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Microbial ecology of deep-sea sunken wood: quantitative measurements of bacterial biomass and cellulolytic activities

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Abstract: When deposited in marine sediments, sunken wood and large animal remains can undergo sufficiently steady decay for oxygen to be depleted, attracting anaerobic living forms. Chemosynthetically living communities have recently been identified around whale skeletons and sunken woods. The phylogenetic resemblance and overlap in species of metazoans living in these habitats with those of highly reduced environments like hydrothermal vents and vent seeps has led to the hypothesis that deep-sea organic rich matter deposits could play a major role in the adaptation and evolution of chemoautotrophic communities at the ocean basin. Until present little attention has been paid to the free-living microbial diversity and activities in large organic falls like sunken woods and whale bones. In this communication we outline a series of methods to quantitatively study microbial biodiversity and degradation processes in sunken wood. Cellulose is the most abundant component of plant material and it can only be degraded by fungi and bacteria. We present results from cellulolytic activities in a long-term *ex-situ* experiment on samples from naturally and experimentally immersed sunken wood. We also have developed methods to quantitatively measure microbial cell numbers in wood chips. Further studies at the molecular level in combination with the methods reported here will broad our narrow knowledge on the microbial biofilms that develop on and within sunken woods and give clues on the ecological importance of these deep-sea organic islands.

Keywords: Microbial ecology • Chemosynthetic Ecosystems • Deep sea • Sunken woods • Organic fall • Bacterial biomass • Cellulolytic activity

Introduction

Sunken wood and the remains of marine mammals represent attractive food sources for a variety of organisms at the seafloor. Oxygen can become locally depleted during the decay of these large local inputs of organic matter attracting anaerobic life forms. Anaerobic degradation of organic compounds results in the production of volatile fatty acids and reduced compounds such as hydrogen sulfide, hydrogen and methane. For this reason, chemosyn-

thetic communities composed of invertebrates with sulfide-reducing endosymbionts, as well as mats of the giant sulfide oxidizing bacterium *Beggiatoa*, inhabit whale skeletons and sunken wood (e.g. Deming et al., 1997). The phylogenetic resemblance of metazoans living in these habitats with those of highly reduced deep-sea environments such as hydrothermal vents and cold seeps (Baco et al., 1999) and the overlap in species among whale falls, sunken wood, vents and seeps (Marshall, 1994; Smith & Baco, 2003; Glover et al., 2005; Smith, in press; Sheador et al., unpublished results) is consistent with the hypothesis that large organic falls (e.g., whale and wood falls) may have played a role as stepping-stones in the adaptation, dispersal and evolution of chemoautotrophic communities found at vents and seeps (Smith et al., 1989; Baco et al., 1999; Distel et al., 2000).

Despite their potential importance for the biodiversity of deep-sea life and the provision of anoxic niches in the ocean, little attention has been paid to the *free-living* microbes that degrade sunken wood and whale bones producing reduced compounds that serve as energy sources for chemosynthetic life. Recently, Tringe et al. (2005) performed a metagenomic study of three deep-sea whale-fall samples, demonstrating different genomic functions in the whale-fall habitat compared to other marine and terrestrial ecosystems. At present, virtually nothing is known about microbial diversity and activity on deep-sea sunken wood. Basic questions concerning microbial colonization and degradation of sunken wood include the following. Which microbial populations initially colonize sunken wood to begin degradation of its major components, i.e. cellulose and lignin? Are these microbial populations dominated by fungi or prokaryotic cells? How do invertebrates interact with cellulolytic microbes to utilize wood as a food source? Does microbial endosymbiosis in invertebrates play a major role in the degradation of wood (e.g. Distel & Roberts, 1997) in the deep sea or are free-living microbes the primary source of nutrition for wood-fall invertebrates (Marshall, 1988; Glover et al., 2005)? These and other questions are now studied within the framework of international projects like BOA (Bois coulés et Organismes Associées), DIWOOD (DIversity, establishment and function of organisms associated with marine WOOD falls), ChEss (Biogeography of deep-sea Chemosynthetic Ecosystems) and other activities.

Here we describe a set of methods proposed to quantitatively study the biodiversity and metabolic processes of free-living prokaryotes that degrade cellulose. We report cellulolytic activities measured in wood samples under *ex-situ* conditions in a long-term experiment to study the development of wood degrading biofilms. We also have developed methods for quantitative, reproducible cell counts from wood chips. The next step is to develop protocols for Fluorescent In Situ Hybridization (FISH) identification of microorganisms in biofilms and wood tissue.

Material and Methods

Sunken wood samples

Samples used for cellulase measurements included commercially available coconut, mangrove, pine and fir wood purchased for the BOA program. In addition, two wood samples were collected in 2004 off southern California (Northeast Pacific) from douglas fir (*Pseudotsuga menziesii*) parcels experimentally implanted on the seafloor at a depth of 1243 m for 4.5 yr, and at 1675 m for 1.8 yr (named TD656 and TD652, respectively, in Fig. 1). Finally, we also sampled natural wood falls and other plant materials recovered at 1200 m depths by trawling off Noumea (West Pacific) during

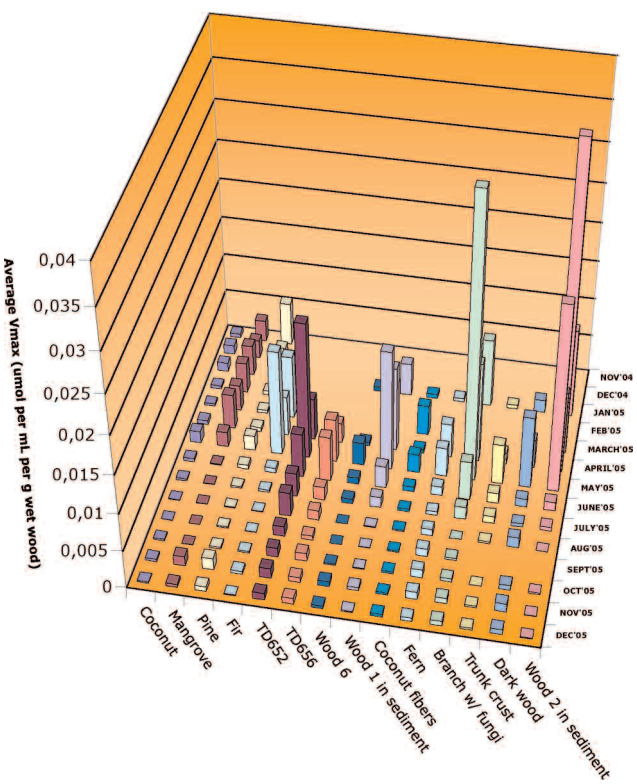

Figure 1. Cellulolytic activity measurements in sunken woods from an ex-situ ongoing experiment undertaken in the laboratory beginning November 2004. See text for details on samples. Empty columns indicate data not obtained.

Figure 1. Mesures de l'activité cellulolytique dans des bois coulés lors d'une experience ex-situ effectuée au laboratoire début novembre 2004. Voir texte pour détails sur les échantillons. Les colonnes vides correspondent à une absence de données.

the 2004 BOA program cruise. These samples consisted of a variety of woods, one fern, and coconut fibers as indicated in Figure 1. The development of quantitative protocols required large amounts of wood material (more than was available from our marine samples) so we used a naturally immersed wood from the nearby University Lake (Bremen) for protocol development.

Cellulolytic activity measurements

Bacteria secrete extracellular hydrolytic enzymes for the degradation of polymers. Microbial extracellular cellulolytic activity in environmental samples can be quantified using fluorogenic substrates (Hoppe, 1993). We used methylumbelliferone (MUF)-ß-glucopyranoside as model substrate of cellobiose to measure the activity of extracellularly excreted β-glucosidase enzyme by cellulolytic fungi and bacteria. Our aim was to demonstrate the utility of this method for cellulolytic activity measurements in immersed woods.

Pine, coconut, and mangrove samples were maintained in closed bottles filled with Mediterranean seawater in the dark at 14ºC beginning in November 2004 (Fig. 1). Fir was added to this experiment in January 2005. Noumea and California samples were kept in original water at 4ºC and two single measurements were undertaken in January and

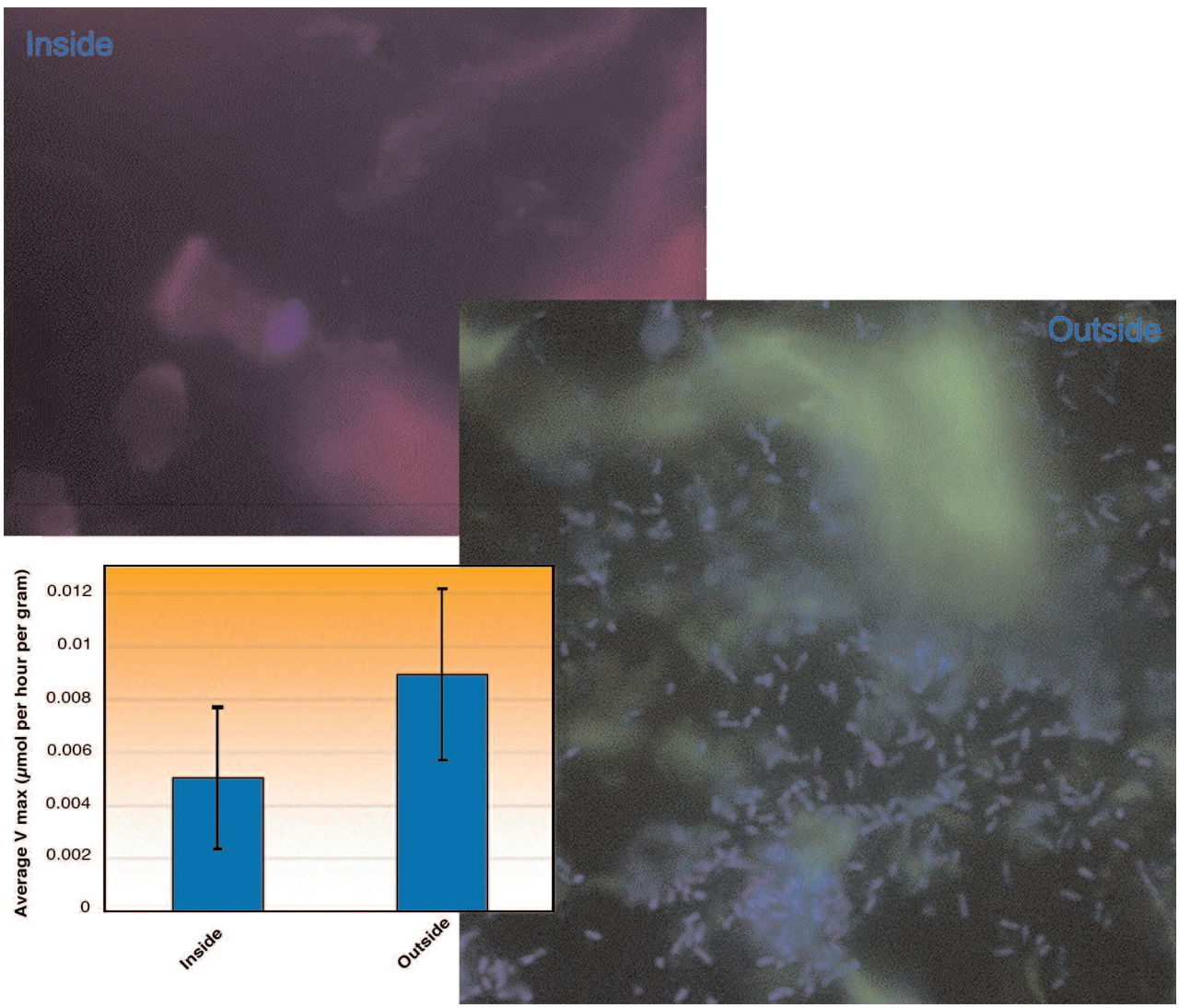

Figure 2. DAPI cell stains (grey dots) of outside and inside artificially submerged wood from sample TD652 California. Grey structures result from wood autofluorescence. The histogram shows cellulolytic activity measurements in these same wood samples.

Figure 2. Coloration au DAPI des cellules (points gris) de l'extérieur et de l'intérieur de bois immerge artificiellement (échantillon TD652, Californie). Les structures grises résultent de l'autofluorescence. L'histogramme montre l'activité cellulolytique de ces échantillons.

March 2005. In April 2005, an experiment was carried out incubating the samples at 14ºC in bottles loosely covered with aluminum foil and shaken at 250 rpm to obtain oxic conditions. Measurements were taken every week for two months and then at monthly intervals.

Procedures for bacterial cell detachment

We tried several methods for detaching bacteria from wood samples preserved in 5 mL of 4% formalin for at least 10 hours.

First, we used two different methods to chip off wood samples:

- Ultra-Turrax tissue homogenizer (IKA TP18-10; fixed speed of 20 000 rpm [2013°ø g]) fitted with a standard axis (type N-18G),
- Custom built wood corer described in here (Fig. 3) produced wood chips of 2.5 mm (H) x 7 mm (W).

After cutting replicate wood chips, we performed sonication to detach cells from the wood, using an ultrasonic probe (Bandelin Electronics Typ. UN200) connected to a standard resonator and operated at an actual output of 200 W (Set: 30% cycle, 72/D frequency). Samples were treated for varying time periods ranging from 5 to 20 min at 5 min intervals. To minimize sonication time for cells that had already been detached, the fixative was collected in an extra vial and replaced with new fixing solution after each 5-min interval. Cooling of samples on icewater was necessary to avoid temperatures over 15ºC. We also tested the use of pyrophosphate prior to sonication to even out the distribution of cells in plant materials (Velji & Albright, 1986). Samples were left for at least 1 h at 4ºC in 10% sodium pyrophosphate; the sodium phosphate was included in fixative for sonication. We also tried the improved procedure by Lunau et al. (2005) for counting cells in sediments by treating the fixed sample with 10% Methanol, followed by 15 min incubation at 35ºC, and then either placement in an ultrasonic bath for 35 min (Bandelin, Sonorex 320 W) or sonication for 20 min as above.

After detachment, the collected solutions containing the cells were diluted to obtain 20-150 bacteria cells (0.5-2.0 µm size) per counting grid. We followed Buesing & Gessner (2002) "Bacterial counts" protocol for staining and

Figure 3. The wood corer can be used in conjunction with a pressure manifold (or just with a hammer) to cut cores from wood (steps 1 and 2). Then, we have designed and built an additional tool (3) with which we are able to precisely cut wood chips as thin as 2.5 mm in height from a 7-mm diameter wood core without disturbing the biofilms covering the wood. An accurate wood chip size helps to standardize methodologies, such as the cell counting procedure described in this paper, and to obtain wood cores from on shipboard during research cruises.

Figure 3. L'échantillonneur de bois peut être utilisé avec une presse manuelle (ou un marteau) pour couper des carottes dans le bois (étapes 1 et 2). Ensuite, nous avons conçu et construit un outil supplémentaire (3) permettant de couper avec précision des tranches de bois aussi fines que 2,5 mm en hauteur à partir d'une carotte de bois de 7 mm de diamètre sans perturber le biofilm couvrant le bois. Une épaisseur de bois précise permet de standardiser la procédure, comme le comptage des cellules décrit dans l'article, et d'obtenir les échantillons de bois à bord pendant la campagne.

visualizing bacteria, except that vigorous shaking after detachment was followed by sample settling for 30 seconds before removing aliquots for filtering. This was necessary to decrease the presence of wood chips on the filter, which interfered with counting due to the high autofluorescence of wood in all ranges of visible light. Settling did not visually affect cell counts. A total of 20 grids (~ 1000 bacteria) per filter were counted for statistical calculations.

Results and Discussion

Cellulolytic activities tend to decrease with time in ex-situ conditions

Cellulose, the major component of plant biomass, is in soluble and resistant to hydrolysis. Only a few bacteria and fungi have developed cellulases to specifically hydrolyze cellulose bonds (Lynd et al., 2002). Therefore, cellulose and lignin hydrolysis might be the main rate-limiting steps in wood degradation. We expected to be able to measure specific cellulolytic activity associated with woods with the MUF method described in Material and Methods as for other aquatic samples (Hoppe, 1993). Figure 1 is a monthly summary of specific cellulolytic-activity measurements undertaken since November 2004 using this method for two experiments, open and capped bottles, in different samples (see above). Results of cellulolytic activities vary greatly with the wood source and its original location. Measured activity rates of wood samples exceeded the negative control at least at the beginning of the experiment (0.01-1.35 µM MUF.h^{-1} in wood vs. 0.0003 in artificial seawater or 0.001 in E. Mediterranean seawater, data not shown) and remained higher than the wood-free control for all samples after one year (Fig. 1). Although not statistically significant, preliminary results indicate a correlation between cellulytic activity and cell numbers (Fig. 2). These results suggest that cellulolytic activity measurements are possible using Hoppe's MUF method. Enzyme activities were higher at the initial time points in both experiments and either reached a specific level or decreased, as happens in the aerated experimental conditions. Fixed samples are available from the beginning of each experiment, which will allow concomitant bacteria cell counts to determine if they correlate with cellulolytic activities.

Bacteria cell counts in woods is highly improved by the use of pyrophosphate in conjunction with sonication

Efficient detachment of cells is crucial for assessing bacterial abundance in solid substrata. We tested different detachment instruments and procedures found in the literature to determine their performance for the release of bacteria associated with wood.

Although Ultra-Turrax has been widely used for tissue homogenization, these homogenizers should not be used for production of wood chips because chips became trapped in the blades, which eventually resulted in inaccurate cell counts. In contrast, the wood corer used in this study (Fig. 3) proved very useful because it made clean cuts to produce woods chips of accurate size without disturbing the wood surface, which is normally covered with bacterial biofilms (Fig. 2).

Methanol treatment followed by sonication, or sonication alone, did not favor cell detachment as efficiently as pyrophosphate pre-incubation. A series of rigorously designed experiments were undertaken with the pyrophosphate-plus-sonication treatment to determine:

(1) *The number of sonication time intervals necessary to obtain a maximum cumulative number of cells*. After 5 min of sonication, half of the maximum number of cells was recovered. After five 5-min sonication the number of cells recovered decreased to 7% of the total. We can conclude that 20 min of sonication is sufficient to maximize cell recovery.

(2) *Statistical deviation of the cell counts with this treatment in replicate cores from the same sample, and in comparison with sonication alone*. Fig. 4 shows the range of differences in bacterial cell counts among different wood cores from the same sample (2-5). Moreover, although not statistically significant, the tendency indicates higher number of bacteria cells obtained with the pyrophosphate treatment (treatment 1, Fig. 4) but with a high variation in cell numbers among samples. Our results suggest that serial counts are necessary to accurately determine cell numbers in sunken woods.

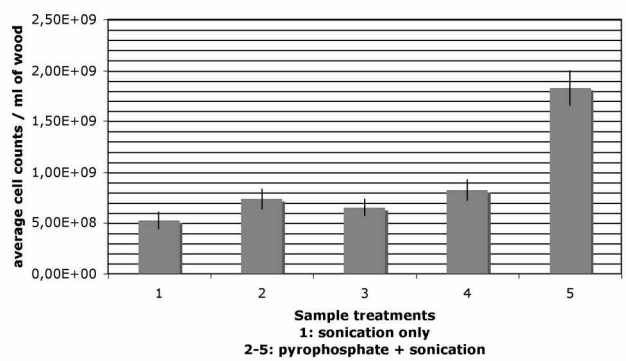

Figure 4. Average cell counts per milliliter of wood with pyrophosphate-plus-sonication treatment in replicate samples of the same wood. In sample 1, pyrophosphate was omitted.

Figure 4. Nombre moyen de cellules par ml de bois après un traitement au pyrophosphate plus une sonication dans des replicats provenant du même bois. Pour l'échantillon 1, le pyrophosphate n'a pas été utilisé.

The pyrophosphate-plus-sonication protocol for cell detachment will also be of great interest for the application of FISH to the study of microbial diversity on sunken woods because the interference of natural autofluorescence from wood components with fluorescent FISH probes can be avoided.

Outlook

The next steps in the development of methods to study the microbial ecology of sunken woods is to apply a combination of 16S ribosomal DNA methods to achieve a quantitative identification of microbial diversity specifically associated with wood, in biofilms on the wood, as well as within the wood tissue itself. We are planning further experiments to test whether there are differences between microbial community biomass and activity related to the type of wood submerged. An interesting question remains as to whether invertebrate-microbial associations are important in wood degradation.

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