'incubation, rendant impossible la détection de cette première phase.

Cette étude a permis de montrer que le bois est un substrat possédant plusieurs microniches et par conséquent, nous recommandons aux futures études sur le bois coulé de tenir compte de cette variabilité en indiquant précisément le lieu d'échantillonnage à l'échelle de l'échantillon. De façon extensive, nous pensons qu'une telle variabilité intervient également dans les autres systèmes chimiosynthétique profonds et qu'une attention particulière doit être portée lors de la mise en place d'un plan d'échantillonnage afin d'éviter une mauvaise interprétation du système. Cette variabilité observée dans les échantillons de bois coulé est liée à un gradient à la fois vertical (dans le sens des vaisseaux conducteurs) dû à la pénétration des composés inorganiques de l'eau de mer, et latéral (perpendiculairement au sens des vaisseaux conducteurs) dû à la lignification et au vieillissement progressif des vaisseaux conducteurs (Sjostrom, 1993).

En nous basant sur la présence d'un gradient latéral, nous émettons l'hypothèse que la première vague de colonisation bactérienne opportuniste dégrade les composés labiles des cellules vivantes du parenchyme et du cambium, provoquant la diminution des lipides et une production de composés dérivés de la fermentation tels que l'hydrogène et l'acétate. Ces dérivés de fermentation sont alors rapidement utilisés par une population de bactéries sulfato-réductrices réduisant les sulfates en sulfure. Par la suite, comme développé dans la discussion de l'article, le recrutement de macrofaune (foreurs) et de microfaune (champignons Ascomycetes et Bactéries potentiellement cellulolytiques) provoque une diversification des niches écologiques. Cette diversification induit une augmentation de la diversité phylogénétique traduisant une potentielle diversification des métabolismes qui atteint son maximum après 120 jours dans le cas du pin et 240 jours dans le cas du chêne. Ce retard observé pour le chêne peut être lié à une plus grande difficulté des larves de foreurs à attaquer le chêne que le pin (Voight, 2015) et par conséquent, cela retarderait cette phase de diversification des communautés bactériennes.

## L'effet des foreurs sur la communauté microbienne (Articles II et IV)

Nous avons vu que la structure et la chimie du bois avaient une influence sur la composition des communautés microbiennes. Cependant, le rôle joué par la colonisation des bivalves foreurs ainsi que leur impact sur la communauté restent incertains. Afin de valider les observations faites en aquarium, nous nous sommes focalisés sur la première phase d'apparition de l'hydrogène sulfuré décrite par Yücel *et al* (2013) et dont nous avons analysé la communauté associée. Grâce à une expérience d'exclusion, nous avons empêché la colonisation du bois par les foreurs pendant les deux premiers mois d'incubation en milieu naturel dans le canyon. Nous avons montré que les foreurs, par leur présence dans les premiers centimètres du bois, favorisaient le recrutement de communautés bactériennes sulfatoxydantes à l'intérieur du bois. Ce résultat va à l'encontre des hypothèses développées à partir de bois incubés une année ou plus (Voight, 2015; Bienhold *et al.*, 2013). Ces études supposaient que les foreurs, en accumulant des résidus de dégradation dans leur tunnel, aboutissaient à la mise en place de niches anoxiques au bout de 6 à 12 mois. Grâce à cette expérience, nous avons montré qu'en milieu naturel, les bivalves xylophages facilitaient au contraire la pénétration de l'oxygène dans le bois induisant ainsi la mise en place d'un système chimiosynthétique à l'intérieur du bois. Cette expérience a aussi mis en évidence que, comme observé dans l'aquarium, après 1-2 mois, la communauté du centre des échantillons de pin est dominée par un Otu assigné à la famille des *Vibrionaceae*. Grâce au métagénome de la communauté du centre du bois, nous avons découvert que ce vibrio ne possédait pas d'enzymes connues liées à la dégradation de la cellulose. Toutefois, celui-ci possédait toutes les enzymes indispensables à la dégradation de composés tels que le sucrose ou le glucose. Cette découverte renforce l'hypothèse qu'une première dégradation des composés les plus labiles du bois soutient la production d'hydrogène sulfuré par les bactéries sulfato-réductrices. Nous émettons l'hypothèse qu'une fois les composés labiles utilisés, le rôle des *Xylophaga sp.* devient primordial dans le maintien du système chimiosynthétique. En effet, la comparaison entre des échantillons de bois non forés et les fèces des foreurs montre que les bactéries sulfato-réductrices ainsi que des *Bactéroidetes* et des *Alphaproteobactéries* (connues pour être des bactéries fermentatrices) sont recrutées préférentiellement dans les tunnels remplis de fèces que dans le bois non dégradé par les foreurs. Les fèces contenaient également des Otu proches des symbiontes de térebrant (Luyten *et al.*, 2006). Cette découverte est assez



surprenante car on sait que les symbiontes des térébrants sont stockés dans les branchies (Distel and Roberts, 1997) et si le fonctionnement est le même chez les *Xylophaga sp.* que chez les térébrants, seules les endoglucanases produites par les symbiontes se retrouvent en contact avec les fèces (O'Connor *et al.*, 2014). Le fait de retrouver ces symbiontes dans les fèces de *Xylophaga sp.* pourrait indiquer que chez cette espèce, les symbiontes sont cultivés dans la lamelle intermédiaire des branchies (Distel and Roberts, 1997) puis transférés dans le caecum afin de digérer le bois. Enfin, ces bactéries seraient rejetées avec les composés non assimilés par le bivalve. De plus amples études sur la biologie de ce bivalve, notamment en aquarium, permettraient de comprendre comment les symbiontes présents dans les branchies sont utilisés lors de la dégradation du bois.

L'écosystème sulfurique bois coulé est donc initié par une population bactérienne s'appuyant vraisemblablement sur les composés labiles dès les premiers mois d'immersion. Puis, une fois ces composés utilisés, c'est l'action des Xylophages qui va maintenir la présence de dérivés de dégradation de la cellulose via leurs fèces. La fermentation de ces composés produira alors des donneurs d'électrons, comme l'acétate utilisée par les communautés sulfato-réductrices. Afin de valider définitivement cette étude, il faudrait pouvoir doser les composés labiles (Zule and Može, 2003) ainsi que les composés pariétaux (cellulose hémicellulose et lignine) (Kaar *et al.*, 1991) (Dence, 1992) et suivre leur dégradation au cours du temps. De plus, l'isolement de ce taxa bactérien dominant dans le centre du bois permettrait de tester sa capacité à dégrader la cellulose. Si tel était le cas, nous serions face à une nouvelle enzyme de dégradation absente à l'heure actuelle des bases de données.

## **Le développement d'un biofilm chimiosynthétique (Article III)**

Le développement de biofilm microbien a été documenté dans l'ensemble des systèmes chimiosynthétiques profonds associés à la production de composés réduits des carcasses de baleine (Smith and Baco, 2003) aux sources hydrothermales (Alain *et al.*, 2004; CD Taylor *et al.*, 1999), en passant par les zones de suintements froids (Grünke *et al.*, 2011; Gentz and Schlüter, 2012). Le point commun à ces environnements est la présence d'une interface où se rencontraient un fluide réduit et l'eau de mer. Comme nous l'avons vu précédemment, le bois représente une source de sulfure d'hydrogène actif après seulement 1 mois d'immersion. La présence d'un biofilm blanc sur des bois immergés a été noté lors des campagnes effectuées

par le LECOB. Toutefois, les tentatives de remonter ces biofilms n'ont jamais abouti en raison du lessivage de ces derniers lors de leur remontée. Grâce au dispositif expérimental en aquarium, nous avons pu montrer que la colonisation de la surface du bois par un biofilm au cours du premier mois d'immersion était reproductible.

De plus, grâce à l'utilisation de la métagénomique, nous avons mis en évidence une transition entre deux populations d'*Arcobacter*. La première population colonisant le bois serait chemolithohétérotrophe, c'est-à-dire que sa source d'énergie est inorganique (en l'occurrence l'oxydation de l'hydrogène sulfuré en soufre élémentaire) et que la source de carbone, elle, est organique et proviendrait du bois. La seconde, apparaissant après 45 jours, serait chemolithoautotrophe et fixerait le carbone inorganique dissous ( $\text{CO}_2$  et  $\text{HCO}_3^-$ ) présent dans l'eau. Cette fixation du carbone est permise par l'utilisation du cycle de Krebs inversé (Hügler and Sievert, 2011; Hügler *et al.*, 2005) permettant, grâce à l'énergie issue de l'oxydation de l'hydrogène sulfuré, de fixer le carbone inorganique dissous. Afin de valider les fonctions associées au biofilm révélé par le métagenome, il faudrait utiliser une technique comme la NanoSIMS permettant de suivre l'incorporation de substrats marqués (Morono *et al.*, 2011) au sein des populations bactériennes. Nous émettons l'hypothèse que ce changement de communauté intervient en raison du développement d'une zone anoxique à proximité du bois hébergeant une communauté de bactéries sulfato-réductrices. Ces bactéries utilisant les composés organiques issus de la fermentation, rentrent donc en compétition avec les bactéries sulfo-oxydantes. Nous émettons l'hypothèse que cette compétition est à l'avantage des bactéries sulfato-réductrices car celles-ci occupent une niche anoxique au contact du bois et donc de la source de composés organiques. La présence de ces bactéries chimioautotrophes fait que le biofilm, en plus de devenir chimiosynthétique, joue un rôle dans la production de l'hydrogène sulfuré. Cette zonation a été validée grâce à des microprofils validant la présence d'une zone oxygène en surface du biofilm qui disparaît après *ca.* 1 mm, laissant place à du sulfure d'hydrogène augmentant au fur et à mesure que l'électrode s'approche du bois. Ce type de microprofils a déjà pu être observé dans d'autres biofilms formés par des *Arcobacter* (Grünke *et al.*, 2011). De plus, les biofilms chimiolithoautotrophes à *Arcobacter* sont connus pour être les premiers colonisateurs des parois des sources hydrothermales. Mais ce biofilm, en plus de son rôle dans la fixation du carbone, pourrait jouer un rôle essentiel dans l'écosystème bois coulé. En effet, il a été montré que la présence d'un biofilm à la surface d'un objet favorisait la colonisation larvaire (Yebra *et al.*, 2004). Dans le cas du bois, le type d'assemblage bactérien jouerait un rôle dans la détection du substrat bois par les larves (Pachu *et al.*, 2012). Nous pensons donc que la mise en place de ce biofilm est une des clés

pour la compréhension du mécanisme de détection du substrat bois coulé par les organismes liés à la dégradation du bois (les foreurs) mais aussi par les organismes possédant des symbiontes chimiosynthétiques (*Idas sp.*).

## Conclusion générale

Nous pouvons donc, sur la base de nos travaux, proposer une succession écologique des communautés bactériennes colonisant les bois coulés.

1. Lorsque le bois est immergé, la fraction labile du bois est fermentée par des bactéries probablement proches des *Bacteroidetes*, des *Vibrionaceae* et des *Spirochaetes* essentiellement au niveau du cambium.
2. Après *ca.* 30 jours, la population de fermenteurs de composés labiles soutient une population de bactéries sulfato-réductrices proches des *Desulfovibrionaceae* et des *Desulfobulbaceae*. Cette population engendre un dégagement d'hydrogène sulfuré aboutissant à la mise en place d'un biofilm opportuniste chimiolithohétérotrophe d'*Arcobacter*.
3. Après *ca.* 45 jours, une communauté de *Desulfovibrio* se développe dans la partie inférieure du biofilm et entre en compétition avec les *Arcobacter* chimiolithohétérotrophes du biofilm pour l'utilisation des produits de fermentation. Cette compétition est en faveur des *Desulfovibrio* et engendre le remplacement de la communauté chimiolithohétérotrophe d'*Arcobacter* par une communauté chimiolithoautotrophe d'*Arcobacter*. En parallèle, le développement d'un biofilm à la surface du bois favoriserait le recrutement des larves pélagiques de la macrofaune associée au bois.
4. Après *ca.* 85 jours, les terriers des Xylophages recrutés atteignent déjà 2.6 cm de profondeur, ce qui entraîne la formation de niches oxiques permettant le développement de bactéries potentiellement chimiosynthétiques dans les premiers centimètres du bois. Les fécès de Xylophages ajoutent de la matière labile au bois permettant le maintien de la production de sulfure d'hydrogène.
5. Après *ca.* 120-240 jours, en fonction de l'essence d'arbre, la diversité des niches entraîne une diversification de la communauté microbienne. On émet l'hypothèse qu'à ce stade, sont présents conjointement les Xylophages et des bactéries dégradant directement la cellulose des parois telles que des *Bacteroidetes* ou des *Clostridiales*. Les champignons, bien que présents au départ, semblent jouer un rôle mineur car ils sont essentiellement détectés lors des premiers mois de colonisation.

6. Nous émettons l'hypothèse que lorsque l'intégralité du bois est colonisée par les foreurs et qu'il n'y a plus de matériel à dégrader, les Xylophages meurent et entraînent un dernier apport de matière labile provoquant un dernier pulse d'hydrogène sulfuré. Cette hypothèse pourra être vérifiée par l'utilisation à long terme de capteurs in situ sensibles au sulfure d'hydrogène.

Le travail développé dans ce manuscrit a donc permis de mettre en évidence le rôle joué par les microorganismes lors de la mise en place de l'écosystème chimiosynthétique bois coulé. Nous avons pu montrer l'intérêt d'utiliser une approche ex-situ en laboratoire afin d'étudier les variations de la communauté microbienne à petite échelle temporelle, révélant des variations rapides de la diversité. L'intérêt de pouvoir disposer d'un système chimiosynthétique en laboratoire permettra à l'avenir d'approfondir les fonctions et les interactions des communautés constituant cet écosystème ainsi que de valider la succession écologique proposée dans cette thèse.

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Résumé :

Lorsqu'un morceau de bois atteint le fond de l'océan, il provoque la mise en place d'un écosystème capable de se développer en absence de lumière. Cet écosystème est qualifié de chimiosynthétique du fait de la présence d'une faune pouvant fixer le carbone inorganique présent dans l'eau de mer. De plus, ce système attire une faune ultra-spécialisée qui utilise des symbiotes bactériens pour digérer le bois. Avant ces travaux, la plupart des études s'intéressaient principalement à la macrofaune et le rôle des microorganismes libres demeurait inconnu. Nous avons pu démontrer dans cette thèse le rôle essentiel que jouent les microorganismes libres dans la mise en place de cet écosystème. Nous avons prouvé que des communautés de microorganismes se succédaient au cours de la première année de colonisation et que cette succession était influencée par le type de bois et l'environnement dans lequel il se trouve. La première phase de cette succession aboutit au développement après un mois, d'une population de bactéries sulfato-réductrices produisant de l'hydrogène sulfuré et ce, même en l'absence d'organismes foreurs. Cette production d'hydrogène sulfuré est à la base (1) du développement rapide d'un biofilm chimolithoautotrophe et (2) du recrutement d'espèces possédant des symbiotes chimiosynthétiques. Nos résultats ont permis d'aboutir à la proposition d'une succession d'étapes clés liées permettant la transformation d'un substrat terrigène en un écosystème qui, il y a plusieurs millions d'années, aurait permis à la faune chimiosynthétique de coloniser les grands fonds.

Mots clés : bois coulé, biofilm, chimiosynthèse, dégradation, foreurs

**Dynamics and role of microorganisms in the deep-sea sunken wood ecosystem**

Abstract :

When wood sinks to the deep-sea floor it creates a new ecosystem that does not depend directly on energy from sunlight. This ecosystem is called chemosynthetic because of the presence of a fauna associated with symbiotic bacteria that can assimilate inorganic carbon from seawater. Furthermore this system is colonized by specialized fauna that use symbiotic bacteria to digest the wood matrix. Previous studies mostly focused on these symbiotic macroorganisms and the role played by non-symbiotic microorganisms in the sunken wood ecosystem remains unknown. We demonstrate in this thesis the important role played by non symbiotic microorganisms during the sunken wood ecosystem establishment. We reveal the ecological succession of microorganisms driven by time and wood structure. The first step of this succession is characterized by a microbial population able to produce hydrogen sulfide after one month of immersion. This hydrogen sulfide production is the basis for (1) a chemolithoautotroph biofilm development on the wood surface and (2) a recruitment of species associated with chemoautotrophic bacteria. Our results suggest a succession of different phases that transform a terrigenous substrate into an environment that may have helped, million years ago, the colonization of the deep sea by chemosynthetic species.

Keywords : sunken wood, mat, chemosynthesis, degradation, wood borers