In-depth analyses of deep subsurface sediments using 454-pyrosequencing reveals a reservoir of buried fungal communities at record-breaking depths

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Keywords
454 pyrotag; Canterbury basin; deep subsurface; fungi; microeukaryotes.

Abstract
The deep subseafloor, extending from a few centimeters below the sediment surface to several hundred meters into sedimentary deposits, constitutes the deep biosphere and harbors an unexpected microbial diversity. Several studies have described the occurrence, turnover, activity and function of subseafloor prokaryotes; however, subsurface eukaryotic communities still remain largely undetected. Ribosomal RNA surveys of superficial and near-surface marine sediments have revealed an unexpected diversity of active eukaryotic communities, but knowledge of the diversity of deep subseafloor microeukaryotes is still scarce. Here, we investigated the vertical distribution of DNA and RNA fungal signatures within subseafloor sediments of the Canterbury basin (New Zealand) by 454 pyrotag sequencing of fungal genetic markers. Different shifts between the fungal classes of Tremellomycetes, Sordariomycetes, Eurotiomycetes, Saccharomycetes, Wallumiomycetes, Dothideomycetes, Exobasidiomycetes and Microbotryomycetes were observed. These data provide direct evidence that fungal communities occur at record depths in deep sediments of the Canterbury basin and extend the depth limit of fungal presence and activity, respectively 1740 and 346 mbsf. As most of the fungal sequences retrieved have a cosmopolitan distribution, it indicates that fungi are able to adapt to the deep subseafloor conditions at record-depth and must play important ecological roles in biogeochemical cycles.

Introduction
Exploration of the marine subsurface was initiated in the 1930s (ZoBell & Anderson, 1936) and since that time it has been demonstrated that abundant and diverse microbial communities inhabit this ecosystem with the potential to impact large-scale biogeochemical cycles (Fredrickson & Balkwill, 2006; Edwards et al., 2012; Anderson et al., 2013). The deep subseafloor biosphere hosts large numbers of living microbial cells, estimated at 2.9 × 10^29 cells, as revealed by recent cell counts (Kallmeyer et al., 2012) and thus represents a significant biome structured by several spatial, physical and energetic constraints (e.g. confinement, pressure, temperature, refractory organic matter, etc.). Studies of subsurface microbial communities (Parkes et al., 2000; Fry et al., 2008; Orcutt et al., 2011) and metabolisms (D’Hondt et al., 2002, 2004; Biddle et al., 2006, 2011) have provided a foundation for understanding the ecological roles of subsurface microorganisms. Prokaryotes remain the common targets of investigations that aim to study subsurface microbial diversity, and debate continues whether Archaea or Bacteria predominate (Schippers et al., 2005; Biddle et al., 2006; Briggs & Biddle, 2012).

Microeukaryotes remain understudied although their presence in marine extreme environments is increasingly
documented. Culture-based and culture-independent methods have demonstrated their occurrence in hydrothermal vents (Edgcomb et al., 2002; Burgaud et al., 2009, 2010; Le Calvez et al., 2009), anoxic environments (Stoeck & Epstein, 2003; Takishita et al., 2005; Jebaraj et al., 2010), deep hypersaline anoxic basins (Alexander et al., 2009; Stock et al., 2012), cold seeps (Takisita et al., 2007; Nagano et al., 2010; Nagahama et al., 2011) and associated with sunken wood (Barghoorn & Linder, 1944; Dupont et al., 2009). The search for microeukaryotes in the deep subseafloor has been delayed by the prevailing viewpoint that spatial constraints were an obstacle to the growth of larger cells or multicellular microorganisms. However, deep systems with cavities of several microns appear large enough to support microeukaryotic life (Ciobanu et al., 2014). Recent studies have definitely demonstrated microeukaryotic presence, activity and metabolisms in the marine subsurface at depths from 5 to 159 m below seafloor (mbsf; Edgcomb et al., 2011; Orsi et al., 2013a, b). Among microeukaryotes, fungal communities appear not to be diverse (Edgcomb et al., 2011; Xu et al., 2014) but consistently dominate, and thus may have significant ecological roles in the deep biosphere. Fungi revealed in marine sediments are typically widespread in terrestrial environments (Richards et al., 2012), indicating that terrestrial and surface-dwelling fungi may be capable of adaptation to deep biosphere conditions and thus may be capable of colonizing the deep subseafloor. Ciobanu et al. (2014) recently extended the boundaries of microbial life using a record-depth (1922 m) sediment core from the Canterbury basin (New Zealand).

That sediment core was investigated to test hypotheses regarding the limits of the deep biosphere, and revealed the occurrence of Bacteria, Archaea and Eukarya at these record depths in the subseafloor. In our study, we conducted an in-depth investigation of microeukaryotic communities from Canterbury basin sediment cores to provide further insights on the nature and extent of subsurface fungal reservoirs in the deep biosphere. We used a 454-pyrosequencing approach targeting eukaryotic small subunit (18S) ribosomal RNA and DNA, and fungal ITS1 regions in order to analyze the presence and activity of the different fungal operational taxonomic units (OTUs) present at the species level, a prerequisite to the understanding of the ecological roles of fungi in the deep biosphere.

Here we show that fungal signatures appear at record-depth in the deep subseafloor, although species richness is extremely low, with only 18 OTUs detected. Based on rDNA and rRNA sequences detected at 1740 and 346 mbsf, respectively, fungi may be viewed as a third microbial component (after Bacteria and Archaea) with potentially important ecological roles in the deep biosphere.

Materials and methods

Site description and sediment sampling

Sediment samples were collected from the Canterbury basin, on the eastern margin of the South Island of New Zealand, during IODP Leg 317 Expedition (RV JOIDES Resolution). A sediment core was drilled at Site U1352 (44°56′26.62″S, 172°1′36.30″E; Fig. 1) in 344 m water depth, and the sediment core had a depth of 1927.5 mbsf, spanning the Holocene to late Eocene periods. The core lithology and environmental parameters at the time of sampling are described in Ciobanu et al. (2014).

Nucleic acid extractions, PCR amplifications and 454 sequencing

DNA extractions were performed from nine samples collected along the core (Table 1). To avoid contamination, all manipulations were carried out in a PCR cabinet exclusively dedicated to low biomass sediment samples (PCR cabinet; Captair® Bio, Erlab). DNA was extracted from 5 × 0.5–1 g frozen samples (―80 °C), where no fluorescent microspheres could be retrieved, with the FastDNA® Spin Kit for Soil (#6560-200; MP Biomedical®, following the manufacturer’s instructions. Ten microliters of linear acrylamide (5 mg mL⁻¹; Ambion/Applied Biosystems) were added to the protein lysis buffer in order to favor DNA precipitation in subsequent stages. At the final step, DNA was eluted in a 50-µL volume. Concentration of extracted DNA was measured with a NanoDrop 1000 Spectrophotometer (Thermo-Scientific). Negative controls (reaction mixture without DNA or cDNA) were included in each set of PCR reactions. In addition, a negative control (e.g. negative DNA or cDNA extraction) was prepared for each work stage to ensure that no contamination with exogenous amplifiable DNA or cDNA occurred during the different stages of sample treatment.

For each DNA extract, four independent 25-µL PCR amplifications were performed using the universal eukaryotic primers Euk 42F (López-Garcia et al., 2003)/Euk 516R (Amann et al., 1990) and fungal primers ITS1 (Gardes & Burns, 1993)/ITS2 (White et al., 1990). Nested PCR amplifications followed with fusion primer sets Euk 82F (Dawson & Pace, 2002)/Euk 516R and ITS1F/ITS2 (White et al., 1990). All PCR reactions were performed in 25-µL volumes containing 1 x Taq DNA polymerase buffer with MgCl₂ (2 mM), 1 mM of additional MgCl₂, 240 µM dNTP, 0.4 µM of each primer, 1 volume of
5× GC-rich buffer, 1 unit of FastStart Taq DNA polymerase, and 1 µL of DNA template. The first PCR assay for ITS1 region started by initial denaturation step at 95 °C for 5 min, followed by 20 cycles of 1 min at 95 °C, 1 min at 56 °C and 1.5 min at 72 °C and a final extension step of 7 min at 72 °C. The second PCR amplification was performed in the same conditions using 10 cycles. PCR products were pooled two by two so as to have two independent replicates for pyrosequencing. The PCR program for 18S and sequencing strategy were described in detail previously (Ciobanu et al., 2014).

RNA extractions were performed using three samples collected along the core, including 12, 346 and 931 mbsf (Table 1). RNA was extracted from 2 × 4 g for the 12-mbsf sample and around 2 × 8 g for 346- and 931-mbsf samples of uncontaminated frozen samples (−80 °C) with the RNA PowerSoil® Total RNA Isolation Kit (#12866-25; MO BIO Laboratories), following the manufacturer’s instructions. To purify the RNA extracts, the MEGAclear RNA purification kit (Life Technologies) was used according the manufacturer’s instructions. To remove any potential DNA contamination, RNA extracts were treated

Table 1. List of studied samples and depths

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Core depth below the seafloor (m)</th>
<th>Study performed</th>
<th>Target marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>2H</td>
<td>12</td>
<td>cDNA</td>
<td>18S rDNA</td>
</tr>
<tr>
<td>42X</td>
<td>346</td>
<td>DNA/cDNA</td>
<td>18S rDNA/ITS</td>
</tr>
<tr>
<td>68X</td>
<td>583</td>
<td>DNA</td>
<td>18S rDNA/ITS</td>
</tr>
<tr>
<td>73X</td>
<td>634</td>
<td>DNA</td>
<td>18S rDNA</td>
</tr>
<tr>
<td>34R</td>
<td>931</td>
<td>DNA/cDNA</td>
<td>18S rDNA/ITS</td>
</tr>
<tr>
<td>87R</td>
<td>1367</td>
<td>DNA</td>
<td>18S rDNA</td>
</tr>
<tr>
<td>110R</td>
<td>1577</td>
<td>DNA</td>
<td>ITS</td>
</tr>
<tr>
<td>122R</td>
<td>1690</td>
<td>DNA</td>
<td>ITS</td>
</tr>
<tr>
<td>125R</td>
<td>1711</td>
<td>DNA</td>
<td>ITS</td>
</tr>
<tr>
<td>128R</td>
<td>1740</td>
<td>DNA</td>
<td>18S rDNA</td>
</tr>
</tbody>
</table>

Fig. 1. Sampling site.
using the Turbo DNA-free kit (Life Technologies). Removal of contaminating DNA was confirmed by the absence of visible amplification of small subunit ribosomal RNA genes using bacterial hypervariable V4 region primers (Cole et al., 2009) after 40 cycles of PCR using the RNA extracts as template. Total extracted RNA was immediately reverse-transcribed to cDNA using the QuantiTect Transcription Kit (Quiagen) according to the manufacturer’s instructions. The universal primers TAR-eukFWD1 and TAR-eukREV3 (Stoeck et al., 2010) were used to amplify the V4 region (c. 380 bp) of the eukaryotic 18S rRNA gene. All PCR reactions were performed in 25-μL reaction volumes containing 1× GoTaq Buffer, 0.2 mM dNTPs, 0.4 μM of each primer, 2.5 mM of MgCl₂, 1 U of GoTaq polymerase and 1 μL of cDNA. The PCR assay started by initial denaturation step at 95 °C for 4 min, followed by 35 cycles of 35 s at 95 °C, 45 s at 48 °C and 50 s at 72 °C and a final extension step of 7 min at 72 °C. Amplicons were purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research) according to the manufacturer’s instructions. Barcodes were added by ligation by the sequencing company (Molecular Research LP, Texas) and pyrosequencing was performed on a GS FLX+ platform (Roche).

**Quality control, clustering and taxonomic assignment of 454 pyrotags**

**DNA- and RNA-based 18S data processing**

The raw sff files were converted to FASTQ format. A quality filtering was performed with USEARCH using the maximum expected error filtering method. Sequences for which all base pairs had a Phred quality score under 15 were removed. A minimum read length retained after trimming was set at 200 bp. The clustering was performed using USEARCH v7 (Edgar, 2010) and the UPARSE function (Edgar, 2013). The removal of duplicated sequences was performed using USEARCH v7 and the de-rep_fulllength command with the sizeout option set at 64. Dereplicated reads were sorted by decreasing abundance with the sortbysize command. OTUs were delineated at a 97% identity threshold using the cluster_otus command. Only OTUs present in both sequencing duplicates were retained. Chimeric sequences were identified and removed using Silva_111 (Quast et al., 2013) as the reference database for eukaryotes within UCHIME (Edgar et al., 2011). The OTU table generated from the UPARSE pipeline was processed and analyzed using the QIIME pipeline (Caporaso et al., 2010). Taxonomic assignment was performed using the assign_taxonomy.py command within QIIME. The taxonomic mapping file was generated with the assign_taxonomy.py command. Sequence representatives of each OTU were aligned using PYNAST (DeSantis et al., 2006) and classified using the silva_111 rep set. The alignment was filtered using default parameters. The 10% most variable positions within the alignment and positions that contained >80% gaps were removed. A eukaryotic phylogenetic analysis was performed with the make_phylogeny.py command within QIIME. Both alpha and beta diversity metrics were determined using the core_diversity_analyses.py command in QIIME. Alpha diversity was assessed by calculating the richness estimator Chao 1 (Chao, 1984) and the Simpson and Shannon diversity indices (Simpson, 1949; Shannon & Weaver, 1963). Beta diversity patterns of samples were assessed using unweighted and weighted UniFrac metrics (Lozupone & Knight, 2005).

**DNA-based ITS1 data processing**

Data processing used for the ITS1 marker was similar to 18S data processing from raw sff files to the dereplication step. Chimeric sequences were identified and removed using UNITE (Abarenkov et al., 2010) as a reference database for fungi (Kõljalg et al., 2013), within UCHIME (Edgar et al., 2011). OTU table generated from UPARSE pipeline were processed and analyzed using QIIME pipeline (Caporaso et al., 2010). Taxonomic assignment was performed using UNITE database with assign_taxonomy.py command. Taxonomic mapping file was performed with the assign_taxonomy.py command. Both alpha and beta diversity metrics were determined using the core_diversity_analyses.py command in QIIME. Alpha diversity was assessed by calculating the richness estimator Chao 1 (Chao, 1984) and the Simpson and Shannon diversity indices (Simpson, 1949; Shannon & Weaver, 1949). Beta diversity patterns of samples were assessed using unweighted and weighted UniFrac metrics (Lozupone & Knight, 2005).

**Statistical analyses**

Multiple factorial analyses (MFA) and principal component analyses (PCA) were processed to elucidate relationships between fungal community structure and some selected environmental parameters acquired during IODP Expedition 317 (Fulthorpe et al., 2011). MFA allows the mapping of sediment samples on a two-dimensional plane showing the contribution of the different parameters (OTUs, Depth, Organic Carbon, Inorganic Carbon, Porosity, Methane, Ethane and Depth). PCA analyses are based on different OTUs obtained and environmental parameters for each sample. All statistical analyses were performed using XLSTAT (Addinsoft, USA, New York, NY).
Results

Fungal diversity

DNA-based eukaryotic gene dataset based on V1–V3 region of 18S rRNA gene

A total of 28,868 sequences were generated by the 454 pyrosequencing for the nine depths from 346 to 1740 mbsf. After quality control, 48% of the sequences were analyzed (14,036 sequences) and were grouped into 13 fungal OTUs. For each sample, the total number of fungal 454 reads, the number of cleaned fungal 454 reads, and the number of fungal OTUs are summarized in Table 2. Fungi in this DNA-based 18S dataset represent 54–100% of all sequences recovered. Chao richness estimators (Table 3) are consistent with the numbers of observed OTUs, indicating a complete coverage of the fungal DNA diversity. The Shannon diversity index computed for every depth ranged from 0 to 2.11 (Table 3), suggesting a low species diversity of fungal communities along the core.

RNA-based eukaryotic gene dataset based on V4 region of 18S rRNA gene

A total of 164,743 sequences were generated for RNA-based 454 pyrotags for three depths, 12, 346 and 931 mbsf. After quality control, 39% of sequences were analyzed (63,564 sequences) and grouped into 185 OTUs, among them only one fungal OTU was observed at 12 and 346 mbsf.

DNA-based eukaryotic gene dataset based on fungal ITS1 region

A total of 17,672 sequences were generated by 454 pyrosequencing for five samples from 346 to 1711 mbsf. After quality control, 59% of ITS1 sequences were analyzed (10,421 sequences) and after removal of singletons were grouped into 18 OTUs. For each sample, the total number of fungal 454 reads obtained, the number of reads retained after quality control, and the resulting number of OTUs are summarized in Table 2. Chao richness estimators are consistent with the numbers of observed OTUs, indicating a complete coverage of the fungal DNA diversity. The Shannon diversity index computed for every depth ranged from 0 to 1.86, suggesting, as for the DNA-based 18S dataset, that the fungal communities using ITS1 are also weakly diversified along the core.

Distribution patterns of fungal communities

Variations in the structure of the fungal communities were determined MFA coupled with a PCA (Figs 2 and 3). MFA represents a convenient tool for comparing several samples characterized by the same subset of factors. Using MFA, it was possible to get an overall picture of the common structure emerging from the dataset. Differences between samples were deduced based on the superimposed representations (Figs 2a and 3a) on which samples are represented as centers of gravity of the different variables. MFA clearly positioned depth and porosity as strong structuring parameters with low depth/high porosity samples on the right of the plot and high depth/low porosity samples on the left (Figs 2a and 3a). It also clearly highlighting the complexity of the diversity with complex vs. not complex samples, as detailed below. MFA allowed us to infer general distribution patterns. Then, PCA was then used to detail community distribution.

DNA-based 18S dataset

The MFA representation allowed samples with close values to be clustered, i.e. 1367-, 634- and 1740-mbsf samples for Table 2. Number of sequences and number of OTUs in the DNA-based datasets

<table>
<thead>
<tr>
<th>Depth</th>
<th>346 mbsf</th>
<th>583 mbsf</th>
<th>634 mbsf</th>
<th>931 mbsf</th>
<th>1367 mbsf</th>
<th>1577 mbsf</th>
<th>1690 mbsf</th>
<th>1711 mbsf</th>
<th>1740 mbsf</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>6425</td>
<td>5868</td>
<td>5868</td>
<td>3789</td>
<td>6392</td>
<td>3789</td>
<td>4521</td>
<td>4521</td>
<td>2318</td>
</tr>
<tr>
<td>ITS</td>
<td>4625</td>
<td>4625</td>
<td>4625</td>
<td>3789</td>
<td>4521</td>
<td>4521</td>
<td>4521</td>
<td>4521</td>
<td>4521</td>
</tr>
</tbody>
</table>

Table 3. Diversity indices for the 18S surveys calculated based on the fungal OTUs

<table>
<thead>
<tr>
<th>Depth</th>
<th>346 mbsf</th>
<th>583 mbsf</th>
<th>634 mbsf</th>
<th>931 mbsf</th>
<th>1367 mbsf</th>
<th>1577 mbsf</th>
<th>1690 mbsf</th>
<th>1711 mbsf</th>
<th>1740 mbsf</th>
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<tbody>
<tr>
<td>18S</td>
<td>6425</td>
<td>5868</td>
<td>5868</td>
<td>3789</td>
<td>6392</td>
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<td>4521</td>
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<td>2318</td>
</tr>
<tr>
<td>ITS</td>
<td>4625</td>
<td>4625</td>
<td>4625</td>
<td>3789</td>
<td>4521</td>
<td>4521</td>
<td>4521</td>
<td>4521</td>
<td>4521</td>
</tr>
</tbody>
</table>

-richness (Chao1)

Diversity (Shannon)

Evenness (Simpson)
inorganic carbon and 346-, 1740- and 931-mbsf samples for organic carbon, as well as differentiating some samples, for example, 583-, 634- and 1367-mbsf samples were inversely correlated with 346-, 931- and 1740-mbsf samples for organic carbon. MFA clearly indicates that 346-, 931- and 1740-mbsf samples represented a relatively organic-rich depth compared with 583-, 634- and 1367-mbsf samples. MFA allowed differentiation of sediment samples based on diversity, that is, complex or not complex. The 346- and 583-mbsf samples, with long-length OTU vectors, represented complex samples compared with the other sediment samples with short-length OTU vectors, demonstrating that fungal diversity was higher in the first sediment layers analyzed. Using the OTU vector, MFA also clearly indicated a strong diversity at the different depths. The lengths and also the different OTU vector directions at different depths confirm that the first sediment layers analyzed were more complex and diverse. PCA allowed visualization of three clusters: (I) *Wallemia muriae*, *Filobasidium globisporum*, *Cryptococcus surugaensis*; (II) *Trichosporon mucoides*, *Malassezia pachydermatis*, *Meyerozyma guilliermondii*, *Pleurostomophora richardsiae*, *Exophiala dermatitidis*, and (III) *Leptosphaerulina chartarum*, *Fusarium solani*, *Trichoderma sp.*, *Cryptococcus curvatus* and *Cyberlindnera jadinii*. The first cluster was mainly composed of OTUs from the deeper sediment horizon.

Fig. 2. MFA and PCA based on 454-pyrosequencing of 18S reads and environmental parameters. (a) Superimposed representation individuals (sediment samples) with the contribution of OTUs and the different environmental parameters. D, depth; E, ethane; Ic, inorganic carbon; M, methane; Oc, organic carbon; P, porosity. (b) Distribution of sediment samples, OTUs and environmental parameters. C.c, *Cryptococcus curvatus*; C.j, *Cyberlindnera jadinii*; C.s, *Cryptococcus surugaensis*; E.d, *Exophiala dermatitidis*; F.g, *Filobasidium globisporum*; F.s, *Fusarium solani*; L.c, *Leptosphaerulina chartarum*; M.g, *Meyerozyma guilliermondii*; M.p, *Malassezia pachydermatis*; P.r, *Pleurostomophora richardsiae*; T.m, *Trichosporon mucoides*; T.sp, *Trichoderma sp.*; W.m, *Wallemia muriae*. Sample depths are given in Table 1.
depth, whereas cluster III contained only OTUs from the shallowest depth. In cluster III, *C. curvatus* appeared significantly correlated with methane. Cluster II appeared negatively correlated with organic carbon, indicating that *T. mucoides*, *M. pachydermatis*, *E. dermatitidis*, *P. richardsiae* and *M. guilliermondii* were OTUs only found in sediment samples with a low organic carbon concentration.

**DNA-based ITS1 dataset**

Consistent with the 18S data, MFA indicated that 346-, 583- and 931-mbsf samples, with long-length OTU vectors, were more complex than 1577- and 1711-mbsf samples. As found for 18S data, the different OTU vector directions at the different depths clearly indicate contrasted fungal communities along the sediment core. PCA clearly differentiated three clusters: (I) *Cryptococcus saitoi*, *Rhodosporidium kratochvilovae*, *Rhodotorula* sp., *Tremella moriformis*, *Leucosporidiella muscorum*; (II) *P. richardsiae*, *Chaetothyriales* sp., *Exophiala spinifera*, *Penicillium* sp., *Batcheloromyces leucadendri*, *Elmerina caryae*, *Rhinocladiella* sp., and (III) *Cryptococcus pseudolongus*, *C. jadinii*, *Galactomyces candidum*, *L. chartarum*, *Trichosporon* sp., and *M. guilliermondii*. Those three clusters appeared depth-
specific, and most of the OTUs were only found at a given depth. Many OTUs belonging to clusters II and III on the right of the PCA were inversely correlated with organic matter. Members of the cluster III, some of which were affiliated to the *Cryptococcus* genus, appeared to be correlated with methane and ethane.

Of the 13 OTUs obtained with eukaryotic primers, six appear to be depth-dependent: OTU 2, OTU 3, OTU 6, OTU 9, OTU 10, OTU and OTU 13 (Table 4). A maximum of four OTUs are shared between samples, observed in the same sample type: OTU 4, OTU 5, OTU 8 and OTU 11. In the ITS1 dataset, the 346-mbsf sample shared OTU 3 and OTU 6 with the 583-mbsf sample and OTU 3 with the 931-mbsf sample. The 583-mbsf sample shared OTU 3 with the 931-mbsf sample and OTU 2 with the 1711-mbsf sample (Table 4).

**Taxonomic composition**

To evaluate the taxonomic composition of each sample, the representative reads were compared against SILVA_111, UNITE and GenBank databases.

### Table 4. List of fungal operational taxonomic units found in deep-sea sediment using eukaryotic V1–V3 primers (a) and fungal ITS1 primers (b)

<table>
<thead>
<tr>
<th>OTU Id</th>
<th>Sample depth (mbsf)</th>
<th>Most similar sequence</th>
<th>$E$ value</th>
<th>Identity (%)</th>
<th>GenBank accession number of the most similar sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU_1</td>
<td>583, 634, 931, 1367, 1690</td>
<td><em>Meyerozyma guilliermondii</em></td>
<td>2.00E-131</td>
<td>261/262 (99)</td>
<td>JQ698913.1</td>
</tr>
<tr>
<td>OTU_2</td>
<td>931</td>
<td><em>Trichosporon mucoides</em></td>
<td>3.00E-130</td>
<td>259/260 (99)</td>
<td>AB001763.2</td>
</tr>
<tr>
<td>OTU_3</td>
<td>583</td>
<td><em>Pleurostomophora richardsiae</em></td>
<td>7.00E-176</td>
<td>338/338 (100)</td>
<td>AY729812.1</td>
</tr>
<tr>
<td>OTU_4</td>
<td>346, 583, 931, 1690</td>
<td><em>Cryptococcus curvatus</em></td>
<td>2.00E-131</td>
<td>261/262 (99)</td>
<td>AB032626.1</td>
</tr>
<tr>
<td>OTU_5</td>
<td>346, 583, 1740</td>
<td><em>Cryptococcus surugaensis</em></td>
<td>0.0</td>
<td>360/361 (99)</td>
<td>AB100440.1</td>
</tr>
<tr>
<td>OTU_6</td>
<td>346</td>
<td><em>Leptosphaerulina chartarum</em></td>
<td>3.00E-174</td>
<td>338/339 (99)</td>
<td>HM216185.1</td>
</tr>
<tr>
<td>OTU_7</td>
<td>346, 1740</td>
<td><em>Filobasidium glabsporum</em></td>
<td>2.00E-131</td>
<td>261/262 (99)</td>
<td>AB075546.1</td>
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<tr>
<td>OTU_8</td>
<td>346, 583, 634</td>
<td><em>Cyberlindnera jadinii</em></td>
<td>3.00E-44</td>
<td>101/101 (100)</td>
<td>KF751183.1</td>
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<tr>
<td>OTU_9</td>
<td>583</td>
<td><em>Exophiala dermatitidis</em></td>
<td>4.00E-129</td>
<td>257/258 (99)</td>
<td>X79317.1</td>
</tr>
<tr>
<td>OTU_10</td>
<td>1740</td>
<td><em>Walleraeum munae</em></td>
<td>1.00E-124</td>
<td>246/246 (100)</td>
<td>AY741381.1</td>
</tr>
<tr>
<td>OTU_11</td>
<td>346, 583</td>
<td><em>Trichoderma sp.</em></td>
<td>2.00E-176</td>
<td>339/339 (100)</td>
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<td>AB473810.1</td>
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<th>OTU Id</th>
<th>Sample depth (mbsf)</th>
<th>Most similar sequence</th>
<th>$E$ value</th>
<th>Identity (%)</th>
<th>GenBank accession number of the most similar sequence</th>
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<td>7.00E-46</td>
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DNA-based eukaryotic 18S dataset

Within the fungal kingdom, only the Dikarya were detected in our DNA-based libraries, with 63% of Ascomycota and 37% of Basidiomycota. The reads were classified into seven classes. Signatures of Saccharomycetes and Tremellomycetes were the most abundant recovered, with 38% and 35% of the reads, respectively. Sordariomycetes, Eurotiomycetes, Dothideomycetes, Wallmiomycetes, and Exobasidiomycetes were less well represented, with 15%, 6%, 3%, 2% and 1% of the reads, respectively. Signatures of Saccharomycetes and Tremellomycetes had a broad distribution pattern, with no apparent specificity for one depth. Dothideomycetes, Sordariomycetes, and Eurotiomycetes were only detected in the first samples from 346 to 583 mbsf. Sequences affiliated to Wallmiomycetes were seen exclusively in our deepest sample from 1740 mbsf. OTUs with a species-level taxonomic assignment are presented in Table 4. Fungal species appear to be unique at the different depths analyzed. Fusarium solani and L. chartarum were only found in the 346-mbsf sample. Cyberlindnera jadinii, E. dermatitidis, Trichoderma sp. and P. richardiae were detected in the shallowest samples up to 634 mbsf. Signatures of T. mucoides were only detected in the 931-mbsf sample, whereas M. guilliermondii, C. curvatus and C. surraequensis were detected in all samples analyzed. Malassezia pachydermatis was only detected at 931–1690 mbsf. Filobasidium globisporum and W. muriae were only found in the deepest layer (Fig. 4).

V4 region of the RNA-based eukaryotic 18S rRNA dataset

Within the fungal kingdom, only one fungal OTU was detected in the samples analyzed at 12, 346 and 931 mbsf using an RNA-based approach, and this was assigned to the basidiomycete yeast Malassezia. Occurrence of reads decreased with depth, with 11 reads (of 27988 eukaryotic reads that passed quality control) observed at 12 mbsf and one read (of 18897 eukaryotic reads) at 346 mbsf. No fungal signatures were detected in the 931-mbsf sample (of 17397 eukaryotic reads).

DNA-based fungal ITS1 dataset

Sequences recovered in the ITS1 dataset were affiliated to the Dikarya. Dominance of Basidiomycota was observed with 61% of the sequences, while Ascomycota communities

Fig. 4. Distribution of fungal OTUs at the species level based on DNA-based V1–V3 SSU (a) and ITS1 region (b). Sample depths are given in Table 1.
were less well represented with 39% of the sequences. *Wallemiomycetes* and *Exobasidiomycetes*, which were previously found in the DNA-based 18S dataset, were absent in the ITS dataset. In contrast, *Microbotryomycetes* were only found with ITS1 primers. Signatures of *Tremellomyces* and *Saccharomyces* were the best represented at the class level with 57% and 26% of the reads, respectively. *Sordariomycetes*, *Microbotryomycetes*, *Eurotiomycetes* and *Dothideomycetes* represented 10%, 4%, 2% and 1% of the reads, respectively. The *Saccharomyces* and *Tremellomyces* displayed a broad distribution pattern, with no apparent specificity for one depth. *Dothideomycetes*, *Sordariomycetes* and *Eurotiomycetes* have been detected in the upper samples down to 582 mbsf, a result consistent with the DNA-based 18S data. OTUs and a species-level taxonomic assignment are given in Table 4. Signatures of *Penicillium* sp., *L. chartarum*, *Chaetothyriales* sp., *E. caryae*, *Rhinocladiella* sp., *B. leucadendri*, *M. guilliermondii*, *P. richardsiae* and *E. spinifera* dominated the upper horizons down to 582 mbsf. *Trichosporon* sp. was detected down to 931 mbsf. Sequences affiliating with *Leucosporidiella muscorum*, *Rhodotorula* sp., *T. moriformis*, *C. saitoi* and *R. kratochvilovae* were only recovered from the 931-mbsf sample, while *C. pseudolongus* and *C. jadinii* appeared to dominate the deepest layers analyzed (Fig. 4).

**Discussion**

The aim of this study was to investigate the vertical distribution of fungal communities occurring in deep subsurface sediments of the Canterbury basin. Toward that aim, we used a DNA-based analysis of eukaryotic rRNA and ITS1 signatures in samples from nine different depths from subsurface sediments collected in the Canterbury basin spanning 346 to 1740 mbsf. In addition, in a separate study included here, we applied an RNA-based analysis of RNA genes from two depths along this transect and one complementary shallow layer sampled at 12 mbsf. These datasets provide insights into the different fungal taxa colonizing the subseaﬂoor. The entire dataset was submitted to the European Nucleotide Archive under the study accession number PRJEB6764.

**Controlling contamination**

Accurate studies of the deep biosphere require strict quality controls of the samples analyzed regarding contamination risks, since the presence of exogenous cells or nucleic acids may lead to erroneous results. During the IODP 317 Expedition, the potential for microbiological contamination of samples was investigated. Different tests during drilling were performed onboard the R/V *Joides Resolution* to quantify fluorescent microbeads mimicking microbial cells that were incorporated into drill fluids during drilling. The exteriors of cores were subsequently investigated using fluorescence microscopy to determine the potential for contamination from seawater and drilling fluids. Onboard, whole-round sediment cores were subsampled from within the core interior, under sterile conditions, and these subsamples were immediately frozen at −80 °C for onshore molecular analyses. Potential contamination of the interior of the core sample is very low and was estimated at 5–11 cells g⁻¹ of sediment, based on observation of fluorescent beads and average densities of 1.85 g cm⁻³ in sediments and 1.99 g cm⁻³ in sedimentary rocks at site U1352 (Ciobanu et al., 2014). In addition, precautions were taken during analysis in the laboratory to avoid contamination with the use of (i) sterile materials dedicated to low biomass samples, (ii) as stringent a data processing strategy as possible (two independent pyrosequencing replicates) and (iii) the analysis of a contaminant library to remove any potential contaminant and to present a conservative picture of subsurface communities.

**Comparison of the DNA-based V1–V3 SSU and ITS1 markers for understanding fungal community diversity**

Among the DNA-based 18S and fungal ITS1 datasets, only the *Dikarya* were detected, suggesting absence or really low abundance of early diverging lineages in these samples. The ratio of *Ascomycota* to *Basidiomycota* was quite different, with 1.7 of the total reads using universal eukaryotic primers and 0.64 of the total reads using the fungal specific primers. However, high numbers of OTUs belonging to *Basidiomycota* in the ITS dataset are consistent with previous studies where basidiomycete yeasts were found to be the dominant fungal forms in deep-sea environments (Takishita et al., 2006; Bass et al., 2007; Singh et al., 2011). Signatures of many common genera were detected in the two datasets — *Cryptococcus*, *Meyerozyma*, *Exophiala*, *Trichosporon*, *Pleurostomophora* and *Leptosphaerulina* – which showed the same distribution pattern along the core. *Leptosphaerulina* was found only at 346 mbsf. Similarly, *Pleurostomophora* and *Exophiala* were only detected at 583 mbsf. These genera, associated with the lower depths, seem to be correlated with high porosity and high organic carbon concentration. Sequences affiliated to *M. guilliermondii* formed the most abundant OTU in the eukaryotic 18S dataset and signatures of this organism were present throughout the sediment core. By contrast, signatures of this taxon were only present in the upper sedimentary layers with the fungal ITS1 marker. *Cryptococcus* was detected all along the core with the eukaryotic 18S marker, but only in the deeper layers with the fungal marker. These differences between datasets were not
surprising, since biases of primers are well known and each primer set favors recovery of specific taxonomic groups of fungi. Also, although ITS1 and ITS2 share many properties, the ITS2 marker is less variable in length compared with ITS1 and is also well represented in databases (Lindahl et al., 2013). Multiple-primer and multiple-marker approaches thus appeared more efficient for capturing a broader picture of fungal diversity.

Fungal communities in deep sediments are not diverse and are close to terrestrial taxa

Using the tag-encoded 454 pyrosequencing approach, we discovered that the fungal diversity is quite low in these samples. Fungal communities appear to be different at different depths since unique OTUs were detected at each sediment depth. Our study identified 22 different genera among the Dikarya. Interestingly, these deep sediment fungi are phylogenetically close to known terrestrial fungi, suggesting that fungi are able to colonize deep-sea habitats. Indeed, it has been shown that the fungi are able to change their membrane composition to tolerate in situ conditions such as a high hydrostatic pressure (Simonato et al., 2006). One of the most abundant OTUs in our dataset was affiliated to Cryptococcus. This is consistent with another study of microeukaryotic diversity in deep-sea methane-rich sediments. Takishita et al. (2006) identified C. curvatus as the dominant eukaryote in 18S rRNA gene libraries from Kuroshima Knoll methane seep. This genus was also detected in subsurface sediments down to 48 mbsf in an RNA-based study of Peru Margin sediments (Orsi et al., 2013a). Cyberlindnera yeasts were found in our study down to 1711 mbsf. These yeasts have already been shown to have the ability to colonize marine environments, including acidic waters (Gadano & Sampaio, 2006) and subsurface sediments (Orsi et al., 2013a). Other yeasts, including Meyerozyma, Malassezia, Rhodosporidium, Trichosporon and Filobasidium have previously been detected in marine sediments (Kutty & Philp, 2008; Edgcomb et al., 2011; Orsi et al., 2013a). Fusarium, present down to 346 mbsf in our samples, is a genus that includes plant pathogens and mycotoxin producers. It has been reported in river and seawater (Palmero et al., 2009) and it may also be an opportunistic pathogen of deep-sea animals (Ramaiah, 2006). This suggests that fungi known in terrestrial environments may also persist at greater sediment depths and might have an ecological role in the deep biosphere.

Distribution patterns

Although fungal communities exhibited quite low overall phylogenetic diversity, diversity was greater in the samples from 346 and 583 mbsf than in deeper samples. After the transition from sediment to sedimentary rock, which occurs around 931 mbsf, diversity appears to decrease. Fungal communities likely depend on a greater amount of organic material than is available in rocky subsurface horizons. Indeed, PCA of OTU distribution supports the correlation with depth and organic matter, with some communities negatively correlated with organic matter that may indicate an adaptation to oligotrophic conditions. This distribution pattern was observed with ITS1 and 18S markers. Also, a positive correlation with representatives of the Cryptococcus genus and methane has been revealed and supports the idea that such basidiomycetes appear to be dominant in deep-sea marine methane-rich environments (Takishita et al., 2006) and that they might be indirectly involved in the deep subseafloor methane cycle. A correlation with ethane has also been revealed with Cryptococcus and might indicate some interactions between this fungus and methanogenic/ethanogenic prokaryotes in deep marine sediments (Hinrichs et al., 2006). The poor overlap of fungal OTUs between the different depths suggests a spatial differentiation of fungal communities according to available resources at different depths, but also supports the observation that contamination is unlikely. Fungal communities appear thus mostly depth-specific with complementary environmental parameters – here organic matter and methane concentration – as structuring parameters likely to influence the distribution of the microeukaryotic communities in marine sediments.

Many fungal OTUs may represent dormant taxa

Surprisingly, only one fungal OTU was detected in our RNA-based 18S dataset compared with 13 fungal OTUs in our DNA-based 18S dataset. As a first conclusion, fungi do not appear to be very diverse or active in the Canterbury basin, in contrast to Peru Margin sediments (Edgcomb et al., 2011; Orsi et al., 2013a, b). Since the RNA-based analyses were an unanticipated addition to our study, and given primers and protocols used for the RNA- and DNA-based analyses were different, variations in recovery of particular fungal OTUs must be interpreted with caution. Fungal OTUs revealed from both DNA- and RNA-based methods affiliated exclusively with the ubiquitous yeast Malassezia. The most ubiquitous species within the class Exobasidiomycetes are related to the genus Malassezia in deep-sea environments (Nagano & Nagahama, 2012). Although Malassezia species are well known as causative agents of skin diseases, this yeast is also frequently recovered by DNA-based analysis of marine samples, indicating that this taxon likely occupies a wider range of niches than previously thought, and that
Malassezia may be common in marine environments that include deep-sea water columns and sediment samples (Amend, 2014). Gao et al. (2008) revealed a high diversity of Malassezia lineages associated with marine sponges and invertebrates, suggesting that they may also be opportunistic pathogens of deep-sea mammals. Their recovery in RNA-based clone libraries suggests that they may also survive on buried organic matter at greater sediment depths (Edgcomb et al., 2011). Although molecular studies suggest the ubiquitous presence of Malassezia phylotypes in deep-sea environments, no cultures have been obtained from deep-sea water and deep-sea sediments. Future studies employing culture-dependent and culture-independent approaches should reveal useful information on the ecological significance of the Malassezia group in marine environment.

Although our RNA- and DNA-based 18S datasets are not strictly comparable, the significantly lower recovery of taxonomic diversity in the RNA-based datasets suggests that the V4 primers could be less complementary to fungal targets. The majority of subsurface fungi in this Canterbury basin subsurface sediments appear slightly active and few persistent fungal taxa seem to colonize the deep subsurface.

Conclusion

To the best of our knowledge, this work is the first dedicated to the specific description of fungal communities in the deep subseafloor. We found an unexpected fungal diversity down to a record depth of 1740 mbsf using DNA-based pyrotag sequencing. RNA provided evidence of active fungi down to 346 mbsf. The fact that some of the fungal sequences obtained in this work have been previously reported from marine ecosystems supports the hypothesis that fungal communities have an important ecological role in this ecosystem. The deep subseafloor fungi revealed in the Canterbury basin are known to be widespread in terrestrial environments, indicating that fungi are highly adaptable organisms, potentially able to colonize and have an ecological role in the deep subseafloor.

Acknowledgements

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