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Development of Environmental Metabarcoding Analysis for Use in Ecological Studies of Aquatic Fungal and Oomycete Communities Using Nanopore Sequencing

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DEVELOPMENT OF ENVIRONMENTAL METABARCODING ANALYSIS FOR USE IN ECOLOGICAL STUDIES OF AQUATIC FUNGAL AND OOMYCETE COMMUNITIES USING NANOPORE SEQUENCING

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by

Douglas Marvin Bennett

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CENTRAL WASHINGTON UNIVERSITY

Graduate Studies

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ABSTRACT

DEVELOPMENT OF ENVIRONMENTAL METABARCODING ANALYSIS FOR USE IN ECOLOGICAL STUDIES OF AQUATIC FUNGAL AND OOMYCETE COMMUNITIES USING NANOPORE SEQUENCING

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One of several factors contributing to amphibian decline are organisms called water molds (oomycetes), which parasitize and kill amphibian and fish eggs, larvae, and occasionally adults. Amphibian decline is one of the most difficult issues facing ecologists and conservationists to date; half of amphibian species are in severe decline and one third of amphibian species are facing extinction, threatening the stability of ecosystems globally. Multiple oomycete species are known to cause the disease saprolegniasis and contribute to amphibian decline; however, the full range of species involved is not presently known. This study evaluated the Oxford Nanopore minION DNA sequencer (R9.2 chemistry) for use in DNA barcoding multiplexed samples. This study aimed to develop new methods of environmental metabarcoding analysis to assess aquatic oomycete and fungal communities using the internal transcribed spacer (ITS) DNA barcode. A proof-of-concept survey was performed to assess community composition and diversity of aquatic oomycetes and Fungi in 4 freshwater habitats with

saprolegniasis present. Samples were taken April 15, 2016 at 4 sites along Interstate-90 near Snoqualmie Pass (Wenatchee National forest, Washington) during *Rana cascadae* and *Anaxyrus boreas* breeding season near eggs with saprolegniasis.

The minION produced high but uneven throughput across all four sites. The sequence accuracy and error type prevented chimera checking and phylotype assignment. Sequences were matched using NanoOK to the UNITE database to assign operational taxonomic units (OTUs). FUNGuild was used to assign trophic modes to OTUs to evaluate functional diversity. The recovered oomycete OTUs were low (~1% of all reads) and represented plant pathogenic phyla. Fungal recovery was broad with aquatic taxa represented heavily. Diversity analysis showed little difference between sites, with evidence that uneven throughput affected beta-diversity indices from assessing compositional differences. Trophic mode analysis showed little difference between sites.

Overall, this study found that the minION sequence data presented multiple bioinformatic challenges for metabarcoding using the ITS region. With low recovery of oomycetes, further study is needed to determine the availability of oomycete zoospores for collection via the sampling methods used in this study. Additionally, oomycete specific primers are recommended for increased recovery.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Oomycete Taxonomy and Ecology

Water molds (oomycetes) are fungus-like protists belonging to the kingdom Chromista; but were originally characterized as Fungi, in the now defunct Class Phycomycetes, due to their morphology (Dick 1976; 2001). Phylogenetic analysis placed the oomycetes evolutionarily closer to diatoms, brown-algae, and other protists in the kingdom Chromista (Beakes et al. 2012). As a group, the Chromista are unified by the production of heterokont zoospores with one tinsel type flagellum and production of chlorophyll a & c containing chloroplasts among photosynthetic representatives (Cavalier-Smith and Chao 2006). The oomycetes have lost their chloroplasts and the production of chlorophyll over time (Cavalier-Smith and Chao 2006).

Oomycetes are mostly pathogens of plants (Huxley 1881; Lucas et al. 1991; Kiesecker et al. 2001; Beakes and Thines 2017). Oomycetes of the order Saprolegniales are mostly saprotrophs that decompose organic matter, making them important contributors to nutrient cycling; however, several species are implicated in causing the amphibian and fish disease saprolegniasis (Huxley 1881; Kiesecker et al. 2001; Beakes and Thines 2017). The Peronosporales includes plant pathogenic genera *Pythium* and *Phytophthora* (Lucas et al. 1991). *Phytophthora infestans* has been identified as the pathogen causing late blight disease; an outbreak of which led to the potato famine in

1840s Europe (Yoshida et al. 2013). Overall, members of the oomycetes impact the ecology of numerous organisms, contribute to nutrient availability, and can be devastating to industry.

Saprolegniasis and Amphibian Decline

Oomycetes have also been implicated causing the disease saprolegniasis in salmonids and other fish dating back to the late 1800s (Huxley 1881) and have been shown to affect egg clutches as well as juveniles, particularly in aquaculture, as a disease of the fins and body (Earle and Hintz 2014). Fish hatcheries globally are susceptible to *Saprolegnia parasitica* due to the fish dense ponds with low water flow (Willoughby and Roberts 1992; Earle and Hintz 2014).

Saprolegniasis has been shown to be a major cause of amphibian decline, specifically in Anurans (frogs), and a minor factor in Caudata (salamanders) decline (Blaustein et al. 1994). Eggs and embryos are the most common life stage where saprolegniasis occurs. Larvae and adult frogs can also have saprolegniasis on their skin, though this is less common than egg infections (Kim et al. 2008). As high as 80% clutch mortality has been observed in severe cases of amphibian egg saprolegniasis, so mitigation of this disease is important to reducing amphibian decline (Kiesecker et al. 2001).

Understanding amphibian decline represents a major challenge facing ecologists and conservationists; half of all species are in severe decline and one third of all amphibian species are facing extinction, threatening the stability of ecosystems globally

(Blaustein and Kiesecker 2002; Stuart et al. 2004). Amphibian decline is thought to be the result of anthropogenic factors, such as deforestation, habitat fragmentation, and habitat use, as well as biological factors of parasitism and disease (Beebee and Griffiths 2005). Anthropogenic factors are being monitored regularly as they affect a larger number of organisms; however, there is still a lack of understanding of the biological factors impacting decline, such as the diseases saprolegniasis and chytridiomycosis (Bienentreu and Lesbarrères 2020). Amphibians are important predators of insects and are food sources for freshwater fish, birds, and mammals. Increased study of saprolegniasis is not only beneficial for preserving the amphibians affected but also the ecosystems to which these amphibians belong.

Factors Affecting Amphibian Disease Severity

Amphibian disease severity varies widely and relatively little is known about how species of oomycetes may interact with each other and other organisms (Kiesecker and Blaustein 1997). It is known that disease severity is affected by several environmental factors such as: temperature, ultraviolet light, and pH level; the response to these factors depends upon the water molds present (Banks and Beebee 1988; Kiesecker and Blaustein 1995; Hatch and Blaustein 2000; Kiesecker et al. 2001; Blaustein et al. 2003). Infection timing in embryonic development has also been shown to affect egg survival (Fernández-Benéitez et al. 2011). Phylogenetic studies have identified at minimum seven and as many as twelve different phylotypes of water mold associated with active infection of amphibian eggs (Johnson et al. 2008; Petrisko et al. 2008). Further evidence

suggests more species than initially indicated may contribute to pathogenicity and egg mortality (Fernández-Benéitez et al. 2008; 2011; Brady 2010; Ault et al. 2012). A succession pattern was also found in saprolegniasis infections, finding that *Saprolegnia ferax* and *Saprolegnia declina* are typically among the first colonizers on an amphibian egg with the number of phylotypes found increasing as the eggs age (Ault et al. 2012).

Inventory and Identification of Aquatic Oomycetes

Historically, conducting biodiversity inventories of the oomycetes required the isolation of axenic cultures from water or soil samples. While relatively inexpensive, this approach is very labor and time intensive. These methods include baiting for motile water mold zoospores in small water samples (20-30 mL) or from obviously infected materials in sterile water. Commonly used baits include seeds, salmon eggs, and snakeskin (Sparrow 1960; Stevens et al. 1974). The baits chosen for a study may select for different species potentially limiting the recovery of slower growing or rare species (Stevens et al. 1974). Overall, these methods tend to select for the most aggressive and fastest growing species in any sample. As a result, many species may fail to be recovered, limiting the understanding of saprolegniasis and aquatic ecosystems.

Morphological identification of pathogens from infected eggs or materials is also problematic. Morphological identification relies upon reproductive structures and spores to be present (Johnson 1956; Seymour 1970). However, recent molecular phylogenetic studies suggest that the morphology of the oomycetes does not accurately reflect phylogeny (Dieguez-Uribeondo et al. 2007; Johnson et al 2008). Due to the

difficulty finding morphological traits that accurately identify monophyletic groups, identification of water molds in many studies is currently done primarily using DNA sequence data and phylogenetic analysis (Johnson et al. 2008; Sandoval-Sierra 2014). This approach allows one to more accurately identify morphologically variable or cryptic species that are difficult to assign to groups morphologically (Johnson et al. 2008).

The phylogenetic identification approach is exemplified in studies using DNA barcoding, the use of a short universal sequences which can be used to determine species boundaries as well as evolutionary relationships (White et al. 1990, Hebert et al. 2003). For oomycetes and Fungi, the primary barcode is the internal transcribed spacer (ITS) region which codes for spacers between ribosomal subunits and a portion of the rRNA (Leclerc et al. 2000; Seifert et al. 2007; Robideau et al. 2011, Schoch et al. 2012; Choi et al. 2015, Riit et al. 2016). Subsequent phylogenetic studies, using DNA barcoding, have identified water molds by phylotype; which is an identification based upon less than 97% similarity by genetic distance between individuals and may represent a more accurate assignment of individual species given the lack of clarity in water mold taxonomy (Parrent et al. 2006; Ault et al. 2012).

Environmental Metabarcoding and eDNA Sampling

Environmental metabarcoding is a relatively new approach which involves taking environmental DNA (eDNA) samples from soil, air, water or other materials, extracting all the DNA from the sample, and sequencing the DNA to identify species of interest (Ogram et al. 1987; Lydolf et al. 2005; Lodge et al. 2012). Environmental metabarcoding

is used in bacterial research as well as for cryptic amphibian species (Taberlet et al. 2012; Sapp et al. 2016). This approach has been used effectively in soil environments to detect terrestrial oomycetes; however, it has not yet been employed for use in the study of aquatic oomycetes (Schmidt et al. 2013).

The primary use of eDNA is to use targeted primers to detect specific individuals or selected groups of individuals; although, a wide range of applications have emerged (Taberlet et al. 2012). The targeted approach has been used to detect cryptic amphibian species in streams (Goldberg et al. 2011) and for detecting invasive fish (Davison et al. 2016). Since there is a lack of clarity of the identities and taxonomy of pathogenetic oomycetes, species specific primer sets have not been developed (Fernández-Benéitez et al. 2008; 2011; Brady 2010; Ault et al. 2012). As a result, the targeted detection of pathogenic oomycetes was not available at the time of this study.

The alternative approach, used in this study, is to utilize general barcoding primers and optimize PCR conditions to maximize return of oomycete sequences. The environmental metabarcoding approach removes the need for cultures and thus removes bait selection and competition as a barrier for identification. Considering the bias of traditional isolation methods, the limitations of morphological identification, and the lack of clarity of oomycetes involved in saprolegniasis, the environmental metabarcoding approach is highly appealing for the study of oomycetes as it removes major limitations of previous studies.

DNA Sequencing Technology

In early studies, the ITS region was sequenced for individual isolates using Sanger sequencing (Horton and Bruns 2001; Hulvey et al. 2007). This was done because, at the time, this was the only sequencing technology available. It also has the advantage of relatively long read lengths (~1000 bp), which enabled the sequencing of each strand with a single reaction. However, this type of sequencing requires either pure (axenic) cultures (Sanger and Coulson 1975; Sanger et al. 1977) or cloned PCR fragments that can be sequenced individually (e.g. Arcate et al. 2006; Hassan et al. 2011; Ault et al. 2012). In order to sequence eDNA recovered from mixed soil and water samples, PCR fragments had to be subcloned into bacteria in order to isolate individuals. Culture and subcloning methods were time consuming, labor intensive, and expensive limiting larger studies of the ecology of microbial environments.

Second-generation sequencing technology, such as Illumina and Pyrosequencing, have increased throughput for whole genome sequencing but with shorter read lengths than Sanger sequencing. These technologies simultaneously sequence an array of individual DNA strands via synthesis with modified nucleotides, allowing for the sequencing of mixed samples (Ahmadian et al. 2006). This lowered the cost and time investment per sample dramatically from the labor-intensive approach required with Sanger sequencing. The other advantage to this second-generation approach is that a relative abundance of individuals in the sample can be estimated based upon the number of sequences that are read, with some consideration to amplification bias if the

samples have undergone PCR (O'Brien et al. 2005; Lamb et al. 2019). As a result, second-generation sequencing has reduced the time and labor of environmental metabarcoding studies (Jumpponen et al 2010; Schmidt et al. 2013).

The Oxford Nanopore minION is the third generation DNA sequencer which takes a novel approach to DNA sequencing (Laver et al. 2015; Weirather et al. 2017). The minION uses sequencing by shape rather than by synthesis. The DNA is drawn through a Nanopore via motor protein as buffer flows through the pore. The change in ion flow caused by the unique shape of each nucleotide as it passes through the pore is detected by an electrode downstream of the pore. This technology can sequence individual strands of DNA without amplification. Additionally, Nanopore sequencing offers much greater read length (average ~2 kbp) which allows the sequencing of the entire ITS while also having multiple samples in the same run. Making the minION ideal for this type of metabarcoding study. The drawback of this technology is lower accuracy, the chemistry used in this experiment was known to have an average accuracy of ~90% at the time of this study.

At the time this study was conducted the average read length of Illumina was less than 300 bp and would not be able to sequence the entire ITS (Quail et al. 2008). Pyrosequencing was capable of around 600 bp at the time of this study, however it was prohibitively expensive (Ahmadian et al. 2006). For this study, the choice was between sequencing the entire ITS region at an expensive cost or roughly half the ITS at a much lower cost when considering second-generation technology. The Oxford Nanopore minION at the time of this study was roughly half the cost for sequencing the entire ITS

compared to Pyrosequencing. The minION was selected for this study as it presented the most cost-effective solution for the environmental metabarcoding approach used in this study. One question of this study was whether the lower accuracy of the minION could be overcome by increased coverage of the ITS region for identification of species or phylotypes.

Study Design

Most of the early studies of amphibian saprolegniasis focused on identifying sites and severity of disease conditions; and used morphology to identify the pathogens responsible (e.g. Banks and Beebee 1988; Blaustein et al. 1994). As a result of the problems with morphological identification methodology, *Saprolegnia ferax* and commonly just *Saprolegnia sp.* were the most common species identified as causing most infections (e.g. Banks and Beebee 1988; Kiesecker and Blaustein 1995; 1999). Subsequently, there is a lack of clarity as to how the presence of individual species of oomycetes affects disease severity. Recent studies have identified several distinct phylotypes associated with amphibian embryos and demonstrated some of these are also pathogenic (Fernández-Benéitez et al. 2008; Johnson et al. 2008; Petrisko et al. 2008; Brady 2010; Ault et al. 2012). Given that traditional isolation techniques are biased for the fastest growing individuals or may misidentify organisms present due to poor taxonomy, a survey of the oomycetes in habitats where saprolegniasis occurs is warranted.

Locally, the cascades frog (*Rana cascadae*) and western toad (*Anaxyrus boreas*) are both affected by saprolegniasis (Hatch and Blaustein 2000; Kiesecker et al. 2001). The western toad is currently considered a state candidate for preservation in Washington; although, globally the population is listed as least concern (IUCN 2020). The cascades frog is near threatened globally (Hammerson and Pearl 2004). These species can both be found in the high alpine lakes in the Cascade mountains in Washington (Cohen and Stebbins 1995).

Western toads and cascades frogs utilize habitat differently. Western toads are a migrating species that rely upon temporary ponds from melting snow to make the journey from overwintering sites to breeding ponds (Bartelt et al. 2004). Cascades frogs are typically found in or near small ponds, lakes, and occasionally in temporary pools near streams (Cohen and Stebbins 1995; Resetarits 1996). Cascades frogs typically hibernate in the mud in shallower portions of ponds and lakes (Cohen and Stebbins 1995). These lifestyle dissimilarities could influence exposure to oomycetes during non-breeding months.

Egg laying behavior is also slightly different between the western toad and cascades frog. Western toad egg clutches are laid in long strings, which could be a behavioral adaptation to prevent the spread of saprolegniasis (Kiesecker and Blaustein 1997). Cascades frogs lay large egg masses and females will group together to lay eggs making them more susceptible to saprolegniasis (Kiesecker and Blaustein 1997). Zoospore recovery near infected eggs of each species could vary between species as egg density varies, as well as differences in host specificity of pathogenic species.

Reproduction of oomycetes can be asexual, producing motile zoospores, or sexual, producing non motile oospores (Wardrip et al. 1999; Hardham 2001). Zoospores are flagellate and are produced in a zoosporangium from the hyphal stage of water mold. Zoospores can encyst multiple times prior to the germination of hyphae; these cysts can persist in harsh environmental conditions and show chemotaxis to hosts and substrates (Ho 1975). The potential presence of zoospores, near amphibian eggs with saprolegniasis, swimming chemotactically to reinfect the egg masses or search for a new host, provides an opportunity for collection. Spores can be filtered from this sample using commonly available filters, as the spores are a similar size to bacteria (Stevens et al. 1974; Dick 2001).

Both the western toad and cascades frog being affected by saprolegniasis, their overlapping locality, and lifestyle differences provides an excellent opportunity to study oomycetes related to saprolegniasis. Potential capture of zoospores by water sample limits potential biases observed in traditional study methods. The eDNA sampling approach is also less invasive than identification of pathogens on infected eggs, as collection of infected materials often disturbs neighboring eggs. Considering these factors, a proof-of-concept study was performed using water samples collected near eggs of these two local amphibians with saprolegniasis present.

Study Aims

The aim of these experiments was to evaluate the use of Oxford Nanopore sequencing for DNA barcoding of the ITS region and eDNA sampling methods to study

oomycetes. The minION presented the most cost-effective approach at the time of this study; as well as, potentially providing more accurate identification due to complete sequencing of the ITS region, despite expected lower basecalling accuracy, compared to other technologies. The study sites selected for this experimental survey represent a variety of habitat conditions where saprolegniasis was present and, if successful, could have provided a baseline for future studies of amphibian saprolegniasis. An ecological analysis was also performed to assess the community recovered and evaluate any potential problems presented by the sequencing and sampling methods used in this study.

CHAPTER II

METHODS

Study Area

This study focused on the central Cascades near Snoqualmie Pass, WA along the Interstate-90 (I-90) corridor. Four freshwater ponds were selected for the presence of breeding western toads and cascades frogs and presence of saprolegniasis. All 4 sites were sampled on April 15, 2016, during the spring snow melt. Snoqualmie pass regularly sees frozen conditions from November to March, with the main snow melt typically occurring around the first week of April. The 4 ponds selected were: Swamp Lake, Swamp Creek, Mardee Lake, and Gold Creek Stockpile as shown in Figure 1.

Swamp Lake is about 0.69 km north of I-90 located immediately east of Kachess Lake Rd, at the time sampled the water was about 0.5-2 m in depth with remnants of emergent vegetation from the previous year still present. Swamp Lake had both cascades frog and western toad breeding occurring at the time of sampling, with some sightings of pacific chorus frog (*Pseudecaris regilla*) eggs and northwest salamander (*Abystoma gracile*) eggs which showed no signs of disease. It is possible other salamanders were present, but at the time of sampling none were observed.

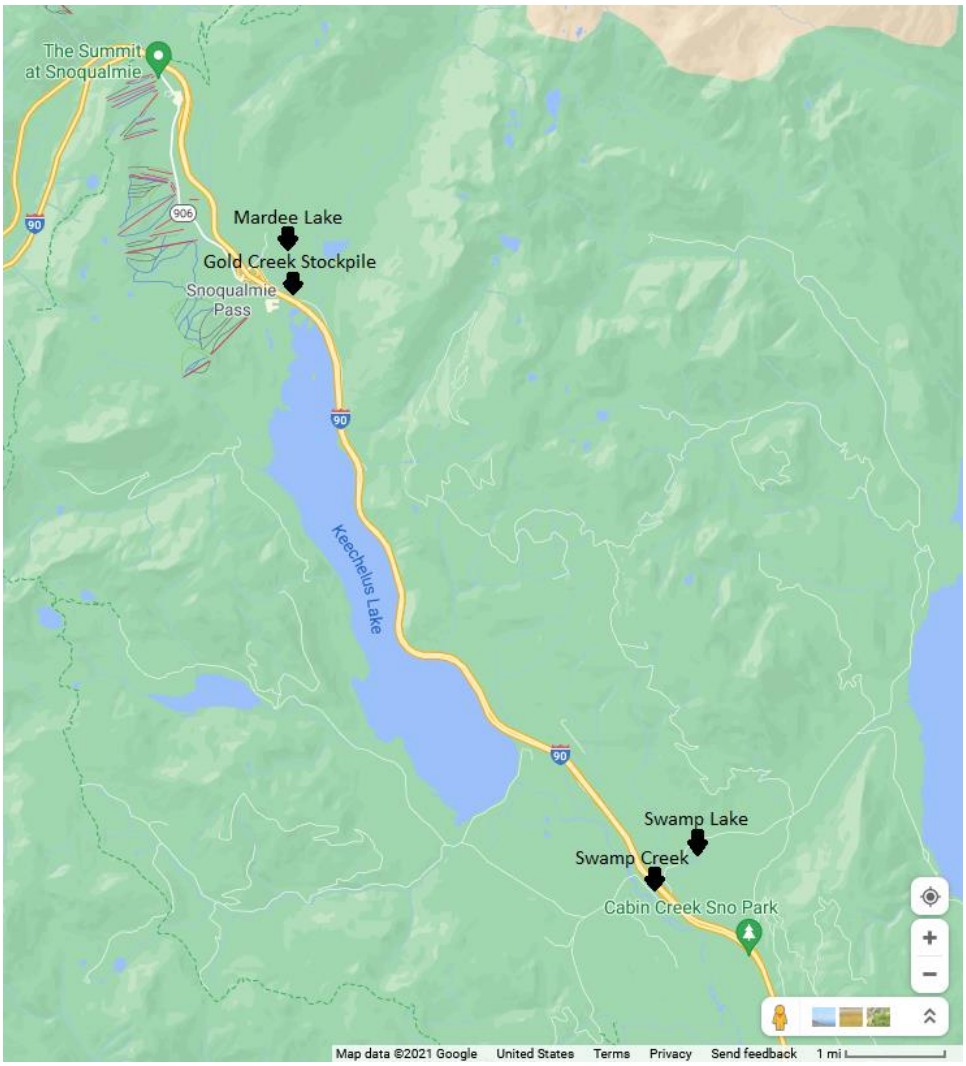

Figure 1: Study area with individual ponds indicated by arrows. (http://www.google.com/maps)

Swamp Creek are located approximately 15.6 km southeast of the Snoqualmie Pass summit. Swamp Creek is located less than 80 m south of I-90 and located north of Stampede Pass Rd, this was the most heavily polluted pond of the 4 selected due to its proximity to I-90. Swamp Creek features shallow (<0.5 m depth) and deep (>1 m depth) sections, samples were taken in the shallow section approximately 20 m north of Stampede Pass Rd where cascades frog eggs were located.

Gold Creek Stockpile was a recently renovated site which, in the summer of 2015, was restored to natural habitat after the removal an asphalt parking lot. Gold Creek Stockpile is located approximately 3 km southeast of the Snoqualmie Pass summit, 0.1 km north of Lake Mardee Rd (NF-4832), approximately 0.18 km north of I-90. Gold Creek Stockpile consisted of 2 ponds at the time of sampling, with the ground mostly free of foliage, cascades frogs were found breeding in the southern most pond which was on average about 1 m in depth and lacked riparian or in water vegetation. The egg mass where samples were taken was partially dehydrated and spread on the bank of the pond, showing the water level had reduced substantially since the egg mass was deposited. Two other egg masses were present at the time of sampling; however, the egg mass with the most eggs submerged with the highest signs of infection were chosen for sampling.

Mardee Lake was the most remote site, located approximately 3.1 km southeast of Snoqualmie pass summit, approximately 0.4 km east of NF-9090 Rd, 0.64 km north of I-90. Mardee Lake was very similar to Swamp Lake in terms of size, depth, and foliage. Only Cascade frog eggs were found at Mardee Lake with the largest egg mass being approximately 2 m long x 1 m wide x 0.5 m deep.

Sampling and Sequencing Preparation

Water samples were taken as close to the edge of the egg mass as possible near areas of active infection, avoiding the capture of tadpoles and plant debris, using two sterilized 300 mL centrifuge bottles per site. Active infection was identified as eggs with

large hyphal structures present, samples were taken where multiple eggs were showing infection. When western toad and cascade frog eggs were both present, one sample was taken at the highest infection site for each species. Samples were then filtered using Millipore HV sterile 0.45 µm vacuum filtration cups (EMD Millipore, Burlington, MA). The filter was aseptically removed from the cup, cut into quarters, and separated into 1.7 mL sterile tubes. Samples were stored at 20^{o}C for 24-48 hours prior to DNA extraction and PCR.

Extraction was performed using a modified hotSHOT technique (Truett et al. 2000), with the addition of 0.1 mL of .05 mm sterile glass bead and two 2 mm sterile glass beads to each sample; followed by a one minute of bead beating prior to incubation at 95^{o}C for one hour. Following extraction, samples were then amplified using ITS5 and ITS4 primers to select for oomycetes (White et al. 1990). PCR conditions were optimized by a preliminary experiment using a DNA extract from a mixed culture of oomycetes isolated from Swamp Lake. Water samples were taken from this mixed culture and extracted using the same method as the pond samples. From these samples several temperature protocols and primers were tested and the amount of DNA from extraction was varied to maximize DNA amplification. PCR was performed using a touchdown temperature protocol decreasing annealing temperature from 58°C to 50°C, stepping down 2°C every 5 cycles, for a total of 40 cycles. This approach prioritizes individuals that matched the primers well, while allowing for amplification of less abundant individuals (Hecker and Roux 1996). PCR was performed in 25 µL aliquots using PCR buffer at recommended concentration, 2.5 mM $MgCl_2$, 200 µM of dNTPs, 0.4

µM of each primer (ITS5 and ITS4), 1.25 units Taq DNA polymerase, and 5 µL of each DNA extract. Following PCR, samples were run on a 1% agarose gel with ethidium bromide and imaged using a UV transilluminator. For each site, the four 25 µL PCR products showing the highest amplification visually were combined and purified using QiaQUICK PCR Purification Kit (Qiagen LLC, Germantown, MD). The DNA samples were eluted in 45 µL of TE buffer for library preparation.

Samples were prepared following a modified library preparation protocol supplied by Oxford Nanopore for whole genome sequencing with the minION sequencer (Oxford Nanopore Technologies, Oxford, UK). Each sample was modified using NEBNext Ultra II end repair mix (New England Biolabs, Ipswich, MA) to leave TA ends on each DNA strand. Following end modification, Oxford Nanopore sequencer adapters were attached to the sample DNA using TA ligase (New England Biolabs, Ipswich, MA). The adapter mix included what is necessary to pull the DNA through the pore and a loop connecting both complimentary strands so that the sequencer would be able to read the forward and reverse strands of each sample. Sequencing both complimentary strands results in two dimensional (2D) reads with higher accuracy than single stranded (1D) reads (Jain et al. 2016). The DNA was then purified using magnetic streptavidin beads (New England Biolabs, Ipswich, MA) to remove any non-nucleotide molecules and remaining small fragments of DNA, including unbound TA-adapters. DNA was eluted from the beads using Oxford Nanopore running buffer. Sequencing was performed using Oxford Nanopore minION sequencer using an R9.2 flow cell. Each site was allotted 24

hours of run time, the flow cell was flushed between samples using a flow cell wash kit (Oxford Nanopore Technologies, Oxford, UK).

Sequence Processing

Minknow (Oxford Nanopore Technologies, Oxford, UK) was used to collect the DNA sequences from the Nanopore sequencer. Sequence data was then uploaded to Metrichor (Oxford Nanopore Technologies, Oxford, UK) for basecalling and initial quality filtering. Sequences that were 1D or failed Metrichor quality filtering were removed from the dataset. Poretools (Loman and Quinlan 2014) was used to then extract the sequences into .fasta file format for input into FungalITSPipeline (Nilsson et al. 2009) and NanoOK (Leggett et al. 2016). This analysis pipeline is shown in Figure 2.

FungalITSPipeline was used as a means of reducing the UNITE fungal database (public release 28.06.2017) to a usable size for NanoOK. Each sample was aligned to sequences in the UNITE database resulting in a possible 10 best matches by e-score for the ITS 1 and its 2 regions individually. Every reference sequence which passed the pipeline's quality filter was then extracted from the UNITE database by Samtools (http://www.htslib.org/) to generate a truncated database for input into NanoOK. NanoOK is a program designed to deal with Nanopore data using optimized LAST (https://gitlab.com/mcfrith/last, Tokyo, Japan) alignments to align each sequence. LAST is an alignment tool that has been shown to be more accurate than, widely used, Basic local alignment search tool (BLAST) (NCBI, Bethesda, MD) when dealing with Nanopore data as it penalizes less for insertions and deletions that are prevalent in Nanopore data

compared to BLAST along with other alignment optimizations for Nanopore data (Jain et al. 2015).

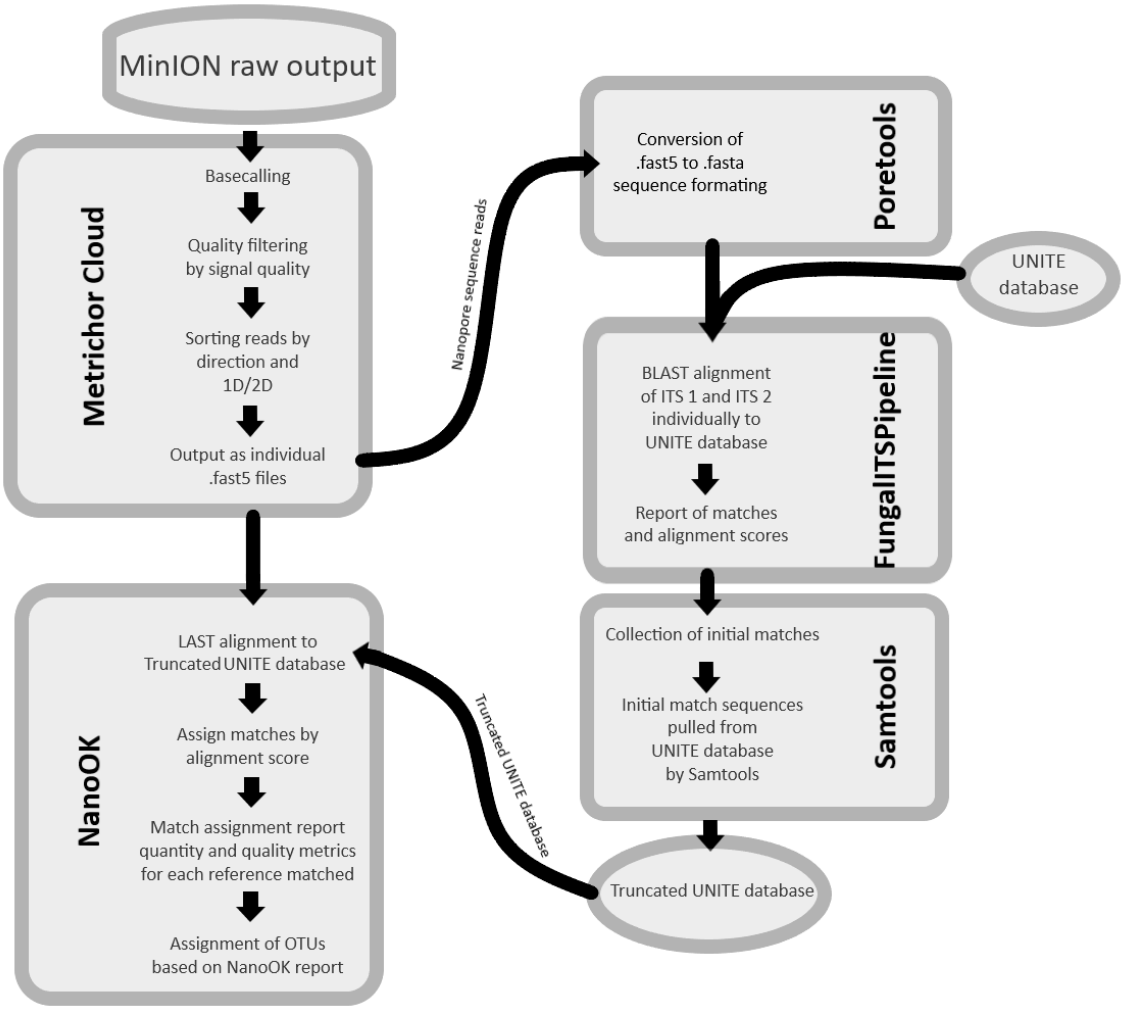

Figure 2: Sequence analysis flowchart.

NanoOK reports the number of sequences that match each reference from the UNITE database. Due to the low quality of the sequencing data and the lack of ability to check for chimeric sequences, there is low confidence in the identification to species level assigned by NanoOK. Instead, each group assigned by NanoOK was treated as an

operational taxonomic unit (OTU) most likely representing a unique group, as standard distance-based assignment of phylotype was unavailable. NanoOK calculates alignment scores for each sequence and assigns identity based upon the highest alignment score. Ecological evaluations were made from the output of NanoOK by treating each match to an OTU as a proxy for relative abundance (O'Brien et al. 2005). In order to ensure accurate recovery of oomycetes, it was confirmed that oomycete reference sequences were included in the truncated database prior to analysis. Additional oomycete references were added to the database from Genbank (NCBI) after initial analyses found poor recovery, and NanoOK analyses were rerun.

Community Structure Analysis

For this experiment, multiple traditional measures of biodiversity were used to evaluate the communities at each site. R version 4.1.0 (R Core Team 2020) was used in the Rstudio (Rstudio team 2021) environment with biodiversityR (Kindt and Coe 2005) and vegan (Oksanen et al. 2020) packages for all diversity index calculations. A species accumulation curve was calculated with error bars sampled randomly 1000 times using biodiversityR (https://cran.r-project.org/web/packages/BiodiversityR/index.html) and vegan (https://cran.r-project.org/web/packages/vegan/index.html) to evaluate OTU recovery with sampling effort (Ugland et al. 2003; Kindt and Coe 2005; Oksanen et al. 2020). A Renyi diversity profile plot was generated using biodiversityR to assess relative diversity as indices increase in dominance influence (Kindt and Coe 2005).

Alpha diversity measures were used to evaluate the overall health of sampled habitats by looking at OTU richness and evenness. Shannon's diversity is one of the most common diversity indices used across ecological studies (e.g. Magurran 1988; Krebbs 2001). Shannon's diversity was used for this study as the data fit the assumptions of this index. Oomycete and Fungal spores are indefinite in the water column and, based upon initial results, nearly all available individuals sampled were recovered. Inverse-Simpson's index was used as a dominance measure to evaluate diversity weighted by the most abundant individual OTUs present in each sample (Simpson 1949; Magurran 1988).

Beta diversity indices were used to evaluate the compositional differences between sampling sites. For this purpose, the Sorenson-Dice index (Southwood 1978) and the Jaccard Distance index were calculated (Janson and Vegelius 1981). Both indices do not consider the abundance of any individuals found, but rather compare the richness of OTUs found at each site.

Functional Diversity

For this experiment, the trophic modes of the OTUs represented at each site were evaluated. Using the tool FUNGuild (Nguyen et al. 2016) trophic modes were assigned to OTUs based upon the taxonomic data available from the UNITE database and is referenced to a curated database of trophic mode identifications. The FUNGuild database assigns trophic modes and guilds to Fungi using published research of individual taxa. While assignments using FUNGuild are reliable, they are limited due to the incomplete nature of the current database. Trophic mode was determined to be

more appropriate for this application, as ecological guilds produced highly nested results and provided little clarity for the dataset. This analysis provides a look into the different ecological niches of OTUs recovered in this survey.

CHAPTER III

RESULTS

Sequencing

Initial quality filtering began during the basecalling process where the output from the sequencer is converted into the actual DNA sequence. Metrichor gives a quality rating based upon the signal quality of each read; additionally, this program sorts sequences by strand direction and 2D reads. Only 2D sequences from the basecalling procedure were used to remove redundancy from having matching strands and as a quality filter, 2D sequences have ~10% higher accuracy on average. Even with higher accuracy, still only 10.93% of the 322 711 reads passed initial quality filters across all sites as shown in Table 1. Swamp Creek and Mardee Lake produced lower pass percentage and lower overall throughput than the other two sites.

Table 1: Metrichor quality filtering during basecalling of 2D sequences.

Site	Pass	Fail	Total Reads	Pass %
Swamp Lake	15 377	107 008	122 385	12.56
Swamp Creek	760	24 773	25 533	2.97
Mardee Lake	3 136	65 225	68 361	4.58
Gold Creek	16 011	90 421	106 432	15.04
Total	35 284	287 427	322 711	10.93
Mean	8 821	71 857	80 678	

The passed sequences were then used to query the UNITE fungal database using FungalITSPipeline in order to produce a truncated database for use with NanoOK. The

FungalITSPipeline does include the ability to chimera check sequences since it queries the ITS1 and ITS2 regions separately. This step is very important when using the ITS region for DNA barcoding as chimeras, DNA sequences made from more than one individual, are generated regularly during the PCR process (Wang and Wang 1997). However, the lower accuracy of the Nanopore sequences made standard chimera checking unreliable with FungalITSPipeline.

A Nanopore specific chimera checking tool was unable to be found or did not exist at the time of this experiment. Nanopore data is prone to insertion, deletion, and gap errors which are heavily penalized by traditional alignment tools used to perform chimera checking. The BLAST alignment tool used by the pipeline program is one of these unoptimized tools. The alignments produced by BLAST were not ideal but the individual matching of ITS1 and ITS2 reduced the effect of lower accuracy sequences and increased the recovered potential matches for the modified database.

NanoOK

NanoOK assigned a total of 20 479 reads matched across all sites with an average of 67% of sequences aligned. NanoOK was used as a means of OTU assignment through alignment score to the UNITE database of reference sequences. NanoOK identified 566 OTUs across all sites, representing 17 phyla. As a consequence of lower throughput, Swamp Creek had the lowest matched sequences with only 586. Of all the sites Gold Creek had the lowest match rate at 29.75% with the other three sites ranging between 77.11%-81.96% as shown in Table 2. This discrepancy could indicate that the sequences

are either of low quality, high in chimeras, or that the individuals represented were not included in the UNITE database. Most likely the latter being the case.

Table 2: NanoOK quality filtering statistics.

Site	Matched	Unmatched	Total	Match %
Swamp Lake	12 603	2 774	15 377	81.96
Swamp Creek	586	174	760	77.11
Mardee Lake	2 526	610	3 136	80.61
Gold Creek	4 764	11 247	16 011	29.75
Total	20 479	14 805	35 284	
Mean	5 119.75	3 701.25	8 821	67.38

The phyla found at each site are summarized in Figures 3-6. Each site shows a mixture of terrestrial and aquatic phyla primarily from the kingdom of Fungi. The Chromista, includes water molds, was strongly represented at each site, with Mardee Lake showing exceptionally lower Chromista than the other sites.

Unfortunately, the Chromista reference sequences matched lacked further taxonomic information, water. All 3 544 Chromista sequences recovered matched to 2 reference sequences from uncultured individuals recovered from 2 different soil studies (Hollister et al. 2010; Lan 2015). Additional oomycete references were added to the truncated database and analyses rerun, this resulted in less than 5% of the previously matched Chromista sequences being identified mostly as members of *Pythium* and *Phytophthora*, plant pathogenic water molds typically found in soils or plants (Lucas et al. 1991; Arcate et al. 2006). It is possible that zoospores were not collected, and a close relative of the oomycetes was instead recovered. Without further study, it cannot be

determined if the lack of recovery is due to low sequence quality or a problem with sampling.

The fungal taxa recovered were highly aquatic as expected and in the rough proportions to be expected as well (Goh and Hyde 1996; Shearer et al. 2007; Hu et al. 2013). A low-level recovery of terrestrial fungal phyla was observed across all sites. While there was some variation between sites, the Ascomycota and Chytridiomycota were the highest represented phyla overall. One surprise is the recovery of Rozellomycota in such high numbers, this basal group of Fungi are pathogens to a number of micro-organisms including other Fungi, oomycetes, and arthropods (Corsaro et al. 2016).

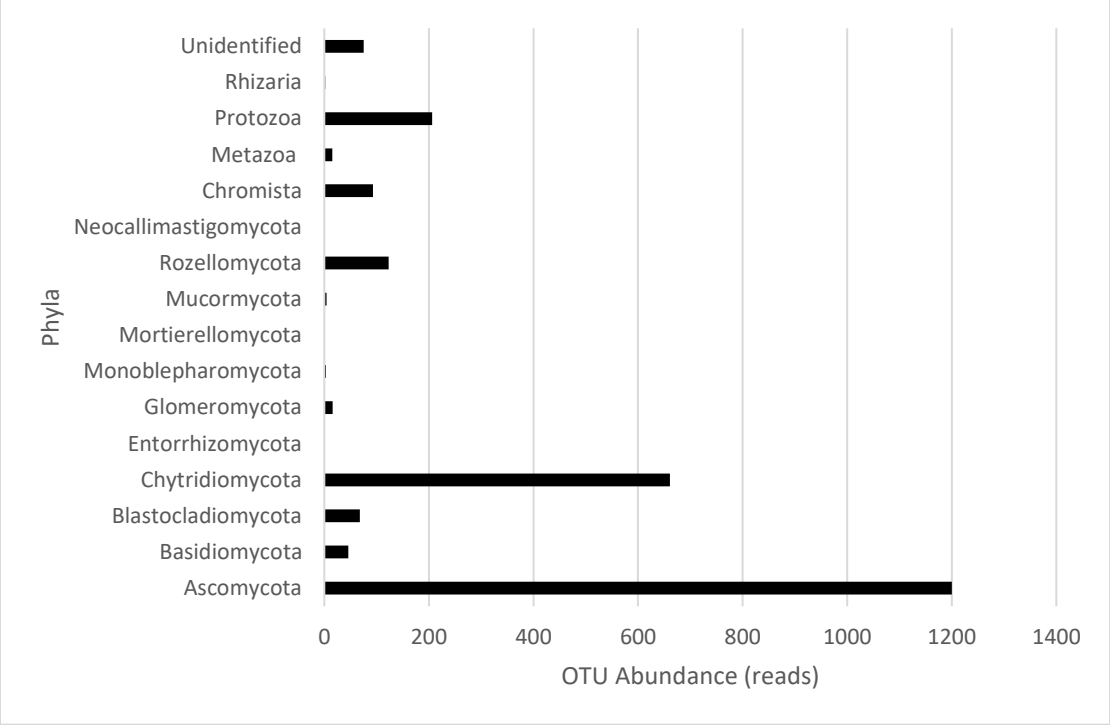

Figure 3: OTU Abundance of phyla at Mardee Lake. X-axis varies by site.

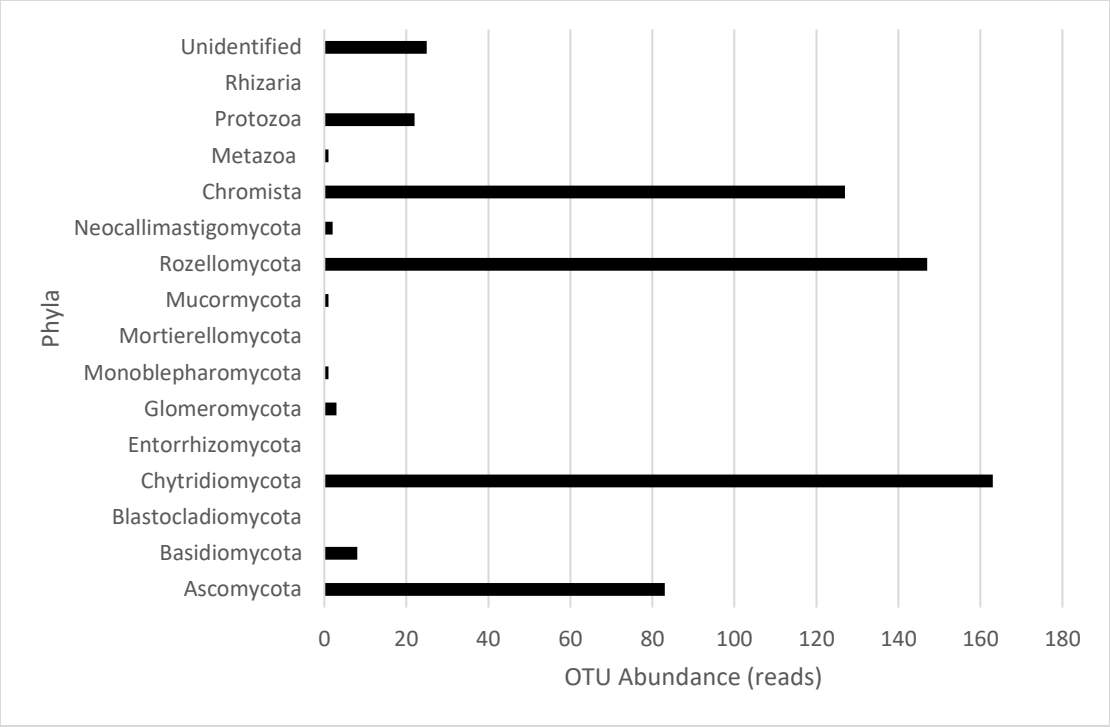

Figure 4. OTU abundance at Swamp Creek. X-axis varies by site.

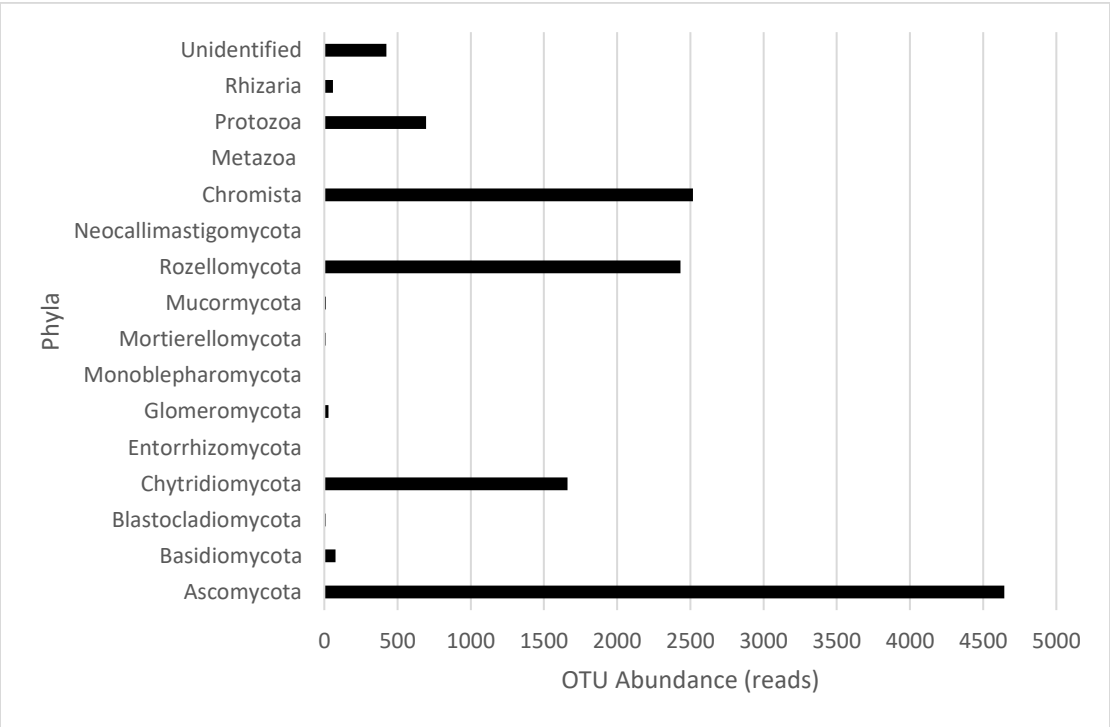

Figure 5. OTU abundance at Swamp Lake. X-axis varies by site.

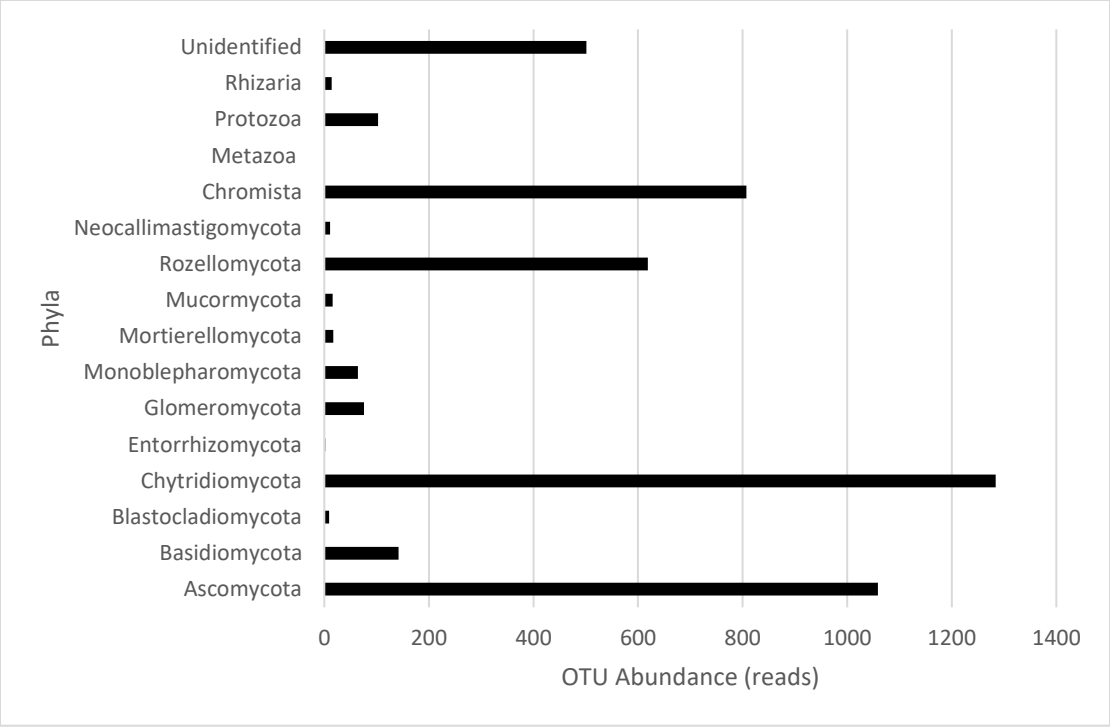

Figure 6. OTU abundance at Gold Creek Stockpile. X-axis varies by site.

As a survey of the Snoqualmie Pass corridor, the species accumulation curve shown in Figure 7 shows that, with only four samples, the data is approaching the OTU plateau; suggesting that a significant amount of the sampled population was recovered. The species accumulation curve indicates that as a survey method, the metabarcoding approach is an efficient method to observe the sampled habitats.

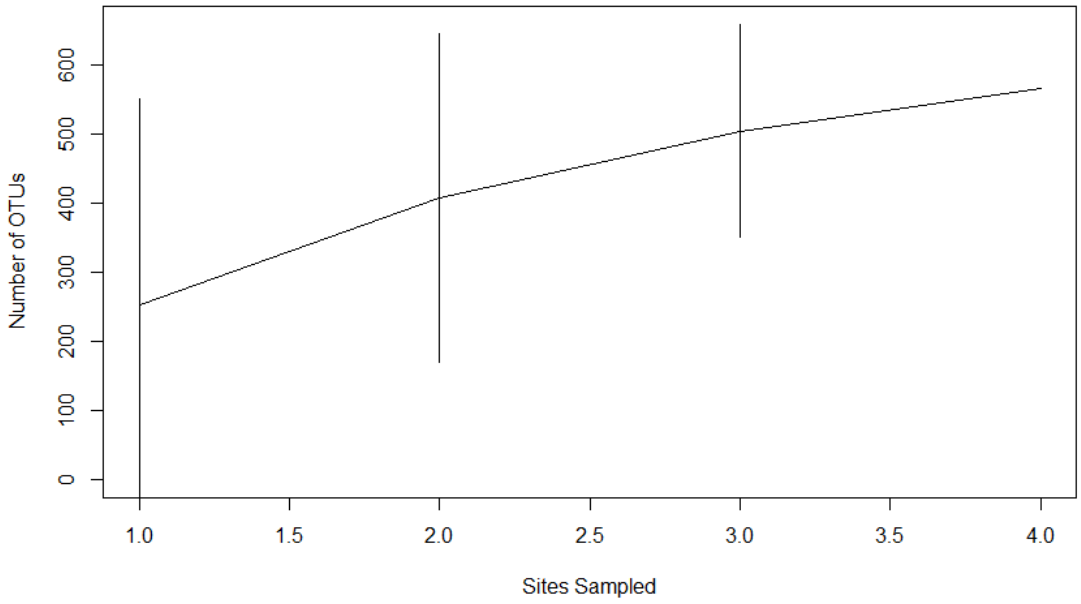

Figure 7: OTU accumulation curve of all sites. Error bars sampled randomly 1000 times.

Traditional Diversity

In terms of alpha diversity measures, shown in Table 3, lower richness and abundance were observed at Swamp Creek. Swamp Creek is comparable to Mardee Lake and Gold Creek Stockpile in mean reads per OTU at 7.3, 12, and 10 respectively. Swamp Lake had the highest mean individuals per OTU but scored the lowest in both diversity indices, indicating that this mean was likely heavily skewed by high abundance of the dominant OTUs. Gold Creek Stockpile was the most diverse site by both diversity indexes, likely influenced by it having the highest richness. Of note, Shannon's index was not much higher for Gold Creek Stockpile compared to the other sites, but the Inverse-Simpson's index indicates that it is likely not as heavily dominated as the other sites which all scored similarly.

Table 3: Alpha diversity indices measured at each site.

Index	Sites			
	Mardee Lake	Swamp Creek	Swamp Lake	Gold Creek Stockpile
Richness	210	80	237	477
Abundance	2 528	586	12 603	4 764
Mean individuals per OTU	12.0	7.3	53.2	10.0
Shannon's diversity	3.0	2.8	2.5	4.3
Inverse-Simpson's	8.0	8.7	7.2	21.0

The Renyi diversity profile plot shown in Figure 8, compares the diversity values of each site as dominance increases to assess community structure. Gold Creek Stockpile at all points is higher at all points than the other three sites as a result of higher abundance. The similarity of the curves observed indicate that the community structures at each site were also similar. Swamp Creek had a slightly flatter profile, indicating that is slightly more evenly distributed.

The Sorenson-Dice index (Table 4) and Jaccard distances (Table 5) evaluate the beta-diversity of the four sites. These indices identify compositional differences between sites. While Gold Creek Stockpile differed most from Mardee Lake by Jaccard distance, but Swamp Creek by Sorenson-Dice index. Swamp Creek overall was the least similar to all other sites by Sorenson-Dice index, this could be due to Swamp creek having the lowest overall richness of only 80 OTUs, all other sites had greater than 210 OTUs.

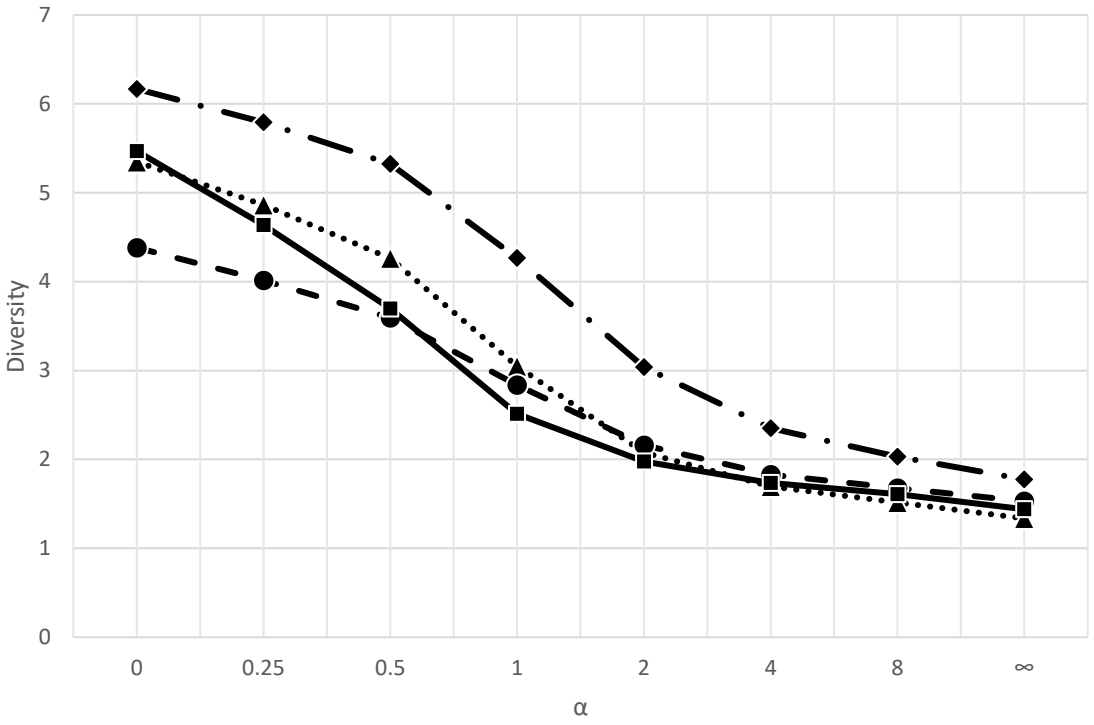

Figure 8: Renyi diversity profile plot showing each site as alpha increases on the x axis comparing diversity on the y axis. As alpha increases, the diversity is less weighted by richness and more heavily weighted by dominance of individuals. Sites shown are: Mardee Lake (triangle with dotted line), Swamp Creek (circle with dashed line), Swamp Lake (square with solid line), and Gold Creek Stockpile (diamond with dashes and dotted line).

Based upon Jaccard distance, Swamp Creek was the most similar to Swamp Lake than any other pairing of sites. The least similar sites by Jaccard distance were Gold Creek Stockpile and Mardee Lake, which showed the second highest similarity by Sorenson-Dice. Overall, the compositional comparison shown in Table 3, indicates all 4 sites represent very similar communities.

Table 4: Sorenson-Dice index matrix comparing each pair of sites.

Sorensen-Dice	Mardee Lake	Swamp Creek	Swamp Lake
Swamp Creek	0.3399		
Swamp Lake	0.4838	0.3123	
Gold Creek Stockpile	0.4865	0.3037	0.5014

Table 5: Jaccard distance index matrix comparing each pair of sites.

Jaccard Distance	Mardee Lake	Swamp Creek	Swamp Lake
Swamp Creek	0.8262		
Swamp Lake	0.8444	0.9528	
Gold Creek Stockpile	0.7525	0.8700	0.8250

Functional Diversity

The functional diversity at each site was determined using FUNGuild to assign trophic modes to each OTU. Gold Creek showed more than double the proportion of symbiotrophic individuals compared to the other 3 sites as shown in Figure 9. Symbiotrophic individuals represent mycorrhizal Fungi and lichenized Fungi which are symbionts with another species; symbiotrophs are typically hard to detect due to limited spore production compared to other Fungi. Mycorrhizal and lichenized Fungi are almost exclusively found in terrestrial habitats. Saprotrophic individuals were the most heavily represented overall, showing the highest proportion of assigned individuals. Microfungi make up the majority of representatives from the pathotroph-saprotroph-symbiotroph mode; this group is the second most represented across all sites and represents individuals that are adaptable to their environment, capable of occupying a

variety of habitat niches. However, 91.22% of all individuals were not able to be assigned by FUNGuild due to lack of information in the underlying database which relies upon published research for trophic mode assignments. OTU's aligned with sequences for *Cladosporium sp.,* the most common genus of fungal spore in the air, was unassigned despite being found more frequently than any other Fungi due to a lack of research supporting an assignment.

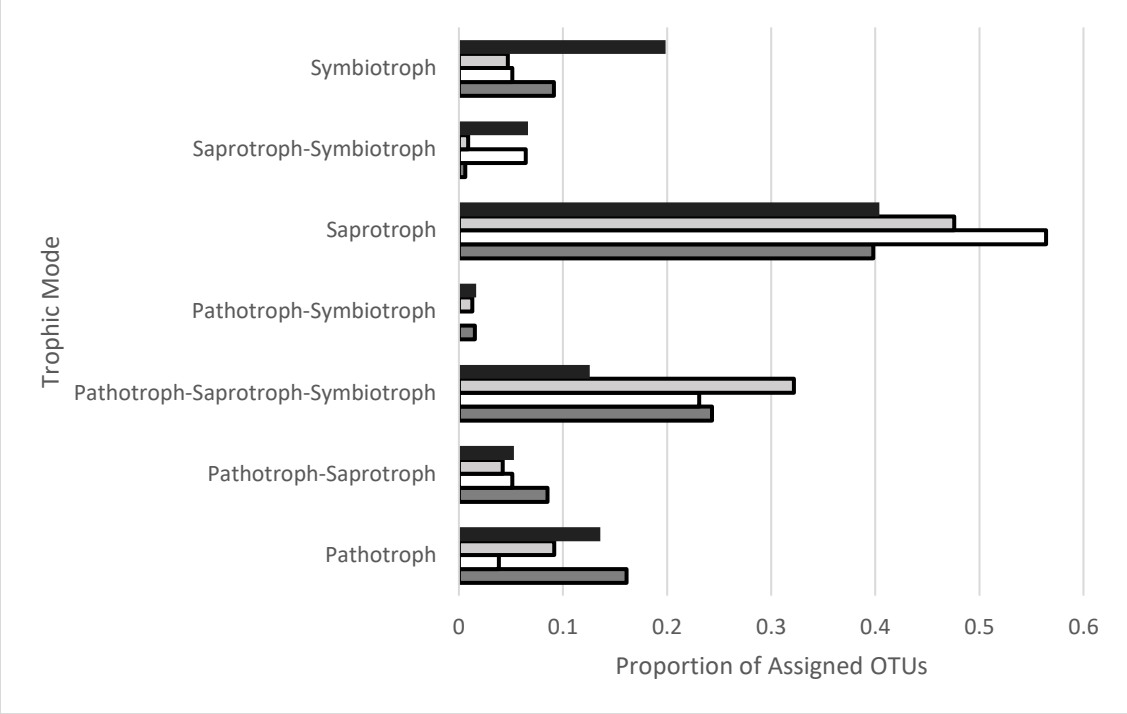

Figure 9: Proportion of assigned OTUs for each trophic mode at each site. Gold Creek Stockpile is black, Swamp Lake is light grey, Swamp Creek is white, and Mardee Lake is dark grey.

CHAPTER IV

DISCUSSION

Nanopore Metabarcoding

The Oxford Nanopore MinION was chosen for its ability to sequence the entire ITS region at a lower cost compared to Pyrosequencing at the time of this study. The lower basecalling accuracy of the minION (~90% for the R9 chemistry used in this study) compared with its competitors Illumina (99.5%) and Sanger sequencing (99.9%) also presented challenges for bioinformatics and reduced confidence in OTU assignments. (Jain et al. 2016; Winand et al. 2020).

The inability to perform traditional sequence quality checks, for example chimera checking, contributed to this lack of confidence. The low basecall accuracy also hindered phylogenetic distance analysis to assign phylotype, as sequences are only as close as the accuracy of the sequencer allows. Phylotype assignment typically occurs at 97% similarity, at ~90% accuracy all samples fall outside of that threshold just by random error, so nearly all individuals would appear as a unique phylotype. The high insertion, deletion, and gap error in Nanopore data prevented the use of standardized bioinformatics tools, such as Qiime (http://qiime.org/) and Mothur (https://mothur.org/), forcing analysis to rely on Nanopore specific tools still in development.

The throughput achieved in this experiment was 322 711 reads, which is considerably less than the yield from current Illumina chemistry of greater than 1 million

paired end reads per sample, but significantly greater than achievable with Sanger sequencing which only produces one sequence per sample (Sanger 1977; Winand et al. 2020). However, differences in throughput between samples created problems with ecological analyses. This could be due to variations in pore availability between the two flow cells used or due to non-nucleotide contamination in prepared libraries. All sequencing libraries were processed using the same reagent lots, so reagent and preparation variability is minimal. Ultimately, throughput inequality showed to be an influential factor in ecological analyses performed in this study.

As used in this study, the minION was determined to be less than ideal for ITS metabacoding; however, the minION has been shown to be better suited for genome sequencing where the depth of coverage and long read lengths can help to error correct and aid alignment to templates (Laver et al. 2015; Greig et al. 2019). While advancements in chemistry have increased accuracy, currently 98.3%, from the R9.2 chemistry used in this experiment, the lack of Nanopore specific bioinformatic tools for barcoding is a hinderance for its use for ITS metabarcoding (Morrison et al. 2020).

Variability in flow cell throughput makes consistency difficult and the increased cost of using a new flow cell for every sample makes this technology comparable in cost to Pyrosequencing. Illumina sequencing has come down considerably in cost and current read lengths allow for full sequencing of the ITS (Rocchi et al. 2017). These factors make Illumina a much more promising technology for metabarcoding moving forward. Sequence data generated by the Illumina chemistry can also be used with most bioinformatics tools currently available.

eDNA Methods

The methods used in this study, eDNA sampling with a single filtration step, were designed to have as little bias as possible. By performing a single filtration, spore loss due to repeated filtration was reduced as well as cost. While members of Chromista were captured, the water molds present near western toad and cascades frog mating sites were unable to be assessed.

The Chromista OTUs accounted for 17.3% of all reads, with 3 544 individuals matched across all sites. The only 2 Chromista OTU references initially matched are from uncultured DNA found in 2 different soil studies (Hollister et al. 2010; Lan 2015). No other taxonomic information was available for these sequences. The follow up analysis recovered low levels of *Phyophthora* and *Pythium* (<5% of previously matched Chromista sequences) which are plant pathogenic oomycetes. One possibility is the lack of recovery is due to poor sequence quality and that the sampling methods used in this experiment captured zoospores. Another possibility is that close relatives of the oomycetes were recovered and not the oomycetes themselves since oomycete references were confirmed to be included in the database used for OTU assignment. The last possibility is that the chemotactic ability of oomycetes keeps individuals closer to the egg mass then can be sampled (Van West et al. 2002). However, without more accurate sequence data, it is unclear why the survey failed to recover oomycetes.

A comparison study was considered using culture methods or collection of infected eggs at the same time water samples were taken, there was sufficient funds

available to Sanger sequence any isolates. Morphological identification, as discussed in the introduction, is problematic, at best individuals could have been identified to genus. Previous studies at Swamp Lake could have been used as a reference; however, the lack of recovery of oomycetes means such a comparison is unnecessary.

Fungal Community Recovery

As a survey, the environmental metabarcoding of aquatic samples used in this study recovered a wide range of Fungi. The phyla recovered were in expected proportions based upon known aquatic habitat usage among Fungi (Goh and Hyde 1996; Shearer et al. 2007; Hu et al. 2013). The Chytridiomycota and Ascomycota were the two most abundant groups across all sites; chytrids are a water adapted group which produces motile spores, and the Ascomycota includes ubiquitous saprotrophs capable of utilizing a wide range of habitats. The recovery of terrestrial phyla could be due to the lakes, ponds, and creeks sampled being shallow enough to allow terrestrial Fungi to grow or they could be acting as a spore sink for airborne spores. Lakes have been shown to act as pollen and spore sinks allowing study of past flora from waterbed sediments (Mudie et al. 2021). The methods used in this study could provide an avenue for the survey of aquatic fungal communities and to a lesser degree the survey of spore availability in the surrounding forest.

The survey conducted in this experiment is a proof of concept for the eDNA methods and minION sequencer used. As shown in Figure 7, the majority of OTUs present collected by the methods used were recovered in just 4 samples. However, it is

likely that the number of OTUs captured is likely smaller than what the data suggests. Due to the basecalling error rate of the minION chemistry used in this study, the alignment accuracy of the sequences likely produced false OTUs. The ITS region being 600-800bp long, does not require a large difference in sequence to identify an organism; so, it is likely that the random error of the Nanopore sequence data could cause a false match to an OTU not actually present in the sample. Additionally, the inability to perform chimera checking during data analysis means there are likely some OTUs that represent chimeric sequences or closely related species not present in the sites sampled.

The analysis workflow used in this experiment generally follows workflows from established pipelines such as PIPITS for Illumina data (Gweon et al. 2015). The main difference being the separation of the ITS1 and ITS2 sequences for each read done by PIPITS allowing chimera checking and determining OTUs based upon distance between sequences rather than by match quality to refence sequences. As stated above, the phylogenetic distance from Nanopore data is only as close as the error rate allows us to observe. Assignment of OTUs based upon match to reference, as opposed to phylogenetic distance, is less representative of the individuals present; especially with high error causing closely related species to be matched falsely. These factors likely produced an overly diverse appearing dataset, with higher species richness than present in the water column.

Traditional Diversity

Ecological diversity observed was very similar among the sites sampled. Gold Creek Stockpile stood out in most diversity measures, as the site with the highest richness. Inverse-Simpson's index indicated that Gold Creek Stockpile was less dominated by few OTUs than the other sites. The Renyi diversity profile plot in Figure 8 showed that all sites had similar community structure. The experimental design of this project, as a result of funding limitations, did not allow for sample replication; as such, traditional comparisons of diversity indices, such as ANOVA or Kruskal-Wallis test, were not able to be performed.

Compositionally, all 4 sites appeared very similar by Sorenson-Dice and Jaccard distance measures. It is possible with more accurate sequencing, and subsequent OTU assignment, that variances detected by diversity indices would equalize. More even throughput could also influence the diversity index values; especially the Sorenson-Dice index which appeared to be more sensitive to the differences in richness observed. Statistical methods have been proposed for correcting for unequal throughput when statistically comparing index values between sites and could improve the fidelity of Nanopore data when evaluating community structure (Willis 2019).

Functional Diversity

The trophic mode assessment performed using FUNGuild, confirmed that the individuals observed at Gold Creek Stockpile were slightly anomalous compared to the

other 3 sites. Symbiotrophic individuals were higher at Gold Creek Stockpile. These individuals are almost exclusively terrestrial mycorrhizae and lichenized Fungi. As expected, most individuals recovered were saprotrophic, almost all Fungi utilize some amount of saprotrophism. Microfungi were the second most abundant group found belonging to the pathotroph-saprotroph-symbiotroph mode. Microfungi produce a large number of spores and can utilize a wide range of habitats and niches. Overall, there was not much difference in functional diversity between the 4 sites. This result was not particularly surprising given the similarity in sites sampled for this experiment. However, the incomplete nature of the FUNGuild database left the majority of OTUs, 91.2% overall, with unassigned trophic modes. A more complete database could provide a more accurate evaluation of the trophic distribution of OTUs recovered.

Future Research

Further PCR optimization could produce better throughput for the oomycetes; however, a more effective approach would probably be to use ITS primers more specific to oomycetes. Subsequent to this study, oomycete specific ITS primers have been developed (Riit et al. 2016). With better targeted ITS primers combined with a more accurate sequencing technology, the eDNA sampling and filtration methods used in this study could be an effective tool for studying water molds and Fungi (Riit et al. 2016, Ruiz-Gomez et al. 2019).

A follow up to this study is necessary to determine if the eDNA sampling used in this study can collect oomycete zoospores near an active site of amphibian

saprolegniasis. A study using oomycete specific primers with Illumina chemistry, should provide sufficient data to determine if pathogenic oomycetes can be recovered via the sampling methods used in this study. If successful, future studies should explore the sampling biases of this approach, such as sampling depth, location, and time of collection, as an important next step before more complex ecological comparisons can be made using this study's methodology.

If unsuccessful at capturing zoospores near infections, sampling locality could be varied to determine if potential pathogens are available for capture away from active saprolegniasis; if they are, the absence of individuals near infections if found consistently elsewhere could be inferred as an indicator of pathogenicity. Confirmation of this could be determined by an infected egg collection at the same time of sampling and metabarcoding analysis of individuals recovered to confirm their presence at infection.

Future fungal ecological studies, using the environmental metabarcoding methods of this experiment, are certainly promising. Fungal specific ITS primers and optimized PCR conditions could further improve recovery of Fungi and more accurate basecalling could provide better fidelity of aquatic fungal communities. As with the oomycetes, exploration of sampling biases is an important next step before more complex ecological comparisons can be made using this study's sampling and filtering methodology.

Conclusions

In conclusion, the minION sequencer and environmental metabarcoding methods used in this study failed to survey the oomycetes. The R9.2 flow cell chemistry used in this experiment did not provide basecalling accuracy or subsequent sequence matching sufficient for confident OTU assignment to species or genus. Throughput inconsistency between samples also influenced ecological analyses. Overall, the Nanopore sequencer was not optimal for the environmental metabarcoding approach used in this study. To improve oomycete recovery, the PCR optimization strategy used in this study could be improved by using oomycete specific ITS primers that had not been developed at the time of this study (Riit et al. 2016). Improved oomycete DNA amplification combined with a more accurate sequencing platform, such as Illumina or Pyrosequencing, could be used to determine if the sampling approach used in this study is effective at capturing zoospores.

Fungal recovery in this study was higher than anticipated. Phyla composition reflects high abundance in aquatic taxa with lower recovery of terrestrial taxa. Alpha and beta diversity analyses were affected by throughput inequality. As a result, all four sites appeared very similar in composition and community structure. Trophic mode analysis also returned expected results based upon taxa recovered. Further expansion of the FUNGuild database is important to increase confidence trophic modes and guilds represented in analyses are close to sampled populations.

Future studies should refine methodology for both oomycete and Fungi. Exploration of methodological biases is an important step to more complex ecological questions of aquatic habitats.

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