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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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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 underexplored. 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, Wallemiomycetes, 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.

Keywords : 454 pyrotag, Canterbury basin, deep subseafloor, fungi, microeukaryotes

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 *et al*., 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 (Takishita *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 meters below seafloor (mbsf) (Edgcomb *et al*., 2011; Orsi *et al*., 2013a, 2013b). 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 (1922m) 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

1. 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).

2. Nucleic acid extractions, PCR amplifications and 454 sequencing

DNA extractions were performed from 9 samples collected along the core (Table 1). In order 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 x 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 Biomedicals®), 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, 4 independent 25 µL PCR amplifications were performed using the universal eukaryotic primers Euk 42F (López-García *et al*., 2003)/Euk 516R (Amann *et al*., 1990) and fungal primers ITS1 (Gardes & Bruns, 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×Taq DNA polymerase buffer with $MgCl_2$ (2mM), 1 mM of additional $MgCl_2$, 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. PCR program for 18S and sequencing strategy were described in detail previously (Ciobanu *et al*., 2014).

RNA extractions were performed using 3 samples collected along the core, including 12, 346, and 931 mbsf (Table 1). RNA was extracted from 2 x 4 g for 12 mbsf sample and around 2 x 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 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 the manufacturer's instructions. The universal primers TAReukFWD1 and TAReukREV3 (Stoeck *et al.*, 2010) were used to amplify the V4 region (~380 bp) of the eukaryotic 18S rRNA. All PCR reactions were performed in 25 µL reaction volumes containing 1X GoTaq Buffer, 0.2 mM dNTPs, 0.4 µM of each primer, 2,5 mM of $MgCl_2$, 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).

3. 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 derep_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 Silva_111 database and including only eukaryotic sequences with 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 greater than 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 (Koljalg *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, 1963). Beta diversity patterns of samples were assessed using unweighted and weighted UniFrac metrics (Lozupone & Knight, 2005).

4. 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 2-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).

Results

1. 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 9 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 to 100 % of total 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. A total of 164,743 sequences were generated for RNA-based 454 pyrotags for 3 depths, *i.e.* 12, 346 and 931 mbsf. After quality control, 39% of sequences were analyzed (63,564 sequences) and were grouped into 185 OTUs, among them only 1 fungal OTUs 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 5 samples from 346 mbsf 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 DNA-based 18S dataset, that the fungal communities using ITS1 are also weakly diversified along the core.

2. Distribution patterns of fungal communities

Variations in the structure of the fungal communities were determined by Multiple Factorial Analysis (MFA) coupled with a Principal Component Analysis (Fig. 2, Fig. 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 (Fig. 2A, Fig. 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 on the left (Fig. 2A, Fig. 3A). It also clearly highlighting the complexity of the diversity with complex versus not complex samples, as detailed below.

MFA allowed us to infer general distribution patterns. Then, Principal Component Analyses were used to detail communities distribution.

DNA-based 18S dataset. The MFA representation allowed to cluster samples with close values, *i.e.* 1367 mbsf, 634 mbsf and 1740 mbsf samples for inorganic carbon, 346 mbsf, 1740 mbsf and 931 mbsf samples for organic carbon and differentiation of some samples, *e.g.* 583 mbsf, 634 mbsf and 1367 mbsf samples inversely correlated with 346 mbsf, 931 mbsf and 1740 mbsf samples for organic carbon. It clearly indicates that 346 mbsf, 931 mbsf and 1740 mbsf samples were relatively organic-rich depth compared to 583 mbsf, 634 mbsf and 1367 mbsf samples. MFA allowed differentiation of sediment samples based on diversity, *i.e.* complex or not complex. 346 mbsf and 583 mbsf samples, with long length OTU vectors, representing complex samples compared to the other sediment samples with short length OTU vectors and demonstrates that fungal diversity is higher in the first sediment layers analyzed. Using the OTU vector, MFA also clearly indicated that diversity was highly contrasted at the different depths. Indeed, the lengths but 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 3 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 depth, while cluster III contained only OTUs from the shallowest depth. In cluster III, *Cryptococcus 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 low organic carbon concentration.

DNA-based ITS1 dataset. Consistent with the 18S data, MFA indicated that 346 mbsf, 583 mbsf and 931 mbsf samples, with long length OTU vectors, were more complex than 1577 mbsf and 1711 mbsf samples. As for 18S data, the different OTU vector directions at the different depths clearly indicate contrasted fungal communities along the sediment core.

PCA clearly differentiated 3 clusters: (i) *Cryptococcus saitoi, Rhodosporidium kratochvilovae, Rhodotorula* sp., *Tremella moriformis, Leucosporidiella muscorum*; (ii) *Pleurostomophora richardsiae, Chaetothyriales* sp., *Exophiala spinifera, Penicillium* sp., *Batcheloromyces leucadendri*, *Elmerina caryae*, *Rhinocladiella* sp., and (iii) *Cryptococcus pseudolongus, Cyberlindnera jadinii, Galactomyces candidum, Leptosphaerulina chartarum, Trichosporon* sp., and *Meyerozyma guilliermondii*. Those 3 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 affiliated to the *Crypococcus* genus, appeared correlated with methane and ethane.

Of the 13 OTUs obtained with eukaryotic primers, 6 appear to be depth dependent, OTU 2, OTU 3, OTU 6, OTU 9, OTU 10, OTU and OTU 13 (Table 4). A maximum of 4 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).

3. Taxonomic composition

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

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 7 classes. Signatures of *Saccharomycetes* and *Tremellomycetes* were the most abundant recovered, with 38% and 35% of the reads, respectively. *Sordariomycetes*, *Eurothiomycetes, Dothideomycetes*, *Wallemiomycetes* and *Exobasidiomycetes* were less 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 of the depths. *Dothideomycetes, Sordariomycetes*

and *Eurotiomycetes* had only been detected in the first samples from 346 to 583 mbsf. Sequences affiliating with *Wallemiomycetes* are 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 *Leptosphaerulina chartarum* were only found in the 346 mbsf sample. *Cyberlindnera jadinii, Exophiala dermatitidis, Trichoderma* sp. and *Pleurostomophora richardsiae* were detected in the shallowest samples up to 634 mbsf. Signatures of *Trichosporon mucoides* were only detected in the 931 mbsf sample, while *Meyerozyma guilliermondii, Cryptococcus curvatus* and *Cryptococcus surugaensis* were detected in all samples analyzed. *Malassezia pachydermatis* was only detected at 931 to 1689 mbsf. *Filobasidium globisporum* and *Wallemia 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 1 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 (out of 27,988 eukaryotic reads that passed quality control) observed at 12 mbsf and 1 read (out of 18,897 eukaryotic reads) at 346 mbsf. No fungal signatures were detected in the 931 mbsf sample (out of 17,397 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 were less 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 *Tremellomycetes* and *Saccharomycetes* were the most 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 *Saccharomycetes* and *Tremellomycetes* 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., *Leptosphaerulina chartarum*, *Chaetothyriales* sp., *Elmerina caryae*, *Rhinocladiella* sp., *Batcheloromyces leucadendri*, *Meyerozyma guilliermondii*, *Pleurostomophora richardsiae* and *Exophiala spinifera* dominated the upper horizons down to 582 mbsf. *Trichosporon* sp. was detected down to 931 mbsf. Sequences affiliating with *Leucosporidiella muscorum*, *Rhodotorula* sp., *Tremella moriformis*, *Cryptococcus saitoi* and *Rhodosporidium kratochvilovae* were only recovered from the 931 mbsf sample, while *Cryptococcus pseudolongus* and *Cyberlindnera jadinii* appear 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 9 different depths from subsurface sediments collected in the Canterbury basin spanning 346 mbsf to 1740 mbsf. In addition, in a separate study that we include here, we applied an RNA-based analysis of rRNA genes from two depths along this transect and one complementary shallow layer sampled at 12 mbsf. These data sets 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.

1. 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* in order 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) data processing strategy as stringent as possible (2 independent pyrosequencing replicates) and (iii) the analysis of a contaminant library in order to remove any potential contaminant and to present a conservative picture of subsurface communities.

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

Among the DNA-based 18S and fungal ITS1 data sets, 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 between 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 affiliating with *Meyerozyma guillermondii* formed the most abundant OTU in the eukaryotic 18S data set 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 data sets were not surprising, since biases of primers are well known and each primer set favors recovery of specific taxonomic groups of fungi. Also, if 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.

3. 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 affiliated with *Cryptococcus.* This is consistent with another study of microeukaryotic diversity in deep-sea methane-rich sediments. Takishita *et al.,* (2006) identified *Cryptococcus curvatus* as the dominant eukaryote in 18S rRNA gene libraries from Kuroshima Knoll methane seep and this genus was 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 were already shown to have the ability to colonize marine environments including acidic waters (Gadanho & Sampaio, 2006) and subsurface sediments (Orsi et al., 2013a). Other yeasts, including *Meyerozyma, Malassezia, Rhodosporidium*, *Trichosporon* and *Filobasidium* were previously detected in marine sediments (Kutty & Philip, 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.

4. 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 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.

5. Many fungal OTUs may represent dormant taxa

Surprisingly only 1 fungal OTU was detected in our RNA-based 18S dataset, vs. 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, 2013b). 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 the 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). Whereas 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 data sets suggests that the V4 primers could be less complimentary to fungal targets. The majority of subsurface fungi in this Canterbury basin subsurface sediments appear slightly active and few persistent fungal taxa seem 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.

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Figure 1. Sampling site.

Figure 2. Multiple Factorial Analysis (MFA) and Principal Component Analysis (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.

Figure 3. Multiple Factorial Analysis (MFA) and Principal Component Analysis (PCA) based on 454-pyrosequencing of ITS 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. B.l: *Batcheloromyces leucadendri*, C.j: *Cyberlindnera jadinii*, C.p: *Cryptococcus pseudolongus*, C.s: *Cryptococcus saitoi*, C.sp: *Chaetothyriales* sp., E.c: *Elmerina caryae*, E.s: *Exophiala spinifera*, G.c: *Galactomyces candidum*, L.c: *Leptosphaerulina chartarum*, L.m: *Leucosporidiella muscorum*, M.g: *Meyerozyma guilliermondii*, P.r: *Pleurostomophora richardsiae*, P.sp: *Penicillium* sp., R.k: *Rhodosporidium kratochvilovae*, Rhi.sp: *Rhinocladiella* sp., Rho.sp: *Rhodotorula* sp., T.m: *Tremella moriformis*, T.sp: *Trichosporon* sp. Sample depths are given in table 1

Figure 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.

Table 1. List of studied samples and depths.

Table 2. Number of sequences and number of OTUs in the DNA-based datasets.

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

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

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

	346 mbsf		**583 mbsf**		**634 mbsf**	**931 mbsf**		**1367 mbsf**	**1577 mbsf**	**1690 mbsf**	**1711 mbsf**	**1740 mbsf**
	18S	ITS	18S	ITS	18S	18S	ITS	18S	ITS	18S	ITS	18S
Number of reads	6425	4625	5868	3789	7646	4521	1485	2318	4377	2866	3399	2659
Retained quality reads	2491	2366	4747	1448	2973	2260	983	919	3568	1809	2056	1240
Number of fungal OTUs	7	3	7	10	2	4	6	1	1	3	2	3

	346 mbsf		583 mbsf		634 mbsf	931 mbsf		1367 mbsf	1577 mbsf	1690 mbsf	1711 mbsf	1740 mbsf
	18S	ITS	18S	ITS	18S	18S	ITS	18S	ITS	18S	ITS	18S
Richness (Chao1)	7	4	10	11	2	4	6	1	1	3	2	3
Diversity (Shannon)	2.11	1.04	1.21	1.71	0.43	0.36	1.86	0	0	1.28	0.07	1.44
Evenness (Simpson)	0.73	0.42	0.51	0.47	0.16	0.12	0.64	0	0	0.54	0.02	0.60

(a)

OTU Id	Sample depth (mbsf)	Most similar sequence	E value	Identity	GenBank accession number of the most similar sequence
OTU_1	583, 634, 931, 1367, 1690	*Meyerozyma guilliermondii*	2,00E-131	261/262(99%)	JQ698913.1
OTU_2	931	*Trichosporon mucoides*	3,00E-130	259/260(99%)	AB001763.2
OTU_3	583	*Pleurostomophora richardsiae*	7,00E-176	338/338(100%)	AY729812.1
OTU_4	346, 583, 931, 1690	*Cryptococcus curvatus*	2,00E-131	261/262(99%)	AB032626.1
OTU_5	346, 583, 1740	*Cryptococcus surugaensis*	0.0	360/361(99%)	AB100440.1
OTU_6	346	*Leptosphaerulina chartarum*	3,00E-174	338/339(99%)	HM216185.1
OTU_7	346, 1740	*Filobasidium globisporum*	2,00E-131	261/262(99%)	AB075546.1
OTU_8	346, 583, 634	*Cyberlindnera jadinii*	3,00E-174	338/339(99%)	EF550447.1
OTU_9	583	*Exophiala dermatitidis*	4,00E-129	257/258(99%)	X79317.1
OTU_10	1740	*Wallemia muriae*	1,00E-124	246/246(100%)	AY741381.1
OTU_11	346, 583	*Trichoderma* sp.	2,00E-176	339/339(100%)	AB491674.1
OTU_12	346, 1690	*Malassezia pachydermatis*	2,00E-147	327/345(95%)	DQ457640.1
OTU_13	346	*Fusarium solani*	5,00E-118	237/238(99%)	AB473810.1

(b)

OTU Id	Sample depth (mbsf)	Most similar sequence	E value	Identity	GenBank accession number of the most similar sequence
OTU_1	1577	*Cryptococcus pseudolongus*	3,00E-55	121/121(100%)	AB051048.1
OTU_2	583, 1711	*Cyberlindnera jadinii*	4,00E-98	201/202(99%)	FJ865435.1
OTU_3	346, 583, 931	*Trichosporon* sp.	9,00E-100	204/205(99%)	JX270559.1
OTU_4	583	*Pleurostomophora richardsiae*	3,00E-44	101/101(100%)	KF751183.1
OTU_5	931	*Cryptococcus saitoi*	1,00E-133	262/262(100%)	JX188127.1
OTU_6	346, 583	*Meyerozyma guilliermondii*	6,00E-136	266/266(100%)	KC119207.1
OTU_7	931	*Rhodosporidium kratochvilovae*	1,00E-92	188/188(100%)	JN662395.1
OTU_8	346	*Leptosphaerulina chartarum*	3,00E-104	214/216(99%)	AM231400.1
OTU_9	583	*Exophiala spinifera*	9,00E-50	111/111(100%)	JX966556.1
OTU_10	583	*Penicillium* sp.	2,00E-86	177/177(100%)	KC464351.1
OTU_11	931	*Leucosporidiella muscorum*	7,00E-86	187/192(97%)	FR717869.1
OTU_12	931	*Rhodotorula* sp.	4,00E-40	106/112(95%)	AM901696.1
OTU_13	583	*Chaetothyriales* sp.	3,00E-35	153/183(84%)	HQ634635.1
OTU_14	583	*Elmerina caryae*	3,00E-24	125/152(82%)	JQ764655.1
OTU_15	583	*Rhinocladiella* sp.	3,00E-59	128/128(100%)	KF811431.1
OTU_16	931	*Tremella moriformis*	3,00E-60	132/133(99%)	AF042426.1
OTU_17	1711	*Galactomyces candidum*	4,00E-73	161/164(98%)	JN974290.1
OTU_18	583	*Batcheloromyces leucadendri*	7,00E-46	122/131(93%)	JF499832.1

OTU Id	Sample depth (mbsf)	Most similar sequence	E value	Identity	UNITE accession number of the most similar sequence
OTU_10	583	*Penicillium minioluteum*	7,00E-89	100%	SH225847.06FU
OTU_12	931	*Rhodotorula minuta*	2,00E-47	97%	SH227573.06FU
OTU_15	583	*Rhinocladiella similis*	8,00E-62	100%	SH210380.06FU