Environmental Drivers for Freshwater Fungal Communities Across a Gradient of Land Uses In Agriculturally Dominated Watersheds

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AAFC	Agriculture and Agri-Food Canada
AG_nK_2014	Percentage of area covered by agricultural land use in basin up to *n* km upstream from the site. *n* = 2, 5, 10, 20 (%)
AMIA_AMN	NH_3, NH_4^+ concentration (mg L^{-1})
ANOVA	Analysis of Variance
ASV	Amplicon Sequence Variant
avg_temp_c_nd	Average temperature recorded at WEBs meteorological station over *n* days prior, including sampling date. *n* = 1, 2, 3, 5, 7. (°C)
CONDUCTIVITY_MSC	Water specific conductivity read with YSI mini sonde in water (µS cm-1)
CV	Coefficient of Variation
dbRDA	Distance-Based Redundancy Analysis
DEVELP_nK_2014	Percentage of area covered by developed land use in basin up to *n* km upstream from the site. *n* = 2, 5, 10, 20 (%)
DISS_OXYGEN_MGL	Dissolved oxygen read with YSI mini sonde in water (mg L^{-1})
DNA	Deoxyribonucleic Acid
eDNA	Environmental Deoxyribonucleic Acid
DOC	Dissolved organic carbon concentration (mg L^{-1})
D_REA_PHOS	Dissolved reactive phosphorus concentration (mg L^{-1})
ITS	Internal Transcribed Spacer
NCBI	National Center for Biotechnology Information
NITRATE	Nitrate concentration (mg L^{-1})
NITRITE	Nitrite concentration (mg L^{-1})
ORP_MV	Electronic measurement of oxidation-reduction (redox) potential (mV)
OTHER_nK_2014	Percentage of area covered by all other undescribed land uses in basin up to *n* km upstream from the site. *n* = 2, 5, 10, 20 (%)

OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
perMANOVA	Permutational Analysis of Variance
PH	pH read with the YSI mini sonde in water
PLS-DA	Partial Least Squares Discriminant Analysis
rain_mm_nd	Total rainfall recorded at WEBs meteorological station over *n* days prior, including sampling date. *n* = 1, 2, 3, 5, 7. (mm)
RDP	Ribosome Database Project
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RU_DISM3S	Discharge of Castor River at Russell station (02LB006), daily mean water discharge ($m^3 s^{-1}$)
site_type	Sampling site land use class (Agriculture, Mixed, Forest)
SNR	South Nation River
strahler	Strahler stream order of the sampling site
SW-TD	Shannon-Weiner True Diversity
TEMP_C	Temperature read with the YSI mini sonde in water (°C)
TOC	Total organic carbon concentration (mg L^{-1})
TOTKN	Total Kjeldahl Nitrogen (mg L^{-1})
TOTPHO	Total phosphorus concentration (mg L^{-1})
TREE_nK_2014	Percentage of area covered by tree/forest land use in basin up to *n* km upstream from the site. *n* = 2, 5, 10, 20 (%)
TURBIDITY_NTU	Turbidity measurement with YSI mini sonde in Nephelometric Turbidity Units (NTU)
WATER_nK_2014	Percentage of area covered by water in basin up to *n* km upstream from the site. *n* = 2, 5, 10, 20 (%)

Abstract

Freshwater fungi are vital to the aquatic food web, essential for nutrient cycling, energy flow, and ecosystem regulation. Their distribution is particularly contingent upon agricultural runoff, which can carry agrochemicals capable of influencing the freshwater mycobiota and potentially impacting the ecosystem services which they provide. While such impacts are well documented for freshwater bacterial communities, fungal communities are critically understudied. Here, we address this research gap by assessing the impact of anthropological and environmental perturbations on the freshwater mycobiota in the agriculturally dominated South Nation River basin in Eastern Ontario, Canada. We undertook biweekly water sampling from 2016-2021, complemented by rich ancillary data including water properties, hydrology, weather conditions, and fungal ITS2 metabarcoding. Our study yielded 6,571 Operational Taxonomic Units from 503 water samples, spanning 15 fungal phyla, dominated by Ascomycota, Basidiomycota, and Chytridiomycota. Agricultural land use decreased the mycobiota alpha diversity and distinct fungal communities were observed at agricultural ditch and mixed-use sites compared to the forested site. Notably, river discharge emerged as a predominant influencer of both alpha and beta diversity, likely transporting fungi via precipitation, especially from plant-rich catchment basins. Intriguingly, environmental data only explained a fraction of fungal community variation, underscoring the significance of unmeasured factors such as fungicide application, alongside stochastic community assembly processes. This work highlights the complex interplay of factors influencing the freshwater fungal community in agriculturally impacted watersheds and shows the need for further investigation for a more comprehensive understanding of the freshwater fungal ecology.

Résumé

Les champignons d'eau douce sont essentiels au réseau trophique aquatique, essentiels au cycle des nutriments, au flux d'énergie et à la régulation des écosystèmes. Leur répartition dépend particulièrement du ruissellement agricole, qui peut transporter des produits agrochimiques capables d'influencer le mycobiote d'eau douce et potentiellement impacter les services écosystémiques qu'ils fournissent. Bien que de tels impacts soient bien documentés pour les communautés bactériennes d'eau douce, les communautés fongiques sont extrêmement sous-étudiées. Ici, nous comblons cette lacune en matière de recherche en évaluant l'impact des perturbations anthropologiques et environnementales sur le mycobiote d'eau douce dans le bassin de la rivière Nation Sud, dominé par l'agriculture, dans l'est de l'Ontario, au Canada. Nous avons entrepris un échantillonnage de l'eau toutes les deux semaines de 2016 à 2021, complété par de riches données auxiliaires, notamment les propriétés de l'eau, l'hydrologie, les conditions météorologiques et le métabarcoding fongique ITS2. Notre étude a donné 6 571 unités taxonomiques opérationnelles à partir de 503 échantillons d'eau, couvrant 15 phyla fongiques, dominés par les Ascomycota, les Basidiomycota et les Chytridiomycota. L'utilisation des terres agricoles a diminué la diversité alpha du mycobiote et des communautés fongiques distinctes ont été observées dans les fossés agricoles et sur les sites à usage mixte par rapport au site forestier. Notamment, le débit des rivières est apparu comme un influenceur prédominant sur la diversité alpha et bêta, transportant probablement des champignons via les précipitations, en particulier à partir de bassins versants riches en plantes. Curieusement, les données environnementales n'expliquaient qu'une fraction de la variation de la communauté fongique, soulignant l'importance de facteurs non mesurés tels que l'application de fongicides, ainsi que les processus stochastiques d'assemblage de la communauté. Ce travail met en évidence l'interaction complexe des facteurs influençant la communauté fongique d'eau douce dans les bassins versants touchés par l'agriculture et montre la nécessité d'investigations plus approfondies pour une compréhension plus complète de l'écologie fongique d'eau douce.

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Contributions

The contributions of collaborators and supervisors for the thesis monograph are as follows: Conceptualization, Wen Chen; Methodology, Phillip Pham, Wen Chen, Izhar Khan, David Lapen; Software, Phillip Pham, Wen Chen; Formal analysis, Phillip Pham; Data curation, Phillip Pham, Mark Sunohara, Emilia Craiovan, Izhar Khan, Wen Chen; Writing – original draft preparation, Phillip Pham; Writing – reviewing and editing, Wen Chen, Stéphane Aris-Brosou, Yichao Shi, David R. Lapen; Visualization, Phillip Pham; Supervision, Wen Chen, Stéphane Aris-Brosou; Project administration, Wen Chen, David R. Lapen; Funding Acquisition, Wen Chen, Izhar Khan, David R. Lapen, Stéphane Aris-Brosou.

Chapter 1: Introduction

1.1 Freshwater fungi: ecological roles and environmental influencers

Freshwater ecosystems, spanning streams, rivers, and lakes, provide vital ecosystem services such as water purification, nutrient cycling, and energy cycling (Vári et al., 2022), all of which are facilitated by a diverse range of organisms, including aquatic fungi (Seena et al., 2022). Aquatic fungi are defined as fungi which spend a part of, or their whole life cycle in aquatic environments (Grossart et al., 2019). Depending on the degree of adaptation, the constituents of the aquatic fungal community can be categorized as indwellers, which spend their whole lives in an aquatic habitat and are constantly active in this environment; periodic immigrants, which regularly enter aquatic environments from events such as leaf fall but are only periodically active; and versatile immigrants that do not regularly enter aquatic environments, but are able to survive and derive nutrients from the aquatic environment (Park, 1972; Grossart et al., 2019).

Aquatic fungi are found in over 46 classes spanning 13 phyla in the fungal kingdom (Calabon et al., 2023). The most extensively studied group among freshwater fungi is hyphomycetes, also known as Ingoldian fungi, encompassing over 300 described species (Krauss et al., 2011; Bärlocher, 2016; Grossart et al., 2019). Freshwater hyphomycetes are traditionally defined as anamorphic fungi, lacking visible fruiting bodies, and releasing multiradiate or sigmoidal conidia in running freshwaters, which facilitate their attachment to decaying leaves or wood, allowing them to thrive in flowing waters (Krauss et al., 2011; Bärlocher, 2016; Barros and Seena, 2022). These fungi are a polyphyletic group, encompassing both Ascomycota (mainly in subphylum Pezizomycotina) and Basidiomycota (Baschien et al., 2013; Qiao et al., 2018). The polyphyletic nature of freshwater hyphomycetes suggests independent evolutionary adaptations for fungi to survive in aquatic environments (Baschien et al., 2013). Freshwater hyphomycetes

hold a significant ecological role in freshwater ecosystems as saprotrophs. They play a crucial role in converting allochthonous organic materials, such as leaf litter from herbaceous plants and wood, into more accessible nutrients for other trophic levels in the aquatic food web, like zooplankton and bacteria (Grossart and Rojas-Jimenez, 2016; Ittner et al., 2018; Grossart et al., 2019). Besides aquatic hyphomycetes, Chytridiomycetes (chytrids) is another important group of aquatic fungi. Chytrids generally produce zoospores with one or more flagella to facilitate movement of spores in aquatic environments and can be parasitic (Gleason et al., 2008). These fungi help to mitigate or prevent algal blooms, while also serving as a high-quality food source for zooplankton and ciliates (Kagami et al., 2007). Therefore, freshwater fungi, collectively, play a critical role in the aquatic food web, contributing significantly to nutrient and energy cycling in the ecosystem. Recent discoveries have even unveiled the capability of freshwater fungi to decompose pollutants stemming from agricultural field treatments, including pesticides and fungicides. This ability positions them to contribute regulatory services within the ecosystem (Oliveira et al., 2015; Ittner et al., 2018).

Within freshwater environments, the fungal community exhibits responses to environmental perturbations, whether originating naturally or from anthropogenic activities (Krauss et al., 2011; Grossart et al., 2022). Water parameters, recognized for their influence on the freshwater mycobiota, encompass water temperature, pH, nutrient load like nitrogen, phosphorus and organic carbon content, and hydrological features like connectivity, discharge, and flow rate. However, interpretations regarding the impacts of water temperature on the diversity and biomass of freshwater fungi are disparate. Earlier studies indicated reduced aquatic hyphomycetes diversity with elevated water temperatures (Bärlocher et al., 2008), while fungal biomass on leaf litter and corresponding decomposition rate increased (Geraldes et al., 2012). In

contrast, Fenoy et al. (2022) reported that an increase in water temperature of at least 4°C might decrease the overall functional diversity of the mycobiota, resulting in reduced leaf litter decomposition. Water pH similarly shapes the fungal community composition (Cudowski et al., 2015; Pietryczuk et al., 2018), though most aquatic fungi are able to grow in a broad range of pH (4.0 – 9.0, Calabon et al., 2023). The capacity of freshwater fungi to decompose leaf litter is also linked to the dissolved oxygen content in the water. The absence of dissolved oxygen diminishes their aptitude for leaf litter decomposition and impacts diversity (Pascoal and Cássio, 2004; Medeiros et al., 2009; Bruder et al., 2016). The influence of nutrient content in water, like nitrogen and phosphorus, has been observed to augment the bioactivity of freshwater fungi, reflected in increased leaf decomposition rates (Gulis et al., 2006; Biasi et al., 2017; Gulis et al., 2017), and enhanced fungal diversity (Duarte et al., 2017; Bai et al., 2018). Biotic contributions from the surrounding environment, such as leaf litter input to streams, also reverberate within the freshwater fungal community. The increase in plant diversity and density surrounding freshwater environments provide additional substrate for freshwater fungi, and generally increase the dissolved organic content available in the water which facilitates the growth of freshwater fungi (Aitkenhead-Peterson et al., 2003; Hagen et al., 2010; Fernandes et al., 2013; Danger et al., 2016). Outside of the stream, freshwater fungi can survive in diverse habitats, including the forest floor, and be carried into streams through surface runoff from precipitation (Chauvet et al., 2016). Limited precipitation not only curtails the transport of organic carbon and fungi into the stream but also during prolonged dry periods, streams might desiccate, resulting in diminished diversity and species turnover: i.e., fungi better adapted for non-aquatic environments could replace freshwater fungi as those more suited to survival outside of aquatic environments prevail (Arias-Real et al., 2021).

Beyond changes in water nutrients and physicochemical properties, an often-neglected factor influencing the freshwater mycobiota is the use of fungicides within the watershed (Ittner et al., 2018). Fungicides are employed as seed treatments or field application sprays to mitigate crop losses due to plant diseases. However, these substances can permeate freshwater systems, leading to unintended toxic effects on freshwater fungi (Dijksterhuis et al., 2011; Zubrod et al., 2019). Currently, research concerning the repercussions of fungicides on freshwater fungi remains constrained. Nonetheless, the review conducted by Ittner et al. (2018) suggests that freshwater fungi exhibit heightened vulnerability to triazole-based fungicides. These substances interfere with fungal growth and reproduction by disrupting the synthesis of ergosterol, the most abundant sterol in fungal cell membranes which is responsible for maintaining the permeability and fluidity of the cell membrane (Dijksterhuis et al., 2011; Rodrigues, 2018). Consequently, this imparts a selection pressure upon the freshwater fungal community, fostering an environment where solely fungicide-tolerant members can survive. This scenario leads to a loss of diversity and compositional shifts within the mycobiota (Baudy et al., 2021).

1.2 Impact of agricultural land uses on freshwater mycobiota in watersheds

Watersheds are defined as the land surface area where water from precipitation-derived water converges to form streams, contributing to larger tributaries and rivers (Allan, 2004; Brooks et al., 2012; Government of Canada, 2020). Agricultural land use exerts influence on stream communities through nutrient and pesticide inputs within the watershed, while also diminishing allochtonous organic inputs to streams due to landscape development (Watzin and McIntosh, 1999). Pollution from nitrogen and phosphorus in freshwater systems is mediated by surface runoff or discharge from nearby farmlands and livestock pastures, resulting in water nutrient

enrichment or eutrophication, leading to algal blooms that deplete oxygen in affected waters (Carpenter et al., 1998; Kato et al., 2009). Agricultural practices also significantly alter landscapes, leading to substantial forest cover loss and reduced biodiversity (Sumit et al., 2012), which can reduce the quantity and diversity of allochthonous organic matter entering streams (Hagen et al., 2010).

The environmental risks stemming from agricultural land use have adverse impacts on the ecological health of freshwater ecosystems, whose well-being is gauged by their capacity to provide ecosystem services (Hernández-Blanco et al., 2022). This is partly a consequence of the environmental perturbations introduced by agricultural activities, which may influence the biodiversity and community composition of freshwater fungi within the watershed. Such disruptions can affect the ecosystem services offered by the freshwater mycobiota (Barros and Seena, 2022; Seena et al., 2022). The pivotal role of freshwater fungi in ecosystems is to provide regulation services through nutrient cycling by decomposing leaf litter and organic waste (Seena et al., 2022). While some studies have suggested that this role of fungi in freshwater ecosystems could be jeopardized by biodiversity losses, potentially undermining ecosystem resilience and leading to reduced organic waste decomposition (Duarte et al., 2006; Duarte et al., 2008), other research proposed that under environmental stress from agricultural activities, freshwater fungi might exhibit functional redundancy, enabling them to continue as decomposers of leaf litter (Martínez et al., 2020; Baudy et al., 2021). Despite the recognized ecological importance of freshwater fungi, limited attention has been directed towards comprehending freshwater fungal communities in the context of environmental risks posed by agricultural land uses (Bai et al., 2018; Ittner et al., 2018; Ortiz-Vera et al., 2018; Baudy et al., 2021). To bridge this knowledge gap, further research is required to better understand the responses of freshwater fungal

communities to agricultural disturbances and the potential repercussions for the health of freshwater ecosystems. One promising approach involves utilizing DNA metabarcoding as a biomonitoring technique for the assessment of the freshwater mycobiota.

1.3 Biomonitoring the freshwater mycobiota using a metabarcoding approach

Freshwater biomonitoring is a technique which measures biological communities in freshwater ecosystems to evaluate the impact of anthropogenic activities, such as agricultural land use, on watershed ecosystem health (Sagova-Mareckova et al., 2021). Advances in high-throughput sequencing (HTS) technologies in the past decade have facilitated the use of environmental DNA (eDNA) metabarcoding as a cost-effective approach for biomonitoring, particularly for microorganisms like bacteria and fungi. These microorganisms serve as valuable bioindicators for water quality and ecosystem health (Keck et al., 2017; Sagova-Mareckova et al., 2021; Warnasuriya et al., 2023). This technique estimates species presence, biodiversity, and relative abundance by amplifying and sequencing a universal DNA marker region like the internal transcribed spacer (ITS) region of the fungal ribosomal RNA (rRNA) gene. This yields information on the biotic community composition in an environment (Acharya-Patel et al., 2023).

A DNA marker region selected for DNA metabarcoding is based on several criteria. First, the barcoding region must exhibit sufficient genetic variation to differentiate taxa at lower taxonomic levels, preferably at species or subspecific levels, while maintaining minimal variation within members of the same species (Schoch et al., 2012). Second, it must also have a high amplification and sequencing success rate, allowing universal primers to amplify a broad spectrum of taxonomic groups in an eDNA sample (Schoch et al., 2012). Additionally, the

selected marker region should have a well-curated reference databases with broad taxonomic coverage for annotating barcode sequences (Chen et al., 2022a). Meeting these criteria, the most commonly used DNA marker regions for fungal metabarcoding include the ITS1 or ITS2, the 18S or 28S rRNA gene regions, and the cytochrome oxidase subunit 1 gene region (Schoch et al., 2012; Xu, 2016; Tedersoo et al., 2022). The ITS region is currently recognized as the universal barcode region for fungi due to its high copy number in fungal genomes, increasing the success rate of amplification (Schoch et al., 2012; Lofgren et al., 2019; Nilsson et al., 2019a; Tedersoo et al., 2022), though it can lead to overestimation of abundances in species with exceptionally high ITS copy numbers (Taylor et al., 2016). In this study, the ITS2 region (Figure 1) was chosen due to its lesser length variation compared to ITS1 (Yang et al., 2018), along with its ability to recover greater richness of operational taxonomic units (OTUs) from environmental samples (Tedersoo et al., 2015).

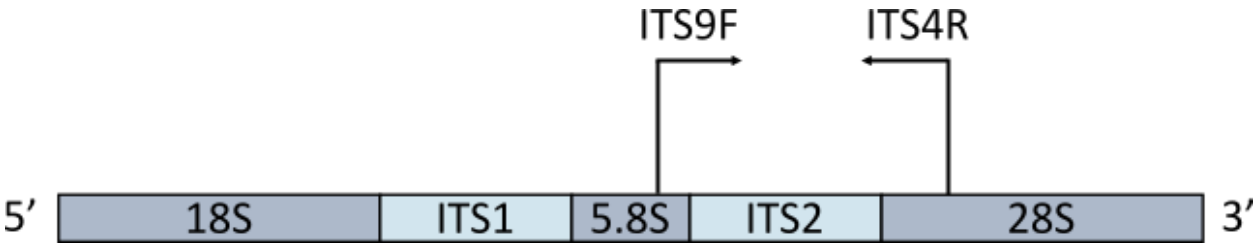


Figure 1. Schematic illustration of the ITS marker region. The fungal rRNA gene contains three major ribosomal subunits: small or 18S, 5.8S, and large or 28S. The ITS2 region, located between the highly conserved 5.8S and 28S subunits is amplified using the ITS9F/ITS4R primer pair in this study.

Sequencing ITS2 amplicons necessitates a HTS platform capable of reliably sequencing the ITS2 region, the length of which ranges from 14 bp to 730 bp with an average length of 182 bp (Yang et al., 2018). Platforms such as Illumina MiSeq have the capacity to generate paired-end short reads of up to 300 bp, which can be merged to improve coverage and sequencing

quality. The consensus or merged paired-end sequences are then used for sequence clustering and taxonomic inference. This makes them suitable for analyzing fungal communities using either ITS1 or ITS2 marker regions (Schmidt et al., 2013). For fungal ITS data, Tedersoo et al. (2022) has suggested that sequences should be clustered at the 97% sequence identity threshold, forming OTUs. This is because sufficient variation exists within the fungal ITS region, and utilizing Amplicon Sequence Variants (ASVs) as representative sequences could lead to an overestimation of fungal diversity (Estensmo et al., 2021). The representative sequences of OTUs are then matched to reference databases using sequence classification algorithms such as Naïve Bayesian classifiers like the Ribosome Database Project (RDP) classifier (Wang et al., 2007), and q2 feature classifier (Bokulich et al., 2018), or non-Bayesian methods such as USEARCH SINTAX (Edgar, 2016a) or BLASTn (Camacho et al., 2009) classification. The effectiveness of this process heavily relies on the quality of the reference databases, which must be well curated to minimize incorrect classification (Keck et al., 2023). The UNITE fungal ITS database for ITS metabarcoding (Nilsson et al., 2019b), is one such database, currently being the most extensively curated collection of ITS sequences and their corresponding taxonomy (Tedersoo et al., 2022).

Ongoing advancements in HTS technology, reference databases, and classification algorithms have paved the way for uncovering the vast and previously unknown diversity of aquatic fungi, thereby broadening the horizons of environmental community ecology studies. To date, around 1.5 million fungal species are estimated to exist, with only about 120,000 species having been described (Hawksworth and Lucking, 2017). A relatively small portion, approximately 3000 to 4000 species, of these known fungi are currently recognized as aquatic fungal species (Shearer et al., 2007; Ittner et al., 2018; Lepere et al., 2019; Calabon et al., 2023). The limited discovery of aquatic fungi can be attributed to the challenges in traditional methods

of fungal identification and classification, such as culturing and *in situ* morphological characterization (Bärlocher, 2016). Through eDNA metabarcoding with markers like 18S and ITS, the detection of previously unknown putative fungal species in freshwater has become possible – these are referred to as "dark matter fungi" due to their presence in the environment without proper annotation (Grossart et al., 2016). As an illustration, in a meta-analysis performed by Lepere et al. (2019), they recovered 25,000 putative fungal species by compiling 18S HTS data from studies across 25 lakes and ponds, as well as four rivers, revealing the current gap in aquatic fungi identification. Furthermore, integrating the freshwater fungal communities into environmental risk assessment studies has revealed the influence of anthropogenic activities, such as urban or agricultural land uses, on the freshwater mycobiota (Bai et al., 2018; Warnasuriya et al., 2023), and its potential repercussions for ecosystem services (Seena et al., 2022). Previous research has primarily focused on larger river systems, investigating environmental drivers associated with diverse anthropogenic activities (Bai et al., 2018; Ortiz-Vera et al., 2018; Pietryczuk et al., 2018). Recent studies, like Baudy et al. (2021), that examined freshwater fungi in agriculturally intensive freshwater systems have concentrated their analysis on selected community representatives, without employing a metabarcoding approach to assess the impact on the entire stream mycobiota. To the best of our knowledge, only a limited number of studies have specifically centered on the effect of agricultural land use on the freshwater fungal communities using a metabarcoding approach, underscoring the necessity for further investigations into the freshwater mycobiota within this context.

1.4 Objective of the thesis

The objective of the thesis is to explore and characterize the diversity, community composition, and functionality of the freshwater mycobiota and their response to agricultural land use. We also aimed to identify aquatic fungal taxa reflecting ecological services and land use impacts. We hypothesized that the freshwater fungal communities are sensitive responders to agricultural land use, meteorological, and other environmental perturbations. To test this hypothesis, we incorporated biweekly water sampling in the agriculturally dominated South Nation River (SNR) basin, situated in Eastern Ontario, Canada, along with a rich ancillary data (water physicochemical properties, hydrology, weather conditions) and metabarcoding of the ITS2 region of the fungal rRNA gene, with the ultimate goal of identifying the environmental drivers of the spatial and temporal dynamics of the aquatic fungal community as a function of land use. Additionally, the effect of land use on the functionality of the fungal community was assessed to gain valuable insights into the ecological consequences of agricultural practices on freshwater ecosystems.

Chapter 2: Methods

2.1 Experimental sites, sampling strategy, and metadata collection

This study was conducted in the SNR basin in Eastern Ontario, Canada. The SNR basin is approximately 3,900 km^2 in size, and is characterized by agricultural land use, constituting around 60% of the total land area, while also featuring scattered small urban establishments throughout the region. Eight sampling sites were carefully chosen to capture a gradient of land use features (Figure 2), stream orders, and functions, informed by prior studies within the same water basin (Lapen et al., 2016; Chen et al., 2018).

The four agricultural sites (SN_18, SN_19, SN_20, SN_21) are man-made agricultural drainage ditches excavated between farm fields and receive controlled sub-surface tile drainage. Consequently, these stream sites are directly influenced by agricultural land use practices (Sunohara et al., 2016). These sites are characterized by low stream order (small streams) and represent areas profoundly impacted by agricultural activities. While agricultural drainage ditch sites SN_18 and SN_20 were sampled from the outset in 2016, sites SN_19 and SN_21 were later included in 2017 and the following sampling seasons.

The mixed-use sites (SN_5, SN_6, SN_10) are tributaries with higher stream order (larger streams), surrounded by a mixture of land uses including urban development, forested land, and agricultural fields. Lastly, the forested stream site (SN_24) is located in a forested wetland with an upstream catchment area of < 5 km^2, and represents relatively undisturbed site with no known upstream or surrounding agricultural land use, and is characterized by dense surrounding vegetation (Lapen et al., 2016). Due to the challenges associated with accessing forested sites and the requirement to process samples within a 24-hour window of collection, we could only incorporate a single forested site in this study.

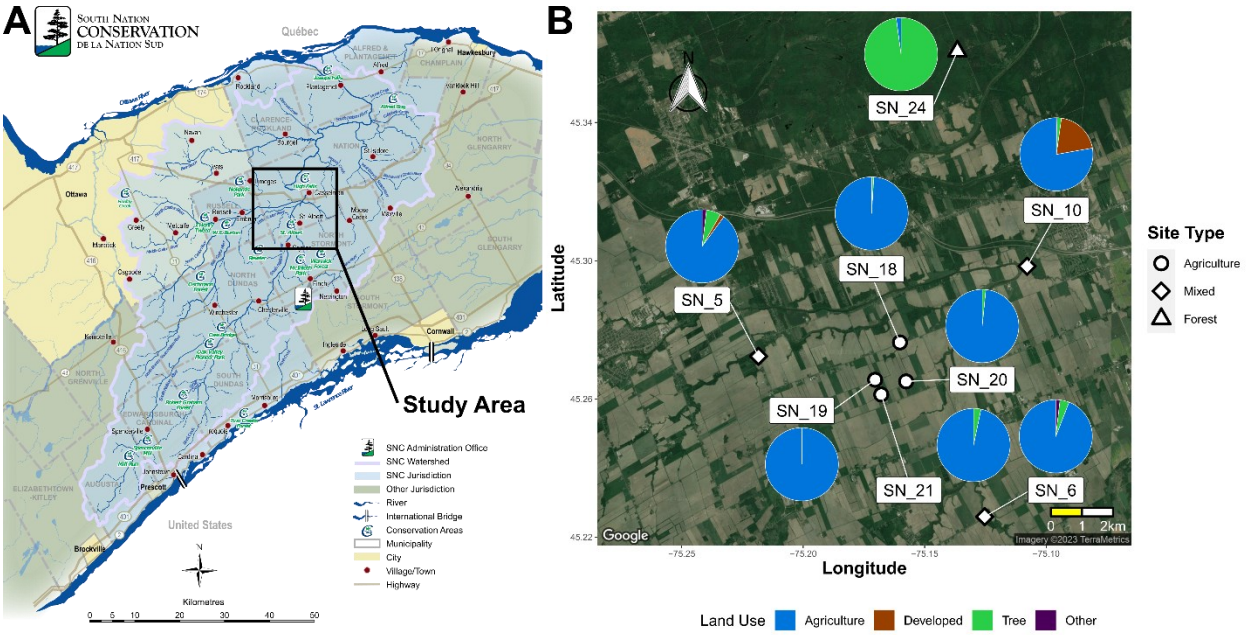


Figure 2. Geographic locations of sampling sites within the SNR basin with land use characterizations extending up to 5 km upstream from each site. (A) Map of the SNR basin, with the study area highlighted, situated in the East of Ottawa, Ontario, Canada, acquired from the South Nation Conservation (South Nation, 2020). (B) SN_18, 19, 20, and 21 are agricultural drainage ditch sites surrounded by agricultural fields, where SN_18/SN_19 and SN_20/SN_21 are respectively located within the same watershed. SN_5, 6, and 10 are influenced by mixed land uses and are situated at larger tributaries with nearby urban and agricultural lands and areas with tree cover. SN_24 is a small, forested stream characterized by minimal anthropogenic land use upstream. The satellite image of the region was acquired using the Google Maps API (Google, 2023).

The mixed-use sites (SN_5, SN_6, SN_10) and the forested site (SN_24) are located on different watersheds, making them independent sampling sites, uninfluenced by autocorrelation effects. In contrast, the agriculture sites are distributed across two agricultural drainage ditches. SN_18 is located ~ 1.45 km downstream of SN_19, and SN_20 is ~ 1 km downstream of SN_21,

thus constituting two pairs of pseudo replicates. Consequently, SN_18 + SN_19, and SN_20 + SN_21 were considered paired sampling blocks, whereas all other sites were deemed independent sampling blocks (n = 6, SN_5, SN_6, SN_10, SN_18 + SN_19, SN_20 + SN_21, SN_24). These blocks were later used as random effect in the mixed effects models for repeated measures, as by Shi et al. (2023) for the same sites (see Section 2.5).

Surface water samples were collected biweekly from each sampling site between April and November over a period of six years from 2016 to 2021. The samples were collected 0 – 50 cm below the surface and were transported on ice to the Ottawa Research and Development Centre of Agriculture and Agri-Food Canada (AAFC) for processing within 24 hours of collection. No water samples were collected during periods when the sampling sites were effectively dry, often with some disconnected puddles, which occurred during dry periods in the summer months. The methods for the analysis of water physicochemical properties and land use characterization have previously been described in detail by Wilkes et al. (2011). Briefly, water temperature, pH, dissolved oxygen, specific conductivity, and turbidity were measured during water sample collection using a YSI 6600 multi-parameter water quality sonde (YSI Inc., Yellow Springs, OH). A portion of each water sample was reserved and sent to the Robert O. Pickard Environmental Centre (ROPEC, Ottawa, ON) laboratory for ammonia, ammonium, dissolved reactive phosphorus, total phosphorus, nitrite, nitrate, and total Kjeldahl nitrogen analysis. The analysis of total and dissolved organic carbon was performed based on the APHA 2540D method described by Rice et al. (2012). Land use for each of the sampling sites was characterized by the percent of water catchment area for a sampling site with a maximum upstream distance of 2, 5, and 10 km from the site (Table A.1), using geographic information system methods described in detail by Wilkes et al. (2011). Stream order was determined using roadside survey, remote

sensing, and digital elevation model databases (Lyautey et al., 2010). Meteorological data including air temperature, precipitation, and solar radiation, were collected daily at a weather station equipped with a HOBO data logger (Onset Computer Corp., Bourne, MS) near SN_20. Cumulative temperature and precipitation were retrieved for the day of sampling, and over 2-, 3-, 5-, and 7 days prior, including the sampling day. River discharge rate measured from the Castor river running through the Russell station (45°15'45" N, 75°20'37" W), was obtained from the Water Survey of Canada online archives as an additional proxy for regional precipitation (WSC, 2023).

2.2 DNA extraction, sequencing library preparation, Illumina MiSeq sequencing

The DNA extraction, library preparation, and sequencing protocols were established and have been used in other metabarcoding studies conducted by AAFC in the SNR watershed (Chen et al., 2018.; Shi et al., 2023). In brief, to extract DNA from the water samples, 500 mL of each sample was passed through a two-filter system to minimize clogging. First, water samples were passed through a 0.7 μm borosilicate glass filter (Thermo Fisher, Ottawa, ON), where the majority of the fungal spores were captured at this step. Then, the filtrate was passed through a 0.22 μm sterile nitrocellulose filter (Millipore, Billerica, MA, USA). Each filter was subjected to total DNA extraction using DNeasy PowerSoil® DNA extraction kits (Qiagen; formerly MoBio), following the manufacturer's protocol. The concentration of the extracted DNA was quantified using a Qubit 3.0 fluorometer, and its purity was assessed by gel electrophoresis using 1% agarose gel with 1X TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 7.8) buffer. The DNA was then stored at -80°C until further use.

Amplification of the ITS2 region was performed by polymerase chain reaction (PCR) using the universal primer pair ITS9F (forward primer, 5' - GAA CGC AGC RAA IIG YGA - 3'), ITS4R (reverse primer, 5' - TCC TCC GCT TAT TGA TAT GC - 3', White et al., 1990; Menkis et al., 2012), with Qiagen HotStar MasterMix (Toronto, ON). The PCR reactions consisted of an initial template denaturation step at 95°C, followed by 30 cycles of amplification (30-second denaturation at 95°C, 30-second annealing at 45°C, 30-second extension at 72°C), and a final 5-minute extension at 72°C. The resulting PCR products were purified using NucleoMag NGS Clean-up and Size Select beads (Takara Bio Inc., Japan). The libraries were then pooled in equimolar ratios, and the diluted pools were then prepared for sequencing following the manufacturer's MiSeq System Denature and Dilute Libraries Guide. Sequencing was performed on the Illumina MiSeq system using a 500-cycle MiSeq Reagent Kit v2 at the National Research Council-Plant Biotechnology Institute (NRC-PBI, Saskatoon, SK), generating paired-end sequence reads of 250 bp in length.

2.3 Metabarcoding data processing

The processing of metabarcoding data, from sequence retrieval to the commencement of data analysis followed a series of steps outlined in Figure A.1. First, the raw sequencing data was processed using Quantitative Insights Into Microbial Ecology 2 (QIIME2, ver. 2021.11, Bolyen et al., 2019). The quality of the raw sequencing reads was assessed using the q2-demux plugin to identify base positions where median sequencing quality exceeded a Phred score threshold of 35, indicating a base call accuracy above 99.96%. To meet this quality threshold, sequences were trimmed at position 20 bp and truncated at position 240 bp (Figure A.2). Subsequently, denoising was performed using DADA2 *denoise-paired* QIIME2 plugin (Callahan et al., 2016) to merge

paired reads and infer ASVs. This denoising step employed an expected error threshold of 2.0 bases per read, and a chimera-fold abundance threshold of 2.0.

The representative sequences of the ASVs were further clustered at a 97% similarity threshold using CD-HIT-EST (ver. 4.8.1, Fu et al., 2012) to OTUs. This clustering step was performed because ASVs tend to overestimate fungal diversity due to some fungal species exhibiting intraspecific variation within the ITS region, leading to multiple ASVs representing a single fungal species (Estensmo et al., 2021; Tedersoo et al., 2022). The resulting OTU representative sequences were processed using fungal ITS extractor (ver. 2010.11, Nilsson; et al., 2010) to extract the ITS2 sequences and exclude non-target amplifications. The command *uchime_ref* in UCHIME2 (ver. 4.2, Edgar, 2016b) was employed, utilizing the high confidence configuration, to remove remaining chimeras, by comparing with the UNITE fungal ITS database (ver. 9.0, Nilsson et al., 2019b). Finally, taxonomic assignments for OTU representative sequences were conducted using the RDP Naïve Bayesian classifier, implemented as the *classify.seqs* command in mothur (ver. 1.48, Wang et al., 2007) against the UNITE fungal ITS database (ver. 9.0, Nilsson et al., 2019b) with a minimum bootstrap confidence of 80%.

OTU table curation and normalization were performed in the following steps. First, the OTUs obtained from 0.22 µm and 0.7 µm filters for each sample, along with any re-sequenced samples, were combined by summing the read counts. Next, the resulting OTU table was filtered to remove any OTUs with a total read count below ten (10), therefore eliminating potential chimeras or sequences arising from PCR or sequencing errors (Edgar, 2016c; Tedersoo et al., 2022). Additionally, OTUs that were not assigned to a known fungal phylum were removed from the OTU table. Then, samples with a total read count of less than 1000 were excluded from the dataset, as those with abnormally low read counts are often due to technical errors and do not

reliably represent a community. Total sum scaling, a normalization method commonly used in the analysis of compositional data, was then applied to transform the OTU table for community-level comparisons (McKnight et al., 2019). Notably, OTU table rarefaction was not performed to avoid the unnecessary removal of rare taxa from further analysis (McMurdie and Holmes, 2014).

2.4 Identification of known freshwater fungi and functional guilds

To identify fungal genera containing known freshwater fungal species from the sequencing dataset in this study, the names and habitat information of the aquatic fungal genera and species were compiled from the Freshwaterfungi.org (https://freshwaterfungi.org/outline.php), and the Freshwater Ascomycetes database (https://fungi.life.illinois.edu/species_monographs) using a custom Python-based HTML text parsing script, available at (https://bitbucket.org/wenchen_aafc/aquatic_mycobiota_pham/src/development/freshwater_ascomycetes_scraper/). The taxonomic lineages associated with the fungal genera were retrieved from the National Center for Biotechnology Information (NCBI) taxonomy database using the *myTAI* package (ver. 0.9.3, Drost et al., 2018). Any fungal genera missing NCBI taxonomy were manually cross-referenced with the Index Fungorum (http://www.indexfungorum.org) to obtain their hierarchical taxonomic lineage information. Freshwater fungal genera without a known taxonomic lineage from NCBI or Index Fungorum were excluded from the dataset used to search for aquatic fungi in the current study. OTUs assigned to a fungal genus potentially containing known freshwater fungal species were determined by matching their taxonomic assignments with the compiled list of known aquatic fungi at the genus level.

The FUNGuild database, accessed in R using the FUNGuildR package (ver. 0.2.0.9000, Nguyen et al., 2016; Furneaux, 2021), was employed to assign OTUs to functional guilds. All

FUNGuild annotations, including “Possible”, “Probable”, and “Highly probable” confidence rankings, were retained. Additionally, fungal taxa were aggregated into “saprotroph”, “animal pathogen”, and “plant pathogen” categories, representing the highest total relative abundance in this study.

2.5 Statistical analyses

All statistical analyses were performed in R (ver. 4.2.1, R Core Team, 2022). The custom scripts used for data analysis can be accessed on Bitbucket repository through the following link (https://bitbucket.org/wenchen_aafc/aquatic_mycobiota_pham/src/development/). To impute missing water physicochemical metadata and minimize discarded data points due to missing values, random forest imputation via the missForest package (ver. 1.5, Stekhoven and Buhlmann, 2012) was employed. *P* values were adjusted for multiple comparison using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) unless stated otherwise. A significance level of $P \leq 0.05$ was interpreted as statistically significant.

To visualize the differentiation of sampling sites based on their water physicochemical properties, partial least squares discriminant analysis (PLS-DA) implemented by the *DiscriMiner* package (ver. 0.1-29, Sanchez, 2013) was utilized. To evaluate the sampling adequacy and estimate OTU diversity across sampling sites, the iNEXT package (ver. 2.0.20, Hsieh et al., 2016) was employed to generate rarefaction curves. Analyses of both alpha- and beta diversity indices were conducted using the vegan package (ver. 2.6-2, Oksanen et al., 2020). For species diversity estimation, the Shannon-Weiner (SW) diversity index was computed using the *diversity* function in vegan and then transformed into true diversity (TD) using the equation: SW-TD = exp(SW), derived by Jost (2006). The true diversity index, SW-TD, was used as measure of

alpha diversity, which represents within-sample community species richness (Whittaker, 1972). The environmental drivers of alpha diversity were determined by assessing variable importance in random forest models, implemented using the randomForest package (Liaw and Wiener, 2002).

Community ordination in relation to the water physicochemical properties and meteorological conditions was performed using distance-based redundancy analysis (dbRDA) implemented in vegan. The community abundance data, i.e. the OTU table, was Hellinger-transformed and community dissimilarities were computed using Bray-Curtis distances (Bray and Curtis, 1957; Legendre and Gallagher, 2001).

To assess the significance of land use categories on community structure, permutational analysis of variance (perMANOVA) implemented as *adonis2* in vegan was employed. Assessment of environmental variables driving the changes in community compositional structure, i.e. beta diversity, was performed using the gradientForest package (ver. 0.1-34, Ellis et al., 2012). Community change between sampling time points were estimated using the *multivariate_change* function from the codyn package (ver. 2.0.5, Hallett et al., 2016). Spearman correlation (ρ) was used to compute correlation. To investigate differences in alpha diversity, relative abundance of taxa, and water physicochemical properties in relation to different land use categories, linear mixed effects models were employed using the *lme* function from the nlme package (ver. 3.1-162, José Pinheiro, 2023). In these models, land use categories were treated as fixed effects, while sampling date and blocks (also see Section 2.1) were treated as random effects. Analysis of variance (ANOVA) testing was performed to assess the significance of factors in linear mixed effects, and other models used in this study. Post-hoc pairwise tests between land use classes in the linear mixed effects models were performed using the *emmeans*

function in the emmeans package (ver. 1.8.4-1), with the ‘tukey’ method for *P* value adjustment for multiple comparisons (Lenth, 2023). The Tukey test was also performed for other multiple pairwise comparisons using the *TukeyHSD* function (R Core Team, 2022). To visualize the seasonal dynamics of alpha diversity and relative abundance of taxa, data points were plotted using the *stat_smooth* function in ggplot2 (ver. 3.4.1), applying local polynomial regression or ‘loess’ smoothing (Wickham, 2016).

Chapter 3: Results

3.1 Water physicochemical properties of the SNR basin

First, we evaluated the impact of the land use classes on the water physiochemical properties of the stream samples. Some of these properties differed significantly between the forested site and the mixed-use or agricultural sites but were similar between the mixed-use and agricultural ditch sites (Figures 3, A.3, A.4, Table 1). ANOVA analyses revealed that total and dissolved organic carbon were observed at significantly greater concentrations at the forested site compared to the agricultural and mixed-use sites ($P < 0.05$), while water conductivity showed an opposite trend (Figure 3, Table 1). Water conductivity, nitrate concentration, temperature, and turbidity were either similar or marginally significant across all land uses classes (Table 1). Notably, nitrate concentrations at the agricultural and mixed-use sites peaked in the spring and fall, maintaining higher levels than at the forested site throughout the year (Figure 3, Table 1). This trend aligned with liquid manure application regimes in the study region (per. comm. D. Lapen).

In this study, dissolved oxygen levels were 9.21 ± 5.50 (MEAN ± SD) mg L^{-1}, while the oxidative-reductive (redox) potentials were 192.16 ± 95.15 mV, though redox measurements were influenced by a measurement probe change in 2018 (Figure 3). These two parameters are closely related in water quality contexts. Redox potential measures the stream's ability to undergo oxidation or reduction (Søndergaard, 2009). A positive redox potential value falling between +200 to +500 mV, combined with dissolved oxygen levels at or above 5 mg L^{-1}, suggests the presence of sufficient dissolved oxygen and a prevalence of oxidation reactions. These indicators collectively point to good freshwater quality (Søndergaard, 2009).

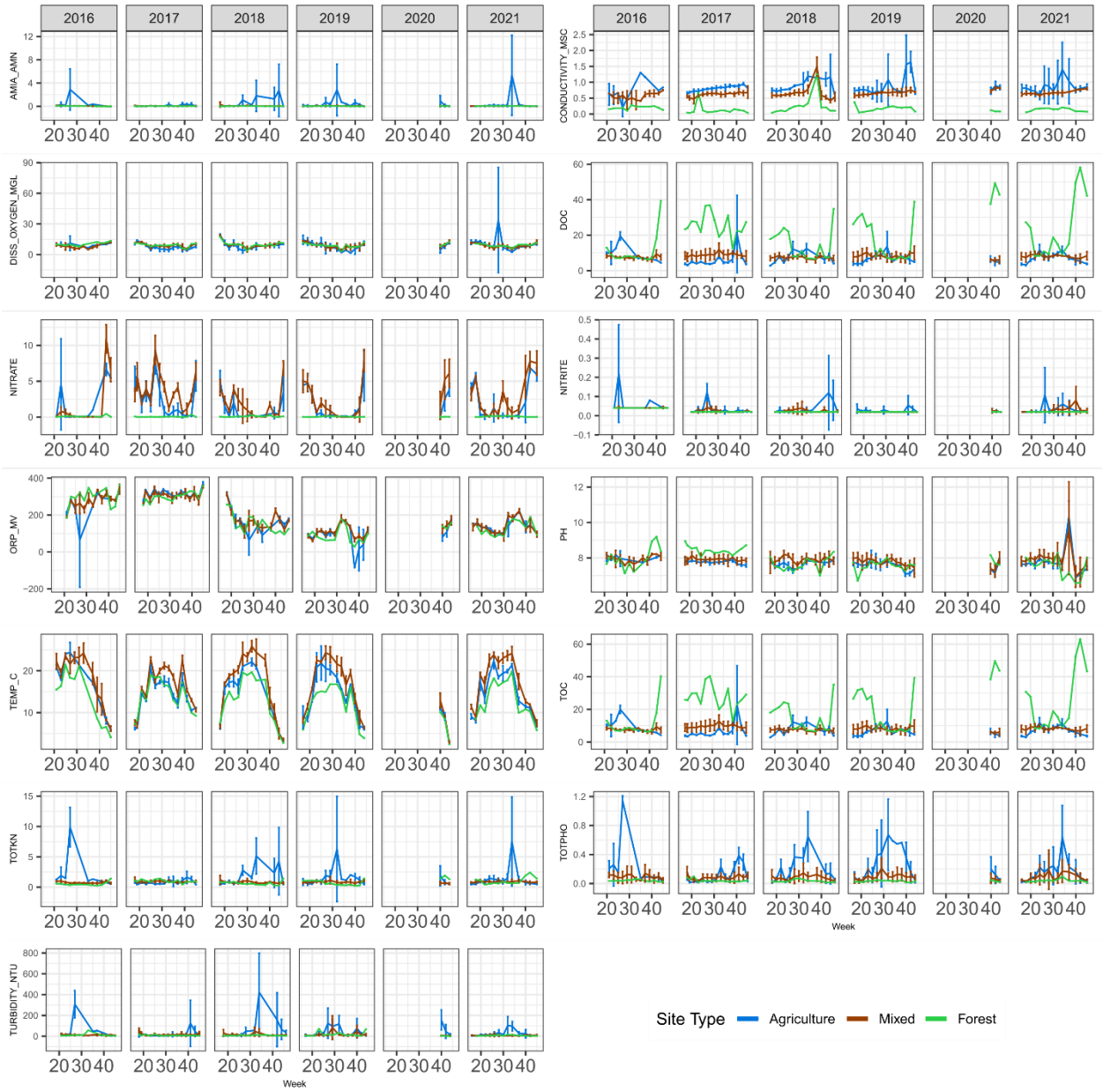


Figure 3. Temporal variation of water physicochemical properties across land use classes. The error bars represent the standard error around the data points. No error bars are reported for forested sites due to $n = 1$ replicate per time point.

Table 1. Comparison of water physicochemical properties by land use class across all sampling years using linear mixed effects model. ANOVA was performed on a linear mixed effects model where land use class was a categorical fixed effect; sampling date and sampling block were random effects. Pairwise comparisons were performed by estimated marginal means with Tukey *P* value adjustment. Significance levels are denoted as *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; and ****, $P \leq 0.0001$.

Water Property	**ANOVA *P* Value**	**Pairwise comparison between land use classes**		
		Agr-Mixed *P* Value	**Agr-Forest *P* Value**	**Mixed-Forest *P* Value**
AMIA_AMN	0.1145	0.0681	0.1311	0.9799
CONDUCTIVITY_MSC	0.0815	0.2012	0.0168*	0.0344*
DISS_OXYGEN_MGL	0.8751	0.9994	0.8753	0.8839
DOC	0.0370*	0.5970	0.0059*	0.0065*
NITRATE	0.0919	0.4269	0.0589	0.0256*
NITRITE	0.5373	0.5920	0.4745	0.8467
ORP_MV	0.6984	0.6953	0.9988	0.7503
PH	0.5021	0.3631	0.6960	0.9273
TEMP_C	0.0919	0.0821	0.3979	0.0417*
TOC	0.0370*	0.5889	0.0060*	0.0066*
TOTKN	0.1584	0.1041	0.1891	0.9919
TOTPHO	0.4839	0.4543	0.3562	0.8014
TURBIDITY_NTU	0.0957	0.0517	0.0860	0.8996

As rainfall affects the stream hydrology, we measured the water discharge at the Castor River, which runs through the sampling region. This discharge data can be used as a proxy for regional precipitation. In 2016, rainfall was sparse and often manifested as isolated, substantial events, rendering this year unusually dry compared to the other sampling years. This dryness was evident in both the cumulative precipitation and river discharge data (Figure A.5), leading to the exclusion of samples from shallow agricultural ditch sites (SN_18, 19, 20, 21) and forested site (SN_24). In contrast, 2017 experienced a wet year with frequent and heavy rainfall events (cumulative rainfall > 600 mm). The remaining years saw more consistent rainfall patterns based on the river discharge data, resulting in minimal sample loss attributed to stream drying (Table A.2).

3.2 Fungal communities in the SNR basin

Next, we examined the freshwater mycobiota diversity and compositional structure from the different land use classes. In this study, a total of 503 stream samples were collected (Table A.2), yielding 10,021,213 high quality paired-end reads. On average, each sample retained 19,922 ± 20,737 (MEAN ± SD) reads. These sequences were clustered into 6,571 OTUs at a 97% sequence identity threshold. The OTU rarefaction curves indicated nearly complete recovery of OTUs for each sampling site (Figure A.6), suggesting that further sampling is unlikely to significantly enhance OTU diversity at these sites.

The majority of recovered OTUs belonged to *Ascomycota* (86.85% of total reads, 73.95% of total OTUs), *Basidiomycota* (8.53%, 18.55%), and *Chytridiomycota* (4.26%, 5.07%). The remaining 2.43% of the OTUs, accounting for only 0.37% of total reads, were assigned to 12 known fungal phyla and an unassigned clade GS01 (Table A.3). Ascomycota exhibited higher

abundance in agricultural and mixed-use sites compared to the forested site, while Basidiomycota displayed the opposite pattern (Figure A.7). Out of all the recovered OTUs, approximately one-third (1,720 OTUs) were shared among all three land use classes (Figure A.8), collectively representing 89.72% of the total abundance.

To identify the genera containing known freshwater fungal species, we compiled 284 aquatic fungal genus names from the Freshwaterfungi.com and the Freshwater Ascomycetes databases. Of these genera, the hierarchical taxonomic lineages of 215 were retrieved from NCBI taxonomy database and/or Index Fungorum database. Due to the limited taxonomic coverage of the two freshwater fungal databases, we assigned 138 OTUs (accounting for 3.38% in total abundance) to 58 genera that contain known freshwater fungal species. The majority of these OTUs were assigned to Dothideomycetes and Sordariomycetes in Ascomycota, and were dominated by the genera *Phaeosphaeria*, *Biappendiculispora*, *Trichoderma*, *Myrmecridium*, *Nigrograna*, *Crassiclypeus*, *Periconia*, *Trematosphaeria*, *Nectria*, and *Lentithecium*. In summary, the taxa identified as true freshwater fungi constituted only a small fraction of the overall stream mycobiota. These genera were distributed across all land use classes and did not exhibit significant differences in abundance (Table 2).

3.3 Impact of land uses on stream fungal diversity

Previous studies in the SNR basin have demonstrated that water with pristine status harboured greater bacterial diversity (Chen et al., 2018). In the present study, we aimed to determine whether pristine freshwater environments would similarly exhibit a more diverse fungal community. Alpha diversity, measured using Shannon-Wiener True Diversity (SW-TD), was highest at the forested site, followed by mixed-use sites (forest-mixed, $P = 0.0531$), and

agricultural sites (forest-agr, $P = 0.0369$, Figure 4A). The seasonal dynamics of alpha diversity varied significantly among sampling years and land use classes. Notably, in 2016, stream fungal diversity was low at all sampling sites, particularly at the forested site (small head stream, Figure 4B). Nonetheless, the yearly coefficient of variation (CV) of fungal alpha diversity was not significantly affected by the land use classes, where CV (MEAN ± SD) was (0.62 ± 0.17) for the forested site, followed by (0.71 ± 0.17) at mixed-use sites, and (0.68 ± 0.11) at agricultural sites. This suggests that the relative magnitude of variation in diversity was not impacted by land use across sampling years. Notably, there were prominent spikes at the beginning of the sampling season in 2017, 2018, 2019, and 2021 at the forested site, as well as in 2017 and 2018 at mixed-use sites. Additionally, spikes were observed during week 30-34 at forested site, especially in 2018 and 2021 (Figure 4B). These spikes were possibly associated with snowmelt or heavy rainfall events, as river discharge was elevated during these time periods (Figure A.5B).

To determine the water physicochemical and hydrological properties driving alpha diversity, we fitted random forest models for each land use class (Figure 4C). The explanatory power was higher for mixed-use ($R^2 = 0.4167$) and forested ($R^2 = 0.4604$) sites but dropped for the agricultural sites ($R^2 = 0.2023$). This suggests that alpha diversity at the latter sites was mostly influenced by additional environmental drivers (such as fungicides) yet to be discovered. River discharge emerged as primary driver of fungal diversity, and it exhibited a positive correlation with alpha diversity (agriculture, $\rho = 0.36$, $P < 0.0001$; mixed, $\rho = 0.52$, $P < 0.0001$; forest $\rho = 0.67$, $P < 0.0001$, Figure A.9). Water nitrate concentration was the second most important factor influencing the stream fungal diversity at agricultural and mixed-use sites, but it held less significance for the forested site. This is due to limited anthropological interventions, leaching, and runoff of nitrate at the forested site (Figure 3). In contrast, conductivity, total and

dissolved organic carbon, and total nitrogen concentration emerged as important factors for the forested site, suggesting a sensitive response of the freshwater fungal diversity to these factors.

Table 2. Habitat descriptions of most abundant fungal genera that contain known aquatic fungal species.

Genus	# of OTUs	Total Relative Abundance (%)	Mean Relative Abundance (%)[1]			*P* Value[2]	Trophic Mode	Fungal Guild	Habitat Description[4]
			Agr	Mixed	Forest				
Phaeosphaeria	12	2.4055	2.6633	1.1552	0.7691	0.0509	Pathotroph-Saprotroph	Fungal Parasite-Plant Pathogen-Plant Saprotroph	Diverse genus, with diverse habitats, can be found in freshwater environments
Biappendiculispora	2	0.1646	0.3882	0.0892	0.1530	0.2774	Pathotroph-Saprotroph	Plant Pathogen-Undefined Saprotroph	Submerged wood or dead herbaceous grass stems
Trichoderma	9	0.1421	0.0947	0.0117	0.1768	0.3813	Pathotroph-Saprotroph-Symbiotroph	Animal Pathogen-Endophyte-Epiphyte-Fungal Parasite-Plant Pathogen-Wood Saprotroph	Diverse genus with diverse habitats, including soil, wood, freshwater, and marine water
Myrmecridium	7	0.1242	0.1915	0.0711	0.0483	0.0718	Saprotroph	Undefined Saprotroph	Submerged decaying wood, leaf litter, stems, and leaves of herbaceous plants
Nigrograna	3	0.0853	0.0661	0.1822	0.0152	0.4058	Pathotroph	Animal Pathogen	Submerged wood from twigs of shrubs and trees
Crassiclypeus	2	0.0759	0.1464	0.0891	0.0200	0.6879	Pathotroph-Saprotroph	Plant Pathogen-Undefined Saprotroph	Submerged dead twigs of woody plants
Periconia	7	0.0749	*NA*[3]	*NA*[3]	*NA*[3]	*NA*[3]	Pathotroph-Saprotroph-Symbiotroph	Endophyte-Plant Pathogen-Wood Saprotroph	Decaying wood in terrestrial, freshwater, mangrove, and marine environments
Trematosphaeria	2	0.0448	0.0091	0.1454	0.0006	0.6304	Saprotroph	Undefined Saprotroph	Terrestrial or submerged wood in freshwater
Nectria	1	0.0409	0.1427	0.0305	0.0146	0.5021	Pathotroph-Saprotroph-Symbiotroph	Animal Pathogen-Endophyte-Fungal Parasite-Lichen Parasite-Plant Pathogen-Wood Saprotroph	Diverse genus with diverse habitats, including wood bark, dead wood, submerged twigs
Lentithecium	1	0.0320	0.0071	0.0586	0.0491	0.2102	Saprotroph	Wood Saprotroph-Plant Saprotroph	Submerged wood, grass in freshwater or terrestrial environments

[1] Mean relative abundance computed by estimated marginal mean from linear mixed effects model.

[2] *P* values represent the effect of land use class on difference in mean relative abundance using linear mixed effect modeling.

[3] NA values were introduced due to insufficient data to run linear mixed effects model.

[4] References for description of genera habitat available in Table A.4

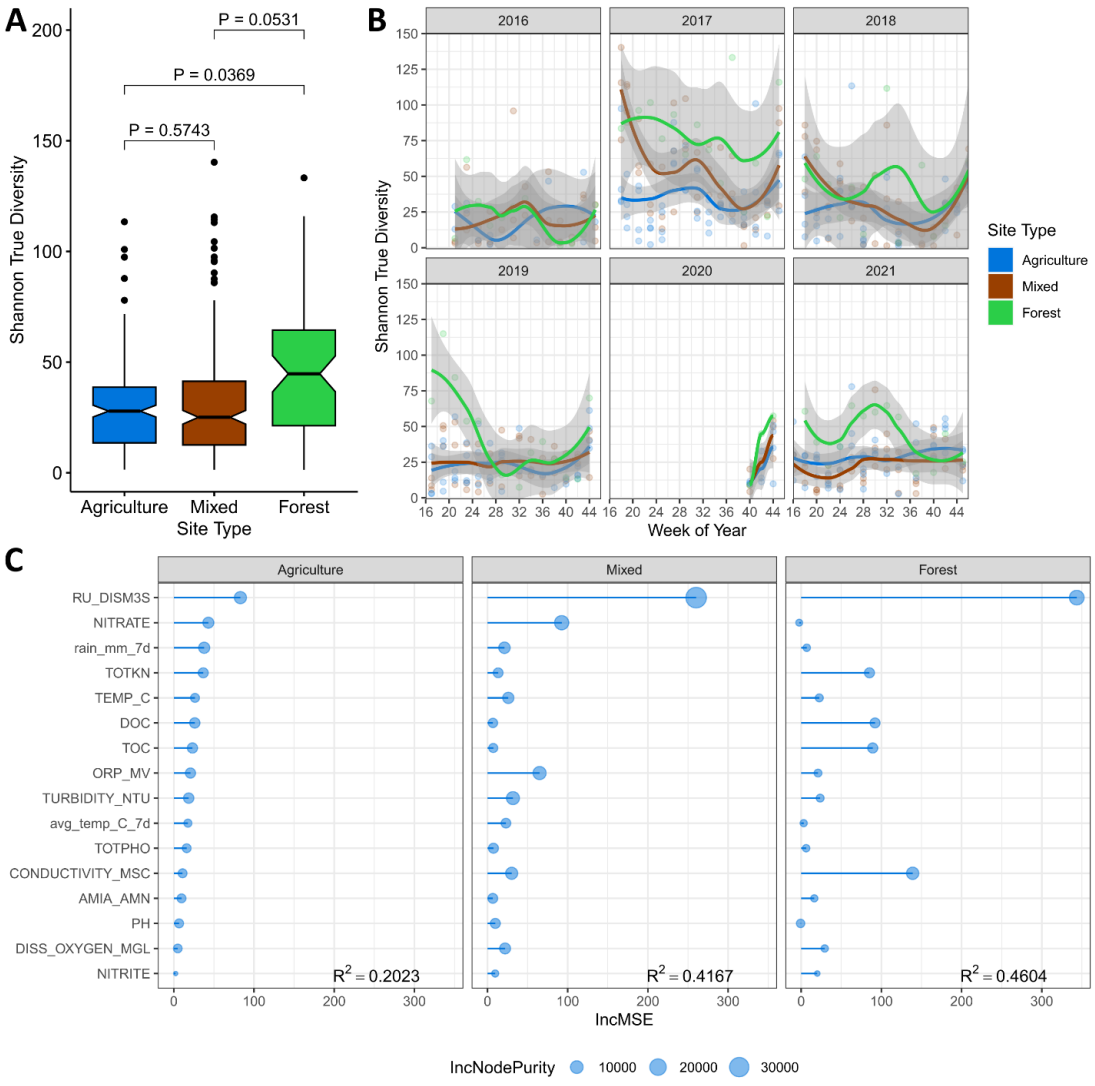


Figure 4. Alpha diversity of surface water samples. (A) Mean alpha diversity, measured using the SW-TD index. Differences in means between agricultural, mixed-use, and forested land use classes were compared using linear mixed effects models. (B) The seasonal dynamic trend of Shannon-Weiner True Diversity index (SW-TD) fitted using local polynomial regression across all sampling years. (C) The importance of environmental variables and their contribution to alpha diversity evaluated using random forest regression, computed for each land use class. The importance of environmental variables in the agricultural sites was ranked based on their Increase in Mean Squared Error (IncMSE), which indicate the rise in mean square error when a specific environmental variable is omitted from the model. The R^2 value of the final random forest model is reported for each land use class.

3.4 Impact of land use on stream fungal community compositional structure

The land use classes had a significant impact on the compositional structure of the fungal community, as indicated by the perMANOVA result (ADONIS2, $F = 16.15$, $P < 0.001$). The dbRDA plot (Figure 5A) shows that samples from the forested site formed a distinct community cluster, while most of the samples from the agricultural and mixed-use sites (both influenced by agricultural activities) were grouped together in a larger cluster, separate from the forested site. This suggests that agricultural activities have an impact on the freshwater fungal community compositional structure. Gradient forest analysis (Figure A.10) identified the most important environmental variables influencing the shift in community compositional structure, including river discharge, 7-day mean temperature, water temperature, water conductivity, and total organic carbon. However, the explanatory power of these environmental parameters was small, both in the gradient forest ($R^2 < 0.02$), and dbRDA ($R^2 = 0.1555$) analyses, indicating that factors other than water physicochemical properties and land use have a greater influence on the beta diversity of stream mycobiota in the study area.

To move beyond this static picture and understand temporal changes in the freshwater mycobiota, we analyzed the rate of community composition shift in each sampling year (Figure 5B), excluding year 2020 due to undersampling during the COVID-19 lockdowns. Based on Bray-Curtis dissimilarities between community centroids at adjacent time points, ANOVA analyses revealed that land use impacted community composition changes in all sampling years (2016, $P = 0.0004$; 2017, $P < 0.0001$; 2018, $P < 0.0001$; 2019, $P = 0.0003$; 2021, $P < 0.0001$; Figure 5B). The difference in the rate of change between the agricultural and mixed-use sites was not significant except in 2016, where agricultural sites (shallow drainage ditches) showed a greater composition change than the mixed land use sites (larger tributaries, $P = 0.0027$). In

2016, the agricultural and forested sites (small and shallow head stream) shared similar levels of community change, while in other years, the forested site exhibited a significantly greater rate of community composition change compared to agricultural sites (Tukey test; 2016, $P = 0.9984$; 2017, $P < 0.0001$; 2018, $P = 0.0010$; 2019, $P = 0.0028$; 2021, $P < 0.0001$). Across the sampling years, agricultural sites exhibited higher community composition change in 2016 (drought year) compared to other sampling years (Tukey test, $P < 0.0001$). In contrast, the forested site did not show differences in composition change rate throughout all sampling years.

3.5 Impact of land use on fungal community trophic modes and function

To investigate how land use may impact the trophic modes and ecological functions of the stream mycobiota, we utilized FUNGuild database (Nguyen et al., 2016) to assign functional guilds to 2,498 OTUs (representing 801 genera) out of 6,571 OTUs. Among these functional guilds, saprotrophs, animal pathogens, and plant pathogens were most abundant across all sites, but their abundances did not differ significantly among the three land use classes (Figure A.11). Both saprotrophs and plant pathogens exhibited seasonal trends, with higher abundance observed later in the sampling season for each year, typically starting around at week 30 (late July – early August) and peaking at the end of the sampling season in autumn (Figure 6). Notably, *Cladosporium*, *Ramularia*, and *Alternaria* were the most abundant saprotrophs and may contain plant pathogens. In contrast, animal pathogens did not exhibit obvious seasonal trends except for a peak in summer 2018 across all sites, primarily due to the enrichment of *Sarocladium* spp. (Figure A.12).

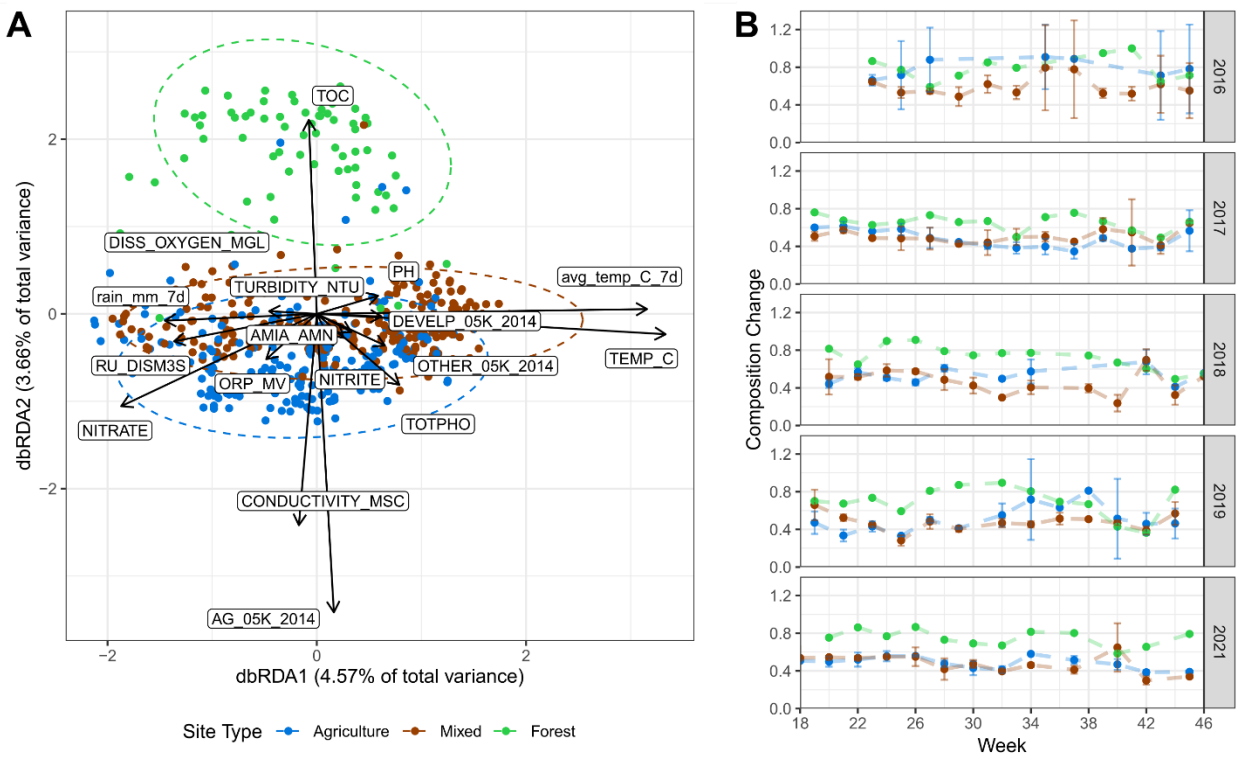


Figure 5. Changes in stream fungal community compositional structure. (A) dbRDA of the surface water fungal community in the SNR basin based on Bray-Curtis distance of Hellinger-transformed OTU table and standardized environmental metadata, showing the contribution of dbRDA axes to the total community variation. The overall adjusted R^2 of the dbRDA model was 0.1555. (B) Changes in community compositional structure between sampling time points, expressed as Bray-Curtis distance between centroids of replicates. Error bars represent the dispersion change or average distance between replicates and their centroids at each time point. No error bars are reported for forested sites due to $n = 1$ replicate per time point. Year 2020 was excluded from the analysis of compositional structure changes due to undersampling during the COVID-19 lockdowns.

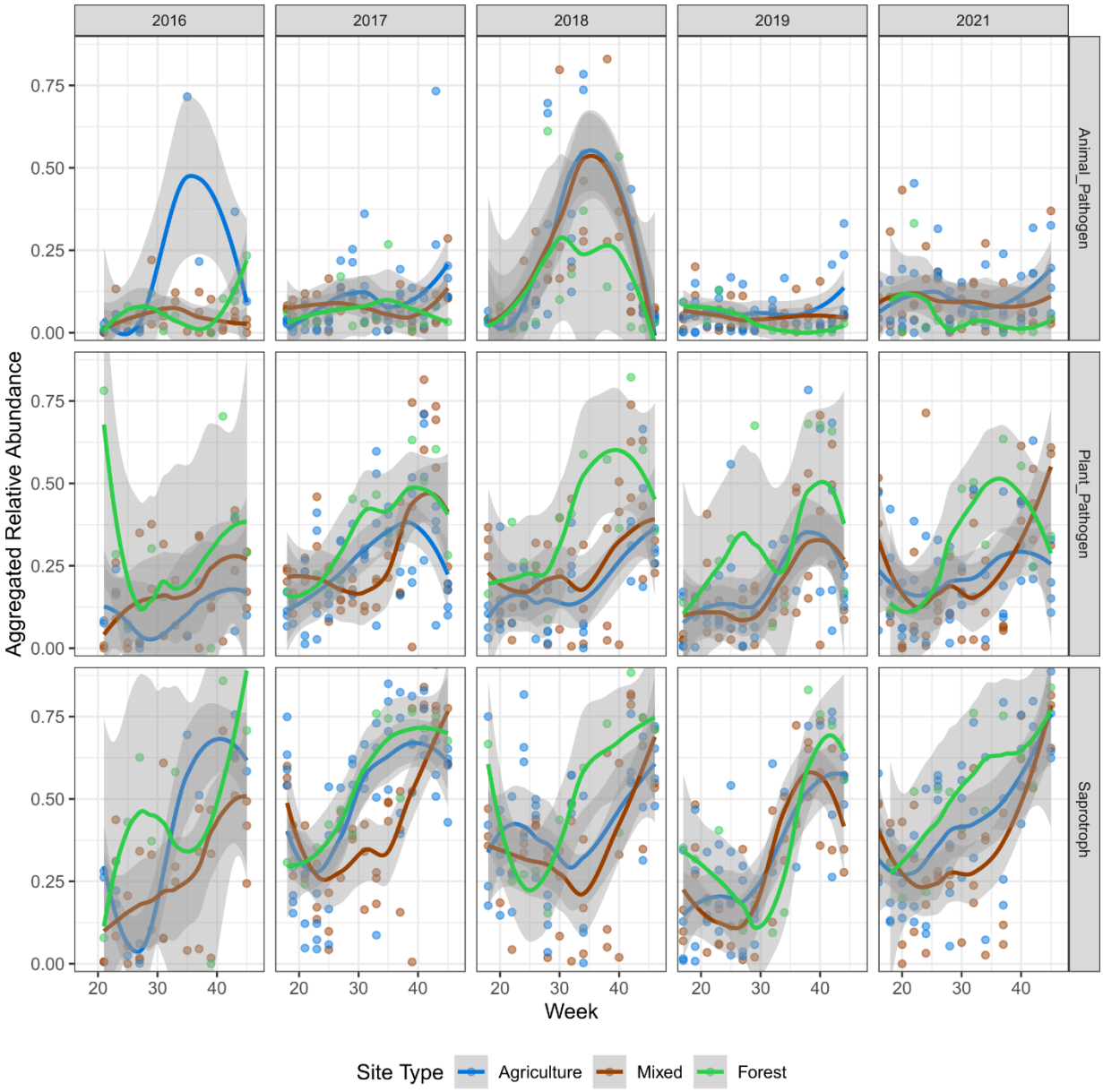


Figure 6. Dynamics of functional guild abundance. OTUs annotated using FUNGuild with guilds containing 'animal pathogen', 'plant pathogen', or 'saprotroph', were aggregated together, resulting in an aggregated relative abundance measure. Trend lines were fitted using local polynomial regression, and the shaded area represents the standard error. The year 2020 was not plotted due to undersampling during the COVID-19 lockdowns.

3.6 Stream fungi as indicators for land use classes

To identify potential indicators in stream mycobiota for different land use classes, we selected the ten most abundant fungal genera recovered from each land use. These genera exhibited high prevalence among sites and year, making their detection easy and reliable – two essential criteria for effective indicators (Warnasuriya et al., 2023). *Ramularia* stands out as the top candidate indicator for two reasons. First, it is the only genus that was highly abundant across stream sites of all three land use classes and exhibit significant differences in abundance between these classes (Figure 7A-C). Its relative abundance decreased from the forested site (mean relative abundance of 10.67%) to mixed-use (4.01%, $P = 0.0357$) and agricultural sites (1.91%, $P = 0.0204$). Its abundance peaked during summer (past week 30, late July to early August) at all three land-use sites, occurring earlier at the forested site except in 2016, which could be attributed to the relatively dry season in 2016 (Figures 7D, A.5A). This summer peak is driven by a decrease in water temperature at both agricultural ($\rho = -0.43$, $P < 0.0001$) and mixed-use sites ($\rho = -0.60$, $P < 0.0001$) with the change of season (Figure 3). In addition to water temperature, particularly at mixed-use sites, *Ramularia*'s abundance was moderately correlated with ammonia and ammonium concentration ($\rho = -0.32$, $P = 0.0002$), water conductivity ($\rho = 0.21$, $P = 0.0435$), nitrate concentration ($\rho = 0.32$, $P = 0.0001$), and total Kjeldahl nitrogen concentration ($\rho = -0.24$ $P = 0.0112$). Note that the abundance of *Ramularia* was not correlated with any water properties at the forested site.

Neosetophoma stands out as the second top candidate indicator for agricultural land use, ranking second in abundance (3.79%) at the agricultural drainage ditch sites, appearing early or in the middle of the sampling season, and being significantly less abundant at the mixed-use sites (1.29%, $P = 0.0445$) and the forested site (0.33%, $P = 0.0437$). Other candidates include

Venturiocistella, *Mycosphaerella*, and *Peniophora,* which were among the most abundant genera at the forested site compared to mixed-use and agricultural sites ($P < 0.05$). *Venturiocistella*'s abundance showed a clear seasonal pattern at the forested site, increased from week 40 (early October) when water temperature started to drop ($\rho = -0.60$, $P < 0.0001$). *Mycosphaerella* showed a weaker seasonal trend, with its relative abundance increasing after week 30 at the forested site; however, there was no significant correlation between *Mycosphaerella* relative abundance with water temperature. Lastly, *Peniophora'*s relative abundance increased in the middle of the sampling season at week 30, but this trend did not consistently occur, as 2019 had a low relative abundance of *Peniophora* throughout the sampling year.

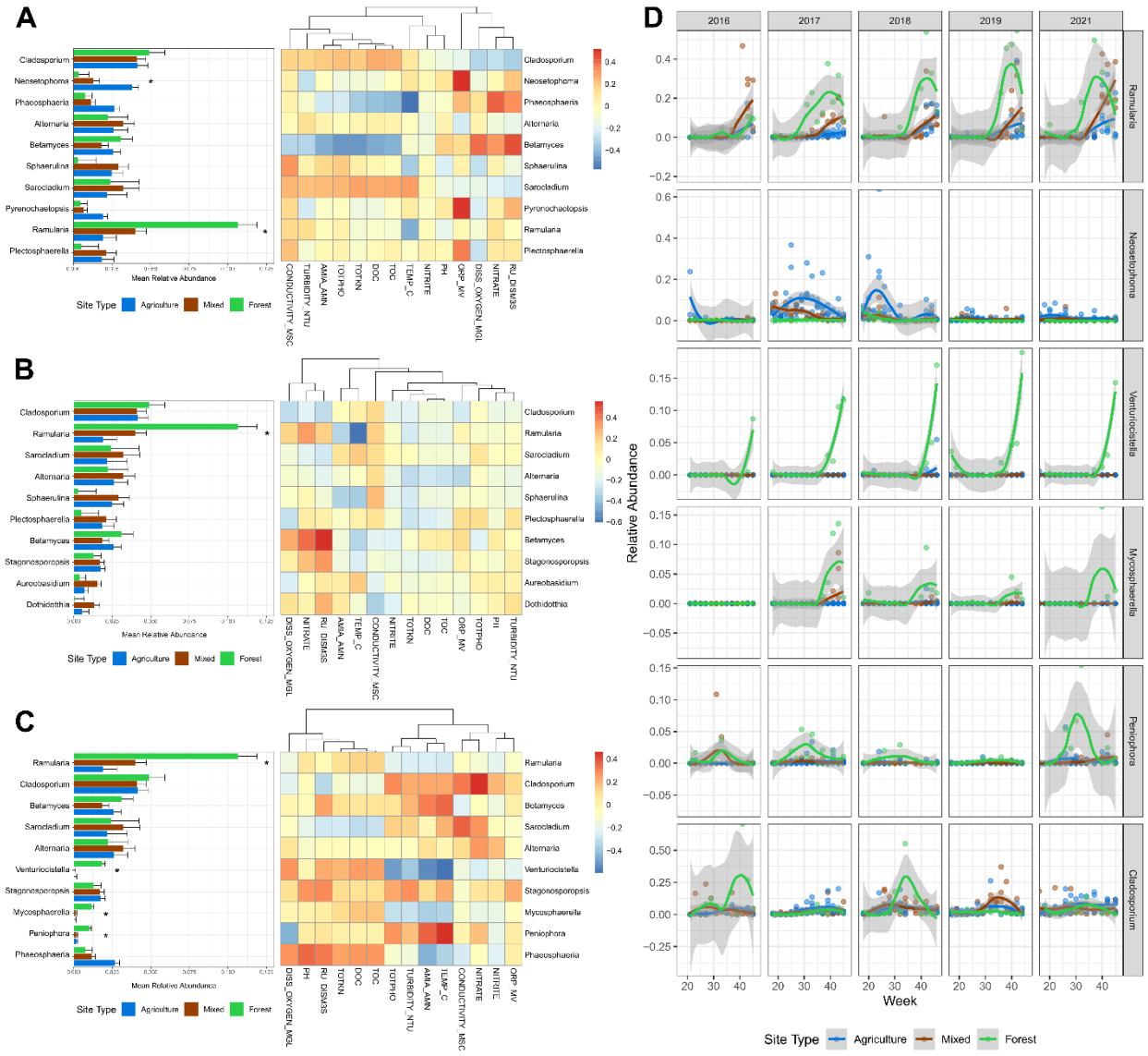

A
B
C
D
Mean Relative Abundance
Site Type
Agriculture
Mixed
Forest
Relative Abundance
Week
2016
2017
2018
2019
2021
Ramularia
Neosetophoma
Venturiocistella
Mycosphaerella
Periophora
Cladosporium

Figure 7. Relative abundance and seasonal dynamics of the most abundant genera with correlations to water physicochemical properties between land use classes. (A - C) Relative abundance of the top ten most abundant genera with correlation heatmap of relative abundance with water physicochemical properties at (A) agriculture sites, (B) mixed-use sites, and (C) forested site. The mean relative abundance was computed with estimated marginal mean of the linear mixed effect models, where whiskers denote standard error. Differences in mean relative abundance were computed with linear mixed effects models, where significant difference in mean relative abundance due to the effect of land use class are denoted *, $P < 0.05$. The columns of the correlation heatmap were clustered using the 'hclust' method for each land use class. (D) Temporal variation in selected genera over time by land use class with a fitted local polynomial regression line. Fungal genera were selected due to a significant difference in mean relative abundance, except for *Cladosporium*, which was selected due to having the highest mean relative abundance. Year 2020 was not plotted for analysis of temporal variations due to undersampling during the COVID-19 lockdowns.

Chapter 4: Discussion

By integrating fungal metabarcoding data with information on land use, hydrology, weather, and water physicochemical attributes across agricultural drainage ditches and streams within the agriculturally dominated SNR basin in Eastern Canada, we revealed substantial disparities in fungal diversity and community compositional structure among agricultural, mixed-use, and forested land uses. Remarkably, despite the acknowledged significance of hydrological, weather, and water physicochemical factors, they only accounted for a minor proportion of fungal community's variance. This implies that unaccounted variables such as fungicide application and potentially stochastic processes may wield a more pivotal influence over the dynamics of the stream fungal community. Additionally, we pinpointed specific terrestrial fungal genera capable of serving as indicators for agricultural land use, suggesting their potential introduction into the stream through runoff, soil erosion, and plant residues from neighboring agricultural area.

4.1 Discovery of stream mycobiota in the agriculturally influenced SNR basin in Eastern Ontario

The present study first sought to identify fungal genera containing known freshwater fungal species utilizing two databases: the Freshwater Ascomycetes database (Shearer and Raja, 2010) and freshwaterfungi.org (Calabon et al., 2020). Due to limited taxonomic coverage in these two databases (Calabon et al., 2023), we only identified 138 OTUs across 58 freshwater fungal genera, representing 3.38% of total abundance in our sequencing dataset, which is likely an underestimate. The remaining recovered fungal sequences represent fungi not annotated in freshwater fungal databases. These may include potential unannotated true aquatic fungi and terrestrial fungi. The fungi identified as freshwater aquatic exhibited no significant variations in

abundance among land use classes (Table 2), suggesting their widespread presence in the study area. These fungi are known to colonize submerged wood or herbaceous grass stems (Calabon et al., 2020), aligning with the key ecological role of decomposing organic matter in the stream environments.

In terms of fungal communities composition, Ascomycota dominated across all land use classes (78.4 – 87.9% mean relative abundance), in contrast to other metabarcoding studies in freshwater settings where they constitute less than 40% of mean relative abundance (Bai et al., 2018; Lepere et al., 2019; Chen et al., 2020). The majority of the recovered stream mycobiota comprised terrestrial fungi such as *Cladosporium* or *Ramularia*. Unlike studies emphasizing Chytridiomycota dominance in freshwater ecosystems, we found these to be a smaller fraction (Bai et al., 2018; Lepere et al., 2019; Chen et al., 2020). This discrepancy likely arises from primer bias and the usage of different barcode regions. Our study used the ITS2 region with ITS9F/ITS4R primer pair, while Bai et al. (2018) and Chen et al. (2020) targeted the ITS1 region and Lepere et al. (2019) used 18S rRNA gene region. Chen et al. (2022a) showed that the reverse primer ITS4R in this study only perfectly matched 45.2% of Chytridiomycota ITS2 sequences in NCBI, also suggesting their poor recovery. Enhanced identification of freshwater fungi entails mitigating Chytrids primer bias by including 5.8S in addition to ITS2 or using full length ITS metabarcoding (Heeger et al., 2019; Tedersoo et al., 2022) alongside a more comprehensive database encompassing known freshwater fungal diversity.

4.2 Impact of agricultural land use on freshwater fungal communities

Alpha diversity of freshwater fungi is an essential metric reflecting the community's ecological functions, with greater values indicating a diverse assortment of saprotrophs, stable biofilm

communities, and enhanced resilience to disturbance, enhancing stream ecosystem stability (Besemer, 2015). Our study revealed that stream fungal diversity was highest at the forested site (SN_24) compared to agricultural drainage ditch and mixed-use sites (Figure 4A). This aligns with previous studies suggesting alpha diversity is higher in minimally disturbed environments within mountainous regions or national parks, compared to those affected by water pollution and forest cover changes in urban and agricultural areas (Bärlocher et al., 2010; Bai et al., 2018). This underscores the influence of water physicochemical properties and stream basin landscape on stream fungal community biodiversity, potentially serving as an indicator of freshwater quality due to land use impact (Warnasuriya et al., 2023). Similar patterns were observed with aquatic hyphomycetes (Pascoal et al., 2005; Solé et al., 2008), as well as macroinvertebrates and fish communities (Weijters et al., 2009). Additionally, the compositional structure of stream fungal communities significantly differed between the forested site and the agriculturally impacted sites (agricultural drainage ditches and mixed-use sites; Figure 5A). This concurs with Bai et al. (2018) and Ortiz-Vera et al. (2018), highlighting distinct aquatic fungal community structures in anthropogenically impacted freshwater ecosystems compared to relatively pristine environments. In this study, Ascomycota were significantly more abundant in the agricultural ditch sites and mixed-use sites than the forested site, while Basidiomycota abundance exhibited an opposite trend (Figure A.7). These disparities may reflect nutrient availability and substrate composition at these sites. For example, anthropogenic organic inputs like fertilizers and crop residues at agricultural and mixed-use sites favor Ascomycota proliferation, known to adapt to nutrient-rich environments (Solé et al., 2008). Conversely, forested sites may offer more woody debris, enriching Basidiomycota decomposers (Goodell, 2020). This is in contrast to Bai et al. (2018), who observed no significant differences in Ascomycota and Basidiomycota relative

abundance between less developed mountainous regions and downstream regions with increased urban and agricultural land use in the Chaobai River.

Noteworthy differences in dominant genera emerged between land use classes, suggesting their adaptation to site-specific environmental conditions. At the forested site, *Venturiocistella*, *Mycosphaerella*, and *Peniophora* thrived, whereas they were scarce in agricultural drainage ditch sites and mixed-use sites (Figure 7A-C). These saprotrophic fungi play essential roles in nutrient and organic matter recycling by decomposing plant materials. In particular, *Peniophora* species are associated with wood decay in forests and other natural habitats (Lambevska et al., 2013), and many *Mycosphaerella* and *Peniophora* species are plant pathogens infecting crops and forest trees (Cannon and Kirk, 2007; Crous et al., 2009; Nguyen et al., 2016). Not surprisingly, these genera positively correlated with total organic carbon, elevated in the forested site due to unmanaged vegetation. Abundances of *Mycosphaerella* and *Venturiocistella* correlated negatively with conductivity, nitrate, and phosphate, prevalent at agriculturally-impacted sites (agricultural ditch sites and mixed-use sites), implying sensitivity to these water pollutants. Mondal et al. (2007) similarly noted *Mycosphaerella* species' sensitivity to nitrogen enrichment, reducing their biomass. *Ramularia* was the only genus found in high abundance across all land uses, but notably more so in the forested site. In contrast, *Neosetophoma* and *Sphaerulina* were prominent in agricultural drainage ditch sites and mixed-use sites during certain sampling periods (*Neosetophoma*, weeks 20-30; *Sphaerulina* weeks 30-40; Figure A.12), correlating positively with water conductivity, an indicator of nutrient level. *Neosetophoma* (in *Phaeosphaeriaceae*) and *Sphaerulina* (in *Mycosphaerellaceae*) species can be foliar pathogens with a broad host range (Cannon and Kirk, 2007). Their increased abundance in agricultural sites suggests tolerance to environmental stressors, while potentially being

outcompeted in the forested site despite having access to a broader host range due to a more diverse fungal community exerting pressure by competitive exclusion (Hardin, 1960), resulting in their lower abundance. In summary, agricultural land use is associated with reduced stream fungal community diversity, accompanied by shifts in taxa abundance driven by water physicochemical properties and catchment area landscape differences between forested and agricultural land uses.

4.3 Environmental drivers of the stream mycobiota

Several environmental drivers exerted significant influence on the freshwater fungal community. Notably, river discharge, employed as a regional precipitation proxy, emerged as a foremost environmental driver impacting both alpha- and beta diversity (Figures 4C, A.9). A rationale behind this is the transport of organic matter and fungi facilitated by precipitation. Our study revealed elevated total and dissolved organic carbon levels at the forested site, over two-fold compared to agricultural and mixed-use sites. These parameters were strongly correlated with fungal community alpha- and beta diversity at forested site (Figures 4C, 5A). The abundance of riparian and basin plants in forested regions introduces a rich variety and quantity of organic matter to the stream or adjacent soil surfaces (Aitkenhead-Peterson et al., 2003; Hagen et al., 2010). Given the denser surrounding vegetation at the forested site compared to the agricultural or mixed-use sites, the contribution of organic matter from riparian and basin plants was much greater. Precipitation carries leaf litter and deceased plants, especially at the forested site, into the stream, nurturing diverse organic resources that boost fungal diversity (Danger et al., 2016), corroborating the findings reported by Fernandes et al. (2013). Moreover, dead plant matter on soil surfaces can serve as habitats for freshwater fungi outside the stream, subsequently

transported by precipitation (Chauvet et al., 2016). The role of regional precipitation via river discharge lies in ferrying organic carbon and fungi existing outside the stream, thus its pronounced importance (Figures 4C, A.9). Instances of heightened diversity align with elevated river discharge at the start of the sampling season, particularly in the forested site. Such occurrences transpired during the snowmelt period (weeks 16 – 20, day < 150; Figures 4A, A.5), with analogous diversity spikes in mid-season during rainier summer months, notably in the 2017 sampling year (Figure A.5A). Conversely, reduced diversity in 2016 (Figure 4A) stemmed from decreased terrestrial fungi transport to stream ecosystems due to diminished precipitation, leading to lower water discharge – akin to findings by Arias-Real et al. (2021) in analogous arid conditions.

In contrast to the forested sites, the agricultural and mixed-use sites witnessed limited basin forest cover, escalated nutrient loads, and altered water quality due to agrochemical runoff and leaching (Carpenter et al., 1998). This manifested through dominant physicochemical drivers such as nitrate and conductivity impacting fungal alpha- and beta diversity (Figures 4C, 5A). Nitrate positively correlated with alpha diversity (Figure A.9), acting as a fungal beta diversity driver, as seen in prior research (Duarte et al., 2017; Bai et al., 2018). Nitrogen enrichment fostered fungal bioactivity (Gulis et al., 2017), potentially influencing fungal communities. This influence was mirrored by certain genera, like *Betamyces*, which positively correlated with nitrate levels at agricultural drainage ditch sites and mixed-use sites (Figure 7A-B). Surprisingly, pH did not wield notable significance in our study, unlike other research (Bai et al., 2018; Pietryczuk et al., 2018), possibly due to limited pH fluctuation across land use classes and the study duration (Figure 3, Table 1).

While our study identified environmental drivers impacting the freshwater fungal community, it also uncovered the potential resilience of this community to changing water physicochemical properties. This resilience is reflected in comparatively minor compositional changes over time at agricultural and mixed-use sites compared to the forested site (Figure 5B), despite the former experiencing greater water quality fluctuations. Stronger taxa abundance correlations with water physicochemical properties in the forested site than in agricultural and mixed-use sites corroborate this resilience (Figure 7A-C). For example, *Cladosporium* exhibited robust nitrate correlation in the forested site, unlike in the other sites. This resilience aligns with the findings of Chen et al. (2022b) in soil microbiomes, where fungal communities were less sensitive to nutrient loads compared to bacterial communities. Shi et al. (2023) similarly revealed greater sensitivity of aquatic bacterial communities to water physicochemical changes in agricultural ditches compared to fungal communities in the present study. However, the freshwater fungal community might have undergone a degree of selection favoring pollution-tolerant taxa, akin to findings of Ortiz-Vera et al. (2018) in the Tietê river with elevated nitrogen and phosphorus from agricultural activities. Similar selective pressures, such as nutrient inputs and other unmeasured pollutants like pesticides and fungicides at agricultural drainage ditch sites and mixed-use sites, may explain the lower compositional changes and insensitivity to water quality shifts. This could be due to the presence of a narrower range of fungi tolerant to the conditions in these habitats.

Additionally, stochastic processes or other factors like fungicide application could influence the freshwater mycobiota. The dbRDA analysis indicated a meager 13.5% explained variance in fungal community by water physiochemical and land use variables (Figure 5A). This might diverge from previous work, where water physicochemical properties explained 86.2% of

the fungal community structure (Solé et al., 2008). This discrepancy could result from the narrow focus on aquatic fungal species through conidia examination, carried out by Solé et al. (2008), while our study employed metabarcoding for entire communities. The dissimilarity in explained variance might partly stem from stochastic community assembly, a significant factor in wetland soil bacterial and fungal communities (Huang et al., 2022), alongside environmental influences. Fungicide application might account for observed modest variation in aquatic fungal communities at agricultural and mixed-use sites (Figure 5B), and contribute to overall community variability (Staley et al., 2015; Ittner et al., 2018). Although underexplored for freshwater fungi (Ittner et al., 2018), recent studies showcase fungicide's potent influence on soil and aquatic fungal community composition, diminishing diversity (Baudy et al., 2021; Ma et al., 2021). Given that seed treatment and foliar fungicides application is recommended to farmers in the study region for plant disease management (OMAFRA, 2017; 2021), with non-target effects on freshwater fungi possible (Dijksterhuis et al., 2011; Baudy et al., 2021), differences in fungal diversity and community composition between sites could be attributed to fungicide application, as exemplified by the *Ramularia* abundance trend. This genus, containing potential plant pathogens (Videira et al., 2016), might be targeted by regional farmers with azole-based fungicides (Kiiker et al., 2021), for wheat, corn, or soybean cultivation (OMAFRA, 2017; 2021). Our results showed that *Ramularia* abundance was delayed in agricultural and mixed-use sites compared to the forested site (Figure 7D), suggesting fungal community suppression due to agricultural fungicide use. This positions *Ramularia* as a potential indicator of watershed fungicide impact on the aquatic fungal community, contingent on future water measurements to confirm fungicide presence and quantity in the freshwater ecosystem.

4.4 Stream fungal community functionality may be resilient to environmental stressors

Functional redundancy pertains to the capability of diverse species within an ecosystem to execute analogous roles, safeguarding ecological functionality in the face of species loss (Rosenfeld, 2002). Despite agricultural land use's impact on fungal diversity and community composition, the freshwater fungal community may possess resilience against environmental stressors, potentially sustaining their saprotrophic role. This is indicated by the inconsequential variation in relative abundance of saprotrophic fungi across land classes (Figure 6). This finding aligns with the conclusions of Pascoal and Cássio (2004) who observed persistent leaf litter decomposition rates, even in nitrogen and phosphorus-enriched streams. Bruder et al. (2016), when assessing agricultural land use’s effect on leaf litter decomposition, noted minimal nutrient-induced impacts in agricultural streams, attributing reduced litter decomposition to sediment-induced dissolved oxygen reduction. Pascoal and Cássio (2004) also identified low dissolved oxygen's effect. Given freshwater fungi's decomposition dependence on dissolved oxygen (Medeiros et al., 2009), the lack of dissolved oxygen differences between land uses (Table 1) could explain the absence of saprotroph relative abundance differences. Furthermore, Baudy et al. (2021) showed the aquatic fungal community's leaf litter decomposition persistence even under fungicide stress, underscoring potential role of functional redundancy in the mycobiota. This is congruent with the present study's saprotrophic fungi comparable abundance across land use classes, especially with lower diversity observed at agricultural and mixed-use sites (Figure 4A). Notably, this study did not collect leaf litter decomposition data, merely assuming unaffected saprotrophic function in agricultural and mixed-use sites. Investigating the functional redundancy hypothesis warrants examining if agricultural land use substantially alters freshwater fungi's organic matter decomposition ability through techniques like dry leaf litter mass

measurement (Bruder et al., 2016), or RNA sequencing-based litter decomposition gene expression analysis (Bourne et al., 2020). These methods estimate litter decomposition rate, contrasting this study's potential leaf litter decomposing fungi relative abundance estimation.

Chapter 5: Research Synthesis

5.1 Summary of research

This thesis research aimed to explore the impact of agricultural land use on the freshwater fungal community through the application of ITS2 metabarcoding and long-term sampling in the agriculturally influenced SNR basin (Figure 2). By delving into the intricate interplay between the land use and freshwater fungal communities, this study contributes to a deeper comprehension of the ecological consequences of agricultural practices on freshwater fungal communities. The primary findings for this investigation reveal that alpha diversity was reduced at the agricultural drainage ditch sites and mixed-use sites in comparison to the relatively untouched/pristine forested environment (Figure 4A). Notably, distinct community clusters were discernible among these land use classes (Figure 5A). However, these outcomes underscore the significance of factoring in both measured and unmeasured environmental variables when deciphering the dynamics of such ecosystems. The results also suggest that, despite variations in community composition and alpha diversity, the relative abundance of saprotroph-annotated fungi in freshwater systems remained relatively consistent across different land use classes, implying their functional significance in stream ecosystems.

5.2 Scientific contributions

This study brings original contributions to the burgeoning field of freshwater fungal research. Our examination of the influence of agricultural land uses on the freshwater mycobiota has unveiled distinct communities across a gradient of land use features, most notably between the undisturbed forested stream, and the agricultural drainage ditch sites. Notably, our analysis of environmental drivers aligns with studies by Bai et al. (2018) and Ortiz-Vera et al. (2018),

highlighting shared factors like organic carbon, and nitrogen. However, our work has revealed river discharge as the predominant environmental driver, introducing a novel perspective. In this context, river discharge serves as a proxy for regional precipitation – a factor not previously recognized as significant in shaping freshwater fungal community structure. Our findings propose a compelling connection between regional precipitation and the freshwater mycobiota (Figures 4B, A.9), possibly due to the transport of terrestrial fungi, nutrients, and plant material into the stream through rainfall. This association is reinforced by the strong correlation between river discharge and increased stream fungal diversity (Figure A.9). Furthermore, our research emphasizes that the measured environmental variables explain only a fraction of the variance in the freshwater fungal community (Figure 5A). This suggests that unmeasured factors, such as fungicide application or stochastic community assembly processes, may wield a more pronounced influence on the freshwater fungal community than water physicochemical and hydrological properties.

This research stands out due to its distinctive focus on the repercussions of agricultural land use on the freshwater mycobiota, coupled with its long-term sampling endeavour. Previous metabarcoding studies, like those by Bai et al. (2018) and Ortiz-Vera et al. (2018), tackled the broader freshwater fungal communities, without pinpointing the localized consequences of agricultural land uses. Moreover, temporal dynamics of freshwater mycobiota have received limited attention. Although studies like Matsuoka et al. (2021) highlighted seasonal variations in forested stream mycobiota, these authors did not correlate water physicochemical properties with the fungal community. Hence, our work pioneers insights into temporal variations in the fungal community concerning water physicochemical and hydrological variables.

The outcome of this research project fills a critical knowledge gap in the field of aquatic fungi ecology, specifically under the strain of agricultural land uses. This significance lies in the fact that despite the well-documented ecosystem services rendered by aquatic fungi (Seena et al., 2022), our understanding of the environmental impact posed by agricultural activities on the freshwater fungal community remains limited. Previous research on this topic has predominantly focused on fungicides (Dijksterhuis et al., 2011; Ittner et al., 2018; Baudy et al., 2021). Conversely, our study delves into other environmental drivers linked to agricultural land use, such as nitrogen and phosphorus enrichment, which have received less comprehensive investigation (Vatova et al., 2022). By scrutinizing freshwater fungi within this context and evaluating the extent of agricultural impact on this community, we contribute to the advancement of beneficial management practices. This approach aims to mitigate adverse impacts of agricultural activities on ecosystem services (Power, 2010).

5.3 Limitations of research and future work

Several limitations are pertinent to this research project. The experimental design of this study included only one sampling site for the forested land use class, which resulted in a lack of replicates at each timepoint. In contrast, agricultural and mixed-use sites had four and three replicates, respectively. This disparity reduced the statistical power for comparing the forested site and other land use classes. However, the effect size was notable in some cases, allowing the detection of significant results for measures like alpha diversity and abundances of taxa. The absence of replicates for the forested site was due to practical constraints, as the AAFC sampling teams needed to visit all sites and return to the research centre within a single day. Ideally, an equivalent number of replicates for the forested site which would ensure that the results reflect

an undisturbed forested environment, rather than being indicative of just one specific site. Additionally, the forested site had substantially greater tree cover in the riparian zone and surroundings compared to the agricultural or mixed-use sites. Since vegetation density and diversity influences stream fungal diversity and community compositions, especially among indwelling or immigrant species (Fernandes et al., 2013), a grassland reference site with minimal tree cover may offer a better comparison to agricultural drainage ditch sites. This would help better determine the effects of water physicochemical properties on the fungal community.

DNA metabarcoding possesses inherent limitations despite its cost-effective advantages in environmental community analysis (Keck et al., 2017). One limitation stems from the dependency on databases for taxonomic assignment. Unidentified sequences remain a challenge, resulting in a significant proportion of unclassified reads (Keck et al., 2023). Furthermore, current databases for annotated aquatic fungi are far from complete and do not encompass all known aquatic fungi from the literature (Calabon et al., 2023). Addressing this necessitates updates to the UNITE database (Nilsson et al., 2019b), for fungi annotation and specialized databases like freshwater ascomycetes (Shearer and Raja, 2010) or freshwaterfungi.org (Calabon et al., 2020). Moreover, even with suitable reference databases, the current classification methods for ITS metabarcoding provide reliable taxonomic annotation only up to the genus level. At the species or subspecific levels of fungi, the ITS region often lacks sufficient resolution (Blaalid et al., 2013; Thines et al., 2018; Lücking et al., 2020).

While metabarcoding ITS2 has proven successful in previous research (Röhl et al., 2017; Luo et al., 2020), it has limitations. The ITS region does not effectively resolve certain fungal taxa like *Fusarium* or *Aspergillus* (Balajee et al., 2009), and the universal primers may fail to amplify all fungi (Lücking et al., 2020). In particular, ITS2 by itself is not a good marker for

identifying Chytridiomycota species as discussed previously. Despite these drawbacks, ITS remains a preferred marker due to its broad amplification and sequencing success, encompassing a wide range of fungal taxa and enabling its use in biodiversity studies (Schoch et al., 2012). The choice of marker gene and sequencing technology was determined before 2016 and remained consistent to maintain data integrity (Sun et al., 2021). While platforms like Oxford Nanopore or PacBio Sequel II produce longer reads, enhancing taxonomic resolution and eliminating biases against Chytridiomycota OTU recovery (Heeger et al., 2019; Tedersoo et al., 2022), the study's choice of the Illumina MiSeq platform was maintained for consistency.

Another limitation is that metabarcoding only provides compositional structure information, with uncertainty in estimating absolute abundance due to factors like dead or inactive fungal cells and variable ITS copy numbers between taxa (Carini et al., 2016; Lamb et al., 2019; Lofgren et al., 2019). For this study, this would mean that it would not be possible to estimate the impact of agricultural activities on the freshwater fungal biomass. Other research has shown that impacts from agricultural land use such as stream nutrient enrichment (Biasi et al., 2017; Jabiol et al., 2018) and fungicide runoff (Baudy et al., 2021) can have an influence on fungal biomass. Future work could incorporate fungal biomass using quantitative real-time PCR, but this approach would be limited to selected target species, as it would require species-specific primers (Feckler et al., 2017). Another approach would be to estimate environmental ergosterol concentrations, which has been used extensively to estimate environmental fungal biomass (Pascoal and Cássio, 2004; Biasi et al., 2017; Koivusaari et al., 2019). This method estimates total environmental fungal biomass since ergosterol is a membrane lipid found exclusively in fungi, allowing it to be used as a proxy for environmental fungal biomass, though it may also

over estimate fungal biomass since ergosterol from dead fungi is also detected (Mille-Lindblom et al., 2004).

Finally, functional annotation of fungal communities relied on the FUNGuild database, based on the assumption that fungi in the same genus share similar ecological roles (Nguyen et al., 2016). The majority of recovered OTUs lacked taxonomic annotation for guild assignment. Future studies might benefit from shotgun metagenomics or metatranscriptomics, offering enhanced taxonomic resolution, allowing for species level identification, leading to a more precise community composition profile (Quince et al., 2017; Donovan et al., 2018; Becker and Pushkareva, 2023). Metatranscriptomics, or the sequencing of environmental RNA, could additionally distinguish between active and inactive cells, providing insights into community functional roles (Hempel et al., 2022). These approaches have traditionally been used for bacterial communities, but has recently gained attention in fungal community analysis, with the development of specific pipelines such as FindFungi for fungal species identification from shotgun metagenomics data (Donovan et al., 2018). Both approaches would offer a better understanding of freshwater fungal community function and taxonomy, addressing current limitations.

Appendices

A.1 Supplemental Tables

Table A.1. Summary of land use at each sampling site. Land uses are reported as percent land use in water catchment areas up to 5 km upstream for each sampling site.

Site ID	Land Use	Strahler stream order	Agriculture (% basin)	Developed (% basin)	Tree (% basin)	Other [1] (% basin)
SN_5	Mixed	4	89.91	1.83	6.39	1.87
SN_6	Mixed	5	94.09	0	4.09	1.82
SN_10	Mixed	4	77.75	20.05	1.93	0.27
SN_18	Agriculture	2	99.07	0	1.93	0
SN_19	Agriculture	2	100.00	0	0	0
SN_20	Agriculture	2	98.41	0	1.56	0.02
SN_21	Agriculture	2	96.73	0	3.27	0
SN_24	Forest	1	2.13	0	97.87	0

[1] Other land use category consists of grassland, wetland, and rock covered area.

Table A.2. Sample summary by site, land use class, and year. Sampling periods: May 24 – November 8 in 2016, May 1 – November 27 in 2017, April 30 – November 13 in 2018, April 29 – November 4 in 2019, July 13 – November 2 in 2020, and April 20 – November 8 in 2021.

Site ID [1]	Land Use	Number of Samples (n) [2]					
		2016	2017	2018	2019	2020	2021
5	Mixed	10	15	14	14	3	14
6	Mixed	12	15	14	14	3	14
10	Mixed	11	15	14	14	3	14
18	Agriculture	7	15	11	11	2	13
19	Agriculture	0	15	11	14	3	13
20	Agriculture	5	15	11	10	3	12
21	Agriculture	0	15	9	9	3	13
24	Forest	11	15	14	14	3	13

[1] Sampling at sites SN_19 and SN_21 started in the 2017 sampling season.

[2] Year 2020 was under sampled due to restrictions related to COVID-19 lockdowns.

Table A.3. Summary of the abundance and recovered OTUs for each identified fungal phylum.

Phylum	Recovered Sequences	Proportion of Recovered Sequences (%)	Number of OTUs	Proportion of OTUs (%)
Ascomycota	8,703,053	86.85	4,859	73.95
Basidiomycota	854,365	8.53	1,219	18.55
Chytridiomycota	426,997	4.26	333	5.07
Rozellomycota	10,860	0.11	41	0.62
Aphelidiomycota	9,108	0.09	18	0.27
Olpidiomycota	6,381	0.06	10	0.15
Mortierellomycota	4,314	0.04	32	0.49
Mucoromycota	2,353	0.02	21	0.32
Monoblepharomycota	2,307	0.02	13	0.20
Blastocladiomycota	788	0.01	10	0.15
Basidiobolomycota	445	< 0.01	6	0.09
Glomeromycota	169	< 0.01	4	0.06
GS01	26	< 0.01	2	0.03
Kickxellomycota	21	< 0.01	1	0.02
Entomophthoromycota	15	< 0.01	1	0.02
Entorrhizomycota	11	< 0.01	1	0.02

Table A.4. List of references for freshwater fungi annotation in Table 2.

Genus	Reference
Phaeosphaeria	Guild/Trophic Mode information from FUNGuild Habitat + additional annotation from (Magaña-Dueñas et al., 2021)
Biappendiculispora	Guild/Trophic Mode information from FUNGuild Habitat + additional annotation from freshwaterfungi.org
Trichoderma	Guild/Trophic Mode information from FUNGuild Habitat + additional annotation from (Liu et al., 2016)
Myrmecridium	Guild/Trophic Mode information from FUNGuild Habitat + additional annotation from: freshwaterfungi.org
Nigrograna	Guild/Trophic Mode information from FUNGuild Habitat + additional annotation from: freshwaterfungi.org
Crassiclypeus	Guild/Trophic Mode information from FUNGuild Habitat + additional annotation from: freshwaterfungi.org
Periconia	Guild/Trophic Mode information from FUNGuild Habitat + additional annotation from: freshwaterfungi.org
Trematosphaeria	Guild/Trophic Mode information from FUNGuild Habitat + additional annotation from: freshwaterfungi.org
Nectria	Guild/Trophic Mode information from FUNGuild Habitat + additional annotation from (Salgado-Salazar et al., 2015) and Index Fungorum
Lentithecium	Guild/Trophic Mode information from FUNGuild Habitat + additional annotation from (Dong et al., 2020)

A.2 Supplemental Figures

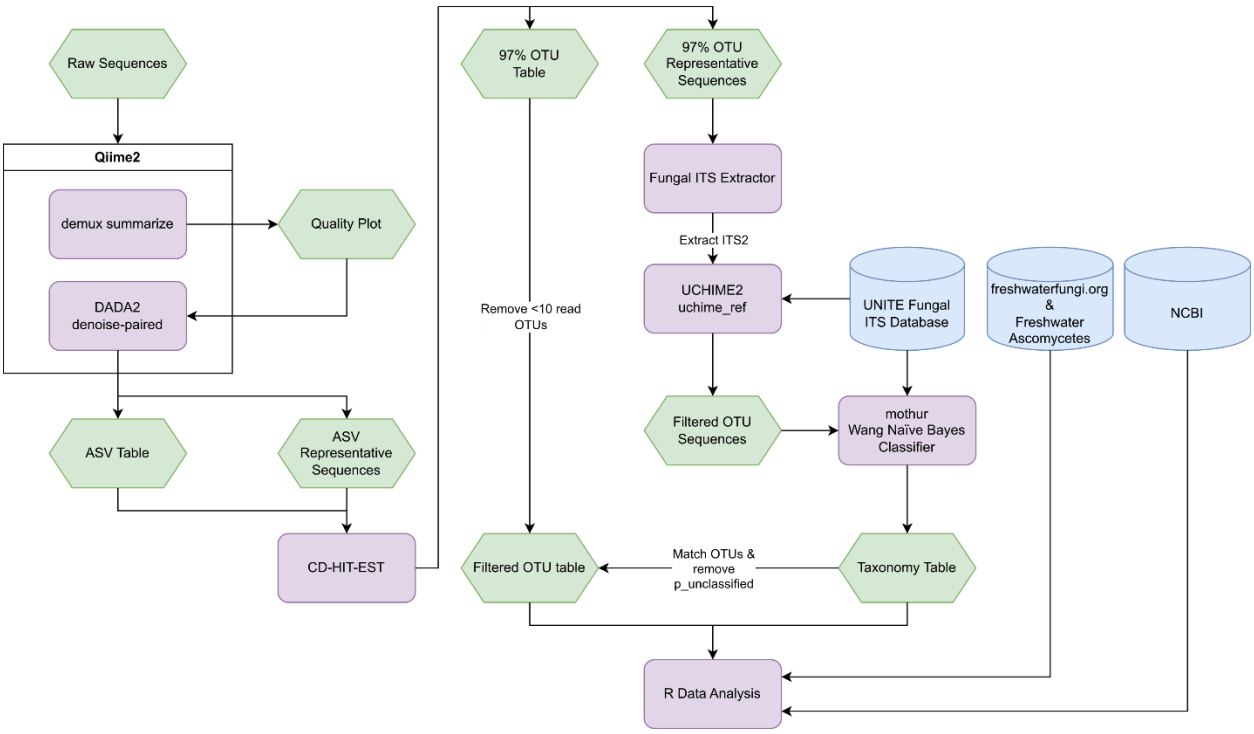


Figure A.1. Summary of metabarcoding data processing steps from sequencing data to data analysis.

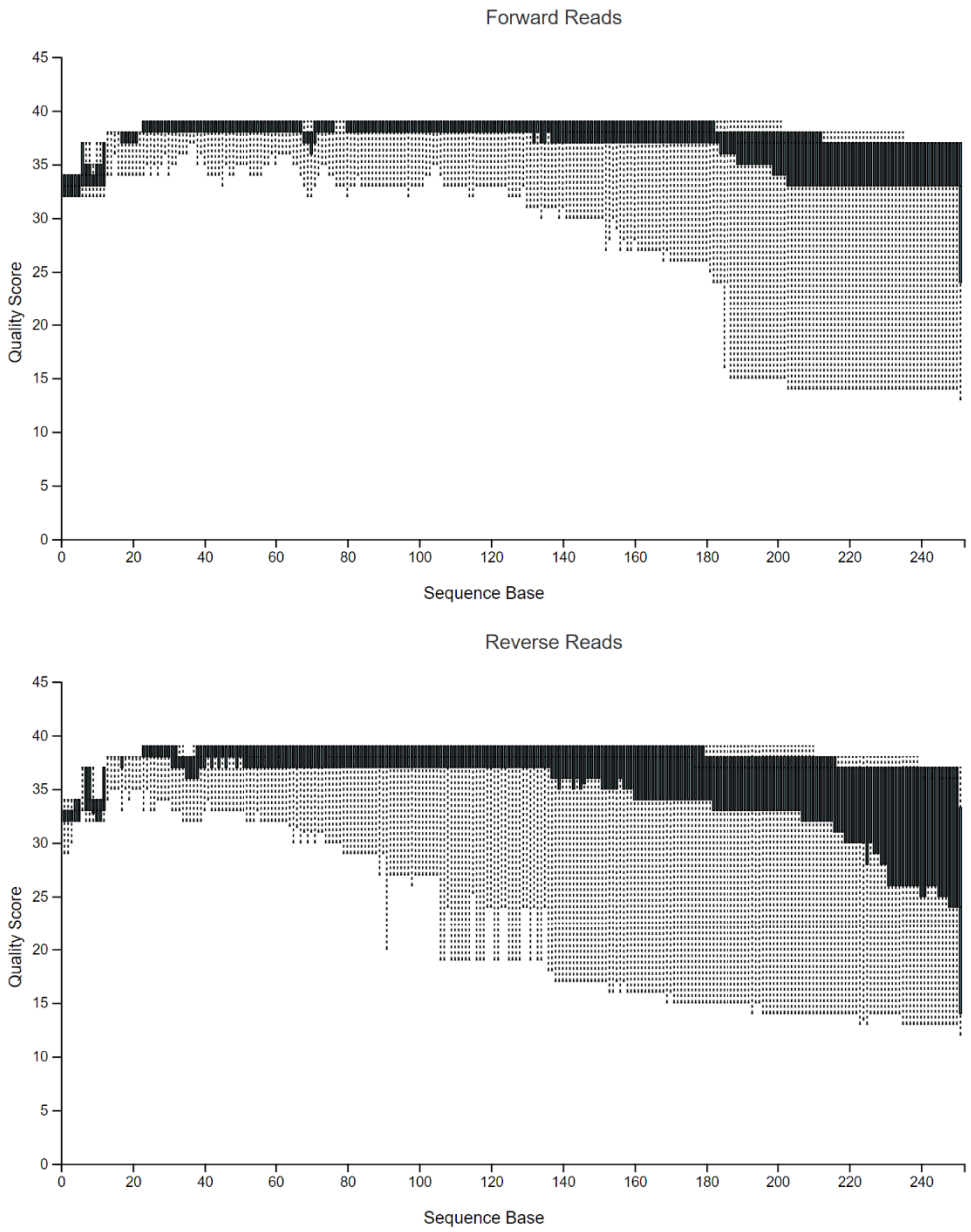
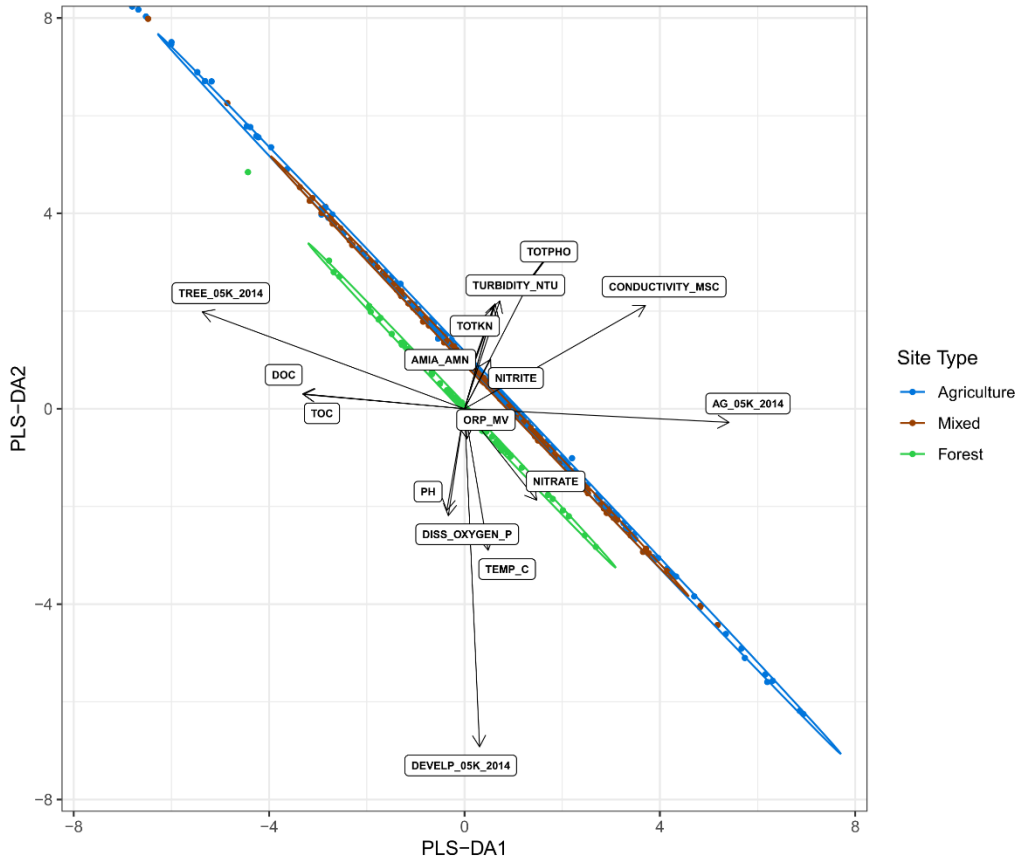


Figure A.2. Raw sequencing data read quality. Quality is expressed using the Phred quality score for each base position, after the removal of adaptors sequences.



Original	Predicted		
	Agriculture	Mixed	Forest
Agriculture	**115**	**105**	**0**
Mixed	**94**	**119**	**0**
Forest	**8**	**15**	**47**

Figure A.3. PLS-DA of sampling sites based on water physicochemical and land use variables, with classification confusion matrix for land use classes. The overall classification error rate is 0.44.

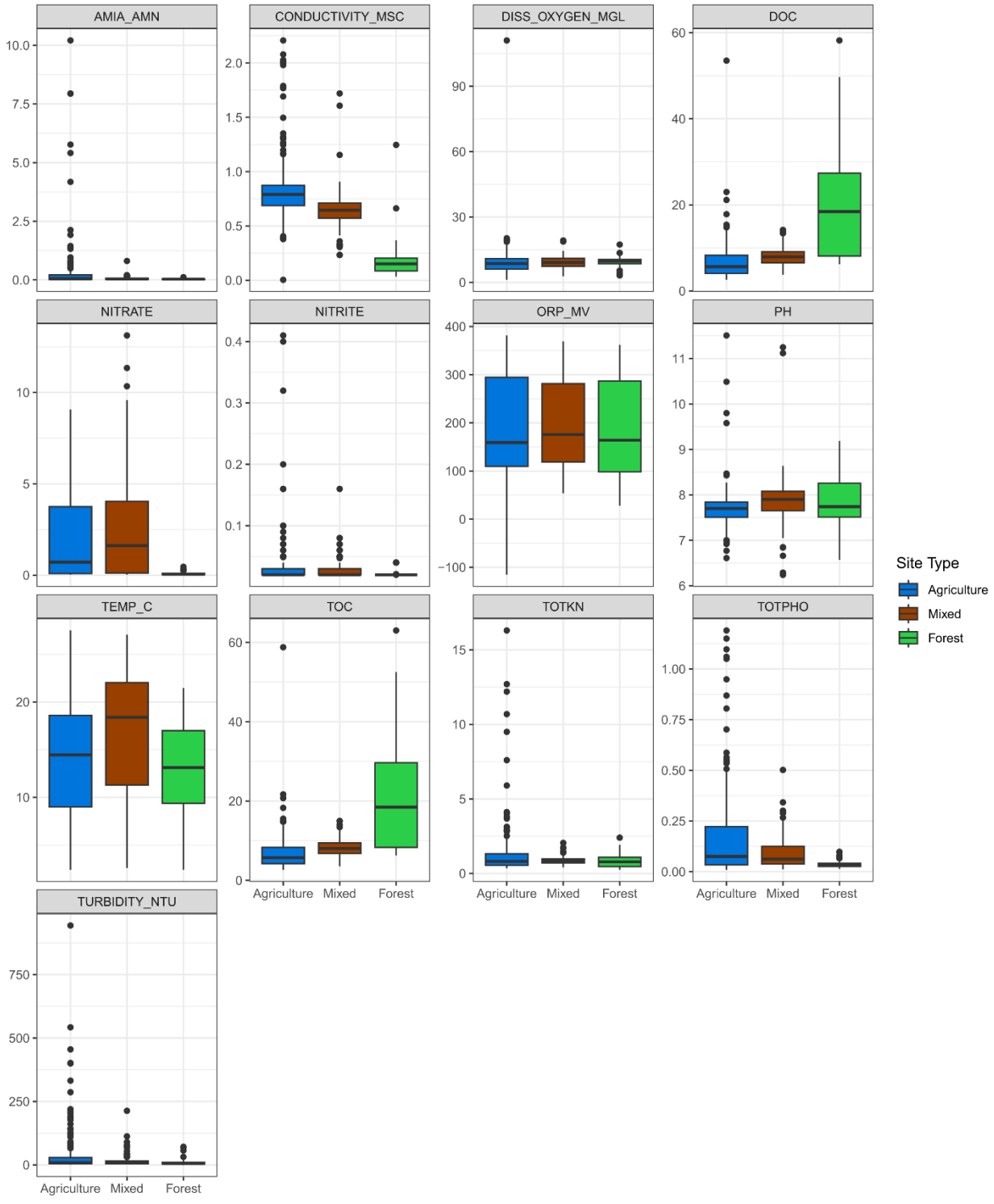


Figure A.4. Mean levels of water physicochemical properties by land use class.

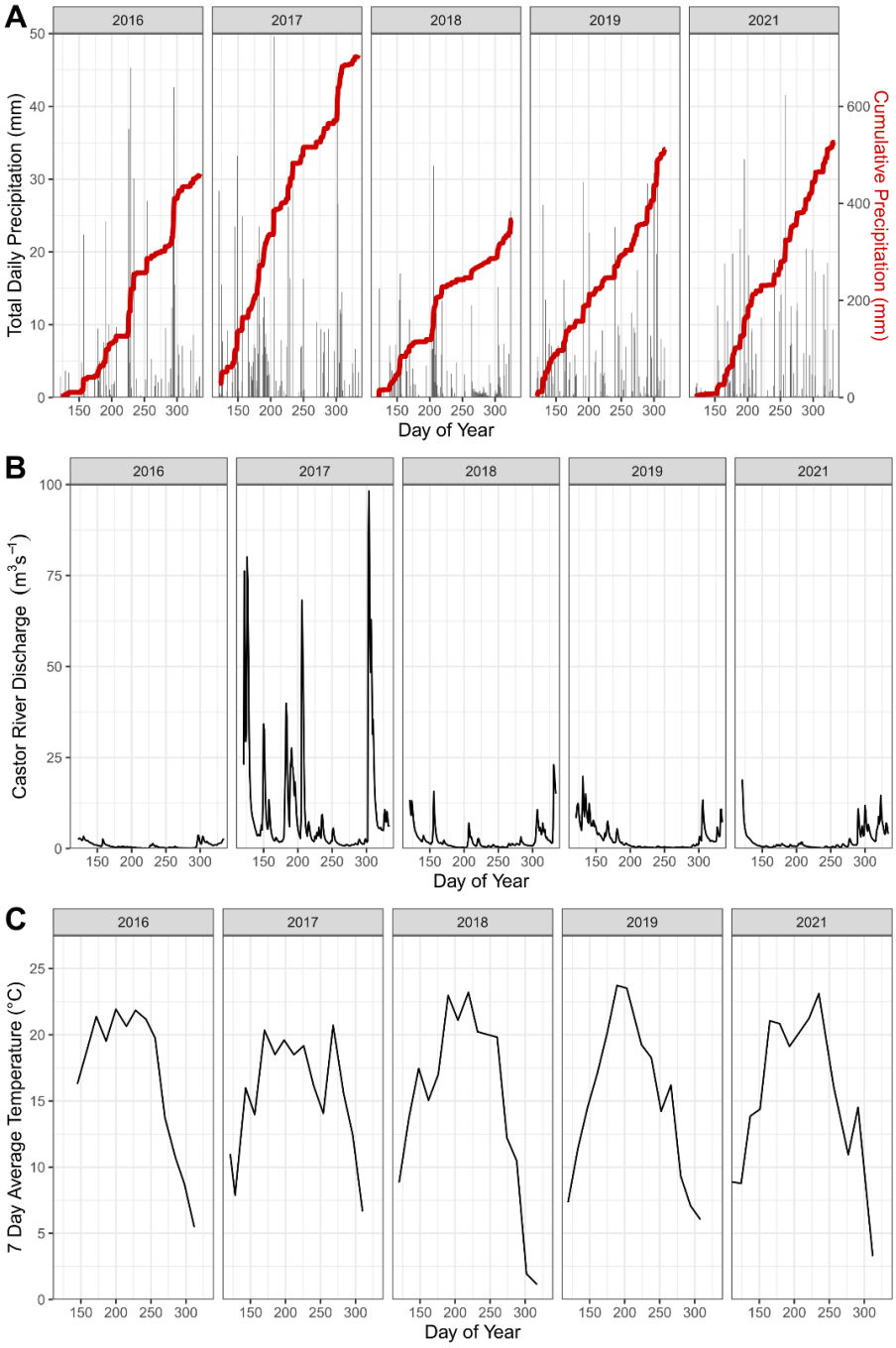


Figure A.5. Weather conditions in the South Nation River basin. (A) Daily cumulative precipitation during the sampling season. (B) Daily mean water discharge of the Castor River, measured at Russell station (02LB006). (C) Mean temperature averaged across 7 days prior to sampling date. Data were collected during the sampling season, ranging from days 110 – 317 of the calendar year (May – November). Year 2020 was not plotted due to undersampling during the COVID-19 lockdowns.

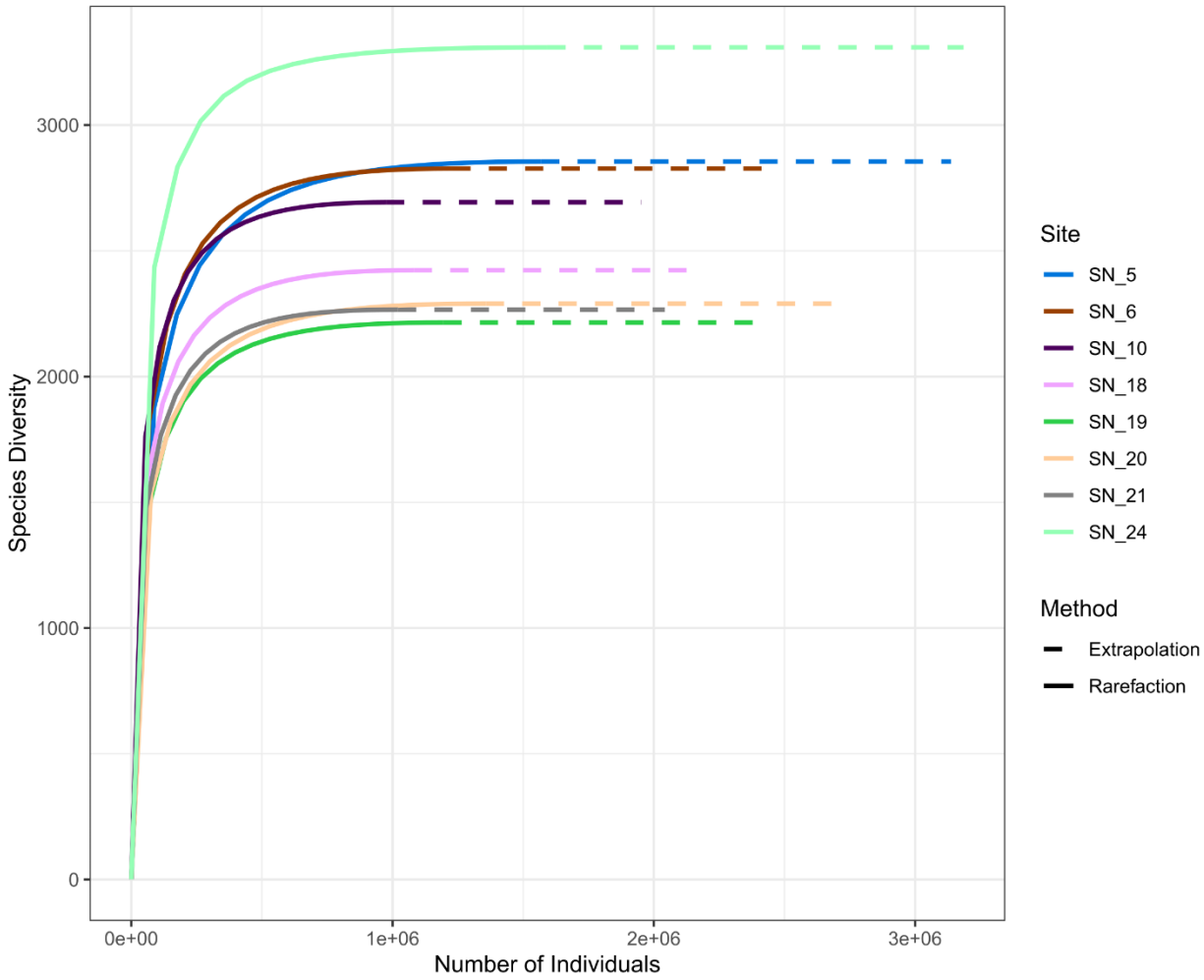


Figure A.6. OTU rarefaction curves by each sampling site. Rarefaction curves of OTU's obtained at each sampling site is plotted with extrapolation curve, predicted using the iNEXT package. Horizontal asymptotes of the rarefaction curves suggest saturated sampling was achieved at each of the eight sampling sites.

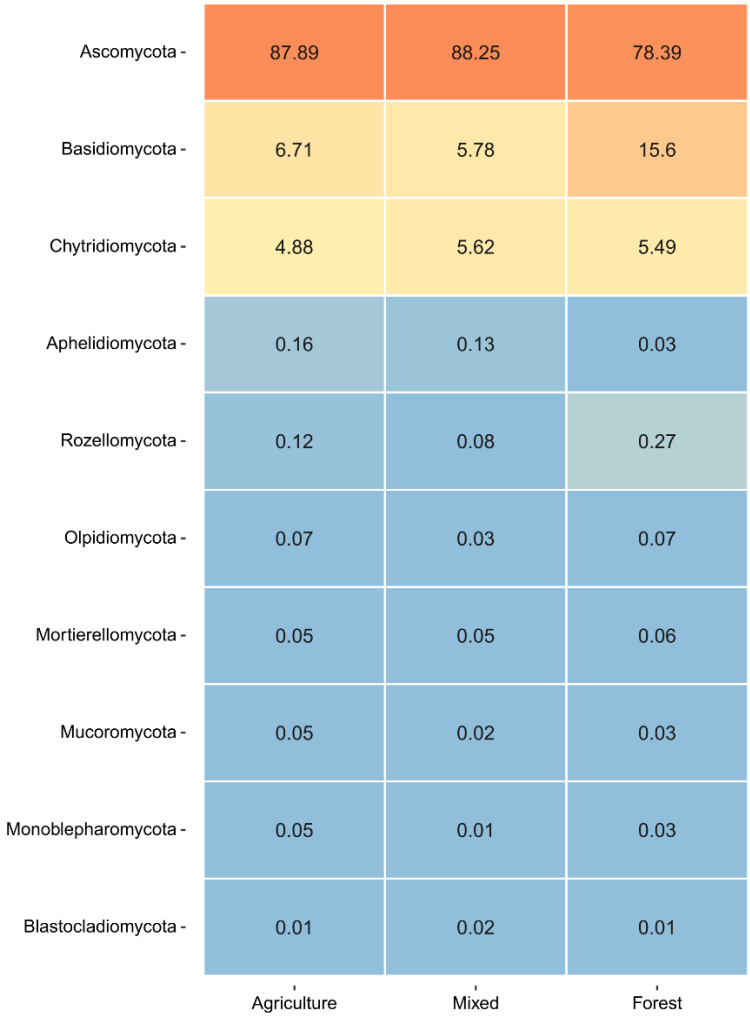


Figure A.7. Mean relative abundance of fungal phyla by land use classes. Differences in mean relative abundance were computed using linear mixed effects modeling, where land use class was a categorical fixed effect; sampling date and sampling block were random effects. Pairwise comparisons were performed by estimated marginal means with Tukey *P* value adjustment. Ascomycota abundance was greater in agricultural and mixed-use sites compared to the forested site (agr-forest, $P = 0.0219$; mixed-forest, $P = 0.0184$), while Basidiomycota abundance was greater in the forested site compared to agricultural and mixed-use sites (agr-forest, $P = 0.0082$; mixed-forest, $P = 0.0058$).

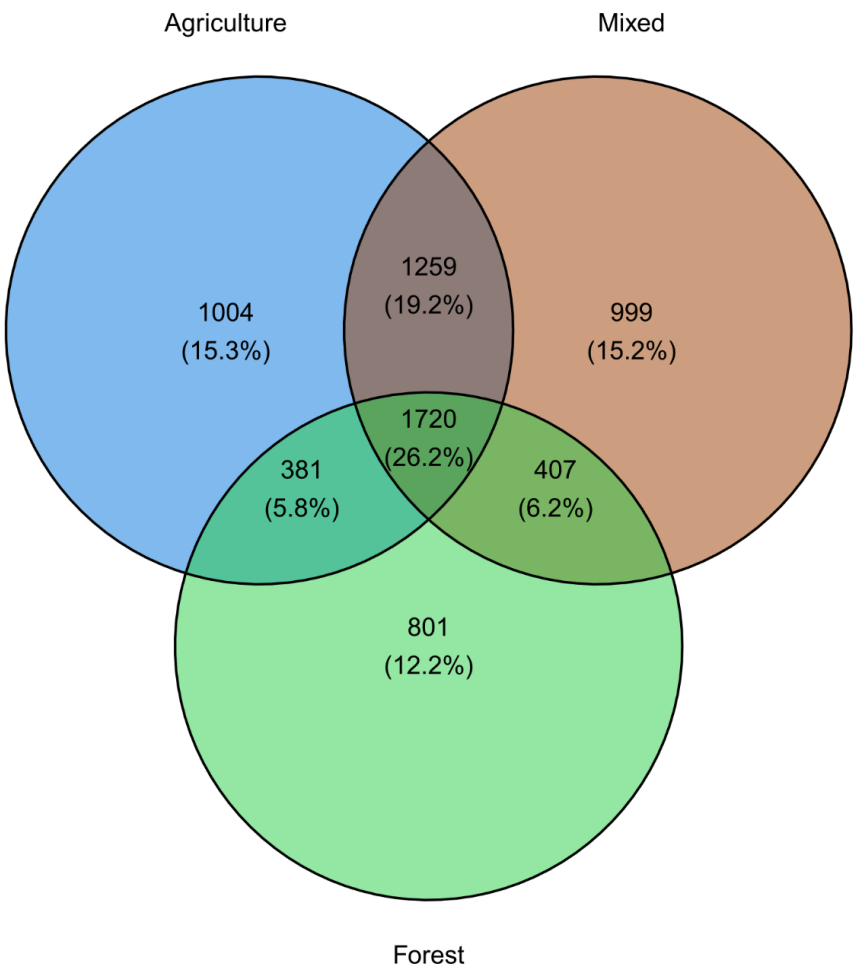


Figure A.8. Number of unique and shared OTUs by land use class.

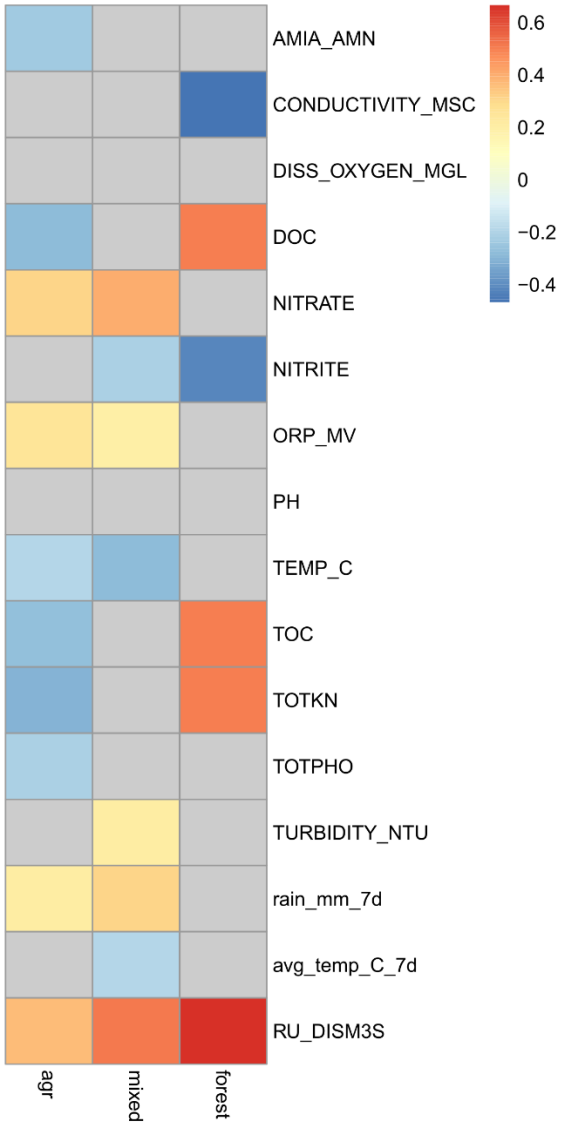


Figure A.9. Correlation between alpha diversity and water physicochemical properties for each land use class. Coloured cells indicate significant correlation at a significance level of $P < 0.05$.

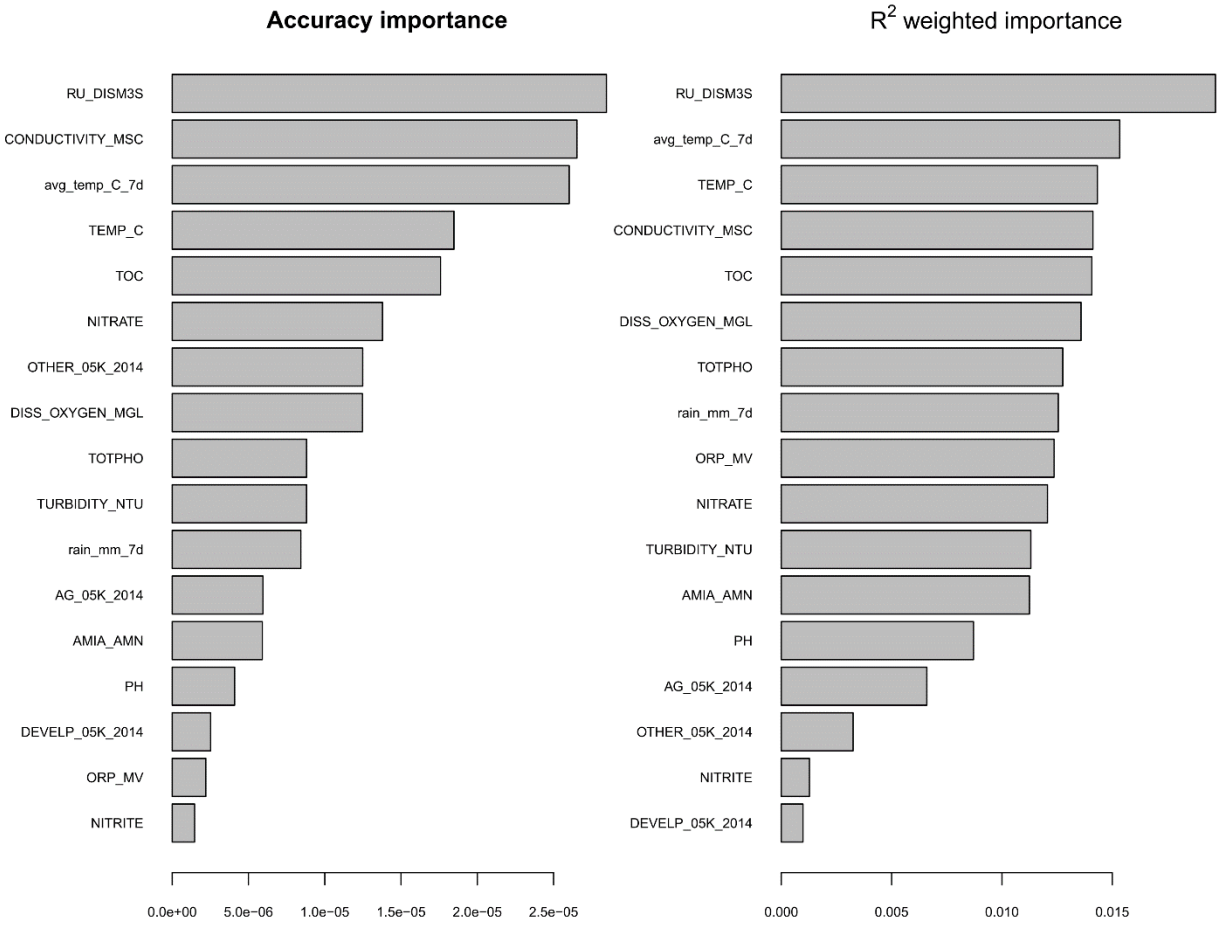


Figure A.10. The importance of environmental variables based on Gradient Forest algorithm.

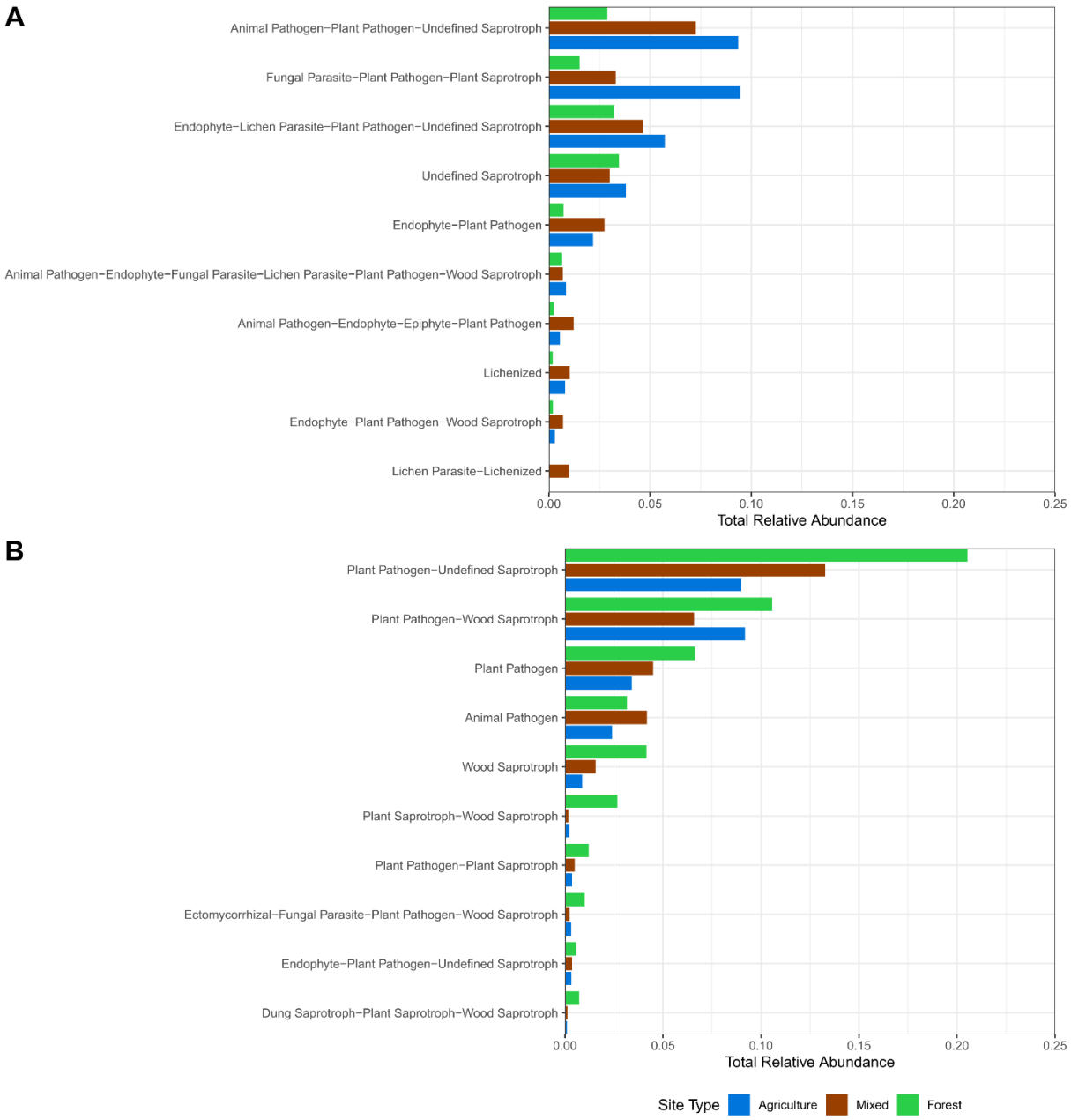


Figure A.11. Total relative abundance of predominant fungal guilds. (A) Dominant guilds with higher relative abundance in agricultural sites compared to the forested site. (B) Dominant guilds with higher relative abundance in the forested site compared to agricultural sites.

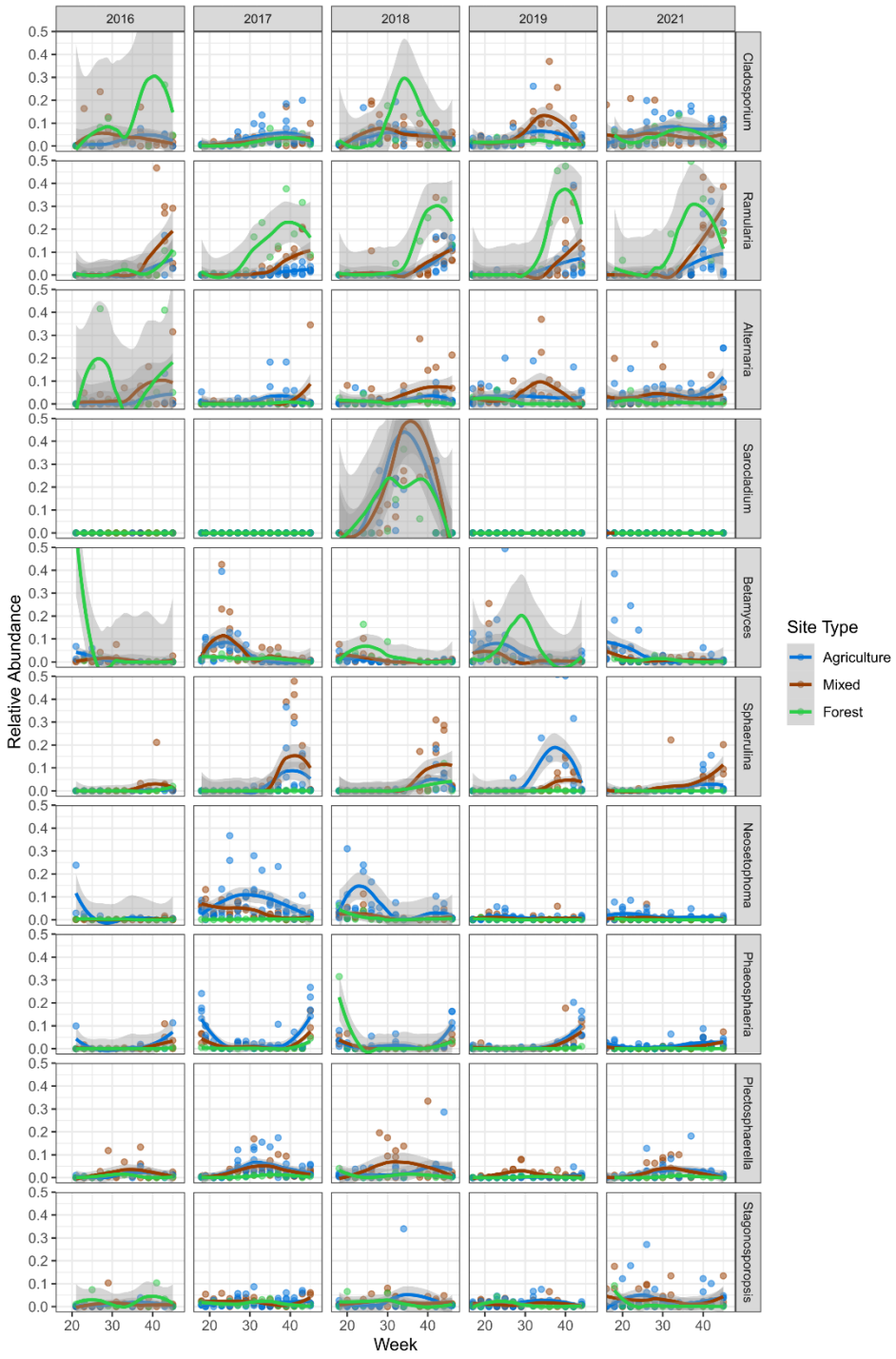


Figure A.12. Temporal variation in the relative abundance of the top 10 most prevalent genera across all land use classes. The year 2020 was excluded from the plot due to limited sampling during the COVID-19 lockdowns.

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