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Arbuscular Mycorrhizal Fungi Colonization Response to Dike Removal in the Salmon River Estuary

by

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requirements for the degree of

Master of Science
in
Geography

Thesis Committee:
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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) form symbiotic relationships with wetland plants and are important to the restoration of coastal estuaries. The purpose of this study was to assess the presence and colonization of arbuscular mycorrhizal fungi in *Distichlis spicata* from three formerly diked areas of the Salmon River Estuary to determine an initial understanding of the mycorrhizal community in tidal wetlands post-restoration. AMF were present in all four sample areas and total colonization ranged on average 39-72%. The reference site had the lowest levels of total colonization, which is contrary to the findings of studies of younger wetland restorations. Most importantly, arbuscular colonization was not significantly different between any sites suggesting that the levels of nutrient exchange in the restoration sites were comparable to reference conditions. Lower soil pH of the restored site due to increased oxidation and acid sulfates could increase AMF nutrient storage and communication with host plants by increasing vesicular colonization. The levels of AMF colonization and soil characteristics showed that the Salmon River Estuary is nearly restored since dike removal. The proximity and connectivity of restoration sites to disturbed sites can potentially influence the AMF community and expedite the restoration process. Existing AMF communities and native fungal inoculum can influence the succession of plant species in restoration sites and should be considered in wetland restoration projects. AMF are incredibly dynamic, resilient, and influential organisms with enormous potential in wetland restoration ecology.

DEDICATION

To my loving parents who have supported me
throughout every dream I wish to follow.
And to my siblings who are out chasing their dreams
and continue to inspire me every day.

ACKNOWLEDGEMENTS

My sincere gratitude goes to numerous people who helped me throughout this project and to Portland State University for being the platform of my education. The motivating force behind the research was my advisor and committee chair, Dr. Martin Lafrenz, who provided his guidance, excellent knowledge of soils and salt marshes, assistance in the field and lab, and literary feedback. Dr. Sarah Eppley was especially helpful with her extensive knowledge of arbuscular mycorrhizal fungi, methodology, and permitting me to spend countless hours processing samples in her laboratory. Thank you to my entire committee, who provided vital comments that helped to improve the manuscript. Thank you to Dr. Yangdong Pan who offered statistical advice while conducting my analyses. Finally, Daniel Bedell, who assisted me in the field and always offered support and advice throughout my degree and thesis study.

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INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are found in the soils of various natural and agricultural ecosystems and establish symbiotic relationships with vascular plants to exchange water and nutrients for photosynthetic sugars (Allen 1991; Bauer et al. 2003; Workman and Cruzan 2016; Wang 2017; Recorbet et al. 2021). Despite their widespread distribution, these organisms are still largely under studied, but are proving to have a major influence on ecosystem dynamics. Mycorrhizae often improve plant growth and survival by facilitating nutrient uptake and increasing resistance to ecological stressors. AMF resource sharing provides vital nutrition (carbon, phosphorus, and zinc) to host plants in highly stressful ecosystems like coastal wetlands (Grime et al. 1987; Carvalho et al. in 2004; Leake et al. 2004; Dolinar and Gaberščik 2010; Dolinar et al. 2016; Ortas and Coskan 2016; Workman and Cruzan 2016). The symbiotic association with AMF can highly influence plant fitness, community distribution, and diversity (Wolfe et al. 2007) and may prove to be an important factor in ecological succession and restoration.

AMF use hyphal strands to invade the cortical cells of vascular plant roots where they form tree-like structures called arbuscules that specialize in nutrient transfer, and balloon-like storage units called vesicles (Figure 1) (Allen 1992; Bauer et al. 2003). This process of AMF entering plant roots and growing structures is referred to as AMF colonization. Hyphal strands of AMF function as root hairs for their host plants, increasing surface area for absorption of water and low mobility nutrients such as phosphorus, copper, and zinc (Braghirolli et al. 2012; Wahl and Spiegelberger 2016). AMF colonization has also been shown to increase plant reproduction, offspring survival, and population size, while also significantly increasing biodiversity, nutrient capture, and productivity with increasing AMF species richness (Van der Heijden et al. 1998; Bohrer et al. 2004).

AMF root colonization of wetland plants is known to increase plant resistance to salinity, drought, pathogens, disease, waterlogging, heavy metals, and cold temperatures (Grime et al. 1987; Leake et al. 2004; Adamus 2005; Roda, Diaz, and Torres 2008; Evelin, Kapoor and Giri 2009; Zhang et al. 2012; Kumar et al. 2015; Zhouying et al. 2016). AMF often form Common Mycelial Networks (Workman and Cruzan 2016) between roots of multiple plant species that mediate competition and abiotic stress (Dolinar and Gaberščik 2010) and promote resource exchange and biodiversity among plant populations (Rillig 2003; Simard and Durall 2004; Smith and Read 2008; Walder et al. 2012; Asmelash, Bekele, and Birhane 2016). The establishment of AMF and their role in ecosystem dynamics is likely vital to the recovery of degraded and disturbed ecosystems.

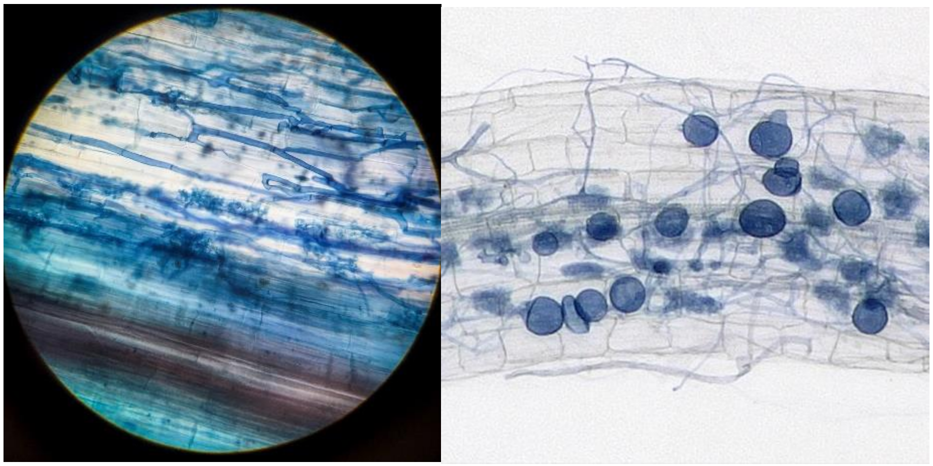

Figure 1: Microscopic photographs of AMF structures: arbuscules and internal hyphae in Distichlis Spicata roots from this study (left) and arbuscles, vesicles, and hyphae example (right) (Evangelisti et al. 2021).

While AMF are found in about 90% of vascular plant roots (Bauer et al. 2003; Dolinar and Gaberščik 2010) and their relationships are generally mutualistic (Wahl and Spiegelberger 2016), plant species vary greatly in the degree in which they establish mycorrhizal associations (Siqueira et al. 1998; Cheeke et al. 2019). The level of mycorrhizal associations ranges from plants that never become hosts for mycorrhizae to species that require AMF colonization (Johnson et al. 1991). As a result, plant competition and succession may occur because of mycorrhizal interactions and the variation in AMF associations with differing plant species (Siqueira et al. 1998). Plant growth has been found to depend on specific AMF species combinations, which can also change community structures throughout succession (Bauer et al. 2003; Cheeke et al. 2019).

Particularly, studies have found that late successional plant species are more sensitive to AMF compositions than early successional species (Cheeke et al. 2019). AMF tend to be not host-specific, however the AMF community is known to significantly shift during early to late plant succession in forest and savanna ecosystems (Johnson et al. 1991). Mycorrhizal infectivity is also known to be significantly correlated with soil characteristics like water soluble carbon and phosphorus (Johnson et al. 1991). The results from previous studies show a close interrelationship between soil properties, plant composition, and AMF colonization. AMF are critical for the establishment and health of plants and could be an important tool for successful restoration of disturbed soils (Cooke and Lefor 1990; Asmelash, Bekele, and Birhane 2016; Zhouying et al. 2016). Although mycorrhizal colonization of wetland plants has been found in roughly

20 countries around the world, little is known about the colonization of AMF in restored tidal wetland ecosystems (Bohrer et al. 2004; Carvalho et al. in 2004).

Wetlands and Agriculture

Wetlands are amongst the most environmentally productive and economically valuable ecosystems in the world providing essential ecosystem services that support communities of plants, animals, and people (Inglett, Reddy, and Corstanje 2005; Moreno-Mateos et al. 2012; Batzer and Sharitz 2014). Wetland tidal and river flooding slows and stores water, regulating accretion of organic and inorganic sediments, nutrient cycling, carbon storage and plant primary productivity (Kadlec and Knight 1996; Dolinar and Gaberščik 2010; Townend et al. 2011; Martínez 2014). Wetland resistance to erosion from flooding depends on the composition and structure of the vegetation and microbial communities, soil type, nutrient availability, and the colonization of plant roots by arbuscular mycorrhizal fungi (AMF) (Dolinar and Gaberščik 2010; Dolinar et al. 2016). Unfortunately, the United States has historically drained and converted tidal wetlands to agriculture or urban use for hundreds of years (Hood 2004; Gedan et al. 2009; Batzer and Sharitz 2014; Hood 2015).

Pacific Northwest wetlands are threatened by a variety of anthropogenic stressors. Land use practices including diking, ditching, logging, and the construction of pipes, pilings, roads, and highways affect both functionality and connectivity (Bertness et al. 2002; Adamus, Larson and Scranton 2005; Smith et al. 2009; Ren et al. 2013). Many salt marshes have been filled or diked to allow for coastal development and agriculture, increasing their susceptibility to eutrophication and pollution potentially reducing resilience to sea-level rise (Aber et al. 2012; Batzer and Sharitz 2014; Schile et al. 2014). Dikes are raised soil embarkments or berms along the tidal channels of rivers or creeks to prevent a terrestrial area from inundation during high tides (Giannico and Souder 2004). Tide gates are steel or wooden flaps mounted to concrete flood boxes or culverts ends that allow upstream fresh water to drain into estuaries or rivers while blocking brackish tidal water from flooding areas above the gate (Giannico et al. 2018).

Dikes and tide gates alter the natural circulation between freshwater and seawater by preventing upland flooding, causing changes in water temperature, soil moisture, sediment transportation, and channel morphology on both sides of the gate. Water stagnation above the gate increases water nutrient concentrations and turbidity and reduces salinity, dissolved oxygen, and pH (Giannico and Souder 2004; Tiner 2013). Excess nutrients, especially nitrogen, promotes eutrophication and algal blooms (Tiner 2013). These physical and chemical changes to the water and soils in turn modify the biological community compositions of animals, aquatic plants, fungi, and bacteria (Giannico and Souder 2004). AMF density and colonization of soils are also highly affected by changes in organic matter, nitrogen, and phosphorus (Silvana et al. 2020). When agriculture encroaches on wetlands, there is a complex impact on fish, macroinvertebrates, waterfowl, and vegetation communities by decreasing diversity, abundance, and obstructing migration to native habitats. For the last 150 years, Pacific Salmon, Trout, and Sturgeon populations have declined to the extent that 17 subspecies are on the federal and Oregon state threatened or endangered species list (Hood 2004; ODFW 2021).

Diking History

Oregon has approximately 120 thousand acres of estuarine wetlands along its coastline and the Salmon River Estuary that covers 440 acres has proportionally more tidal marsh (68%) than any other estuary in Oregon (Adamus 2005; Adamus, Scranton and Larson 2005). The Salmon River estuary was first used for agriculture starting in 1850 when white settlers began grazing cattle on the high marsh when it was dry during the summers and increased in the 1920s with the construction of access roads to the surrounding areas (Beckham 1975 as cited by Mitchell 1981). Today, the conversion of Oregon tidal marshes to pastureland, through the construction of dikes and tide gates, is the largest anthropogenic impact resulting in the loss of 57.9% of historic tidal wetlands across Oregon (Boule and Bierly 1987; Good 2000; Buchner 2004; Scranton 2004; Gray 2005; Adamus, Larsen and Scranton 2005; Tiner 2013; Brophy 2019). From 1954-1974, roughly 75% of the lower Salmon River estuary had been diked for growing hay, livestock grazing, and boat access (Figure 2). The Salmon River marshes were impacted by dikes and tide-gates restricting tidal flow for 17, 26 and 35 years, but historical cattle grazing occurred for nearly 120 years (Beckham 1975; Mitchell 1981; Grey 2005).

In 1961, the U.S. highway 101 was rerouted to cut straight through the estuary essentially, acting as a large embarkment that fragmented the upper and lower reaches of the marsh (Mitchell 1981; Ellingson and Ellis-Sugai 2014; Gray 2005). In 1971, Eilers

(1975) rated the Salmon River Estuary as moderately disturbed amongst 14 other Oregon impacted estuaries specifically due to the relative area of agricultural diking, road, urban, and industrial fill, cattle grazing, and fences. These disturbances highly reduce ecosystem functionality and connectivity by severing habitats for plant and animal species, altering water quality, and increasing vulnerability to invasive species (Adamus, Larsen, and Scranton 2005). The spatial and biological connectivity between wetlands and adjacent systems is a crucial aspect for sustaining many organisms and wetland functions (Amezaga et al. 2002). Bio-geophysical fragmentation impacts water quality and storage, organism dispersal, community composition, and biodiversity (Frenkel and Morlan 1990; Amezaga et al. 2002; Dale et al. 2010; Cohen et al. 2016). These manmade barriers cut off tidal channels obstructing accessibility to migrating fish (Giannico et al. 2018), particularly for many Salmon subspecies who are on the federal and state Endangered species list.

Diking and tide gates reduce or completely inhibit tidal flow and sediment input, which increases soil subsidence and organic oxidation (Frenkel and Morlan 1991), altering sediment deposition patterns and vegetation composition across the marsh surface (Frenkel and Morlan 1991; Buchner 2004; Gray 2005; Tiner 2013; Clifton, Hood and Hinton 2018). However, recent decades have seen a growing appreciation for the importance and restoration of tidal wetland ecosystems in support for salmon and waterfowl populations, commercial fisheries, carbon storage, and providing shoreline protection from storms and sea-level rise (Cooke and Lefor 1990; Beck et al. 2001; Wigand et al. 2001; Gedan et al. 2011; Hood 2015; Clifton, Hood and Hinton 2018).

Beginning in the 1970s, the filling and diking of Oregon tidal marshes was strongly limited by state and federal requirements for permits (Adamus, Larsen, and Scranton 2005). In 1974, Congress passed the Cascade Head Scenic-Research Area Act (Public Law 93-535) creating the Cascade Head Scenic-Research Area (CHSRA), which established a long-term goal of “restoring the Salmon River estuary and its associated wetlands to a natural estuarine system free from man’s developments” (USFS 1977). Return of a wetland to "original conditions" is a common objective of restoration, but reaching that goal is practically impossible. Not only are natural conditions rarely known prior to disturbance, but even the process of restoration adds disturbance and creates novel successional pathways as the ecosystem recovers.

Restoration of the Salmon River Estuary started in 1978 with about half of the 5,000-foot dike removed on the North bank of the Salmon River (Mitchell 1981; Frenkel and Morlan 1991; Ellingson and Ellis-Sugai 2014). This area became formally known as the “Mitchell Marsh” because it was the study site for Diane Mitchell’s doctoral dissertation from 1978-1981. Two other major dikes and tide gates were also removed in 1987 and 1996 (Figure 2). In 1987, the remaining dike material in the Mitchell Marsh was leveled to the historic marsh elevation and a second removal occurred on the South bank (named the “Y” Marsh for proximity to the YWCA camp) (Gray 2005; Mitchell 1981). In 1996, just Northwest of Hwy 101, a third dike and tide gate on Salmon Creek was removed (Grey 2005; Ellingson and Ellis-Sugai 2014; Flitcroft et al. 2016). The three dike removals in 10-yr intervals created a unique “space-for-time” chronosequence that allows researchers and restoration managers to monitor the trajectory of restoration alongside a reference marsh.

Figure 2: Aerial photos of the Salmon River Estuary pre-dike removal in 1975 (top) and post dike-removal in 2012 (bottom) (Ellingson and Ellis-Sugai 2014). Restoration sites indicated by the year of dike removal.

The dike removal process included removing the fill material with small bulldozers and returning it to the ditches where it was originally obtained (Ellingson and Ellis-Sugai 2014) and reconnecting the tidal creeks to the main Salmon River channel (Morlan and Frenkel 1992). No active restoration techniques like planting or grading were implemented in any of the three dike removals (Mitchell 1981; Frenkel and Morlan

1991; Grey 2005). Dike deconstruction is the first phase of restoration and adds another level of disturbance to the wetland on top of the disturbance already caused by dike and tide gate implementation and years of agricultural effects. Dike deconstruction requires the use of large heavy machinery like excavators, bulldozers, scrapers, and dump trucks that can cause soil displacement, rutting, and compaction (Ellingson and Ellis-Sugai 2014). According to Morlan and Frenkel (1992), the 1978 and 1987 Salmon River Estuary restoration projects had relatively low intensive manipulation and good potential for restoration success compared to wetlands that have been converted for urban development. The future ecological character diked tidal marshes will ultimately reflect a heritage of altered hydrology and novel successional pathways during both the period of disturbance and post-restoration.

Frenkel and Morlan (1991), found that 10 years post dike removal in the 1978 marsh, soil elevation had decreased and subsided. This result is attributed to dewatering of soils, organic matter oxidation, compaction by livestock, and buoyancy loss (Grey 2005), and likely dike deconstruction as well. Although the 1978 marsh continues to evolve as sediments are trapped and deposited by the vegetation, Frenkel and Morlan (1991) estimated that it might take another 50 years for the restored wetlands to become similar again to the original high marsh in both vegetation and elevation. In 2017, from personal observations, the 1978 area still had a classic low to high marsh gradient, while the 1987 and 1996 areas were primarily high marsh with little to no low or mid marsh and dramatic banks that fell into the main Salmon River channel. Grey (2005) completed an extensive study on the three dike removals in terms of fish and invertebrate rehabilitation and found that even after nearly 30 years there were still significant disparities between the reference marsh and the restored marshes. A more recent study (Flitcroft et al. 2016), found that after 30 years the vegetation assemblages, soil elevation, and channel morphology of all recovering marshes were well on the trajectory toward reference conditions and that in 1999 the 1978 and 1987 marshes had become statistically similar. Flitcroft also found trajectories toward reference conditions in terms or soil elevation, channel morphology, and vegetation. However, the fungal community and colonization of the Salmon River estuary remains vastly unknown.

AMF in Agriculture

Soil inoculated with fungi is commonly used in agriculture and other restoration practices to improve plant establishment and transplanting (Cooke and Lefor 1990; Bauer et al. 2003; Wang et al. 2009; Zhang et al. 2012; Koziol and Bever 2017; Cheeke et al. 2019). AMF in soil root zones may enhance ecological adaptation of plants, including pioneer plant colonizers, to salt marsh environments (Carvalho, Caçador, and Martins-Loução 2001). Richter and Stutz (2002) found that mycorrhizal inoculation benefited restoration efforts in abandoned agricultural fields in semiarid regions. Inoculation with mycorrhizae also improved the development of grasses during the early stages in the restoration in Poland (Ryszka and Turnau 2007). Bingham and Biondini (2009) reported that AMF inoculation can speed up the regeneration process of grasslands and may be used as an effective biological approach in the restoration of degraded desert ephemeral plant communities (Zhang et al. 2012**).** AMF can increase health and stress tolerance of restoration plants (Cooke and Lefor 1990; Adamus 2005; Roda, Diaz, and Torres 2008; Pratt-Zossoungbo and Biber 2009; Asmelash, Bekele, and Birhane 2016) and improve the development of restoration methods. Currently, no studies have thoroughly documented the presence or functionality of the mycorrhizal community across the Salmon River Estuary. With the three recovering marshes in differing stages and conditions alongside a reference marsh for comparison, the Salmon River estuary provides the ideal location to answer questions regarding AMF colonization and wetland succession in response to dike removal.

Research Context

AMF are critical for the establishment and health of plants and could be an important part of restoration practices and succession of disturbed soils (Cooke and Lefor 1990; Johnson 1991; Bauer et al. 2003; Zhang et al. 2012; Zhouying et al. 2016; Koziol and Bever 2017). The three dikes removed 10-years apart in the Salmon River Estuary (1978, 1987, and 1996) provides a unique opportunity for research on tidal marsh recovery in a catena or chronosequence framework (Gray 2005; Flitcroft et al. 2016). There is a key interrelationship between soil properties, plant composition, and AMF colonization that can impact ecosystem dynamics throughout succession. The primary objective of this study was to determine if colonization by AMF and soil properties differed between restored and reference wetlands across the three temporal restoration periods. Mycorrhizal colonization was expected to be found in each of the wetlands and that levels in the restored wetlands would be lower than the reference site (Cooke and

Lefor 1990; Azi, Sylvia, and Doren 1995; Richter and Stutz 2002; Allen et al. 2003; Haug, Setaro and Suárez 2013; Asmelash, Bekele, and Birhane 2016; Silvana et al. 2020). The purpose of this study was to assess the presence and colonization of AMF in a native grass host plant from the three previously diked areas of the Salmon River Estuary and to determine a base understanding of the mycorrhizal community in tidal wetlands post-restoration. This study can inform restoration managers and environmental conservationists of the value and importance of arbuscular mycorrhizal fungi in restoration practices and advance the knowledge of mycelium in coastal wetland ecosystems.

METHODS AND MATERIALS

Study Area

The Salmon River Estuary (45° 01' N, -123° 58' W) is located immediately south of Cascade Head, approximately 6 kilometers north of Lincoln City, Oregon and lies within the historic Cascade Head Scenic-Research Area (Figure 3). This estuary is approximately 194 km^2 and with an 800-hectare estuary, half of which is tidal marsh and has proportionately more tidal marsh (68%) than any other Oregon estuary (Adamus et al. 2005; Grey 2005). Congress created the Cascade Head Scenic-Research Area in 1974 to maintain and enhance the scenic and ecological qualities of the areas (Ellingson and Ellis-Sugai 2014). Frenkel and Morlan (1991), noted that like many estuaries in the Pacific Northwest, 75.4 % of the historic marshes along the lower Salmon River were altered through the installation of dikes in the early 1960s to create pasturelands (Gray 2005). Since 1976, three dikes have been removed, initiating restoration of 150 acres of tidal marsh (Gray 2005; USFS 2006).

Tidally controlled hydrology can be easily reestablished so drained and diked agricultural lands became good prospects for restoration to recover losses in coastal wetlands (Kusler and Kentula 1989). In the Pacific Northwest, estuarine marshes are habitats of restoration interest because of their important ecosystem functions for salmonid populations (Grey 2005). However, little to no studies have looked at the effect of these restoration projects on mycelium colonization and none provide the chronological sequence of restored habitats that are available at the Salmon River estuary. The U.S. Forest Service removed dikes from three different sites of the Salmon

River Estuary in 1978, 1987 and 1996. The Salmon River estuary was chosen for this study because of the unique history that offers an opportunity to address knowledge gaps in the patterns and dynamics of estuarine restoration and its effects on the marsh community, particularly arbuscular mycorrhizae fungi. The resulting series of restored marshes enabled us to study soil composition and mycelium abundance over a 28-year span and compare functional equivalency to an adjacent, un-diked reference site. In this study, marsh sites are referred to by the year the dike was removed, and restoration began (i.e., 1978 as “D78”, 1987 as “D87”, and 1996 as “D96”).

Sampling

Suggested sampling months varied throughout other studies from anywhere between March and September (Cooke and Lefor 1990; Miller 2000; Eppley 2009; Wilde 2009). A study by Eppley (2009), suggested sampling *Distichlis spicata* during flowering in July, while a study by Wilde et al. (2009) suggested that spore formation and AMF richness was higher in September than in June (Netherlands and Northern Germany). For this study, soil and root samples were collected mid- to late August of 2018. A 100-meter transect was run parallel to the main Salmon River channel and 15-meters landward of the high-water level within the reference and three restored wetland areas (1978, 1987, and 1996) of the Salmon River Estuary (Figure 2). Ten samples were taken along each transect at random distances between 0-100m (none closer than 5m). Each sample consisted of soil and the full *Distichlis spicata* plant for laboratory processing and examination of soil characteristics (organic matter, salinity, pH, and nitrate-nitrogen) and root AMF colonization. Sample sites may be referred to in abbreviation by their year of dike removal (i.e. D76, D87, and D96) and the reference site as "REF" throughout the remainder of the paper.

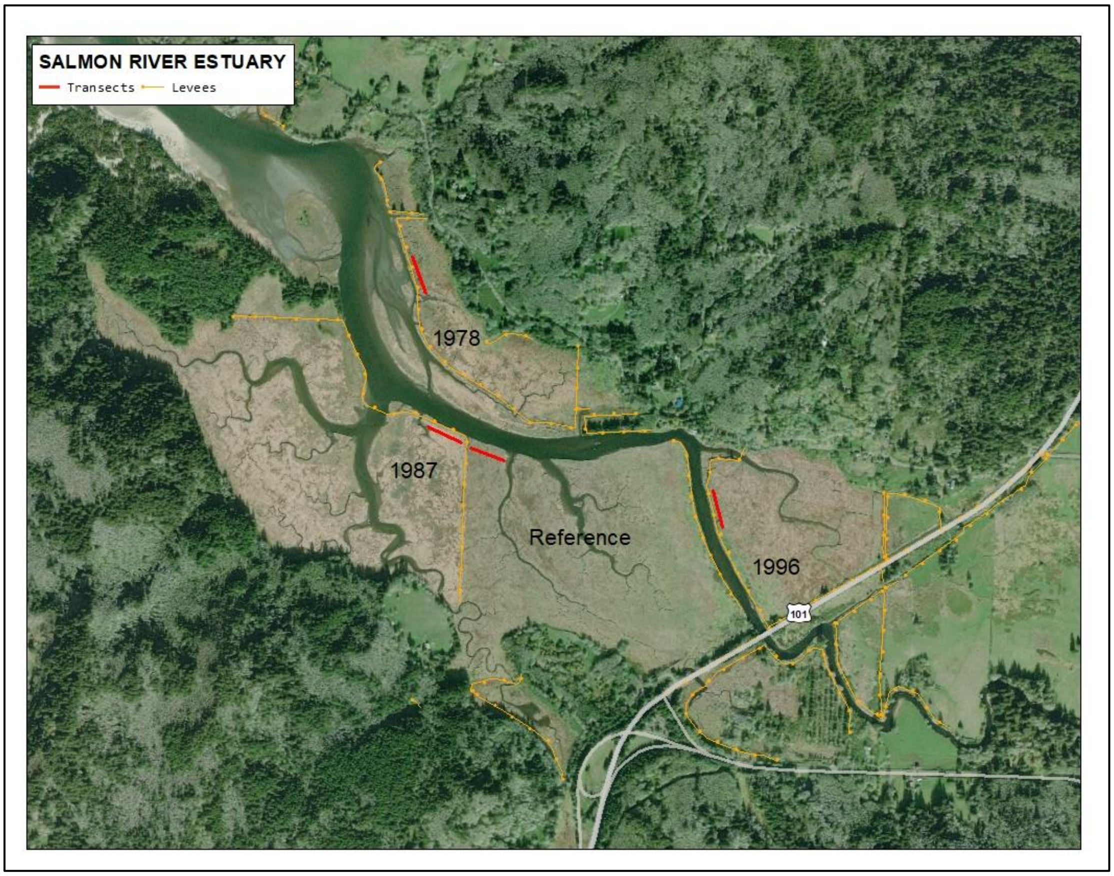

Figure 3: Aerial map of the Salmon River Estuary historical dikes (orange lines), project transects (red lines), and the date of dike removal per area.

Soil Parameters

Organic Matter

Soil samples were tested for soil gravimetric moisture and organic matter content using the Weight Loss on Ignition method. Wet soil samples were measured to 5mL, weighed, and dried in an oven overnight at 110° C and then reweighed to determine dry weight. The inverse value of the dry weight was used as the gravimetric moisture content. Samples were then placed in a cold muffler oven to be baked at 400° C for at least 16 hours or overnight to burn off the organic matter. Samples were cooled and weighed immediately.

Conductivity and pH

Conductivity and pH were measured through aqueous extraction (Tanji 1990; USDA 1993; HACH Company 1992; Staff 2014) by creating a 1:5 soil-water solution (5mL soil to 25mL DI water) for each sample taken along each transect. The soil and water were combined and agitated for one hour prior to measurement to ensure full integration. Samples were then immediately tested for conductivity (mS) and pH.

Nitrate-Nitrogen

For each soil sample, a 1:5 soil-water mixture (5mL soil to 25mL deionized water) was made for nitrate-nitrogen (NO_3-N) testing. Soil-water mixtures were agitated for 1 hour to ensure fully integrated samples. The soil solutions were then filtered using a vacuum system and 2.5µm membrane filters. The extracted water samples were then tested for nitrate-nitrogen (ppm) using a LeMotte Nitrate-Nitrogen test kit.

AMF Colonization

Distichlis spicata, a salt marsh hay, which grows throughout Pacific Northwest marshes was analyzed for colonization of AMF because of its dominant presence throughout the estuary. Root samples were rinsed with deionized water and preserved in 70% ethanol at 2˚C until chemical processing. Roots were then cleared by 10% KOH for 20 min, and neutralized in 1% HCl for 30 min. Roots were then stained using Trypan Blue (0.05% in lactoglycerin) for 60 min following Gemma and Koske (1989). Dyed roots were rinsed with deionized water for elimination of undesired dye particles and preserved in lactoglycerol. From the processed roots, fifty 1-cm root segments were randomly selected and mounted in glycerin on microscope slides.

AMF colonization was quantified using the magnified intersection method of McGonigle et al. (1990) and examined under the microscope at x200 magnification. Each root slide was examined for 100 intersections in a random order with the identity of the roots unknown to the reader. Each intersection was counted as either “negative” (no AMF structures), “arbuscules” (at least one arbuscule), “vesicles” (at least one vesicle), or “hyphae” only (at least one hyphae, but no arbuscules or vesicles).

Arbuscular colonization (AC) and vesicular colonization (VC) was calculated by dividing the count of their respective categories by the total number of intersections examined. Hyphal colonization (HC) was calculated as the proportion of non-negative intersections. Percent total colonization (TC) was calculated as the total number of intersections minus the non-negative counts divided by 100. Lab processing was performed at the Lafrenz Soil and the Center for Life in Extreme Environments (CLEE) Eppley Labs at Portland State University in Oregon, USA.

Statistical Analyses

Data of concentrations of soil chemical (organic matter, nitrate-nitrogen, conductivity, and pH) and AMF colonization (internal hyphae, vesicles, arbuscules, and total) per sites were subjected to an analysis of variance. Differences between means were compared using Tukey's test ($p < 0.05$). The transect data was analyzed using an analysis of variance (ANOVA) test to determine if there was a significant response to dike removal in mycelium colonization and soil compositions between the reference and dike removal areas. The null hypothesis of the ANOVA test was that there was no significant difference ($p < 0.05$) between the reference and dike removal areas. Differences between means were compared using Tukey's test ($p < 0.05$). The relationship between the percentages of total colonization and soil chemical factors were analyzed using a simple linear correlation analysis (Pearson). Correlation between variables were considered significant when the probability associated with the correlation coefficient was $p < 0.05$.

RESULTS

Soil Characteristics

Average soil parameters at the sampling sites are shown in Table 1. Values of organic matter, conductivity, and nitrogen-nitrate were similar at each site. Organic matter was on average between 18.27 and 18.69 in D78, D96 and REF. Organic matter on average measured highest in D87 with 20.58%. Conductivity was highest in D96 with a mean of 19.31mS and lowest in D87 with mean of 16.31mS. The most neutral pH was found in the REF site with a mean of 6.5 pH, which was significantly different than the restored sites where more acidic soils were on average 5.7 pH in D87 and D96. On average, NO_3-N was highest in D87 with 0.72ppm and lowest in D78 with 0.11ppm. Boxplots were created to graphically illustrate the variance of the upper and lower quantiles and standard error of each soil characteristic (Figure 4).

Table 1: ANOVA results with f-statistic and p-value for soil factors per site. All other values expressed as mean ±standard error (n=10). Values with the same letters are not significantly different (Tukey's test, p < 0.05).

	OM (%)	*F*	*P*	COND (mS)	*F*	*P*
REF	18.28 ±2.25 a	0.22	0.88	17.09 ±0.91 a	2.41	0.08
D78	18.30 ±2.2 a			17.10 ±0.84 a		
D87	20.58 ±2.58 a			16.31 ±0.85 a		
D96	18.70 ±2.27 a			19.31 ±0.7 a		
	pH			NO_3-N (ppm)		
REF	6.507 ±0.89 a	10.14	**5.54E-05**	0.41 ±0.13 a	2.15	0.11
D78	6.01 ±0.16 b			0.11 ±0.09 a		
D87	5.77 ±0 b			0.72 ±0.32 a		
D96	5.783 ±0.11 b			0.18 ±0.1 a		

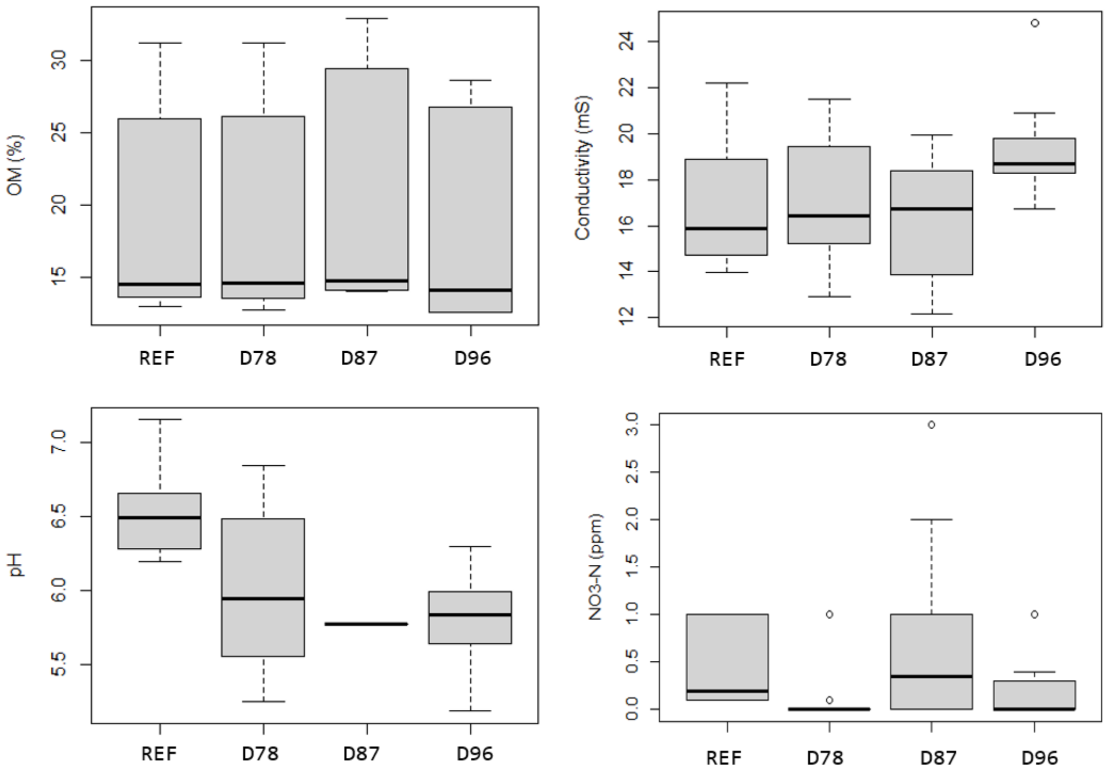

Figure 4: Boxplots of soil characteristics at each sample site with range, median, and standard error bars.

AMF Colonization

Mycorrhizal structures associated with root colonization in *Distichlis spicata* were internal hyphae, vesicles, and arbuscules. Average percentage of total colonization and internal structures of AMF were evaluated according to the four sites (Figure 5). Average total mycorrhizal colonization was 39% at the reference site and ranged from 53% (D87) to 72% (D78) in the three restored sites. The reference site had the lowest total colonization on average at 14% less than the lowest restored site (D87) and area D78 had the highest by 13% more than area D96 at 59%.

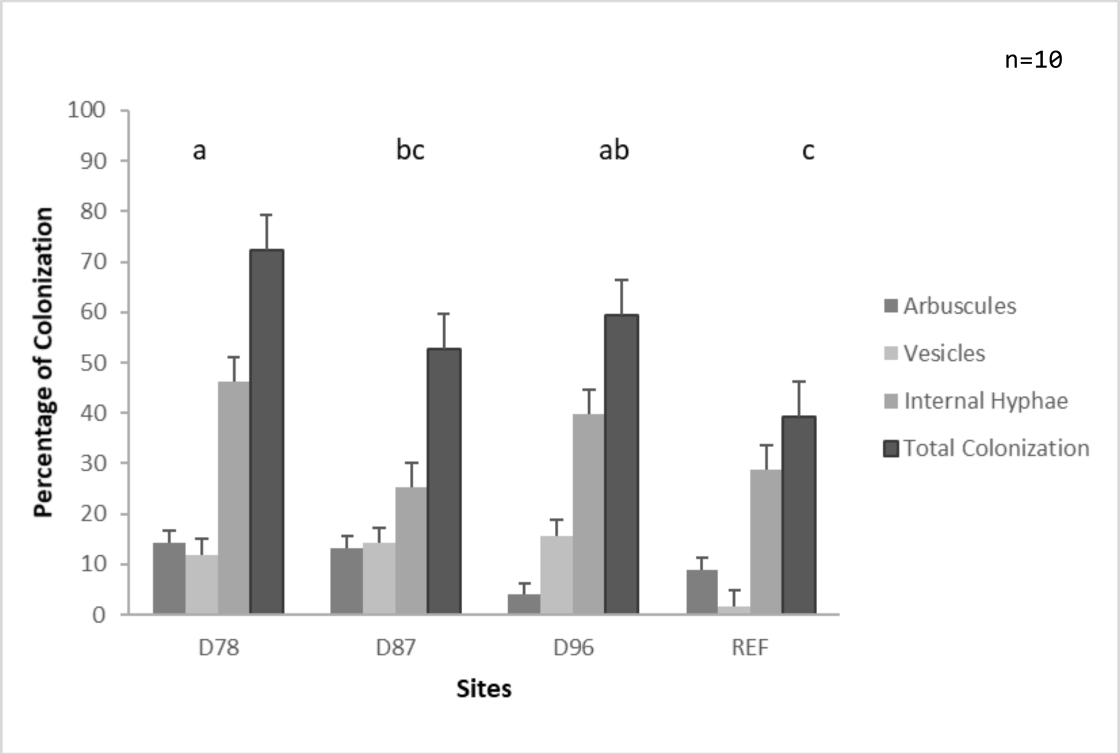

Figure 5: Percentage of root colonized (arbuscules, internal hyphae, vesicles, and total colonization) in Distichlis spicata at the four sites analyzed. Values are the means for the sampling sites with error bars. The same letter above bars indicates that values do not differ significantly by ANOVA and Tukey's test (p <0.05).

The reference site also ranked lowest in average percent of internal arbuscles and vesicles with 9% and 2% respectively. Areas D78 and D87 were each just under an average of 15% arbuscles, which was 4-10% higher than D96 and the reference site. Restored area D96 had the lowest average arbuscules with 4% and D78 had the highest with 14%. All three restored areas had 10-14% higher vesicles than the reference site. Average internal hyphae ranged from 25-46% with the highest in D78 and the lowest in D87.

In the ANOVA results across all four sites and between paired sites, significant differences in the total colonization, internal hyphae, and vesicles were found ($p < 0.05$) (Table 2). Arbuscular structures were not found significantly different across all sites or between sites. Significant interactions varied for both total colonization and AMF structures (internal hyphae, vesicles, and arbuscules) in the paired site analysis. Total colonization at the reference site was significantly different from both D78 and D96 restored sites. Site D78 also had significantly different total colonization from the D87 site.

When considering only the percentages of internal hyphae and vesicles there were significant differences across all sites and between some sites. For internal hyphae area D78 was the only restored site to be significantly different from the reference site. Within the restored sites, area D87 was significantly different from both D78 and D96, but the latter two areas were not statistically different when paired together. Vesicles were significantly different across all sites and only between the reference site and D96. No sites showed significantly different in terms of percent arbuscules. The percentage of total colonization, internal hyphae, and arbuscules were not significantly correlated ($p<0.05$) with any soil characteristics. However, vesicles were negatively correlated and significantly different with pH (Table 3).

Table 2: Analysis of variance for the percentage of total AMF colonization and mycorrhizal structures (internal hyphae, vesicles, and arbuscules) in Distichlis spicata across all sites and between sites. Values with (*) are significantly different (Tukey's test, p <0.05).

	Internal Hyphae			Vesicles		
	df	*F*	*P*	*df*	*F*	*P*
All Sites	3	7	**<0.05***	3	3.39	**<0.05***
REF-D78			**<0.05***			0.17
REF-D87			0.92			0.06
REF-D96			0.15			**<0.05***
D78-D87			**<0.05***			0.96
D78-D96			0.62			0.86
D87-D96			**<0.05***			0.99
	Arbuscules			Total Colonization		
	df	*F*	*P*	*df*	*F*	*P*
All Sites	3	2.08	0.12	3	7.76	**<0.05***
REF-D78			0.64			**<0.05***
REF-D87			0.79			0.24
REF-D96			0.71			**<0.05***
D78-D87			0.99			**<0.05***
D78-D96			0.13			0.27
D87-D96			0.21			0.77

Table 3: Pearson correlation coefficients between soil characteristics and percentage of total AMF colonization and mycorrhizal structures (p<0.05).

	Hyphae	Vesicles	Arbuscules	Total
OM (%)	- 0.27	0.18	0.18	0.01
CON (mS)	0.23	0.01	- 0.21	0.06
pH	- 0.05	**- 0.41****	0.02	- 0.27
NO_3-N (ppm)	- 2.1	- 0.01	0.01	- 0.15

** p<0.05; ** p<0.01*

DISCUSSION

This study confirms the presence and association of AMF with *Distichlis spicata* in both the reference site and the three restored sites of the Salmon River Estuary. On average total AMF colonization across all sites was 39-72%, which is consistent with earlier studies of fall collected *Distichlis spicata* AMF colonization rates (Richter and Stutz 2002; Bauer et al. 2003; Eppley 2009). Similar to studies in Indiana and Florida (Aziz, Sylvia, and Doren 1995; Bauer et al. 2003), the lowest levels of AMF colonization were found in the reference site (14-33% lower than restored sites), which is contrary to the findings of the many other wetland restoration studies. However, samples collected in this study were 30-50 years post restoration, while samples from contrary studies were typically taken ≤10 years post restoration (Cooke and Lefor 1990; Azi, Sylvia, and Doren 1995; Richter and Stutz 2002; Allen et al. 2003; Haug, Setaro, and Suárez 2013; Asmelash, Bekele, and Birhane 2016; Silvana et al. 2020). The results of this study are supported by similar studies whose findings suggest that the AMF colonization and community composition could be a key driver of long-term plant community succession.

Aziz, Sylvia, and Doren (1995) postulated that everglade vegetation and AMF community complexity would stabilize and decrease as species richness of AMF and plants peak in disturbed areas and site conditions became more stable. This proposed successional progression was found in savanna and forest ecosystems from a 1-60yr chronosequence study in Minnesota (Johnson et al. 1991). A greenhouse study of woody plants (Siqueira et al. 2012) also found that AMF infectivity was inversely related to succession. In that early successional species were highly susceptible to AMF colonization, while late successional species had low susceptibility to infection and low fungal colonization. This study suggested that the high levels of AMF colonization in early successional species was due to their high demand for nutrients (particularly in degraded soils), while late successional species didn't have the same reliance on AMF (Siqueira et al. 2012). Furthermore, Koziol and Bever (2017) found that later successional grasses that strongly benefit from specific AMF taxa and that early successional grassland species were less affected by AMF species composition. *Distichlis spicata* was a late successional species post-restoration of the three Salmon River Estuary areas (Frenkel and Morlan 1991) and while the colonization by AMF varied the overall levels of AMF colonization across all sites exhibited healthy mycorrhizal communities.

Total colonization was significantly different across all sites and between some sites. The reference site was significantly different ($p<0.05$) from the 1978 marsh and the 1996 marsh, which may be explained by their physical separation by the main Salmon River channel. Both the 1978 marsh and the 1996 marsh are located on the North bank of the Salmon River, while the reference marsh is located on the South. The reference site was not significantly different from the 1987, marsh which was not only adjacent to the reference site but also only separated by a minor intermittently inundated channel ~20ft wide (Figure 2). Neighboring plant populations with common mycelial networks can initiate colonization, accelerate succession, and determine plant community structures of surrounding disturbed areas (Richter and Stutz 2002; Püschel, Rydlová, and Vosátka 2007; Asmelash, Bekele, and Birhane 2016; Torrez et al. 2016; Workman and Cruzan

2016; Koziol and Bever 2017; Recorbet et al. 2021). The physical proximity and biological connectivity of the 1987 marsh to the reference site probably influenced and accelerated the colonization of AMF through a common mycelial network (Workman and Cruzan 2012), root fragments, and spore dispersal via wind, water, or animal vectors (Bauer et al. 2003).

The 1978 marsh has only a very small relatively undisturbed marsh just north of where the dike was breached. Immediately post-removal in the 1978 marsh, the existing plant community of upland pasture species was decimated and the high marsh plants slowly recolonized (Frenkel and Morlan 1992). It's possible that when the plant community experienced a rapid and extreme shift post-removal, the AMF community experienced a similar shift as all their existing plant hosts were wiped out. Then as the early successional marsh plants began to recolonize, a different AMF community would colonize the influx of new plant species and produce a novel successional pathway for that marsh area. Frenkel and Morlan (1992) distinguished two categories of plant succession recolonizing the 1978 marsh: ephemeral and persistent. Initially there was ephemeral on-native weedy plants, Saltmarsh sandspurry (*Spergularia marina*), dwarf alkaligrass (*Puccinellia punula*) and brass buttons (*Cotula coronopifolia*). The persistent native colonizers pickleweed (*Salicornia virginica*), Lyngbye's sedge (*Carax lyngbyei*) gradually dominated by 1988. Saltgrass (*Distichlis spicata*) was a late successional species, likely due to the need for specific fungal symbionts as described by Koziol and Bever (2017).

The 1996 marsh was probably the most disturbed of the three marshes included in this study due to sustained fragmentation on two sides. On the NW edge, the marsh is bounded by another dike (built 1996), separating private property still used for agriculture. On the SW edge, there's the impact of highway 101, which has had a berm fail twice causing subsidence. These current impacts could slow the restoration process and explain in part why the 1996 marsh had significantly different total colonization from the reference. From personal observation during field sampling, the 1996 marsh was still very much colonized by reeds and upland pasture plant species.

Internal hyphae and vesicles were both significantly different across all sites and between some sites, however arbuscules were not significantly different between any sites. Internal hyphae and vesicles are important AMF structures for root infiltration and nutrient absorption and storage, however arbuscules are the key structure in nutrient and water exchange between mycorrhizae and the host plant (Cooke and Lefor 1990; Allen 1991; Bauer et al. 2003). The lack of significant difference in arbuscules across all sites and between sites suggested similar levels of AMF colonization in the restored and reference wetlands. Therefore, the levels of arbuscular colonization in the three restored wetlands are at or approaching the natural conditions of the reference wetland. This could suggest that the levels of nutrient exchange between *Distichlis spicata* and AMF in the restored sites are comparable to the reference site. This study did not differentiate AMF species that are known to have varying levels of colonization. A much more extensive study of the fungal community by differentiating by AMF species would be necessary to accept the null hypothesis and determine whether the AMF community was truly similar between the restored and reference wetlands.

Percentage of total, arbuscular, and hyphal AMF colonization did not significantly correlate with any of the tested soil characteristics (OM, conductivity, pH and NO_3-N) between sites. However, percentage of AMF vesicles had a significant negative correlation with pH. This result was also found with the AMF *Acaulospora* in the savanna and forest chronosequence study in Minnesota (Johnson et al. 1991). Vesicles are the sack-like structures that store excess nutrients and possibly regulate the cell-to-cell communication between the plant and the fungi (Rizzo 2020). One study found an increase of fungal vesicle growth with an increase in lead (Pb) stress levels (Yang et al. 2016), which suggests that in this study an increase in vesicle growth could be an indicator of stress in more acidic soils.

All three restored sites had significantly more acidic soil than the reference site, which is directly due to prolonged periods of without flooding (Tiner 2013; VanZomeren et al. 2020). The oxidation of estuarine sediments and soils leads to an accumulation of acid sulfates, which can decrease soil pH <4 and is a common result in degraded coastal estuaries (VanZomeren et al. 2020). The acidification of soil can also be seen in degraded savanna and forest ecosystems going through successional restoration (Johnson et al. 1991). Soil pH had a significant negative correlation with vesicular colonization and pH was significantly more basic in the reference site, which had the lowest levels of vesicular colonization by more than 10%. There's potential that the increased accumulation of acid sulfates in the soils of the restored sites influenced AMF nutrient storage and AMF communication with host plants by increasing vesicular colonization.

Furthermore, pH was the only soil factor to be significantly different from the restoration sites, which may suggest homogeneity of the other soil parameters (OM, conductivity, and NO_3-N) in the soils across all sites and pH within the three restored sites. These results suggest that some soil characteristics in the high marsh of the three restored sites are at or approaching reference conditions as Frenkel and Morlan also stated in their 1991 study. However, AMF are known to be significantly affected by other soil properties not included in this study like carbon, phosphorus, nitrogen, and potassium (Johnson 1991). More extensive soil and AMF sampling would be required to truly accept the null hypothesis, the results of this study show that in terms of the measured soil characteristics and AMF colonization of *Distichlis spicata*, the three restored sites in the Salmon River Estuary are nearly recovered since the dikes were removed. This is synonymous with Frenkel and Morlan's (1991) prediction that it would take roughly 50 years for the Salmon River Estuary to recover from the damage caused by the dikes and agriculture.

Limitations

While this analysis has proven the presence and association of AMF with *Distichlis spicata* with both differences and similarities of mycorrhizal structures across the restoration sites, we acknowledge some assumptions and limitations in the methodology. AMF colonization of *Distichlis spicata* and other marsh grasses were highest early in the growing season and decreases as the season progresses (Azi, Sylvia, and Doren 1995; Ryszka and Turnau 2007; Eppley 2009). Sampling could have been taken across multiple seasons to account for variability in AMF colonization levels. Soil samples were taken in areas with varying biodiversity and each sample was dense with root mass, which made identifying *Distichlis spicata* roots from other plant species difficult to confirm. A larger sampling size for both roots, soil, and seasons could have decreased the standard error and better estimated the confidence intervals.

Eppley (2009), found a significant difference in AMF colonization between male and female *Distichlis spicata* plants in the Salmon River Estuary. Future studies should account for plant sex when testing for AMF colonization. The function and infectivity of AMF with host plants also varies between fungal species, identifying species, spore density and morphology (Johnson et al. 1991; Evelin 2009), could help distinguish levels of colonization and the AMF community structure in wetland grasses (Evelin 2009; Wilde et al. 2009; Lee et al. 2013). The variation in AMF species colonization also changes from early to late succession (Johnson et al. 1991). AMF colonization influences various levels of ecosystem communities and their behaviors like photosynthesis, biodiversity, nutrient and carbon cycling, and soil structure (Rillig 2004; Wang et al. 2009; Asmelash, Bekele, and Birhane 2016). Researching AMF at the community level is less prominent due to the multitude of variability, but it's potentially more beneficial to the efficacy of wetland restoration at the ecosystem scale (Rillig 2004; Wilde 2009).

Assessments of AMF in restoration should incorporate multilevel spatial/temporal scale and biotic-abiotic factors like: seasonal variability and rainfall (Carvalho et al. 2001; Silvana et al. 2020), plant diversity, composition, richness (Aziz, Sylvia, and Doren 1995; Wolfe et al. 2006), and sex-specific variation (Eppley 2009), plant succession (Asmelash, Bekele, and Birhane 2016; Koziol and Bever 2017), and soil feedback systems (Turner and Friese 1998; Bauer et al. 2003; Wolfe 2005; Ryszka and Turnau 2007). Each of these variables have been found to affect AMF colonization in salt marshes around the world, therefore including them in further studies would assist in determining how AMF succession occurs and influences the restoration of tidal wetlands over time.

AMF in Restoration

AMF are pivotal to mineral nutrient cycling along the biotic-abiotic interface between plants and soil substrates (Rillig 2004). Because of this AMF have major potential in accelerating and strengthening restoration in disturbed coastal wetlands where salinity, inundation, and soil degradation adversely affect plant growth and productivity (Bauer et al. 2003; Singh, Gill and Tuteja 2011). The reestablishment of critical processes like sediment accretion are critical to whether vegetation and AMF communities will approach “natural conditions” or turn into a novel ecosystem. Reintroduction of AMF to grassland restoration treatments has improved native plant diversity, richness, relative abundance, and productivity while simultaneously inhibiting less desirable weedy species (Zhang 2012; Koziol and Bever 2017). A study from 2021

(Huang et al.), found that AMF inoculation dramatically increased various wetland plant's growth under chromium (Cr) stress at differing water depths and increased chlorophyll, K, and P contents. Specifically, diverse native AMF inoculum is beneficial to early successional grass species and necessary for late successional species growth (Koziol and Bever 2017; Cheeke et al. 2019).

Furthermore, inoculation with spores from native AMF species derived from the area surrounding the restoration site also promotes mutualistic AMF associations with transplanted native grasses or seedlings. This increases the area of mycelial networks and the overall biological connectivity and productivity, resistance to invasive species (Asmelash, Bekele, and Birhane 2016; Koziol and Bever 2017). Increasing AMF richness with locally derived inoculum can increase infection effectiveness, plant production and resilience and may prove to play an important functional role in wetland plant succession and restoration (Cooke and Lefor 1990; Turner et al. 2000; Bauer et al. 2003; Koziol and Bever 2017).

Late successional grasses in disturbed ecosystems are sensitive to variation in AMF community composition particularly where traditional practices negatively impact soil communities. In early succession of temperate and boral ecosystems, AMF are the primary association with plants, while in older soils and late successional species there's a shift to colonization by ectomycorrhizal fungi (Piotrowski 2008), this shift could be due to the increase of soil nutrients as degraded soil remediate during late succession. In the case of dike removal, the impacts of restoration like soil compaction and vegetation destruction could also alter the course of plant succession. However, Thorne et al. (2013)

and Sales et al. (2018) both found that high density soil compaction had no significant effect on AMF colonization of five different grasses species. Therefore, the AMF in the Salmon River estuary might not have been affected by the compaction of soils during the dike removal construction and would allow the AMF community to better assist in plant reestablishment.

Future of Coastal Wetlands

Great progress has been made in the USA since the 1960's to control human impact by restoring and protecting coastal wetlands. Preserving structural integrity and resilience of tidal wetlands has never been more imperative. As the oceans heat and expand and polar ice continues to melt, sea level rise is a significant threat to the physical integrity of tidal wetlands. Increased fluctuation and duration of tidal flooding will slowly drown coastal wetlands as they exist today without room to shift. Between low-lying urban development and the rising oceans, wetlands only have two options, to move landward or slowly disintegrate and disappear. To keep pace with rising sea levels, Oregon's tidal marshes will need a sustained supply of sediment (Adamus, Larson, and Scranton 2005). Wetland structural integrity depends on physical and biological connectivity to support critical ecosystem functions like plant and animal habitat, carbon storage, nutrient cycling, and shoreline stabilization as sea level rise resistance. Wetland preservation and restoration is the only way to effectively ensure these ecosystems will persist.

CONCLUSION

The results of this study suggest that while total AMF colonization varied significantly, the arbuscular colonization and therefore nutrient exchange between AMF and *Distichlis spicata* in all three restoration sites of the Salmon River Estuary were similar to reference conditions. The acidic soils in the restored areas, due to oxidation and the accumulation of acid sulfates could increase the growth of AMF vesicles within plant roots due to stress. Additionally, the proximity and connectivity of reference sites to disturbed sites can potentially influence the AMF and plant community and expedite the restoration process. The spatial and biological connectivity of wetlands and adjacent systems is a crucial aspect for sustaining and restoring wetland ecosystems. However, large scale restoration practices can change the ecological trajectory and create novel successional pathways. Existing AMF communities and locally derived inoculum could be the defining factor in wetland restoration success. Future assessments should include various spatial and temporal scales along with additional biotic and abiotic factors to comprehensively evaluate the status of the wetland restoration. Differentiating AMF by species would better inform how AMF responds to restoration and the status of the overall mycorrhizal community of the Salmon River estuary. Overall, the levels of AMF colonization and soil characteristics in this study showed that the Salmon River estuary is nearly restored since dike removal.

AMF are essential to mineral nutrient cycling, water availability, and resilience for wetland plants and have major potential to accelerate and reinforce restoration practices of coastal wetlands. Inoculation of diverse native AMF to wetland restoration treatments could improve native plant diversity, richness, and productivity and inhibit invasive species. Wetland plant resistance to salinity, drought, pathogens, disease, waterlogging, heavy metals, and cold temperatures are all influenced by AMF colonization. AMF could have an important role in establishing and sustaining wetland communities as well as determining the direction of vegetation succession post-disturbance. Degraded coastal wetlands are continuing to be restored from extensive damage and AMF could be critical to the success of restoration practices. Studies like this are necessary to inform restoration managers and environmental conservationists of the value of mycorrhizae in restoration practices and advance the knowledge of mycelium in wetland environments.

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