AN *IN-SITU* STUDY DESIGN TO ASSESS REMOVAL OF EXCESS NUTRIENTS FROM AGRICULTURAL RUNOFF BY FUNGI IN CONSERVATION BUFFERS

by

Bailey Jo Campbell

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ABSTRACT

Nutrients from agricultural runoff are a leading source of freshwater pollution. Numerous rivers and lakes in Montana contain elevated nutrient levels, increased harmful algae blooms, and disrupted aquatic ecosystems. Conservation buffers are a widely accepted strategy to intercept runoff and mitigate nutrient loading to aquatic systems. Fungi are also known for facilitating key ecosystem functions such as pollutant removal and nutrient cycling; therefore, fungal enhancement of conservation buffers may increase nutrient uptake and reduce nutrient loads in aquatic systems. Yet, fungi are not commonly incorporated into conservation buffers, and their role in enhancing phytoremediation of elevated nutrient concentrations remains unclear. However, numerous studies have demonstrated that excess nutrients decrease above- and below-ground biodiversity, which impacts plant productivity, nutrient acquisition, water retention, and pathogen and disease resistance. Thus, despite naturally occurring microbial communities, conservation buffers within a degraded landscape might need enhanced with fungi to promote or maintain productivity.

The objective of my paper is to present an *in-situ* experimental design intendent to resolve this knowledge gap. I conducted a watershed analysis to find a location within the Upper Missouri River Watershed suitable for restoration based on land use, land ownership, hydrologic context, water quality, and amount of overland flow across agricultural land. I synthesized over 35 articles to create a site-specific design intended to experimentally test whether incorporating fungi into conservation buffers will (1) promote nutrient uptake in plants, despite nutrient-saturated conditions and (2) reduce soil nitrate leaching. The metrics for evaluation are foliar nitrogen and phosphorus concentrations and soil nitrate levels using ion exchange resin capsules. In this design, five of ten conservation buffers are treated with fungi and monitored long term to quantify and compare the response ratios of the treatment to the control plots. The response ratios will reveal the degree of effectiveness of the treatment and whether buffers need enhanced with fungi to retain nutrients. *In-situ* studies of fungi-enhanced phytoremediation are lacking, and fungi are greatly underutilized and overlooked as an effective tool in restoration of excess nutrients. As a headwaters state that ranks high in agricultural production, Montana's contribution to water quality degradation is increasingly being unveiled. Results from this study could be used as a remediation tool to improve conservation buffer effectiveness to retain nutrients and mitigate nutrient pollution.

INTRODUCTION

Nutrient Pollution in Freshwater Systems

Degradation of water quality in freshwater systems is a major environmental concern worldwide. Climate change, land use alteration, and increasing population growth are expected to accelerate water contamination. Agricultural runoff is the leading cause of non-point source pollution to streams, rivers, and lakes (Nottingham & Messer, 2021; USEPA, 2021). Among the many pollutants in agricultural runoff, nitrogen and phosphorus from inorganic and organic fertilizer are the most common source of impairment of ground and surface water (Nottingham & Messer, 2021). Nutrient pollution has become the most "widespread, costly, and challenging environmental problems" in the United States (USEPA, 2023).

Unlike other freshwater pollutants, nutrients are a naturally occurring, essential component to ecosystems. Yet, in excess, nutrients cause eutrophication of water bodies, biodiversity loss, harmful algal blooms, and hypoxic conditions that are devastating to aquatic ecosystems. Consistently high concentrations of nitrogen (N) and phosphorus (P) directly affect communities of phytoplankton, periphytic algae, and grazers, all of which play vital roles in structuring the food web. The resulting change in ecosystem structure has cascading effects on soil productivity, vegetation, human health, and the economy. Streams and rivers in agricultural settings have among the highest N and P concentrations (Munn et al., 2018). The elevated concentrations demands special attention to nutrient loading of these systems, and the development of effective restoration efforts that alleviate the environmental effects of agricultural activities.

Montana has been called the 'Headwaters of the Continent' because it is the only state that sends water to three oceans (MT DEQ, 2017). Originating in Montana, the Missouri River

and its tributaries cover over 200,000 square kilometers and contains approximately 56% of the state's water (MT DEQ, 2017). This cold, oxygenated river provides critical habitat for many important species, and a foundation for valuable ecosystem services. According to the Montana Department of Labor and Industry (2022), boating and fishing generated $160 million in gross domestic product (GDP), accounting for over 25% of GDP from outdoor recreation in Montana (Hendrix, 2022). Yet, this crucial resource is being threatened from large fluxes of N and P from agricultural sources. In Montana, commercial fertilizer purchased in 2017 amounted to over 200,000 kilograms of N and over 80,000 kilograms of P (**Table 1**). Nutrient levels on the Upper Missouri River have been trending upward, and often exceed the Montana Department of Environmental Quality (DEQ) water quality numerical standards. A water quality report in 2020 revealed that 35% of rivers and 22% of lakes in Montana are impaired due to elevated nutrient concentrations (MT DEQ, 2021).

Commercial Fertilizer Purchased	**2003**	**2005**	**2007**	**2009**	**2011**	**2016**	**2017**
Nitrogen (1000 kg)	117,047	106,200	158,226	138,305	228,141	214,046	208,606
Phosphorus (1000 kg)	60,173	60,008	73,602	65,801	69,681	91,176	84,825

Table 1: *Amounts of fertilizer purchased in Montana (in 1,000 kg). Fertilizer information is reported by state fertilizer control offices and excludes livestock manure, liming materials, peat, potting soils, soil amendments, soil additives, and soil conditioners. Source: U.S. EPA (2022).*

Excess nutrients cause a rapid overgrowth of algae in waterbodies. Harmful algal blooms are increasing in prevalence and occurrence, shutting down waterways statewide. For example, in August of 2022, the Montana DEQ submitted a proposal to the U.S. Environmental Protection Agency (EPA) to list 40-50 miles of the Gallatin River as impaired due to excessive algal growth (Dore, 2022). This degradation of water quality at the headwaters of the Missouri River exposes

Montana's contribution to the global freshwater pollution crisis. Nutrients continue to accumulate along the 3,710-mile stretch of the Missouri-Mississippi River system and eventually are released into the Gulf of Mexico. These toxic nutrient loads in the Gulf are devastating to marine life and the coastal economy, producing a 'dead zone' measuring 4,280 square miles in size (NOAA, 2022). Addressing these nutrient pollution issues and protecting or restoring water resources requires collaboration among numerous public and private entities (MT DEQ, 2017).

Decreasing nutrient loads is challenging due to the fundamental connections among nutrient pollution, fertilization, and food production. The global human population is expected to increase to 9.3 billion by 2050 (Basit et al., 2021; Spinelli et al., 2021), with an expected rise of 60% in food production (Spinelli et al., 2021). According to the National Agricultural Statistics Service (2019), the total monetary value of Montana agriculture production is just over $4 billion, and agricultural production covers 58 million acres or 62% of Montana (Haynes et al., 2020). Addressing agricultural runoff in the face of increasing demand on crop production and environmental stressors in a manner that is economically feasible will require a comprehensive approach (Juncal et al., 2023), including strategies for reducing excess fertilization such as precision agriculture and cost-effective, environmentally sound, best management practices (BMPs) that use bioremediation to reduce nutrient movement into aquatic systems.

Bioremediation

Bioremediation is an attractive tool that has gained considerable attention as a strategy to detoxify contaminants in soil, water, or air. Phytoremediation involves using existing or planted vegetation to remediate contaminated sites (Rubin & Gorres, 2020). For example, vegetative buffers, filter strips, constructed wetlands, and bioretention cells are methods developed to mitigate freshwater pollution in urban and rural areas. These conservation buffers are intended to

slow, intercept, and filter surface or groundwater between a pollutant source and an aquatic system to prevent water quality degradation. A conservation buffer is a biofiltration method and a BMP that is widely accepted as an effective remediation strategy for reducing freshwater pollution. This strategy is particularly attractive because it is non-invasive, requiring no engineering controls or excavation (USEPA, 2006). Instead, these buffers stimulate natural processes and provide a barrier between anthropogenic activities and waterways.

Fungi have also been widely explored for their capacity to filter or remove toxins from a contaminated environment, a process termed 'mycoremediation'. Fungi are known to break down environmental pollutants including complex hydrocarbons, dioxins, polyaromatic carbons, petroleum, dyes, detergents, pharmaceuticals, and heavy metals (Purohit et al., 2018; Basit et al., 2021; Mohapatra et al., 2021). These highly diverse, eukaryotic organisms are ideal for bioremediation because of their adaptability to harsh environments (pH and temperature), vast mycelial network, ability to colonize easily, and production of potent enzymes capable of transforming recalcitrant compounds (Mohapatra et al., 2021, Spinelli et al., 2021). Many fungal species develop a tolerance to prolonged exposure to a pollutant (Mohapatra et al., 2019). These qualities may be attributed to a short generation time and a high rate of evolutionary adaptation (Vaksmaa et al., 2023).

There are inherent challenges and limitations to studying fungi *in-situ* on a landscape scale. Prior studies of mycoremediation of freshwater systems have primarily been restricted to controlled, laboratory settings. Despite knowledge that suggests fungi are key players in combating pollution, fungi are greatly underutilized and overlooked as an effective tool in restoration efforts. Although they involve vastly different organisms, phytoremediation and mycoremediation may be fundamentally connected. More than 80% of plants have a mutualistic

symbiotic relationship with arbuscular mycorrhizae fungi (Rubin & Gorres, 2020). Arbuscular mycorrhizae fungi facilitate bidirectional exchange of nutrients and water, as well as stabilize soil, increase photosynthesis, and stimulate pathogen and drought resistance. Terrestrial plants invest nearly 20% of their photosynthates in maintaining symbiosis with arbuscular mycorrhizae fungi for improved N and P acquisition (Tiruvaimozhi et al., 2018). Given this mutualistic relationship, many ecosystem processes that are associated with plants may also be influenced by plant-fungi interactions.

Incorporating arbuscular mycorrhizae fungi as a 'bio-fertilizer' has documented benefits for plant productivity, species richness, and soil health (Gilliam, 2019; Stanescu & Maherali, 2017). Further, fungi reduce nutrient leaching and promote nutrient cycling (Taylor et al., 2018; Dalecka et al., 2020; Thomas et al., 2009; Xu et al., 2021; Mo et al., 2015; Mei et al., 2019; Kohler et al., 2018). These studies largely involve manipulating rates of N and P applications, many of which are greenhouse experiments involving a single plant species in a setting where environmental variables are removed. Existing, *in-situ* mesocosm studies on mycoremediation of excess nutrients have produced inconsistent results (Thomas et al., 2009; Taylor et al., 2018., Dalecka et al., 2020). One possible explanation is that nutrients are essential to a healthy ecosystem, so research is complicated by a multitude of factors relative to other pollutants. Therefore, our understanding of how nutrient cycles and plant communities respond to the use of fungi as a bio-fertilizer in a nutrient-saturated environment is limited.

Although conservation buffers are a widely accepted strategy to mitigate freshwater pollution, to my knowledge there are no studies that have examined the potential use of fungi to enhance conservation buffers and reduce nutrient pollution. There is reason to believe that fungi may play a vital role, but data are lacking. Therefore, the objective of my paper is to present an

experimental design intendent to resolve this knowledge gap. In this paper, I review studies on the response of fungi and plants to excess nutrients. Using prior findings, I designed a study that is intended to reveal the role that fungi have in nutrient cycling and leaching in buffers. If fertilizing conservation buffers with fungi enhances retention of nutrients, then nutrient loss via runoff to streams would also be mitigated.

LITERATURE REVIEW

Plant-Fungi Mutualism

It is widely accepted that plant diversity improves critical ecosystem functions, such as plant and soil productivity. Therefore, maintaining above and below ground biodiversity is essential in the effectiveness of conservation buffers. However, numerous studies have shown that excess N to soil decreases plant diversity. For example, Gilliam (2019) monitored the effects of N additions on the herbaceous layer in a forested ecosystem. Nitrogen significantly decreased species diversity after 20 years. Specifically, the competitive advantage of nitrophilic species resulted in a loss of N-efficient species. Soons et al. (2017) compared the results of 189 nutrient enrichment experiments to determine the effect on plant species richness in natural and semi-natural landscapes. Nitrogen additions and combined N and P additions reduced species richness across all experiments by an average of 16%, whereas P additions did not have an effect.

Although elevated nutrients may decrease plant diversity, numerous studies suggest that fungi promote plant species diversity. For example, Stanescu & Maherali (2017) found that enhancing soil with arbuscular mycorrhizae fungi greatly reduced the competitive ability of strong competitor plant species, which increased overall species diversity. However, inputs of

nutrients negatively affect microbial biodiversity and abundance (Gilliam, 2006; Tiruvaimozhi et al., 2018; Mo et al., 2015; Kohler et al., 2018; Van Der Heijden, 2010), consequently reducing plant diversity and nutrient cycling. The enhancement of arbuscular mycorrhizae fungi in impacted systems may therefore be necessary to support or restore diverse plant communities under elevated nutrient conditions. Because soil degradation from fertilizer overuse and monocrop planting depletes naturally occurring microbial communities (Zhen et al., 2014). bio-fertilizing buffers with fungi could improve colonization potential of host plants and stimulate symbiosis.

Bioretention of Nutrients

The potential for fungi to enhance phytoremediation of elevated nutrient concentrations has been documented in previous experiments. Many such studies of fungi-enhanced phytoremediation involve bioretention cells in urban areas. Bioretention cells allow surface water to collect, slowing the retention time and filtering toxins using a range of materials, media, drainage systems, and biota. However, bioretention cells are unnatural and often require excavation, engineering, and alterations to hydrology. Many state and federal water quality policies prohibit these activities in close proximity of a stream. Hence, bioretention cells are not a realistic remediation strategy in a vast agricultural landscape.

Despite the limited applicability of bioretention cells in rural landscapes, experiments in these structures have recently been used to investigate the role of fungi and other microorganisms. *Stropharia rugoso-annulata* (garden giant fungi) incorporation into urban bioretention cells slowed the release of total P, based on water samples collected through the system (Taylor et al. 2018). Similarly, *Trametes versicolor* (white-rot fungus) removed total P from municipal wastewater after six hours of incubation (Dalecka et al., 2020).

A comparative mesocosm study aimed to test whether integrating fungi into a bioretention cell would influence nutrient concentrations in surface water (Thomas et al. 2009). In this study, surface water from agricultural land adjacent to a tidal wetland was redirected to flow through twin biofiltration cells. Throughout the study, nutrient reduction results varied. However, total N was reduced in outflow to a higher degree in the fungi-treated cell during the third phase compared to the control plot. Alternatively, total P was consistently exported through both cells, but to a lesser extent in the fungi-treated cell. They attributed the increase in nutrient removal in the treated cell to the addition of mycorrhizal fungi to the plants, which increases nutrient absorption capacity of root systems and enhances soil structure.

Nutrient Exchange

In addition to the community level effects, elevated nutrient concentrations are known to alter plant foliar N:P ratios. A meta-analysis found that in over 134 fertilization studies, N addition significantly increased foliar N concentrations by 19 %, yet decreased foliar P by 3% (Jinhua et al. 2020). Similarly, Xu et al. (2021) conducted a N addition experiment and evaluated the foliar nutrient stoichiometry in various plants in a boreal forest. Over six years, the leaf N concentration increased, and the leaf P concentration decreased (foliar N:P ratios increased) across all taxonomic groups. Mo et al. (2015) found when only P was added to a forest environment, leaf P concentrations were significantly increased. Yet, when N and P were combined, the foliar P ratio was reduced. Notably, they also observed a decrease in soil fungal biomass with the co-addition experiment, which they suggested to have “limited P uptake in plants despite high soil availability”.

Mei et al. (2019) conducted an *in-situ* study to determine whether arbuscular mycorrhizae fungi influence nutrient uptake in temperate meadow plant species under nutrient rich conditions.

The fungi promoted P uptake in plants, which lowered the foliar N:P ratio. In another study, increased ectomycorrhizal fungi richness and diversity increased P uptake in European beech saplings under a global change study that simulated increased N addition, warming, and drought (Kohler et al., 2018). Both studies suggest that increased N concentrations increase the dependency of plants on their fungal symbionts. Other studies suggest that P-limited ecosystems will become increasingly more prevalent amidst global change (Liu et al., 2021, Jinhua et al., 2020; Mo et al., 2015; Mei et al., 2019; Van der Heijden, 2010). Furthermore, acidic soils from N deposition, drought, and rising temperatures reduce soil mobility and plant uptake of P (Kohler et al., 2016; Liu et al., 2021). These studies are significant given that P is a non-renewable resource that is vital for plant growth (Rubin & Gorres, 2020).

Microbes are far more efficient at obtaining P from soil compared to plants (Mei et al., 2019). In the presence of dead plant tissue or naturally occurring phosphates in soil, fungi break down organic P compounds via enzymatic activity, releasing it into a soluble form for use (Thomas et al., 2009; Liu et al., 2021). Once orthophosphate is taken up by the hyphae, it is transformed into polyphosphate, creating a P gradient that further enhances uptake by plants and reduces soil acidification (Mei et al., 2019). Arbuscular mycorrhizae fungi and ectomycorrhizal fungi can increase plant P uptake several-fold (Rubin & Gorres, 2022). Fungi can store P when it is in excess, to trade with plants when needed (Muerdter et al., 2018). During plant senescence, nutrient uptake declines and P is cycled back into the environment from leaf litter. Fungi accelerate the decomposition of litter, retaining the nutrients from leaf litter in the soil and mitigating the loss to aquatic systems via surface flow. Thus, fungi are chief regulators of nutrient cycling.

Nutrient Leaching

Enhanced bioretention of nutrients in conservation buffers may also alter the amount and availability of N in soil and groundwater. Nitrate is negatively charged, highly mobile in soil, and persistent in waterways making it the majority (95-99%) of N leached (Kucova et al., 2016; Luo et al., 2020; Munn et al., 2018). In addition, conventional tilling increases the production of nitrate via mineralization, resulting in higher nitrate leaching. Thus, most studies use nitrate as an indicator of nutrient leaching or limitation.

Direct measurement of nitrate leaching is difficult due to the spatial and temporal complexity of nitrate concentrations and groundwater movement. Instead of directly measuring nitrate concentrations in soil or groundwater, Ion Exchange Resin (IER) devices are often used to measure cumulative nutrient-leaching in soil (Grahmann et al., 2018; Sullivan & Jiang, 2001; Kucova et al., 2016). In contrast to direct soil sampling methods, IER devices reflect how nutrients and other elements in the rhizosphere are available for biological use over time (UNIBEST, 2019). Vizuete-Jaramillo et al. (2022) tested the efficiency of IER samplers to monitor nitrate fluxes in a semi-arid environment. They concluded that IER samples are effective at accumulating nitrate over an entire growing season.

Chen et al. (2015) used IER bags to assess nitrate leaching and found that soil nitrate concentration was higher after N and combined N and P additions to the field plots. Grahmann et al. (2018) estimated nitrate leaching on agricultural land under different tillage systems using IER samplers. They determined that nitrate leaching varied by crop species but was significantly higher under tillage systems. In both studies, higher concentrations of nitrate accumulated in IER bags corresponded with an event which increases N leaching.

Plant uptake of nitrate from the soil system may be enhanced by the activity of fungi, which also have the necessary enzymes to directly utilize nitrate as a N source. As a result, ammonia can be produced by the fungal body and transformed or transferred to the host plant as an additional N source (Thomas et al., 2009). Arbuscular mycorrhizae fungi can store vast amounts of N in their spores and extraradical hyphae, enhancing uptake by plants and reducing nitrate leaching (Fang et al., 2020). For example, *Allium porrum* (leek) inoculated with *Claroideoglomus claroideum* (arbuscular fungi) reduced nitrate leaching in the potted plants by almost 85% compared to the control pots (Kucova et al., 2016). This was detected using discs with mixed ion exchange resin placed in the bottom of experimental pots. Similarly, Fang et al. (2020) studied AMF- inoculated *Populus* spp. (poplar) under high N applications. Their results showed nitrate and ammonium declines of 20% and 68%, respectively compared to the control. The combined results from these studies expose how plants and fungi respond to or remediate sustained inputs of N and P fertilizer.

STUDY DESIGN OBJECTIVES

My experimental design is based on the understanding that: (1) N + P additions to soil decrease foliar P in plants, which increases plant foliar N:P ratio, (2) excess N decreases plant and microbial richness and diversity, (3) excess N reduces P availability to plants, a scenario exacerbated by N deposition and climate change, (4) fungi inoculation increases P mobility, availability, and plant uptake, which lowers the N:P ratio, and (5) fungi inoculation increases above- and below-ground diversity.

In this mesocosm design, ten conservation buffers (‘plots’ here after) distributed in pairs will be installed on opposite sides of two tributaries of the Upper Missouri River near Toston, Montana to test whether fungi: (1) facilitate nutrient uptake in conservation buffers, despite

nutrient-saturated conditions and (2) reduce nitrogen leaching. The metrics for evaluation are (1) foliar nutrient stoichiometry and (2) soil nitrate (NO3-) concentration. With the fungal treatment, I hypothesize that (1) nutrient availability and plant uptake would be increased, evident by an increase in leaf P concentrations and a lower leaf N:P ratio, and (2) nitrate concentrations in the soil would be lower compared to the control plots.

EXPERIMENTAL DESIGN

Study Area

I identified two tributaries to the Upper Missouri that flow through an extensive agricultural area on the west side of the Missouri River: Crow Creek and Warm Springs Creek (**Figure 1**). One study site will be located on Lower Crow Creek at the confluence of Crow Creek and Warm Springs Creek (**Figure 2**), and an additional two study sites will be located along each tributary at an interval of 2 kilometers or greater, for a total of 5 study sites. At each of these sites, a control and treatment plot will be placed on opposite banks of each of the tributary (**Figure 2**). The sites will be located along the tributaries at locations where hydrologic flow paths indicate a high magnitude of agricultural runoff entering the tributary and floodplain. The process for selecting the Lower Crow Creek site is described in detail below; the process for selecting sites along the other four tributaries will follow a similar process.

I performed a spatial analysis to determine site suitability between Three Forks and Townsend, Montana within the upper Missouri River watershed. This area was chosen because it is a broad agricultural valley close to the headwaters of the Missouri River. I conducted a watershed analysis in QGIS to delineate the area that contributes runoff to this stretch of the Missouri River. Next, I used land ownership and land cover layers to differentiate cultivated land from other land cover types (**Appendix A**). I used flow accumulation and flow direction tools to

determine the location within the basin that has the greatest magnitude of groundwater movement across cultivated land. This site was found to be a large floodplain at the confluence of Crow Creek and Warm Springs Creek at the Missouri River (**Figure 1**).

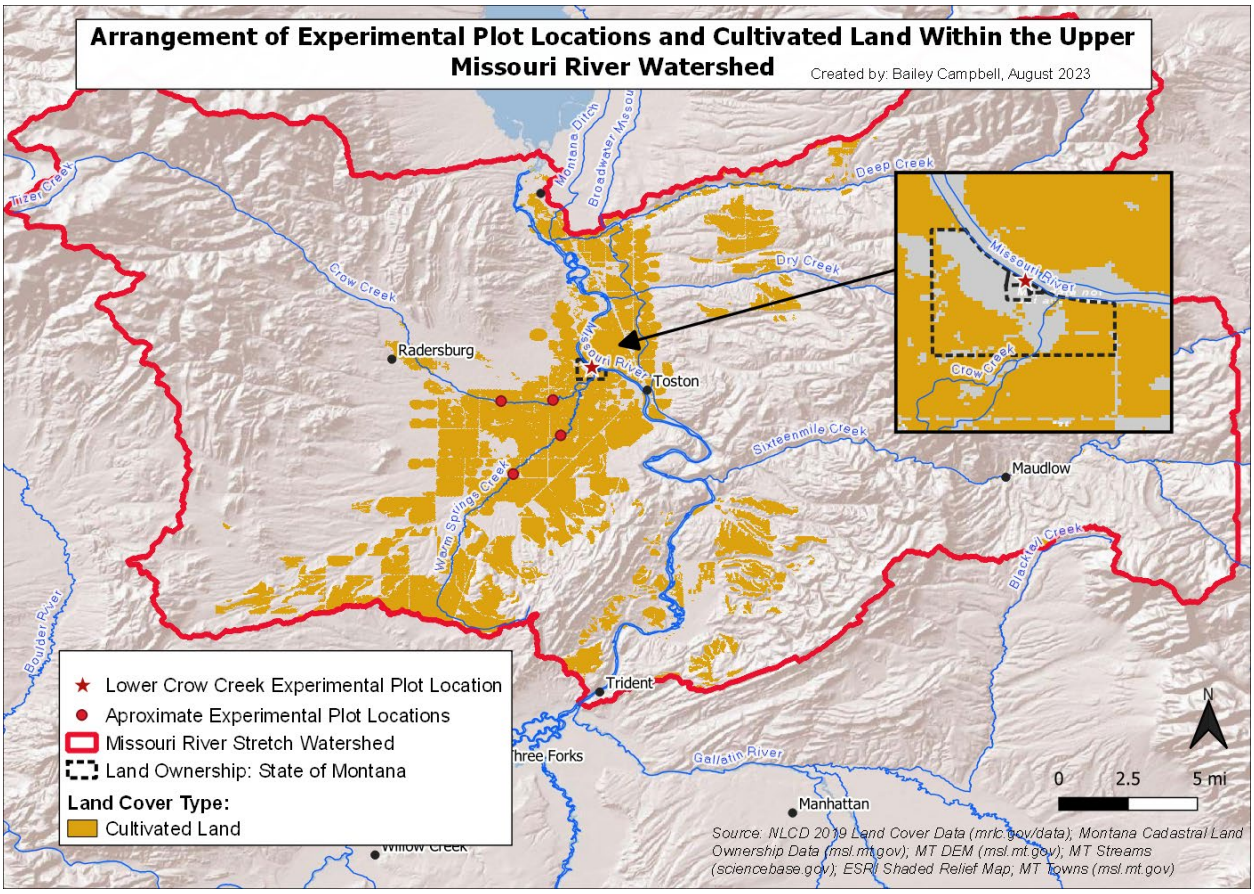


Figure 1*: Location map of study area. A watershed analysis was conducted in QGIS to find the major source of agricultural runoff within the Upper Missouri River watershed. The lower Crow Creek experimental plot location is shown in the inset map. Specifically, the lower Crow Creek plots would be located at 46° 11'29" N, -111°29'28" W.*

A waterbody report from 2021 indicates that Crow Creek is listed as impaired (Category 5 under the Clean Water Act) and needs a restoration plan (MT DEQ, 2021). The probable sources contributing to impairment are listed as grazing and agriculture resulting in excess N, P, and sedimentation (MT DEQ, 2021). The adjacent 22.6 mile-stretch of the Missouri River

between Toston Dam and Canyon Ferry Reservoir is also listed as impaired from various sources. Warm Springs Creek has not been assessed by the DEQ at the time of this paper.

A Montana Cadastral layer shows this 417-acre parcel to be owned by the State of Montana that is used for grazing and irrigated for alfalfa. I performed a remote sensing supervised image classification on Landsat8 images from June and August of 2017 to assess temporal variation in soil moisture and vegetation greenness within the parcel (**Appendix B**). From this, I determined that this floodplain location is occasionally saturated and able to support a diverse plant community, ranging from riparian to semi-arid species.

Treatment Plot Locations

A diversion dam is located 0.25 stream miles from the Missouri River in Crow Creek to provide water to an irrigation canal running parallel to the Missouri River. Five treatment plots and five control plots will be installed in pairs and located on opposite sides of the creek and upstream of the dam, to capture temporal saturation (**Figure 2**). Placing the paired plots on opposite sides of the river reduces the likelihood of the treatment plot influencing the control plot for each of the five paired plots. Additionally, I considered whether the paired plots would have a similar likelihood of capturing high-water events, such as if the site is located at an oxbow bend in the creek (**Figure 2**). The slope of the selected study area is 0-2% with an elevation range of 1188 to 1200 meters above sea level. This area is ideal and consistent with U.S. EPA guidelines because groundwater and surface water flow are slower, which increases nutrient retention time through the system and improves performance.

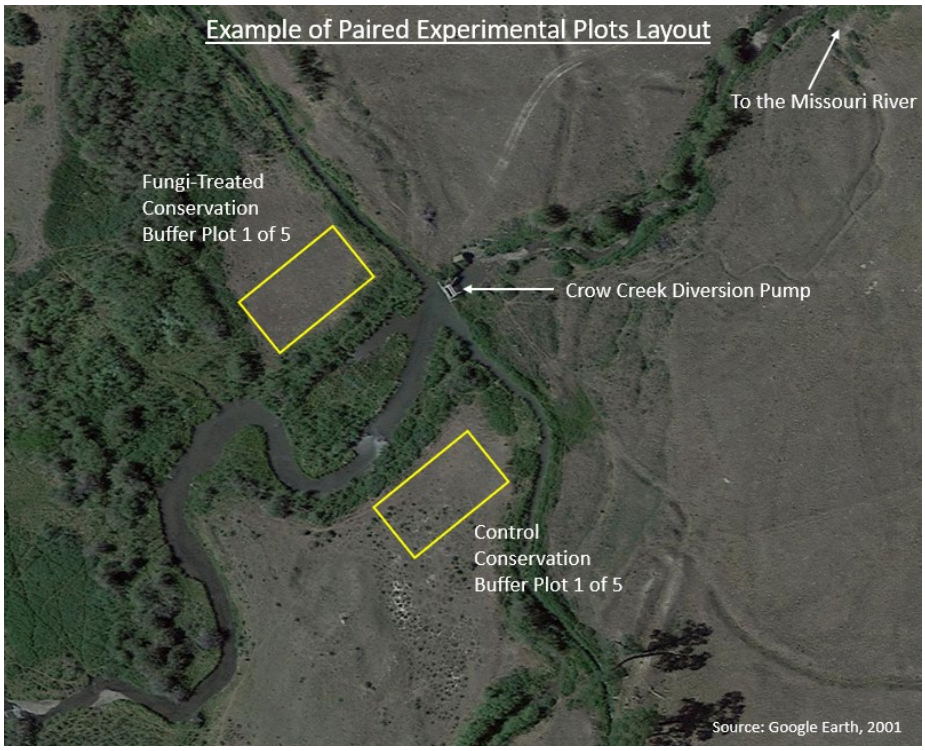


Figure 2. *Example of paired experimental plot layout. Paired plots are comprised of one treatment plot and one control plot. These paired plots represent similar site characteristics as reasonably feasible, using the methods described above. Source of imagery: Google Earth, 2001*

The USDA soil map of this site reveals that soil on both sides of the creek (location for plots) is classified as Fluvaquentic Haplaquolls (**Appendix C**). This soil type is characteristic of drainageways and has a typical soil profile of up to 46 inches of silt loam, which is poorly drained and has a moderately high to high capacity of the most limiting layer to transmit water. The weighted average pH for this soil type is 7.2. Access to the site is via a two-track road off Smith Lane and MT Highway 12 that is utilized for maintenance on the dam.

Site Preparation

There is an abundance of resources available to guide conservation buffer design and layout, vegetation components, and planting guidelines which this paper does not discuss in

detail. Rather, I elaborate on components that are unique to this experimental, site-specific design.

As stated previously, conservation buffers are a suitable best management practice because they are non-invasive, semi-natural, and require minimal site preparation. The numerous confounding variables in the design are considered inherent components that demonstrate the practicality of a "real-world" setting. Using the measuring tool in Google Earth, I determined the appropriate plot size to be 100 feet by 50 feet (**Figure 2**). This follows USDA guidelines (2008) discussed in, "*Conservation Buffers Design Guidelines"*, which suggests a width of at least 100 feet is necessary when targeting nutrients to improve water quality.

Soil

The goal of this experimental design is to determine whether the addition of fungi *enhance* buffer effectiveness at the selected sites to demonstrate practicality in restoration. Thus, soil and existing vegetation are not manipulated (e.g., sterilized) understanding that native microbial communities are present. Soil sterilization alters soil chemistry (Asghari & Cavagnaro, 2012), soil invertebrate communities, and soil infiltration rates (Kucova et al., 2016). Sterilizing or other soil manipulations would therefore introduce additional sources of variation in the control plot.

Vegetation

The five treatment and five control plots will be planted with grasses, herbs, and shrubs which are identical in composition and size, as reasonably feasible. Vegetation will be planted in a grid-like pattern with each plant uniquely labeled for sampling. Ultimately, the paired plots will be mirrored, despite naturally-occurring (volunteer) vegetation. Planting will take place after the risk of last frost in the spring, which is June 11-June 20 for the study area. Two years will be

allocated to site preparation and bi-weekly monitoring to ensure planted vegetation has been established. In this timeframe, dead individuals will be replaced.

In accordance with best management practices, only native vegetation will be planted in the plots. Various online and local resources will be used to guide selection of vegetation. Known, N fixing species will not be planted to avoid introducing an additional source of N into the plots. Plants with deeper, more extensive root systems (such as bunchgrass) are suggested to be the most efficient in nutrient removal (Muerdter et al., 2018), and will be incorporated in the design. In general, the habitat type of the study area is primarily *Artemesia tridentata* (big sagebrush) / *Agropyron spicatum* (bluebunch wheatgrass) with adjacent habitat type of *Pseudotsuga menziesii* (Douglas Fir) / *Agropyron spicatum* (Pfister, 1977). However, plants that tolerate riparian habitats will be used nearest to the stream bank.

Mulch

An equal weight of wood chip media (Douglas Fir or Ponderosa Pine) from a local sawmill will be spread evenly across the plots. Wood chips will be used because commonly used alternatives (i.e., straw and sawdust) are easily dispersed in water, potentially contributing to freshwater contamination (Stamets, 2020). The woodchip substrate for both the treatment and control plots will first be autoclaved for sterilization.

Fungal Treatment

In the third year, the wood chip media in the treatment plot will be inoculated with *Pleurotus pulmonarius* (common oyster)*, Pleurotus populinus* (aspen oyster), and *Agaricus campestris* (meadow mushroom). These species are native to Montana and are found in riparian areas yet are also capable of thriving in arid conditions (Cripps et al., 2016), and have been the subject of numerous mycoremediation studies. Fungal spores, mycelia, or colonized substrate are

readily and commercially available for these species at various local and regional sources. In addition to the inoculated wood chip substrate, arbuscular mycorrhizal fungi and ectomycorrhizal fungi inoculant sticks will be set into the soil and spaced according to manufacturer recommendations across the five treatment plots. The fungal treatments (inoculations) will continue to take place in the spring of each following year, to give the mycelium more time to colonize and grow (Stamets, 2020) and to continually augment the population throughout the study.

Sampling Procedures

Baseline Site Characteristics

Initial site characteristics will be recorded biweekly from April to October for years 1 and 2 (before fungal treatment). These data would serve as a baseline against which to compare the effects of the fungal treatment. Sampling would include (1) deploying and collecting IER capsules and (2) collecting leaves for laboratory analysis, as described below.

Soil Sampling Using Ion Exchange Resin (IER) Capsules

This study will use manufactured IER capsules purchased from UNIBEST, Inc. in Bozeman, MT (**Figure 3**). These spherical capsules are encompassed by a mesh plastic and measure 2 cm in diameter and weigh less than 10 g (Sullivan & Jiang, 2001).

Figure 3: *Drawing depicting how ion-exchange resin capsule captures nutrient cycling patterns. Source: UNIBEST, International.* https://www.unibestinc.com/technology

Four permanent soil sampling locations would be constructed within each plot (40 total samplings sites). Construction would take place after vegetation has been planted during the first year. Deployment, retrieval, and replacement of IER capsules will take place at the same sites throughout the duration of this study. Deploying the IER capsules will follow the methodology of Sullivan & Jiang (2001), who used IER capsules to monitor nitrate leaching in golf courses and sod farms. This approach minimizes the disturbance while allowing for long-term monitoring. Other methods of IER capsule deployment and retrieval used in agricultural systems may capture seasonal nitrate dynamics more directly, however such methods are unsuitable for this application due to the increased soil disturbance.

To facilitate IER capsule deployment, retrieval, and replacement, a bottomless box with a removable lid, measuring 30 cm in width, length, and height, will be buried in the soil so that the lid is just below ground surface. Four holes will be drilled into the sides at depths of 7, 14, 21, and 28 cm below ground surface. These holes will hold the permanent, horizontal ‘access tubes’ for setting and retrieving each of four IER capsules per box. Each resin capsule will be

fastened to a custom spring-loaded rod inserted into the access tubes. The box will be covered with a lid to close the system (**Figure 4**). Capsules will be collected and replaced monthly throughout the growing season to capture the accumulated value of nitrate passing through the soil profile. Retrieved capsules will then be sent to the UNIBEST laboratory for analysis.

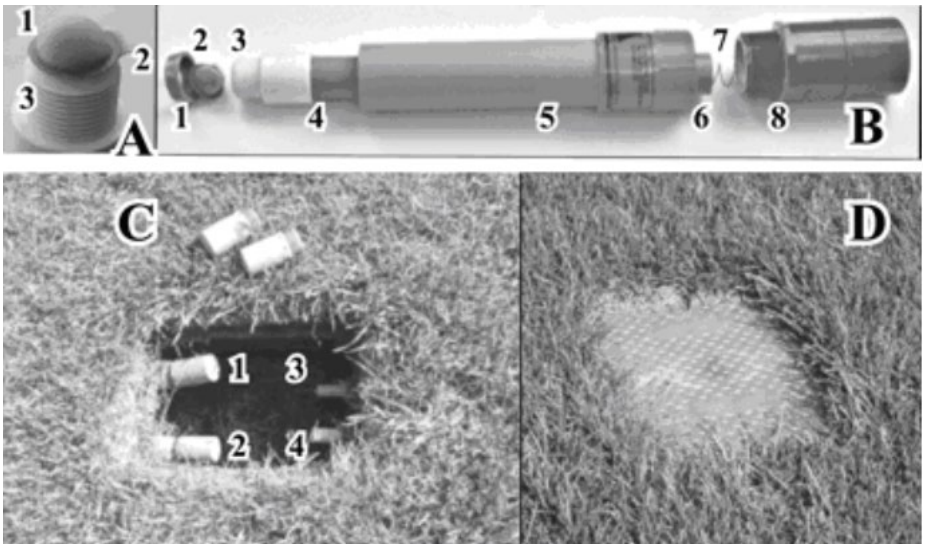


Figure 4*: Ion-exchange resin capsule installation procedure, courtesy of Sullivan & Jiang (2001). (A) The UNISET resin capsule with attachment pieces. (B) A spring-loaded tube with attachment for capsule for deployment. (C) A buried installation box with 4 access tubes running horizontal into soil at varying depths. (D) Installation box covered with lid.*

Leaf Collection

Foliage will be collected biweekly from each labeled plant following the same protocol as Xu et al. (2021). Protocols for foliage collection will vary by plant type but will remain consistent across plots and sampling frames. As older leaves have been found to be more responsive than newly emerged leaves to nutrient availability (Mo et al., 2015), three mature leaves from each species that were planted will be randomly collected and grouped according to species. Laboratory analysis of leaf N and P content will follow procedures by Xu et al. (2021),

and will include oven-drying, milling, and measuring nutrient contents using an elemental analyzer.

ANALYSIS METHODS

Nitrate Leaching Analysis

The concentration of nitrate found in each IER capsule (as determined by laboratory analysis) at each sampling site (site number) and depth (capsule number) will be compared against baseline conditions for each of the 10 plots to get a response ratio. This analysis of the within-plot changes in nitrate availability will directly account for interannual variability, such as variation in fertilizer application rates or environmental factors. The response ratio for each plot is calculated using the following equations:

$$Response\ Ratio_{\ Treatment} = \frac{Nitrate\ at\ capsule\ \#1\ at\ Site\ A\ at\ Month\ at\ Baseline}{Nitrate\ at\ capsule\ \#1\ at\ Site\ A\ at\ Month\ at\ Year}$$

$$Response\ Ratio_{\ Control} = \frac{Nitrate\ at\ capsule\ \#1\ at\ Site\ A\ at\ Month\ at\ Baseline}{Nitrate\ at\ capsule\ \#1\ at\ Site\ A\ at\ Month\ at\ Year}$$

where ‘nitrate’ is the measured nitrate mass from the laboratory analysis from the appropriate capsule, ‘capsule number’ refers to the soil depth the IER capsule was placed (4 depths per plot), ‘site letter’ refers to the sampling site location (4 sampling sites per plot), ‘month’ and ‘year’ refer to the date the IER capsules were collected (current year versus baseline data). The response ratio compares soil nitrate concentrations within a plot to detect differences before and after the treatment. The response ratio compares within-plot difference over time to account for variations across sites. As the study progresses, statistical analysis will include prior years data in addition to baseline conditions to detect the effectiveness over time. The response

ratios for the paired treatment plots and control plot will then be compared to determine the soil nitrate ratio after the fungal treatment using the following equation:

$$Soil\ Nitrate\ Ratio = \frac{Response\ Ratio_{\ Treatment}}{Response\ Ratio_{\ Control}}$$

The soil nitrate ratio compares the response ratio of a treatment plot to a control plot to determine whether fungi had an influence on nitrate leaching. In other words, the soil nitrate ratio measures the level of effectiveness (i.e., removal rate) of the treatment plot compared to the control plot. This method accounts for the expected decrease in soil nitrate concentrations in both plots, regardless of the fungal treatment, because of the vegetation alone. Additionally, comparing the response ratios of paired plots (five total pairs) reduces the variability expected across sites. In this equation, if the soil nitrate ratio is < 1 (the treatment plot had a mean response ratio that was less than the control plot), results would suggest that nitrate leaching was mitigated after incorporating fungi into the buffer.

Plant Foliar N:P Ratio

The N and P concentrations measured in the foliage of each sampled plant will be compared against baseline conditions for the ten plots to get a response ratio. As with the nitrate analysis, this within-plot comparison is to account for interannual variability, such as changes in phenology. The response ratio is calculated using the following equation:

$$Response\ Ratio_{\ Treatment} = \frac{N{:}P\ for\ Species\ A\ at\ Month\ at\ Baseline}{N{:}P\ for\ Species\ A\ at\ Month\ at\ Year}$$

$$Response\ Ratio_{\ Control} = \frac{N{:}P\ for\ Species\ A\ at\ Month\ at\ Baseline}{N{:}P\ for\ Species\ A\ at\ Month\ at\ Year}$$

In the equation, ‘N:P’ is the calculated nutrient ratio in the leaves collected from individuals for the appropriate species from the laboratory analysis, ‘species’ refers to the scientific name, ‘month’ and ‘year’ refer to the date the foliage was collected (current year versus baseline data). The response ratio compares foliar N:P ratios for a particular species at a specified time in the growing season within a plot to detect differences before and after the treatment. The response ratio compares within-plot differences over time to account for variation across sites. As the study progresses, statistical analysis will include prior years data in addition to baseline conditions to detect the effectiveness over time. The response ratios for each species in the treatment and control plots will be compared to determine the nutrient uptake effectiveness ratio after the fungal treatment using the following equation:

$$Nutrient\ Uptake\ Effectiveness\ Ratio = \frac{Response\ Ratio_{Treatment}}{Response\ Ratio_{Control}}$$

Nutrient uptake effectiveness ratio compares the response ratio of a treatment plot to a control plot to determine whether fungi had an influence on nitrate uptake. In other words, the nutrient uptake effectiveness ratio measures the level of success (i.e., uptake rate) of a treatment plot compared to a control plot. This approach accounts for the expected variation between plots, regardless of the fungal treatment, because of characteristics of individual plants alone. In the equation, if the N:P response ratio for each species in the treated plot is less than the ratio for the control plot (i.e., P concentrations were higher in the treatment), results would indicate that fungi facilitated nutrient uptake in plants.

Statistical Analysis

I will use a statistical approach to determine the significance of any observed effects of the treatment as measured by the soil nitrate ratio and nutrient effectiveness ratio. This approach will use a one sample t-test to test the hypothesis that the population mean for these values is less than 1 (indicating a treatment effect). The null hypothesis for each analysis is that there is no statistical difference in the treatment compared to the control, indicated by the absence of a significant difference between the calculated ratios and 1. The *p*-value produced by this analysis will indicate the probability of getting the observed results (or results indicating a stronger treatment effect) if the fungi did not alter nutrient uptake and leaching, and the differences between the treatment and the control populations were due to other factors not associated with the treatment. Therefore, a low *p*-value (< 0.05) will indicate that the observed results are likely to be cause by the treatment. In contrast, a high *p*-value will indicate that the treatment did not significantly change the observed results.

DISCUSSION

Nitrate Leaching

The quantity of nitrate in the IER capsules indicates the extent to which soil nitrate concentrations exceed the ability of the soil-microbe-plant system to use nitrate as a nitrogen source (Sullivan & Jiang, 2001). Therefore, larger concentrations of nitrate in the capsules are indicative of a greater potential for leaching into the environment. If significant decreases in nitrate concentrations in the IER capsules are observed in the treatment plots, results suggest that fungi reduced nitrate leaching. In other words, nutrient uptake by one or more components in the microbiome-plant system was enhanced with the addition of fungi. This interpretation is consistent with other studies on nitrate loss using IER bags, samplers, or capsules (Sullivan &

Jiang, 2001; Grahmann et al., 2018; Vizuete-Jaramillo et al., 2022; Kucova et al., 2016). If observed, it is also possible that nitrate is lost to the atmosphere via denitrification from anerobic conditions. Regardless of the mechanism, results would suggest that fungi play a role in mitigating nitrate leaching in vegetative stream buffers and preventing loss to Crow Creek and the Missouri River.

Alternatively, if the soil nitrate ratio does not differ between the treatment plots and the control plots, the results would indicate that fungi did not impact nitrate leaching. Possible explanations for this include: (1) the naturally occurring microbial community is fully functional and does not need enhancement, (2) the introduced fungi did not colonize or form a symbiosis with host plants, (3) confounding variables influenced results, or (4) fungi do not play a role in the mitigation of nitrate leaching. Although contrary to the broader understanding of the role of fungi in plant-fungi-soil systems, such a result would be consistent with the findings of Van der Heijden (2010), who showed that arbuscular mycorrhizal fungi had no impact on nitrate leaching but lowered P leaching by 60% in grassland microcosms. They noted that other species of fungi may be more efficient in acquiring N.

If the fungi treatment increase nitrate leaching (soil nitrate ratio is >1), there are potential explanations. First, it is possible that confounding variables influenced results. Second, numerous factors in the soil microbiome and plant-soil system influence nitrate leaching. It is possible that the additions of fungi could stimulate N-fixation in the treatment plot, influencing results. Such results would be consistent with those of Xiao & Lu (2022), who found that arbuscular mycorrhizal fungi-inoculated *Trifolium repens* (white clover) increased nitrate leaching but decreased P loss. However, the N fixation capabilities of this plant species could have influenced results. Although nitrogen-fixing species were not incorporated in my

experimental design, it is possible that 'volunteer' nitrogen-fixing species are naturally and randomly occurring across sites. Lastly, it remains possible that unforeseen site characteristics between plots varied greatly, despite efforts in the study design to mitigate the issue by replicating the experiment and analyzing site characteristics prior to site selection.

Foliar Nutrient Composition

Analyzing changes in nutrient ratios is thought to be a more reliable method for assessing current soil conditions, compared to single element concentrations (Jinhua et al., 2020). Various studies have shown that sustained inputs of combined N and P fertilizer decreased foliar P concentrations, consequently increasing the foliar N:P ratio. Likewise, many have demonstrated that fungi promote P availability, mobility, and uptake in plants. Therefore, if the N:P response ratio for each species in the treated plots is less than the ratio for the control plots (i.e., P concentrations were higher in the treatment), results would indicate that fungi facilitated nutrient uptake in plants. Furthermore, increased nutrient uptake in the soil-microbe-plant system would imply that nutrients were bioavailable, utilized, and were not lost to Crow Creek and the Missouri River.

Alternatively, if there is not a statistically significant difference in the N:P response ratios for the treated plots compared to the control plots, it is possible that fungi did not facilitate nutrient uptake in the plants within the plots. If there is not a difference between the paired plots, this observation could support the "optimal allocation strategy," in which plants invest less carbon in fungi when soil nutrients are sufficient. However, many studies suggest that increased additions of N can make P less biologically available to plants (Tiruvaimozhi et al., 2018; Liu et al., 2021). In this P-limited system, plants allocate more energy to roots and fungal partnerships (Tiruvaimozhi et al., 2018; Liu et al., 2021). Other potential explanations include: (1) fungi did

not colonize host plants, (2) P is not the limiting nutrient, (3) sampling error or environmental variables influenced results, or (4) the naturally occurring microbial community does not need to be enhanced.

Future Research

Fungi and the soil microbiome are inherently difficult to study *in-situ.* Likewise, nutrient cycling is highly complex, involving numerous biological mechanisms that are influenced by countless environmental variables. A landscape-level, mesocosm study of this caliber that explores both above- and below-ground processes is likely to come with inherent challenges and possible error. For example, hydrology, herbivory, vandalism, or climatic events could impact plot characteristics. Most importantly, planted vegetation and/or inoculated fungi that were selected might not be suitable for the study area. Thus, a one-year timeframe will be allocated to monitoring site conditions and troubleshooting. In addition, the duration of the study will largely impact confidence in results. Fertilizer application rates, precipitation events, and agricultural practices vary over time. Vegetation and microbial communities will likely show improved ecosystem services the longer they are established. Hence, comparing data across numerous years as a long-term study will reduce error.

Major knowledge gaps exist between successful bench-level findings, landscape-scale experimental work, and the utility in restoration. Thus, future work should be directed to applying controlled, laboratory findings to *in-situ,* natural field settings. Numerous components within this study design are worth exploring further. For example, what plant-fungus species combinations are most efficient at nutrient removal? What variables influence the success of mycorrhizae-mediated phytoremediation? Why are fungi not commonplace in restoration? Lastly, there is far less relevant research available that was conducted in North America, let

alone Montana. Hence, future research is needed in areas that reflect similar ecological, climatic, and geographic conditions.

CONCLUSION

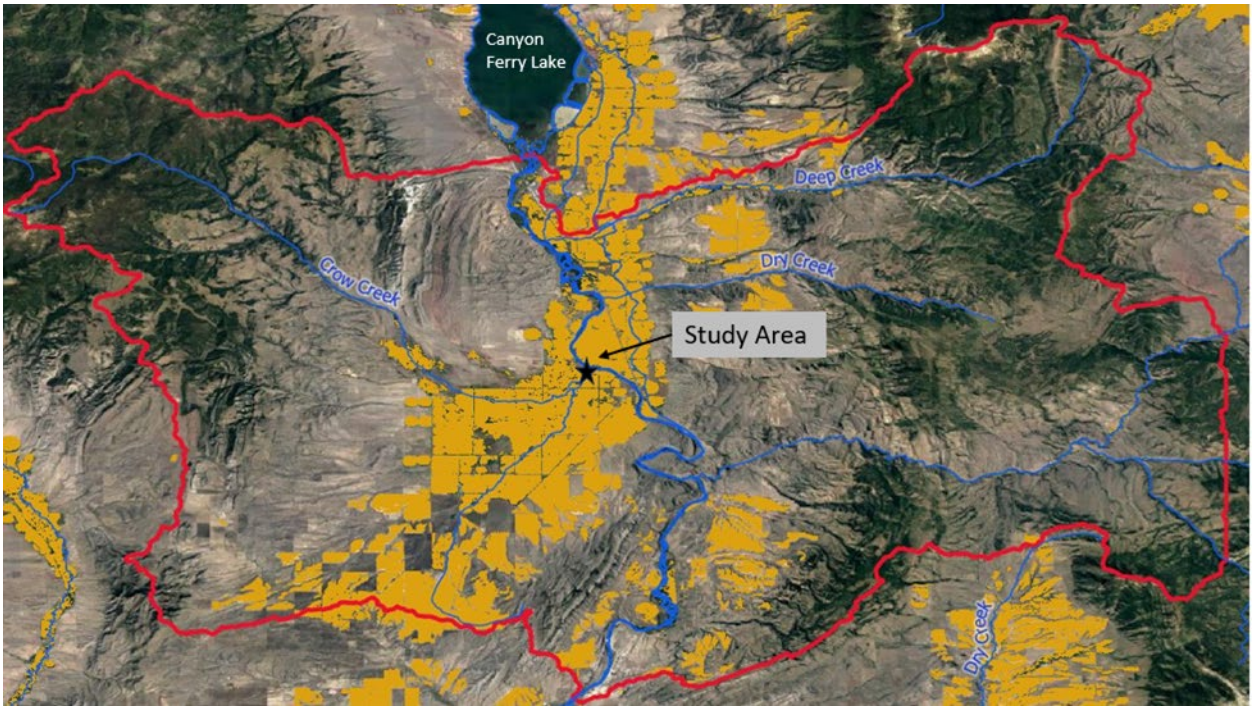
Our Earth's freshwater supply is being degraded faster than it can be restored, and nutrient pollution from human activities is a leading cause. The rapidly declining health of aquatic habitats has profound effects on entire ecosystems and the services they provide. Only 28% of rivers and streams in the United States are considered "healthy" based on their biological communities (US EPA, 2021) and agricultural runoff is the leading cause of deterioration. As a headwaters state that ranks high in agricultural production, Montana's contribution to nutrient pollution is increasingly being unveiled. On a smaller scale, Broadwater County is experiencing increasing water quality and quantity issues while vegetation strips along waterways are decreasing (NRCS, 2019). Population growth and climate change necessitate the use of agrochemicals to meet the regional and national food demand. Ultimately, if agrochemical use and nitrogen deposition are not reduced, emphasis must be placed on improving restoration efforts to protect freshwater ecosystems and human health.

Conservation buffers and mycoremediation are both independent practices well known for their necessary ecosystem services, but approaches for combining these strategies remain largely overlooked in research and in restoration. Despite inherent challenges associated with understanding soil-fungi-plant interactions on a landscape level, there is an urgent need to explore whether fungi can enhance bioremediation efforts and mitigate freshwater pollution. Climate change and increased N deposition from anthropogenic activities is likely to increase the dependency of plants on their fungal symbionts (Mei et al., 2019; Kohler et al., 2018). This study provides a pathway for revealing the extent to which fungi facilitate nutrient uptake and reduce

leaching when utilized as a biofertilizer to enhance conservation buffers. If successful, this study could motivate increased consideration and implementation of mycorrhizae-mediated phytoremediation of nutrients, with widespread benefits for the management and restoration of water resources.

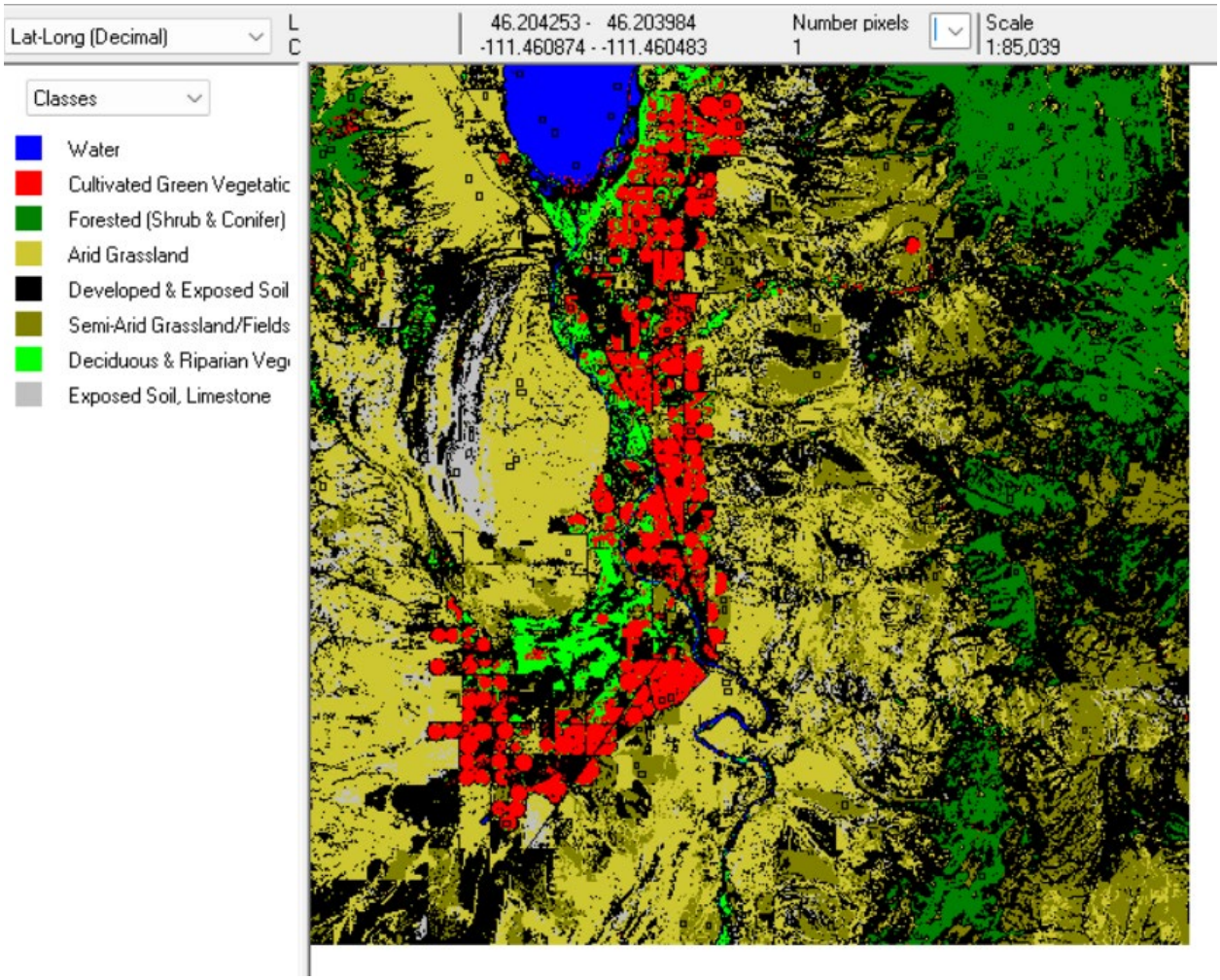
APPENDICES

Appendix A



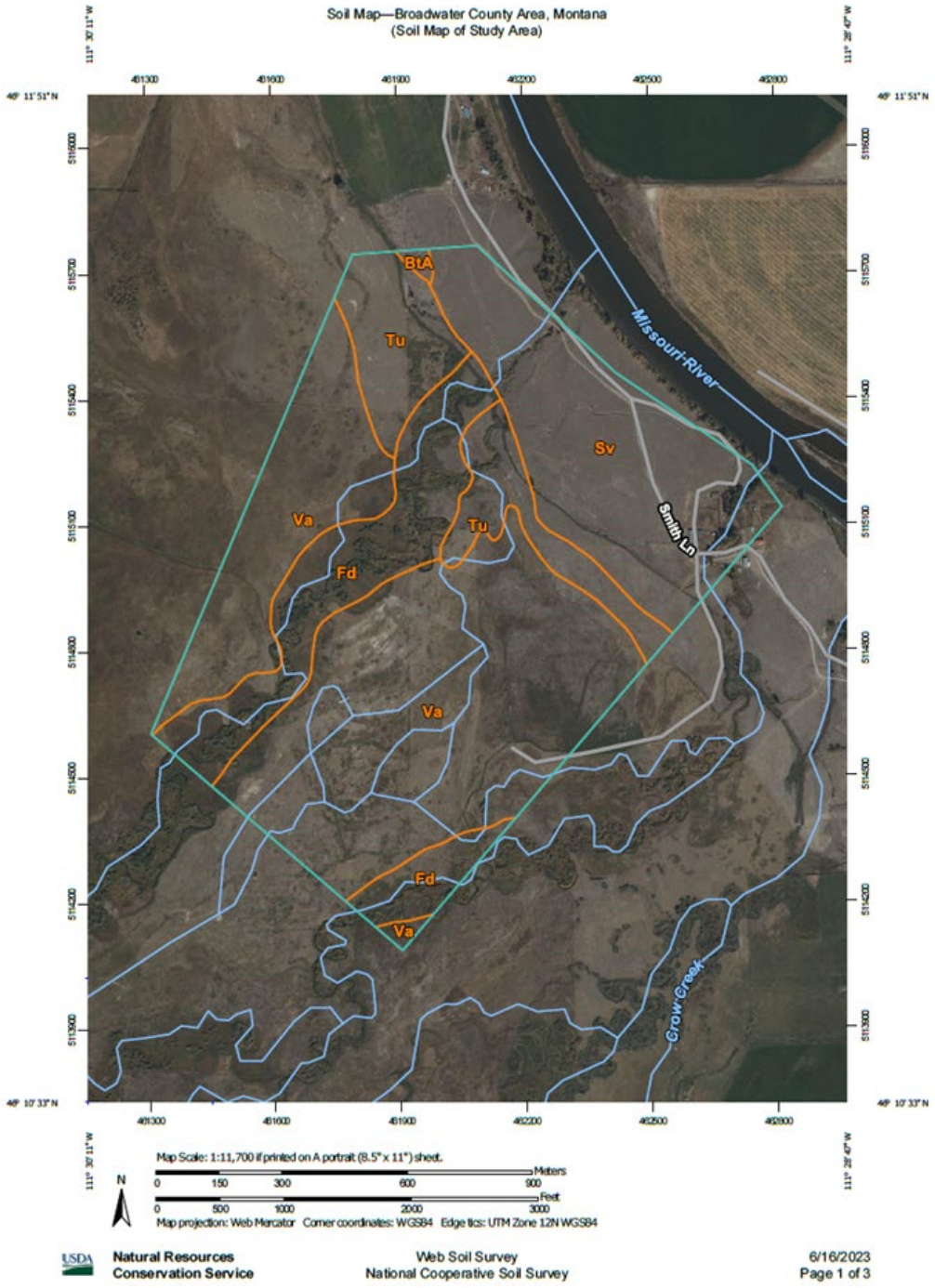
Appendix A: Location Map created in QGIS using the watershed analysis tool. Yellow areas represent cultivated land use areas. The red outline shows the boundary for the Upper Missouri River watershed between Three Forks and Townsend, Montana. The study area is the location of major agricultural runoff based on the watershed analysis (depicted with a star).

Appendix B



Appendix B: Product of supervised classification of Landsat8 imagery of study area from June 2017 showing moisture and greenness in landscape features (labeled above). Area surrounding the study site shows cultivated crops, riparian and deciduous vegetation, and arid grassland. When compared against imagery from different months of a growing season, temporal variation in vegetation can be assessed.

Appendix C



Appendix C: USDA NRCS Web Soil Survey map (2023) showing the same soil type for the Lower Crow Creek paired plot units. This is a suitable method for reducing site variation when selecting the paired plot site locations.

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