Water Research 145 (2018) 50–61

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

First insights into the living groundwater mycobiome of the terrestrial biogeosphere

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ARTICLE INFO

Article history:
Received 28 February 2018
Received in revised form
2 June 2018
Accepted 27 July 2018
Available online 28 July 2018

Keywords:
Groundwater
Living fungi
Fungal diversity
Subsurface biosphere
ITS
Critical zone exploratory

ABSTRACT

Although fungi play important roles in biogeochemical cycling in aquatic ecosystems and have received a great deal of attention, much remains unknown about the living fractions of fungal communities in aquifers of the terrestrial subsurface in terms of diversity, community dynamics, functional roles, the impact of environmental factors and presence of fungal pathogens. Here we address this gap in knowledge by using RNA-based high throughput pair-end illumina sequencing analysis of fungal internal transcribed spacer (ITS) gene markers, to target the living fractions of groundwater fungal communities from fractured alternating carbonate-/siliciclastic-rock aquifers of the Hainich Critical Zone Exploratory. The probed levels of the hillslope multi-storey aquifer system differ primarily in their oxygen and nitrogen content due to their different connections to the surface. We discovered highly diverse living fungal communities (384 Operational Taxonomic Units, OTUs) with different taxonomic affiliations and ecological functions. The observed fungal communities primarily belonged to three phyla: Ascomycota, Basidiomycota and Chytridiomycota. Perceived dynamics in the composition of living fungal communities were significantly shaped by the concentration of ammonium in the moderately agriculturally impacted aquifer system. Apart from fungal saprotrophs, we also detected living plant and animal pathogens for the first time in this aquifer system. This work also demonstrates that the RNA-based high throughput pair-end illumina sequencing method can be used in future for water quality monitoring in terms of living fungal load and subsequent risk assessments. In general, this study contributes towards the growing knowledge of aquatic fungi in terrestrial subsurface biogeosphere.

1. Introduction

In terrestrial environments, the subsurface is markedly subdivided into unsaturated zones (spanning organism-rich soils, less diverse subsoils, parts of bedrock, caves) and saturated zones (mainly bedrocks including aquifers). The saturated zones and the aquifers hold prime importance by suppling tremendous amounts of freshwater (30%) (Griebler and Avramov, 2015) to more than 25% of the world population for domestic, agricultural and industrial use (Ford and Williams, 2013; Martin and White, 2008). Because of their incredible importance to human lives, aquifer systems have been studied to investigate the presence of different microbial groups and their potential roles in nutrient cycling by employing both culture dependent and culture-independent high throughput methods. However, most of such studies with the focus on geomicrobiology in subsurface aquifer systems have targeted prokaryotes (bacteria and archaea) (Flynn et al., 2013; Gray and Engel, 2013; Herrmann et al., 2015; Lazar et al., 2017; Opitz et al., 2014; Shabarova and Pernthaler, 2010; Shabarova et al., 2014). The potential presence, activity and ecological importance of microbial eukaryotes, such as fungi have largely been overlooked with only a

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https://doi.org/10.1016/j.watres.2018.07.067

few studies specifically targeting fungi in pristine carbonate-rock aquifers (Lategan et al., 2012; Nawaz et al., 2016; Schwab et al., 2017).

Fungi are a wide spread group of microbial eukaryotes with 2.2–3.8 million species (Hawksworth and Lucking, 2017) that not only play significant roles in ecosystem functioning but also influence humans and human related activities (Mueller and Bills, 2004). Owing to the characteristic lifestyle (decomposers, symbionts and pathogens), the ability to develop long hyphal networks, produce spores and grow as single yeast-cells, fungal omnipresence has widely been accepted in terrestrial (Tedersoo et al., 2014), marine (Ivarsson et al., 2016; Xu et al., 2014) and freshwater (Nawaz et al., 2016; Wurzbacher et al. 2010, 2011) ecosystems. Like in terrestrial and marine environments, the ecological importance of fungi in freshwater systems is also significant in several key areas e.g., degradation and decomposition of organic and inorganic materials, which results in subsequent recycling of nutrients and energy and ultimately impacts the downstream food web dynamics (Goh and Hyde, 1996; Kagami et al., 2014; Kuehn, 2016; Wurzbacher et al., 2011). When referring to the subsurface freshwater systems in fractured bedrock regions (supplying fresh water for domestic purposes), apart from the presence of saprotrophs, the quality of the water is also heavily depending on the presence of potential pathogens (Hageskal et al., 2009) that can infect animals, plants and ultimately humans. Therefore, to access the significance of fungi in ecosystem functioning of subsurface aquifers, we need to develop a comprehensive picture of the actual metabolically active fungal communities.

In a previous study, we analyzed total DNA from groundwater samples of the Hainich Critical Zone Exploratory (Hainich CZE) well transect that accesses a pristine multi-storey aquifer system hosted in thin-bedded carbonate/siliciclastic alternations (Nawaz et al., 2016). The sole purpose of our previous DNA based study was to get an evidence on the existence/presence of fungi in the subsurface aquifer system of Hainich CZE and when yes assess their community composition. Along with the evidence of fungal presence in this ecosystem, we found that the two aquifers assemblages of the Middle Triassic bedrocks exhibit distinct fungal communities across different relief positions and depths along the Hainich hillslope that differ in their respective oxygen and nitrate concentrations (Küsel et al., 2016). Nevertheless, it is important to consider that the previous studies in subsurface aquifers have used environmental-DNA (eDNA) to target the unexplored biodiversity of aquatic fungi and their potential roles in ecosystem functioning (Lategan et al., 2012; Nawaz et al., 2016; Panzer et al., 2015; Risse-Buhl et al., 2013). Although the eDNA inventory gives an overall impression of the microbial biodiversity, it bears the possibility that the retrieved DNA also reflects metabolically inactive or even dead species (Rajala et al., 2011). Therefore, to get a better understanding of the functioning of subsurface aquifer system, there is a need to capture the fractions of the fungal communities, which are actually metabolically active rather than detecting the recoverable fungal communities.

In this study, we aimed to extend the scope of available information regarding the presence of different fungal groups and community composition in terrestrial subsurface aquifers from total fungal populations to the level of living fractions. We used culture independent RNA-based paired-end illumina sequencing analysis of fungal internal transcribed spacer (ITS2) region to target the metabolically active fraction of the fungal community in two pristine aquifer assemblages in Hainich CZE. Specifically, considering three sampling campaigns in different seasons, we aimed to: **i)** investigate spatiotemporal variability in the diversity of living fungi in two superimposed bedrock aquifer assemblages, **ii)** identify abiotic factors shaping the living groundwater fungal community composition, **iii)** identify the living functional groups including the detection of potential animal and plant pathogens.

2. Materials and methods

2.1. Site description

The study took advantage of the groundwater monitoring wells of the Hainich CZE within the framework of Collaborative Research Centre AquaDiva (CRC AquaDiva). The Hainich CZE covers parts of the eastern Hainich hillslope, including parts of the Hainich National Park in the northwest of the federal state of Thuringia, Germany, as described in more detail in Küsel et al. (2016). The forested Hainich low-mountain ridge and agriculturally used, gently inclined (2°) eastern hillslope function as the recharge area for regionally used groundwater resources in the multi-storey aquifer system of Triassic bedrock (Kohlhepp et al., 2017). The ~6 km long downslope well transect accesses the subsurface habitats of sloping fractured limestone/mudstone alternations at several relief positions and depths or at different distances to preferential surface-recharge (outcrop) areas (Kohlhepp et al., 2017), respectively (Fig. 1).

In the Upper Muschelkalk (Germanic Triassic lithostratigraphic subgroup) strata, the multi-storey aquifer system can be aggregated to the lower aquifer assemblage (HTL), representing one aquifer in the Trochitenkalk formation (moTK), and to the upper aquifer assemblage (HTU), hosting nine aquifer storeys that in turn aggregate the hydrostratigraphy of the thin-bedded alternations of the Meissner formation (moM) (Kohlhepp et al., 2017) (Fig. 1). Marked differences in the two aquifer assemblages (HTL and HTU) in hydrochemistry (oxicity, nutrient supply) due to the aquifer configuration, overburden and land use were reported in Küsel et al. (2016) and were reconstructed in Kohlhepp et al. (2017). Specifically, the HTU aquifers are generally oxygen-deficient and the HTL aquifer is oxygen-rich.

2.2. Groundwater sampling

Groundwater samples for physico-chemical and biological analysis were collected during regular four-weekly joint sampling campaigns in December 2014, March 2015, and June 2015 representing winter (groundwater low-stand), spring (groundwater high-stand) and summer, respectively. Water samples from sites H3, H4 and H5 were collected from seven permanently water-bearing monitoring wells namely H32, H43, H52 and H53 (HTU) and H31, H41, H51 (HTL). The hilltop/upper slope sites H1 and H2 were not sampled because of very low groundwater quantities due to seasonal or overall low local saturation. Prior to sampling, the groundwater was pumped out and discarded, using a submersible sampling pump (MP1, Grundfos, Denmark) until the physico-chemical parameters i.e., pH, dissolved oxygen, redox potential and specific conductivity were stabilized. For molecular analysis (RNA extraction), the collected groundwater was transferred to sterile glass bottles, stored at 4 °C and transported to laboratory within 1 h. Approximately 2–3 L of groundwater was filtered through 0.2 μm polycarbonate filters (Nuclepore, Whatman; Merck, USA). The filters were then transferred into sterile reaction tubes, embedded in dry ice within 1 min and stored in laboratories at −80 °C until subsequent nucleic acid extraction.

2.3. Physico-chemical analysis

For all water samples, the physico-chemical parameters i.e., extraction temperature, pH, dissolved oxygen, redox potential and specific electrical conductivity were measured on site in a flow-

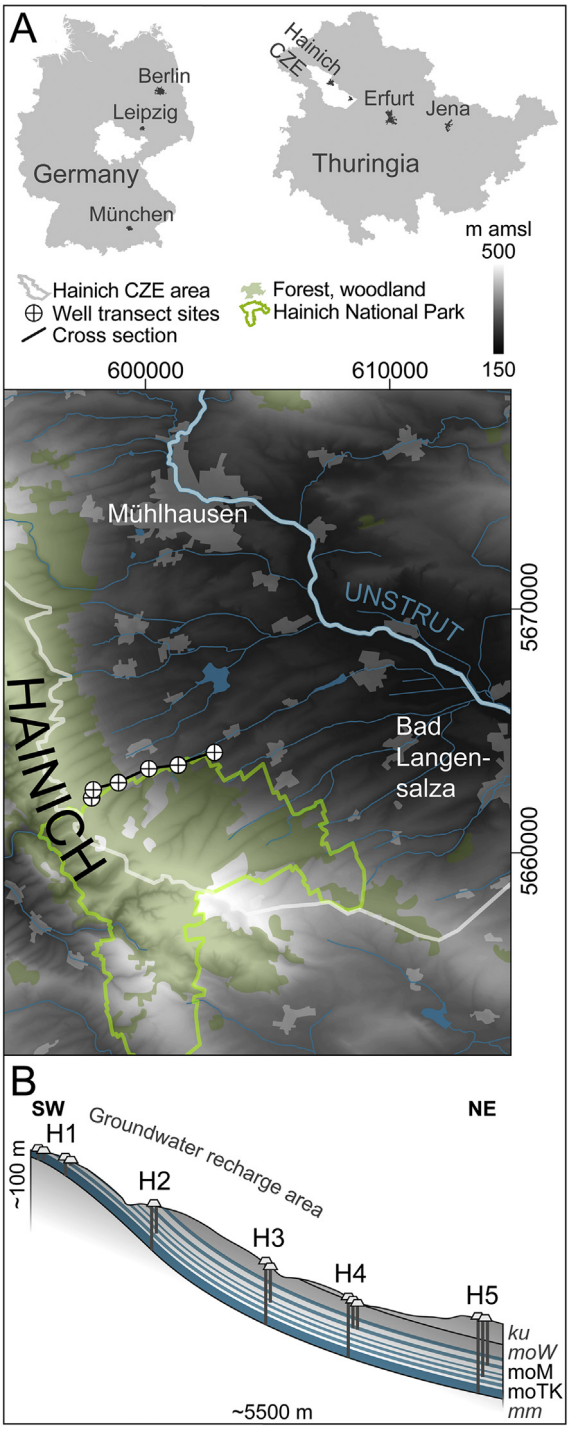

Fig. 1. Site description of Hainich Critical Zone Exploratory. (A) Location of the study site in the Hainich Critical Zone Exploratory (Hainich CZE) (data sources: DEM © GDI-Th, DLM250 ©GeoBasis-DE/BKG 2016; dl-de/by-2-0, http://www.govdata.de/dl-de/by-2-0; Coordinate system: ETRS89/UTM, zone 32N); (B) Schematic cross section of the well transect; blue levels mark the schematic multi-story hydrostratigraphy; lithostratigraphic units (Germanic Middle Triassic): mm Middle Muschelkalk, moTK Trochitenkalk fm., moM Meissner fm., moW Warburg fm., ku Lower Keuper. The groundwater wells target different depths in the subsurface and in different surface locations (forest, grassland, agriculture cropland). Groundwater samples were collected and analyzed from the permanent water bearing wells in HTL (H31, H41, H51) and HTU (H32, H42, H43, H52 and H53) aquifer assemblage. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

through cell using respective probes as explained in Küsel et al. (2016). In situ groundwater temperatures were obtained by permanently installed data-loggers (see Kohlhepp et al., 2017). For nitrate, nitrite and ammonium concentrations, groundwater was filtered through 0.2 μm pore size sterile syringe filters for subsequent analysis. Concentration of sulfate ions was determined by ion chromatography (IC 20 system, Dionex, CA, USA) equipped with an IonPac AS11-HC column and an IonPac AG11-HC precolumn, and total organic carbon (TOC) concentrations were determined using a TOC analyzer (AnalytikJena, Jena, Germany).

2.4. RNA extraction, amplicon library and Illumina MiSeq sequencing

Extraction of total RNA was carried out from polycarbonate filter disks by using the PowerWater RNA Isolation Kit (MO BIO Laboratories Inc., USA) according to the manufacturer's protocol. In addition to on-column DNase treatment included in the protocol of the PowerWater RNA Isolation kit, RNA extracts were treated with TurboDNA free (Thermo Fisher Scientific, USA) and checked for PCR amplification using universal fungal primers to ensure the absence of any traces of DNA. The RNA extracts were then transcribed to cDNA using the NebNext first strand synthesis kit (including random haxamers) (New England BioLabs, USA) following the manufacturers protocol. The cDNA extracts were purified using MinElute columns (Qiagen).

To target the living fungal community, an ITS2 library was constructed using the primer combination fITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990), which anneal to the 5.8S and LSU rRNA genes, respectively. The PCR reaction mix included 5 ng of cDNA extract as template, 15 pmol of each forward primer (fITS7) and reverse primer (ITS4 in 20 μL volume of MyTaq buffer containing 1.5 units MyTaq DNA polymerase (Bioline) and 2 μl of BioStabII PCR Enhancer (Sigma, USA). For each sample, the forward and reverse primers had the same 8-nt barcode sequence. PCRs were carried out for 30 cycles using the following parameters: 2 min 96 °C pre-denaturation; 96 °C for 15s, 50 °C for 30s, 72 °C for 60s and a final extension at 72 °C for 5 min. The concentration of amplicons was determined by gel electrophoresis. When needed, PCR products showing low yields were further amplified for 3–7 cycles. cDNA samples that failed to give PCR products were diluted 10 times and the PCR reaction was repeated. About 20 ng amplicons of each sample were pooled for up to 48 samples carrying different barcodes. The amplicon pools were purified with one volume AMPure XP beads (Agencourt) to remove primer dimer and other short mispriming products, followed by an additional purification on MinElute columns (Qiagen). About 100 ng of each purified amplicon pool was used to construct Illumina libraries using the Ovation Rapid DR Multiplex System 1–96 (NuGEN). Illumina libraries were pooled and size selected by preparative Gel electrophoresis. Sequencing was done on an Illumina MiSeq using V3 Chemistry (Illumina) at LGC Genomics Berlin.

2.5. Sequence processing and bioinformatic analysis

Sequential bioinformatic analysis was performed to filter out high quality reads from the paired-end sequences generated by the Illumina MiSeq sequencing platform using MOTHUR (Schloss et al., 2009) and OBI Tools (Boyer et al., 2016) software suits. Briefly, read pairs were extracted from raw libraries if the two reads hold the expected primer (forward primer for forward library, reverse primer for reverse library) at its 5' end. Forward and reverse raw reads from the same sample were assembled by using the simple-bayesian algorithm with a threshold of 0.6 and a minimum overlap of 20 nucleotides as implemented in PANDAseq (Masella et al.,

2012). To extract high-quality reads, all the assembled reads were trimmed with the following parameters: i) minimum average Phred score of 25 on the trimmed length, ii) no ambiguities in the sequence length and iii) maximum length of 10 homopolymers in the sequence. The reads were then pre-clustered using CD-HIT-EST, allowing a maximum of 1% of dissimilarity and with only one base allowed per indel (Niu et al., 2010), in order to merge those reads arising likely from sequencing errors (Huse et al., 2010). Chimeric sequences were detected using the UCHIME algorithm (Edgar et al., 2011) as implemented in MOTHUR. After removal of chimeric sequences, reads from each sample were pooled together and were dereplicated into unique sequences and sorted by decreasing abundance. The resulting reads were then clustered into operational taxonomic units (OTUs) using the CD-HIT-EST algorithm (Fu et al., 2012) at a threshold of 97% sequence similarity. The OTU representative sequences (the most abundant sequence in each OTU) were taxonomically assigned against the reference sequences from the unite database (version unite.v7) (Koljalg et al., 2013) using the naive bayesian classifier (Wang et al., 2007) as implemented in MOTHUR using the default parameters. The sequences identified as fungi were further classified against the full version of the UNITE (v7.0) database to improve their taxonomic annotation. Finally, all the sequences identified as fungi were again classified against fungal sequences of the UNITE database augmented with non-fungal eukaryotic sequences from NCBI (version 211) (Benson et al., 2013) in order to detect sequences from non-target organisms.

In order to assess the effect of the removal of rare OTUs, which potentially might originate from artificial sequences (Kunin et al., 2010), we performed a Mantel test using Bray–Curtis dissimilarities to assess the correlations between the whole matrix and a matrix excluding the rare OTUs (singletons, doubletons, and tripletons) (Nawaz et al., 2016). The result indicated that the removal of rare OTUs from the total community had no effect on the fungal community composition ($R_{Mantel} = 0.999$, $P = 0.001$). Therefore, the final dataset without rare OTUs was used for further statistical analysis unless otherwise stated.

The fungal OTUs (taxa) retrieved in this study (living fungi) were compared to the fungal OTUs from our previous study (Nawaz et al., 2016) using the CD-HIT-EST-2D algorithm at 97% sequence similarity threshold (http://weizhong-lab.ucsd.edu/cd-hit/). Finally, representative sequences of the dominant fungal OTUs, removing the rare taxa, were assigned to functional or ecological groups using the FUNGuild database (Nguyen et al., 2016). The fungal ITS2 raw sequence dataset are deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under bioproject number PRJNA436133.

2.6. Statistical analysis

The R (R Development Core Team, 2015) and PAST (v2.17) software- (Hammer et al., 2001) were used for the data analysis. The OTU matrix was randomly normalized to the smallest number of reads per sample to get minimum common sequencing depth of 1024 reads. For the generation of rarefaction curves and alpha diversity calculation, the OTU data matrix (including single, double and triple-tons) was used, whereas for all the other analyses, the abundant OTU data (without single, double and triple-tons) matrix was used, unless otherwise stated.

Sample-based individual rarefaction curves (Hurlbert, 1971) were generated for all the groundwater samples from lower and upper aquifer assemblages to access the sampling efforts by using the function “diversity” in PAST. In this study, we used the observed fungal OTU richness as the measure for fungal diversity. Differences in the species richness between the sampling seasons (spring, summer and winter) were compared using ANOVA followed by Tukey *post hoc* test. Whereas the differences in the species richness between two aquifer assemblages (lower and upper aquifer assemblages) were compared using Two-sample T-test in PAST. To minimize the effect of differences in the abundance measure of different OTUs in the data set, we used presence/absence data for the community composition analysis using Jaccard's dissimilarity index by using the vegan package (Oksanen et al., 2015) implemented in R. To visualize the fungal community composition in the lower and upper aquifer assemblages across the different sampled seasons, we used two-dimensional Non-metric multidimensional scaling (2D-NMDS) ordination based on Jaccard dissimilarity index using the vegan package. To assess similarities in the fungal community composition of lower and upper aquifer assemblages, we performed non-parametric multivariate analysis of variances (NPMANOVA) using PAST. We also tested for differences in the homogeneity of multivariate dispersion between the two groups (HTL and HTU) using betadisper function in the Vegan package of R.

Physico-chemical parameters of the groundwater samples from both aquifer assemblages were fitted to the NMDS ordination plot using the “envfit” function of the vegan package as implemented in R. Goodness-of-fit statistics (R^2) were calculated with P values based on 999 permutations. Based on the Goodness-of-fit statistics ($P < 0.05$), all significant physico-chemical parameters were further analyzed by distance-based redundancy analysis (dbRDA) using the Jaccard dissimilarity index with the vegan package (function “capscale”) to determine the most influential physico-chemical parameter describing the living fungal community composition. For all the analysis, a P-value of <0.05 was considered as significant and P-values between 0.05 and 0.1 were considered as marginally significant. Core genus was defined as the genus that consistently appeared in at least 90% of the samples (Caporaso et al., 2011; Huse et al., 2012; Loudon et al., 2014) being considered for the analysis.

3. Results

3.1. Physico-chemical parameters based clustering of groundwater wells

The milieu of the two aquifer assemblages is characterized by neutral pH values between 7.2 and 7.4 and temperatures ranging from 8.8 to 10.1 °C. Similarly, HCO_3^- (measured as acid-neutralizing capacity) and TOC (~2 mg L^{-1}) of the samples from the lower and upper aquifer assemblages did not differ significantly. The mean specific electrical conductivity from both aquifer assemblages ranges between 514.7 and 770.3 µS cm^{-1}. In the lower aquifer assemblage, the mean oxygen concentrations were higher (H31: 7.6 mg L^{-1}, H41: 6.0 mg L^{-1}, H51: 2.7 mg L^{-1}) than in upper aquifer assemblage except for well H32 (2.0 mg L^{-1}). The mean nitrate concentrations in the oxic wells (H31, H41, H51) were higher compared to the anoxic wells (H42, H43, H52 and H53), except for well H32 which had the highest measured mean nitrate concentration (31.85 ± 3.4 mg L^{-1}). Similarly, higher mean concentrations of total sulfur (S_t = 30.10 ± 0.57–102.97 ± 3.35 mg L^{-1}) and sulfate (89.4 ± 0.1–296.1 ± 15.9 mg L^{-1}) were also observed in wells of lower aquifer assemblage. Contrary to this, water samples from the anoxic HTU wells have a higher mean concentration of ammonium (0.8 ± 0.1 mg L^{-1}), sodium (16.63 ± 0.3 mg L^{-1}), potassium (14.05 ± 0.25 mg L^{-1}) and total iron (0.16 ± 0.02 mg L^{-1}) compared to water samples from HTL wells. The physico-chemical parameters of the extracted groundwater samples are summarized in Table S1.

Principal component analysis (PCA) using the physico-chemical parameters of groundwater samples used in this study separated the samples into four distinct clusters (Fig. 2), which explained 71.47% of total variance by the first two principal components (PCs):

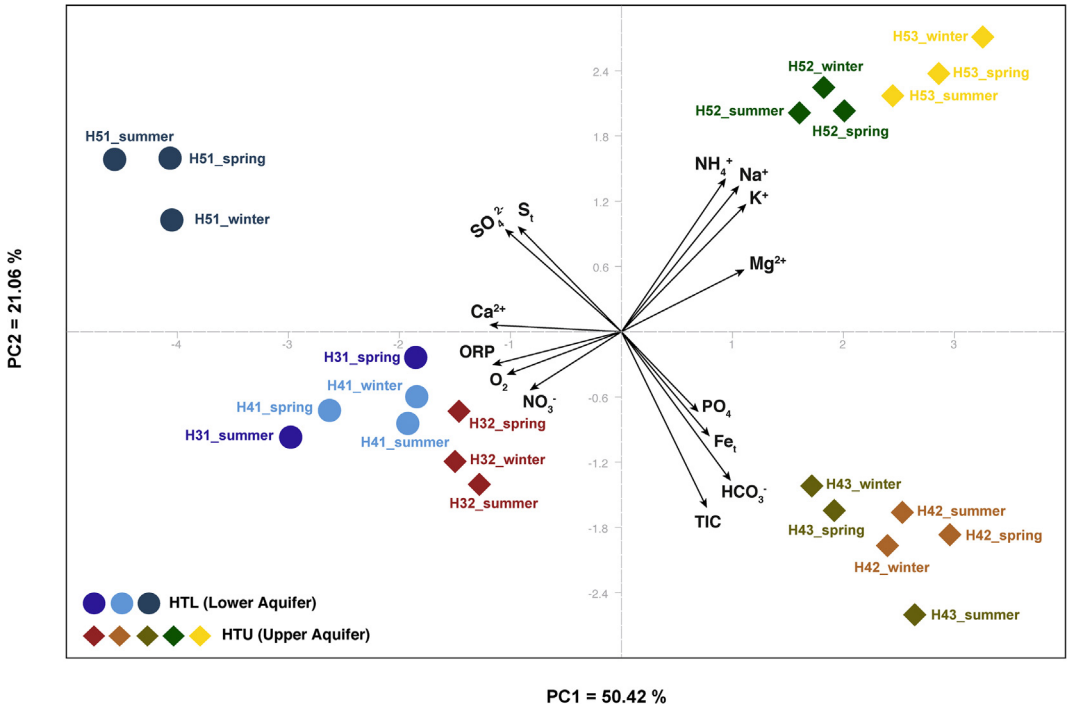

Fig. 2. Principal component analysis (PCA) of physico-chemical parameters of extracted groundwater used in this study. The length of the vectors indicates the strength of the correlation of respective parameter with the samples. Filled circles represent the samples from lower aquifer assemblage, filled diamonds represent the samples from upper aquifer assemblage and different colors represent different sampling seasons. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

PC1 = 50.42% and PC2 = 21.05%. The overall strength of correlations between the groundwater samples (PCA axis scores) and their physico-chemical parameters are summarized in Table 1. Total inorganic carbon (TIC), K^+, NH_4^+, Fe_t, Mg^{2+} and Na^+, and HCO_3^- showed a significant ($p < 0.05$) positive correlation with PC1, whereas, specific electrical conductivity, redox potential, concentrations of dissolved O_2, Ca^{2+}, S_t, SO_4^{2-} and NO_3^- showed a significant ($p < 0.05$) negative correlation with PC1 (Fig. 2). Specifically, the concentrations of SO_4^{2-} and St showed a significant ($p < 0.05$) positive correlation with PC2, which separated the samples from well H51 in the lower aquifer assemblage from the rest. Furthermore, the significant ($p < 0.05$) positive correlation of the concentrations of NH_4^+ and Na^+ with PC 1 separated the anoxic wells (H42, H43, H52, H53) from H4 and H5. Groundwater samples from well H42 and H43 have higher TIC and HCO_3^- but lower NH_4^+, K^+ and Na^+ compared to the groundwater samples from the wells H52 and H53 of the upper aquifer assemblage.

3.2. Overview of bioinformatic analysis

A total of 447,613 raw reads were generated by paired-end Illumina MiSeq sequencing of the water samples from lower and upper aquifer assemblages at sites H3, H4 and H5. After trimming and removal of chimeric sequences, a total of 149,295 (33.35%) high quality fungal ITS2 reads were used in the downstream analysis. Sequence clustering at $\geq$ 97% similarity followed by the removal of non-target sequences (7 OTUs represented by 59 reads) resulted in separating out a total of 1443 fungal OTUs represented by 17,963 reads. The removal of rare OTUs (single-, double- and triple-tons) resulted in the final dataset with 382 OTUs comprising of 16,701 reads. Mantel test based on the Bray-Curtis distance measures with 999 permutations ($R_{Mantel} = 0.999$, $P = 0.001$) indicated no impact of the removal of rare OTUs from the dataset. The number of reads after different bioinformatic steps are summarized in Table S2.

3.3. Distribution of metabolically active fungi in the lower and upper aquifer assemblages

The final dataset from the two aquifer assemblages contained 382 fungal OTUs which could be taxonomically assigned to 4 phyla, 12 classes, 34 orders, 54 families, and 83 genera. Out of these 382 identified fungal OTUs, 94 and 195 OTUs were exclusively present in the lower and upper aquifer assemblages, respectively, while 93 OTUs (24.3%) were shared between the two aquifer assemblages. The sample-based individual rarefaction curves for all the samples from the upper and lower aquifer assemblage were close to saturation, indicating towards the undiscovered community in the system (Figure S1). We used the observed OTU richness (number of OTUs) as a measure of the diversity of living fungi (Purahong et al., 2016). We found that the living fungal diversity differed between the two aquifer assemblages (HTL and HTU) and within the aquifer assemblages (wells: H3, H4 and H5) (Fig. 3a). Specifically, when all the HTL wells (H31, H41, H51) were considered together, the highest number of living fungal OTUs was observed in the groundwater of well H41 in winter (153 OTUs), while the lowest number of living fungal OTUs was observed in the well H41 in summer (68 OTUs). Similarly, for the HTU wells (H32, H42, H43, H52 H53), the highest number of living fungal OTUs was observed in well H53 in winter and the lowest number of living fungal OTUs was found in well H32 in summer. Overall, in the HTL and HTU groundwater wells, the highest living fungal diversity was observed in winter (Fig. 3a). Likewise, when all the samples from HTL and HTU aquifer assemblages were unified according to the sampling seasons, ANOVA followed by a Tukey *post hoc* pair wise comparison showed the fungal species richness in winter was significantly different ($P < 0.05$, ANOVA) from those in summer and spring

Table 1
Heat-map showing the strength of the correlations between water physico-chemical properties and Principal Component (PC) axis. Significant variables ($P < 0.05$) are shown in bold and marginally significant variables ($P < 0.10$) are shown in italics.

Physico-chemical Properties of Water Samples	PC 1 (50.42%)	PC 2 (21.05%)
K^+	**0.86**	0.27
NH_4^+	**0.79**	*0.36**
Fe_t	**0.77**	-0.29
Mg^{2+}	**0.76**	0.28
HCO_3^-	**0.76**	**0.50**
Na^+	**0.70**	*0.39**
TIC	**0.60**	**-0.70**
PO_4	**0.49**	*-0.37**
NO_3^-	**-0.69**	0.13
SO_4^{2-}	**-0.78**	**0.54**
Reduction Potential	**-0.79**	-0.14
S_t	**-0.80**	**0.53**
O_2	**-0.84**	-0.01
Ca^{2+}	**-0.89**	-0.23

(Fig. 3b).

Considering the fungal taxonomic distribution, a total of four fungal phyla (Ascomycota, Basidiomycota, Chytridiomycota and Zygomycota) were found in the Upper Muschelkalk groundwater samples (Fig. 4). The living fungal communities were primarily dominated by three phyla together accounting for 58% of the total relative abundance. The phyla Ascomycota and Basidiomycota were the two most frequently observed phyla in both aquifer assemblages, accounting for 55% of the total relative abundance, whereas Chytridiomycota and Zygomycota contributed little to the overall taxonomic distribution at phylum level (<4% of the total relative abundance). It is important to consider that the phylum Chytridiomycota (typical of lake and sediment ecosystems) was only detected in the groundwater samples from wells H32 in the oxic domain of the upper aquifer assemblage (Fig. 4). In the final dataset, according to the UNITE database 40% of the abundant OTUs were identified as fungi but without further taxonomic resolution.

Among the oxic environments of the lower aquifer assemblage, in well H31 the phylum Ascomycota was highly represented (89%) with small fractions of the phylum Zygomycota (2%) in spring. In contrast, in summer, the phylum Basidiomycota appeared with 12% relative abundance along with Ascomycota (73%). The groundwater samples in winter from the well H41 were dominated by members of phylum Basidiomycota (44%) followed by Ascomycota (23%) and Zygomycota (6%), whereas in spring, the phylum Zygomycota was not observed at all and the relative abundance of Ascomycota and Basidiomycota also decreased slightly (23%–10% and 44%–41% respectively). Interestingly in summer, the phylum Ascomycota was highly (61%) and Basidiomycota was least detected (23%) when compared to the winter and spring samples (Fig. 4). In well H51 (associated with more sulfate and calcium ions), the phylum Ascomycota was highly detected (53% relative abundance), with only 7% of Basidiomycota in winter. Surprisingly, the relative abundance of Ascomycota and Basidiomycota decreased enormously in spring to 4% and 5%, respectively. Similarly, in the HTU well H32 (associated with higher nitrate concentrations), the living fungal taxonomic distribution was primarily contributed by the phyla of Ascomycota and Basidiomycota in winter and spring. In summer, the phylum Ascomycota was highly detected (60%) while Basidiomycota contributed only little (17%). The phylum Chytridiomycota was only detected in the groundwater samples form well H32 in spring (Fig. 4).

Among the anoxic environments of the upper aquifer assemblage, wells H42 and H43 showed different distributions of the living fungal phyla compared to the wells H52 and H53 (Fig. 4). In winter, the phylum Ascomycota was highly detected in well H43 (54%) compared to well H42 (8%). Surprisingly in well H43, the relative abundance of the phylum Ascomycota was reduced considerably in spring to 8% compared to winter (54%). In the anoxic groundwater wells at site H5 (H52 and H53), the phylum Zygomycota was highly detected (23% in H52 and 27% in H53) in winter compared to all other wells either in lower or upper aquifer assemblage.

3.4. Well and aquifer assemblage specific core fungal genera

Considering the sampling seasons together for the individual groundwater wells in the lower and upper aquifer assemblages, we identified the core genera associated with each specific well (Table 2). In the HTL, we identified seven and three genera from the wells H31 and H51, respectively, which accounted for 38.52% and 8.74% of the total sequences in their respective wells. In the HTU, core genera ranging between one and three were identified from wells H32, H42, H43, H52 and H53 and the fraction of sequences associated with these genera were 14.21%, 3.31%, 1.16%, 3.61% and 1% of the total sequences in their corresponding wells. When all the groundwater wells in the lower aquifer assemblage were considered together, *Mycosphaerella* was identified as core genus in the HTL, which was represented by 5.5% of the total sequences. In contrast, no core genus could be identified in the HTU across all wells.

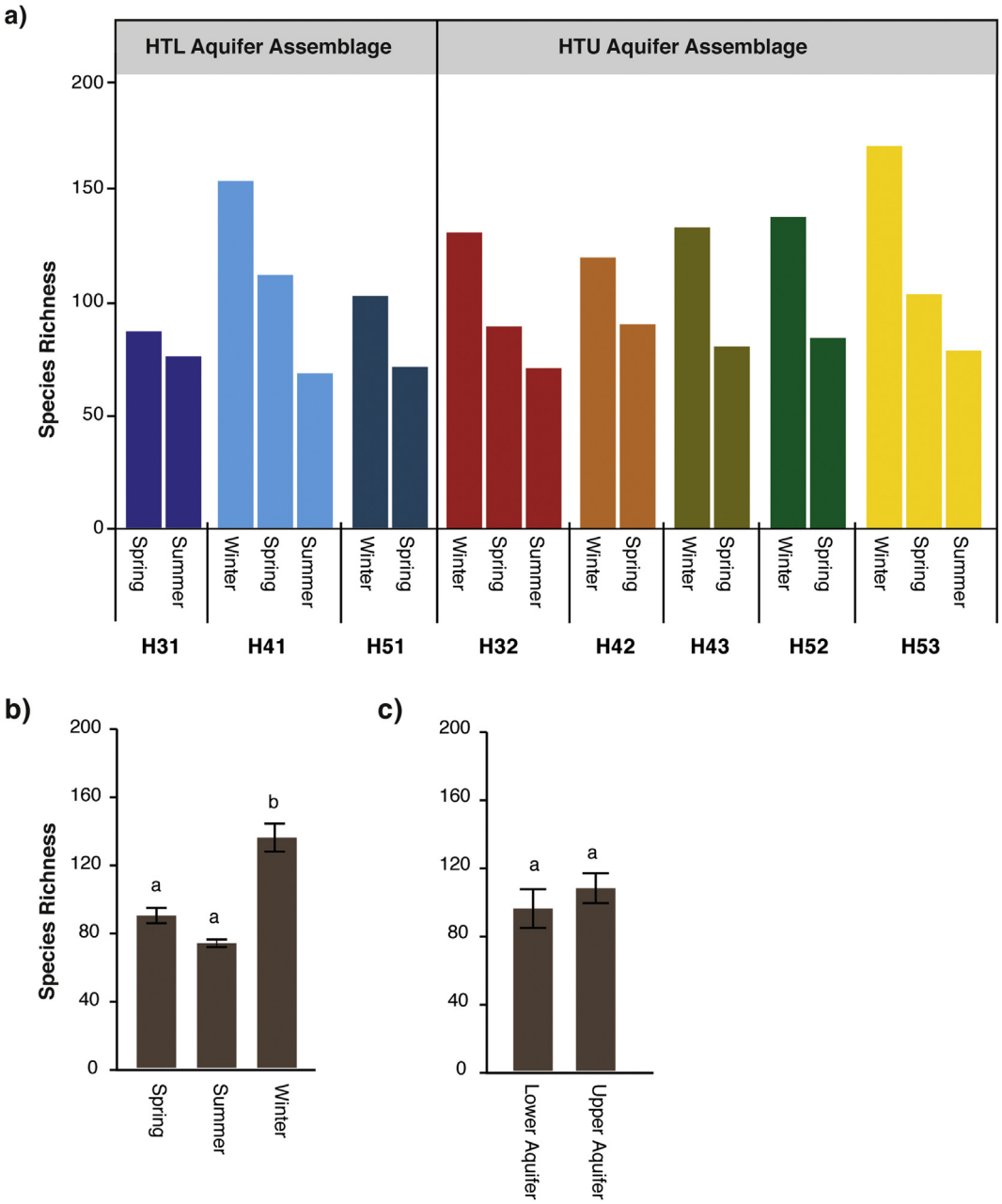

Fig. 3. Changes in the observed fungal species richness (number of OTUs) in, a) groundwater wells from lower and upper aquifer assemblages across different sampling seasons, b) when all the samples were combined according to the season (spring, summer and winter) and c) when all the samples were combined according to the aquifer assemblages (HTL and HTU aquifer assemblage). Different letters above bars indicate significant differences in the species richness between the seasons ($P<0.05$) based on a Tukey *post hoc* pairwise comparison.

3.5. *Fungal community composition and physico-chemical parameters*

The living fungal community composition in the lower and upper aquifer assemblages is presented in two-dimensional non-metric multidimensional scaling (2D-NMDS) ordination based on presence/absence data using Jaccard distance measures (Fig. 5). The NPMANOVA analysis showed that the living fungal community composition of the two aquifer assemblages are significantly different from one another ($F = 1.623, P = 0.003$), with no significant effect on multivariate dispersion between the groups (betadisper, $F = 0.77$, $P = 0.39$). In the NMDS ordination, the groundwater samples from the HTU except from H42 and H43 appeared to be in close proximity, separating the samples from the HTL (Fig. 5). This indicated that fungal communities of the respective aquifer assemblages are most similar. The physico-chemical parameters of the groundwater samples important for fungal growth were correlated with the living fungal community composition to identify the factors that played significant roles in shaping the community in the two aquifer assemblages. We found that a set of physico-chemical parameters (environmental abiotic variables), were significant predictors of the living fungal communities in the aquifers. The significant and marginally significant physico-chemical parameters are presented in Table 3. These parameters included: extraction temperature, pH, total inorganic carbon (TIC) and concentrations of ammonium, orthophosphate, total iron, and

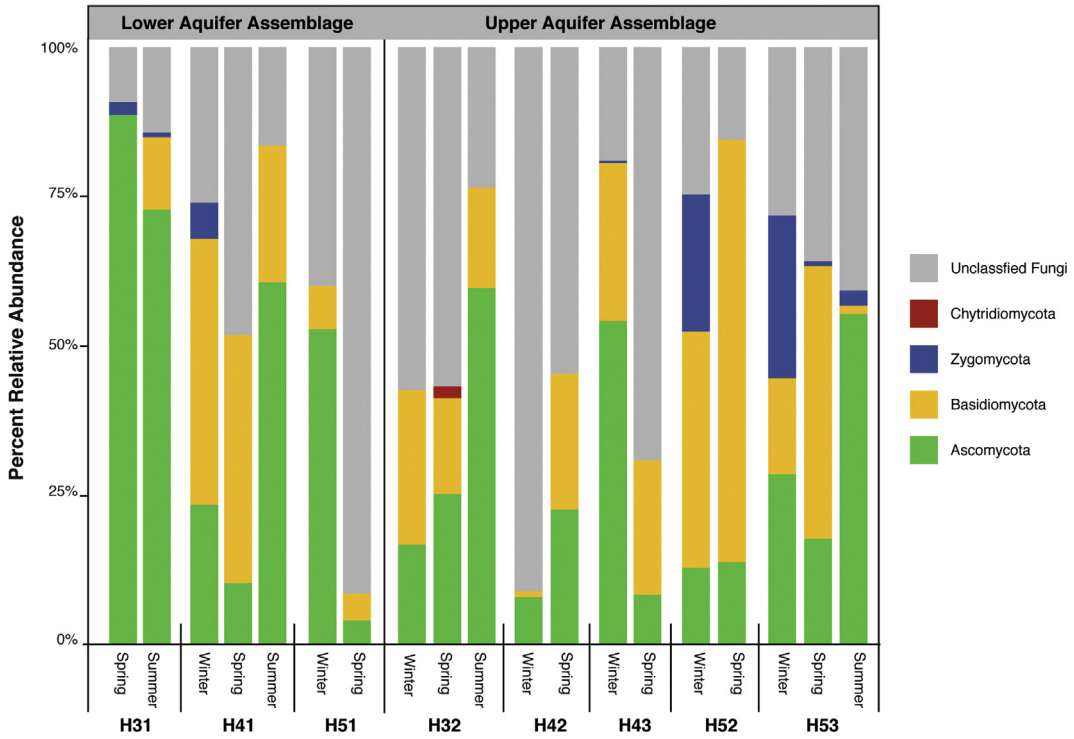

Fig. 4. Taxonomic distribution (percent relative abundance) of living fungi at phylum level in all the groundwater wells in lower and upper aquifer assemblages across different sampling seasons.

Table 2
Core fungal genera associated with specific wells of HTL and HTU aquifer assemblage and their collective percent of the sequences.

	Well	Genus	% of total sequences		Well	Genus	% of total sequences
HTL Aquifer Assemblage	**H31**	Cladosporium Mycosphaerella Clavispora Beauveria Mortierella Wickerhanomyces Lecanicillium	38.52%	**HTU Aquifer Assemblage**	**H32**	Cladosporium Aureobasidium Clavispora	14.21%
					H42	Cladosporium Clavispora Alternaria	3.31%
					H43	Didymella	1.16%
	H51	Mycosphaerella Metschnikowia Malassezia	8.74%		**H52**	Candida Resinicium	3.61%
					H53	Malassezia	1%

potassium. Among all the parameters, the differences in the living fungal community composition between the two-aquifer assemblages were significantly explained by ammonium (NH_4^+) ($F = 2.0742$, $P = 0.001^{***}$) determined by distance-based redundancy analysis (dbRDA).

3.6. Functional groups in the lower and upper aquifer assemblages: detection of potential animal and plant pathogens

To get meaningful ecological and functional categories of the identified fungal OTUs in our dataset, we used FUNGuild (an annotation tool for parsing fungal NGS datasets). In general, from the two aquifer assemblages, fungi belonging to three different trophic modes i.e., saprotroph (57 OTUs, 15%), pathotrophs (34 OTUs, 9%) and symbiotrophs (9 OTUs, 2.4%) were identified. Within each trophic mode, we also identified fungi belonging to different ecological guilds. Fungi within the pathotrophic mode were subcategorized into animal (16 OTUS, 47%), plant (17 OTUs, 31%) and fungal pathogens (3 OTUs, 9%). These ecological functional groups were distributed along different fungal phyla. Animal and plant pathogens were highly represented by the phylum Ascomycota (89–100%). In contrast, ectomycorrhizal fungi and fungal pathogens were highly represented by the phylum Basidiomycota (60–100%), and saprotrophs were represented by three different phyla i.e., Ascomycota (37%), Basidiomycota (31%) and Zygomycota (28%). The fungal OTUs with unidentified ecological functional groups made up the largest fraction in the dataset (259 OTUs, 68%).

4. Discussion

When carbonate-rock aquifers in the terrestrial subsurface biosphere are explicitly taken into account, a huge information gap exits about fungal diversity, community composition and roles of environmental factors in shaping these communities. This paucity of knowledge is primarily due to the fact that in the past, the most common methods used for studying aquatic fungal diversity were either based on the microscopic identification of asexual spores or culture based identifications (Grossart and Rojas-Jimenez, 2016). All

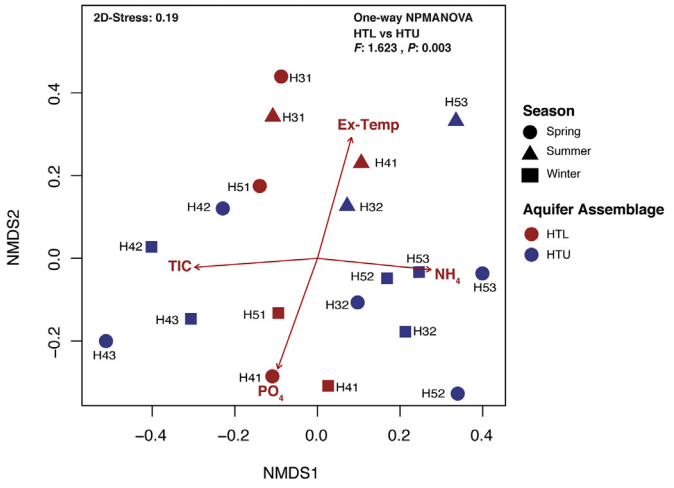

Fig. 5. Two-dimensional non-metric multidimensional scaling (2D-NMDS) ordination of living fungal communities in HTL (red) and HTU (blue) aquifer assemblage in three different sampling seasons i.e., spring (filled circles), summer (filled triangles) and winter (filled squares). The NMDS ordination (stress 0.19) was fitted with the physico-chemical parameters of the extracted water samples (only significant factors $P < 0.05$ are shown).

Table 3
Goodness-of-fit statistics (R^2) for factors fitted to the two-dimensional non-metric multidimensional scaling (2D-NMDS) ordination of living fungal community composition.

Factor	R^2	P
Extraction temperature	0.3635	**0.031** *
Electric conductivity	0.0375	0.736
pH	0.2358	*0.087* .
O_2	0.0810	0.488
Fe^2	0.1357	0.322
NH_4^+	0.3013	**0.042** *
PO_4	0.3176	*0.050* .
TIC	0.3506	**0.034** *
NO_3^-	0.0611	0.610
SO_4^{2-}	0.0006	0.997
Cl^-	0.2752	*0.070* .
Ca^{2+}	0.1133	0.366
Fe_t	0.2462	*0.089* .
K^+	0.2708	*0.068* .
Mg^{2+}	0.1163	0.316
Na^+	0.1907	0.166
S_t	0.0006	0.995

Significant P values ($P < 0.05$*) are indicated in bold.
Marginally significant ($P < 0.1$·) are indicated in italics.

such methods have their intrinsic limitations, which results in incomplete description of the actual fungal diversity in an ecosystem (Jeewon and Hyde, 2007; Schmit and Lodge, 2005). Keeping in mind the potential inherent biases of culture independent techniques (Lucking et al., 2014; Tedersoo et al., 2010), this work provides an in-depth picture and novel insights about living fungal diversity, community composition and fungal functional groups in pristine aquifer assemblages of the Hainich CZE in the terrestrial subsurface biosphere. Along with that it also encompasses the role of environmental factors in shaping such community compositions.

4.1. Aquifers harbor highly diverse living fungal communities

To our knowledge, despite the oligotrophic and pristine conditions of the studied subsurface ecosystem, we have reported a diverse pool of living aquatic fungi than what has been published in previous studies from bedrock aquifers or related freshwater habitats. However, a direct comparison of our findings about living fungal diversity and community composition with previously published studies is difficult because there are few studies about aquatic fungi in subsurface pristine aquifer systems. For instance, by using culture based techniques, Lategan et al. (2012) have reported 83 fungal strains from shallow unconfined aquifers in Australia and Hageskal et al. (2006) isolated 24 species from Norwegian groundwater sources. Specifically from the same aquifer system in Hainich CZE, up to 437 fungal OTUs have been reported by sequencing 18s RNA gene fragments Risse-Buhl et al. (2013) and fungal ITS regions Nawaz et al. (2016). However, it is important to consider that the studies mentioned above were DNA based, which target the total communities. Whereas, in this work targeting the living fractions of the fungal communities from the groundwater samples, we reported 382 fungal OTUs. Such huge numbers and diversities of living fungal OTUs reflect the fact that subsurface aquifer system is much more vibrant from the mycological point of view than thought before. It is also important to consider that 40% of the retrieved fungal OTUs could only be identified as fungi but without further taxonomic resolution. This indicates either insufficient representation of the aquatic fungal sequences in the actual databases, or that these fungal species may not have been characterized before and could be considered as potentially novel fungal groups specialized for aquatic habitats. In either case, this points towards an unexplored pool of fungal species in aquifers.

Interestingly, by comparing the living fungal OTUs detected in this study with the fungal OTUs from our previous DNA based study using 454-sequencing (Nawaz et al., 2016), we found an overlap of only 5.8% of the living fungal OTUs with the ones previously reported. This shows that there exists a marked difference between recoverable fungal community and the community that is living in the environment. Consequently, this signifies the importance of the RNA based approach used in this study to target the living players of the fungal communities to better understated the role of microbes in the ecosystem functioning of such habitats.

The fungal diversity varies across the different sampling campaigns, which represent different seasons (spring, summer and winter). Higher living fungal diversity of the winter samples from both aquifer assemblages, compared to spring and summer samples indicate the temporal variability and natural seasonal dynamics of the fungal communities. This trend could be associated with the groundwater low and high stand in the respective seasons which points to the dilution effect of the fungal biomass. But to confirm this hypothesis, repeated measurements in the same season would be needed in future to confirm such seasonal dynamics in the studied aquifer system.

Interestingly, within the same aquifer assemblage different sampling wells showed variable distributions of the fungal phyla. This finding is in good agreement with our previous study (Nawaz et al., 2016). Specifically, when all the groundwater wells in the lower oxic aquifer (HTL) are considered together, the members of Dothideomycetes were most frequently identified in wells H31 and H41. Fungi from the class Dothideomycetes were also reported to be abundant in the aquifers (Korbel et al., 2017). This is the most diverse and largest fungal class of the phylum Ascomycota (Kirk et al., 2008), which is a heterogeneous group of organisms that persists in most of the habitats where fungi can be found. Additionally, in our dataset, we also found a fraction of fungal OTUs belonging to family Nectriaceae in anoxic groundwater wells. It has been reported that in the absence of oxygen, facultative anaerobic microscopic fungi from family Nectriaceae are capable of using nitrate as alternative terminal electron acceptor in their respiration process (Kurakov et al., 2008).

In the lower aquifer assemblage, the genus *Mycosphaerella* was

identified as core genus. The members of the genus *Mycosphaerella* are generally classified as endophytes, saprotrophs and symbionts but most of the members are plant pathogens causing heavy loss to the agricultural crops worldwide (Crous et al., 2001). Moreover, by comparing the reported core fungal genera in this study with the previously published studies, we identified that the genera *Alternaria*, *Aureobasidium*, *Beauveria* and *Mortierella* were reported before in groundwater, surface-water and lakes (Oliveira et al., 2013; Wurzbacher et al., 2016) and the genus *Malassezia* from deep sea sediments (Amend, 2014; Singh et al., 2012) and deep groundwater bedrock fractured zones (Sohlberg et al., 2015). Whereas, the rest of the genera observed in this study were reported for the first time in terrestrial subsurface aquifers. Some of the fungal genera we identified in this study, have also been reported in different terrestrial habitats, which suggests that those fungi may have non-aquatic origin or there is a life style transition in their life cycle (Shearer et al., 2007; Vijaykrishna and Hyde, 2006).

4.2. Factors corelating the living fungal community composition

The groundwater wells accessing the two aquifer assemblages in Hainich CZE showed different environmental conditions as indicated by distinct clustering patterns based on their physico-chemical parameters (Fig. 2). This result is supported by previous studies which also reported marked differences in the oxygen and nitrate contents along with the concentrations of sulfate and iron in the two aquifers (HTL and HTU) (Kohlhepp et al., 2017; Küsel et al., 2016; Schwab et al., 2017). Generally, differences in physico-chemical parameters of the water samples are important in shaping aquatic fungal community compositions (Gulis and Suberkropp, 2004; Manoharachary and Ramarao, 1981; Medeiros et al., 2009). In our case, we found differences in the living fungal communities in the lower and upper aquifer assemblages and these variations in the overall community composition was significantly correlated to the ammonium (NH_4^+) concentration. Similarly, strong correlations between fungal diversity and ammonium and nitrate concentrations have also been previously reported in deep fractured zones (Sohlberg et al., 2015), in marine environments (Tisthammer et al., 2016) and anoxic sub-seafloor sediments (Orsi et al., 2013). Although the employed methods, set of measured environmental variables, the studied ecosystem and the geographic locations of the studies mentioned above are not directly comparable with our study, but they all agree that the environmental filtering is important in the aquatic fungal community composition. In oxic conditions, fungi use oxygen as substrate to produce energy for their biological activities. But the studied aquifer system in the Hainich CZE also has sub-oxic to anoxic domains in the upper aquifer assemblage (Kohlhepp et al., 2017; Küsel et al., 2016). To support their growth under anoxic conditions, filamentous fungi (members of sub-kingdom Dikarya) have been reported to induce a set of genes to produce O_2-independent energy-producing metabolic mechanisms (Takasaki et al., 2004). By such oxygen independent metabolic mechanisms, fungi can reduce nitrate to ammonium to gain energy (Takasaki et al., 2004; Zhou et al., 2010). This phenomenon has been previously attributed to the facultative or anaerobic bacteria and archaea, but growing evidence suggesting that this mechanism is also widely distributed among many eukaryotic microbes including different fungal groups (Kamp et al., 2015; Zhou et al. 2002, 2010). Such fungal abilities to thrive in the anoxic conditions of the Hainich aquifer systems points towards their versatile mode of survival in adverse conditions and their potential roles in the nutrient cycling in the terrestrial subsurface aquifers.

4.3. Diverse functional groups inhabit the aquifers

In the past, fungi have been thought to have negligible ecological roles in aquatic ecosystems. But it has been established that apart from terrestrial environments, fungi also play important roles in element cycling and mineralization in aquatic ecosystems (Ivarsson et al., 2016). We found that in our studied aquifer system, after unidentified fungal functional groups, saprotrophs were highly detected. In an ecosystem, saprotrophs are considered as backbone of the elemental cycling processes. Therefore, the living fungal saprotrophs in the studied oligotrophic aquifer system may contribute to the solubilisation of minerals and decomposition of natural compounds into simpler substance due to their broad spectrum of enzymatic capabilities (Giraud et al., 2001; Junghanns et al., 2005). Subsequently, those simpler substances could be used further by other organisms in the aquifer food web.

Apart from the fungal groups involved in elemental cycling in subsurface aquifers, the pathotrophs, including animal and plant pathogens are also important to consider. Such aquifers around the world are considered major source of water for domestic purposes (Martin and White, 2008). Therefore, the presence of potential animal and plant pathogens in such aquifer systems cannot be overlooked, as exposure to pathogenic fungi would have health concerns for humans (Pereira et al., 2010). Specifically, in the analyzed groundwater samples, we found fungal OTUs that are known animal, plants and human pathogens i.e., *Rhodotorula mucilaginosa* (Opportunistic human pathogen in immune-compromised patients) (da Cunha et al., 2009; Kaur et al., 2007), *Paecilomyces lilacinus* (human and animal pathogen) (Luangsa-Ard et al., 2011) *Lecanicillium lecanii* (entomopathogenic fungus) and *Blumeria graminis* (plant pathogen) (Cowger et al., 2018; Ilyas et al., 2014) Pathogenicity is one aspect, but fungal presence in water sources is also associated with taste and odor problems (Doggett, 2000; Pereira et al., 2010).

In our previous DNA based study (Nawaz et al., 2016), fungal plant pathogens were also detected. However in DNA based approaches, it is quite possible to detect the dead/dormant species as well (Rajala et al., 2011). But the detection of fungal plant pathogens in this RNA based study confirms that these groups are not only present in the aquifers but also are also alive. Considering the geological/structural setting of Hainich CZE, our targeted subsurface aquifer system is primarily recharged by the infiltration of rainfall and snow-melts and the recharge areas are located in different land-use types (i.e., forest, grassland and cropland) but dominated by forest land-use (Kohlhepp et al., 2017; Küsel et al., 2016). During the recharge events, the location of the recharge areas not only impacts the kind of organic matter that is being transported with water but also the type of microbes (Lehman, 2007; Somaratne, 2014). The detection of living plant and animal pathogens in our dataset, points to a surface-origin of unknown percentage of the living fungal groups which along with infiltrating water might have been transported from the surface to the subsurface (considering the fractured zones in the bedrock aquifer system). Nevertheless, the fate of surface-sourced fungi in the aquatic subsurface oligotrophic environment is relatively unknown in the literature.

5. Conclusions

Following conclusions can be drawn within the framework of present study:

- Investigating subsurface groundwater aquifers using high-throughput Illumina sequencing, allowed us to identify a

diverse (both taxonomic and functional) pool of metabolically active fungal communities.
- The living fungal communities in the shallow aquifers in the mixed carbonate/siliciclastic alternations were significantly corelated with ammonium and TIC supply.
- An overlap of only 5.8% of the living fungal OTUs with the ones in the DNA based study, highlighted a marked difference between total fungal community and the community that is living in an environment.
- In the targeted subsurface aquifer system, we detected living fungal OTUs that are known plant pathogens in the literature and the databases. The presence of these fungal groups in such environments, points towards the transitional ability of fungi in their life styles (lifestyle switching). But further studies are required in the future to comprehensively test such hypothesis in aquatic habitats.
- We suggest that the fungal meta-barcoding approach used in this study, can be used to monitor and assess the quality of water supplies considering the presence of potential fungal pathogens which could be harmful for animals, plants and humans.
- Furthermore, the role of fungal saprotrophs in such oligotrophic subsurface environment is suggested to be studied in future studies.

Author contribution

AN, TW and FB planned the study. KT designed the field site and planned the field installation. AN and MH performed the lab work. AN, WP and TW analyzed the data and perceived the manuscript concept. AN wrote the manuscript. TW, KK and FB acquired the funding. WP, RL, MH, KK, KT, TW and FB reviewed the manuscript.

Acknowledgments

This study is part of the Collaborative Research Centre AquaDiva (CRC 1076 AquaDiva) of the Friedrich Schiller University Jena, funded by the Deutsche Forschungsgemeinschaft (SFB 1076). The responsible state environmental offices of Thüringen issued fieldwork permits. The authors would like to thank Heiko Minkmar, Bernd Ruppe, and Falko Gutmann for groundwater sampling and on-site measurements/sample preparation, Christine Hess and Maria Fabisch for scientific coordination and the Hainich National Park.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.07.067.

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