Fungal communities in Scandinavian lakes along a longitudinal gradient

Maryia Khomich[1,2*], Marie L. Davey[2], Håvard Kauserud[2], Serena Rasconi[3], Tom Andersen[1]

[1]Section for Aquatic Biology and Toxicology, Department of Biosciences, University of Oslo, P.O. Box 1066 Blindern, 0316 Oslo, Norway

[2]Section for Genetics and Evolutionary Biology, Department of Biosciences, University of Oslo, P.O. Box 1066 Blindern, 0316 Oslo, Norway

[3]WasserCluster–Biological Station Lunz, Inter-university Centre for Aquatic Ecosystem Research, A-3293 Lunz am See, Austria

***Corresponding author:** Department of Biosciences, University of Oslo, P.O. Box 1066 Blindern, 0316 Oslo, Norway

Tel.: +47-22845979, Fax: +47-22854726

E-mail address: maryia.khomich@ibv.uio.no, marykhomich@gmail.com

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Abstract

This study investigates the diversity and distribution of fungal communities in 77 oligotrophic lakes in southern Norway and Sweden using 454-sequencing with fungal-specific primers targeting ITS2 region of the rRNA gene. A total of 232 operational taxonomic units (OTUs) belonging to four recognized phyla were detected. A large proportion (70.69%) of the detected OTUs was Dikarya (Ascomycota and Basidiomycota), while Chytridiomycota dominated quantitatively (63.37% reads). The most abundant aquatic fungi were taxonomically assigned to Chytridiomycota, whose members are known to be saprobes on a large variety of substrates and parasites of phytoplankton, zooplankton, fungi and invertebrates, suggesting that resident fungi strictly depend on surfaces and, therefore, are closely associated with other types of aquatic organisms. Our results indicate that surface waters of oligotrophic lakes harbour a diverse pool of fungi, both with tentative terrestrial and true aquatic origin. Longitude and environmental factors were important in structuring the fungal community composition.

Keywords: aquatic fungi, Chytridiomycota, ITS region, freshwater, diversity, 454 pyrosequencing

1. Introduction

Fungi are an ecologically and functionally diverse kingdom of eukaryotic organisms that have evolved a wide array of mutualists (e.g. lichens, mycorrhizal fungi, endophytic fungi), parasites and saprotrophs. Fungal communities have been shown to be both large and highly diverse in soils and plant-associated habitats (Blaalid et al., 2012; Botnen et al., 2014; Tedersoo et al., 2014). However, the current known biodiversity of aquatic fungi is poorly documented and the number of aquatic species constitutes only a tiny fraction of those reported from terrestrial environments. Nevertheless, fungi are common inhabitants of aquatic ecosystems and act as key players in the turnover of both allochthonous and autochthonous organic matter, serving as important mediators of energy and nutrient transfer to higher trophic levels (Kuehn, 2016). Freshwater fungi are a taxonomically and morphologically diverse group found in various aquatic habitats including lakes, ponds, rivers, streams, sediments, submerged substrata, freshwater algae and invertebrates, as has been demonstrated by diversity studies from temperate, tropical and subtropical regions (Duarte et al., 2016; Hyde et al., 2016). Additionally, fungal sequences detected in aquatic environments span a large variety of novel, deep-branching, and yet uncultured, fungal lineages, which have been termed ‘dark matter fungi’ (Grossart et al., 2016).

Freshwater ecosystems have traditionally been subdivided into lentic (standing waters: lakes, ponds, wetlands) and lotic (running waters: streams, rivers). In contrast to well-studied lotic systems (Duarte et al., 2015; Graça et al., 2016), where fungi are mainly recognized as litter decomposers (Duarte et al., 2015), lentic freshwater fungal diversity is only starting to be unveiled using high-throughput sequencing (Comeau et al., 2016; Monchy et al., 2011), which has identified a large number of unknown fungal lineages (Ishida et al., 2015). Lakes, the biggest

freshwater reservoirs, are structured into littoral and pelagic zones. The former is a ‘hotspot’ for all kinds of fungi providing diverse ecological niches, whereas the latter can both harbour highly specialized species and serve as a medium for propagule dispersal (Wurzbacher et al., 2010). The Chytridiomycota, an early divergent fungal lineage, represents the best studied aquatic fungal group, and occurs primarily in lakes where they are well adapted to the aquatic lifestyle, acting both as saprotrophs and parasites of a wide range of hosts (Kagami et al., 2007; Kagami et al., 2014; Rasconi et al., 2012; Sime-Ngando, 2012; Wurzbacher et al., 2014). Parasitism by chytrids is an important ecological driving force in the aquatic food web dynamics (Rasconi et al., 2012; Sime-Ngando, 2012). The transfer of nutrients from phytoplankton to zooplankton occurs via the zoospores of parasitic chytrids through the ‘mycoloop’ (Kagami et al., 2014). In addition, other possible mycoloops may exist in freshwater food webs, with saprotrophic chytrid zoospores released from pollen and consumed by zooplankton (Kagami et al., 2014). Aquatic hyphomycetes are common inhabitants of lakes (Chauvet et al., 2016; Wurzbacher et al., 2010). Filamentous fungi that require solid substrata are widespread in the littoral zone of lakes where there is substantial leaf litter input from the terrestrial vegetation (Wurzbacher et al., 2010). Some studies to date suggest that yeast forms appear to dominate the known diversity of aquatic fungi in the pelagic zone of lakes, as well as in marine environments (Bass et al., 2007; Richards et al., 2012; Richards et al., 2015; Tisthammer et al., 2016). However, this view contradicts with recent surveys in freshwater and marine ecosystems (Comeau et al., 2016; Hassett et al., 2016; Hassett and Gradinger, 2016) reporting the dominance of Chytridiomycota.

The number of truly aquatic lichens is small, with 270 lichen and lichenicolous species occurring regularly in freshwater of an estimated total of 13 500 lichens (Jones et al., 2014; Thüs et al., 2014). Terrestrial filamentous fungi (e.g. endophytic and air-borne fungi) are often passively

introduced into lakes in the form of spores and fragments of mycelia via inflowing streams, rainwater, wind and soil particles (Voronin, 2014). However, it is often unclear whether such fungi are terrestrial or truly aquatic (Wurzbacher et al., 2010). In most cases, the minimal abundance of the spores of the terrestrial fungi is in the middle water layer and the maximal is near the bottom, where fungal propagules accumulate during sedimentation (Voronin, 2014). Different factors have been shown to influence the community composition of freshwater and marine fungi. For example, temperature, pH, conductivity, altitude, differences in the riparian vegetation, seasonality in the temperate regions and the presence of various pollutants affected the diversity and distribution of aquatic hyphomycetes (Duarte et al., 2016). In contrast, sample depth, oxygen and nitrate concentrations explained 73% of the total variance in comparison to 18% explained by geographic location for marine fungal communities on a global scale (Tisthammer et al., 2016).

In this study, we aimed to investigate the diversity and abundance of freshwater fungi in the epilimnion of 77 ultra-oligotrophic to mesotrophic boreal lakes (Fig. 1) over a 750 km longitudinal diversity gradient across southern Scandinavia (Ptacnik et al., 2010; Ptacnik et al., 2008) using the internal transcribed spacer (ITS2) marker. These boreal lakes represent a good model to study compositional variation from a perspective of multiple communities connected by dispersing organisms (Hortal et al., 2014; Leibold et al., 2004), with species richness in a given site strongly linked to metacommunity dynamics and dispersal from adjacent sites (Ptacnik et al., 2010). Ptacnik et al. (2010) assessed the relative importance of local versus regional factors as predictors of local genus richness in unicellular phytoplankton across Scandinavian lakes and showed that phytoplankton metacommunities integrated richness of local communities across environmental gradients on a scale between 100 and 400 km. However, the Scandinavian

diversity gradient is complex and not fully resolved as it coincides both with major changes in landscape productivity, altitude and soil depth, as well as the main dispersal routes for freshwater organisms after the glacial retreat (Khomich et al., in press). Recurring glaciations in boreal areas can be considered an important, though neglected, historical climatic factor influencing biota (Soininen, 2012). Lakes for our study were carefully selected to be as similar as possible with respect to properties other than longitudinal position and local productivity (Table S1). Our objectives were as follows: (i) to analyse taxonomic composition of aquatic fungal communities across a known biodiversity gradient, (ii) to characterise the ecology of the detected fungal taxa hypothesizing that both resident and transient components of aquatic communities are simultaneously present, (iii) to explore the patterns of variation in fungal OTU composition across lakes in this gradient to confirm whether it follows the same longitudinal pattern, as has earlier been shown for phyto- and zooplankton diversity with non-molecular methods (Hessen et al., 2006; Ptacnik et al., 2010) and 18S rDNA amplicon sequencing of eukaryotic communities in these lakes (Khomich et al., in press).

2. Materials and methods

2.1. Site description

Lakes for this study were selected from the 'Rebecca' (Solheim et al., 2008) and 'Nordic lake survey 1995' (Henriksen et al., 1998) data sets on Norwegian and Swedish lakes to generate a subset of lakes fulfilling the following criteria: longitude 5–18 °E, latitude 58–62 °N, altitude < 600 m, surface area > 1 km^2, total phosphorus (TP) < 30 μg L^{-1}, total organic carbon (TOC) < 30 mg L^{-1} and pH > 5. The lakes were chosen to create a representative subset of boreal lakes with best possible coverage and orthogonality with respect to gradients of TP, TOC and

longitudinal position. The former two represent two major effects on aquatic productivity (Thrane et al., 2014), while the latter reflects the regional diversity gradient (Ptacnik et al., 2010). The three gradient variables were split in two factor levels (high/low), giving eight different combinations of TP, TOC and longitude. A total of 12 lakes were randomly sampled from each of the eight combinations. Sampling was performed mainly by hydroplane in July to August 2011 (Thrane et al., 2014). Because of unfavorable weather conditions during sampling the number of sampled lakes was eventually reduced to 77 (Fig.1).

2.2. Sampling program

Water samples were collected from the lake epilimnion (0 - 5 m) using an integrating water sampler (Hydro-BIOS, Germany) in the central part of each lake during daytime. For DNA analysis, up to 15 L of water was pre-filtered on 100 µm mesh to remove metazoans and filtered onto 47 mm 2 µm Isopore TTTP membrane filters (Millipore Corp., MA, USA) taken in 3x3 replicates. The filters were stored at -20 °C in cryovials until DNA extraction. Samples for nutrients were collected as described in Thrane et al. (2014). Concentrations of TP, TOC and total nitrogen were determined using standard techniques (for details, see Thrane et al., 2014).

Chemical characteristics of the water (e.g. nutrients, pH and ionic strength) are the most relevant environmental factors determining changes in plankton community composition. TOC and TP were chosen as proxies in the study design to reveal regional environmental gradients and local nutrient supply variability, respectively. The third variable, conductivity, is directly related to the concentration of ionic solutes, and therefore serves as an indicator of soil depth and landscape productivity that is less affected by local pollution than TP (Ryder, 1982). It is important to take into account that not all predictor variables are completely independent (Thrane et al., 2014).

Pearson correlation coefficients for the relationship between TOC and TP was 0.61, and for TP and conductivity was 0.54 ($P < 0.00001$; all variables log transformed) (Fig. S1, Table S1).

2.3. DNA extraction, amplification and 454-sequencing of the ITS2 region

DNA was extracted from the filters using NucleoSpin® Plant II Kit (Mackerey-Nagel, Düren, Germany) according to the protocol from the manufacturer and quantified using Nanodrop (NanoDrop Technologies Inc, DE, USA). The fungal specific modified forward fITS7a ('A' is inserted instead of 'R' at position 5) and reverse ITS4 primers (Ihrmark et al., 2012; White et al., 1990) were used to amplify ITS2. Fusion primers for 454 pyrosequencing incorporating these sequences were designed according to the protocol by Roche by adding adaptors A and B, a key (TCAG) and 10-bp unique tags (MIDs in Roche technical bulletin 005 - 2009) to the forward and reverse primers, respectively. The fusion primers were used in PCR amplifications performed on a PTC-200 DNA Engine Cycler (BioRad, USA) in 20-µl reaction volumes containing 4 µl of DNA template (i.e. 5 - 10 ng), 1x Phusion HF buffer, 0.2 mM dNTPs, 0.25 µM of each primer, 0.02 U/µl Phusion HotStart II polymerase (Finnzymes, Vantaa, Finland), 3% DMSO and 1 mg ml^{-1} BSA (New England BioLabs, Auckland, New Zealand). The amplification program was as follows: 30 s at 98 °C, followed by 30 cycles of 10 s at 98 °C, 30 s at 53 °C and 30 s at 72 °C, with a final extension step at 72 °C for 5 min before storage at -20 °C. PCR products were cleaned with a Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), using a Sequalprep Normalization Plate (96) Kit (Invitrogen, Paisley, UK) and pooled into equimolar amplicon libraries. Ten samples were sequenced twice (technical replicates) to test for sequencing consistency. The 454 Titanium sequencing of the tagged amplicons was performed using GS FLX Titanium (Lib-A chemistry) at the Norwegian Sequencing Centre at the

University of Oslo (Norway) on 1/2 of a 454 FLX Titanium sequencing plate (454 Life Sciences, Branford, CT, USA). The raw 454 reads with corresponding mapping files were deposited in Dryad (doi:xx.xxxx/dryad.xxxxx).

2.4. Bioinformatics

A total of 434 603 (average length 424.7 nt) reads from 87 samples were quality-filtered, denoised, and processed using QIIME v. 1.8.0 (Caporaso et al., 2010) on the Abel cluster at the University of Oslo. All reads with mismatched forward and/or reverse tags were removed to avoid false positives in amplicon data set (Carlsen et al., 2012). Sequences with length < 200 nt and > 550 nt, average Phred quality score of < 25, mismatches in the tags, homopolymers exceeding 6 nt, ambiguous base calls > 1 and > 1 mismatch in the primers were discarded. In addition, reads were checked for quality by using a 50-nt sliding window (average quality score > 25) to identify regions of low-sequence quality and truncated to the last good window. The resulting sequences (280 502) were denoised using DeNoiser v. 091 (Reeder and Knight, 2010), as implemented in QIIME v. 1.5.0. ITSx 1.0.11 (Bengtsson-Palme et al., 2013) was used to remove the flanking 5.8S and 28S rRNA gene fragments for optimal resolution of ITS2 clustering and removal of compromised and non-target sequences. As filtering removed most of the partial sequences (83 909), we retained only sequences > 99 nt in length (175 853 reads), as suggested by Tedersoo et al. (2014). Reads were clustered into OTUs using the UCLUST algorithm (Edgar, 2010) with a 97% similarity threshold. The 97% similarity cut-off has been widely used to delineate fungal OTUs in most comparable aquatic studies (Duarte et al., 2015; Gutiérrez et al., 2015), although it has been shown that ITS region is not equally variable among five fungal phyla with intraspecific ITS variability ranging from zero to 24.2% (Nilsson et al.,

196 2008). Global singletons (OTUs represented by only a single sequence across the entire data set)
197 were considered probable sequencing errors and removed (Kunin et al., 2010; Quince et al.,
198 2009; Tedersoo et al., 2010). The most abundant representative sequence per OTU was selected
199 and subjected to BLAST searches against the NCBI-nr/nt database (version 2.2.29). Taxonomic
200 assignments were made by comparing the representative sequence of each OTU against
201 reference databases NCBI-nr/nt and UNITE v. 7 (unite.ut.ee). For a broad taxonomic annotation,
202 taxonomy was assigned at the level of order and family. When the top BLAST match was to
203 unclassified or uncultured fungus, the top 10 matches (if available) were screened for
204 concordance and if possible, taxonomy was assigned based on the subsequent best hits meeting
205 the minimum thresholds of > 80% sequence similarity and > 70% coverage. All those OTUs
206 with best BLAST matches to non-fungal organisms, or a best match with < 80% sequence
207 similarity and < 70% coverage to a reference sequence assigned to the kingdom Fungi were
208 discarded as non-fungal or unidentifiable OTUs. OTUs were considered putative chimeras and
209 discarded when matching the two criteria: (i) being identified as chimeric by both UCHIME and
210 PERSEUS (Edgar et al., 2011; Quince et al., 2011) and (ii) having a top BLAST match with
211 < 90% coverage and < 90% sequence similarity to a reference sequence assigned to the kingdom
212 Fungi (Mundra et al., 2015). For lower rank taxonomy assignment (i.e. genus level) the RDP
213 Naïve Bayesian rRNA Classifier Version 2.11 against the Warcup Fungal ITS training set 2 with
214 95% confidence threshold was used, as suggested by Deshpande et al. (2016).
215
216 *2.5. Statistical analyses*
217 To minimize the effect of abundance measure inconsistencies, community composition analyses
218 were conducted on presence/absence data using Jaccard's dissimilarity index (function *vegdist* in

vegan package). Downstream statistical analyses were performed in R version 3.2.2 (R Development Core Team, 2015) using the package vegan (Oksanen et al., 2013) for multivariate and species richness analyses unless otherwise noted. Rarefaction curves were constructed by applying the *rarecurve* function in vegan.

Ordinations by non-metric multidimensional scaling (NMDS) (Minchin, 1987) were used to describe patterns of variation in fungal OTU composition along the longitudinal gradient. Similarity of NMDS ordinations with two (k = 2) and three (k = 3) dimensions was evaluated by Kendall's rank correlation coefficient τ between NMDS axes when matching the two criteria: $|\tau| > 0.4$ and the corresponding P-value < 0.05 (Liu et al., 2008). Since the majority of dissimilarity indices used to estimate β-diversity across sites can vary due to changes in the other two components (α- and γ-diversity), we compared the performance of several distance metrics (Bray-Curtis, Jaccard, Gower and Raup-Crick, as implemented by the 'bray', 'jaccard', 'gower' and 'raup' options for the *vegdist* function in vegan) on our data set in eight different variations using NMDS ordination (k = 2). Assessment of metrics' validity was done by Procrustes correlation run in 999 permutations (function *procrustes* in package vegan). In addition, NMDS ordinations were conducted on a subset of the matrix representing ten technical replicates to confirm that sequencing-induced variation was smaller than biological variation in the samples. Permutation-based significance tests by the *envfit* function were used to fit spatial (longitude, latitude, altitude) and environmental (TOC, TP and conductivity, all log transformed) factors to the NMDS ordination (k = 2). The *ordisurf* function in vegan was used to fit response variables (TOC and TP, both log transformed) as contour lines to the NMDS ordinations (k = 2). To account for sequencing bias, NMDS was conducted on the subset of lakes (with > 150 total reads and > 10X coverage; coverage = total reads / OTUs richness per lake) resulting in a data set of

30 lakes. A standard Mantel test on geographic location and environmental variables to investigate correlation between lakes (function *mantel* in vegan) was run using Raup-Crick dissimilarity index between aquatic fungal communities and 999 permutations. Raup-Crick distance is robust to the differences in α-diversity than other dissimilarity metrics (Birtel et al., 2015; Chase et al., 2011). In addition, partial Mantel test (function *mantel.partial* in vegan) to analyse solely the effect of local environment (TOC, TP and conductivity, all log transformed; Euclidean distance) by partialing out the effect of space (longitude, latitude and altitude) was done.

The aquatic fungal communities were split into core (abundant) and transient (occasional or rare) OTUs based on the position of each OTU within the log-normal species abundance distribution (SAD) using persistence-abundance plots as described in Kostovcik et al. (2015).

3. Results

3.1. Data characteristics

After quality filtering and denoising, 280 502 reads of the original 434 603 reads were retained. Of these, 175 853 reads were > 99 nt long ITS2 sequences. The resulting sequences clustered into 3808 OTUs, of which 1857 had no BLAST hit in the NCBI-nr/nt and UNITE databases, 1026 matched to non-fungal organisms, and 209 OTUs had poor matches to fungi (< 80% sequence similarity and < 70% coverage in the BLAST analysis). All these OTU groups were regarded as non-fungal and discarded, leaving 716 fungal OTUs. Among these, an additional 484 were removed as singletons or chimeras, leaving a final, curated dataset of 232 fungal OTUs comprising 18 738 reads (4.3% of the initial reads), including the ten technical replicates used for checking sequencing consistency. The ten technical replicate pairs had more similar OTU

265 composition than random pairwise comparisons between samples (Fig. S2), demonstrating little
266 influence of biases introduced during PCR and sequencing on community composition measures.
267 After removal of the technical replicates, a total of 16 513 sequences (3.8% of initial raw reads)
268 representing 232 OTUs for the 77 lake samples were used for downstream analyses.
269
270 *3.2. Total fungal richness*
271 Rarefaction curves of OTU richness for each lake indicated that the total fungal diversity was not
272 recovered in most of the lakes (Fig.2). A significant relationship between richness and
273 sequencing depth was observed (both log transformed; $P < 0.001$, Pearson correlation coefficient
274 = 0.40) (Fig. S3). In other words, the ordinations showed structuring by sequencing depth, which
275 likely reflects the under-sampling bias.
276
277 *3.3. Taxonomic fungal diversity*
278 Environmental fungal sequences obtained in our study clustered within the major fungal phyla
279 Ascomycota, Basidiomycota and Chytridiomycota. A very few sequences belonging to
280 Zygomycota were retrieved, and phyla like Cryptomycota and Glomeromycota either had no
281 representation in our data set, or remained unassigned. Representative sequences of the OTUs
282 were subjected to two independent similarity searches. First, we assigned taxonomy against the
283 NCBI nr/nt database containing both identified and unidentified sequences (version 2.2.29). To
284 account for possible misclassification of aquatic fungal sequences by GenBank, taxonomic
285 assignment was also done against the curated, quality-checked fungal ITS sequence database
286 UNITE (version 7), where many of the sequences undergo rigorous filtering and classification to
287 species hypothesis using phylogenetic evaluation (Kõljalg et al., 2013). Finally, a representative

sequence for each OTU was classified using the RDP Naïve Bayesian rRNA Classifier v.2.11 against the Warcup Fungal ITS training set 2 to assign taxonomy below the order level, as suggested by Deshpande et al. (2016). A total of 36 orders of fungi were detected (Tables S2 - S3). A total of 44.83% of the OTUs (15.21% of reads) belonged to Basidiomycota, while the Ascomycota accounted for 25.86% OTUs (5.43% of the reads). Chytridiomycota was represented by 20.26% OTUs (63.37% of the reads), while a small proportion of OTUs (2.16%, 0.19% of reads) belonged to Zygomycota, and the remaining 6.90% OTUs (15.81% of the reads) were not assigned at the phylum level. The 20 most frequently observed OTUs represented 82.23% total reads (Fig. 3A).

Sequences from Ascomycota matched 12 known orders, whereas Basidiomycota was represented by 20 known orders. The dominant basidiomycete orders were the Agaricales (9.91% OTUs), Tremellales (4.31% OTUs), Polyporales (3.88% OTUs) and Russulales (3.88% OTUs). The most common ascomycete orders were the Pleosporales (8.62% OTUs), Helotiales (3.45% OTUs) and Hypocreales (2.59% OTUs). The chytrid diversity in the lakes was represented by the orders Rhizophydiales (1.29% OTUs), Chytridiales (0.86% OTUs) and Spizellomycetales (0.43% OTUs). 17.67% chytrid OTUs remained unclassified at the order level. Zygomycota diversity was exclusively represented by Mortierellales (2.16% OTUs) (Table S2). Interestingly, OTU 1013 had the closest match (98% similarity) to the parasitic chytrid strain Rhizophydiales sp. Chy-Lys2009 (FR670788; 4 sites, 120 reads) isolated from *Planktothrix*-dominated lake Lyseren in southern Norway.

The closest matches to the following fungal genera were obtained in the present study: *Cryptococcus* (9 OTUs), *Cortinarius* (8 OTUs), *Rhodotorula* (5 OTUs), *Taphrina* (4 OTUs), *Exidia* (4 OTUs), *Microdochium* (3 OTUs), *Mycena* (3 OTUs), *Sistotrema* (3 OTUs), *Leccinium*

(3 OTUs), *Xylodon* (3 OTUs), *Alternaria* (2 OTUs), *Malassezia* (2 OTUs), *Sporobolomyces* (2 OTUs), *Trichosporon* (2 OTUs), *Gymnopus* (2 OTUs), *Ceratobasidium* (2 OTUs), *Itersonilia* (2 OTUs), *Mastigobasidium* (2 OTUs), *Heterobasidion* (2 OTUs), *Peniophora* (2 OTUs), *Lactarius* (2 OTUs), *Cladosporium* (1 OTU), *Exophiala* (1 OTU), *Chytriomyces* (1 OTU), *Rhizoclosmatium* (1 OTU), *Betamyces* (1 OTU), *Globomyces* (1 OTU) and *Powellomyces* (1 OTU). The detailed taxonomic assignments for the final 232 OTUs are presented in Table S3 (NCBI/UNITE assignment) and Appendix 1 (RDP Naïve Bayesian Classifier).

3.4. Ecology of aquatic fungi

The fungi detected in this study included both presumed resident and transient components of aquatic communities. Fig. 3B shows the partition between core and transient fungal OTUs based on the position of each OTU within the log-normal SAD using persistence-abundance plot according to Kostovcik et al. (2015). Occupancy (number of sites in which each OTU was present) was plotted against persistence (maximum read abundance of each OTU across all sites). The occupancy threshold for the split between core and transient OTU groups was found by minimizing the Akaike Information Criterion (AIC) for the fit of the core group to a log-normal rank-abundance distribution. The identified core community consisted of 9 OTUs (colored dots on the right in Fig. 3B). The remaining OTUs were classified as primarily transient and presumably of terrestrial origin (Fig. 3B, left) based on their taxonomic affinities to well-defined groups of terrestrial fungi. However, it is possible some low abundance and rare aquatic taxa could be captured in the 'transient' category as well. The members of 10 most abundant fungal orders, i.e. members of Rhizophydiales (including unassigned OTUs), Capnodiales, and Tremellales were quantitatively more abundant than expected, whereas Pleosporales, Agaricales,

Malasseziales, Polyporales and Sporidiobolales had a large number of relatively low-abundance OTUs (Fig. 3C).

In order to investigate the richness effect on β-diversity in NMDS analyses, we compared several dissimilarity indices (i.e. Bray-Curtis, Jaccard, Gower and Raup-Crick) on eight subsets of sites (Fig. S4). Jaccard, Raup-Crick and Bray-Curtis dissimilarity indices produced very similar results, with Gower being the least robust metrics. Since NMDS axes for two and three dimensions were highly correlated (NMDS1: $P < 0.00001$, $\tau = 0.89$; NMDS2: $P < 0.00001$, $\tau = 0.87$, respectively), we will focus on the two first NMDS dimensions. To test the robustness of our NMDS analysis, ordinations based on subsets with minimum total reads from 50 to 150 (38 to 64 lakes) were compared (all with Procrustes significance probabilities = 0.001 on 999 permutations, and Procrustes correlations = 0.73-0.99), suggesting that the conservative choice of > 150 reads is probably valid for much wider subsets (Fig. S5). The NMDS analysis performed for the subset of studied lakes (with > 150 total reads and > 10X coverage) found significant correlation between aquatic fungal community composition and longitude ($P = 0.001$) as well as local environmental factors (TOC ($P = 0.005$), TP ($P = 0.001$) and conductivity ($P = 0.002$), all log transformed) (Fig. 4A, 4B and Table S4).

Community dissimilarity increased significantly with geographical distance (Mantel correlation = 0.20, $P = 0.001$ on 999 permutations) and local environment (Mantel correlation = 0.17, $P = 0.002$ on 999 permutations). The significant effect of the local environment (TOC, TP and conductivity, all log transformed) persisted after spatial adjustment by a partial Mantel test (Mantel correlation = 0.12, $P = 0.03$ on 999 permutations).

4. Discussion

357 *4.1. Fungal diversity in freshwater*

358 Fungi in freshwater have varying ecological roles, e.g. as decomposers, pathogens or parasites of
359 sponges, fish, crustaceans, algae or other fungi (Gleason et al., 2008; Ishida et al., 2015; Kagami
360 et al., 2007; Wurzbacher et al., 2010) and can occur as residents (adapted to aquatic
361 environments) or transients (occurring in water fortuitously) (Shearer et al., 2007). The relatively
362 low number of fungal OTUs (232) detected in our study may be due to the exclusion of most
363 basidiomycetes and zygomycetes, to the lower species diversity of plant hosts in aquatic habitats,
364 to environmental restrictions on growth of fungi in the water column, or dominance of fungal
365 groups (i.e. Cryptomycota) that are underrepresented in ITS2 databases (Bärlocher and Boddy,
366 2016; Shearer et al., 2007). Moreover, it is difficult to compare OTU numbers between studies
367 due to the fact that bioinformatics processing parameters can significantly impact OTU detection
368 and richness estimates (Gihring et al., 2012; Kunin et al., 2010; Quince et al., 2009; Schloss,
369 2010). Richards et al. (2015) suggest that the DNA extraction protocols used are likely biased
370 against the recovery of fungal sequences (in particular, filamentous forms with robust cell walls).
371 However, assuming the low levels of diversity recovered here do represent a species-poor
372 community, the drivers responsible for this may be similar to those in marine ecosystems as, for
373 example, low nutrient levels, absence of substrates for fungal cell attachment, and the dominance
374 of free-floating single-celled plankton in the photic zone (Richards et al., 2012). Fungal
375 community composition varied both with longitude and local environmental factors suggesting
376 that fungi, like protists, respond to local and metacommunity scale productivity gradients
377 (Ptacnik et al., 2010). TOC (i.e. the sum of suspended particulate and dissolved organic matter)
378 will probably reflect resource availability for osmotrophs and saprotrophs, and may, as such,
379 influence the fungal community composition. In our study we aimed at selecting boreal lakes

within the TOC and TP range typical for this region. Since dissolved organic matter (which constitutes > 90% of TOC in these lakes) contains a small, but non-zero, amount of phosphorus, there will be a weak, but unavoidable covariation between TOC and TP. We excluded lakes with particularly high TOC since these will not be equally available across the spatial gradient (i.e. brown-water lakes are less common in the west). We also excluded lakes with particularly high TP since these typically reflect local pollution rather than regional trends. We deliberately constrained the climatic variation in our study by making the longitudinal gradient three times longer (750 km) than the latitudinal (Khomich et al., in press). With this study design we find a strong longitudinal signal of the same magnitude as in earlier studies with non-molecular methods (Hessen et al., 2006; Ptacnik et al., 2010). The results of Mantel test suggest that adjacent lakes tend to be compositionally more similar. Moreover, effects of the local environment on aquatic fungal communities were still present after partialing out spatial factors.

The fungi detected in this study included a large fraction of putatively terrestrial taxa and taxa known to occur in both terrestrial and aquatic ecosystems. This concurs with other studies that have detected both resident and transient components of fungal communities in aquatic ecosystems (Gutiérrez et al., 2015; Zhang et al., 2015), although conclusions regarding the terrestrial or aquatic status of the OTUs recovered here must be drawn with caution, as high confidence taxonomic assignments to the genus and species level are severely hampered by under-populated reference databases, and low abundance or rare aquatic species may be classed as 'transient' components of the community using SAD analyses. However, based on their taxonomic affinity to known groups of terrestrial fungi, the putative terrestrial fungal OTUs likely are fungal structures that have been washed into aquatic habitats where they are not active contributors to the community, but still can be detected. In general, overlap between species in

freshwater and terrestrial habitats can be high compared to between marine and freshwater, or marine and terrestrial habitats (Shearer et al., 2007; Zhang et al., 2015), reflecting the evolutionary history of aquatic fungi, which has been suggested to include multiple transitions from terrestrial to aquatic forms (Vijaykrishna and Hyde, 2006).

The most abundant OTUs were taxonomically assigned to Chytridiomycota (Fig. 3A and 3B), whose members are known to be saprobes on a large variety of substrates and parasites of phytoplankton, zooplankton, fungi and invertebrates (Gleason et al., 2008; Kagami et al., 2014; Sime-Ngando, 2012). Although the pre-filtering of water samples on 100 μm mesh could potentially exclude some filamentous fungi or fungi attached to phytoplankton and thus reduce the detected chytrid diversity, our findings are largely in agreement with literature sources (Jobard et al., 2012; Lefèvre et al., 2012) and confirm the importance of basal fungal groups in aquatic food webs (Kagami et al., 2014; Sime-Ngando, 2012). These planktonic fungi are strictly dependent on surfaces and, therefore, are closely associated with all types of aquatic organisms (e.g. algae, copepods) and most likely also lake snow particles (Tang et al., 2006; Wurzbacher et al., 2010). However, most surveys on freshwater parasite dynamics are focused on hosts (e.g. cyanobacteria and diatoms) (Sime-Ngando, 2012), with several studies reporting chytrid parasitism as an important ecological factor for determining abundance of the filamentous cyanobacterium *Planktothrix* in lakes in southern Norway (Kyle et al., 2015; Rohrlack et al., 2015). Interestingly, one OTU recovered here had the closest match (98% similarity) to the chytrid strain Rhizophydiales sp. Chy-Lys2009 (FR670788) which was isolated from the *Planktothrix*-dominated lake Lyseren in southern Norway. According to Sønstebø and Rohrlack (2011), this chytrid strain showed high infectious capability for *Planktothrix* sp., but failed to infect other filamentous cyanobacteria.

426

427 *4.2. Dikarya (Ascomycota and Basidiomycota)*

428 Members of Basidiomycota occurred frequently in the surface waters of oligotrophic lakes, but
429 with lower abundance than those of the Ascomycota. Our results are not in concordance with
430 previous studies in which early diverging fungal lineages were found to be the dominant fungal
431 forms in freshwater habitats (Lefèvre et al., 2012). Although Dikarya is the dominant fungal
432 group in marine environments (Bass et al., 2007; Edgcomb et al., 2011; Tisthammer et al., 2016;
433 Zhang et al., 2015), 'basal' lineages are thought to dominate in fresh and brackish waters
434 (Lefèvre et al., 2012; Richards et al., 2012; Shearer et al., 2007). In particular, Ascomycota
435 (including aquatic hyphomycetes), Chytridiomycota and other true fungi comprise most of the
436 documented freshwater fungal diversity (Bärlocher and Boddy, 2016; Shearer et al., 2007). The
437 inconsistency between our findings and the prevailing opinion in the literature may partially be
438 explained by possible primer bias, since the primers used in our data set have not been evaluated
439 against members of the basal fungal lineages (Ihrmark et al., 2012), and were designed to
440 amplify Dikarya. Alternatively, the high diversity of Basidiomycota detected in our study may
441 reflect detection of the transient component of the fungal community. Basidiomycota are
442 dominant fungal community components in terrestrial environments (Buée et al., 2009) and the
443 most commonly identified orders of Basidiomycota in this study were common terrestrial
444 lineages like Agaricales, Polyporales and Russulales. Their occurrence in these aquatic
445 ecosystems is likely due to transient introductions through transport of allochthonous organic
446 material (Bärlocher, 2016) and deposition of wind-dispersed spores. In addition, a significant
447 proportion of Basidiomycota detected in the studied lakes belonged to ectomycorrhizal fungal
448 orders, e.g. Agaricales and Thelephorales. As with invertebrates and plants (Green et al., 2008), a

major vector for long distance dispersal of fungal spores is likely migratory water birds, which may explain their presence in the surface waters of lakes (Hyde and Soytong, 2008).

Among Ascomycota the most commonly detected orders were Pleosporales, Helotiales and Hypocreales, all of which include members with aquatic lifestyles. However, it is difficult to determine if these fungi represent transient or resident members of the fungal community. Members of the Pleosporales and Hypocreales are known to be lignicolous (growing on submerged woody debris) in both aquatic and terrestrial habitats (Hyde et al., 2016; Hyde et al., 2013; Maharachchikumbura et al., 2015; Wijayawardene et al., 2014), so it is unclear whether the detected OTUs represent transient contributions of spores of terrestrial species or the signature of resident populations of fungi decomposing material in the littoral zone. Similarly, leaf-degrading fungi from ascomycete genera *Aureobasidium, Cladosporium, Alternaria* and *Phoma* detected in this study play an initial role in emergent macrophyte decomposition (Wurzbacher et al., 2010) and cannot be easily classed as resident or transient community members. Some ascomycetes categorized as freshwater fungi have also been reported from other habitats (e.g. terrestrial and marine), indicating possible ubiquitous distribution of some species, which is consistent with evolutionary reconstructions that support a terrestrial origin for both freshwater ascomycetes and marine ascomycetes (Kodsueb et al., 2016; Vijaykrishna and Hyde, 2006).

4.3. Yeast-like forms

The majority of higher fungi occurring in aquatic environments have unicellular yeast growth forms. Interestingly, yeasts may account for more biomass and diversity than filamentous fungi in these systems. Yeast-like growth forms are expected to be more abundant in freshwater

environments than in seawater, comprising < 100 cells/l in unpolluted lakes versus < 10 cells/l in open ocean waters (Hagler and Ahearn, 1987).

Yeast-like forms detected in our study belonged to the basidiomycetous orders Tremellales, Sporidiobolales, Leucosporidiales, Malasseziales and Trichosporonales (altogether 10.34% OTUs), and ascomycetous orders Taphrinales, Saccharomycetales and Dothideales (altogether 3.44% OTUs). Similarly, basidiomycetous yeasts often constitute the bigger fraction of the total yeast population in oligotrophic oceanic waters (Nagahama, 2006). Earlier it was assumed that yeasts are transients washed in from the phylloplane or the littoral zone, but there is now clear evidence for their more or less permanent residence in open waters. Members of Tremellales (*Cryptococcus* species) have previously been reported in lake surface waters (Rosa et al., 1995; Van Uden and Ahearn, 1963; Wurzbacher et al., 2010). An opportunistic black yeast pathogen genus *Aureobasidium* (Dothideales) found in our study is considered to enter aquatic environments with plant material, and the red yeast *Sporobolomyces* spp. (Sporidiobolales) is similarly introduced by the fallen leaves of terrestrial plants (Libkind et al., 2009; Nagahama, 2006). The genus *Rhodotorula* (Sporidiobolales) includes ubiquitous saprophytic yeasts isolated from different aquatic habitats and invertebrates (Nagahama et al., 2003), and it was unsurprisingly detected widely in our samples. Two OTUs belonging to Malasseziales were found in our study systems. Culture-independent studies of fungi from environmental samples show that *Malassezia* (Malasseziales) is a cosmopolitan lipophilic yeast widely distributed in deep-sea sediments, hydrothermal vents, stony corals, fish guts, Antarctic soils and in the exoskeleton of soil nematodes (Amend, 2014; Bass et al., 2007; Edgcomb et al., 2011; Gao et al., 2008). It is currently unclear whether the diversity of *Malassezia*-like organisms may reflect similar ecological diversity with trophic strategies ranging from saprotrophy to biotrophy

(Amend, 2014). One OTU was assigned to the black yeasts genus *Exophiala* (Chaetothyriales), which includes ubiquitous, opportunistic pathogens causing both superficial and systemic mycoses in marine and freshwater fish (e.g. halibut, salmon, cod), although it can also be isolated from substrates such as soil, sediments, decaying wood and plant material (Gjessing et al., 2011; Overy et al., 2015). Similarly, other known yeast-like pathogens recovered in this study closely related to terrestrial fungi and associated with disease in aquatic environment include *Trichosporon, Taphrina, Ustilago and Exophiala* (Higgins, 2000; Richards et al., 2012).

4.4. Chytridiomycota, Cryptomycota and Zygomycota

Our results show a significant fraction of unclassified fungal sequences in surface waters of lakes (up to 16% of total reads) suggesting that surface waters of oligotrophic lakes can be a source of unknown fungal diversity. Richards et al. (2012) hypothesize that aquatic environments host a significant number of unclassified novel groups branching below the Dikarya radiation, thus reflecting an ancient transition from aquatic to terrestrial ecosystems. Among the currently recognized basal fungal lineages, chytrid fungi were detected in high abundances in our study, and are known to be ubiquitous in aquatic environments, both as saprotrophs and obligate parasites of phytoplankton (James et al., 2006; Kagami et al., 2007; Shearer et al., 2007; Voigt et al., 2013).

The three known Chytridiomycota orders, namely Rhizophydiales, Chytridiales and Spizellomycetales, were all detected, with a large fraction of sequences matching poorly to the available ITS2 references. Importantly, identification of Chytridiomycota increasingly relies on a combination of both ultrastructure and molecular data suggesting a polyphyletic nature for many chytrid genera (Grossart et al., 2016; Karpov et al., 2014; Letcher et al., 2008a; Letcher et al.,

2012; Letcher et al., 2008b; Letcher et al., 2008c). Possible mismatches in reference databases limit our ability to infer the ecological role of these chytrid fungi based solely on environmental sequences. In addition, the great majority of Chytridiomycota have not been cultured and studied, and are thus classified as 'uncultured' in sequence databases (Grossart et al., 2016).

However, not only parasitic, but also saprotrophic fungal lifestyles can be of ecological relevance. For example, saprotrophic Chytridiomycota are commonly found on pollen, which occurs in huge quantities in lakes, particularly during the clear-water phase in spring when organic matter and nutrients are low (Wurzbacher et al., 2014). It is unclear whether the Chytridiomycota detected in this study represent saprotrophic or parasitic taxa. We hypothesize that both functional groups may be present in the aquatic environment occupying narrow ecological niches (Gleason et al., 2008; Rasconi et al., 2011).

Representatives of a single order in Zygomycota, Mortierellales, were detected in this study at low frequency and abundance. Zygomycota are known to be relatively rare in aquatic habitats (Shearer et al., 2007) and are most commonly saprobic soil-inhabiting fungi on decaying organic material (Wagner et al., 2013) that most likely enter the aquatic environment transiently with wind or soil particles.

Cryptomycota are known to occur in freshwater environments, as well as in marine and soil ecosystems, however the group was notably absent from our samples. While the group may in fact be rare in surface waters, we hypothesize that our inability to detect Cryptomycota in the studied lakes is more likely attributable to primer bias, as the primers used have not been tested against the group, or as a result of the paucity of ITS2 sequences for this group in public databases (< 1% sequences in the UNITE database) which may cause Cryptomycota sequences to be incorrectly identified as non-fungal or classified only to the Kingdom level.

4.5. Methodological considerations

The fungal-specific primer (fITS7a) used in this study, was tested for members of the Ascomycota, Basidiomycota, and Zygomycota, and focused on the exclusion of the plant Kingdom (Ihrmark et al., 2012). As a result, the primer's specificity for other groups, including basal fungal lineages and animals, remains largely unknown. The high proportion of OTUs in this study that remained unassigned (48.77%) or matched non-target organisms (26.94%) (e.g. Chlorophyta, Cryptophyta and Ciliophora) suggests that the primer combination fITS7a/ITS4 is not fungal-specific in aquatic environments. This substantially reduced the effective sequencing depth for fungal taxa, and as a result, the full diversity of the aquatic fungal communities was not recovered in this study (see Fig.2).

The high proportion of unidentified sequences in our data set may reflect poor ITS database coverage, as some of the taxa that were expected to be dominant (Chytridiomycota and Cryptomycota) have very little ITS information available for them. An alternative would be to use the more conserved LSU/SSU regions which have the advantages of inferring higher-level phylogenetic relationships and identifying novel fungal lineages, though they cannot discriminate between closely related fungal species. Therefore, a combination of several gene markers achieves a higher and more reliable phylogenetic inference, as has recently been established for Chytridiomycota (Grossart et al., 2016; Lefèvre et al., 2012; Letcher et al., 2008c). Few studies have shown that both ITS and LSU regions provided comparable accuracy in estimating fungal diversity and taxonomic assignments to the genus level (Brown et al., 2014; Porras-Alfaro et al., 2014). Nevertheless, the ITS approach remains useful when targeting well-

563 defined taxonomic groups where species level OTU identification is important (Brown et al.,
564 2014; Richards et al., 2012).
565 Another concern is that detection of rare species (or OTUs) can be particularly difficult in low
566 density populations, as would be expected in surface water fungal communities. In agreement
567 with previous surveys (Lefèvre et al., 2012), a majority of the taxonomically assigned true fungi
568 detected in our study were only found in few lakes suggesting they are likely rare. Therefore, an
569 intense sampling of aquatic fungal communities should be considered to improve the exploration
570 of rare taxa which may play an important role by becoming dominant in response to
571 environmental changes, as well as representing a novel source of diversity. Moreover, an
572 alternative could be to sample monthly or seasonally to track the presence of particular aquatic
573 species, as well as to obtain a better understanding of the main factors regulating pelagic fungal
574 communities, as has recently been shown for small eukaryotes in freshwater ecosystems (Mangot
575 et al., 2013; Simon et al., 2015).
576
577 *4.6. Concluding remarks*
578 In conclusion, our results suggest that surface waters of oligotrophic lakes harbour many fungal
579 taxa, but many of these seem to be transient and likely transported from the surrounding
580 terrestrial environment by wind, water and/or migratory birds. Importantly, there are several
581 limitations to our study that can inform future surveys of freshwater fungi using amplicon
582 sequencing. First, fITS7a/ITS4 performed poorly as a fungal-specific primer combination in an
583 aquatic environment. Non-target amplification hampered sequencing depth to the extent that we
584 were unable to capture the total fungal diversity. Secondly, the scarcity of taxonomic references
585 for basal fungal lineages in public ITS2 sequence databases creates difficulties in identifying

586 OTUs below the phylum level, such that targeting more conserved gene regions may be more
587 appropriate under some circumstances.
588
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597

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859

860 **Figure legends**

861 **Fig. 1.** A map of sampled lakes (*n* = 77). The mountain ridge extends S-N around 8 ºE. The
862 subset of lakes (*n* = 30) used in the NMDS ordination is indicated by dark red colour.

863

864 **Fig. 2.** Rarefaction curves for 77 sampled lakes describing the number of fungal OTUs as a
865 function of the number of reads.

866

867 **Fig. 3. A)** The relative abundance of top 20 fungal OTUs. The identity number of the respective
868 OTU is shown below the bars. Colours represent the fungal phyla. **B)** Core (right) and transient
869 (left) aquatic community members based on discontinuity in persistence/abundance distribution
870 of fungal OTUs. Dashed line represents the threshold between transient and core groups,
871 identified by minimizing the AIC of a log-normal rank-abundance fit to the core group. **C)** Top
872 10 most abundant fungal orders.

873

874 **Fig. 4.** Nonmetric multidimensional scaling (NMDS) plot of the subset of lakes (*n* = 30) scaled
875 by OTU richness, coloured by longitude with contour lines indicating change in **A)** TOC and **B)**
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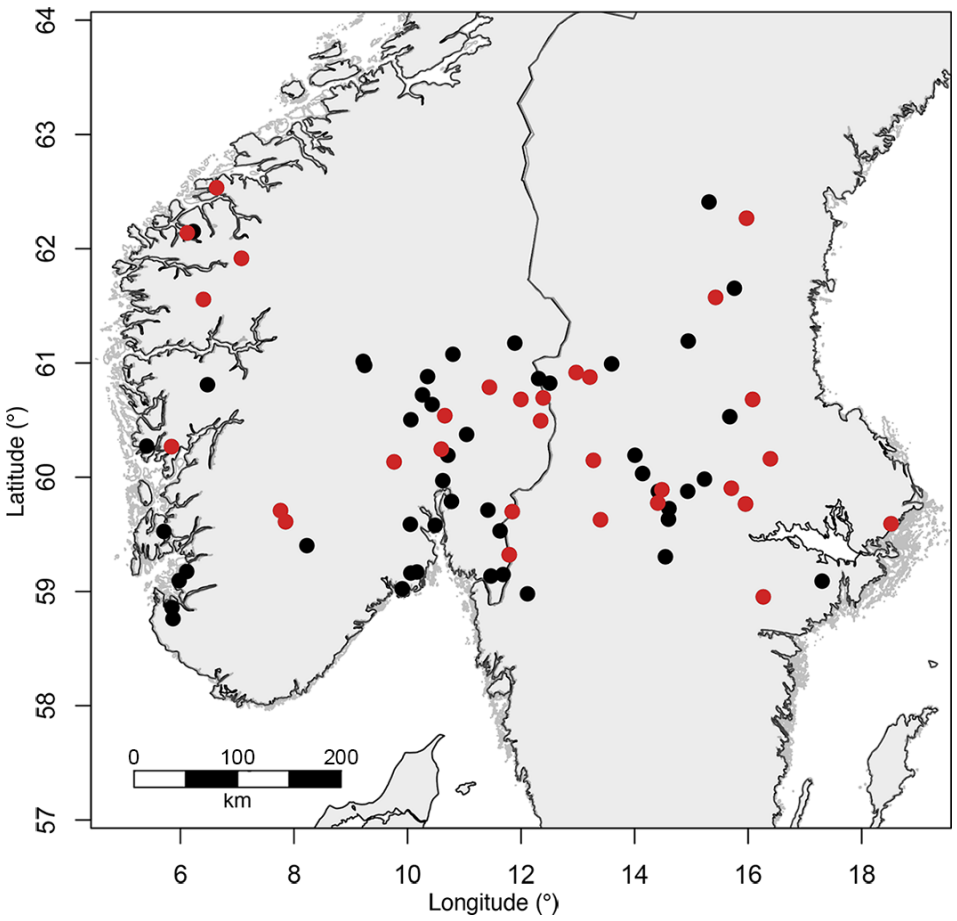

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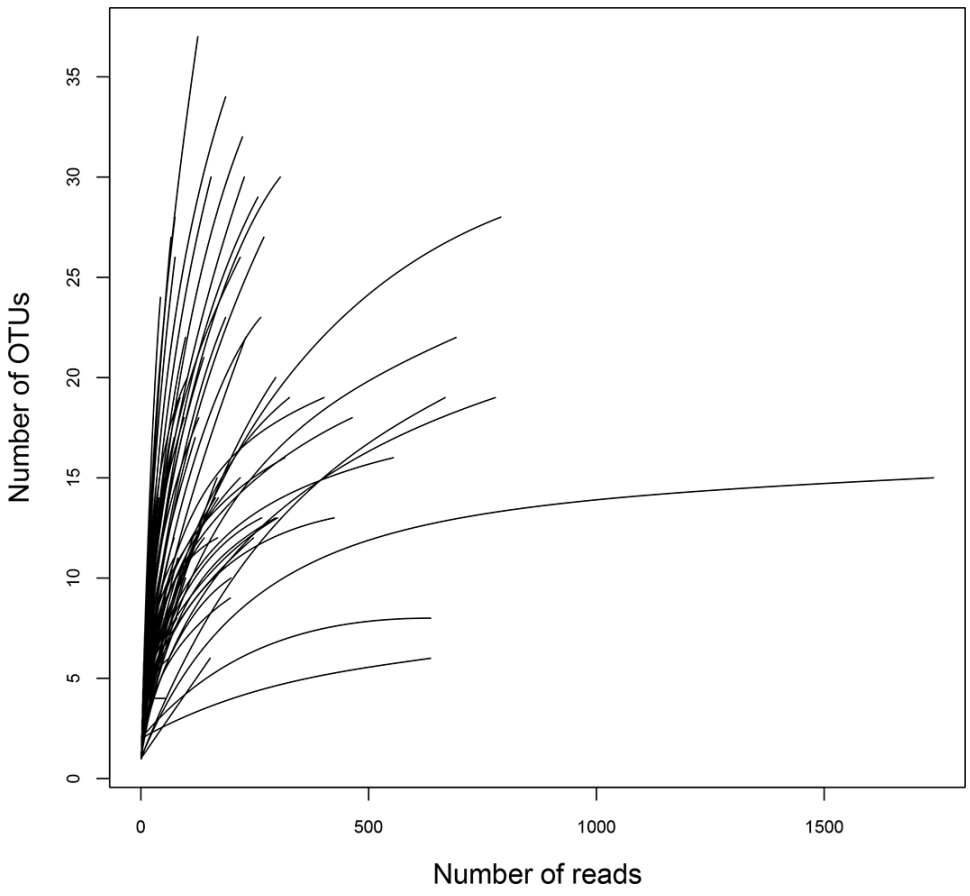

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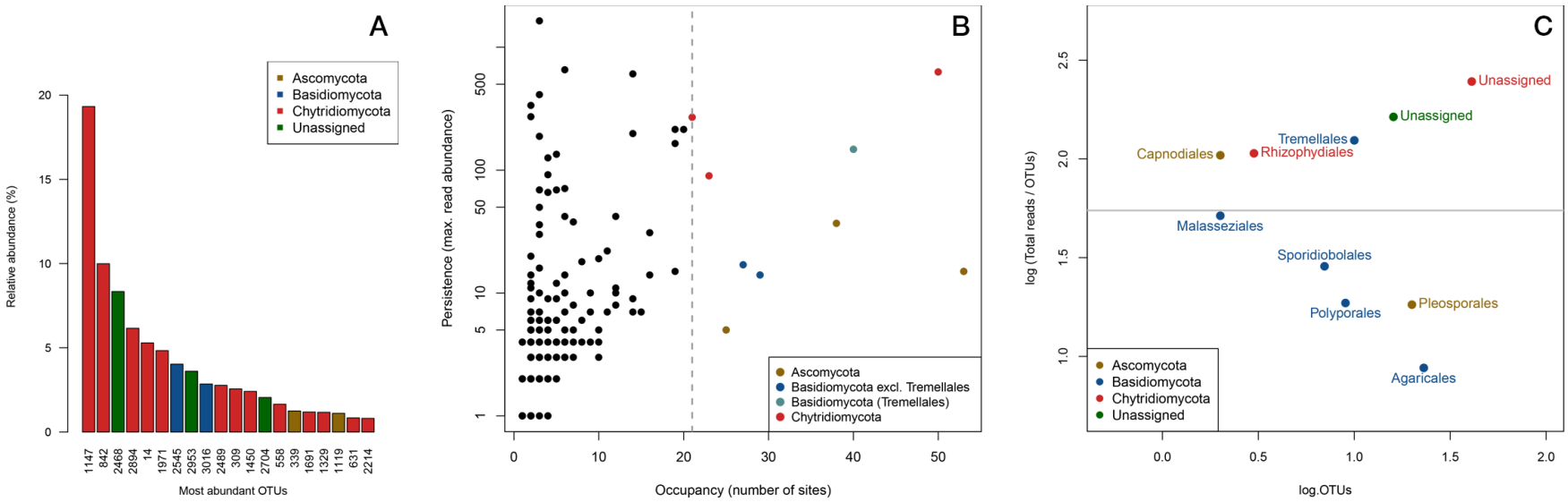

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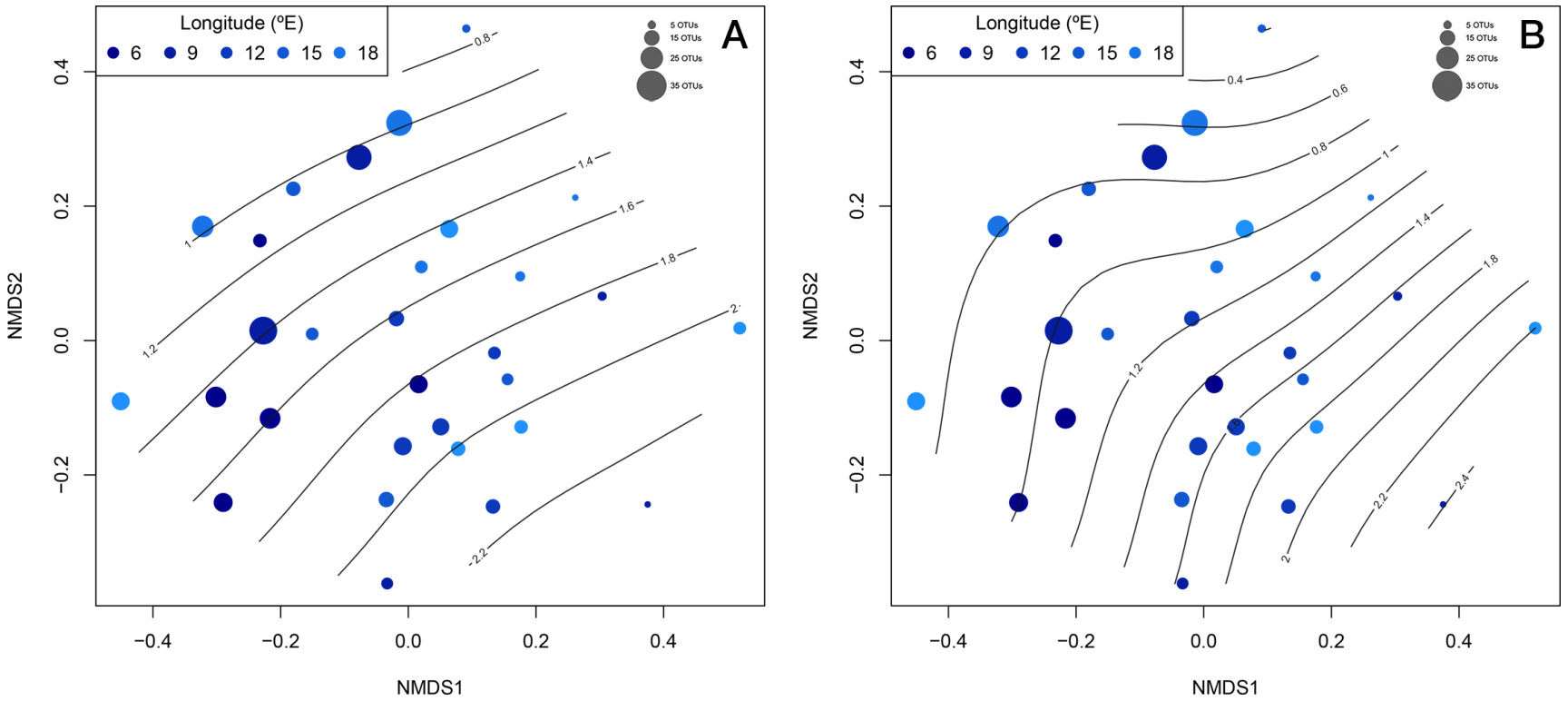

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