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# Shifts in species richness and species composition of grassland fungi along environmental gradients in semi-natural grasslands in Norway

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Master of Science in Fungal Ecology

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## Abstract

Semi-natural grasslands created through extensive agricultural practices function as hotspots for biodiversity. However, changes in land-use, and particularly accelerated intensification, have caused a drastic decline of this ecosystem in Europe since the mid 20th century. The rapid decline highlights the urgency of increasing our knowledge of these biodiverse ecosystems to improve conservation efforts. As grassland fungi constitute a significant portion of the biodiversity in semi-natural grasslands, this thesis investigates how species richness and species composition of CHEGD fungi – an acronym of fungal taxa associated with semi-natural grasslands: *Clavariaceae*, *Hygrocybe* s.l, *Entoloma*, *Geoglossaece/ Microglossum*, and *Dermoloma/Pseudotricholoma*, varies across environmental gradients. Data from soil samples and fruitbodies in semi-natural grasslands across Norway allows for investigation of CHEGD fungal responses to three environmental gradients: calcareousness in soil, bioclimatic sections (oceanic-continental gradient) and bioclimatic zones (elevational/latitudinal gradient). Relationships between environmental gradients and CHEGD fungal diversity are further compared across two sampling methods: fruitbody surveys and eDNA from soil samples. Potential differences in ecology within three species complexes: *Hygrocybe conica* coll., *Cuphophyllus virgineus* coll., and *Gliophorus psittacinus* coll., are also examined.

Shifts in species richness and species composition of CHEGD fungi were identified along the environmental gradients, and the different sampling methods captured several similar patterns. Species richness increased with higher calcareousness, and species composition differed between weakly and strongly calcareous soils in both fruitbody and eDNA data. Interactions between gradients found indirect effects of climate on edaphic conditions, and moderate conditions along the elevational/latitudinal gradient were associated with high diversity in both datasets. The eDNA data found high species richness to associate with intermediate oceanic influence and little variation along the rest of the gradient. The fruitbody data revealed in contrast a positive relationship between oceanic influence and species richness. Still, species composition showed variation between low and high oceanic influence in both datasets. Ecological variation within the three species complexes was not significantly explained by environmental gradients, but associations between taxa and specific environmental conditions was found.

The findings of this study contribute to a deeper understanding of the ecology in semi-natural grasslands and highlight the importance of climatic and edaphic factors, along with their interactive effects on fungi associated with this habitat type. These insights are important for ensuring representative conservation measures that preserve fungal diversity in semi-natural grasslands. The ecological variation within species complexes shows great potential for further species delimitation, and promising signals for differences in habitat preferences for future descriptions of other cryptic species as well. Future mycological surveys in semi-natural grasslands should therefore include data collection from both fruitbodies and environmental-DNA. Increasing the knowledge of fungal diversity associated with semi-natural grasslands is essential for a deeper understanding of the ecology in these threatened habitats.

## Sammendrag

Semi-naturlige enger er skapt gjennom langvarig ekstensiv hevd og fungerer som hotspots for biologisk mangfold. Endringer i arealbruk, og spesielt den kraftige økningen av intensivt arealbruk, har imidlertid ført til en rask tilbakegang av denne naturtypen i Europa siden midten av 1900-tallet. Den kraftige tilbakegangen understreker viktigheten av å øke kunnskapen om denne artsrike naturtypen for å bedre bevaringstiltak. Beitemarkssopp utgjør en betydelig del av det biologiske mangfoldet i semi-naturlig eng og denne masteroppgaven undersøker hvordan artsrikdom og artssammensetning av CHEGD-sopp – et akronym for sopp særlig tilknyttet semi-naturlig eng i gruppene: *Clavariaceae* (fingersopper) *Hygrocybe* s.l. (engvokssopper i vid forstand), *Entoloma* (rødsporer), *Geoglossaece/Microglossum* (jordtunger) og *Dermoloma/Pseudotricholoma* (grynmusseronger), varierer langs miljøgradienter. Data fra både jordprøver og fruktlegemer i semi-naturlige enger over hele Norge, gjør det mulig å undersøkelse hvordan beitemarkssopp påvirkes av tre ulike miljøgradienter: kalknivå i jord, bioklimatiske seksjoner (oseanisk-kontinental gradient) og bioklimatiske soner (lavland–fjell og sør-nord gradient). Sammenhenger mellom miljøgradienter og beitemarkssopp blir videre sammenlignet mellom to innsamlingsmetoder: feltundersøkelser av fruktlegemer og miljø-DNA (mDNA) fra jordprøver. Potensielle forskjeller i økologi innad i tre artskomplekser: *Hygrocybe conica* coll. (kjeglevokssopp-gruppen), *Cuphophyllus virgineus* coll. (krittvokssopp-gruppen), og *Gliophorus psittacinus* coll. (papegøyevokssopp-gruppen), blir også undersøkt.

Endringer i artsrikdom og -sammensetning av beitemarkssopp ble identifisert langs alle miljøgradienter, og de ulike innsamlingsmetodene fanget opp flere lignende mønstre. Antall arter økte med høyere kalkinnhold, og sammensetningen av arter viste ulikheter i svak og sterk kalkrik jord i dataen fra både jordprøver og fruktlegemer. Interaksjoner mellom gradientene viste til indirekte effekter av klima på jordforhold, og moderate forhold langs lavland-fjell (og sør-nord) viste sammenheng med høy diversitet i begge datasett. Data fra jordprøvene viste til et høyt antall arter ved intermediær oseanitet og ellers lite variasjon langs gradienten, mens data fra fruktlegemer viste en klar positiv sammenheng mellom oseanitet og artsrikdom. Begge metoder fant imidlertid variasjon i sammensetningen av arter i høy og lav oseanitet. Økologisk variasjon innenfor de tre artskompleksene ble ikke signifikant forklart av miljøgradientene, men det ble likevel funnet assosiasjoner mellom artshypoteser og spesifikke miljøforhold.

Funnene i denne oppgaven bidrar til en dypere forståelse av økologien i semi-naturlig eng og understreker betydningen av klimatiske faktorer, jordforhold, og deres interaktive effekter for sopp knyttet til denne naturtypen. Disse innsiktene er viktige for å sikre representative bevaringstiltak som beskytter mangfoldet av sopp i semi-naturlig eng. Den økologiske variasjonen i artskompleksene viser et stort potensial for videre artsavgrensning og lovende tegn for bruk av variasjon i habitat-preferanser i fremtidige beskrivelser av andre kryptiske arter også. Videre mykologiske undersøkelser i semi-naturlig eng bør derfor inkludere innsamling av data fra både fruktlegemer og miljø-DNA. Økt kunnskap om soppdiversiteten knyttet til denne naturtypen er avgjørende for en dypere forståelse av økologien i disse truede habitatene.

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# Introduction

Human societies have influenced terrestrial ecosystems for thousands of years with varying ecological impacts depending on the intensity and scale of different practices (Ellis et al., 2021). Traditional management practices, especially those resembling natural disturbances (e.g., large mammal grazing, wildfires), have been found to promote ecosystem function by enhancing species richness and heterogeneity (Billeter et al., 2007; Ellis et al., 2021; Middleton, 2013) ). However, land-use change has remained the dominant threat to global biodiversity for more than fifty years due to accelerated intensification (IPBES, 2019), and biodiverse cultural landscapes that prior societies have maintained over millennia is negatively affected (Ellis et al., 2021; Lasanta et al., 2017; Plieninger et al., 2016)

Continued use of extensive agricultural practices in former forested areas in Europe have generated semi-natural grasslands functioning as biodiversity hotspots (Billeter et al., 2007; Janssen et al., 2016; Middleton, 2013). Semi-natural grasslands are characterized by regular grazing or mowing, minimal to no use of fertilizer, and absence of recent plowing (Halvorsen et al., 2016). These ecosystems function as key habitats for many native species adapted to the landscape structure maintained by low-intensity farming systems (Billeter et al., 2007; Bratli et al., 2011). Today semi-natural grassland is evaluated as a threatened habitat type in Europe due to the reduction in both area and quality caused by intensification of agricultural practices and abandonment (Aune et al., 2018; Janssen et al., 2016). In the last seventy-five years it is estimated that 90 % of semi-natural grasslands have disappeared across Western Europe (Griffith et al., 2013). In Norway alone, 60 % of semi-natural grasslands have experienced a decline in ecological condition, reflecting their deviation from intact ecosystems, and more than a 50% reduction in area has been estimated for the last fifty years (Aune et al., 2018; Hovstad et al., 2018; Høitomt et al., 2025). The extent of these detrimental changes have let to semi-natural grassland being assessed as critical endangered in Norway (Høitomt et al., 2025) and emphasizes the importance and urgency of studying the biodiversity that depends on these habitats.

Fungal communities are a significant component of the biodiversity associated with semi-natural grasslands. Grasslands rich in “waxcaps” (*Hygrocybe spp*.) was familiar among mycologists already in the 18th century as “waxcap grasslands” (Griffith et al., 2013). Nitare (1988) found that several fungal groups showed a strong association with semi-natural grasslands. These fungi were later referred to as CHEGD fungi in Europe - an acronym encompassing taxonomically diverse groups with seemingly ecologically related species from *Clavariaceae*, *Hygrocybe* s.l, *Entoloma*, *Geoglossaece/Microglossum* and *Dermoloma/Pseudotricholoma* (Griffith et al., 2013). Although species within other fungal taxa also inhabit semi-natural grasslands, less is known about their dependence on these habitats (Jordal et al., 2016). In contrast, CHEGD fungi are strongly specific to semi-natural grasslands (Griffith et al., 2002; Jordal et al., 2016), and metabarcoding of soil fungal communities have shown that they represent the most abundant groups in undisturbed grasslands (Caboň et al., 2021; Detheridge et al., 2018). Their high abundance suggests that they play an important role in these ecosystems, yet our understanding of their functional ecology remains incomplete (Halbwachs et al., 2018).

CHEGD fungi are highly sensitive to phosphorus and/or nitrogen levels and soil tillage, which highlights their value as indicators of semi-natural grasslands (Caboň et al., 2021; Detheridge et al., 2016; Griffith et al., 2013). Long-term reduction of CHEGD diversity in response to management intensity, especially fertilization and soil disturbances, makes them relevant also for assessing the impacts of management practices (Boertmann, 2010; Ejrnæs & Bruun, 1995; Halbwachs et al., 2018). Levels of soil calcareousness, reflecting variation in base-richness and nutrient availability has also been linked to fungal distributions and habitat preferences in grasslands (Boertmann, 2010; Brandrud et al., 2023; Griffith et al., 2002; Nitare, 1988). However, it remains unclear how calcareousness shapes entire CHEGD fungal communities, particularly in combination with other environmental factors. Furthermore, the underlying processes behind the response of grassland fungi to management intensity and edaphic factors are not established. Increasing evidence suggest that several grassland fungi, particularly species within *Hygrocybe* s.l and non-lignicolous *Clavariaceae* have a biotrophic life strategy, but the mechanisms are poorly understood (Birkebak et al., 2013; Halbwachs et al., 2013; 2018; Seitzman et al., 2010; Tello et al., 2014). Nevertheless, the extent to which grassland fungi are influenced by environmental factors beyond management regimes and land-use history is largely unexplored, and the substantial climatic variation in Norway offers an ideal setting to study fungal responses to environmental gradients.

DNA barcoding of fruitbodies and analyses of eDNA from soil samples have significantly enhanced our knowledge of fungal diversity in semi-natural grasslands, revealing a higher species richness than previously recognized in Europe, including Norway (Brandrud et al., 2023; Crous et al., 2021; Jordal & Larsson, 2021; Noordeloos et al., 2022). Annual field surveys and DNA analyses continue to enhance DNA reference databases which facilitate species delimitation and identification, a trend particularly evident for the genus *Entoloma* where several species new to science have been described recently (Brandrud et al., 2023; Dima et al., 2021; Jordal & Larsson, 2021; Lodge et al., 2014; Noordeloos et al., 2022). DNA analyses have also revealed that species previously treated as single taxa, especially within *Hygrocybe* s.l., is in fact species complexes that have yet to be fully described (Detheridge & Griffith, 2021; Hustad et al., 2013; Lodge et al., 2014). Many of the undescribed taxa are currently only recognized through molecular data as operational taxonomic units (OTUs). Both *Clavariaceae*, *Geoglossaceae* and *Dermoloma* remain understudied with potential for discovering both species not yet recorded in Norway and species new to science (Arauzo & Iglesias, 2014; Fedosova et al., 2018; Kautmanová et al., 2012; Sánchez-García et al., 2021). Taxonomic revisions continue to reshape the classification of grassland fungi, with subdivisions of genera and discoveries of novel linages (Arauzo & Iglesias, 2014; Birkebak et al., 2013; Hustad et al., 2013; Lodge et al., 2014; Sánchez-García et al., 2021). However, many clades remain poorly resolved and more research is needed to further unravel CHEGD taxonomy. The still unresolved diversity of CHEGD fungi and the knowledge gaps regarding their ecology underline the urgency to improve our understanding of these fungi to support conservation efforts, particularly considering the rapid decline of semi-natural grasslands in the last century.

Traditional field surveys of fruitbodies are currently the main method to assess fungal communities in grasslands and emerge as superior for detecting rare species (Frøslev et al., 2019). Still, this method is highly influenced by weather conditions and dependent on the inconsistent production of macro

fruitbodies (Lücking et al., 2020). In comparison, eDNA metabarcoding is slightly less effective at identifying red-listed species but is shown to identify a broader spectrum of species – including those that seldom or never produce visible fruitbodies (Frøslev et al., 2019). Although eDNA metabarcoding show less variance in site richness compared to the traditional collection of fruitbodies, both sampling methods is found to capture similar differences in fungal ß-diversity (Frøslev et al., 2019) However, environmental predictors are shown to explain a larger portion of variation in community composition when based on eDNA data (Frøslev et al., 2019). To capture a broad spectrum of fungal diversity, including species with unpredictable fruiting patterns as well as rare red-listed species, a combination of traditional field work and eDNA analyses of soil stands out as the most effective approach (Frøslev et al., 2019; Lücking et al., 2020).

The aim of this study is to investigate relationships between environmental gradients and species richness and species composition of CHEGD fungi in semi-natural grasslands in Norway. The data analyzed in this study are derived from the project "Hidden fungi of semi-natural grasslands in Norway: combining fruitbody studies and eDNA", funded by the Norwegian Biodiversity Information Centre. The project is a collaboration between the Norwegian University of Life Science (NMBU), the Norwegian Institute of Nature Research (NINA) and Miljøfaglig Utredning (MFU) – which have organized and conducted the collection of data. The data include fungal records collected by both fruitbody surveys and eDNA metabarcoding of soil samples from 72 semi-natural grasslands with varying degrees of calcareousness located across different bioclimatic zones and sections in Norway (Moen, 1999). The wide ecological range of the study sites facilitates investigation of how environmental gradients influence CHEGD fungal diversity in semi-natural grasslands, and the comprehensive sampling regime enables a comparison of the insights gained from the different sampling methods. Operational taxonomic units (OTUs) identified through eDNA metabarcoding of soil also enables investigation of potential ecological variation within three species complexes from *Hygrocybe* s.l: *Hygrocybe conica* coll., *Cuphophyllus virgineus* coll., and *Gliophorus psittacinus* coll.

# Methodology

## Study sites

A total of 72 open semi-natural grasslands in Norway (Fig.1; Table A1) were chosen by the project partners (NMBU, NINA, MFU) in 2022. The selection was based on available data from *Naturbase*, a national database developed by the Norwegian Environment Authority (NEA), providing mapped information of selected areas for nature and outdoor recreation (Miljødirektoratet, n.d). This information in addition to previous records of CHEGD fungi in *Artskart* (Artskart, 2022), a service that provide geospatial species information, was used to prioritize semi-natural grasslands likely to support a high diversity of CHEGD fungi. Still, some sites were excluded due to outdated information and significant quality decline from discontinued management or fertilization. In such cases, other sites were added to ensure the intended spatial coverage of the project.

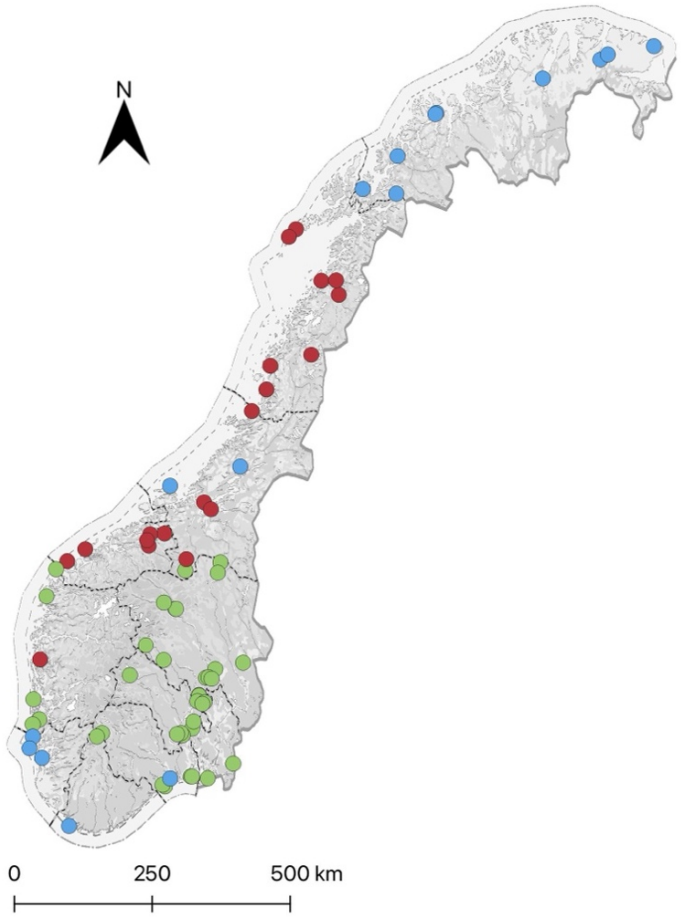


Figure 1: Map showing the 72 sites included in the project with colors representing the year of sampling (red dots represent sites sampled in 2022, green dots represent sites samples in 2023, and blue dots represent sites sampled in 2024).). Topographic background map from the Norwegian Mapping Authority (Nieploch, 2023).

The selected study sites represent semi-natural grasslands of varying size located in different bioclimatic zones and sections (Moen, 1999) across Norway. This ensured coverage of environmental gradients from highly oceanic to slightly continental, boreonemoral to low alpine, and from weakly to strongly calcareous soils. The classification of bioclimatic sections are determined by variations in humidity and seasonal temperature differences, reflecting an oceanic-continental gradient (from west to east), and includes highly oceanic (O3), markedly oceanic (O2), slightly oceanic (O1), indifferent (OC), and slightly continental section (C1) (Moen, 1999). The classification of bioclimatic zones are based on variations in temperature of the growing season and altitude above sea level and reflects an elevational and latitudinal gradient, and includes nemoral (N), boreonemoral (BN), southern boreal (SB), middle boreal (MB), northern boreal (NB), and low, mid and high alpine zone (LA, MA, HA) (Edvardsen et al., 2024; Moen, 1999). The single study site in the nemoral zone (N) was reassigned to boreonemoral (BN) to ensure consistency in the dataset. The boreonemoral zone (BN) thus represent the lowest elevation in this study, and the low alpine zone (LA) represents the highest. Degrees of calcareousness was classified as weakly (2), intermediate (3) and strongly (4) calcareous soils primarily based on site descriptions from *Naturbase*, with adjustments made after field visits based on indicator species from generalized species datasets (Halvorsen, 2015).

## Data collection

Field surveys was conducted in 2022, 2023 and 2024 and involved the systematic collection of fruitbodies and soil samples, as the combination of approaches proved to be beneficial for assessing fungal communities (Frøslev et al., 2019). Miljøfaglig utredning (MFU) was responsible for the fieldwork in most sites, except for nine sites surveyed by the Norwegian Institute for Nature Research (NINA) in 2023. Field assistants and/or landowners often contributed to the fieldwork.

In 2022, nineteen sites across northern, central, and western Norway were surveyed in September and October (Table A1; Fig. 1). These regions were generally warmer and wetter this year compared to the 1991–2020 average (Grinde et al., 2023). However, most sites were surveyed in September which was recorded as a particularly dry month. In 2023, thirty-seven sites were surveyed in eastern and western Norway from late August to mid-October (Table A1). The general trend that year was that eastern Norway experienced very wet conditions, whereas western Norway had more typical to dry conditions (Gangstø et al., 2024). Most sites were surveyed in September which was recorded as a very warm month that year, while sites visited in October faced unusually dry conditions (Gangstø et al., 2024). The year 2024 was recorded as the third warmest and third wettest on record in Norway - and the warmest year ever recorded in northern Norway (Gangstø et al., 2025). This year, sixteen sites were surveyed between late August and late September (Table A1). Most sites experienced warmer and wetter conditions compared to the reference period this year (Gangstø et al., 2025). However, the northernmost sites faced unusually warm and dry conditions, resulting in drought well into autumn (Gangstø et al., 2025).

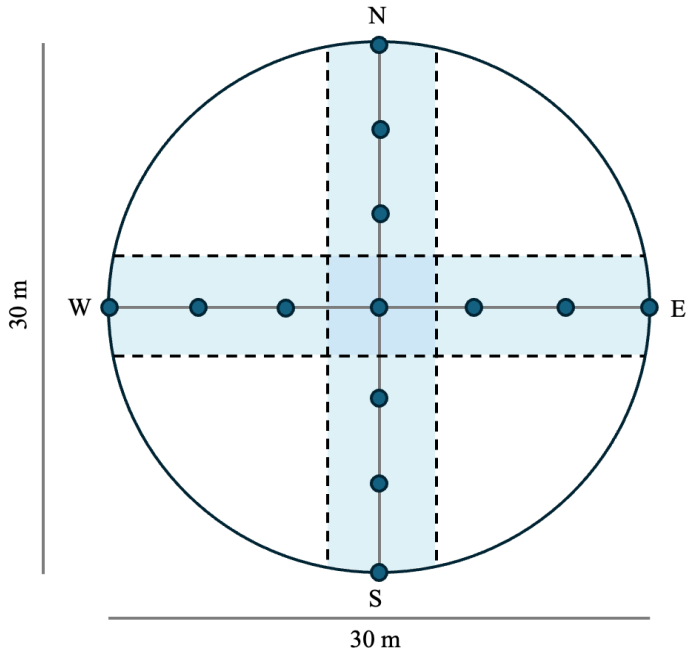


Figure 2: Schematic representation of a soil sampling area. Blue dots illustrate the areas where sub-samples of soil were conducted at five-meter intervals along the transects, while the light blue zone indicates the one-meter belt along the transects where fruitbodies were registered and potentially collected inside the sampling areas.

To capture small-scale variations within each grassland while maximizing the efficiency of data collection, a dual approach combining a grid-based and transect-based method was applied. At each grassland site, sampling areas were established in suitable habitats with potential for high fungal

diversity. Each sampling area consisted of two intersecting transects. The transects were 30 meters long and were laid out in a cross configuration where the initial transect was oriented north to south, with the intersecting transect (oriented west to east) crossing it perpendicular at the midpoint (Fig.2). The number and configuration of sampling areas depended on the site size to prevent oversampling of small grasslands and under-sampling of large grasslands. Sites smaller than 15 decares (da) included one sampling area, while three sampling areas were established in grasslands larger than 100 da. Sites of intermediate size featured two sampling areas. Furthermore, a minimum distance of 15 meters was maintained between sampling areas to ensure a comprehensive coverage.

## Soil sampling and field surveys of fruitbodies

Within the established sampling areas, soil sub-samples were collected every 5 meters along the transects using a soil core sampler with a diameter of 3 cm (Fig.2). Seven sub-samples were collected along the transect in the N-S direction at 0, 5, 10, 15, 20, 25, and 30 meters. Subsequently, three sub-samples were collected 5,10, and 15 meters from the midpoint in the E-W direction. All sub-samples from a site were combined into a single “zip-lock” bag. To ensure soil homogenization, the sample was thoroughly mixed before it was dried for at least 12 hours, depending on the soil structure, at 35 °C. Dried samples were stored frozen after field work until they were transferred to the NINA Center for Biodiversity Genetics (NINAGEN) for DNA analysis by the end of the season (October) each year.

Traditional field surveys of fruitbodies were conducted by searching within a one-meter-wide area on each side of the transects (Fig.2) within the sampling areas. Additionally, a search for fruitbodies was done throughout each site to detect species outside the sampling areas. Species with distinctive macro characteristics were identified in the field, while species difficult to identify were examined under a microscope for accurate identification. A selection of cryptic species and collections of potential interest were dried and transferred to NorBOL, a network of Norwegian researchers and institutions dedicated to DNA barcoding of Norway's biota, for DNA barcoding. Each barcoded collection was assigned an ID, photographed, and accompanied by metadata according to NorBOL standards. The collections were then deposited in the fungarium at the Oslo Natural History Museum (O).

## DNA extraction and DNA metabarcoding of soil samples

DNA extraction and metabarcoding of soil samples were performed at the NINA Center for Biodiversity Genetics (NINAGEN). For each sample, 3-5 grams of soil were transferred into individual 50-mL Garnet Lysing Matrix tubes containing 9.8 mL of PSB buffer (protein solubilization buffer) and 1.2 mL of MT buffer (a lysis buffer). These samples were then homogenized using a FastPrep96 machine (MP Biomedicals, Santa Ana, California, USA) at 4.0 rpm for 40 seconds. Subsequently, 1 mL of supernatant was collected as a subsample and transferred to a 2 mL tube with 250 µL PPS. DNA extraction was performed uniformly across all subsamples using an extraction kit specifically designed for isolating DNA from bacteria, fungi, plants, and animals in soil and other environmental samples (MPBio FastDNA Spin Kit for Soil). Amplification of the internal transcribed spacer 2 (ITS2) region of the ribosomal DNA operon (ITS2 rDNA) was conducted using the fITS7-ITS4 primer set (Ihrmark et al., 2012; White et al., 1990) following a standard Illumina protocol. The initial PCR included primers with overhang adaptor sequences and was followed by a second PCR to add Illumina indices. The first PCR reaction involved

heating at 94 °C for 5 minutes, then 35 cycles at 94 °C for 30 seconds, 56 °C for 30 seconds, and 72 °C for 30 seconds, concluding with a final extension at 72 °C for 7 minutes. The second PCR was conducted according to Illumina's specifications for DNA/RNA DU Indexes. Each PCR product was quality-checked using a 4200 TapeStation machine (Agilent, Santa Clara, California, USA) and purified with magnetic beads (MAG-BIND RXN PURE PLUS). Finally, the samples were normalized and pooled into a library for sequencing on a NovaSeq machine (Illumina, San Diego, California, USA) at the Norwegian Sequencing Centre in Oslo.

## Bioinformatic analyses of environmental DNA sequences

Sequence data were further quality filtered, trimmed, error-corrected, merged, and chimera checked using the cutadapt v3.7 (Martin, 2011) and dada2 v1.9 (Callahan et al., 2016) algorithms. Primers were removed from both the 5' and 3' ends of reads with a minimum length match of 17 base pairs (bp) and 0.15 expected errors in the primer region. We removed sequences from the dataset that contained ambiguous bases, >2 expected errors in the forward or reverse directions, or with length <50 bp after truncation at the first instance of a base with a quality score <10. Error rate models with enforced monotonicity were estimated for forward and reverse sequences and after correction, reads were merged with a minimum overlap of 30 bp. Amplicon sequence variants (ASVs) were inferred for each sample and chimeric sequence variants were assessed on a per-sample basis, as chimeric events occur at the individual PCR level. If a sequence variant was flagged as chimeric in more than 90% of the samples in which it occurred, it was removed. Although the fITS7-ITS4 primer set are designed to preferentially amplify fungal DNA, non-specific amplification of DNA from other organisms occurred to some extent. To verify that the ASVs belonged to fungal species, a BLAST search (Camacho et al., 2009) was conducted of all ASVs against NCBI's "nucleotide nonredundant" database (Sayers et al., 2022). Any ASVs that matched the GenBank database with a similarity >95% and a coverage >80% to an organism outside the fungal kingdom were classified as "off-target" and excluded from the dataset. Finally, we used the IDTAXA algorithm (Murali et al., 2018) and the UNITE database v.10 (Nilsson et al., 2019) to classify all remaining ASVs to the lowest possible taxonomic level receiving >70% confidence and removing any ASVs that could not be assigned to the fungal kingdom at this confidence level.

## DNA barcoding of fruitbodies

DNA extraction and barcoding of fungal fruitbodies were coordinated by NorBol and performed at the Canadian Centre for DNA Barcoding (CCDB), University of Guelph. DNA was extracted using a glass fiber plate-based protocol, where homogenized tissue samples were lysed in a buffer solution containing Proteinase K and incubated at 56 °C (Ivanova et al., n.d). The lysates underwent binding, washing, and elution steps, with purified DNA eluted in prewarmed $ddH_2O$ and stored at -20 °C (Ivanova et al., n.d). PCR amplification targeted the internal transcribed spacer (ITS) region of the ribosomal DNA operon using the ITS1-F (Gardes & Bruns, 1993) and ITS4 primers (White et al., 1990). The PCR thermocycle program is identical to the program used for PCR of soil samples (Kuzmina & Ivanova, n.d). Amplified products were pooled and sequenced using high-throughput platforms, following CCDB's standardized DNA barcoding protocols (Ivanova & Grainger, n.d).

## Data processing: the challenges of CHEGD taxonomy

The taxonomy of CHEGD fungi is under development, meaning that the latest classifications are not yet available in international databases. To ensure the most accurate and updated taxonomy for this study, sequencing data from soil samples and fruitbodies sequenced by NorBol were cross-checked and revised using recent phylogenetic reconstructions created by researcher Bálint Dima, Eötvös Loránd University (ELTE) and researcher Ivona Kautmanová, Slovak Natural History Museum (SNM). These phylogenetic trees include the latest taxonomy of a selection of CHEGD genera which allows for the most recent classification for the taxa included in the reconstructions. Cryptic species (including taxa within unresolved species complexes) and other undescribed taxa are represented in the dataset as operational taxonomic units (OTUs) with unique numbers and/or provisional "species" numbers (sp.). Collections submitted for sequencing in 2024 were not processed in time for this study. As a result, not all sequenced collections were cross-checked with the latest taxonomy. However, the latest taxonomy was used where possible and sequencing data not included in recent phylogenetic reconstructions were classified using the UNITE database v.10 (Nilsson et al., 2019).

To capture a broader diversity of species, all records of fruitbodies from the field surveys (including those recorded both within and outside the sampling areas) as well as previous records of CHEGD fungi from each site were included in this study. The previous records were downloaded from *Artskart* (Artskart, 2022) and incorporated into the dataset as fluctuating climate and weather during the field seasons caused significant variation in fruiting conditions. However, most of the previous records have not been sequenced. As a result, most cryptic species identified through traditional fruitbody surveys lack the latest classification. For the fruitbody data, operational taxonomic units (OTUs) in the phylogenetic reconstructions were included when calculating species richness per site, but cryptic species were assigned to their traditional species units when investigating species composition. For example, *Hygrocybe conica* 2 (sp.45) was renamed as *Hygrocybe conica* (coll.) in analysis of species composition based on fruitbody data. This was done to reduce the differences between sites as the amount of sequenced collections of fruitbodies varied across sites.

Due to the differences in species classification between records from fruitbody surveys and records from eDNA metabarcoding of soil samples, analyses were conducted separately for the two sampling methods. The separation improves reliability of the results and allows for comparison of methods.

## Statistical analyses

The statistical analyses were conducted using R version 4.4.2 (Pile of Leaves) (R Core Team, 2023) within the RStudio integrated development environment, version 2024.12.0+467 (R Core Team, 2023). Data wrangling and preparations were performed using the *readxl* package (Wickham & Bryan, 2025) and various functions from the *tidyverse* package (Wickham, 2023). In the process of statistical analyses, assistance with coding and statistical methods was sought using OpenAI's ChatGPT (GPT-4-turbo), an AI-based language model (OpenAI, 2024). All statistical analyses were carried out separately for data obtained through fruitbody surveys and by eDNA metabarcoding of soil samples.

*Species richness*

To investigate the relationship between species richness and environmental variables, four negative binomial regression models were fitted with species richness as the response variable. For each sampling method, two models were fitted: an additive model (without interactions) and an interaction model (including interaction terms). The additive models included environmental variables (bioclimatic section, bioclimatic zone, and degree of calcareousness) and covariates to account for variation in sampling eff ort. The interaction models included (in addition to the main effects) significant interactions between environmental variables that according to model selection criteria (see section below) improved model fit.

Model selection was conducted by comparing Akaike Information Criterion (AIC) calculated with functions from the base *stats* package in R (R Core Team, 2023). AIC evaluate model fit and complexity by providing a balance to prevent overfitting (Burnham & Anderson, 2004). A difference in AIC (ΔAIC) of ≥ 2 between two models indicated that the model with the lower AIC had the best model fit. Likelihood ratio tests (LRTs) were used to statistically assess the significance of added interaction terms, covariates and random effects. Residual diagnostics were performed using functions from the *DHARMa* package (Hartig, 2024). Interaction terms were included only if LRTs indicated significant model fit improvement ($p < 0.05$), and AIC values ($\Delta AIC \geq 2$) as well as residual diagnostics supported the improvement. Model selection criteria indicated that the interaction models had the best model fit, but due to missing combinations of independent environmental variables in the data (resulting in singularities), the additive models (showing the second-best fit according to model selection criteria) were also retained for comparison and interpretation.

For species richness based on eDNA metabarcoding, the additive model was fitted as a generalized linear mixed-effect model (GLMM) using the *glmer.nb* function from the *lme4* package (Bates et al., 2015). To account for variations in sampling effort, sequencing depth and number of sampling areas established per site were included. The additive eDNA model included the three environmental variables and the log-transformed sequencing depth as fixed effects, while the number of soil sampling areas per site was included as a random effect. The interaction eDNA model was fitted as a generalized linear model (GLM) using the *glm.nb* function from the *MASS* package (Venables & Ripley, 2002) and included three significant interaction terms (calcareousness × section, calcareousness × zone, and section × zone) in addition to the additive effects. Number of sampling areas and sequencing depth was included as fixed effects in the interaction model as it improved model fit according to model selection criteria.

For species richness based on fruitbody surveys, both models (additive and interaction) were fitted as generalized linear models (GLMs) using the *glm.nb* function from the MASS package (Venables & Ripley, 2002). To account for variations in sampling effort, visit frequency (calculated as the number of unique dates per site for the records) was included as fixed effects in both models. The additive fruitbody model included the three environmental variables and visit frequency categorized into four levels: 0-2 (very low), 3-5 (low), 6-15 (medium), >15 (high). The interaction fruitbody model included one interaction (calcareousness × zone ) in addition to the additive effects as this was the only interaction that significantly improved model fit according to model selection criteria. Given the correlation between the number of established sampling areas per site and site size (1 = <15daa, 2 = 15-100daa, 3 = >100daa),

number of sampling areas was tested in the fruitbody models both as a random and a fixed effect. However, due to minimal explanatory value and lack of model improvement, it was ultimately excluded from the final models.

The relationships between environmental variables and species richness were visualized using boxplots of model predictions alongside raw data points using the *ggplot* function (ggplot2 package; Wickham, 2016) with plot layouts arranged using the *patchwork* package (Pedersen, 2024). Plot designs were adjusted using the *RColorBrewer* package (Neuwirth, 2022) and the *extrafont* package (Chang, 2023)

### *Species composition*

Non-metric multidimensional scaling (NMDS) ordination was applied to study relationships between species composition of CHEGD fungi and environmental gradients using the *metaMDS* function (vegan package; Oksanen et al., 2025). The *scores* function was used to extract axes scores for sites and species (vegan package; Oksanen et al., 2025). NMDS ordination was performed separately for the two sampling methods. The fruitbody data was analyzed using species presence-absence, while the eDNA data was analyzed using Hellinger-transformed abundances calculated as follows:

$$Y'_{ij} = \sqrt{\frac{X_{ij}}{\sum X_{ij}}}$$

where $X_{ij}$ is the raw abundance (number of sequences) for each species $j$ in sample $i$, $\sum X_{ij}$ represents sequencing depth (total number of sequences per sample), and $\frac{X_{ij}}{\sum X_{ij}}$ represents proportional abundance.

Relationships between environmental variables and NMDS ordination patterns were assessed using the *envfit* function (with permutations = 999) from the *vegan* package (Oksanen et al., 2025). Results were visualized using the *ggplot* function (ggplot2 package; Wickham, 2016), with species names jittered to avoid overlap using the *ggrepel* package (Slowikowski, 2024), and factor centroids for the environmental variables extracted and displayed as vectors in the ordination space. The *patchwork* package (Pedersen, 2024), the *RColorBrewer* package (Neuwirth, 2022), and the *extrafont* package (Chang, 2023) were used to improve plot layout.

To compare ordination methods, both NMDS and detrended correspondence analysis (DCA) were performed in parallel, following the recommendations of Økland (1996). DCA was conducted using the *decorana* function, while the *scores* function was used to extract axes scores (vegan package; Oksanen et al., 2025). To assess the similarity between the two ordination methods, Kendall´s rank correlation (τ) was used to assess the correlation between NMDS and DCA axes, while Procrustes analyses and Protest were applied to evaluate the overall geometric similarity between the two ordination spaces. Strong correlations ($\tau > 0.65$ and p-value $< 0.001$) and visual examination supported the use of the NMDS analyses.

Canonical correspondence analysis (CCA) was used to test the overall effect of environmental variables on species composition while controlling for variation in sampling effort using the *condition* function. Separate models were fitted for eDNA data and fruitbody data, and model selection was conducted by assessing likelihood ratio tests (LRT). The relative importance of each environmental variable was

evaluated through marginal Permutations tests which assess each variable's effect while keeping the other variables constant. The number of sampling areas (correlating with site size) were included as a covariate in both CCA-models, while sequencing depth (log-transformed) was an additional covariate in the eDNA model, and site visit frequency in the fruitbody model.

Indicator species analysis (ISA) was performed separately for the two sampling methods to identify species associated with the environmental gradients using *multipatt* function from the *indispecies* package (Cáceres et al., 2025). Only species occurring in more than five sites were included in the analysis to enhance reliability of the results. For each environmental variable, a set of indicator species was identified based on the IndVal.g criterion (Dufrêne & Legendre, 1997), which combines the degree to which a species is restricted to a particular group and how consistently it occurs within that group. Permutation tests (with permutations = 999) were used to assess the significance of the indicator values.

### *Environmental variation within species complexes*

To investigate potential ecological variation within species complexes, NMDS ordination analyses were performed on species abundance data obtained through eDNA metabarcoding of soil for three specific species complexes chosen for their high relative abundance: *Hygrocybe conica* coll., *Cuphophyllus virgineus* coll., and *Gliophorus psittiacinus* coll. Only operational taxonomic units (OTUs) occurring in at least five sites were included in the analyses to improve reliability. Further analyses, including NMDS ordinations with environmental fitting (envfit) and DCA ordinations (and tests to evaluate the similarity between ordination methods), and CCA analyses with permutations tests to investigate the effects of environmental variables, were conducted as described for the full dataset.

## Results

### Species richness along environmental gradients

eDNA metabarcoding of soil samples captured higher CHEGD fungal diversity compared to traditional fruitbody surveys with an average of 56 versus 32 species per site (Fig. B1).

Both sampling methods indicated a weak but significant relationship between soil calcareousness and species richness in the additive models, suggesting that higher levels of calcareousness generally support greater CHEGD fungal diversity (Table 1). Compared to weakly calcareous soils, species richness was significantly higher in strongly calcareous soils in the eDNA data (Table 1; eDNA model), and significantly higher in intermediate calcareous soils in the fruitbody data (Table 1; fruitbody model). While the eDNA data showed a gradual increase in species richness with higher calcareousness, the fruitbody data showed a unimodal pattern as species richness peaked at intermediate calcareousness (Fig 3; Table 1).

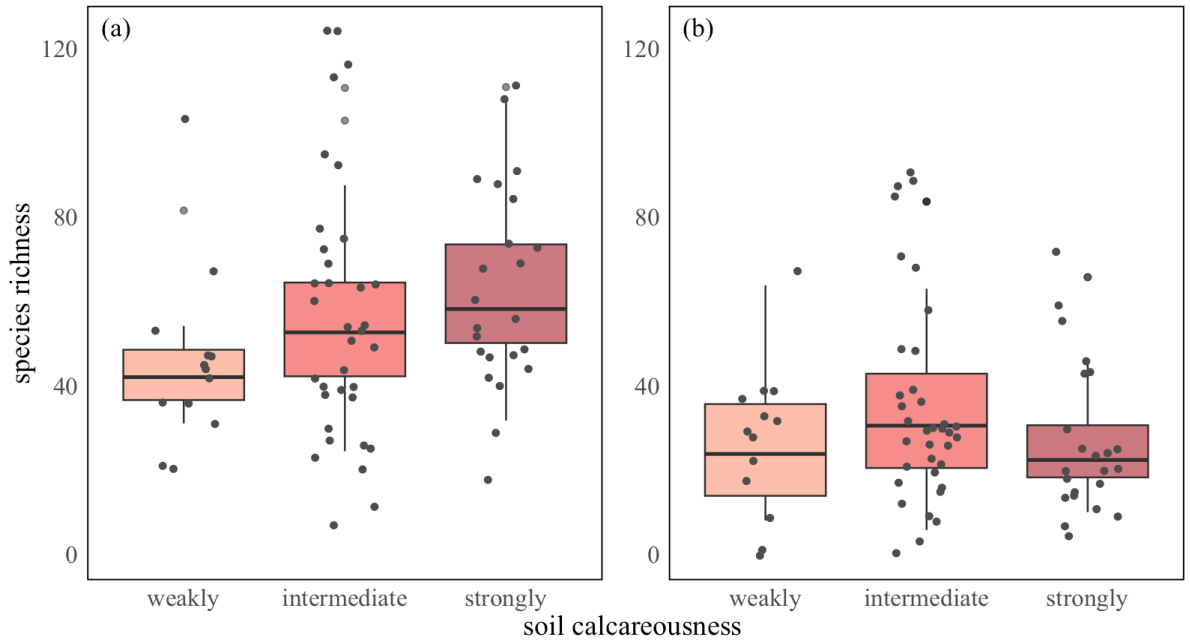


Figure 3: Species richness of CHEGD fungi across three levels of calcareousness representing a gradient from weakly to strongly calcareous soils based on (a) data from eDNA metabarcoding of soil and (b) data from fruitbody surveys. The boxes represent model-predicted interquartile ranges (IQRs) of species richness based on a negative binomial regression models with horizontal lines indicating the mean richness for each level of calcareousness. Individual points show observed species richness for each site.

However, the relationship between calcareousness and species richness was not constant across environmental gradients. The interaction models indicated that the association varied across bioclimatic zones (the elevational/latitudinal gradient) for both sampling methods, while the eDNA interaction model also found variations across bioclimatic sections (the oceanic-continental gradient) (Table B1; Table B2). Species richness in strongly calcareous soils was significantly lower – or showed a non-significant decreasing trend – in the boreal zones (SB, MB, and NB), compared to weakly calcareous soils in the boreonemoral zone (BN) across sampling method (Table B1; Table B2). This pattern, consistent across sampling methods, suggests that the positive association between calcareousness and species richness may decline at higher elevations/latitudes and lower temperatures. Further, the eDNA interaction model found the relationship between intermediate calcareous soils and species richness to be significantly weaker in the slightly oceanic (O1) and indifferent (OC) sections compared to weakly calcareous soils in the highly oceanic section (O3), suggesting that the positive relationship between calcareousness and species richness may decline with lower oceanic influence, and that higher oceanic influence may enhance species richness even in less calcareous soils. (Table B1).

Relationships between bioclimatic sections and species richness were found by both sampling methods, indicating an association between CHEGD diversity patterns and the oceanic-continental gradient (Table 1). Species richness was significantly higher in the slightly oceanic section (O1) compared to the highly oceanic reference section (O3) in the eDNA additive model (Table 1; eDNA model). The eDNA interaction model also found the highest species richness in the slightly oceanic section (O1), supporting a potential diversity peak at intermediate oceanic influence even when interactive effects across environmental gradients are considered (Table B1). In contrast, the fruitbody data showed a significant

reduction in species richness from the highly oceanic section (O3) to the indifferent section (OC) in the additive model (Table 1), and a similar but more progressive decline from highly oceanic (O3) to less oceanic sections (O1, OC) in the interaction model (Table B2).

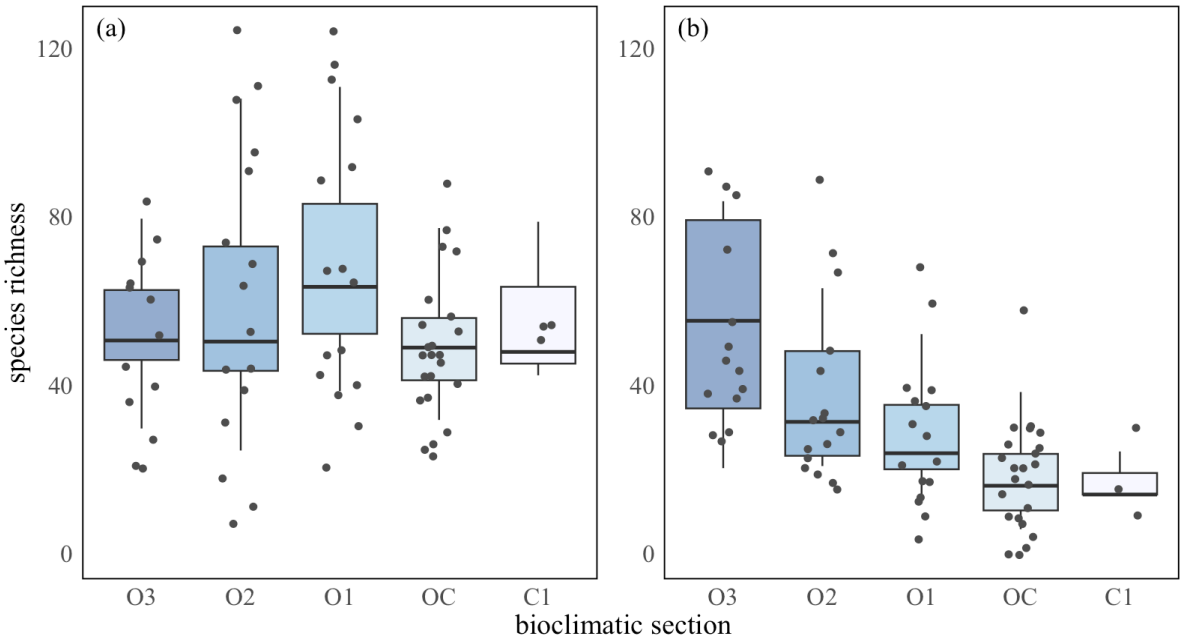


Figure 4: Species richness of CHEGD fungi across bioclimatic sections based on (a) data from eDNA metabarcoding of soil and (b) data from fruitbody surveys. Bioclimatic sections, representing climatic variations in seasonal temperatures and humidity, are ordered from left to right representing an oceanic-continental gradient: highly oceanic (O3), markedly oceanic (O2), slightly oceanic (O1), indifferent (OC), and slightly continental section (C1). The boxes represent model-predicted interquartile ranges (IQRs) of species richness based on two negative binomial regression models with horizontal lines indicating the mean richness in each bioclimatic section. Individual points show observed species richness for each site.

Relationships between bioclimatic zones and species richness were more comparable across sampling methods with the additive models showing a weak, but significant decline in species richness from the boreonemoral (BN) to the low-alpine zone (LA) in the fruitbody data, and a non-significant tendency showing the same trend in the eDNA data (Table 1). This indicates a decline in CHEGD diversity from low to high elevations (and from warmer to colder temperatures). However, interaction models revealed significantly higher species richness at intermediate to high levels along the elevational/latitudinal gradient compared to the boreonemoral reference zone (BN) (Table B1; Table B2). The fruitbody interaction model found significantly higher species richness in the middle boreal zone (MB) (Table B2), and a non-significant tendency showed the same trend in the eDNA interaction model (Table B1). The eDNA interaction model did also reveal a significant increase in species richness in the northern boreal zone (NB) (Table B1), which the high species richness found at high latitudes through eDNA metabarcoding reflects (Fig. B1). Still, the similar relationships observed in both datasets suggests that environmental conditions at intermediate to high elevations/latitudes may be favorable for high CHEGD fungal richness when interactive effects of environmental gradients are accounted for. Furthermore, the combined results from additive and interaction models could suggest a potential unimodal response along this gradient, regardless of sampling method.

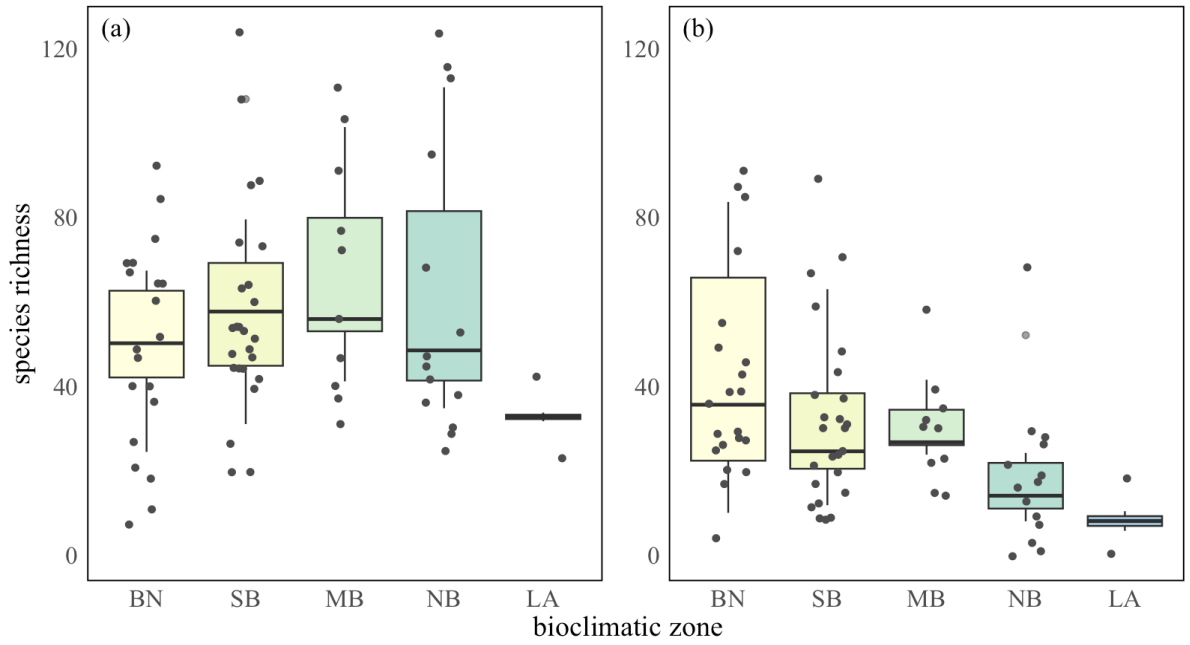


Figure 5: Species richness of CHEGD fungi across bioclimatic zones based on (a) data from eDNA metabarcoding of soil and (b) data from fruitbody surveys. Bioclimatic zones, representing climatic variations in temperature and altitude, are ordered from left to right reflecting a lowland-mountain gradient: boreonemoral (BN), southern boreal (SB), mid-boreal (MB), northern boreal (NB), and low alpine zone (LA). The boxes represent model-predicted interquartile ranges (IQRs) of species richness based on two negative binomial regression models with horizontal lines indicating the mean richness in each bioclimatic zone. Individual points show observed species richness for each site.

eDNA metabarcoding also revealed several significant interactions between bioclimatic zones and sections in relation to species richness of CHEGD fungi (Table B1). Species richness in the southern boreal zone (SB) was significantly higher in the markedly oceanic (O2) and indifferent (OC) sections compared to the boreonemoral–highly oceanic reference combination (BN × O3) (Table B1). This could suggest that species richness of CHEGD fungi may respond positive to decreasing oceanic influence with only a modest increase in elevation. Species richness in the slightly oceanic section (O1) was significantly lower in the boreal zones (SB, MB, NB) compared to the same reference conditions in BN × O3, suggesting that species richness of CHEGD decreases with lower oceanic conditions and increasing elevation and latitude. These results suggest that the effect of oceanic influence on CHEGD richness depends on both the elevational and latitudinal gradient, and that conditions in the slightly oceanic section (O1) may be less favorable at higher elevations/latitudes and lower temperatures.

Table 1: Results from two negative binomial regression models showing estimated effects of calcareousness, bioclimatic zones, and bioclimatic sections on species richness of CHEGD fungi. The eDNA model represent estimates based on data from eDNA metabarcoding, while the fruitbody model is based on data obtained from fruitbody surveys. Estimates represent expected change in species richness for each variable and standard errors (SE) indicate the variability of the estimates. Z-values assess the strength of the effect, while p-values are denoted by stars when significant (*p<0.05, **p <0.01,***p <0.001) and a period (.p <0.1) to highlight non-significant tendencies.

| Variable | Estimate | SE | z value | p value |
|---|---|---|---|---|
| **eDNA model** | | | | |
| (Intercept) | 1.03 | 0.62 | 1.68 | 0.094. |
| Intermediate calcareous soils (calcareousness 3) | 0.06 | 0.13 | 0.48 | 0.634 |
| Strongly calcareous soils (calcareousness 4) | 0.28 | 0.14 | 2.01 | 0.045* |
| Southern boreal zone (SB) | 0.04 | 0.14 | 0.26 | 0.795 |
| Middle boreal zone (MB) | -0.05 | 0.18 | -0.29 | 0.770 |
| Northern boreal zone (NB) | 0.03 | 0.17 | 0.16 | 0.872 |
| Low alpine zone (LA) | -0.65 | 0.34 | -1.91 | 0.057. |
| Markedly oceanic section (O2) | 0.05 | 0.17 | 0.27 | 0.788 |
| Slightly oceanic section (O1) | 0.35 | 0.17 | 2.09 | 0.037* |
| Indifferent section (OC) | 0.37 | 0.22 | 1.73 | 0.084. |
| Slightly continental section (C1) | 0.43 | 0.29 | 1.48 | 0.138 |
| Sequencing depth | 0.24 | 0.05 | 4.45 | <0.001*** |
| **Fruitbody model** | | | | |
| (Intercept) | 2.74 | 0.21 | 13.23 | <0.001*** |
| Intermediate calcareous soils (calcareousness 3) | 0.27 | 0.13 | 2.01 | 0.044* |
| Strongly calcareous soils (calcareousness 4) | 0.03 | 0.15 | 0.20 | 0.841 |
| Southern boreal zone (SB) | -0.07 | 0.14 | -0.55 | 0.582 |
| Middle boreal zone (MB) | 0.16 | 0.18 | 0.92 | 0.356 |
| Northern boreal zone (NB) | -0.19 | 0.18 | -1.09 | 0.278 |
| Low alpine zone (LA) | -0.80 | 0.38 | -2.10 | 0.035* |
| Markedly oceanic section (O2) | -0.21 | 0.16 | -1.32 | 0.186 |
| Slightly oceanic section (O1) | -0.28 | 0.17 | -1.64 | 0.101. |
| Indifferent section (OC) | -0.46 | 0.18 | -2.63 | 0.009** |
| Slightly continental section (C1) | -0.30 | 0.31 | -0.94 | 0.347 |
| Visit frequency (low) | 0.55 | 0.17 | 3.16 | 0.002** |
| Visit frequency (medium) | 0.84 | 0.18 | 4.65 | <0.001*** |
| Visit frequency (high) | 1.42 | 0.19 | 7.40 | <0.001*** |

## Species composition

The NMDS ordinations showed similar overall patterns for both sampling methods with the contrasting environmental conditions along each gradient (e.g. weakly and strongly calcareous soil) having the strongest effect on species composition (Fig. 6a; Fig. 7a; Table C1). CCA analyses found all three environmental gradients to significantly explain variations in species composition (Table 1), suggesting that different levels of calcareousness, the oceanic-continental gradient and the elevational/latitudinal gradient influence species composition of CHEGD fungi regardless of sampling method. Although all environmental gradients contributed to CHEGD community shifts, bioclimatic conditions explained more variation than calcareousness for both fruitbody and eDNA data (Table 1).

Table 1: Results from permutation tests of Constrained Correspondence Analysis (CCA) presenting the marginal effects of explanatory variables based on CHEGD fungal communities. Results are presented separately for two sampling methods: eDNA metabarcoding of soil (left) and fruitbody surveys (right). Columns display the degrees of freedom (Df), ChiSquare, F-values, and p-values with significance level (*p<0.05, **p <0.01,***p <0.001) for bioclimatic sections, bioclimatic zones, and soil calcareousness. For eDNA, sequencing depth and sampling areas were included as covariates (condition terms), while for fruitbody data, visit frequency and sampling areas were included as covariates. The residuals represent unexplained variation.

| | Sampling method | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | eDNA metabarcoding of soil | | | | Fruitbody surveys | | | |
| **Variables** | **Df** | **ChiSquare** | **F** | **p-value** | **Df** | **ChiSquare** | **F** | **p-value** |
| Bioclimatic sections | 4 | 0.97 | 1.17 | 0.001 *** | 4 | 0.36 | 1.13 | 0.016 * |
| Bioclimatic zones | 4 | 0.97 | 1.17 | 0.009 ** | 4 | 0.41 | 1.29 | 0.026 * |
| Soil calcareousness | 2 | 0.47 | 1.12 | 0.012 * | 2 | 0.19 | 1.21 | 0.005 ** |
| Residual | 58 | 12.05 | | | 55 | 4.43 | | |

The NMDS ordination based on fruitbody surveys revealed a taxonomic clustering by CHEGD group with *Entoloma* spp. indicating association with drier, more continental climates, higher elevations /latitudes and calcareous soils (Fig. 7). The indicator species analysis (ISA) identified *Entoloma porphyrogriseum* as a significant indicator species for slightly continental conditions and *E. incanum* for strongly calcareous soil (Table D1; Table D2). Species in the genera *Hygrocybe* s.l, *Clavariaceae*, and *Geoglossaece*/*Microglossum* tended to cluster in the opposite direction compared to *Entoloma* spp., suggesting association with lower elevations, more humid oceanic climates, and weaker calcareousness (Fig. 7). *Clavulinopsis luteoalba* and *Trichoglossum walteri* was found to be significant indicators for oceanic climate, *Hygrocybe reidii* for low levels of calcareousness and *Camarophyllopsis schulzeri* for both oceanic conditions and weakly calcareous soil (Table D1; D2). Species of the genera *Entoloma* and *Hygrocybe* s.l was distributed along the whole calcareousness gradient, suggesting specializations in both weakly and strongly calcareous soils within genera. In the eDNA ordination, taxonomic clustering was less pronounced than suggested by the fruitbody data (Fig. 6). However, many of the same taxa were significantly associated with similar environmental conditions in both fruitbody and eDNA data according to ISA, including the indicator species mentioned above (see Appendix D). For instance, *Pseudotricholoma metapodium* showed a significant association with weakly calcareous soil in both datasets (Table D1).

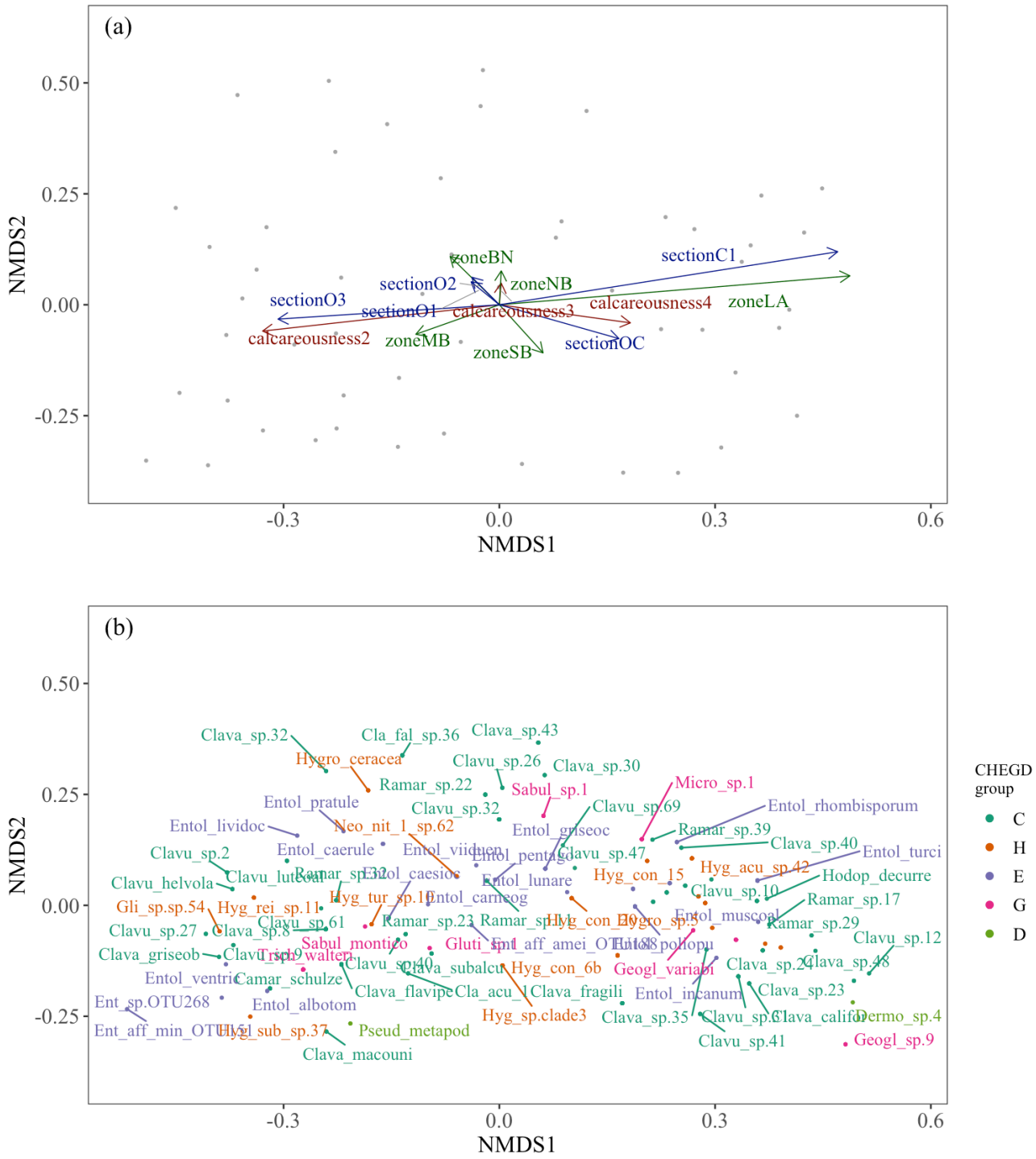


Figure 6: Non-metric multidimensional scaling (NMDS) ordination plot of CHEGD fungal communities based on data from eDNA metabarcoding. (a) NMDS ordination space showing sites as grey points with fitted environmental vectors representing bioclimatic sections (blue), bioclimatic zones (green), and levels of calcareousness (brown). Environmental vectors indicate the direction and strength of the correlation between species composition of CHEGD fungi and the ordination axes (Table C1). (b) NMDS ordination space showing species significantly associated with at least one environmental variable tested by Indicator Species Analyses (see Appendix D), Species labels are colored according to CHEGD-taxa: *Clavariaceae* (C), *Hygrocybe* s.l (H), *Entoloma* (E), *Geoglossaceae/Microglossum* (G), and *Dermoloma/Pseudotricholoma* (D).

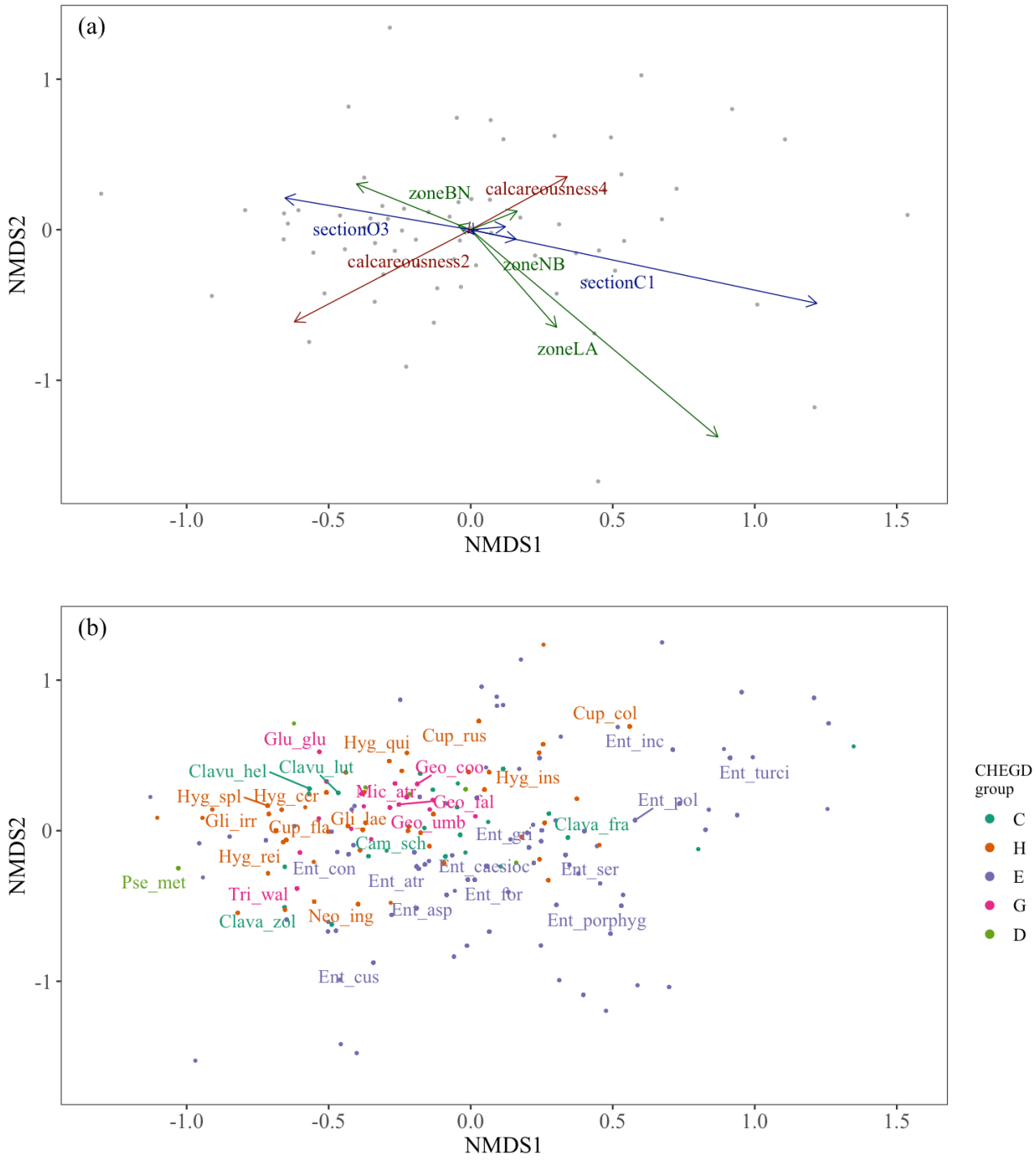


Figure 7: Non-metric multidimensional scaling (NMDS) ordination plot of CHEGD fungal communities based on data from traditional fruitbody surveys. (a) NMDS ordination space showing sites as grey points with fitted environmental vectors representing bioclimatic sections (blue), bioclimatic zones (green), and levels of calcareousness (brown). Environmental vectors indicate the direction and strength of the correlation between species composition of CHEGD fungi and the ordination axes (Table C1). (b) NMDS ordination space showing species significantly associated with at least one environmental variable tested by Indicator Species Analyses (see Appendix D). Species labels are colored according to CHEGD-taxa: *Clavariaceae* (C), *Hygrocybe* s.l (H), *Entoloma* (E), *Geoglossaceae/Microglossum* (G), and *Dermoloma/Pseudotricholoma* (D).

## Ecological variation within species complexes

Separate NMDS ordinations of the species complexes, *Hygrocybe conica* coll., *Cuphophyllus virgineus* coll., and *Gliophorus psittacinus* coll., indicated ecological variations within all three complexes.

Within *Hygrocybe conica* coll. a clear distinction between *H. conica* 2 (sp.45) and other operational taxonomic units (OTUs), particularly *H. conica* 15, was evident along NMDS axis 1, suggesting potential differences in environmental preferences or habitat conditions (Fig. 8). The indicator species analysis (ISA) identified *Hygrocybe conica* 15 as indicator for slightly continental conditions, suggesting potential preference to more oceanic climate for *H. conica* 2 due to indications of opposite distribution patterns in the NMDS ordination space (Table D2; Fig. 8). Similarly, in the NMDS ordination of *Cuphophyllus virgineus* coll., spatial separation, and especially between *C. virgineus* 6 (sp.88) and *C. virgineus* 3 (sp.85), indicated ecological differentiation between OTUs (Fig. 9). *C. virgineus* 4 (sp.86) was identified as an indicator for higher calcareousness than weakly calcareous soil and for slightly continental conditions and showed seemingly different habitat preferences than the other two OTUs mentioned (Table D1; Table D2; Fig. 9). NMDS ordination of *Gliophorus psittacinus coll*. also indicated ecological divergence with *G. psittacinus* 1 (sp.51) and *G. psittacinus* 3 (sp.53) being distinctly separated in the ordination space (Fig. 10). However, none of the OTUs within *G. psittacinus* coll. were identified as indicator species for environmental conditions.

The CCA analyses found no significant relationship between environmental gradients and species composition within the three species complexes (Table E1; Table E2), suggesting that the potential ecological variations are driven by other factors. Few of the OTUs were identified as indicators according to ISA which further supports that other biotic or abiotic factors generated the observed variation within the species complexes (Appendix D).

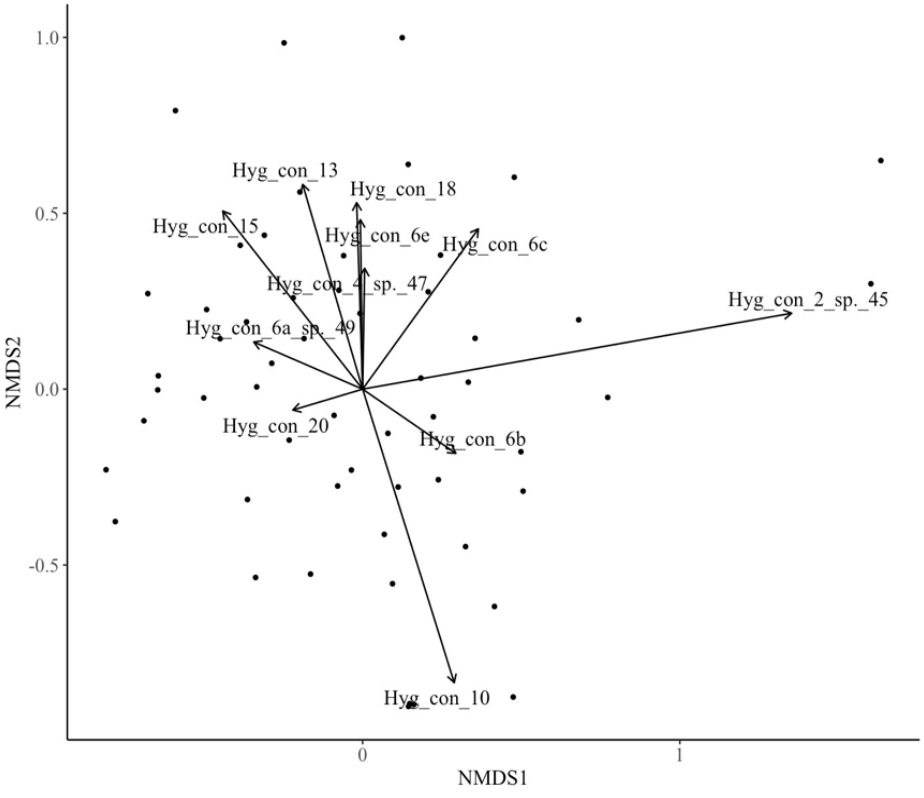


Figure 8: Non-metric multidimensional scaling (NMDS) ordination of sites based on records of the species complex: *Hygrocybe conica* coll., illustrating ecological differentiation among OTUs. Arrows represent fitted vectors showing the direction and strength of association for each OTU across the ordination space. (For distribution of OTUs among *Hygrocybe* s.l, see fig. C4)

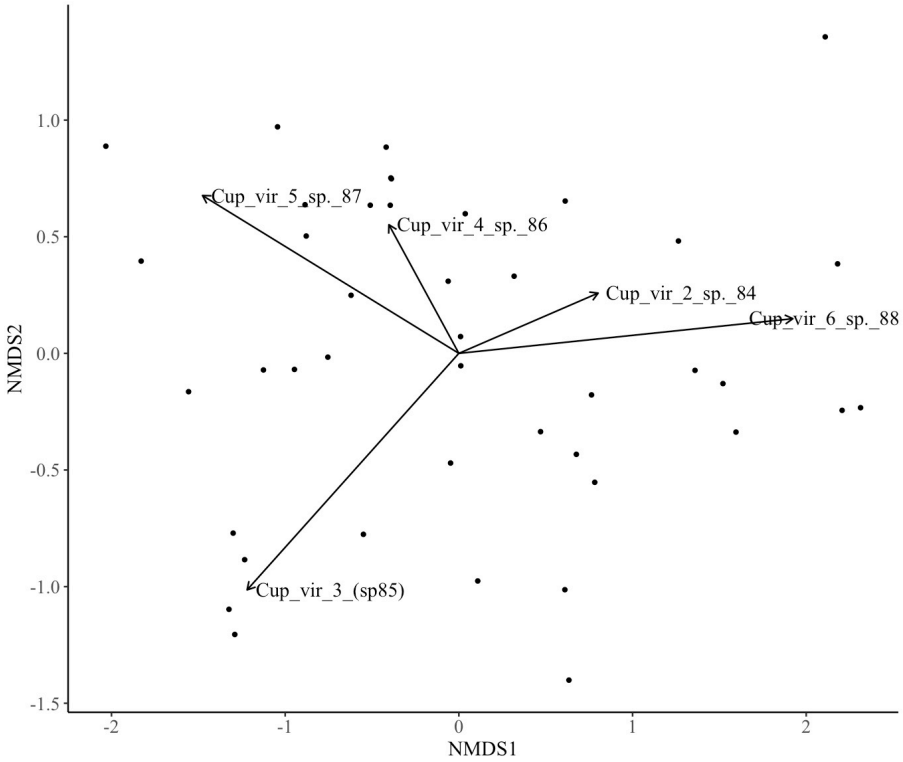


Figure 9: Non-metric multidimensional scaling (NMDS) ordination of sites based on records of the species complex: *Cuphophyllus virgineus* coll., illustrating ecological differentiation among OTUs. Arrows represent fitted vectors showing the direction and strength of association for each OTU across the ordination space. (For distribution of OTUs among *Hygrocybe* s.l, see fig. C4)

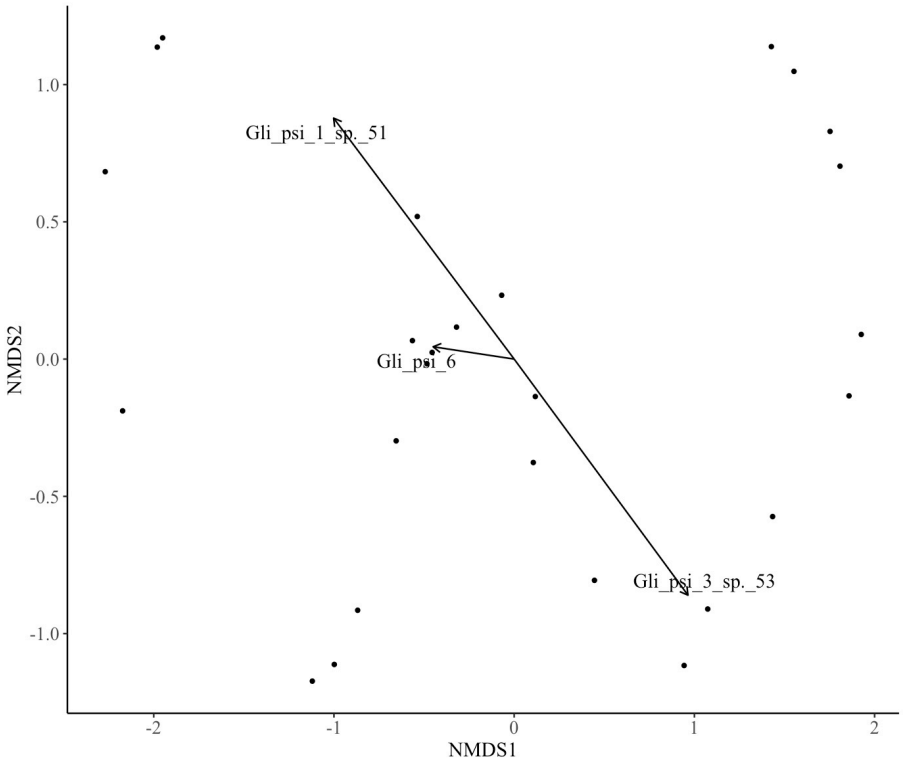


Figure 10: Non-metric multidimensional scaling (NMDS) ordination of sites based on records of the species complex: *Gliophorus psittacinus* coll., illustrating ecological differentiation among OTUs. Arrows represent fitted vectors showing the direction and strength of association for each OTU across the ordination space. (For distribution of OTUs among *Hygrocybe* s.l, see fig. C4)

# Discussion

This study investigated relationships between species richness and composition of CHEGD fungi and three environmental gradients: soil calcareousness, bioclimatic sections (representing an oceanic-continental gradient from west to east), and bioclimatic zones (reflecting an elevational/latitudinal gradient), and compared results from two sampling methods: traditional fruitbody surveys and eDNA metabarcoding of soil. All three environmental gradients were found to significantly influence species richness and species composition of CHEGD fungi across sampling method, and interactions between them indicated that the relationships were context dependent. eDNA metabarcoding also found indications of potential ecological differences within the three species complexes: *Hygrocybe conica* coll., *Cuphophyllus virgineus* coll., and *Gliophorus psittacinus* coll.

## Species richness and species composition along environmental gradients

### *Species richness along the soil calcareousness gradient*

This study found that calcareousness was positively associated with species richness of CHEGD fungi, supporting previous findings linking calcareousness to fungal distribution patterns in semi-natural grasslands (Boertmann, 2010; Brandrud et al., 2023; Griffith et al., 2006; Nitare, 1988; Noordeloos et al., 2022). The positive relationship between calcareousness and CHEGD species richness was found across sampling method, but the pattern of increasing species richness with higher calcareousness was more evident in the eDNA data than in the fruitbody data. The fruitbody data showed an increase in richness at intermediate calcareousness, while the eDNA data showed a clear gradual increase from weakly to strongly calcareous soil. This difference could be a result of methodical differences as calcareous soils correlate with more arid/continental climate and can thus affect fruitbody formation (Bolan et al., 2023). However, the positive relationship between calcareousness and species richness became stronger and more consistent when significant interaction terms were included in the models. This could suggests that the influence of calcareousness is more pronounced when potential interaction effects between edaphic and climatic conditions is accounted for (Tedersoo et al., 2014).

Interactions between calcareousness and bioclimatic gradients indicated that the richness-enhancing effect of higher calcareousness was weakened with colder and drier conditions. This aligns with Tedersoo et al. (2014) finding climate to affect fungal richness indirectly by altering edaphic conditions. However, it remains unclear whether this association reflects ecological patterns or is a consequence of the uneven distribution of study sites as strongly calcareous grasslands were more frequent in the boreonemoral (BN) and southern boreal (SB) zones and declined at higher elevations. Similarly, strongly and intermediate calcareous soils were more common in less oceanic sections, while weakly calcareous soils were more evenly distributed across both bioclimatic gradients. Uneven sampling across gradients, combined with absence of combinations of interactions terms (particularly in the eDNA interaction model) introduces potential uncertainties that call for caution in the interpretations of interaction effects.

The mechanisms behind the relationships between calcareousness and CHEGD fungi remain unresolved. Whether the responses are driven by direct physiological effects of calcium on fungal cellular processes (Jackson & Heath, 1993; Roy et al., 2020) or by indirect effects through soil chemistry, nutrient

availability or biotrophic associations remains unknown (Halbwachs et al., 2018). A hypothesis proposed by Nitare (1988) suggests that the relationship may be linked to low phosphorus availability rather than high calcium levels as phosphorous input has detrimental effects on fungal diversity in grasslands (Caboň et al., 2021; Detheridge et al., 2016). Furthermore, some seemingly calcareous-demanding species are also found in old grasslands where long-term mowing have depleted phosphorus, mimicking the conditions found in naturally phosphorus-limited habitats such as calcareous soils (Nitare, 1988). Tedersoo et al. (2014) found a high nitrogen-phosphorous (N/P) ratio to positively influence the richness of *Geoglossomycetes*, further indicating that phosphorus limitation may play a role in shaping the diversity of CHEGD fungi. This hypothesis could also clarify why some *Hygrocybe* spp. are found to also thrive in rich soils in deciduous forests (Boertmann, 2010; Griffith et al., 2002; Jordal et al., 2016), and why few *Geoglossaceae* species showed associations with the calcareousness gradient in this study. Potential correlations with phosphorous availability could also reflect biotrophic relationships as plants dependency on fungal interactions are regulated by nutrient availability (Gilbert et al., 2009; Halbwachs et al., 2018).

The different levels of calcareousness used in this study is based on the classification system of Nature in Norway (NiN version 2.3) which groups soils in semi-natural grasslands into four levels based on indicator species (Halvorsen et al., 2016), although the lowest level is not represented in this study. The classification reflect underlying variation in base-richness and nutrient availability and is broadly used for mapping of nature in Norway (Halvorsen et al., 2016). However, the classification can introduce uncertainties as it relies on the presence and detection of indicator species and does not capture local spatial heterogeneity. Nevertheless, the significant positive relationships between calcareousness and species richness across sampling methods, found despite these potential uncertainties, underline the relevance of this gradient for assessing fungal communities in semi-natural grasslands.

### *Species richness along the bioclimatic gradient*

Climatic variation from highly oceanic conditions (in the west) to more continental conditions (in the east) played a significant role in shaping CHEGD fungal richness in both fruitbody and eDNA data. The fruitbody data showed a decline in species richness along the gradient from highly oceanic to slightly continental climate, in line with previous findings (Boertmann, 2010; Djemiel et al., 2024). The eDNA data did not find the same linear pattern but showed instead a peak in slightly oceanic areas with less variation across the rest of the gradient. This could suggest that eDNA metabarcoding captures more climate generalists than fruitbody surveys (Djemiel et al., 2024; Frøslev et al., 2019) as generalists tolerate a wider range of conditions while specialists are associated with distinct conditions across environmental gradients. This is not unlikely as eDNA detected higher site richness on average, while the fruitbody data included more threatened species (which often correlates with specializations), aligning with Frøslev et al. (2019). However, variation in the strength and direction of the relationships between sampling methods is likely to reflect methodological differences, as eDNA can detect fungal presence regardless of fruiting, whereas traditional fruitbody surveys rely on fruiting for species detection. Fruiting patterns vary greatly with variations in temperature and precipitation (Eveling et al., 1990; Halbwachs et al., 2018; Krah et al., 2023) and sampling bias is therefore more likely along the oceanic-continental gradient (based on variations in humidity and seasonal differences in temperature) for fruitbody surveys.

While fruitbody surveys are found to be slightly better at detecting threatened species (Frøslev et al., 2019), the detection of fruitbodies may still be more challenging in drier conditions.

The peak in species richness in slightly oceanic sections and the relatively low variance across sections in the eDNA data suggests that intermediate levels of oceanic influence may offer favorable conditions for CHEGD diversity. These findings also highlight the conservation value of drier inland grasslands which may be underestimated in traditional surveys. Barbi et al. (2025) found that root endophytic fungi responded primarily to mean annual temperature and summer precipitation, rather than to climatic seasonality. Given the strong indications of a biotrophic lifestyle in several CHEGD tax (Birkebak et al., 2013; Halbwachs et al., 2018; Seitzman et al., 2010), these findings could help explain the moderate peak observed under slightly oceanic conditions, where temperature variations and seasonal precipitation are less pronounced compared to the extremes of the gradient. However, only three sites were in the most continental section (C1), which may influence the interpretation of the overall gradient effect. Still, the consistency of main effects in both eDNA models (additive and interaction) increase the reliability of the relationship between high species richness and slightly oceanic condition.

Climatic variation from (low elevations in) the south to (higher elevations in) the north also played a significant role in shaping CHEGD fungal richness. The consistent but weak effects in the additive models across sampling methods indicated a weak decline in species richness from the boreonemoral zone (BN) to the low alpine zone (LA) – consistent with the latitudinal/elevational biodiversity gradient and global fungal responses to increasing elevation/latitude (Rahbek, 1995; Tedersoo et al., 2014; Willig et al., 2003). However, different functional groups of fungi have shown different responses to environmental gradients (Barbi et al., 2025; Tedersoo et al., 2014), and interpretation of CHEGD fungal responses to the highest elevation is limited in this study by the low number of sites in the low alpine zone (n = 2). In the interaction models, species richness peaked at intermediate to high elevations/latitudes – specifically in the middle boreal zone (MB) in the fruitbody data and in the northern boreal zone (NB) in the eDNA data. Combined interpretation of significant main effects in both additive and interaction models for both sampling methods thus suggests a potential unimodal response along this gradient, consistent with the intermediate-disturbance hypothesis predicting peak diversity at intermediate environmental stress levels (Menge & Sutherland, 1987). These findings could imply that conditions at intermediate to high levels along this elevational and latitudinal gradient (intermediate to low mean temperatures) in Norway offer ecological niches for a broad range of CHEGD fungi. Griffith et al. (2006) found upland grasslands in Britain to support higher CHEGD diversity than calcareous or mesotrophic lowland grasslands based on fruitbodies, which further supports that intermediate to high elevations/latitudes may provide suitable conditions for these fungi.

Interestingly, eDNA metabarcoding found the highest species richness in semi-natural grasslands in northern Norway which indicate a positive latitudinal trend, aligning with recent findings (Barbi et al., 2025). The bioclimatic zones used in this study are based on temperature variables reflecting a combination of elevational and latitudinal variation which limits the ability to distinguish the relative contribution of the two gradients. Barbi et al. (2025) found species richness of root endophytic fungi to increase with latitude but found no clear trend with elevation. The northern boreal zone (NB) occupies

lowland areas in the northernmost counties (Troms and Finnmark) in Norway (Moen, 1999), and as the most species-rich sites were in the southern (SB), the mid (MB) and the northern boreal (NB) zone in northern Norway, this could indicate that CHEGD fungi respond more strongly to latitude than to elevation. Future studies using separate climate variables and improved representation across elevational zones are needed to clarify these responses. Fruitbody surveys did not reveal the same latitudinal pattern, which may imply that environmental conditions in higher latitudes (and/or elevations) reduce species detection rate through traditional fruitbody surveys as both duration and timing of fruiting is found to decrease with lower temperatures (Krah et al., 2023). This points to a potential underestimation of fungal diversity in semi-natural grasslands in northern Norway. The high species richness recorded through eDNA metabarcoding in northern Norway could imply that many species have yet to be detected further north by traditional fruitbody surveys, and that semi-natural grasslands in this region should be given more attention in future fungal surveys.

The findings of peaking species richness in the middle (MB) and northern boreal (NB) zones, as well as the high richness detected in northern Norway through eDNA metabarcoding, could also reflect unmeasured regional factors rather than climatic factors that favors CHEGD fungal diversity. Different aspects of land-use are shown to considerable impact grassland fungi (Caboň et al., 2021; Detheridge et al., 2016), and much of the middle boreal zone (MB) was historically used for grazing and haymaking in Norway, and the northern boreal zone (NB) cover areas particularly important for traditional summer dairy farming (Moen, 1999). Similarly, the upland vegetation in Britian has been shaped by extensive land-use over thousands of years and have generally been less accessible for agricultural intensification than lowland areas (Averis et al., 2004). The reduced impact of intensive land-use in upland Britain may explain the high fungal richness found in upland grasslands by Griffith et al. (2006) and further support land-use as a plausible underlying factor for fungal diversity patterns in semi-natural grasslands (which is further discussed below).

Underlying factors should also be considered when interpreting interactions between bioclimatic gradients in the eDNA data, suggesting that site-specific environmental conditions influence species richness of CHEGD fungi. Limited representation of several bioclimatic interactions made interpretation of these interactions challenging. Furthermore, the bioclimatic gradients are based on climatic variables which can correlate and thus cause spurious relationships (Byrnes & Dee, 2025). These uncertainties suggest that the interaction effects may not reflect reliable relationships between complex environmental conditions and CHEGD fungal diversity patterns. However, spurious effects could also mask relationships involving unmeasured variables (Byrnes & Dee, 2025). For instance, relationships between the area of a habitat fragment and species richness is well established (MacArthur & Wilson, 1967), and the area of the sites is only indirectly accounted for in this study by the number of sampling areas established – which in the eDNA models mainly reflects the amount of substrate analyzed. Still, uneven sampling across combinations of bioclimatic zones and sections limit the ability to separate interaction effects from sampling bias (Duncan & Kefford, 2021).

To investigate CHEGD fungal responses to climatic variations, this study used the classification of bioclimatic zones and sections by Moen (1999) with some adjustments: the single site in the nemoral (N)

was combined with the boreonemoral (BN) zone, and subsections of the highly oceanic section was merged. The adjustments are supported by later analysis of regional biogeoclimatic variation across Norway which argued for uncertainties around the separation of the nemoral (N) and boreonemoral (BN) zones as well as the separation of strongly oceanic section (O3) into two subtypes (Bakkestuen et al., 2009). Furthermore, the modifications reduced model complexity and ensured consistency with the classification used in the Nature in Norway (NiN) system (Edvardsen et al., 2024), which also represent the framework for the calcareousness gradient applied in this study.

### *Variation in species composition along environmental gradients*

Shifts in species composition of CHEGD fungi was significantly influenced by all three environmental gradients, but bioclimatic conditions explained more of the variation compared to calcareousness, aligning with previous research of fungal responses (Tedersoo et al., 2014). The influence of calcareousness on variation in species composition was stronger for both fruitbody and eDNA data when environmental gradients were assessed individually (using envfit) compared to when the contribution of gradients was assessed simultaneously. These findings could potentially support the interaction effects of calcareousness and bioclimatic gradients on species richness in this study and further highlight the feedback loops between climatic conditions and edaphic factors (Tedersoo et al., 2014).

Bioclimatic zones explained more of the variation in species composition than bioclimatic sections in the fruitbody data, whereas in the eDNA data, both bioclimatic gradients had comparable explanatory strength. The differences between sampling methods suggests that eDNA metabarcoding also detected more climate generalists along the elevational/latitudinal gradient (as previously mentioned in relation to the oceanic-continental gradient), further highlighting methodological differences (Frøslev et al., 2019; Lücking et al., 2020). However, shifts driven by temperature variations along the elevational and latitudinal gradient could also be more prevalent for fruiting patterns than for species occurrences (Krah et al., 2023), but the low number of localities in the low alpine zone (n = 2) limits the reliability of relationships regarding high elevations. This is especially true for the fruitbody data where one of the two sites in the low alpine zone (#79 Persfjorden in Vardø) had no records from traditional surveys due to the unusually dry season in the yar of sampling, and was therefore excluded from the fruitbody ordination analysis.

The significant effect of weakly and strongly calcareous soils shifting species composition in opposite directions, suggest specialization to low or high calcareousness among CHEGD fungi, supported by previous findings (Boertmann, 2010; Brandrud et al., 2023; Griffith et al., 2006; Nitare, 1988; Noordeloos et al., 2022). *Entoloma* showed greatest variation in species preferences along this gradient in the fruitbody data, and *Entoloma incanum* appeared as the clearest indicator species for strongly calcareous soils across sampling methods, suggesting niche specialization. Calciphilous species according to recent taxonomic revisions from the Norwegian *Entoloma* project showed clear associations with the strongly calcareous end of the gradient which further support specialization to different levels of calcareousness among grassland fungi (Fig. C5; Brandrud et al., 2023). Species distribution patterns in relation to the distribution of calciphilous species (Fig. C5) could potentially contribute to future evaluations of habitat preferences of *Entoloma* spp., (including species hypothesis). However, higher calcareousness and

continentality (OC and C1) shifted species composition in similar directions in both ordinations, reflecting the overlap of calcareous soils and more continental climate in Norway.

The NMDS ordination based on fruitbody data showed taxonomic clustering by CHEGD group while there was no obvious clustering of taxa in the NMDS ordination based on eDNA data (Fig. C3-C7). This could indicate that the clustering is associated with variations in fruiting condition preferences across taxa rather than habitat requirements that regulate whether species are present or not. The indications of divergent ecology between taxa are supported by previous studies of CHEGD fungal diversity: Griffith et al. (2006) found variations in microhabitat preferences and fruiting patterns between *Hygrocybe* and *Entoloma* and correlations between species richness of *Hygrocybe* and *Clavariaceae*, while Boertmann (2010) underline that *Hygrocybe* spp. are primarily woodland species outside northwestern Europe as treeless habitats are typically too dry elsewhere. Semi-natural grasslands in oceanic climates therefore serve as suitable habitats (Boertmann, 2010). Still, these previous findings are also based on records of fruitbodies and do not help to unravel the mechanisms behind the observed taxonomic patterns. However, several species showed consistent associations with environmental gradients across sampling methods, increasing the reliability of these relationships. Future ecological studies of CHEGD fungal communities combining traditional fruitbody surveys with eDNA approaches are needed to separate variation of fruiting patterns from other sources of variation.

## Ecological variation in species complexes – a potential tool for future formal descriptions

The species complexes: *Hygrocybe conica* coll., *Cuphophyllus virgineus* coll., and *Gliophorus psittacinus* coll. represent three of the most common taxa in semi-natural grasslands (Boertmann, 2010; Jordal et al., 2016). However, their wide distribution and frequent occurrence is likely to reflect the unresolved delimitations of cryptic species with potential ecological differences that remain unresolved. This study presents strong indications of diverging ecology or habitat preference within all three complexes. Still, the nature of these differences remains unclear as no significant relationships were evident between presence/absence data of the operational taxonomic units (OTUs) and the included environmental gradient. However, non-significant results do not necessarily mean absence of ecological associations. Limited records and potential taxonomic uncertainties in delimitations of taxa can affect the strength of the relationships and obscure underlying patterns. For instance, bioclimatic sections showed the strongest association ($r^2 = 0.019$, $p = 0.111$) for the observed variation within *Cuphophyllus virgineus* coll., suggesting that the oceanic-continental gradient can potentially serve as a relevant factor for further investigation of this species complex.

Nevertheless, some OTUs showed associations to specific environmental gradients in the indicator species analysis (ISA) when all records were included, suggesting potential environmental preferences when assessed together with whole CHEGD communities. *Cuphophyllus virgineus* 4 (sp.86) (probably identical to *C. virgineus* var. *ochraceopallidus*) was associated with intermediate and strongly calcareous soils, aligning with previous habitat descriptions (Boertmann, 2010). *C. virgineus* 4 also showed association with slightly continental climate, together with *Hygrocybe conica* 15. Since strongly calcareous soils occur more frequently in continental climates in Norway – which the distribution of study sites also reflects – these associations could reflect either ecological preferences or geographical

patterns. *Hygrocybe conica* 15 did not show association with calcareousness which could strengthen the indication of greater adaptation to drier climates than other *H. conica´*s. *Cuphophyllus virgineus* 5 (sp.87) was associated with the low alpine zone (LA) but is also found in several lowland sites. With only two study sites representing low alpine conditions, this potential relationship requires further assessment.

Systematic interpretations of OTUs and formal descriptions of new species take time. Taxonomic revisions of *Hygrocybe conica* coll., *Cuphophyllus virgineus* coll., and *Gliophorus psittacinus* coll. (and other species complexes of CHEGD taxa) are incomplete and not yet published. Ecological traits and habitat preferences are an important part of species delimitation and description. The indications of ecological differences between cryptic species in this study may therefore serve as a useful supplement for DNA interpretations and provide valuable insights into the ongoing and future work of formal descriptions. These indications also have great potential for improvement when more sequenced specimens become available.

### Effect of sampling method – "man against machine" or a love story?

eDNA metabarcoding and traditional fruitbody surveys generally revealed similar ecological patterns in CHEGD fungal responses to environmental gradients but differed in overall site richness. eDNA metabarcoding recorded higher species richness per site (mean of 56 versus 32 species), aligning with Frøslev et al. (2019) finding eDNA to capture a broader spectrum of fungal diversity compared to fruitbody surveys. However, the relatively strong correspondence in species richness trends and consistent shifts in species composition across sampling methods in this study suggests that both eDNA metabarcoding and fruitbody surveys have the potential to capture key ecological patterns (Frøslev et al., 2019).

CHEGD fungal diversity is still understudied, resulting in the presence of cryptic and undescribed species which can make species identification challenging without genetic markers (Lücking et al., 2020). eDNA metabarcoding which operates directly with genetic markers could therefore be particularly valuable for capturing the full CHEGD diversity. However, species richness estimates should be interpreted with caution as analysis of eDNA could misinterpret taxonomic units (Lücking et al., 2020). This is especially relevant for lineages with high ITS marker variance (Lücking et al., 2020). For instance, the particularly high number of cryptic OTUs in *Clavariaceae* (Fig. C3) could indicate that the genetic variation within this group is greater than in many other fungal lineages when based on ITS, which call for caution regarding diversity estimates (Lücking et al., 2020). However, previous findings of highly irregular fruiting patterns within *Clavariaceae* (Fadnes, 2023) may also indicate that this group include many taxa that rarely produce fruitbodies. If so, this could potentially support the indications of several undescribed taxa that are currently only known from eDNA. Nevertheless, phenotypic data from collections of fruitbodies is essential to detect potential diagnostic features for species delimitations and formal descriptions (Lücking et al., 2020). Also, the reliability of DNA-based approaches rely on the robustness of reference sequences, and to strengthen reference databases and thus improve the consistency of species identification in the future, voucher specimens are crucial (Lücking et al., 2020). To enhance our understanding of the hidden diversity of CHEGD fungi and to increase the quality of species identifications in the future, both traditional fruitbody surveys and DNA-approaches prove essential.

The combination of molecular and phenotypic data when assessing fungal communities is not only valuable when species classification is incomplete, but also to reduce sampling bias. Data collection by fruitbody surveys is highly influenced by irregular fruiting patterns and climatic variation (Fadnes, 2023; Frøslev et al., 2019; Newton et al., 2003). Fadnes (2023) conducted regular fruitbody surveys in the same semi-natural grassland for 20 years and found that sampling should not only be repeated yearly, but that regular visits throughout the season was important for capturing credible diversity measures. Site richness showed great variance between seasons and new species were detected every year (Fadnes, 2023). These findings underline that sampling bias is likely to occur when fungal diversity measures in grasslands rely solely on fruitbodies. Sampling for eDNA is not limited by irregular fruiting patterns, but rather by sampling coverage as even with an intensive design, soil cores sample only a small fraction of the grassland area (Frøslev et al., 2019). Sampling representation therefore rely on an even distribution of species, and especially rare species with a very limited and/or patchy distribution could easily be missed (Frøslev et al., 2019). Fruitbody surveys allow for more targeted surveys (possibly reflected by better detection of red-listed species), and greater representation of site surface (Frøslev et al., 2019).

While historical records of CHEGD fungi were included in this study, one-third of the study sites had been visited fewer than four times. This is likely to impact ecological interpretation of results as strong significant relationships between higher visit frequency and increasing species richness was evident in the fruitbody models (Fig. B1). This calls for caution in interpretations, especially when results differ between sampling methods as ecological patterns detected through fruitbody records may reflect variations in fruiting requirements rather than true measures of species occurrence.

Nonetheless, the hidden diversity of CHEGD fungi and the incomplete taxonomy highlight the complementary roles of eDNA metabarcoding and traditional fruitbody surveys when assessing fungal communities in grasslands. The high species detection rate relative to the low sampling effort underline the efficiency of eDNA metabarcoding, while fruitbody surveys remain essential to achieve robustness of DNA reference databases, monitor threatened species and formally describe the hidden fungal diversity in grasslands (Frøslev et al., 2019; Lücking et al., 2020). This study shows that both sampling methods have the potential to capture broad ecological patterns of fungal diversity, but the potential of improving accuracy of ecological interpretations increases with more data of both molecular and phenotypic traits. The collaboration between taxonomists and field mycologist is important in future surveys of CHEGD fungi to achieve high quality revisions and increase species knowledge – which is crucial for biodiversity assessments and conservation efforts of semi-natural grasslands.

## Consistency of unexplained variation in species composition

While the environmental gradients included in this study explained significant variation in both species richness and composition, a large proportion of community variation remained unexplained (Table C2). Only 16.6% of variation in eDNA-based species composition and 17.5% in fruitbody-based composition were accounted for by the included environmental gradients. The consistency between sampling methods increases reliability of these results and suggests that approximately 80% of the variation in species composition remains unexplained in this study. Furthermore, the NMDS ordinations of both fruitbody

and eDNA data show clear clustering of species-rich sites (Fig. C2; Fig. C3), suggesting similar environmental conditions that serve as especially favorable for high CHEGD diversity. These indications highlight the importance of other factors in shaping fungal communities in semi-natural grasslands (Ejrnæs & Bruun, 1995).

The large body of evidence supporting a strong association between land-use and biodiversity measures in semi-natural grasslands highlight habitat continuity and management intensity as essential factors for grassland diversity (Barreiro et al., 2022; Boertmann, 2010; Ejrnæs & Bruun, 1995; Griffith et al., 2012; Halbwachs et al., 2018; Johansson et al., 2008; Pornon & Andalo, 2023). Fungal community composition in grasslands are regulated by the continuity of habitats (grassland age) as species accumulate with time, and fungal diversity in old semi-natural grasslands are shown to be distinct from younger ones (Boertmann, 2010; Ejrnæs & Bruun, 1995; Shimono et al., 2024; Öster, 2006). As several archaeological and palaeoecological findings indicate a long history of outfield grazing and summer farming in Norway as well as regional differences in continuity (Bruteig et al., 2003), such variations are likely to play a role in shaping CHEGD communities. However, variation in habitat continuity is not included in this study. Interestingly, while old undisturbed semi-natural grasslands are associated with high fungal diversity, establishment of summer farms in northern Norway (where the most species-rich sites were found) is believed to have happened later than in other parts of the country (Bruteig et al., 2003). However, the dispersal capacity of grassland fungi is found to be relatively efficient (Detheridge et al., 2018), which may have a mitigating effect on the potential lower habitat continuity in northern Norway. Nevertheless, the high species richness of CHEGD fungi detected at high latitudes in this study could also suggest that land-use continuity in the northern Norway have been underestimated or that other factors offset the potential negative effects of low continuity.

Grassland fungi respond negatively to management intensity, and especially their response to nutrient enrichment, soil tillage and grazing/mowing pressure is well studied (Caboň et al., 2021; Ejrnæs & Bruun, 1995; Griffith et al., 2013; Griffith et al., 2012; Halbwachs et al., 2018). Although many of the study sites showed signs of previous fertilization events in varying degrees (personal observation), this factor was not quantified and incorporated in this study. The strong evidence of negative long-term effects of fertilization and soil tillage on fungal diversity in grasslands underlines the importance of accumulating fertilization events for explaining shifts in CHEGD fungal communities (Boertmann, 2010; Griffith et al., 2013; Halbwachs et al., 2018). Previous studies have also found varying relationships between grassland fungi and the intensity of extensive agricultural regimes (Caboň et al., 2021; Ejrnæs & Bruun, 1995; Griffith et al., 2012; Tälle et al., 2016). Grazing pressure or mowing regimes were not investigated in this study, and parts of the unexplained variation is likely to reflect that. The different aspects of land-use are all essential to understand ecological dynamics in semi-natural grasslands but the challenges of defining such factors and the limited knowledge of the past makes them hard to study (Barreiro et al., 2022). However, previous findings in combination with the results of this study highlight the importance of considering edaphic and climatic variations in combination with local land-use factors when assessing fungal diversity in semi-natural grasslands.

The substantial evidence that points to a biotrophic life strategy among CHEGD fungi indicates that vegetation patterns could also have a significant influence on CHEGD fungal communities (Birkebak et al., 2013; Griffith et al., 2014; Halbwachs et al., 2018; Tello et al., 2013). Whether the relationships between environmental gradients and CHEGD fungi in this study also represent indirect effects of biotrophic interactions with plants cannot be certain. However, it is not unlikely given that the gradients used in this study are based on variables strongly linked to vegetation patterns (Halvorsen, 2015; Moen, 1999). Some congruence between fungal diversity and plant diversity, particularly grasses, have been found (Griffith et al., 2014; Öster, 2006). However, findings of high fungal diversity in grasslands with low plant diversity also indicate that these connections are not yet fully understood (Boertmann, 2010; Öster, 2006). Still, just as fungal communities are shown to be strongly influenced by land-use, so are plant communities (Barreiro et al., 2022; Johansson et al., 2008) which makes it challenging to separate potential overlapping effects. The findings of relationships between CHEGD fungi and environmental gradients in this study together with the sum of potential direct and indirect effects discussed, highlight the complexity of semi-natural grassland ecosystems.

## Conclusion

The findings of this study highlight that calcareousness and bioclimatic gradients play significant roles in shaping fungal communities in semi-natural grasslands, both above and below ground. Significant relationships were identified based on gradients reflecting multiple environmental variables, underlining the potential of CHEGD fungi as environmental indicators. However, to enhance interpretation of ecological patterns, future research should separate elevational from latitudinal influences when investigating fungal diversity in grasslands. Furthermore, the relationships found in this study also show great potential of studying the influence of more precise climate and edaphic variables, which would deepen our understanding and improve predictions of how climate change will impact grassland fungi. However, the knowledge gaps regarding fungal diversity in semi-natural grasslands make ecological interpretation more challenging, which highlight the need for both molecular and phenotypic data. Future mycological surveys in grasslands should therefore aim to collect data through both fruitbody surveys and eDNA sampling, and according to this study – semi-natural grasslands in northern Norway show great potential for further investigation of the undescribed diversity of CHEGD fungi.

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# Appendix A – Site information

Table A1: Information about the 72 sites included in the study, arranged in order from north to south. Site ID represent the unique identification code for each site, while Site provides the site name, and Location the respective municipality and county. Sampling date refers to the day data collection occurred, and Sampling are/site size represent the number of sampling areas established, which correlate with the site size (1 = >15daa, 2 = 15-100daa, 3 = <100daa). Environmental variables registered for each site include soil calcareousness(the level of calcareousness in the soil), bioclimatic zone (reflecting the lowland-mountain gradient), and bioclimatic section (reflecting the oceanic-continental gradient).The abbreviations for bioclimatic zones represent: boreonemoral (BN), southern boreal (SB), middle boreal (MB), northern boreal (NB), and low alpine zone (LA). The abbreviations for bioclimatic zones represent: highly oceanic (O3), markedly oceanic (O2), slightly oceanic (O1), indifferent (OC), and slightly continental section (C1). Coordinates provide the respective coordinates for each site (EUREF89 UTM sone 33).

| Site ID | Site | Location | Sampling date | Sampling areas / site size | Soil calcareousness | Bioclimatic zone | Bioclimatic section | Coordinates |
|---|---|---|---|---|---|---|---|---|
| 79 | Persfjorden, Bukkemolltangen | Vardø, Finnmark | 23.08.2024 | 2/15–100daa | intermediate | LA | OC | 1082213 E, 7889529 N |
| 175 | Store Leirpollen | Tana, Finnmark | 22.08.2024 | 1/<15daa | weakly | NB | OC | 997921 E, 7874434 N |
| 223 | Smalfjordsletten | Tana, Finnmark | 22.08.2024 | 1/<15daa | weakly | NB | OC | 984421 E, 7865136 N |
| 151 | Kolvik | Porsanger, Finnmark | 24.08.2024 | 2/15–100daa | strongly | NB | OC | 879148 E, 7830424 N |
| 55 | Hyttefloget - Høystadsletta | Karlsøy, Troms | 28.08.2024 | 3/>100daa | intermediate | NB | O1 | 684848 E, 7767823 N |
| 56 | Nordeidet - Høystadsletta | Karlsøy, Troms | 28.08.2024 | 3/>100daa | intermediate | NB | O1 | 683114 E, 7765481 N |
| 117 | Neveråsen | Senja, Troms | 29.08.2024 | 3/>100daa | weakly | MB | O1 | 614164 E, 7688917 N |
| 154 | Busjhågen | Kvæfjord, Troms | 31.08.2024 | 2/15–100daa | weakly | MB | O1 | 550694 E, 7628824 N |

| Site ID | Site | Location | Sampling date | Sampling areas / site size | Soil calcareousness | Bioclimatic zone | Bioclimatic section | Coordinates |
|---|---|---|---|---|---|---|---|---|
| 119 | Finnesletta | Gratangen, Troms | 30.08.2024 | 3/>100daa | intermediate | NB | O1 | 611857 E, 7620116 N |
| 148 | Skagsanden | Flakstad, Nordland | 08.09.2022 | 3/>100daa | strongly | MB | O2 | 428467 E, 7555295 N |
| 149 | Trolldalen-Helvetestind | Moskenes, Nordland | 08.09.2022 | 3/>100daa | strongly | MB | O2 | 415246 E, 7541429 N |
| 146 | Alvenes | Fauske, Nordland | 07.09.2022 | 1/<15daa | strongly | SB | O1 | 501678 E, 7461792 N |
| 145 | Ytterodden | Bodø, Nordland | 07.09.2022 | 1/<15daa | strongly | SB | O2 | 474692 E, 7461240 N |
| 205 | Ljøneshammersetra | Bodø, Nordland | 06.09.2022 | 2/15–100daa | strongly | NB | O1 | 506514 E, 7435582 N |
| 116 | Leirskaldalen: Fjelldal | Hemnes, Nordland | 06.09.2022 | 2/15–100daa | intermediate | NB | O2 | 456392 E, 7326228 N |
| 93 | Tjøtta: Knausholmen | Alstahaug, Nordland | 05.09.2022 | 3/>100daa | intermediate | SB | O2 | 381617 E, 7305852 N |
| 94 | Kjøllsøya SE | Brønnøy, Nordland | 04.09.2022 | 3/>100daa | strongly | SB | O2 | 374451 E, 7262879 N |
| 1 | Skeisneset, Våttvika | Leka, Trøndelag | 03.09.2022 | 1/<15daa | strongly | SB | O3 | 347880 E, 7223429 N |
| 217 | Hals | Steinkjer, Trøndelag | 09.09.2024 | 2/15–100daa | weakly | MB | O2 | 326492 E, 7121946 N |
| 110 | Nordstaulvika 1 | Frøya, Trøndelag | 20.09.2024 | 2/15–100daa | intermediate | SB | O3 | 198610 E, 7086803 N |
| 121 | Fagerli | Indre Fosen, Trøndelag | 02.09.2022 | 1/<15daa | weakly | SB | O2 | 260458 E, 7056930 N |

| Site ID | Site | Location | Sampling date | Sampling areas / site size | Soil calcareousness | Bioclimatic zone | Bioclimatic section | Coordinates |
|---|---|---|---|---|---|---|---|---|
| 222 | Østmarka sykehus | Trondheim, Trøndelag | 25.10.2022 | 1/<15daa | intermediate | SB | O1 | 273116 E, 7044089 N |
| 221 | Mogstad Austistua: Stortrøa | Surnadal, Møre og Romsdal | 01.10.2022 | 1/<15daa | intermediate | SB | O2 | 188303 E, 6999858 N |
| 89 | Aksneset | Tingvoll, Møre og Romsdal | 29.09.2022 | 1/<15daa | intermediate | SB | O2 | 162143 E, 6998370 N |
| 90 | Solvang | Tingvoll, Møre og Romsdal | 29.09.2022 | 2/15–100daa | intermediate | SB | O2 | 156260 E, 6987840 N |
| 87 | Jordalsøra | Sunndal, Møre og Romsdal | 30.09.2022 | 1/<15daa | weakly | SB | O2 | 159521 E, 6977504 N |
| 88 | Molnes E | Giske, Møre og Romsdal | 27.09.2022 | 2/15–100daa | intermediate | BN | O3 | 43516 E, 6971221 N |
| 8 | Slettvoll W | Oppdal, Trøndelag | 22.09.2022 | 1/<15daa | intermediate | MB | OC | 228129 E, 6952874 N |
| 199 | Nerlandsøya: Mulevika | Herøy, Møre og Romsdal | 26.09.2022 | 2/15–100daa | intermediate | BN | O3t | 10725 E, 6949316 N |
| 213 | Vangrøftdalen: Kløftåsen | Os, Innlandet | 24.08.2023 | 1/<15daa | weakly | NB | OC | 290412 E, 6947171 N |
| 109 | Honningsvågen: Stranda | Stad, Vestland | 26.09.2023 | 2/15–100daa | weakly | BN | O3t | -9688 E, 6934511 N |
| 97 | Ryphuslia | Oppdal, Trøndelag | 23.08.2023 | 3/>100daa | strongly | LA | OC | 226265 E, 6932492 N |
| 214 | Vingill (Vingelen) | Tolga, Innlandet | 24.08.2023 | 1/<15daa | weakly | NB | OC | 285751 E, 6928230 N |
| 108 | Nordbotnen | Bremanger, Vestland | 27.09.2023 | 3/>100daa | weakly | SB | O3 | -27167 E, 6885245 N |

| Site ID | Site | Location | Sampling date | Sampling areas / site size | Soil calcareousness | Bioclimatic zone | Bioclimatic section | Coordinates |
|---|---|---|---|---|---|---|---|---|
| 33 | Helle-Fellese-Lye | Vågå, Innlandet | 01.09.2023 | 3/>100daa | intermediate | SB | C1 | 187348 E, 6873875 N |
| 24 | Granrud | Sel, Innlandet | 20.09.2023 | 1/<15daa | intermediate | SB | C1 | 208419 E, 6862457 N |
| 27 | Josten N | Sel, Innlandet | 20.09.2023 | 1/<15daa | intermediate | SB | C1 | 209424 E, 6862201 N |
| 218 | Sparstad: Nørre Heimebeitet | Vang, Innlandet | 08.09.2023 | 2/15–100daa | intermediate | MB | OC | 154125 E, 6796013 N |
| 91 | Lygra | Alver, Vestland | 04.10.2022 | 2/15–100daa | weakly | BN | O3 | -39030 E, 6769667 N |
| 227 | Grønstølen | Nord-Aurdal, Innlandet | 08.09.2023 | 1/<15daa | intermediate | NB | OC | 186922 E, 6768731 N |
| 220 | Kassiberget I | Elverum, Innlandet | 09.09.2023 | 1/<15daa | intermediate | MB | OC | 331753 E, 6763815 N |
| 136 | Stor-Ile SW | Ringsaker, Innlandet | 07.09.2023 | 1/<15daa | strongly | BN | OC | 280941 E, 6752330 N |
| 219 | Anfinnset | Hol, Buskerud | 29.08.2023 | 1/<15daa | intermediate | NB | OC | 124966 E, 6741232 N |
| 123 | Steinberg NE | Vestre Toten, Innlandet | 06.09.2023 | 1/<15daa | strongly | SB | OC | 268375 E, 6737356 N |
| 30 | Finstad | Vestre Toten, Innlandet | 14.09.2023 | 1/<15daa | intermediate | SB | OC | 264000 E, 6736438 N |
| 126 | Gile, Smiubakken | Østre Toten, Innlandet | 07.09.2023 | 1/<15daa | strongly | SB | OC | 274277 E, 6734720 N |
| 224 | Grindaker | Gran, Innlandet | 25.08.2023 | 1/<15daa | intermediate | SB | OC | 251330 E, 6704750 N |

| Site ID | Site | Location | Sampling date | Sampling areas / site size | Soil calcareousness | Bioclimatic zone | Bioclimatic section | Coordinates |
|---|---|---|---|---|---|---|---|---|
| 225 | Tingelstad kirke | Gran, Innlandet | 25.08.2023 | 1/<15daa | intermediate | SB | OC | 251350 E, 6703825 N |
| 226 | Litlakalsøy | Austevoll, Vestland | 07.10.2023 | 2/15–100daa | strongly | BN | O3t | -51129 E, 6697202 N |
| 84 | Rustad øvre | Jevnaker, Innlandet | 17.10.2023 | 1/<15daa | strongly | SB | OC | 248340 E, 6693852 N |
| 82 | Amundrud | Lunner, Innlandet | 25.08.2023 | 1/<15daa | strongly | SB | OC | 261341 E, 6692010 N |
| 83 | Kjørvensætra | Lunner, Innlandet | 17.10.2023 | 1/<15daa | strongly | MB | OC | 257545 E, 6688847 N |
| 190 | Spyssøya, Myra | Bømlo, Vestland | 30.09.2023 | 2/15–100daa | intermediate | BN | O3t | -40403 E, 6660528 N |
| 157 | Skogen | Bærum, Akershus | 11.09.2023 | 2/15–100daa | intermediate | MB | O1 | 241506 E, 6656526 N |
| 104 | Vestre Vika | Bømlo, Vestland | 30.09.2023 | 2/15–100daa | strongly | BN | O3t | -52583 E, 6651579 N |
| 156 | Solli | Asker, Akershus | 11.09.2023 | 2/15–100daa | intermediate | SB | O1 | 239136 E, 6643959 N |
| 169 | Brekkebråtan | Drammen, Buskerud | 13.09.2023 | 1/<15daa | strongly | BN | OC | 218375 E, 6635769 N |
| 159 | Bjåen W | Bykle, Agder | 30.08.2023 | 1/<15daa | intermediate | NB | O1 | 74151 E, 6635642 N |
| 170 | Halshaugen | Øvre Eiker, Buskerud | 13.09.2023 | 1/<15daa | strongly | BN | OC | 211466 E, 6633733 N |
| 139 | Ryghsetra | Drammen, Buskerud | 13.09.2023 | 2/15–100daa | strongly | SB | O1 | 221670 E, 6632125 N |

| Site ID | Site | Location | Sampling date | Sampling areas / site size | Soil calcareousness | Bioclimatic zone | Bioclimatic section | Coordinates |
|---|---|---|---|---|---|---|---|---|
| 161 | Breive W | Bykle, Agder | 30.08.2023 | 1/<15daa | intermediate | NB | O1 | 64943 E, 6629402 N |
| 228 | Kvalsvik N | Haugesund, Rogaland | 29.09.2024 | 2/15–100daa | strongly | BN | O3t | -52306 E, 6629263 N |
| 144 | Åkra: Nedre Liknes | Karmøy, Rogaland | 26.09.2024 | 3/>100daa | intermediate | BN | O3t | -58539 E, 6607935 N |
| 143 | Knott | Rennesøy, Rogaland | 27.09.2024 | 2/15–100daa | intermediate | BN | O3t | -35449 E, 6590091 N |
| 140 | Bøensætre | Aremark, Østfold | 10.09.2023 | 1/<15daa | weakly | BN | O1 | 313638 E, 6579727 N |
| 72 | Moutmarka: Skjælva E | Tjøme, Vestfold | 12.09.2023 | 2/15–100daa | intermediate | BN | O1 | 236317 E, 6556409 N |
| 74 | Sønstegård W | Tjøme, Vestfold | 12.09.2023 | 2/15–100daa | intermediate | BN | O1 | 238574 E, 6556265 N |
| 216 | Skipstadkilen N | Hvaler, Østfold | 10.09.2023 | 2/15–100daa | strongly | BN | O2 | 267184 E, 6552913 N |
| 1072 | Langøya NW | Bamble, Telemark | 03.10.2024 | 1/<15daa | strongly | BN | O2 | 198814 E, 6552494 N |
| 12 | Gumøy E | Kragerø, Telemark | 16.10.2023 | 1/<15daa | intermediate | BN | O2 | 184940 E, 6541089 N |
| 81 | Jomfruland: Saltstein W | Kragerø, Telemark | 15.10.2023 | 1/<15daa | intermediate | BN | O2 | 189183 E, 6538101 N |
| 60 | Haugestranda | Farsund, Agder | 28.09.2024 | 3/>100daa | intermediate | N | O3 | 14017 E, 6466022 N |

# Appendix B – Supplementary statistics and figures, species richness

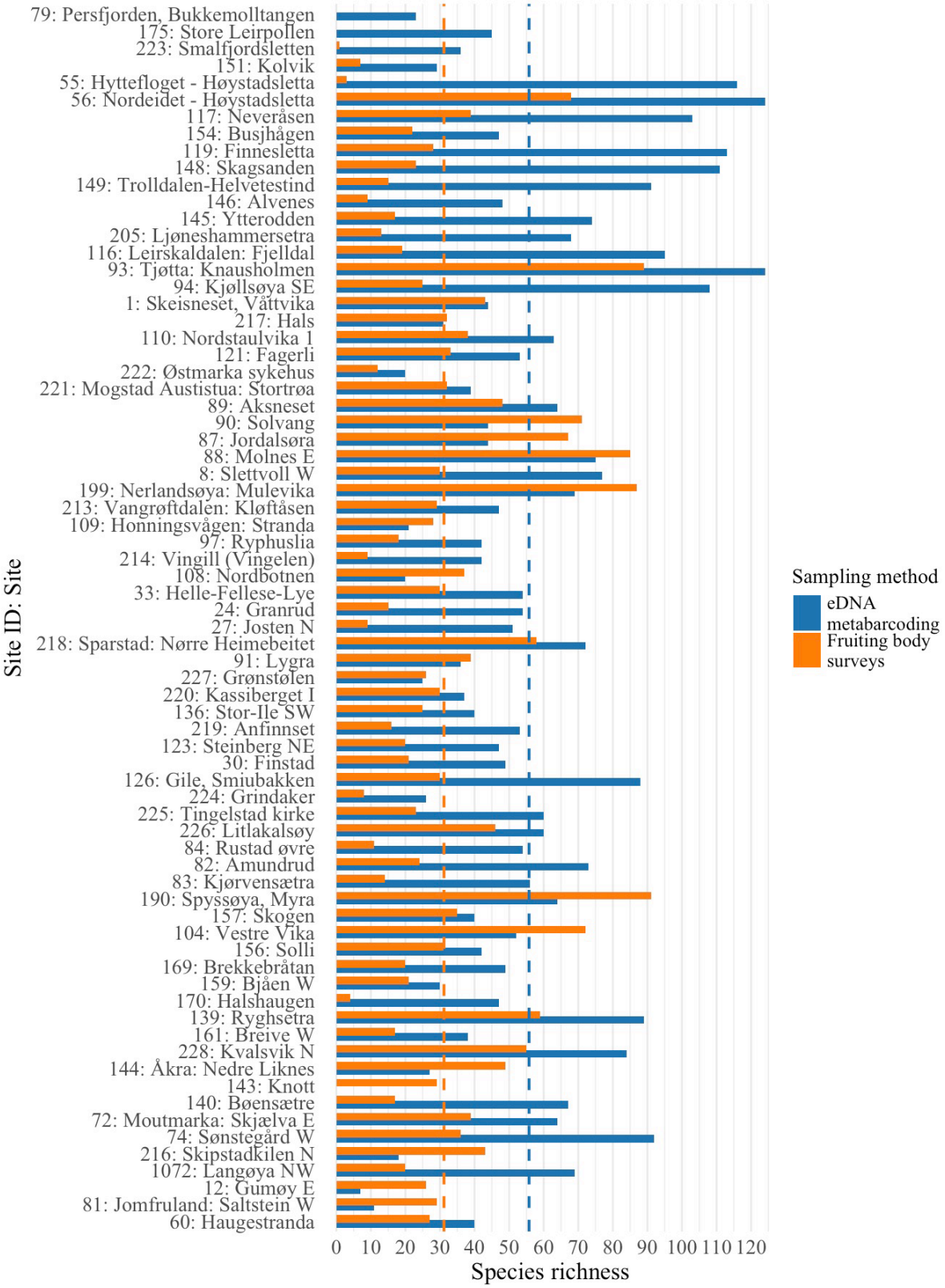



Figure B2: Barplot showing species richness recorded in each site grouped by sampling method: eDNA metabarcoding of soil (blue bars) and fruitbody surveys (orange bars). Study sites are arranged in order from north to south. Dashed lines represent average species richness recorded by each sampling method.

Table B1: Results from a negative binomial generalized linear mixed model based on eDNA data showing estimated effects of bioclimatic zones, bioclimatic sections, calcareousness, interaction terms, sequencing depth and sampling area on species richness of CHEGD fungi. Estimates represent expected change in species richness for each variable and standard errors (SE) indicate the variability of the estimates. Z-values assess the strength of the effect, while p-values are denoted by stars when significant (* p <0.05,** p <0.01,*** p<0.001) and a period (.p <0.1) to highlight non-significant tendencies. Missing or limited combinations of environmental variables in the data are not included in the table.

| **Term** | **Estimate** | **SE** | **z value** | **p value** |
|---|---|---|---|---|
| (Intercept) | 0.56 | 0.56 | 1.00 | 0.316 |
| Intermediate calcareous soils (calcareousness 3) | 0.59 | 0.23 | 2.57 | 0.010* |
| Strongly calcareous soils (calcareousness 4) | 0.94 | 0.24 | 3.85 | <0.001*** |
| Southern boreal zone (SB) | 0.08 | 0.34 | 0.23 | 0.822 |
| Middle boreal zone (MB) | 0.82 | 0.49 | 1.68 | 0.093. |
| Northern boreal zone (NB) | 0.92 | 0.32 | 2.92 | 0.003** |
| Low alpine zone (LA) | -0.30 | 0.34 | -0.89 | 0.374 |
| Markedly oceanic section (O2) | -0.59 | 0.46 | -1.28 | 0.199 |
| Slightly oceanic section (O1) | 1.42 | 0.30 | 4.71 | <0.001*** |
| Indifferent section (OC) | -0.04 | 0.22 | -0.19 | 0.853 |
| Slightly continental section (C1) | 0.28 | 0.28 | 1.02 | 0.306 |
| Sequencing depth | 0.19 | 0.05 | 4.15 | <0.001*** |
| Sampling areas | 0.28 | 0.06 | 4.54 | <0.001*** |
| Calcareousness 3 × SB | -0.02 | 0.39 | -0.06 | 0.951 |
| Calcareousness 4 × SB | -0.84 | 0.45 | -1.84 | 0.065. |
| Calcareousness 3 × MB | 0.30 | 0.38 | 0.80 | 0.422 |
| Calcareousness 4 × MB | -0.54 | 0.47 | -1.14 | 0.254 |
| Calcareousness 3 × NB | 0.19 | 0.43 | 0.45 | 0.655 |
| Calcareousness 4 × NB | -1.55 | 0.37 | -4.15 | <0.001*** |
| Calcareousness 3 × O2 | -0.42 | 0.42 | -1.00 | 0.316 |
| Calcareousness 4 × O2 | 0.26 | 0.45 | 0.59 | 0.557 |
| Calcareousness 3 × O1 | -1.02 | 0.34 | -2.97 | 0.003** |
| Calcareousness 4 × O1 | 0.29 | 0.47 | 0.61 | 0.545 |
| Calcareousness 3 × OC | -0.82 | 0.33 | -2.44 | 0.015* |
| SB × O2 | 1.13 | 0.29 | 3.86 | <0.001*** |
| MB × O2 | -0.09 | 0.38 | -0.24 | 0.812 |
| NB × O2 | 0.33 | 0.48 | 0.68 | 0.494 |
| SB × O1 | -0.80 | 0.33 | -2.43 | 0.015* |
| MB × O1 | -1.50 | 0.45 | -3.36 | <0.001*** |
| NB × O1 | -1.21 | 0.44 | -2.75 | 0.006** |
| SB × OC | 1.13 | 0.34 | 3.28 | 0.001** |

Table B2: Results from a negative binomial generalized linear model based on fruitbody data showing estimated effects of bioclimatic zones, bioclimatic sections, calcareousness, interaction terms and visit frequency on species richness of CHEGD fungi. Estimates represent expected change in species richness for each variable and standard errors (SE) indicate the variability of the estimates. Z-values assess the strength of the effect, while p-values are denoted by stars when significant (*p <0.05,**p <0.01,***p <0.001) and a period (.p<0.1) to highlight non-significant tendencies. Missing or limited combinations of environmental variables in the data are not included in the table.

| Term | Estimate | SE | z value | p value |
|---|---|---|---|---|
| (Intercept) | 2.48 | 0.26 | 9.70 | <0.001*** |
| Intermediate calcareous soils (calcareousness 3) | 0.53 | 0.22 | 2.36 | 0.018* |
| Strongly calcareous soils (calcareousness 4) | 0.51 | 0.24 | 2.15 | 0.031* |
| Southern boreal zone (SB) | 0.40 | 0.28 | 1.39 | 0.164 |
| Middle boreal zone (MB) | 0.63 | 0.29 | 2.16 | 0.031* |
| Northern boreal zone (NB) | 0.05 | 0.33 | 0.14 | 0.890 |
| Low alpine zone (LA) | -0.36 | 0.40 | -0.90 | 0.367 |
| Markedly oceanic section (O2) | -0.24 | 0.14 | -1.71 | 0.087. |
| Slightly oceanic section (O1) | -0.32 | 0.15 | -2.12 | 0.034* |
| Indifferent section (OC) | -0.51 | 0.16 | -3.17 | 0.002** |
| Slightly continental section (C1) | -0.33 | 0.28 | -1.18 | 0.236 |
| Calcareousness 3 × SB | -0.42 | 0.31 | -1.35 | 0.176 |
| Calcareousness 4 × SB | -0.66 | 0.33 | -1.98 | 0.048* |
| Calcareousness 3 × MB | -0.24 | 0.35 | -0.70 | 0.484 |
| Calcareousness 4 × MB | -1.06 | 0.37 | -2.87 | 0.004** |
| Calcareousness 3 × NB | -0.07 | 0.35 | -0.20 | 0.842 |
| Calcareousness 4 × NB | -0.88 | 0.47 | -1.89 | 0.058. |
| Visit frequency (low) | 0.55 | 0.16 | 3.44 | <0.001*** |
| Visit frequency (medium) | 0.77 | 0.17 | 4.56 | <0.001*** |
| Visit frequency (high) | 1.37 | 0.17 | 7.85 | <0.001*** |

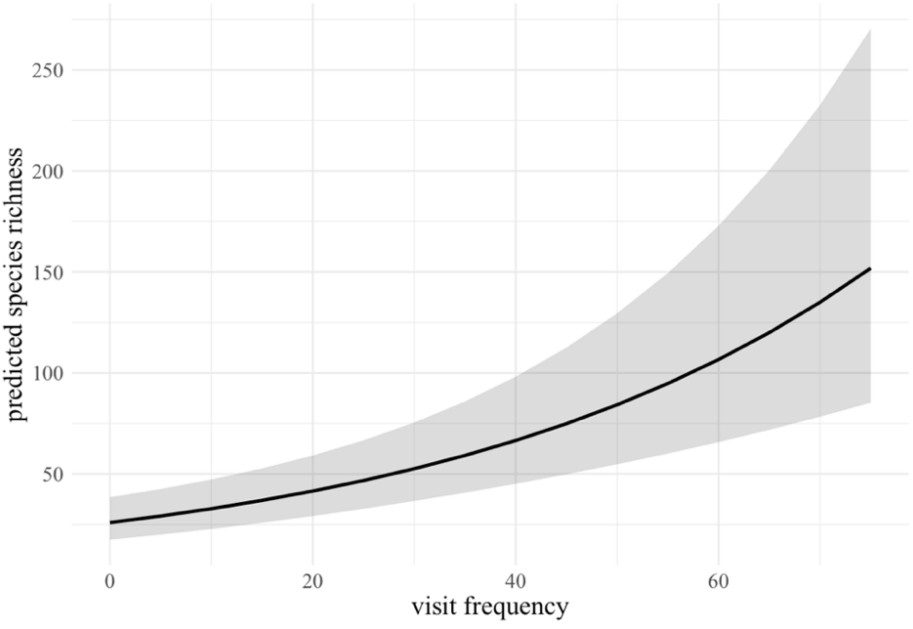



Figure B1: Predicted species richness of CHEGD fungi with increasing visit frequency (number of fruitbody surveys) based on a negative binomial regression model including calcareousness, bioclimatic sections and zones as predictors. The black line shows species richness estimates while the shaded area represents the 95% confidence interval.

# Appendix C – Supplementary statistics and figures, species composition

Table C1: Results from environmental fitting analysis (envfit) showing the relationships between environmental variables and species composition of CHEGD fungi in the NMDS ordination analyses based on eDNA metabarcoding of soils and fruitbody surveys. The analysis was conducted separately for each sampling method. The $r^2$ values indicate the proportion of variation in species composition explained by each variable (goodness of fit). *Non-significant tendencies are presented with a period (.p<0.1) while stars reflect the strength of significant effects (*p<0.05,**p<0.01,***p<0.001).*

| | Sampling method | | | |
|---|---|---|---|---|
| | eDNA metabarcoding of soils | | Fruitbody surveys | |
| **Variables** | **r2** | **p-value** | **r2** | **p-value** |
| Bioclimatic section | 0.1824 | 0.001** | 0.0919 | 0.130 |
| Bioclimatic zone | 0.0930 | 0.102 | 0.1141 | 0.058 . |
| Calcareousness | 0.1489 | 0.001*** | 0.1062 | 0.002** |
| Sampling areas | 0.0044 | 0.867 | 0.0085 | 0.780 |
| Sequencing depth | 0.0045 | 0.835 | | |
| Visits | | | 0.0831 | 0.060 . |

Table C2: Explained variation from Constrained Correspondence Analysis (CCA) models for CHEGD fungal communities detected through eDNA metabarcoding and fruitbody surveys. Total inertia represents total variation in the community data, the constrained inertia represent variation explained by environmental variables (calcareousness, bioclimatic zones and bioclimatic sections) when accounting for sampling effort, and the explained variation shows the percentage of total variation explained by the environmental variables.

| **Sampling method** | **Total inertia** | **Constrained inertia** | **Explained variation (%)** |
|---|---|---|---|
| eDNA metabarcoding of soil | 15.08 | 2.5 | 16.6 |
| Fruitbody surveys | 5.85 | 1.02 | 17.5 |

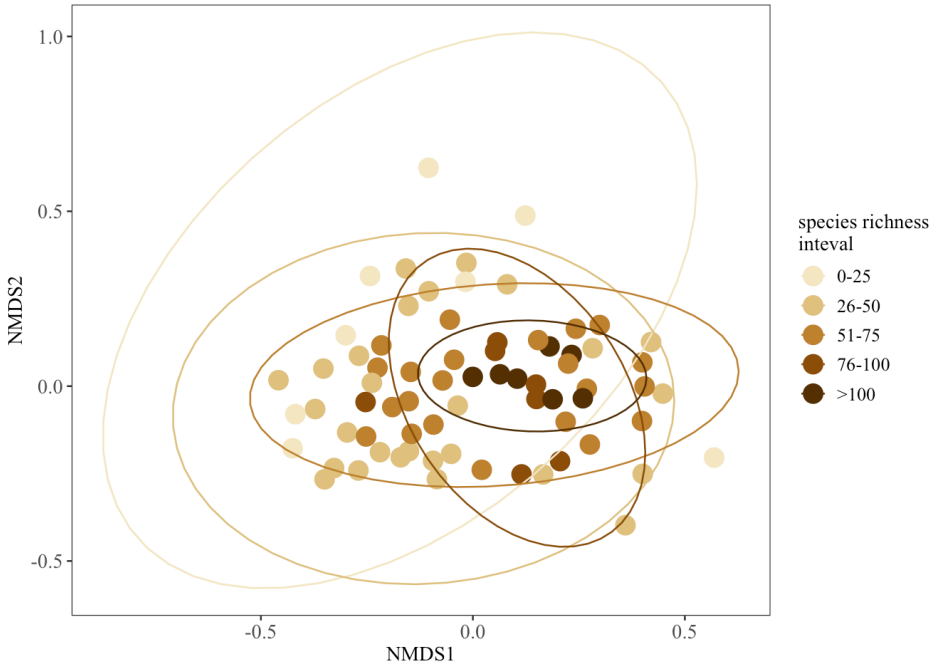
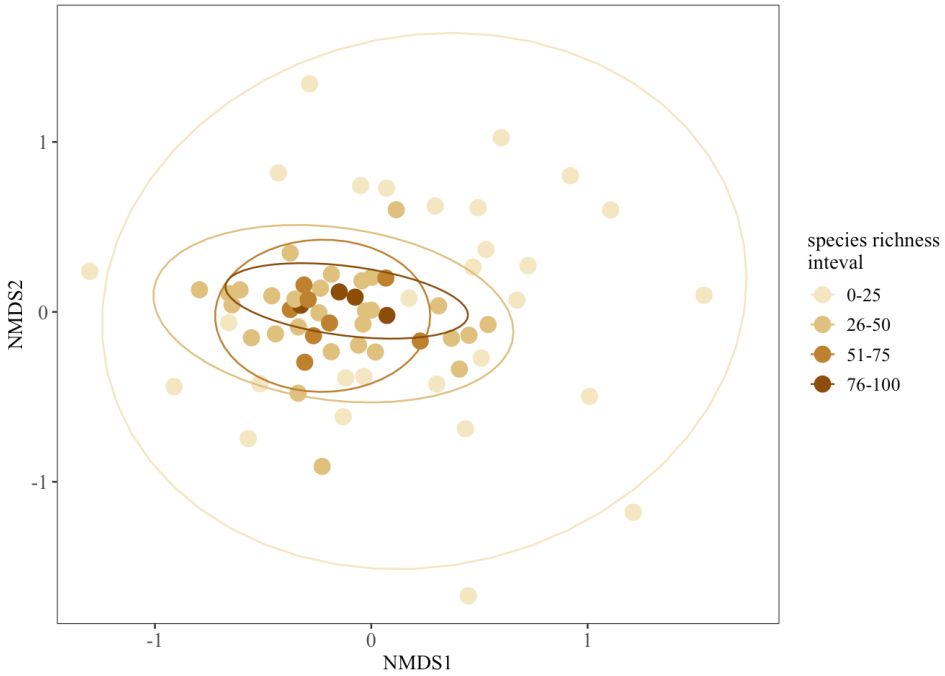



Figure C1: NMDS ordination based on eDNA data showing the study sites colored by species richness intervals (SRI) representing the number of species recorded per site. Study sites with the highest species richness (SRI: >100) cluster closely together in the ordination space, indicating similar species composition and potentially similar environmental conditions. The ellipses represent the 95% confidence interval of each SRI. Site IDs with >100 species records: 55, 56, 93, 94, 117, 119.




Figur C2: NMDS ordination based on fruitbody data showing the study sites colored by species richness intervals (SRI) representing the number of species recorded per site. Study sites with the highest species richness (SRI: 76-100 and 51-76) cluster closely together in the ordination space, indicating similar species composition and potentially similar environmental conditions. The ellipses represent the 95% confidence interval of each SRI. Site IDs with 76-100 species records: 88, 93, 190, 199.

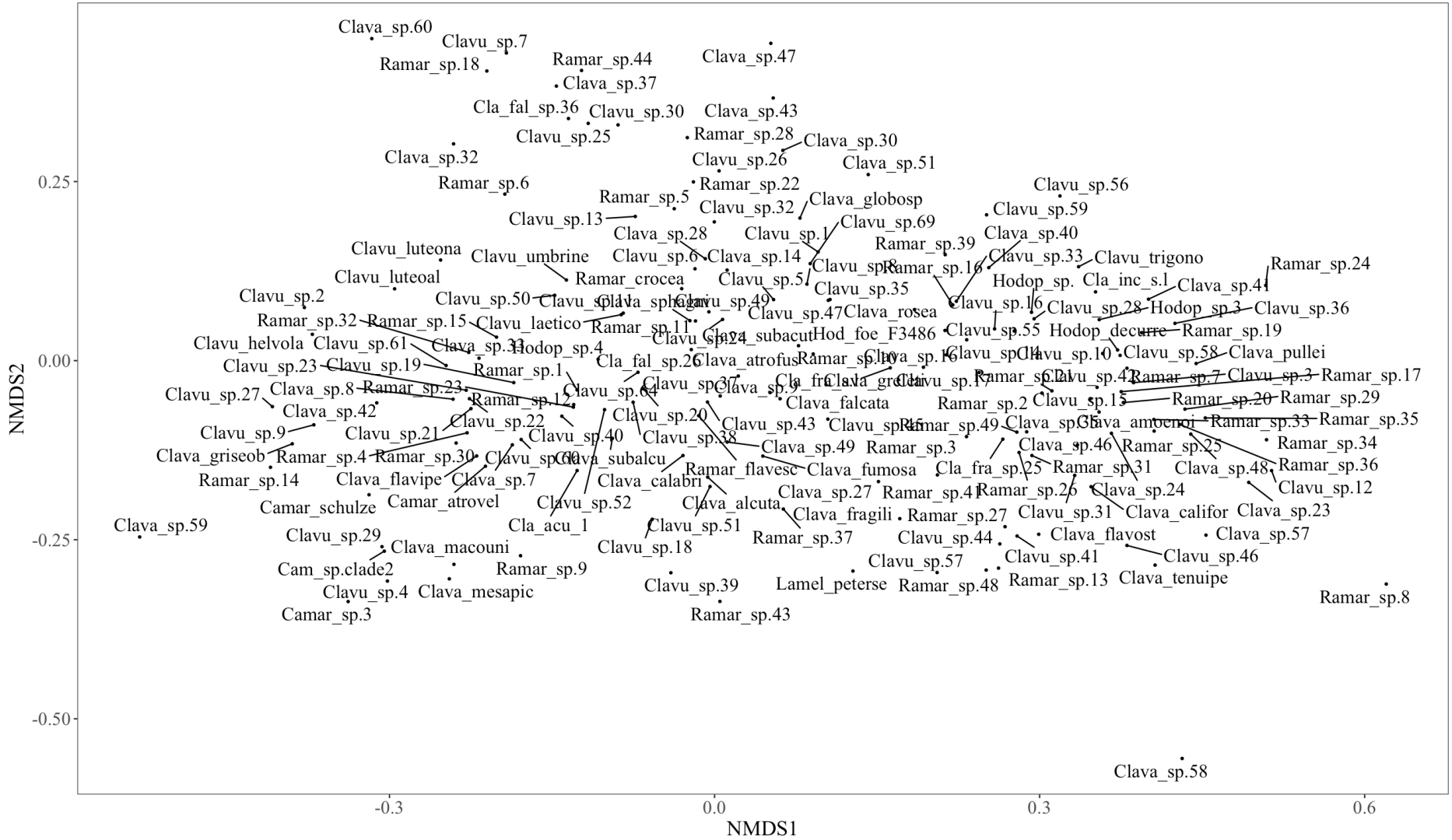



Figure C3: NMDS ordination of the eDNA data including only *Clavariaceae* species occurring in more than one site. Nearby species in the ordination space share similar distribution patterns across the study sites, suggesting similar habitat preferences between species.

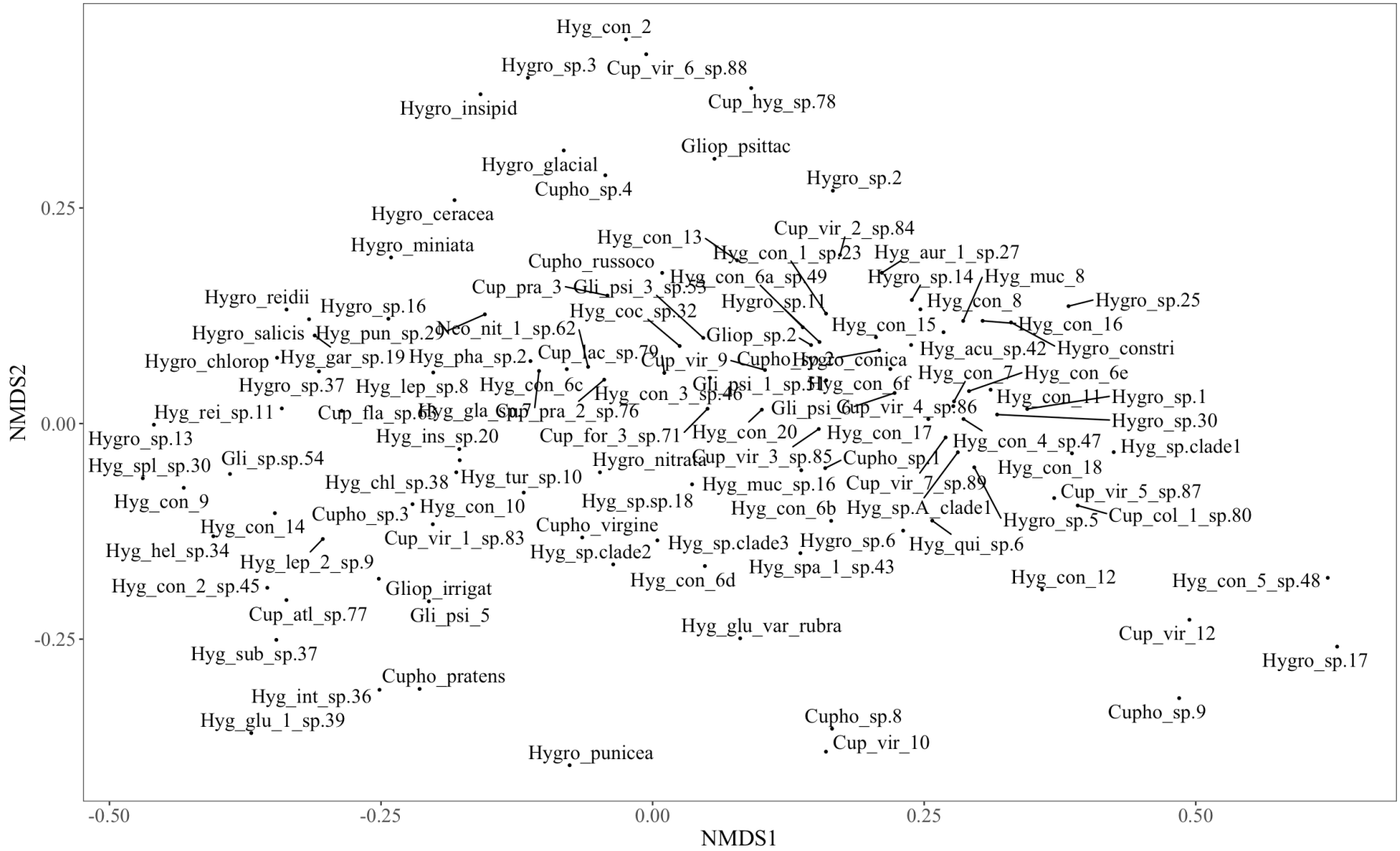



Figure C4: NMDS ordination of the eDNA data including only *Hygrocybe* s.l species occurring in more than one site. Nearby species in the ordination space share similar distribution patterns across the study sites, suggesting similar habitat preferences between species.

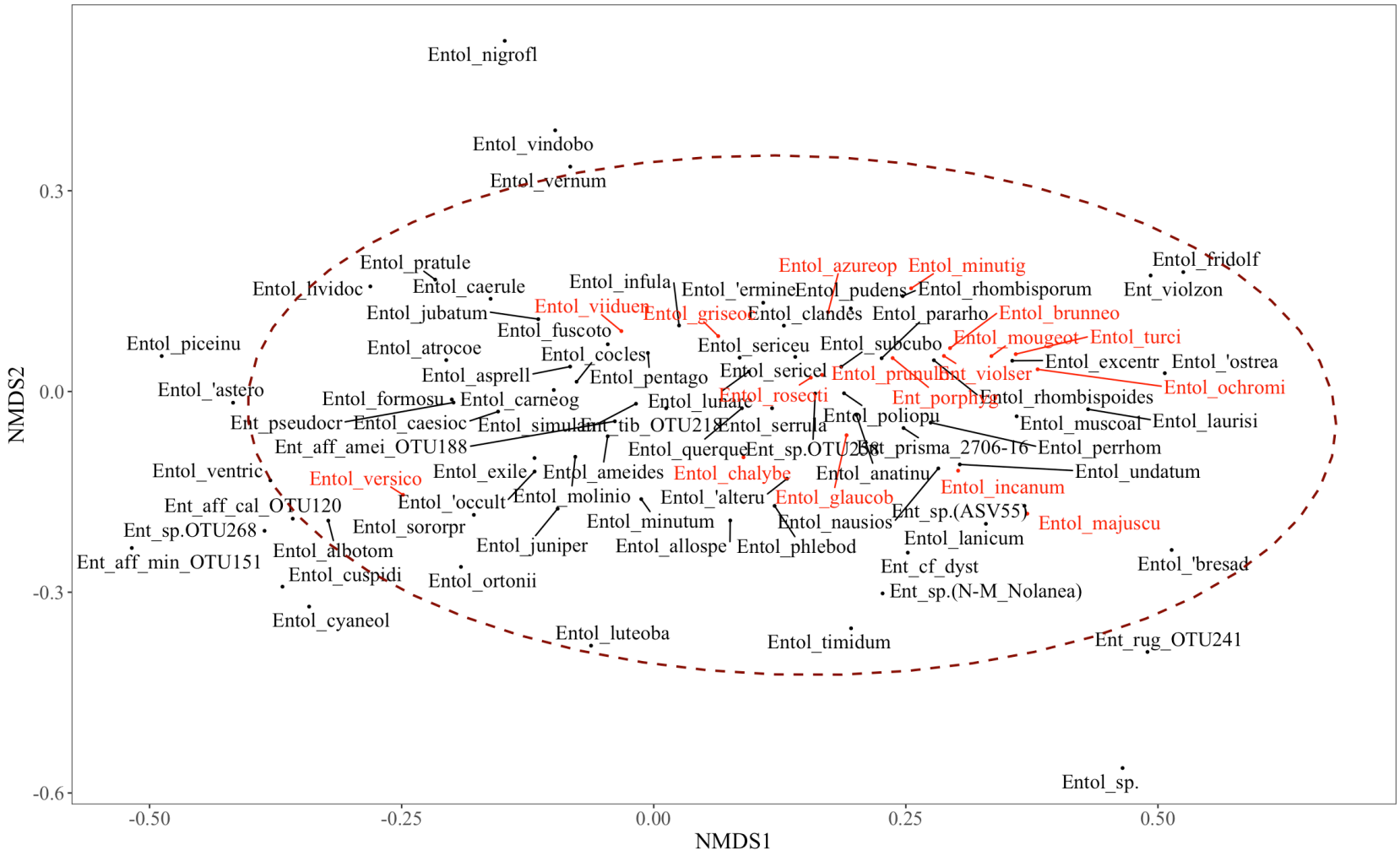



Figure C5: NMDS ordination of the eDNA data showing *Entoloma* species occurring in more than one site. Nearby species in the ordination space share similar distribution patterns across the study sites, suggesting similar habitat preferences between species. Species linked to high calcareousness in previous study (Brandrud et. al., 2023) are colored in red. The ellipse represents the 95% confidence interval of strongly calcareous soils.

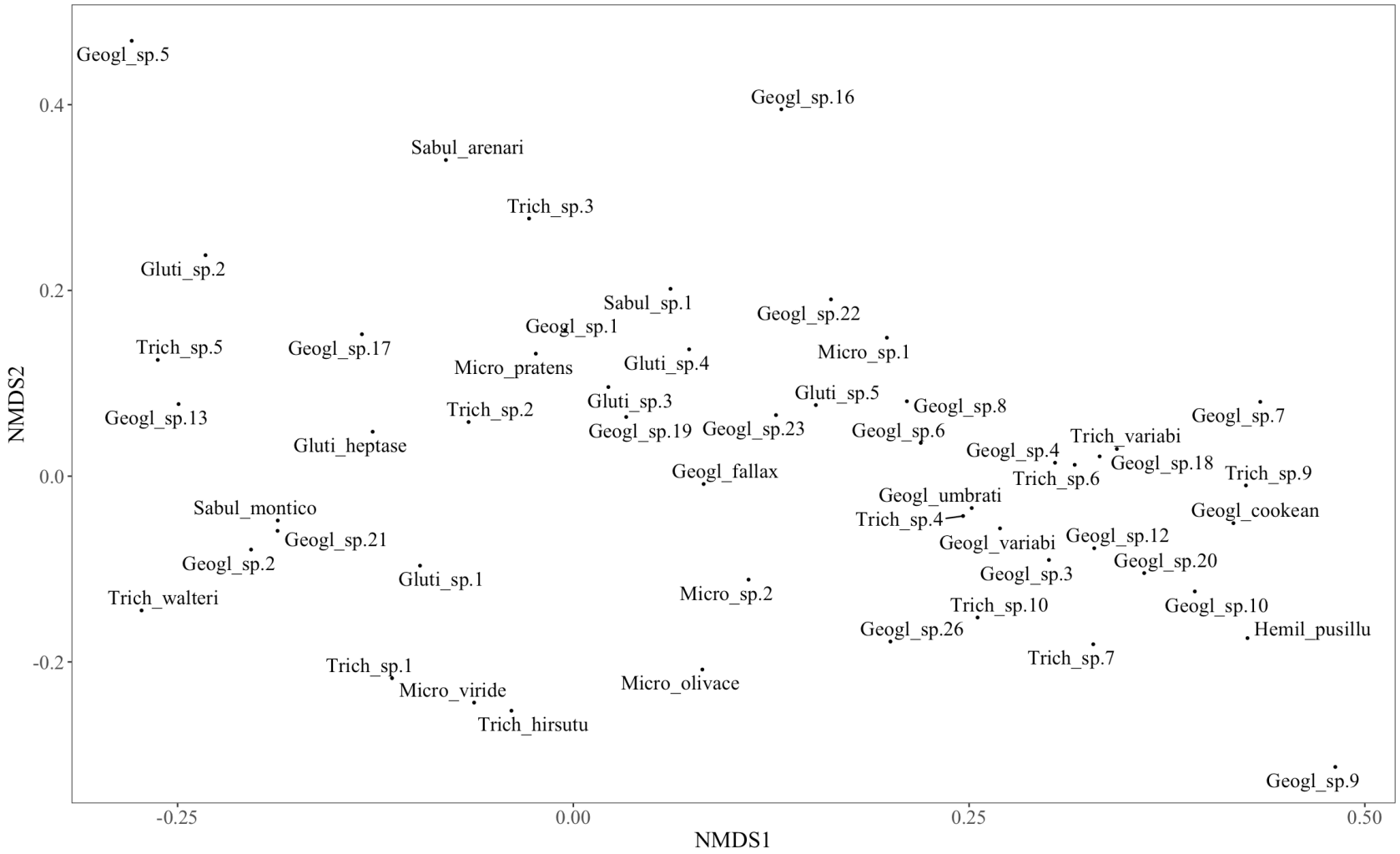



Figure C6: NMDS ordination of the eDNA data including only *Geoglossaece/Microglossum* species occurring in more than one site. Nearby species in the ordination space share similar distribution patterns across the study sites, suggesting similar habitat preferences between species.

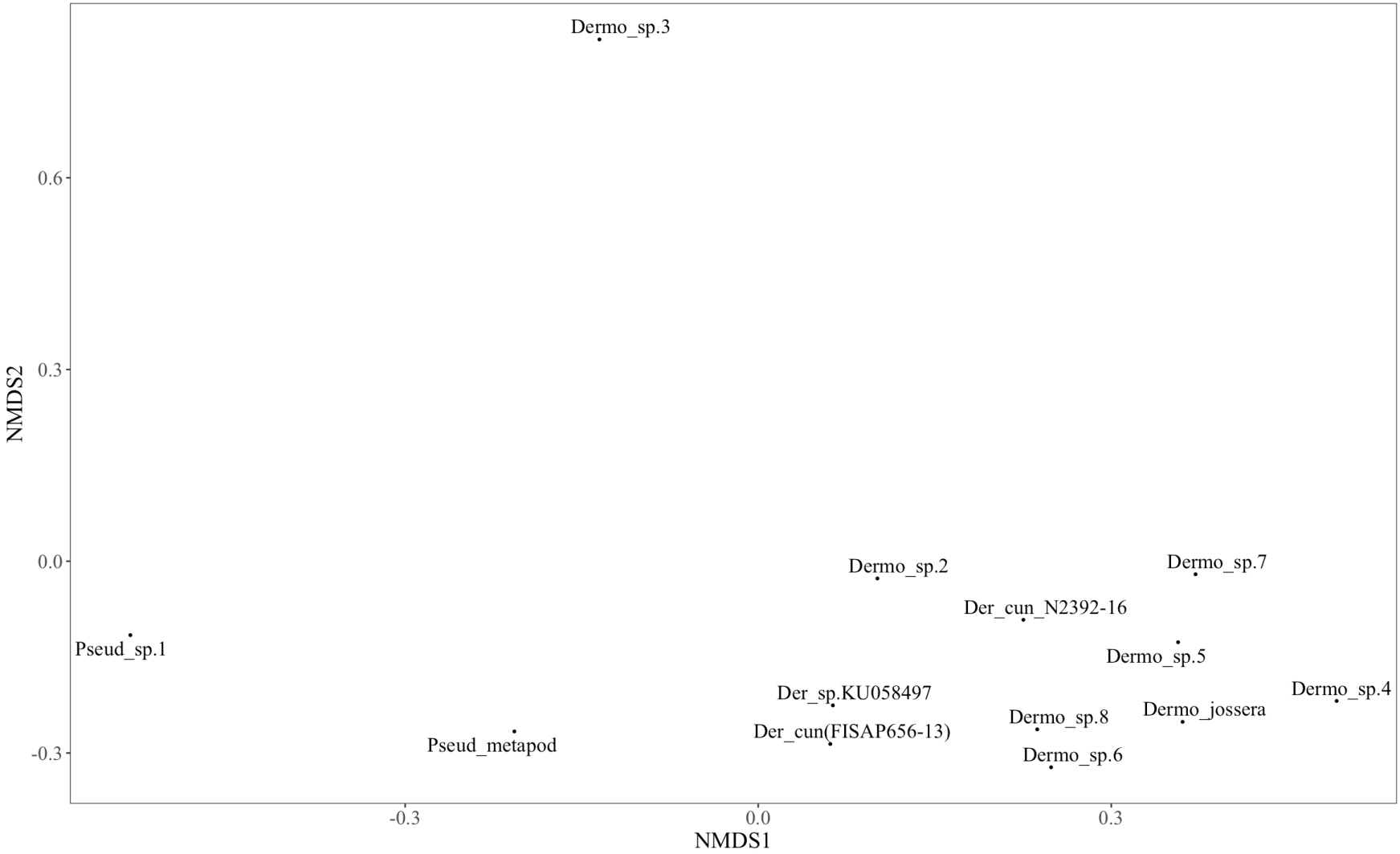



Figure C7: NMDS ordination of the eDNA data showing only *Dermoloma/Pseudotricholoma* species occurring in more than one site. Nearby species in the ordination space share similar distribution patterns across the study sites, suggesting similar habitat preferences between species.

# Appendix D – Indicator species analysis

Table D1: Indicator species for soil calcareousness identified from Indicator Species Analyses (ISA) based on both eDNA and fruitbody data. The table shows species significantly associated with soils of different calcareousness levels (weakly, intermediate, or strongly calcareous). For each species, Red List (RL) status(Artsdatabanken, 2021), indicator value from 0 to 1 (IndVal), and associated p-values. Only species with statistically significant associations ($p < 0.05$) are included.

| Levels of calcareousness | Indicator Species | RL | IndVal | p value |
|---|---|---|---|---|
| | **eDNA data** | | | |
| **Weakly calcareous soils** | *Clavaria flavipes* | VU | 0.734 | 0.003 |
| | *Glutinoglossum sp.1* | | 0.473 | 0.040 |
| | *Clavulinopsis sp.32* | | 0.604 | 0.019 |
| | *Hygrocybe turunda sp.10* | | 0.534 | 0.031 |
| | *Entoloma sp. OTU.268* | | 0.448 | 0.010 |
| | *Clavulinopsis sp.40* | | 0.509 | 0.015 |
| | *Entoloma caeruleum* | VU | 0.636 | 0.001 |
| | *Clavulinopsis sp.69* | | 0.426 | 0.025 |
| | *Entoloma lividocyanulum* | | 0.395 | 0.036 |
| | *Entoloma aff. Minitum OTU151* | | 0.549 | 0.002 |
| | *Entoloma albotomentosum* | | 0.540 | 0.008 |
| | *Hygrocybe helobia* | | 0.441 | 0.049 |
| | *Sabuloglossum monticola* | | 0.454 | 0.009 |
| | *Clavaria sp.32* | | 0.456 | 0.018 |
| | *Pseudotricholoma metapodium* | EN | 0.501 | 0.006 |
| | *Hygrocybe ceracea* | | 0.541 | 0.018 |
| | *Camarophyllopsis schulzeri* | NT | 0.654 | 0.001 |
| | *Clavaria macouni* | | 0.450 | 0.027 |
| | *Hygrocybe reidii sp.11* | | 0.578 | 0.026 |
| | *Hygrocybe substrangulata sp.37* | | 0.439 | 0.044 |
| **Intermediate calcareous soils** | *Entoloma porphyrogriseum* | NT | 0.502 | 0.044 |
| | *Microglossum sp.1* | | 0.547 | 0.024 |
| | *Entoloma pentagonale* | | 0.499 | 0.013 |
| **Weakly + strongly calcareous soils** | *Clavaria fragilis* | | 0.498 | 0.029 |
| **Intermediate + strongly calcareous soils** | *Entoloma poliopus* | | 0.616 | 0.026 |
| | *Clavulinopsis sp.10* | | 0.654 | 0.026 |
| | *Cuphophyllus virgineus 4 sp.86* | | 0.587 | 0.031 |
| | *Hygrocybe acutoconica* | | 0.613 | 0.035 |

| Levels of calcareousness | Indicator Species | RL | IndVal | p value |
|---|---|---|---|---|
| **Strongly calcareous soils** | *Clavulinopsis sp.41* | | 0.527 | 0.008 |
| | *Cuphophyllus colemannianus 1 sp.80* | | 0.549 | 0.030 |
| | *Hygrocybe clade3* | | 0.602 | 0.006 |
| | *Entoloma muscoalpinum* | | 0.519 | 0.029 |
| | *Clavaria californica* | | 0.534 | 0.031 |
| | *Entoloma incanum* | NT | 0.450 | 0.038 |
| | *Hodophilus decurrentior* | | 0.414 | 0.027 |
| | *Clavaria sp.35* | | 0.595 | 0.005 |
| | *Clavaria sp.24* | | 0.523 | 0.020 |
| | **Fruitbody data** | | | |
| **Weakly calcareous soils** | *Clavaria zollingeri* | | 0.671 | 0.001 |
| | *Entoloma cuspidiferum* | | 0.441 | 0.030 |
| | *Pseudotricholoma metapodium* | | 0.616 | 0.006 |
| **Weakly + intermediate calcareous soils** | *Entoloma asprellum* | | 0.630 | 0.017 |
| | *Entoloma conferendum* | | 0.684 | 0.032 |
| | *Hygrocybe reidii* | | 0.710 | 0.014 |
| | *Neohygrocybe ingrata* | | 0.542 | 0.034 |
| **Intermediate + strongly calcareous soils** | *Entoloma turci* | NT | 0.695 | 0.012 |
| | *Hygrocybe insipida* | | 0.680 | 0.048 |
| | *Cuphophyllus russocoriaceus* | NT | 0.649 | 0.032 |
| | *Neohygrocybe ovina* | | 0.572 | 0.034 |
| **Strongly calcareous soils** | *Entoloma incanum* | NT | 0.493 | 0.029 |

Table D2: Indicator species for bioclimatic sections identified from Indicator Species Analyses (ISA) based on both eDNA and fruitbody data. The table shows species significantly associated with bioclimatic sections reflecting climates along the oceanic-continental gradient (highly oceanic (O3), markedly oceanic (O2), slightly oceanic (O1), indifferent (OC), and slightly continental section (C1)). Norwegian Red List (RL) status (Artsdatabanken, 2021), indicator value from 0 to 1 (IndVal), and associated p-values. Only species with statistically significant associations ($p < 0.05$) are included.

| Bioclimatic section | Indicator Species | RL | IndVal | p value |
|---|---|---|---|---|
| | **eDNA data** | | | |
| **Highly oceanic (O3)** | *Clavulinopsis luteoalba* | LC | 0.711 | 0.014 |
| | *Gliophorus sp.54* | | 0.582 | 0.025 |
| | *Clavaria acuta 1* | | 0.542 | 0.028 |
| | *Clavulinopsis helvola* | LC | 0.721 | 0.011 |
| | *Clavulinopsis sp.2* | | 0.755 | 0.006 |
| | *Clavulinopsis sp.61* | | 0.601 | 0.019 |
| | *Clavulinopsis sp.9* | | 0.680 | 0.007 |
| | *Clavaria griseobrunnea* | | 0.648 | 0.016 |
| | *Clavulinopsis sp.27* | | 0.654 | 0.014 |
| | *Entoloma ventricosum* | LC | 0.552 | 0.032 |
| **Highly + markedly oceanic (O3 + O2)** | *Camarophyllopsis schulzeri* | NT | 0.607 | 0.034 |
| **Highly + slightly (O3 + O1)** | *Clavaria sp.8* | | 0.677 | 0.017 |
| **Highly to slightly (O3 + O2 + O1)** | *Clavaria flavipes* | VU | 0.729 | 0.020 |
| | *Entoloma caesiocinctum* | LC | 0.645 | 0.041 |
| | *Trichoglossum walteri* | VU | 0.706 | 0.015 |
| | *Clavaria falcata sp.36* | | 0.697 | 0.029 |
| | *Ramariopsis sp.23* | | 0.628 | 0.038 |
| **Markedly oceanic (O2)** | *Entoloma carneogriseum* | DD | 0.559 | 0.009 |
| | *Neohygrocybe nitrata* | NT | 0.623 | 0.016 |
| **Slightly oceanic climate (O1)** | *Entoloma viiduende* | VU | 0.533 | 0.022 |
| | *Rapariopsis sp.32* | | 0.656 | 0.007 |
| **Slightly oceanic + slightly continental (O1 + C1)** | *Clavaria sp.23* | | 0.570 | 0.046 |
| | *Clavulionopsis sp.26* | | 0.578 | 0.040 |
| | *Entoloma griseocyaneum* | NT | 0.761 | 0.001 |

| Bioclimatic section | Indicator Species | RL | IndVal | p value |
|---|---|---|---|---|
| **Slightly continental + markedly to slightly oceanic (C1 + O2 + O1)** | *Entol_subcubo* | | 0.596 | 0.036 |
| **C1 + OC + O1 + O3** | *Ramariopsis sp.11* | | 0.680 | 0.040 |
| **Slightly continental (C1)** | *Clavaria sp.16* | | 0.659 | 0.018 |
| | *Hygrocybe conica 15* | | 0.706 | 0.011 |
| | *Clavulinopsis sp.10* | | 0.849 | 0.003 |
| | *Clavulinopsis sp.12* | | 0.537 | 0.037 |
| | *Cuphophyllus virgineus 4 sp.86* | | 0.650 | 0.010 |
| | *Entoloma rhombisporum* | | 0.735 | 0.001 |
| | *Ramariopsis sp.22* | | 0.724 | 0.009 |
| | *Ramariopsis sp.29* | | 0.525 | 0.026 |
| | *Clavulinopsis sp.16* | | 0.711 | 0.005 |
| | *Clavulinopsis sp.28* | | 0.547 | 0.025 |
| | *Clavulinopsis sp.47* | | 0.501 | 0.031 |
| | *Entoloma turci* | | 0.728 | 0.009 |
| | *Microglossum sp.1* | | 0.669 | 0.015 |
| | *Ramariopsis sp.17* | | 0.770 | 0.005 |
| | *Dermoloma sp.4* | | 0.778 | 0.003 |
| | *Entoloma porphyrogriseum* | NT | 0.622 | 0.021 |
| | *Ramariopsis sp.39* | | 0.748 | 0.002 |
| | *Hygrocybe sp.5* | | 0.945 | 0.001 |
| | **Fruitbody data** | | | |
| **Highly oceanic (O3)** | *Clavulinopsis helvola* | LC | 0.874 | 0.001 |
| | *Clavulinopsis luteoalba* | LC | 0.812 | 0.002 |
| | *Geoglossum umbratile* | LC | 0.658 | 0.022 |
| | *Gliophorus irrigatus* | LC | 0.864 | 0.001 |
| | *Gliophorus laeta* | LC | 0.833 | 0.002 |
| | *Hygrocybe punicea* | LC | 0.715 | 0.026 |
| | *Hygrocybe reidii* | LC | 0.789 | 0.001 |
| | *Hygrocybe quita* | NT | 0.704 | 0.013 |
| | *Geoglossum fallax* | LC | 0.812 | 0.005 |
| | *Hygrocybe chlorophana* | LC | 0.798 | 0.007 |
| | *Hygrocybe splendidissima* | VU | 0.835 | 0.003 |

| Bioclimatic section | Indicator Species | RL | IndVal | p value |
|---|---|---|---|---|
| | *Hygrocybe miniata* | LC | 0.569 | 0.034 |
| | *Geoglossum cookeanum* | NT | 0.557 | 0.027 |
| | *Glutinoglossum glutinosum* | LC | 0.647 | 0.019 |
| | *Trichoglossum hirsutum* | LC | 0.581 | 0.041 |
| | *Cuphophyllus flavipes* | VU | 0.722 | 0.008 |
| | *Trichoglossum walteri* | VU | 0.535 | 0.036 |
| **Highly + markedly oceanic climate (O3 + O2)** | *Microglossum atropurpureum* | VU | 0.633 | 0.035 |
| | *Cuophophyllus lacmus* | NT | 0.547 | 0.049 |
| | *Clavaria zollingeri* | VU | 0.572 | 0.042 |
| | *Entoloma caesiocinctum* | LC | 0.663 | 0.027 |
| **Highly to slightly oceanic (O3 + O2 + O1)** | *Entoloma atrocoeruleum* | NT | 0.737 | 0.008 |
| | *Entoloma conferendum* | LC | 0.735 | 0.017 |
| | *Camarophyllopsis schulzeri* | NT | 0.643 | 0.037 |
| | *Hygrocybe helobia* | LC | 0.601 | 0.039 |
| | *Cuphophyllus russocoriaceus* | NT | 0.728 | 0.006 |
| **Markedly oceanic (O2)** | *Neohygrocybe ingrata* | VU | 0.638 | 0.041 |
| **Highly + slightly (O3 + O1)** | *Hygrocybe ceracea* | LC | 0.799 | 0.001 |
| **Slightly continental (C1)** | *Entoloma poliopus* | LC | 0.710 | 0.014 |
| | *Entoloma porphyrogriseum* | NT | 0.739 | 0.008 |

*Table D3:* Indicator species for bioclimatic sections identified from Indicator Species Analyses (ISA) based on both eDNA and fruitbody data. The table shows species significantly associated with bioclimatic zones reflecting climates along the lowland-mountain gradient (low alpine (LA), northern boreal (NB), mod boreal (MB), southern boreal (SB), and boreonemoral (BN)). Norwegian Red List (RL) status (Artsdatabanken, 2021), indicator value from 0 to 1 (IndVal), and associated p-values. Only species with statistically significant associations ($p < 0.05$) are included.

| Bioclimatic zone | Indicator Species | RL | IndVal | p value |
|---|---|---|---|---|
| | **eDNA data** | | | |
| **Low alpine climate (LA)** | *Clavaria sp.48* | | 0.639 | 00.13 |
| | *Clavulinopsis sp.31* | | 0.733 | 0.026 |
| | *Clavaria sp.40* | | 0.644 | 0.032 |
| | *Clavulinopsis sp.12* | | 0.677 | 0.017 |
| | *Geoglossum sp.10* | | 0.572 | 0.048 |
| | *Cuphophyllus virgineus 5 sp.87* | LC | 0.625 | 0.037 |
| **Low alpine and northern boreal (LA + NB)** | *Clavaria sp.43* | | 0.575 | 0.049 |
| | *Clavaria sp.30* | | 0.627 | 0.023 |
| | *Entoloma pratulense* | VU | 0.661 | 0.037 |
| **From low alpine to middle boreal (LA + NB + MB)** | *Clavaria subacuta* | | 0.676 | 0.033 |
| | *Sabuloglossum sp.1* | | 0.698 | 0.032 |
| **Northern and middle boreal (NB + MB)** | *Hygrocybe ceracea* | LC | 0.631 | 0.037 |
| | *Hygrocybe conica 20* | LC | 0.655 | 0.044 |
| **Northern boreal (NB)** | *Entoloma caeruleopolitum* | VU | 0.717 | 0.021 |
| | **Fruitbody data** | | | |
| **Low alpine climate (LA)** | *Entoloma sericeum* | LC | 0.762 | 0.035 |
| **South boreal to northern boreal (SB + MB + NB)** | *Entoloma griseocyaneum* | NT | 0.751 | 0.035 |
| **BN + SB + MB + LA** | *Hygrocybe insipida* | LC | 0.725 | 0.029 |

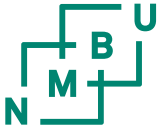
# Appendix E – Supplementary statistics, species complexes

Table E1: Results from Canonical Correspondence Analysis (CCA) showing the marginal effects of three environmental gradients: bioclimatic sections (oceanic–continental). bioclimatic zones (lowland–mountain). and soil calcareousness. on species composition within three CHEGD species complexes (*Hygrocybe conica* coll, *Gliophorus psittacinus* coll., and *Cuphophyllus virgineus* coll.).

| Species complex | Variable | Df | ChiSquare | F value | p value |
|---|---|---|---|---|---|
| *Hygrocybe conica* coll. | Bioclimatic sections | 4 | 0.53 | 1.52 | 0.303 |
| | Bioclimatic zones | 4 | 0.39 | 1.11 | 0.761 |
| | Soil calcareousness | 2 | 0.24 | 1.39 | 0.447 |
| *Gliophorus psittacinus* coll. | Bioclimatic sections | 3 | 0.17 | 1.37 | 0.479 |
| | Bioclimatic zones | 3 | 0.10 | 0.80 | 0.785 |
| | Soil calcareousness | 2 | 0.07 | 0.91 | 0.597 |
| *Cuphophyllus virgineus* coll. | Bioclimatic sections | 4 | 0.43 | 2.04 | 0.156 |
| | Bioclimatic zones | 4 | 0.36 | 1.74 | 0.268 |
| | Soil calcareousness | 2 | 0.13 | 1.29 | 0.490 |

Tabell E2: Results from environmental fitting analysis (envfit) showing the goodness of fit ($r^2$) and p-values for the relationship between environmental variables (and covariates) and species composition (NMDS ordination) for the three species complexes (*Hygrocybe conica* coll., *Gliophorus psittacinus* coll., and *Cuphophyllus virgineus* coll.).

| Species complex | Variable | $r^2$ | p value |
|---|---|---|---|
| *Hygrocybe conica* coll. | Bioclimatic sections | 0.1412 | 0.113 |
| | Bioclimatic zones | 0.0433 | 0.788 |
| | Soil calcareousness | 0.0169 | 0.773 |
| | Sequencing depth | 0.0355 | 0.403 |
| | Sampling areas | 0.0482 | 0.246 |
| *Gliophorus psittacinus* coll. | Bioclimatic sections | 0.0926 | 0.815 |
| | Bioclimatic zones | 0.0833 | 0.642 |
| | Soil calcareousness | 0.0050 | 0.987 |
| | Sequencing depth | 0.0150 | 0.852 |
| | Sampling areas | 0.0269 | 0.731 |
| *Cuphophyllus virgineus* coll. | Bioclimatic sections | 0.1927 | 0.111 |
| | Bioclimatic zones | 0.0898 | 0.494 |
| | Soil calcareousness | 0.0392 | 0.504 |
| | Sequencing depth | 0.0113 | 0.809 |
| | Sampling areas | 0.0062 | 0.895 |



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