Western Coniferous Forest Responses to Reclaimed Water Along a Time Series of Water Reuse Facilities

A Dissertation

Presented in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

with a

Major in Environmental Science

in the

College of Graduate Studies

University of Idaho

by

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May 2023

Abstract

Forest water reclamation is a cost-effective approach of managing treated municipal wastewater or reclaimed water by disposal on native forests. It provides a unique opportunity to combine forest productivity with improved water quality. However, prolonged application may have detrimental effects on both forest health and water quality. This dissertation uses three studies to investigate the long-term effects of reclaimed water land application on forest responses along a four-decade time series of water reuse facilities in northern Idaho. First, tree growth and vegetation diversity responses were investigated using tree rings and vegetation diversity indices. Forest water reclamation substantially improved tree growth responses by 30% to more than 100%. However, a decline in tree growth and understory vegetation diversity occurred with increasing length of treatment, particularly after three decades of treatment. Second, this dissertation investigated soil water nitrogen (N) and phosphorus (P) concentrations and potential leaching rates using drain gauges and porous cup tension lysimeters to capture preferential flow and soil matrix flow. Soil water samples were analyzed using a suite of microplate-based colorimetric assays. The leaching rates were calculated as a product of drainage and nutrient concentrations. Concerning levels of nitrate (NO_3^-) leaching occurred at the long-established facilities, where forests had received more than 30 years of reclaimed water. While phosphate (PO_4^{3-}) leaching losses were minimal, both NO_3^- and PO_4^{3-} leached predominantly through preferential flow paths. Finally, soil CO_2 efflux, exoenzyme activities and amino compounds were quantified to study soil biological responses of coniferous forests to long-term reclaimed water application. Amending forest with reclaimed water had little effect on soil biological responses except for N-releasing chitinase activity and P-releasing acid phosphatase activities. Soil chitinase activities were suppressed by effluent treatment especially at long-established facilities due to a possible shift in microbial composition. The suppression in litter phosphatase activities may indicate an abundance in readily available P supplied in reclaimed water. In addition, higher amino acid uptake for the reclaimed water treated plots at the recently established facilities may indicate N-limitation and reliance on a broader range of organic and inorganic sources for N acquisition from the ecosystem.

Acknowledgments

An immense thank you to my PhD advisor, Dr. Mark Coleman for his invaluable support and guidance, for always encouraging me to take on challenges, for celebrating my small successes, for motivating me when I have failed and for inspiring me to work as hard as he does. Thank you to my committee members, Dr. Daniel G. Strawn, Dr. Erik R. Coats and Dr. Greg Latta for their support and valuable insight on the project. I am eternally grateful to our collaborators without whose support this project would not have been possible. I would like to thank Matthew Plaisted at Idaho Department of Environmental Quality (IDEQ); Ron Hise, Chris Hoosick and Nathan Blackburn at Heyburn State Park; Dex Vogel and Trecy Carpenter at Ellisport Bay Sewer District; Bob Hansen, Don Moore, Stephen Miller and William Valentine at Garfield Bay Water & Sewer District and Bottle Bay Recreational Water & Sewer District; Leanord Johnson and Jeremy Polk at Cave Bay Community Services; Norris Booth from Coeur d'Alene Tribe and Ray Entz from Kalispell Tribe of Indians; and the Ellisport Bay landowners Dale and Gary Vanstone. A special thanks to Madeline Schwarzbach and Zach Sanchez for assisting with field work and analysis, and for making field work fun and memorable. And lastly, thank you to USDA for the NIFA grant (2020-67020-31174), University of Idaho Stillinger Herbarium for the Stillinger Herbarium Expedition Fund and to Meter Group Inc. for the Grant A. Harris Fellowship that were integral to this research.

Dedication

To mom, Audrey, Astha and Maddy— Thank you for all the love and prayers. And in loving memory of my dad, who was always proud of every little thing I did.

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Chapter 1: Introduction

Increasing concerns for dwindling freshwater resources due to drought, over-consumption and anthropogenic contamination has led to exploration of a range of uses for treated wastewater or reclaimed water. Reclamation of treated municipal wastewater using land application systems is a decades-old, well-established approach for managing wastewater globally (Andrews et al., 2016; Asano, 1987; Hamilton et al., 2007). Approximately 34 billion gallons of wastewater is processed daily in the United States (United States Environmental Protection Agency (EPA), 2022), making wastewater one of the largest sources of waste from our civilization. The EPA recommends land-application as a method for further treatment through recycling of nutrients and organic matter, and as an approach to conserve water resources (Al-Jamal et al., 2002). Historically, land application of reclaimed water was limited to irrigation of crops, rangelands, forests, disturbed areas, and recreational areas such as golf courses and parks (Al-Jamal et al., 2002; Sopper & Kardos, 1974). Today, reclaimed water has a multitude of uses in agriculture, silviculture, landscaping, aquaculture, industries, and is even utilized for groundwater recharge; making it a valuable, viable and sustainable resource (Akhoundi & Nazif, 2018; Miller, 2006; Kalavrouziotis et al., 2015).

Many municipal water reuse facilities in Idaho land apply reclaimed water on native coniferous forests during summer or the growing season when surface water flows are low, due to strict quality standards for releasing wastewater directly into water sources. Such forest water land application systems are prominent in smaller communities where construction of tertiary wastewater treatment plants is not financially and technologically feasible. Forest water reclamation has been widely accepted as an effective method of treatment of secondary treated wastewater. Land application has low management and maintenance costs and reduced operational requirements (Miguel et al., 2014). Land application systems utilize low-cost lagoons (Al-Jamal et al., 2002), and tertiary treatment is achieved using established native vegetation and soil that act as natural filters to trap and assimilate the applied nutrients and contaminants (Mexal et al., 2002). Land application can also help to reduce nutrient loads, particularly in areas where surface waters are sensitive to nutrient additions (Barton et al., 2005a; Tomer et al., 2000).

While forests have been known to assimilate applied water and nutrients, potential environmental risks of land application have also been identified. Detrimental impacts include a decline in forest productivity, tree mortality, altered vegetation community structure, nutrient leaching, and detrimental effects on soil physical, chemical and biological health (Aiello et al., 2007; Duan et al., 2011; Magesan et al., 2000; Oswald et al., 2009; Thomas et al., 1999; Toze, 2006; Wallach et al., 2005). The regional forest water reclamation facilities in northern Idaho have been in operation for several years to decades and are located near the iconic regional lakes- Lake Coeur

d'Alene and Lake Pend Oreille. Yet, there is little information regarding long-term effects of reclaimed water application on forest growth and vegetation diversity, potential for soil N saturation and leaching, biological responses and overall implications on regional forest health.

The studies included in this dissertation opportunistically utilize a four-decade time series of forest water reclamation facilities in northern Idaho established between 1978 and 2013. Tree growth responses to reclaimed water have been observed in many forest types. However, detrimental effects on productivity and alteration of vegetation diversity have also been identified. Here, I consider the potential of forest water reclamation to assess the inherent productivity and shifts in vegetation diversity at the regional coniferous forests by studying species-specific tree growth responses. I also examine the implications on overstory and understory vegetation diversity to long-term reclaimed water amendment. Prolonged hydraulic and nutrient loading from reclaimed water application may ultimately exceed the finite assimilation capacity of the receiving forests. Nutrients such as nitrogen (N) and phosphorus (P) from such nutrient-saturated soils may eventually leach into surface and ground water sources resulting in irreversible detrimental impact on environmental quality. Amending forests with reclaimed water containing N and P can also alter soil biological processes such as soil CO_2 efflux, exoenzyme activities, and amino acid and amino sugar product pools, and potentially impact soil quality and forest productivity. I evaluate the soil water nutrient concentrations and leaching rates and investigate the timeline for the onset of nutrient leaching losses. I examine the effects of soil biological responses to reclaimed water amendment and discuss its impacts on soil quality.

1.1 Forest Water Reclamation in Idaho

In Idaho, use of reclaimed water and standards are regulated by the state agency Idaho Department of Environmental Quality (IDEQ). Forest water reclamation (FWR) facilities operate in compliance with IDEQ guidelines and requirements for efficient water reuse and nutrient management (IDEQ, 2007). Municipal wastewater receives primary and secondary treatments which includes aeration using lagoons and disinfection by chlorination before application on forested areas during the growing season (April 1-October 31). Because land application doesn't require high-end treatment nutrient removal technology, it can be a cost-effective approach for managing municipal wastewater and protecting environmental quality (Asano, 1987; Hamilton et al., 2007; Vogeler, 2009). Land application is prominent in smaller communities in northern Idaho. Therefore, diversion to land application is often preferred due to strict State water quality standards and discharge regulations designed to protect regional surface water resources. The alternatives include various artificial nutrient removal processes that can be rigorous and costly.

The Pacific Northwest U.S. has a cool and wet winter season, which receives the majority of the annual precipitation; and a warm and dry summer season, characterized by a hydrologic drought and low streamflow (Kormos et al., 2016). With reduced surface water levels in summer and the stringent water quality discharge regulations (IDEQ, 2014), land application is the most cost-effective avenue for safe disposal. Water quality standards are established to protect public health while encouraging the wastewater reuse and reclamation (Asano, 1987). Compliance with the regulatory framework is mandatory to practice forest water reclamation. Idaho has Recycled Water Rules (IDAPA 58.01.17) with procedures and requirements for issuing reuse permits (IDEQ, 2014). It provides guidance on planning, design, operation, and treatment of wastewater to protect public and environmental health. The Idaho Pollutant Discharge Elimination System (IPDES) Program permits pollutant discharges through Clean Water Act and the Rules Regulating the Idaho Pollutant Discharge Elimination System Program (IDAPA 58.01.25). The primary motivators for reclaimed water land application are stringent discharge regulations, increasing demand for water, and the desire to reduce pollutants in receiving waters.

Many forest water reclamation facilities in northern Idaho are located near Lakes Coeur d'Alene and Lake Pend Oreille. These lakes have sustained indigenous American communities and hold important cultural, recreational, and socio-economic value to the local inhabitants and attract lucrative tourism. Evidence for nutrient enrichment in Lake Coeur d'Alene (Wood & Beckwith, 2008) raises public concerns over degraded water quality among Idaho's iconic lakes (Liao et al., 2016). Although the major causes of increasing lake nutrients are uncertain, some suspect that land application of reclaimed water may play a role. Nutrients in reclaimed water may eventually leach into surface and ground water sources (Cameron et al., 1997), potentially leading to impairment of water quality due to eutrophication and result in loss of ecosystem services (Carpenter et al., 1998; Polglase et al., 1995; Barton et al., 2005; Hook & Kardos, 1978; Muga & Mihelcic, 2008; Daniel et al., 1998; Reed-Andersen et al., 2000; Yang et al., 2008).

1.1.1 Reclaimed Water Classification

The extent of use of reclaimed water is limited by its quality depending on its classification (IDEQ, 2014). Reclaimed water is categorized into five classes (Classes A-E) by the level of treatment. Class A reclaimed water undergoes oxidation, coagulation, clarification, filtration, and disinfection. Class A reclaimed water is the highest quality reclaimed water and receives the most treatment out of all other classes. The treatment process includes secondary treatment followed by filtration and disinfection. Class A water must meet strict requirements on turbidity and pathogens. Because Class A water requires extensive treatment, it can be applied in areas where human contact

can occur such as parks, businesses, homes and recharging potable aquifers. Class B reclaimed water is the second highest in quality and treatment processes are like Class A reclaimed water. However, the turbidity requirements following filtration and disinfection requirements are not as strict as Class A. Despite many uses such as irrigation of crops, pastures, parks, playgrounds, golf courses etc., it cannot be distributed to homeowners for residential irrigation or for aquifer recharge like Class A. Class C system is the most common type of municipal reuse system in Idaho with most number of active Class C reuse permits issued Statewide (IDEQ, 2014). Class C reclaimed water is a secondary treated effluent which involves oxidation and disinfection. Filtration is not required, and disinfection requirements are less stringent compared to classes A and B. Class C reclaimed water still has multitude of uses such as irrigation of forest, fodder, and food crops (additional pathogen removal process). Class D reclaimed water also requires oxidation and disinfection. However, disinfection requirements are an order of magnitude lower compared to Class C. Class E reclaimed water is the least treated of all other classes and only requires primary treatment, which does not involve oxidation or disinfection (IDEQ, 2014).

Table 1.1. Reclaimed water classification.

Classification	Class A	Class B	Class C	Class D	Class E
Oxidized	Yes	Yes	Yes	Yes	No
Clarified	Yes	Yes	No	No	No
Filtered	Yes	Yes	No	No	No
Disinfected	Yes	Yes	Yes	Yes	No
Total coliform (organisms/100 ml)	23	23	230	2,300	No limit
Buffers required	No	Yes	Yes	Yes	Yes

Wastewater Land Application Operators Study and Reference Manual (IDEQ, 2014).

1.2 Reclaimed Water Application on Western Forests

Productivity of western forests are largely limited by moisture and nutrient availability. Northwestern United States is characterized by wet winters with abundant supply of moisture and a very dry growing-season with limited water availability, and soils with low fertility which results in a positive growth response to fertilization (Gessel et al., 1990). Therefore, addressing such limitations through reclaimed water amendments allows us to identify inherent growth potential of trees, which in turn, sets targets for operational management practices and reference points for improved genotypes. Forests are known to have long-term capacity for nutrient storage and for being able to renovate reclaimed water without causing adverse impacts on the ecosystem and water quality (McKim et al., 1982). The effectiveness of a forest ecosystem to assimilate applied wastewater depends on various parameters such as soil biological, physical and chemical attributes, vegetation uptake rate and irrigation management strategies such as constituent and hydraulic loading rates,

reclaimed water characteristics, site conditions and leachate water quality standards (Barton et al., 2005a, McKim et al., 1982). Uptake of nutrients in reclaimed water by vegetation is largely dependent on tree species, stand density, structure and age, and the understory vegetation composition (McKim et al., 1982). Forest water reclamation facilities present an opportunity to quantify growth responses to long-term reclaimed water application and identify inherent growth potential of regional western coniferous forest ecosystems.

1.2.1 *Western Trees and Growth Responses*

Ranging from the eastern slopes of the Cascade Mountains of eastern Washington and Oregon to western slopes of the Rocky Mountains of northern Idaho and western Montana (Jurgensen et al., 1997), the forests of the Inland Northwest U.S. comprises vast areas of temperate forests dominated by a diversity of ecologically and economically valuable coniferous species such as grand fir (*Abies grandis* (Douglas ex D. Don) Lindl.), Rocky Mountain Douglas-fir (*Pseudotsuga menziesii* var. glauca (Beissn.) Mayr), lodgepole pine (*Pinus contorta* Douglas ex Loudon), western redcedar (*Thuja plicata* Donn ex D. Don), western hemlock (*Tsuga heterophylla* (Raf.) Sarg.), ponderosa pine (*Pinus ponderosa* Lawson & C. Lawson), and western larch (*Larix occidentalis* Nutt) (C. Shen & Nelson, 2018). Forest productivity is greater in warm and moist habitats dominated by cedar and hemlock compared to dry Douglas-fir and ponderosa pine inhabited sites (Jurgensen et al., 1997).

Timber harvesting is one of the predominant economic activities of this region (Affleck, 2019), with a timber output from Idaho and Montana alone accounting for 91% of the roundwood production for the Rocky Mountain Region (FIA, 2009). Fastest growth occurs on moist locations with good nutrient availability (Burns & Honkala, 1990) while moisture largely defines productivity of western forests (Hermann & Lavender in Burns & Honkala, 1990) due to major growth limitations imposed by summer droughts. Commercially important species in the Inland Northwest such as Douglas-fir, grand fir, and Ponderosa pine have been found to positively respond to N fertilization treatment, implying that their productivity relies on site nutrient supply, specifically N, and other site characteristics such as water availability (Coleman et al., 2014; Garrison-Johnston et al., 2005; Parent & Coleman, 2016). N has been identified as the most limiting nutrient, particularly in Douglas-fir ecosystems, with positive height growth responses recorded in response to N fertilization (Gessel & Walker, 1956).

N and P are essential soil nutrients limiting ecosystem primary productivity in terrestrial ecosystems forests with plant nutrient accumulation being one of the most important factors in soil and ecosystem development, and forest productivity is highly correlated with nutrient cycling in coniferous forest ecosystems (Howard & McLauchlan, 2015; Cole & Rapp, 1981; Johnson & Turner,

2014; Filippelli, 2008; Lang et al., 2016) . Coniferous forest are limited in N and a strong correlation has been observed between coniferous forest production and nitrogen requirement associated with the annual growth of a forest (D. W. Cole & Rapp, 1981; Compton et al., 2003; Van Miegroet & Cole, 1984). Plants and microbes are only able to take up small molecules such as amino compounds, NH^{4+} and NO^{3-} and since most soil N occurs as insoluble organic polymers, most of the soil N is not readily available for plant and microbial intake (Schimel & Bennett, 2004).

Regular small doses of water and nutrients have been known to improve forest growth (Chappell et al., 1991; Coleman et al., 1998; Ingestad, 1987; 1982). Moreover, regular low dose amendments supplied during the growing season can significantly increase nutrient retention and restrict ecosystem losses (Van Miegroet & Cole, 1984). Supplementing forest stands with regular increments of water and nutrients can double or triple growth rates (Coyle et al., 2016; Iivonen et al., 2006; Weetman et al., 1997). Yet, because of practical restrictions to growing western forests with metered supply of water and nutrients, we have little understanding of the upper limits of productivity in the regions. Significant productivity responses have also been reported for hybrid poplar plantations (Stanton et al., 2002). However, markets for poplar are limited in the Inland northwest and the sawmills are optimized for high-value conifers.

Conifer species such as pines have a very low nutrient requirement (Wehrmann, 1968). Western redcedar has very high nutrient requirements. Studies with ammonium (NH_4^+) and nitrate (NO_3^-) as sources of N have demonstrated that low N demanding species such as *Pinus contorta* (lodgepole pine) and *Tsuga heterophylla* (western hemlock) prefer NH_4^+ while high N demanding species such as redcedar prefer NO_3^-. Intermediate species such as Douglas-Fir have been known to utilize both inorganic forms of N (Bigg & Daniel, 1978; Gosz, 1981). Therefore, low doses of nutrients, particularly N, may significantly improve growth in these regional tree species. Maximum growth potential of western conifer forests is yet to be explored. Forest water reclamation provides an opportunity to assess western conifer forest growth potential in response to regular doses of water and nutrients through reclaimed water during the growing season.

1.2.2 *Tree Growth Responses to Reclaimed Water*

Positive growth responses have been reported in various forest systems that have been treated with municipal reclaimed water such as: poplar (Moffat et al., 2001), bald cypress (Hesse et al., 1998a), western hemlock, Pacific silver fir and western redcedar (Weetman et al., 1993), Douglas-fir and Lombardy poplar (Gessel et al., 1990a), black spruce and Tamarack (USGS), Pitch Pine and Oak (Jordan et al., 1997a) among others. Forests not only have long-term capacity for storing nutrients as plant biomass, but also provide a low-cost and manageable approach for renovating wastewater

(McKim et al., 1982). Thus, wastewater is a potential resource and the filtering effect of vegetation filters can be used for biomass production which in turn can be utilized for generating renewable wood fuel and energy (Aronsson & Perttu, 2001).

Tree rings allow us to study historical growth patterns using chronologies that extend back decades to thousands of years (McCarroll & Loader, 2004). Retrospective assessment of ecosystem dynamics with tree-ring records of site conditions over an extended time can be valuable in forest management (Arenas-Castro et al., 2015; Shikangalah et al., 2020). Annual growth increments from ring-width chronologies for each species facilitate understanding of species-specific responses to increased water and nutrients of the forested sites. The tree-rings are cross-dated and the ring series standardized to remove systematic biological growth trends (Swetnam et al., 1985). Cross-dating of tree rings across different trees from the same species within a site allows comparison of ring width patterns, which should be similar for trees in a site subjected to similar environmental conditions such as precipitation and temperature. Environmental conditions may limit tree growth, which is expressed as synchronous variations in ring width (Fritts, 1974). Despite decades of reclaimed water application, growth in response to reclaimed water amendments at forest water reclamation facilities have only been described in required forest management plans without comparison to non-treated control plots. Therefore, studying growth response at forest water reclamation facilities in comparison to untreated controls using tree rings presents an important opportunity to document long-term effects of supplemental water and nutrients in reclaimed water.

1.2.3 Understory Vegetation Responses

Plant community composition has a significant influence on nutrient retention and cycling, and soil fertility (Binkley & Giardina, 1998; Compton et al., 2003; Hobbie, 1992). Irrigation with reclaimed water addresses moisture limitations in forests and leads to highly productive herb-shrub layer community existing under more mesic conditions. However, plant diversity has been found to significantly decline with opportunistic species replacing the perennial herb-shrub layer vegetation, ultimately resulting in an altered understory vegetation and community simplification (Hunt & Shure, 1980). Reclaimed water application can result in dramatic changes in understory vegetation composition (Jordan et al., 1997b). Thus, increase in moisture may potentially lead to displacement of native vegetation adapted to dry habitat and will be replaced by opportunistic invasive species that are able to thrive in a more mesic environment. In addition, abundant water and nutrients availability during the growing season may lead to denser overstory canopies, which intercept nutrient and light resources and result in an overall decline in understory vegetation diversity (Alvarez-Clare et al., 2013; Ibáñez et al., 2016; Schroth et al., 2015). Understory vegetation plays an important role in the

structure and function of forest ecosystems (P. J. Burton et al., 1992; DeWald & Mahalovich, 1997; Oliver & Lippke, 1993; J. Zhang et al., 2016).

1.2.4 Forest Management Implications

Many facility-specific factors can also play an important role when determining nutrient uptake and growth increment in forest ecosystems. Both uptake and increment have been found to decline sharply after crown closure as the nutrient-rich foliar biomass reaches a steady state (Miller, 1981; Switzer & Nelson, 1972; J. Turner, 1981). Forest management practices can play a key role in stand nutrient improvement. Repeated small dose fertilization can further improve forest growth (Chappell et al., 1991) especially when combined with thinning (Brix, 1992). Furthermore, early stand thinning further directs site resources toward overstory crop trees by removing less competitive intermediate and suppressed trees (Chase et al., 2016). Scanlin & Loewenstein suggest that there is about a 75% chance that thinning a stand of Douglas-fir or grand fir will increase the cubic foot volume growth rate of residual trees (Scanlin & Loewenstein, 1979). Thinning can be an important forest management practice to improve nutrient uptake and tree growth. Fertilization responses have also reported to be higher with vegetation control in young well-spaced stands and stocking control in older stands (Mika et al., 1992). While species such as western redcedar respond well to thinning and can grow relatively well even under moderate competition (O'Callaghan, 2012), Douglas-fir growth responses are greater in vigorous and properly spaced stands with low relative density (Coleman et al., 2014). Because nutrient uptake and productivity are tightly linked (Aubrey et al., 2012), forest management is critical for sustainability of forest water reclamation over the long-term.

1.3 Effects of Land Application on Soil Health

Land application of reclaimed water may have serious implications on soil health such as alteration of physiochemical and microbiological attributes, accumulation of chemical and biological contaminants, decline in soil productivity and fertility, and a potential risk to public and environmental health (Becerra-Castro et al., 2015). Short term effects of land application on soil health include increase in microbial activity (Toze, 2006), increase of soil bulk density and porosity (Aiello et al., 2007; Wallach et al., 2005), accumulation of salt in soil, also known as soil salinity (Duan et al., 2011), which causes decrease in soil permeability, infiltration rate and hydraulic conductivity (Pearson, 2003). On the contrary, several studies have also shown long-term reclaimed water application to improve soil physical properties (Vogeler, 2009) and not have any negative impact on soil chemical characteristics (Duan et al., 2010b). The viability of a land application system is highly dependent on the soil hydraulic properties. Wastewater irrigation can cause decline in

infiltration rate in highly permeable volcanic ash soil due to clogging of soil pores (Cook et al., 1994). The clogging of the soil pores occurs primarily due to the constituents and microbial by-products in wastewater effluent (McAuliffe et al., 1982; Siegrist & Boyle, 1987).

Nonetheless, enrichment with N may lead to increases in nitrification and accelerated NO_3^- leaching loss along with an increase in soil solution acidity, particularly in the upper part of the soil profile (Van Miegroet & Cole, 1984). Protons produced during nitrification displace primarily divalent cations such as magnesium (Mg^{2+}) and calcium (Ca^{2+}) in soil which in long-term may result in gradual depletion of the exchangeable bases along with an increase in soil acidity. In acidic soils with low base saturation, it can lead to potential aluminum (Al^{3+}) toxicity and/or nutrient imbalances, which may ultimately have adverse impacts on overall forest productivity (Kelly et al., 1990; Raynal et al., 1990). However, with proper irrigation management practices, reclaimed water application may improve soil fertility (Mohammad & Mazahreh, 2003). Soil is an important pool in N cycling in forest ecosystem as it serves as one of the major N reservoirs and governs long-term flux of N to water sources (Reed-Andersen et al., 2000). Therefore, proper metering of water and constituent nutrient loading rates at forest water reclamation facilities is critical for preventing adverse effects on soil properties.

1.4 Reclaimed Water Effects on Nutrient Cycling and Potential Leaching Losses

In a natural, undisturbed and closed coniferous ecosystems, N retention is high due to low inputs through precipitation and nitrogen fixation and leaching losses are minimal (Gosz, 1981). However, continuous artificial inputs of nutrients in reclaimed water may alter the forest nutrient cycling processes and open the otherwise closed ecosystem. N saturation may occur in areas where N inputs are greater than the ecosystem demand which may result in accumulation of NH_4^+ to cause an increase in nitrifier i.e., ammonia-oxidizing bacterial abundance. Nitrifiers produce NO^{3-} through the process of nitrification, which is vulnerable which can potentially be lost by leaching to groundwater, lakes and streams (Aber et al., 1998; Katz, 2020). Thus, assimilation of nutrients by vegetation is essential in land application and losses through leaching may pose serious health concerns and directly violate IDEQ permit requirements (IDEQ, 2014).

N in reclaimed water occurs mainly as NH_4^+, nitrite (NO_2^-), NO_3^- and organic N, and raw wastewater is particularly high in NH_4^+ (IDEQ, 2014). High NH_4^+ concentration may lead to increased nitrification and production of NO_3^- (Johnson, 1992). NO_3^- is much less strongly absorbed and readily leached than NH_4^+ (Johnson & Todd, 1988; Van Breemen et al., 1982; Van Miegroet & Cole, 1984; Kinjo & Pratt, 1971), and unless NO_3^- is removed from soil solution via plant uptake, denitrification or microbial uptake, it is very mobile and will readily move through the soil.

Furthermore, NO_3^- is the most commonly identified pollutant in ground water (Freeze & Cherry, 1979). During initial operation of land application, leachate NO_3^- concentrations have been found to often exceed that of applied reclaimed water due to mineralization of organic N lost from litter and humus, therefore making the ecosystem more susceptible to leaching losses (Hook & Kardos, 1978). N mineralization and nitrification rates have been found to be highest in spring and fall and linked with increased leaching through soil (Johnson, 1992; Strader et al., 1989). Nitrification varies seasonally with changes in temperature and moisture regime, with the highest NO_3^- concentration in soil solution in spring because of snowmelt. Soil and soil pore water NO_3^-concentrations typically decrease during the growing season and NO_3^- leaching is lowest during the winter months in majority of the forest ecosystems (Foster et al., 1989; Nadelhoffer et al., 1983; Shepard et al., 1990; Strader et al., 1989). While NO_3^- is naturally low in N-limited western forest ecosystems (Shan et al., 2014), reclaimed water amendments can increase NO_3^- availability, which in turn, increases leaching potential (Polglase et al., 1995) and the risk of ground water contamination (Bond, 1998).

Organic pools of N are also quite important for forest productivity, particularly dissolved organic nitrogen (DON) which is one of the major forms of N lost from soil to freshwater (Jones & Willett, 2006). DON has been found to be particularly important under N-saturated conditions where studies have shown an increase in DON efflux with increased N input (Compton et al., 2003; Fang et al., 2009; McDowell et al., 2004). Significant DON losses has been found to drain from forest floor (McDowell et al., 1998; Qualls et al., 2000). While DON flux with similar inorganic inputs was found to be considerably lower for temperate forests, leaching risk remains under saturated conditions (Fang et al., 2009).

Reclaimed water and constituent nutrients movement in soil affects the assimilation of applied nutrients due to soil biological and chemical processes which take place primarily in the topsoil and is enhanced by increased contact time and reclaimed water-soil interaction (McLeod et al., 1998). Leaching occurs mainly through matrix and preferential flow paths while surface runoff occurs when water inputs exceed the capacity of soil to absorb the water (Reid et al., 2018). While matrix flow facilitates increased contact time due to delayed movement through micropores between soil aggregates, preferential flow occurs around the aggregates due to cracks and worm channels in soil profile, significantly limiting the contact time and retention (McLeod et al., 1998). Significant N losses have been attributed to increased preferential flow which reduces contact between the reclaimed water and soil matrix (Barton et al., 2005b).

Similar to N, P is also vulnerable of being rapidly lost as runoff or leached to surface waters through preferential pathways (Bol et al., 2016; Julich et al., 2017a; Simard et al., 2000). P is a limiting nutrient for primary productivity in terrestrial ecosystems and plant P demand can be one of

the most important drivers of soil and ecosystem development (Filippelli, 2008; Lang et al., 2016). Despite being an important nutrient, natural inputs of P in a forest ecosystem which occurs by atmospheric deposition and mineral weathering, are quite small (Sohrt et al., 2017; Newman, 1995). While northern temperate forests are typically not considered P limited, limitation may occur due to weathering and high soil acidity with low bioavailable inorganic P (DeForest et al., 2012). Soil P availability is also limited due to slow diffusion and high fixation in soil (Schachtman et al., 1998; J. Shen et al., 2011). In a forest ecosystem, the mineral soil, forest floor, vegetation and microbial biomass serve as the most important pools for P storage (Sohrt et al., 2017; Yanai, 1992). Despite being largely adsorbed in soil, P losses have been reported via leaching.

P leaching is generally not considered to be a concern in land application (Barton et al., 2005a). However, P leaching losses have been observed in sandy soil (Aulakh et al., 2007; Iskandar & Syers, 1980; Latterell et al., 1982), sandy loam soil (Mamo et al., 2005), and Gley soil (Barton et al., 2005a). P leaching in reclaimed water irrigated forests has been found to be less than 2 kg P ha^{-1} (Burton & Hook, 1979; Tomer et al., 2000). However, P leaching depends on soil-specific P retention index, vegetation uptake and soil storage (Barton et al., 2005a). P leaching occurs predominantly through preferential flow and thus, it can be reduced by limiting preferential flow by matching P in reclaimed water with vegetation uptake and P storage capacity (Barton et al., 2005a).

Soils vary in their ability to assimilate applied P. Soil properties such as pH, and clay, silt and sand content have been found to be correlated with P retention (Ballard & Fiskell, 1974). P sorption capacity relies on soil's anion exchange capacity and increases with clay content due to large surface area for sorption. Soil mineralogy also has an important effect on P retention with highest P retention observed in volcanic soils rich in amorphous soil minerals such as allophanes (Batjes, 2011).Therefore, it is important to take soil types and irrigation management into consideration to enhance uptake and minimize preferential flow (Barton et al., 2005a). While municipal reclaimed water total phosphorus levels are quite low ranging from 2 to 20 mg L^{-1} (IDEQ, 2014), soil P buildup and losses may be detrimental to environmental quality (Bennett et al., 2001). Toil P storage capacity can be assessed along with hydraulic conductivity and potential for preferential flow (Barton et al., 2005a; Hooda et al., 2000; Nair et al., 2004; Renneson et al., 2015) to identify P loss risk.

To prevent leaching losses, forest water reclamation facilities are required to ensure that there is no growing-season drainage and preferential flow is limited. Greater retention maximizes N uptake and immobilization, decreases leaching potential (Magesan et al., 1998), and increases denitrification (Monnett et al., 1995). Therefore, it is important to identify nutrient retention capacities and monitor NO_3^- and PO_4^{3-} leaching potential of forest water reclamation systems. There is little information regarding when forest water reclamation sites might become N saturated nor on the effects of long-

term water reclamation treatments (Aber & Magill, 2004), especially in western forests. In addition, hydraulic and constituent loading rates for forest water reclamation facilities in the western US are not based on studies at actual facilities. Therefore, evaluation of potential for nutrient leaching is essential to ensure sustainability of forest water reclamation systems, particularly those that have been in operation for decades.

1.5 Soil Biological Responses

Reclaimed water has often been used to improve soil quality due to the nitrogen (N), phosphorus (P) and micronutrient content which can be beneficial for soil productivity (Brzezińska et al., 2001; Brzezinska et al., 2006). However, over the long-term, continuous amendments of N and P may alter nutrient cycling and biological responses of forest ecosystems. Reclaimed water application has been known to increase (Brzezinska et al., 2006; Cairns et al., 1978; Schipper et al., 1996; Chen et al., 2008; Filip et al., 1999; Filip et al., 2000), as well as have mixed effects with both increase and decrease of soil biological activity (Brzezińska et al., 2001; Jian et al., 2016; Meli et al., 2002). Soil biological responses are used as important indicators of adverse effects of reclaimed water application on soil health (Martinez-Salgado et al., 2010; Speir, 2002). Furthermore, changes in soil biological characters are considered sensible indicators of soil quality, and are more sensitive than physical or chemical soil properties (Friedel et al., 2000). Irrigation with wastewater has been found to have a strong impact on soil microbial activities and abundance (Friedel et al., 2000; Jueschke et al., 2008), with effects on enzyme activities and nutrient turnover (W. Chen et al., 2008; Heinze et al., 2014). Wastewater application has also been found to increase bacteria, fungi and actinomycetes (Saber, 1986). Such enhancement in microbial activity has been linked with constant humidity from irrigation (Friedel et al., 2000). The type of soil enzyme activities and the rates at which the substrates are broken down are also influenced by microbial community composition (Chapin et al., 2012). However, others indicate little influence of wastewater irrigation on microbial biomass and enzyme activities (Heinze et al., 2014; Schipper et al., 1996a).

1.5.1 Soil CO_2 Efflux

Soil CO_2 efflux is sensitive to biotic and abiotic environmental conditions such as temperature, moisture, soil, vegetation, substrate availability, composition and activity of microbial community (Y. Luo & Zhou, 2006; Schlesinger & Andrews, 2000; Fér et al., 2022). Additions of inorganic N (NH_4^+ and NO_3^-) has been found to have variable effects on soil CO_2 efflux and microbial activity (Micks et al., 2004). Soil respiration increased (Hopkins et al., 2013) or decreased (Giardina et al., 2003; Sun et al., 2014; Zhou et al., 2014; Olsson et al., 2005; Phillips & Fahey, 2007;

Zhang et al., 2016; Janssens et al., 2010) in response to fertilization. N directly stimulates primary production (Vitousek & Howarth, 1991) resulting in more substrate for soil respiration (Y. Luo & Zhou, 2006). In environments with surplus of N, fertilization could cause N leaching and cause little change in soil respiraiton (Y. Luo & Zhou, 2006). In reclaimed water or chronic N addition studies, soil CO_2 efflux does not increase (Micks et al., 2004; Schipper et al., 1996a) and in some cases decreases over time (Bowden et al., 2004). Soil CO_2 efflux is an important indicator of biological response, but its response to reclaimed water or chronic N amendment suggests that total biological response can be neutral or negative despite accumulations in soil organic matter.

1.5.2 Soil Exoenzyme Activity

Soil enzymes catalyze biogeochemical cycling of nutrients such as C, N and P (Alkorta et al., 2003). These enzymes mediate organic matter decomposition and nutrient cycling in soil and allow for breakdown of biological macromolecules present in litter and soil such as cellulose, hemicellulose, chitin and protein (Allison et al., 2007; DeForest, 2009a; Fog, 1988; Saiya-Cork et al., 2002a). Enzyme activities are reflective of soil microbial activity and substrate availability. Enzyme activities are essential for understanding microbial community responses to resource availability and nutrient turnover in forest soils. N mineralization is mediated by microbes by regulation of activities of cellulase (β-glucosidase), protease (aminopeptidase), and chitinase (β -N-acetyl-glucosaminidase) (Ekenler & Tabatabai, 2004; Tabatabai et al., 2010), while Phosphatase transforms P from unavailable organic form into PO_4^{3-} ions that are easily available for uptake for plants and microbes (Eivazi & Tabatabai, 1977), and can be an indicator of potential P mineralization and biological activity in soils (Dick & Tabatabai, 1993).

Soil extracellular enzymes or exoenzymes are the drivers of organic matter decomposition and nutrient cycling in forest ecosystems and provide important insight on microbial functions for acquisition of nutrients from organic substrates in soil (Bach et al., 2013; DeForest, 2009a; Fatemi et al., 2016; Marx et al., 2001). Exoenzyme activities are sensitive to environmental changes and can be easily measured, which makes them commonly assessed indicators of biological soil quality (Dick et al., 1997; Kotroczó et al., 2014; Yakovchenko et al., 1996). Both inorganic and organic uptake are important for plant N nutrition (Näsholm et al., 2009; Schimel & Bennett, 2004). Depolymerization and N mineralization are important drivers of transforming complex organic molecules to plant-available N (Shan & Coleman, 2020). Depolymerization involves degradation of complex organic polymers into amino compounds mediated by microbial extracellular enzyme activities. Mineralization follows as microbes break down DON, utilize C for energy requirements for growth and maintenance and excrete NH_4^+ as the immediate product (Chapin et al., 2012).

C and N releasing exoenzyme activities help maintain balance between C and N availability in soil and are correlated with P releasing phosphatase activities (Bowles et al., 2014; Fatemi et al., 2016; Shan et al., 2014; R. L. Sinsabaugh & Shah, 2012). This is particularly important in western forests limited by N (Edmonds et al., 1989; Shan et al., 2014). N addition has been shown to increase (Brzezinska et al., 2006; W. Chen et al., 2008; Dong et al., 2015; Filip et al., 2000) and decrease (Fatemi et al., 2016) exoenzyme activity or have both positive and negative effects (Allison & Vitousek, 2005; H. Chen et al., 2018; Jian et al., 2016). Reclaimed water application may change microbial dynamics, community composition and enhance biological activities due to added nutrients (W. Chen et al., 2008). Exoenzyme activity depends on microbial production in response to soil chemical and environmental factors (Bowles et al., 2014; R. G. Burns et al., 2013; R. L. Sinsabaugh et al., 2009).

Contrasting literature exists regarding variation in enzyme activity between soil or litter as a substrate. Exoenzyme activities in temperate deciduous forest are not affected by inputs of litter and woody debris while root removal significantly decreases exoenzyme activities (Kotroczó et al., 2014). Other studies indicate an increase in soil enzyme activities with litter addition due to substrate induction (Bandick & Dick, 1999; R. G. Burns et al., 2013; Debosz et al., 1999; Dornbush, 2007; Weintraub et al., 2013; Yao et al., 2009). The extent of upregulation in enzyme activities with litter addition is highly dependent on litter type (Tian & Shi, 2014). Ai et al. (2023) found that soil enzyme activities to litter and root removal were also found dependent on soil edaphic factors rather than climate, while others have reported both climatic and edaphic variables as controlling factors for soil enzyme activities (Zheng et al., 2018). Removing litter and living roots have been found to decrease exoenzyme activity, while litter addition significantly increased exoenzyme activities, indicating that soil C, N and P-releasing enzyme activities are controlled by plant carbon input and additive effect of litter particularly for C-degrading enzymes (Ai et al., 2023).

1.5.3 Amino Acids and Amino Sugar Pools

Assessment of product pools of amino compounds determines the importance of released compounds relative to their demand. Measuring nutrient demand allows us to understand forest productivity and soil quality as nutrient uptake and forest productivity are tightly linked (Aubrey et al., 2012). Soil amino acids organic N are integral for plant nutrition (Werdin-Pfisterer et al., 2009). Plants are able to take up free amino acids and do not have to completely rely on inorganic N for nutrient acquisition when mineralization is limited (Näsholm et al., 1998; Nordin et al., 2001; Schimel & Stuart Chapin, 1996; Werdin-Pfisterer et al., 2009). However, free amino acids are present in low concentrations in bulk soil likely due to rapid turnover by soil microbes (Jones, Shannon, et

al., 2005). An increase in amino acid uptake during plant and microbial growth cycles can cause decline in soil amino acid concentrations (Jones, Healey, et al., 2005). Seasonal changes may result in disintegration of soil organic matter followed by subsequent flushing of amino acid into soil due to microbial, mycorrhizal and root tissue lysis and physical disintegration of soil organic matter (Ivarson & Sowden, 1966; Lipson & Monson, 1998). N addition has also been shown to inhibit both aminopeptidase and chitinase activity (Allison & Vitousek, 2005; Hernández & Hobbie, 2010; Olander & Vitousek, 2000). Thus, study of amino compounds is necessary for a better understanding of the effects of reclaimed water application on N-cycling in forest ecosystems.

Many land application systems have been in operation for decades. Yet, soil nutrient cycling responses to long-term reclaimed water land application and effects of N enrichment on enzyme activities is poorly understood (H. Chen et al., 2018; Schnecker et al., 2015). The goal of this study is to characterize the soil biological responses to decades of resource amendments in comparison to adjacent non-amended stands. We are not aware of existing literature on the long-term impacts of land application of reclaimed water on soil CO_2 efflux, exoenzyme activities and available product pools. Evaluation of enzyme product pools provides a measure of N and P turnover which is indicative of N and P availability and improved soil quality and is thus an important resource for understanding biological processes.

1.6 Summary of Research

This dissertation investigates the effects of land application of reclaimed water on western coniferous forests in northern Idaho along a four-decade time series of five water reclamation facilities located near Lake Coeur d'Alene and Lake Pend Oreille. To examine the effectiveness of forest water reclamation, this dissertation contains three research manuscript chapters in addition to an introduction and conclusion chapter. Chapter two evaluates the tree growth and vegetation diversity responses at water reclamation facilities practicing forest land application with reclaimed water (Joshi & Coleman, 2023). Inherent productivity potential and shifts in vegetation diversity provide an assessment of vegetation responses to reclaimed water. Chapter three investigates soil water nutrient concentrations and leaching potential. Nutrients can be lost via leaching during forest water reclamation, particularly through preferential flow paths. Nutrient leaching losses are explored in response to long-term reclaimed water amendment. Chapter four examines the biological responses of soil CO_2 efflux, exoenzyme activities and amino acid and amino sugar pools to reclaimed water. The information found in the following chapters can be useful for forest water reclamation facility managers and regulators to understand forest assimilation capacity to current permitted loading rates, demonstrated by tree growth and vegetation diversity assessment. They can know about the

thresholds of hydraulic and nutrient loading limits to prevent nutrient saturation, deep drainage and leaching and long-term effects of reclaimed water application on soil quality. The facility managers and regulators can ultimately protect environmental quality surrounding their communities by implementing sound management practices that involve appropriate loading rates and rigorous monitoring of nutrients to prevent leaching losses.

Chapter 2: Tree Growth and Vegetation Diversity in Northern Idaho Forest Water Reclamation Facilities

2.1 Abstract

Forest water reclamation can improve tree growth and renovate municipal wastewater. Although there are indications that long-term application may exceed forest assimilation capacity, there is limited information on the long-term effects of reclaimed water application on coniferous ecosystems. The purpose of our study was to assess the impacts of prolonged reclaimed water application on forest growth responses and vegetation diversity. We examined the effects of reclaimed water at five water reuse facilities established between 1978 and 2013 in a four-decade time series. We collected tree cores and stem measurements to determine current and retrospective increments. We assessed plant diversity with vegetation surveys. The greatest diameter response observed for reclaimed water amendment compared to controls was 166.1% for western redcedar, while Douglas-fir increased up to 116.4% and ponderosa pine increased up to 100.6%. The minimum response observed was 30.3%. Current annual increments showed that the basal area and volume were significantly greater at long-established facilities for reclaimed-water-amended plots. The understory vegetation diversity declined with application time, while overstory vegetation diversity increased with application time. We conclude that reclaimed water can be a valuable re-source to improve forest productivity, but continued application without stocking control may have detrimental effects on forest growth and vegetation diversity.

Keywords: reclaimed water amendment; time series; forest productivity; vegetation diversity

2.2 Introduction

Reclaimed water is a byproduct of human society that can serve as a reliable re-source to enhance forest growth and production. Coniferous forest growth in the western United States is limited by water and nutrient resources (Al-Jamal et al., 2002; Cole & Gessel, 1992). Regionally, summer drought imposes significant restrictions in water availability, which can be alleviated through supplemental irrigation (Gessel et al., 1990b). Nutrients such as nitrogen (N) and phosphorus (P) also substantially limit the productivity of coniferous forests (Cole & Rapp, 1981; Johnson & Turner, 2014; Lang et al., 2016). Amendments to overcome forest nutrient limitations typically involve one-time, high-dose fertilizer applications (Chappell et al., 1991; Fox et al., 2006), yet regular low doses of supplemental nutrients that match nutrient demand can improve growth by two- or three-fold (Coyle et al., 2016; Iivonen et al., 2006; Ingestad, 1987; Weetman et al., 1997). Moreover, regular

low dose amendments supplied during the growing season can significantly increase nutrient retention and restrict ecosystem losses (Van Miegroet & Cole, 1984). Providing supplemental water and nutrient resources through land application of reclaimed water offers the opportunity to overcome forest resource limitations, improve the inherent productivity potential of regional forests and renovate wastewater to return it safely to the environment.

Forest water reclamation (FWR) using land application systems is a well-established, environmentally sound, and cost-effective approach for managing and renovating wastewater globally (Andrews et al., 2016; Asano, 1987; Hamilton et al., 2007). Land application systems are prominent in smaller communities where the construction of tertiary wastewater treatment plants is not financially and technologically feasible. In such communities, low-cost lagoons are utilized (Al-Jamal et al., 2002), and tertiary treatment is achieved using established native vegetation and soil that act as natural filters to trap and assimilate the applied nutrients and contaminants (Mexal et al., 2002). FWR has been widely accepted as an effective disposal method with environmental benefits (Duan, Sheppard, et al., 2010; Jordan et al., 1997b). It helps to reduce nutrient loads, particularly in areas where surface waters are sensitive to nutrient additions (Barton et al., 2005a; Tomer et al., 2000). Many municipal water reuse facilities in the U.S. annually apply reclaimed water at forested facilities during dry summer months. During winter, these facilities either reserve wastewater in lagoons for summer land application or dispose of it into surface waters when permitted. Diversion to land application is often preferred due to strict water quality standards and discharge regulations designed to protect surface water resources. The alternative includes various artificial nutrient removal processes that can be rigorous and costly.

Reclaimed water containing constituent nutrients is effective in stimulating tree growth (Marron, 2015; Mexal et al., 2002), which also provides economic opportunities (Mexal et al., 2002). Various deciduous and coniferous forest ecosystems have shown an increased tree growth response to reclaimed water application (Gessel et al., 1990b; Hesse et al., 1998b; Jordan et al., 1997b; Moffat et al., 2001; Weetman et al., 1993). Timber harvesting is one of the predominant economic activities in the Pacific Northwest region (Affleck, 2019). Forest water reclamation provides an opportunity to analyze the maximum growth potential of western coniferous forests by alleviating growth limitations and to explore the upper limits of forest productivity for high-value timber species in the Inland Northwest.

While forests are considered to be benign repositories for nutrient storage, potential environmental risks of land application have also been identified. Detrimental impacts include a decline in forest productivity, tree mortality, altered community structure, nutrient leaching, and detrimental effects on soil physical, chemical and biological health (Aiello et al., 2007; Duan et al.,

2011; Magesan et al., 2000; Oswald et al., 2009; Thomas et al., 1999; Toze, 2006; Wallach et al., 2005). Reclaimed water application can also dramatically change under-story species composition with weed invasion as a potential indicator of nitrogen saturation (Jordan et al., 1997b). Irrigation with reclaimed water can lead to a highly productive herb–shrub layer understory that exists in more mesic conditions. However, plant diversity has been found to significantly decline with opportunistic species dominating the perennial herb–shrub layer vegetation and simplifying the community (Hunt & Shure, 1980). In addition, abundant water and nutrients during the growing season may lead to denser overstory canopies, which intercept nutrient and light resources and result in an overall decline in understory species diversity (Alvarez-Clare et al., 2013; Ibáñez et al., 2016; Schroth et al., 2015), which plays an important role in the structure and function of forest ecosystems (P. J. Burton et al., 1992; DeWald & Mahalovich, 1997; C. D. [1. Oliver & Lippke, 1993; J. Zhang et al., 2016).

Scores of water reuse facilities have been practicing forest water reclamation for decades in northern Idaho. Yet, there have been no attempts to understand the facility-specific long-term effects of reclaimed water amendment on tree growth and vegetation diversity. Most of the existing studies on the effects of land application of re-claimed water are decades old and short-term. These require continued investigation to understand the long-term implications for forest ecosystems and environmental quality. Our forest water reclamation study presents a unique opportunity to assess forest responses using a time series of regional water reuse facilities with the longest operation time being over 40 years. We were able to assess the benefits and implications of forest water reclamation on regional conifer forest growth and diversity at decadal time-scales using tools in dendrochronology that allow retrospective assessment of ecosystem dynamics with tree-ring records of site conditions over an extended time and that can be valuable in forest management (Arenas-Castro et al., 2015; Shikangalah et al., 2020). We also used vegetation diversity surveys to assess changes in species composition in response to wastewater irrigation (Jordan et al., 1997b), providing insight into long-term community-level changes in the forest eco-system.

Our main objectives were to assess facility-specific growth responses using dendrochronology (Arenas-Castro et al., 2015; McCarroll & Loader, 2004; Shikangalah et al., 2020) and to investigate vegetation diversity responses to long-term reclaimed water application. Our secondary objectives were to compare the impacts of various lengths of treatment with permitted loading rates at five different forest water reclamation facilities with varying dates of establishment. We hypothesized that regular low doses of growing-season nutrient and water amendment will result in enhanced tree growth. We also hypothesized that vegetation diversity will decline with increasing length of application.

2.3 Materials and Methods

2.3.1 Study Facilities

The study was conducted at five water reuse facilities situated along Lake Coeur d'Alene and Lake Pend Oreille in northern Idaho, United States (Figure 2.1). All facilities were established between 1978 and 2013 to create a four-decade time series (Table 2.1). To determine reclaimed water treatment effects on forest growth, five one-tenth-acre measurement plots were established in management units at each of the five study facilities along with five adjacent control plots (n=50, Figure A1 and Figure A2). Where possible, the control plots selected had comparable soil, stand composition and structure as the treatment plots. The treatment and control plots were established at locations with no more than a 5% slope.

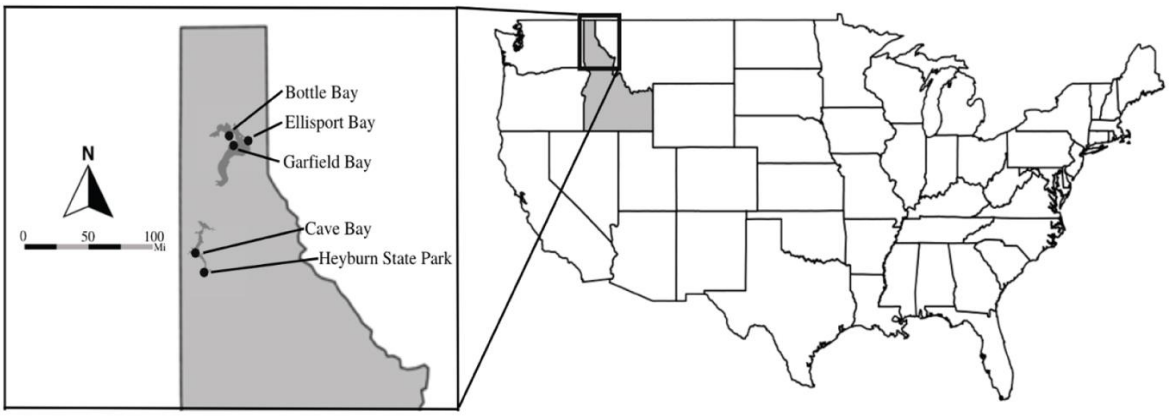

Figure 2.1. Study area: water reclamation facilities in northern Idaho, USA.

Table 2.1. Study facility information, including location, elevation, establishment date, mean annual precipitation, mean annual temperature, average maximum temperature, and average minimum temperature.

Reuse Facility	Coordinates	Elevation (m)	Estd date	MAP[1] (mm)	MAT[1] (°C)	T_{max}[1] (°C)	T_{min}[1] (°C)
Cave Bay	47.4703° N 116.8803° W	711	2013	534.4	8.4	19.6	−5.1
Heyburn State Park	47.3462° N 116.7821° W	769	2010	662.6	8.2	19.4	−5.2
Ellisport Bay	48.2159° N 116.2696° W	659	2000	633.1	7.7	18.8	−5.6
Bottle Bay	48.2018° N 116.4207° W	696	1989	752.4	7.4	27.4	−5.7
Garfield Bay	48.2287° N 116.4384° W	707	1978	708.9	7.4	27.4	−5.8

[1]30-year average data from (PRISM Climate Group). MAP, mean annual precipitation; MAT, mean annual temperature; T_{max}, average maximum temperature; T_{min}, average minimum temperature.

Soils varied between facilities (Table 2.1). Soils at each of the Pend Oreille facilities (Garfield Bay, Bottle Bay and Ellisport Bay) contained the same soil series, which is characterized by parent material of volcanic ash and/or loess over till derived from granite and/or metamorphic rock. The soils are well drained, and the ecological sites are ashy over loamy, glassy over mixed, superactive, frigid Alfic Udivitrands (Soil Survey Staff, 2019). Heyburn State Park soils are moderately well drained, and the ecological site is warm frigid, xeric, unglaciated, loamy and fragipans. Soils at Heyburn facility are fine-silty, mixed, superactive, frigid Vitrandic Fragixeralfs (Soil Survey Staff, 2019). Cave Bay soils are well drained, and the ecological site is warm mesic, xeric, and unglaciated (Soil Survey Staff, 2019).

Table 2.2. Initial soil properties for the top 15 cm of mineral soil depth and facility overstory and understory vegetation composition.

Facility	Soil type	Texture	pH	Bulk Density (g m^{-3})	Dominant tree species[1]	Dominant understory vegetation species[2]
Cave Bay	Lacy	Gravelly loam	6.9 ± 0.18	0.8 ± 0.04	*P. menziesii, P. ponderosa*	*P. malvaceus, H. discolor, S. albus*
Heyburn SP	Carlinton	Silt loam	6.5 ± 0.18	0.99 ± 0.05	*P. ponderosa, P. menziesii*	*P. malvaceus, H. discolor, S. albus*
Ellisport Bay	Pend Oreille	Silt loam	6.4 ± 0.13	0.85 ± 0.04	*T. plicata, T. heterophylla, A. grandis*	*P. munitum, C. alpina, B. aquifolium*
Bottle Bay	Pend Oreille	Silt loam	6.2 ± 0.15	0.68 ± 0.04	*T. plicata, P. menziesii, A. grandis*	*P. malvaceus, S. albus, H. discolor, B. aquifolium*
Garfield Bay	Pend Oreille	Silt loam	6.7 ± 0.17	0.71 ± 0.04	*T. plicata, P. menziesii, A. grandis*	*P. malvaceus, S. albus, H. discolor, B. aquifolium*

Note: [1]P. ponderosa—ponderosa pine (Pinus ponderosa Douglas ex C. Lawson); P. menziesii—Douglas-fir (Pseudotsuga menziesii var. glauca (Mirb.) Franco); A. grandis—grand fir (Abies grandis (Douglas ex D. Don) Lindl.); T. plicata—western redcedar (Thuja plicata Donn ex D. Don); T. heterophylla—western hemlock (Tsuga heterophylla (Raf.) Sarg.).

[2]P. malvaceus—ninebark (Physocarpus malvaceus (Greene) Kuntze); H. discolor—ocean spray (Holodiscus discolor (Pursh) Maxim); S. albus—common snowberry (Symphoricarpus albus (L.) Blake); P. munitum—Western Swordfern (Polystichum munitum (Kaulf.) Presl, Tent.); C. alpina—alpine enchanter's nightshade (circaea alpina L. ssp. pacifica (Asch. & Magnus) P.H. Raven; B. aquifolium—creeping Oregon grape (Berberis aquifolium Pursh. Beaq).

The forest water reclamation facilities apply aerated and disinfected reclaimed water from lagoons on forested facility management units using slow rate land treatment that utilizes sprinklers for uniform distribution of reclaimed water. Reclaimed water containing constituent N and P was applied at the discretion of facility managers ranging from daily to weekly frequencies during the growing season. Average hydraulic loading among the facilities was 30 cm yr^{-1}, while average constituent nutrients were applied at 37 kg N ha^{-1} yr^{-1} and 14 kg P ha^{-1} yr^{-1}. Since each facility was

established on different dates cumulative loads in 2019 ranged from 168 kg N ha^{-1} and 79 kg P ha^{-1} at Cave Bay (est. 2013) to 1752 kg N ha^{-1} and 563 kg P ha^{-1} at Garfield Bay (est. 1978).

2.3.2. Stand Parameters

Individual tree diameter at breast height (DBH) was measured for each tree over 2.5 cm (1 in) diameter in each of the 0.04 ha ($1/10^{th}$ acre) study plots (*n=10* at each facility). Tree DBH measurements were collected in Fall 2019 and 2021. Initial 2019 DBH was used to calculate stand-level estimates of various forestry parameters. Plot basal area (BA) was calculated using Equation (1):

$$\mathrm{BA} = \sum_{i=1}^{t} 0.00007854 * \left(\mathrm{DBH}_i^2\right) \qquad (1)$$

where BA is the basal area per hectare, t is the number of trees in the plot and 0.00007854 is a forester's constant for metric units (Caron et al., 2021). Quadratic mean diameter (QMD)in centimeters was calculated using Equation (2):

$$\mathrm{QMD} = \sqrt{\left(\frac{\frac{\mathrm{BA}}{\mathrm{TPH}}}{0.00007854}\right)} \qquad (2)$$

where TPH is the number of trees per hectare (VanderSchaaf & Burkhart, 2007) and Stand Density Index (SDI) was computed using Equation (3) (Woodall et al., 2005):

$$\mathrm{SDI} = \mathrm{TPH} * \left(\frac{\mathrm{QMD}}{25.4}\right)^{1.605} \qquad (3)$$

Tree heights were measured in Fall 2019 where feasible. We predicted within- and between-year heights for unmeasured trees using height–DBH regression models from measured trees (Hulshof et al., 2015; Wykoff et al., 1982) for the main purpose of calculating volume. Tree volumes were estimated as the product of DBH squared, height and species-specific taper coefficients (Wykoff et al., 1982). Plot level diameter increment, basal area increment, and volume increment between 2019 and 2021 were determined and expressed as annual increments.

2.3.3 Tree-ring Width Data and Series Chronologies

Tree cores were collected in Fall 2019. All tree species occurring within each circular plot were cored at breast height using a 4.3 mm increment borer. Cores from two trees per species were collected from each DBH class present in every plot. The cores were air-dried, mounted, sanded, polished, and scanned at 2400 dpi resolution. The scanned images were examined using *WinDENDRO* software package (Regent Instruments Inc., Quebec, Canada). *WinDENDRO* is a semi-automatic image analysis program that requires manual adjustment of ring boundaries to account for growth anomalies (Maxwell et al., 2011). To ensure that all the rings were accurately detected, each ring width was analyzed. Where necessary, missing rings were added, and false rings were removed

manually. Accurate ring width chronologies were developed using crossdating, which involved matching ring width patterns across trees at each facility.

Statistical accuracy of crossdating was checked using the COFECHA program, which created a master chronology of tree rings and calculated correlation coefficients to indicate how well the interannual variability in the ring widths for any one core correlated with the other ring widths within the series (Arenas-Castro et al., 2015; Bogino et al., 2009; Maxwell et al., 2011; Roversi et al., 1975; Shikangalah et al., 2020, 2021). Bark thickness was measured for all of the cored trees and estimated for unmeasured trees using a species-specific, DBH-based regression model computed for measured trees (Yang & Radtke, 2022). Diameter inside bark (DIB) was determined by subtracting the bark thickness. DIBs for the cored trees were calculated every five years from ring width analysis. Differences between consecutive 5-year DIB measurements were used to determine diameter increments. Plot level mean diameter increment (DI) and basal area increment (BAI) were calculated for each facility. We did not determine height or volume increments from tree-ring data due to unknown stocking and the uncertainty of predicting retrospective heights from measured height–DBH regression models.

2.3.4. Understory Vegetation Survey and Biomass

2.3.4.1. Understory Vegetation Survey

The understory shrub and herb layer species were assessed in early summer 2020 to correspond with peak flowering. Four one-meter-square sampling quadrats were established by randomly locating quadrats at either 2 m or 5 m distance from the plot center along four transects in each cardinal direction (N, S, E, W). Within each sampling quadrat, shrub and herb layer composition were determined by identifying and counting individual plants to species level. Both shrub and herb layer species composition were documented with floristic voucher specimens of herb layer species archived at Stillinger Herbarium, University of Idaho, Moscow, ID. Based on the abundance of understory species including shrubs and forbs, species richness, Shannon–Wiener diversity index and Pielou's evenness were calculated at the plot level (G. G. Wang & Kemball, 2005). Species richness (S) was estimated using Equation (4):

$$S = \Sigma n \qquad (4)$$

where n is the total number of species documented across four sampling quadrats in each plot. Shannon–Wiener diversity index (H) was estimated using Equation (5):

$$H = \sum_{i=1}^{S} p_i \ln p_i \quad (5)$$

where ln p_i is the natural logarithm of the *i*-th species proportion. Pielou's evenness (J) was estimated using Equation (6):

$$J = \frac{H}{\ln S} \quad (6)$$

2.3.4.2. Understory Vegetation Biomass

The herbaceous vegetation in three one-meter-square sampling quadrats was collected in every study plot. Stem diameters for shrubs and seedlings (DBH < 2.5 cm) within the quadrats were measured using a caliper and representative samples of the measured shrubs and seedlings (*n*=8–12) were collected. Allometric equations of dry mass (DM) and caliper measurements were developed to determine dry mass from caliper measurements for samples that were not destructively harvested. Mass from harvested samples and estimated shrub DM were summed for each quadrat and plot-level averages were calculated.

2.4. Statistical Analysis

Analysis of variance was performed on initial stand parameters as dependent response variables in a two-way factorial model that included treatment and facility as class variables. Dependent variables included plot-level mean diameter increment, basal area increment, total volume increment, understory vegetation biomass, Shannon–Wiener diversity index, richness, and evenness. Analysis of covariance was performed on growth increments as dependent variables with facility, treatment and species included as independent class variables. Initial values for each increment were included as covariates in their respective models. If normality and homoscedasticity assumptions for analysis of variance were not met, data were transformed (Box & Cox, 1964) prior to analysis. Differences were considered significant at $\alpha = 0.05$. Tukey's pairwise comparisons were performed to compare least square means between the control and treatment at the five reuse facilities. Statistical analyses were conducted in R version 4.2.1 (Core, 2021).

2.5. Results

2.5.1. Initial Stand Parameters

Initial stand metrics following establishment indicate some structural differences among facilities and between effluent stands in FWR management units and adjacent control stands at each FWR facility. Facilities explained more of the variation in stand variables than treatment differences did (Table 2.3, *F*-statistic for F > *F*-statistic for T). Although, effluent stands in comparison to

controls had greater quadratic mean diameter (QMD), basal area (BA), mean height and total stand volume (Table 2.3, T, $P \leq 0.05$; Table 2.4; Figure 2.2), the stand density index (SDI) was equal (T, $P = 0.26$), and trees per hectare (TPH) were lower (T, $P < 0.01$). These results indicate that tree size was consistently greater in effluent plots, but measures of density were ambiguous. Additionally, the response of TPH to treatment depended upon the facility (Table 2.3, T × F, $P < 0.01$; Figure 2.2a). Due to the in-growth of many saplings in control plots, TPH at establishment was 54% higher in Bottle Bay control plots than in effluent plots. TPH in Cave Bay effluent plots were equal to the controls. When all locations were combined, there was a greater number of small diameter trees in control plots, but there was a lower number of large diameter trees in controls (Figure 2.2 a & b).

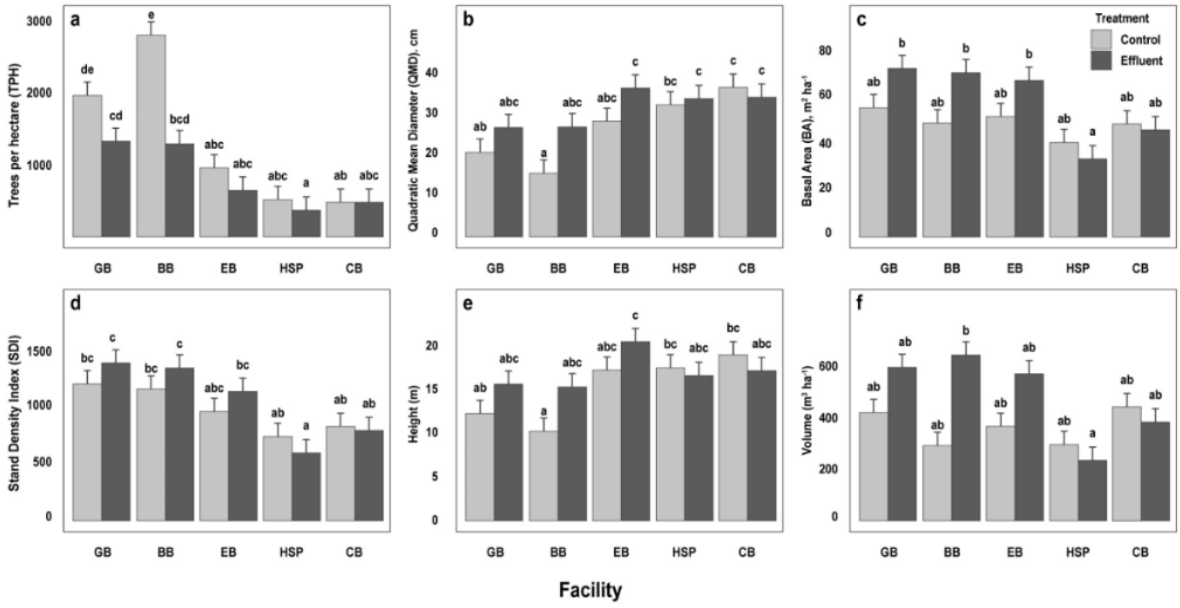

Figure 2.2. Stand parameter plot means and standard errors ($n = 5$) at the water reuse facilities; (a) trees per hectare (TPH); (b) quadratic mean diameter (QMD); (c) basal area(BA); (d) stand density index (SDI); and (e) height; and (f) total volume. Five facilities included: Garfield Bay (GB), Bottle Bay (BB), Ellisport Bay (EB), Heyburn State Park (HSP) and Cave Bay (CB). Same letters over bars indicate no differences between treatment levels at $\alpha = 0.10$. Facilities are arranged in establishment order with GB being first (1978) and CB last (2013).

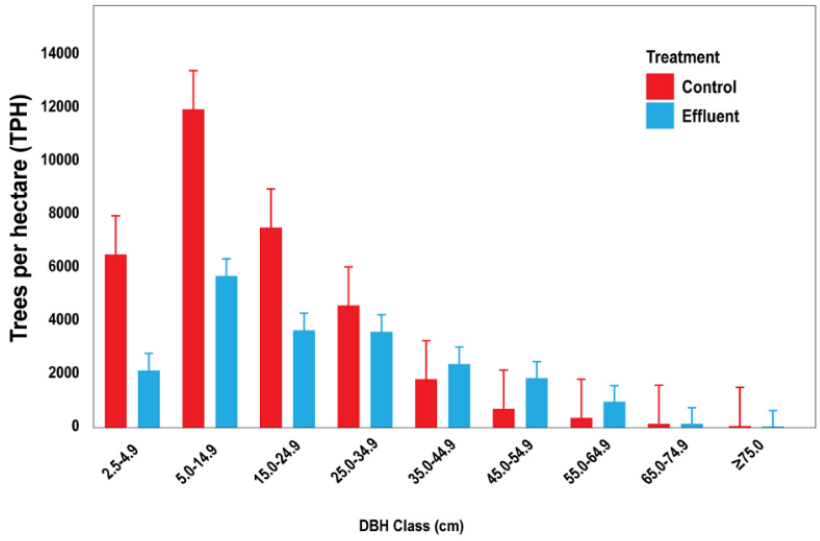

Figure 2.3. Size class distribution of control and effluent treatment plots at the five reuse facilities.

Table 2.3. Analysis of variance results for stand parameters at establishment (2019). Results include *F*-statistic (*F*) and *P*-values (*P*) of the measured effects of reclaimed water treatment (T) and facility (F) for stand parameters: TPH (Trees per hectare); QMD (Quadratic Mean Diameter); BA (Basal Area); SDI (Stand Density Index), Height and Volume. Boldface indicates significance at $P \leq 0.05$.

Effect	TPH		QMD (cm)		BA ($m^2\,ha^{-1}$)		SDI		Height (m)		Volume ($m^3\,ha^{-1}$)	
	F	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***
T	21.0	**<0.01**	8.3	**<0.01**	4.1	**0.05**	1.3	0.26	3.5	**<0.01**	5.3	**0.03**
F	32.5	**<0.01**	10.5	**<0.01**	5.2	**<0.01**	14.8	**<0.01**	6.8	0.06	2.6	**0.05**
TxF	5.7	**<0.01**	2.0	0.11	1.7	0.17	1.2	0.35	1.9	0.12	2.3	0.08

Table 2.4. Descriptive statistics (LS mean ± SE) of stand parameters [TPH (Trees per hectare); QMD (Quadratic Mean Diameter); BA (Basal Area); SDI (Stand Density Index); Height and Volume] by treatment at the five water reuse facilities.

Treatment	TPH		QMD		BA		SDI		Height		Volume	
Control	1335±160	a	26.4±1.7	a	48.3±3.67	a	968±68.2	a	15.3±0.84	a	368±41.2	a
Effluent	817±160	b	31.4±1.7	b	57.1±3.67	a	1044±68.2	a	17.1±0.84	a	488±41.2	b

Note: Same letters with each measurement indicate no differences between treatment levels at $\alpha = 0.10$.

2.5.2. Estimated Historic Diameter Increment Responses from Tree Cores (DI_c)

2.5.2.1. DI_c Responses Across Facilities

Cored tree diameter increment (DI_c) for increment periods 2014–2018, 2009–2014 and 2004–2009 varied by tree diameter, treatment, species, and facility (Table 2.5). The effect of variable diameter among trees, treatments and facilities was effectively accounted for in the model by including initial diameter (D_0) in the model (Table 2.5, $F > 83$, $P < 0.01$). Historic diameter

increments for the effluent plots were notably higher compared to the control plots (Figure 2.4) for each of the increment periods at all facilities except at Garfield Bay. DI_c at the Bottle Bay effluent plot for both the 2014–2018 and 2009–2014 increment periods were, respectively, 70.6% and 89.9% greater than the control (Figures 4a, b). Similarly, DI_c at Ellisport Bay was approximately 45.8% greater in effluent plots for the 2014–2018 increment period and 108.3% greater for 2009–2014. A similar increase was observed for recently established facilities in 2014–2018 with a 99.4% greater response in effluent plots at Heyburn State Park and a 108.8% greater response at Cave Bay. There was a 63.1% increase in diameter increment at effluent plots in Heyburn State Park in 2009–2014. In 2004–2009, greatest differences in diameter increment were observed at Ellisport Bay with a 382.6% increase at effluent plots compared to the controls (Figure 2.4c). The diameter increment was 65.9% greater at the Bottle Bay effluent plots. In contrast, there was a 3% decrease in diameter increment at the Garfield Bay effluent plots compared to the controls.

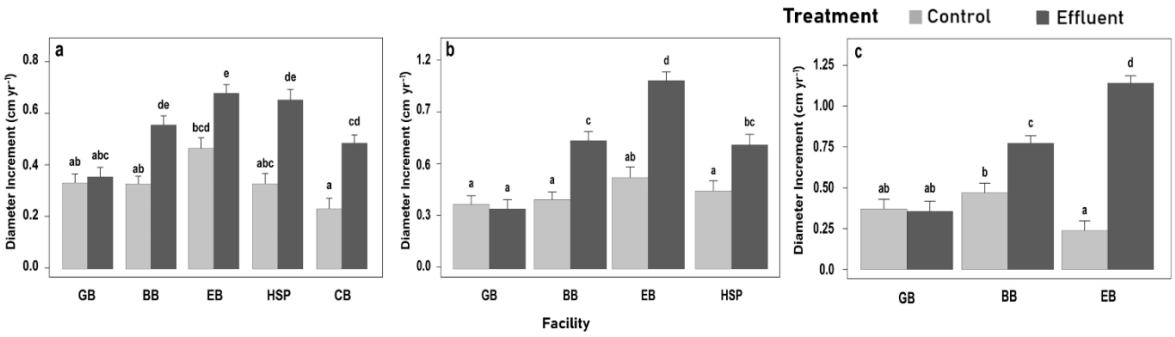

Figure 2.4. Mean and standard error (n = 5) of five-year Diameter Increment during facility operation for (a) diameter increment period 2014–2018; (b) diameter increment period 2009–2014 and (c) diameter increment period 2004–2009 for all species combined. Same letters over bars within each panel indicate no differences between treatment levels at $\alpha = 0.10$. Facility abbreviations are described in Figure 2.2. Facilities are arranged in establishment order with GB being first (1978) and CB being last (2013). Since treatment had not begun at some facilities, the number of facilities decreases for earlier increments.

Table 2.5. Analysis of covariance results for diameter increment. Results include *F*-statistic (*F*) and *P*-values (*P*) for the first three five-year increment periods (2004-2009, 2009-2014, and 2014-2018) at the plot level. Initial diameters D_0 (diameters in 2004, 2009 and 2014) were used as covariates. Boldface indicates significance at $P \leq 0.05$.

Effect	**DI_c (cm)**					
	2014–2018		**2009–2014**		**2004–2009**	
	F	***P***	***F***	***P***	***F***	***P***
T	53.33	**<0.01**	50.24	**<0.01**	58.94	**<0.01**
F	15.41	**<0.01**	33.68	**<0.01**	25.98	**<0.01**
Sp	2.05	**0.03**	2.16	**0.02**	2.49	**<0.01**
TxF	2.88	**0.02**	9.49	**<0.01**	32.77	**<0.01**
D_0	97.55	**<0.01**	84.99	**<0.01**	83.34	**<0.01**

2.5.2.2. Species-specific DI_c Responses

DI_c responses to reclaimed water depended upon the species, facility, and treatment (Table 2.5). Although tree growth declined over time, the diameter increments at Ellisport Bay and Bottle Bay were significantly enhanced with the onset of reclaimed water application (Figure 2.5 c & d). The diameter increments for effluent plots for western redcedar were 41.6% at Garfield Bay, 86.0% at Bottle Bay and 59.8% at Ellisport Bay of that in control plots (Figure 2.6a). The treatment difference in diameter increment for western redcedar was not statistically significant at Garfield Bay. The diameter increments for Douglas-fir at Bottle Bay, Heyburn State Park and Cave Bay were significantly greater for effluent plots compared to the unamended control plots (Figure 2.6b). Bottle Bay had a 69.9%, Heyburn State Park had a 113.6% and Cave Bay had a 116.4% greater diameter response to reclaimed water application compared to controls. The 30.3% difference at Garfield Bay was not statistically significant. The diameter increment responses were significantly greater in reclaimed-water-amended ponderosa pine stands where they increased by 100.6% at Heyburn State Park and by 50.8% at Cave Bay compared to control plots (Figure 2.6c).

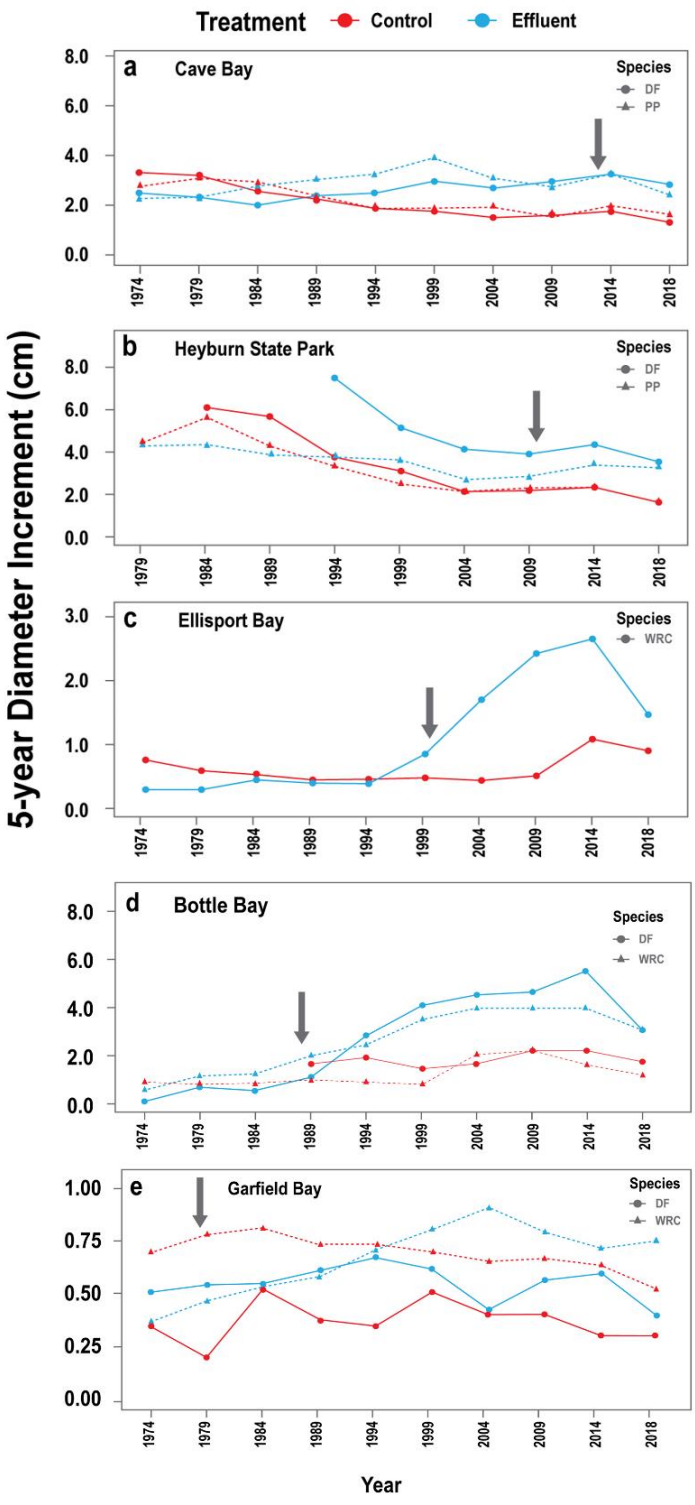

Figure 2.5. Average five-year diameter increments for (a) DF (Douglas-fir)and PP (ponderosa pine) at Cave Bay; (b) PP (ponderosa pine) and DF (Douglas-fir) at Heyburn State Park; (c) WRC (western redcedar) at Ellisport Bay; (d) DF (Douglas-fir) and WRC (western redcedar) at Bottle Bay; and (e) DF (Douglas-fir) and WRC (western redcedar) at Garfield Bay. Arrows indicate the start of reclaimed water application.

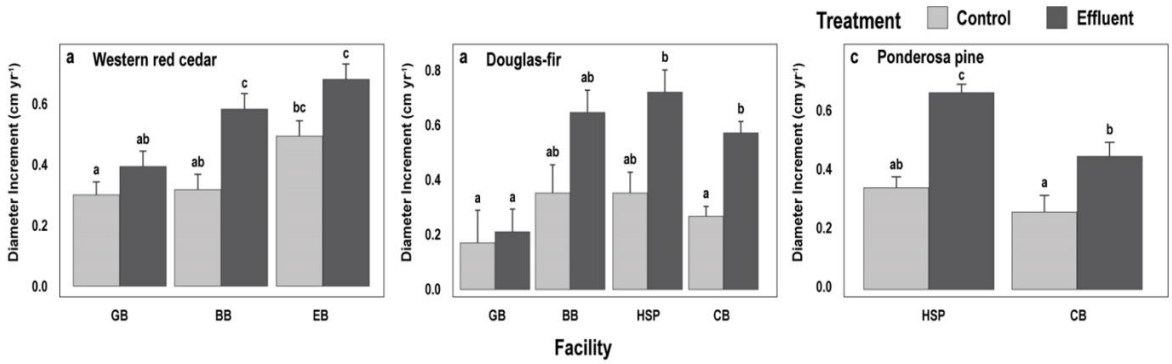

Figure 2.6. Species responses to treatment variations of 2014–2018 mean diameter increment for facilities where those species occurred: (a) western redcedar; (b) Douglas-fir; and (c) ponderosa pine. No species occurred at all facilities. Same letters over bars indicate no differences between treatment levels at $\alpha = 0.10$. Facility abbreviations are described in Figure 2.2. Facilities are arranged in establishment order with GB being first (1978) and CB last (2013).

2.5.3. Growth Increments during Study Period

The growth increment determined from the DBH, and height measurements collected during the study period (2019–2021) varied by initial measurements, treatment, and facility (Figure 2.7). As with tree core increments, diameter increment (DI) was highly dependent upon the initial diameter (Table 2.6, D_0, $F = 230$). Including initial values in the model accounted for the effect of diameter when testing for treatment and facility effects. The DIs for the reclaimed-water-amended plots were consistently higher compared to the unamended controls (Figure 2.7a). The differences were significantly greater at Heyburn State Park, where effluent plots had an approximately 118.4% greater diameter growth than control plots, while DI treatment responses at other facilities were much lower (Table 2.6, TxF, $P < 0.01$).

Basal area increment and volume increment varied by both treatment and facility (Figures 7b and c). Basal area increment and volume increment were consistently higher in reclaimed-water-amended plots compared to the controls (Table 2.6, T, $P < 0.01$) and neither depended on facility (Table 2.6, TxF, $P > 0.71$). Basal area increments for effluent compared to control plots were 85.2% greater at Garfield Bay and 93.6% greater at Ellisport Bay. Similarly, volume increments in effluent plots compared with control plots were 142.9% greater at Garfield Bay, 77.7% at Bottle Bay and 147.4% at Ellisport Bay.

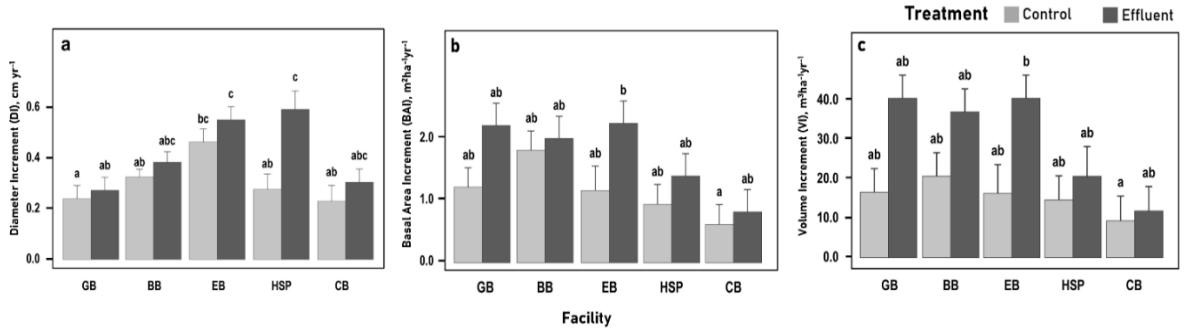

Figure 2.7. Plot means and standard error (n = 5) for 2019-2021annual increments for (a) diameter increment; (b) basal area increment; and (c) volume increment. Same letters over bars indicate no differences between treatment levels at α = 0.10. Facility abbreviations are as described in Figure 2.2. Facilities are arranged in establishment order with GB being first (1978) and CB last (2013).

Table 2.6. Growth increment analysis of covariance results. Plot mean diameter increment (DI), mean annual basal area increment (BAI) and mean volume increment (VI) for 2019–2021. Results include *F*-statistic (*F*) and *P*-values (*P*). Covariates for DI, BAI and VI were initial diameter (D_0), basal area (BA_0), and volume (V_0) measurements in 2019. Boldface indicates significance at $P \leq 0.05$.

Effect	DI (cm yr^{-1})		BAI ($m^2ha^{-1}yr^{-1}$)		VI ($m^3\,ha^{-1}\,yr^{-1}$)	
	F	***P***	***F***	***P***	***F***	***P***
T	9.11	**<0.01**	8.50	**<0.01**	11.06	**<0.01**
F	30.23	**<0.01**	7.35	**<0.01**	5.44	**<0.01**
TxF	10.48	**<0.01**	0.54	0.71	0.47	0.76
Covariate	230.23	**<0.01**	1.17	0.29	4.34	**0.04**

2.5.4. Understory Vegetation Biomass and Diversity Responses

The understory vegetation biomass, Shannon–Wiener diversity index and richness for understory species varied by facility (Table 2.7, Figures 2.8 and 2.9). The understory vegetation biomass was significantly greater for the effluent plots at Heyburn State Park and Cave Bay (Figure 2.8). The Shannon–Wiener index was significantly affected by treatment at only one facility (Table 2.7, TxF, P = 0.05). Evenness was marginally affected by treatment (TxF, P< 0.09). Typically, vegetation diversity, richness and evenness in the reclaimed-water-amended plots were not different from the control. The exception to this was at the Ellisport Bay facility, where in effluent plots, the Shannon–Wiener diversity index was 65% of the controls and evenness was 57.7% of the controls (Figure 2.9). The understory vegetation biomass at the recently established facilities Heyburn State Park and Cave Bay were also orders of magnitude greater than the long-established facilities (Table 2.7, Figure 2.8). The date of facility establishment affected both the Shannon–Wiener diversity index and richness. They were greater at the recently established facilities (Figures 2.9 a and b) and the facility establishment response was consistent for both treatments (Table 2.7, TxE, $P \geq 0.44$).

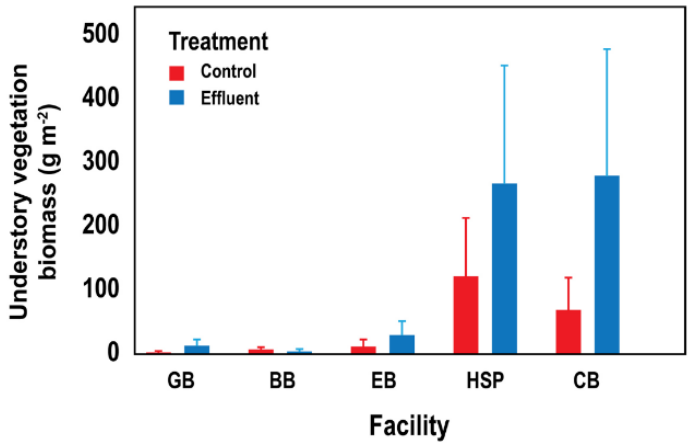

Figure 2.8. Comparison of understory vegetation biomass at the five reuse facilities. Facility abbreviations are as described in Figure 2.2. Facilities are arranged in establishment order with GB being first (1978) and CB last (2013).

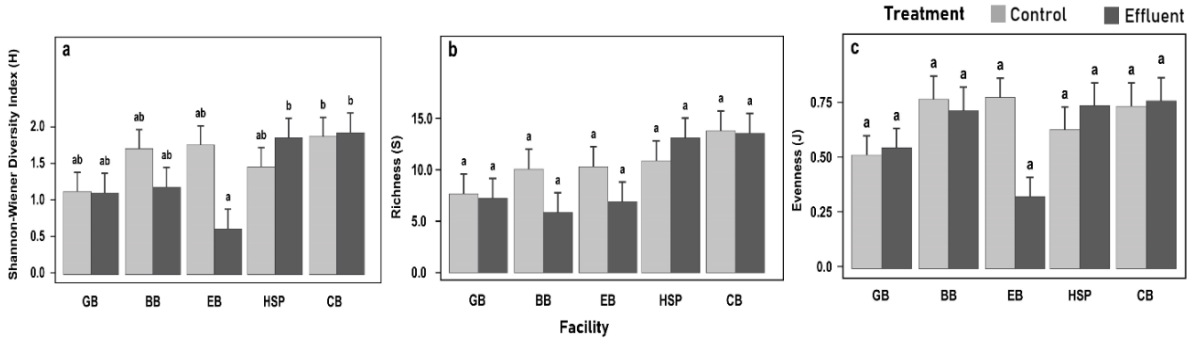

Figure 2.9. Plot mean and standard error (*n*=5) for understory (**a**) Shannon–Wiener diversity index; (**b**) richness; and (**c**) evenness. Same letters over bars indicate no differences between treatment levels at α = 0.10. Facility abbreviations are as described in Figure 2.2. Facilities are arranged in establishment order with GB being first (1978) and CB last (2013).

Table 2.7. Analysis of variance (ANOVA) and analysis of covariance (ANCOVA) results for understory Shannon–Wiener diversity index (H), richness (S) and evenness (J). Results include *F*-statistic (*F*) and *P*-values (*P*). ANOVA assessed effects of reclaimed water treatment (T) and facility (F) for the diversity parameters. The ANCOVA assessed the effects of establishment date (E) as a covariate. Boldface indicates significance at $P \leq 0.05$.

Test	Effect	Biomass (g m^{-2})		Shannon–Wiener diversity index (H)		Richness (S)		Evenness (J)	
		F	***P***	***F***	***P***	***F***	***P***	***F***	***P***
ANOVA	**T**	5.3	**0.03**	2.17	0.15	0.94	0.34	0.94	0.34
	F	20.69	**<0.01**	3.08	**0.03**	3.80	**<0.01**	1.94	0.12
	TxF	1.67	0.17	2.64	**0.05**	0.88	0.48	2.17	0.09
ANCOVA	**T**	0.01	0.91	1.84	0.18	0.95	0.34	0.80	0.38
	E	58.38	**<0.01**	6.88	**<0.01**	12.62	**<0.01**	1.88	0.18
	TxE	0.02	0.90	0.47	0.50	0.60	0.44	0.02	0.88

2.5.5. Overstory Tree Diversity Responses

Similarly, overstory diversity, richness and evenness varied by facility and date of establishment but were largely unaffected by treatment (Table 2.8). In contrast with the understory vegetation diversity, the Shannon–Wiener index, and richness for overstory vegetation were greater in early established facilities compared to the recently established facilities (Table 2.8, F, $P < 0.01$; E, $P < 0.02$), except Cave Bay which was comparable with the early facilities (Figure 2.10 a, b). Heyburn State Park followed by Cave Bay showed the lowest tree diversity and richness, while the highest diversity and richness were observed at Garfield Bay and Bottle Bay. As with understory diversity and richness, the facility establishment response was consistent for both treatments (Table 2.8, TxE, $P \geq 0.75$).

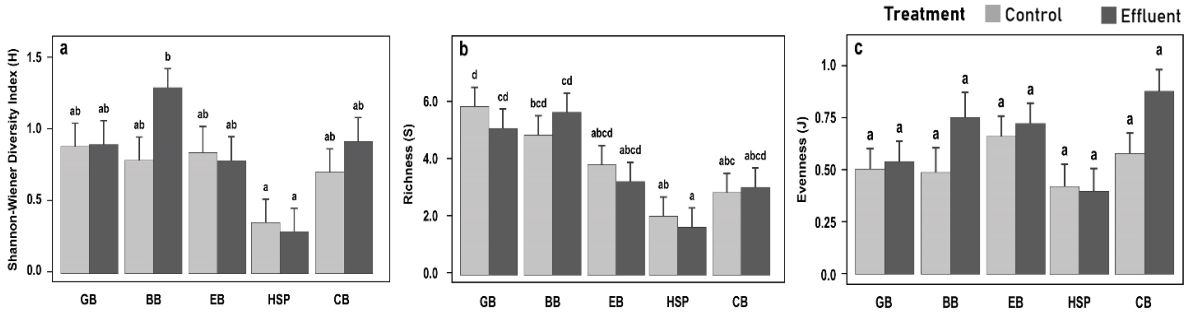

Figure 2.10. Plot mean and standard error (n=5) for overstory (a) Shannon–Wiener diversity index; (b) richness; and (c) evenness. Same letters over bars indicate no differences between treatment levels at $\alpha = 0.10$. Facility abbreviations are as described in Figure 2.2. Facilities are arranged in establishment order with GB being first (1978) and CB last (2013).

Table 2.8. Analysis of variance (ANOVA) and analysis of covariance (ANCOVA) for overstory Shannon–Wiener diversity index (H), richness (S) and evenness (J). Results include *F*-statistic (*F*) and *P*-values (*P*) for the parameters. Facility establishment date (E) was used as the ANCOVA covariate. Boldface indicates significance at $P \leq 0.05$.

Test	Effect	Shannon–Wiener diversity index (H)		Richness (S)		Evenness (J)	
		F	***P***	***F***	***P***	***F***	***P***
ANOVA	**T**	1.49	0.23	0.17	0.68	3.75	0.06
	F	5.67	**<0.01**	12.32	**<0.01**	2.99	**0.03**
	TxF	1.15	0.35	0.56	0.69	0.96	0.44
ANCOVA	**T**	1.16	0.29	0.16	0.69	3.11	0.09
	E	6.43	**0.02**	38.94	**<0.01**	0.18	0.67
	TxE	0.10	0.75	0.05	0.83	0.06	0.81

2.6. Discussion

Our results show that reclaimed water amendments during the growing season had overall positive effects on forest growth responses of coniferous forests in the Inland Northwest and yet had minimal effects on vegetation diversity. We found that the dominant commercial tree species occurring at the study facilities responded positively to reclaimed water, with substantial increases in growth. Furthermore, incremental fertilization and irrigation from reclaimed water application during the growing season sustained growth responses over the long-term. These findings provide insight into long-term effects of reclaimed water application on tree and forest growth responses and community-level changes in understory and overstory vegetation diversity.

2.6.1. Forest Growth Response

These results support our hypothesis that regular growing season amendments of reclaimed water at permitted rates will substantially and continuously increase Inland Northwest tree growth and forest productivity compared to unamended controls. Diameter, basal area, and volume increments were consistently greater in effluent amended plots compared to control plots (Figures 2.4 and 2.7), which agrees with other studies showing sustained growth increases in response to incremental fertilization in combination with irrigation (Bergh et al., 1999; Cromer, 1980; Iivonen et al., 2006; Ingestad, 1987; Pereira et al., 1989; Weetman et al., 1997). Supply of moisture and nutrients in reclaimed water addresses the seasonal summer drought and N limiting characteristic common to the Inland Northwest. The water- and nutrient-deficient trees opportunistically responded to such resource availability through increased annual growth increments, which translate into increased productivity. Tree growth responses at the FWR facilities were consistent with previous studies on forest responses to N fertilization in the Inland Northwest where N is the main growth limiting nutrient (Mika et al., 1992), and thus N contained in reclaimed wastewater was expected to enhance tree growth. Yet, the magnitude of the N fertilizer response within the region is dependent upon the available moisture (Cromer, 1980). Consequently, the addition of both water and incremental nutrients at FWR facilities realized 30 to 116% increases in diameter growth relative to the controls (Figure 2.6). Furthermore, those growth enhancements were sustained year-over-year after facility establishment, while the above regional fertilization studies demonstrate increases up to 39% relative to controls, and that was temporary: lasting up to six years following treatment (Cromer, 1980). Of course, improved soil moisture along with N and other growth-limiting nutrients supplied incrementally in wastewater is expected to be superior to N-only fertilization. Yet, our results suggest that regional productivity potential is far higher than that achieved thus far with modern forest management practices.

2.6.2. Individual Species Responses

The mixed conifer forests in the facilities studied offered an opportunity to consider species-specific responses to wastewater amendments at common locations. Various growth responses to wastewater application have been reported among forest types (Cromer, 1980; Gessel et al., 1990b; Moffat et al., 2001; Weetman et al., 1993). Thus, we expected species-specific treatment differences in tree growth because species are known to respond differently to nutrient and moisture availability (Aubrey et al., 2012). Our study found that all dominant tree species responded positively to reclaimed water treatment (Figures 2.5 and 2.6). The greatest diameter increment response to reclaimed water treatment was observed in western redcedar at Ellisport Bay and Bottle Bay and for Douglas-fir at Bottle Bay (Figure 2.5). A large change also occurred for western redcedar at Garfield Bay; however, the average diameter growth increments among treatment plots at establishment were half those of the controls and, over time, increments in the effluent plots crossed above those of controls (Figure 2.5e). This was analogous to the findings of Hesse et al. (Hesse et al., 1998a), who reported that pretreatment, basal area increments were originally lower in treatment plots, gradually increased with the initiation of reclaimed water amendment and ultimately exceeded the growth of the controls by more than 50%. In contrast, the initial diameter increments in effluent plots were larger for Douglas-fir and ponderosa pine at the recently established Cave Bay and Heyburn state Park facilities compared to the controls (Figures 2.5a, b). However, except for ponderosa pine at Heyburn, they did not diverge and continued to follow similar growth increment differences after establishment. Such responses may be due to other dominating site factors (Wallace et al., 2007), which are outside of experimental control factors in multisite studies.

The overall responses of individual species were also dependent upon the facility at which they were growing. For the three species that occurred in sufficient numbers to make a comparison, each showed a near or above doubling of the average five-year diameter growth increment for at least one of the facilities (Figure 2.5). Other studies show that fast-growing species have even greater responses. For instance, a three-fold increase for Douglas-fir and an eight-fold production increase for poplar was found for wastewater-treated stands in western Washington (Gessel et al., 1990b). Western redcedar and Douglas-fir showed the lowest diameter growth increment at Garfield Bay (Figure 2.6). However, based on previous reports and the responses of these species at the other facilities within our study, we expected to see a much larger increase in diameter increment in response to reclaimed water application. The lack of a growth response among tree species may be due to other dominant site factors affecting tree growth. A similarly limited response was observed in a mixed oak–hickory forest, where it was concluded that the lack of a growth response to fertilization may be from individual tree and microsite characteristics that influence tree growth (Wallace et al.,

2007). The annual application rate will also cause a potential decline. For instance, in a 15-year N-amendment study, a rate of 50 kg N ha^{-1} yr^{-1} continued to stimulate forest growth, while 150 kg N ha^{-1} yr^{-1} caused a decline in productivity and increased tree mortality (Magill et al., 2004). However, facility N loading rates in our study averaged 37 kg N ha^{-1} yr^{-1} with the rate at Garfield Bay being 42 kg N ha^{-1} yr^{-1}, so these rates are moderate and not expected to cause forest decline.

A decrease in tree growth is also linked to stand development. Young, vigorous forests achieve peak stand growth early in development, and that plateaus after canopy closure and maximum leaf area (Binkley et al., 2002; J. R. Foster et al., 2014; Xu et al., 2012). Effluent stands at Garfield Bay are fully stocked with an average of 1300 TPH and an individual tree volume of 0.46 m^3 (Figure 2.2), which means that they are approaching the developmental stage of imminent mortality (Klinka & Brisco, 2009). Indeed, we observed a decline in diameter increment over time in the tree core data (Figure 2.5), which is consistent with growth dynamics in mature forests where inter-tree competition slows growth and ultimately results in mortality of suppressed trees (Oliver & Larson, 1996). Inland Northwest stands that are fully stocked respond poorly to fertilization unless they have been thinned to allow growing space (Scanlin & Loewenstein, 1979). Thus, among the possible reasons for the limited diameter increment response to reclaimed water amendments at Garfield, overstocking is the most likely cause.

2.6.3. Vegetation Diversity Responses

The understory diversity indices at Bottle Bay and Ellisport Bay significantly declined in reclaimed-water-amended plots compared to the controls (Figure 2.9). Stocking increased at these sites but stand development had not yet reached the level of competition that it had at Garfield Bay. Although reclaimed water addition overcomes moisture limitations and leads to a highly productive understory, plant diversity ultimately declines with continued treatment resulting in community simplification (Hunt & Shure, 1980). Forest fertilization also increases leaf area index and light interception (Fox et al., 2007) and decreases plant species diversity (Bobbink et al., 2010; Thomas et al., 1999) by favoring the most shade-tolerant understory species (Alvarez-Clare et al., 2013; Ibáñez et al., 2016; Schroth et al., 2015). Indeed, plant community composition is often used as an indicator of site quality (Binkley & Giardina, 1998; Compton et al., 2003; Hobbie, 1992). Decreased diversity at Bottle Bay and Ellisport Bay in effluent plots is most likely due to increased canopy cover, which is an expected growth response by the overstory to abundant moisture and nutrients during the growing season.

The Shannon–Wiener diversity index of the understory also depends on the initial understory composition and on stand developmental conditions (VanderSchaaf et al., 2000; J. Zhang et al.,

2016). Understory species abundance at Heyburn State Park (est. 2010) and Cave Bay (est. 2013) in our study was dominated by common snowberry and other shrub species (Figure 2.8) with ponderosa pine and Douglas-fir overstories. While reclaimed water amendments at these recently established facilities are expected to ultimately decrease understory diversity through an increase in common snowberry abundance (VanderSchaaf et al., 2000), that transition had not yet occurred during our observations. Conversely, the abundant growth of alpine enchanter's nightshade (*Circaea alpina* L.) in effluent plots at Ellisport Bay (est. 2000) is most likely due to increased moisture availability which corresponded with significant declines in diversity (Figure 2.9). We also noted an increase in the abundance of Rocky Mountain maple (*Acer glabrum*) in the effluent plots at Bottle Bay (est. 1989). This supports observations that continued inputs of N may change forest stands from a slow-growing and slow-N-cycling coniferous forest to a fast-growing and fast-N-cycling deciduous forest (McNulty et al., 1996). Thus, the duration of reclaimed water application treatment and initial prevalence of understory species noticeably affected species diversity.

Our results indicate that stand density and overstory vegetation development also significantly influence understory vegetation responses to reclaimed water. The decline in understory diversity in long-established facilities corresponded with increased overstory diversity (Figure 2.10) and increased stocking (Figure 2.2), as well as increased basal area and volume increments, especially in effluent plots (Figure 2.7). Understory vegetation growth depends on light and nutrient availability (Dirnböck et al., 2014; Gurmesa et al., 2016; Walter et al., 2016). Nitrogen amendments create denser tree canopies leading to lower light and nutrient availability for understory plants (Alvarez-Clare et al., 2013; Ibáñez et al., 2016; Schroth et al., 2015). Lower light availability results in lower understory diversity in old growth forests compared to that found with higher light in selectively logged uneven-aged forests (Scheller & Mladenoff, 2002). Thus, progressive stand development and increased overstory diversity at our long-established facilities may also have resulted in a decline in understory diversity due to decreased resource availability and the selection of tolerant understory plants.

2.6.4. Time series Study Design and Analysis

Our study opportunistically focused on a time series, or a space-for-time substitution, which uses chronosequences as an alternative to long-term, longitudinal studies. Time series have been extensively used to study long-term responses in ecological studies that would otherwise not be possible to practically address (Wogan & Wang, 2018). Time series are challenging to establish because of the strong influence of site conditions on stand metrics which then become confounded with the temporal effect of interest. However, time series studies can be an extremely useful and effective tool for ecosystem management due to the value of long-term data (Banet & Trexler, 2013).

In our study, initial stand metrics demonstrated differences among facilities that were related to establishment date, and effluent plots were often larger in tree and stand dimensions than controls (Figure 2.2). While the rankings of initial parameters are consistent with stand development and treatment responses, it is difficult to be certain that these factors are the cause, and not confounded with site conditions. Despite inevitable tradeoffs inherent in this time series, we were reasonably successful in matching control and effluent stand structure, species composition, soils, and topography. Furthermore, we used initial parameter values as covariates in statistical models that tested growth increments. These covariates largely accounted for differences in size variation within and among stands. Thus, our FWR study appeared to provide a unique and rigorous chance to assess long-term forest responses to reclaimed water application.

2.7. Conclusions

Forest water reclamation offers the prospect of improving forest productivity while renovating applied wastewater and preventing environmental degradation. Increasing demand for renewable wood products combined with decreasing land area for working forests creates a critical need to improve forest productivity on available acreage. Dramatic increases in forest productivity demonstrate the potential productivity in the Inland Northwest and generally for dry western forests.

Our findings suggested that trees respond favorably to reclaimed water irrigation during the growing season at permitted rates, particularly western redcedar and Douglas-fir. However, long-term reclaimed water application may adversely affect tree growth, permanently alter vegetation composition and diversity, and lead to community-level changes in forest ecosystems. Our results also suggest that an increasing length of treatment may lead to a decline in tree growth and diversity. While we concluded that FWR promotes forest growth, evidence from past literature suggests that there are potential environmental risks of nutrient contamination and eutrophication of surface waters in response to long-term land application. Future research on soil nutrient budgets, nutrient cycling, drainage and leaching within this time series will provide more insight into the water and nutrient assimilation capacities of these forest application systems. Despite the potential risks, proper hydraulic and nutrient loading rates in low doses spread across sufficient application areas would optimize forest productivity and prevent nutrient losses.

Forest water reclamation presents environmental, social, and economic implications in the region. Understanding long-term forest responses enables water reuse facility managers to formulate sound forest management strategies to promote productivity and prevent nutrient contamination, and the consequent environmental degradation, while presenting possibilities of generating revenue through timber production. The social liability of reclaimed water can be potentially converted to an

asset that substantially improves productivity. Furthermore, tertiary treatment of reclaimed water by forest ecosystems prevents environmental degradation and helps maintain water quality and sustain communities. When repeated across many municipalities across the Inland Northwest, FWR will be at a scale to maintain regional environmental quality and boost the economic viability of facilities and adjacent timberland owners.

Chapter 3: Nutrient Leaching Potential Along a Time Series of Forest Water Reclamation Land Application Facilities in Northern Idaho

3.1 Abstract

Forest water reclamation is a decades-old practice of repurposing reclaimed water using land-application on forests. Widely accepted as a safe disposal alternative, it has economic, environmental, and social benefits, particularly in areas sensitive to nutrient additions. Long-term land application of reclaimed water may lead to nutrient saturation and subsequent leaching causing irreversible impairment of environmental quality. The goal of this study was to investigate the long-term effects of reclaimed water application on nutrient leaching potential in a time series of forest water reclamation facilities in northern Idaho. Our approach included installation of drain gauges and porous cup tension lysimeters to capture preferential flow and soil matrix flow, and a suite of microplate-based colorimetric assays to quantify drainage and soil water nutrient concentrations to determine net leaching losses of nitrogen and phosphorus species. Analysis of variance and covariance showed a significant effect of treatment, season, and establishment date across the facilities, with a pronounced increase in drainage during the wet-season in reclaimed water amended plots at the long-established facilities. While differences in soil water ammonium, phosphate and dissolved organic nitrogen concentrations between control and effluent treatments in lysimeter samples were not significant, nitrate concentration was notably higher in effluent treated plots. Nutrient concentrations in drain gauge samples were significantly higher than lysimeter samples, indicating occurrence of nutrient losses predominantly through preferential flow paths. Nitrate was found to be most vulnerable to leaching via both matrix and preferential flow paths during the wet season, particularly at those facilities that have been in operation for over three decades. We conclude that while forest water reclamation presents a unique opportunity to manage reclaimed water and improve forest productivity, long-term inputs may potentially lead to nitrogen saturation and nitrate leaching losses from land application systems. Routine monitoring of drainage and nutrient concentrations can be useful indicators of nutrient leaching and can be used for protecting environmental quality.

Keywords: Forest water reclamation; drainage; nutrient leaching; environmental quality

3.2 Introduction

Reclaimed water has a multitude of uses as a source of both water or nutrients in agriculture, silviculture, landscaping, aquaculture, industrial, and groundwater recharge; making it a valuable, viable and sustainable resource (Akhoundi & Nazif, 2018; Miller, 2006; EPA, 2012; Kalavrouziotis et

al., 2015). Land application of reclaimed water or treated municipal wastewater is a decades-old, well-established approach for managing wastewater globally (Andrews et al., 2016; Asano, 1987; Hamilton et al., 2007; Mexal et al., 2002). Low management and maintenance costs, and reduced operational requirements (Miguel et al., 2014) makes land application technologically and financially feasible for small communities that are required to meet discharge regulations in the Pacific Northwest.

The Pacific Northwest U.S. has cool and wet winter season, which receives the majority of the annual precipitation; and a warm and dry summer season, characterized by a hydrologic drought and low streamflow (Kormos et al., 2016). With reduced surface water levels in summer and stringent discharge regulations (IDEQ, 2014), water reuse facilities opt for land application of reclaimed water on native coniferous forests instead of releasing directly into surface waters. Land application on forests, or forest water reclamation, is allowed during summer if the hydraulic and constituent nutrient loading rates do not exceed the soil and vegetation assimilation capacity. Such land application systems utilize low-cost lagoons, vegetation water and nutrient uptake and soil infiltration mechanisms for continued treatment of applied wastewater (Al-Jamal et al., 2002; Mexal et al., 2002). While land application has been well-established, the loading rates and potential environmental implications from prolonged application are still in question.

Reclaimed water is a valuable source of water and soluble nutrients such as nitrogen (N) and phosphorus (P) which are indispensable for tree growth (Mexal et al., 2002).While forests have been considered to serve as benign repositories for nutrient storage, prolonged water and nutrient loading from reclaimed water application may ultimately exceed the nutrient assimilation capacity of forests and increase soil nutrient concentrations. Nutrients may eventually leach into surface and ground water sources (Cameron et al., 1997), potentially leading to impairment of water quality due to eutrophication, followed by loss of ecosystem services (Carpenter et al., 1998; Polglase et al., 1995; Barton et al., 2005; Hook & Kardos, 1978; Muga & Mihelcic, 2008; Daniel et al., 1998; Reed-Andersen et al., 2000; Yang et al., 2008).

Coniferous forest ecosystems are inherently nutrient limited (Compton et al., 2003; Van Miegroet & Cole, 1984), and N and P requirements are largely derived from litter decomposition (Binkley & Fisher, 2013). Ammonium (NH_4^+) and organic N concentrations are comparatively higher relatively to NO_3^- and, nitrification rates are low (Shan et al., 2014) and NO_3^- leaching is therefore minimal (Van Miegroet & Cole, 1984). Continued application of reclaimed water may alter forest nutrient cycling processes and open the closed forest ecosystems to nutrient leaching. N inputs greater than the ecosystem demand causes accumulation of nutrients in soil which are lost to groundwater, lakes and streams (Aber et al., 1998). Scores of forest water reclamation facilities have been in

operation for several years to decades. While application rates are well within the permitted rates, there is a gap in information on long-term soil concentrations and potential nutrient saturation and leaching losses at these forest water reclamation facilities.

Nutrient leaching may occur through movement in matrix and preferential-flow paths (Reid et al., 2018). The movement of applied wastewater in matrix-flow occurs through micropores within soil matrix water in close association with soil physical components, which increases contact time. In contrast to preferential-flow, where gravitational water flows through cracks and worm channels in soil profile (McLeod et al., 1998). Preferential-flow presents an important pathway of P loss from forest water reclamation sites. Tension lysimeters capture the matrix flow of applied nutrients in soil water while drain gauges are used to assess soil water loss through deep drainage via matrix as well as preferential flow. Assimilation of applied nutrients due to soil biological and chemical processes takes place primarily in the topsoil and is enhanced by increased contact time and reclaimed water-soil interaction (McLeod et al., 1998). N and P losses in forest soils occur predominantly through preferential-flow pathways which reduces contact between reclaimed water and the soil matrix (Barton et al., 2005b; Bol et al., 2016).

Nitrogen is primarily lost from soil as nitrate (NO_3^-) due to minimal anion binding sites, high solubility of NO_3^- and low reactivity within the soil profile. Eighty percent of applied N is retained in the forest ecosystem as NH_4^+ stored within the topsoil, but the contact time during preferential transport is insufficient for NO_3^- retention (Hagedorn et al., 2001). While applied NO_3^- is readily available for uptake by plants and microbes, it is also vulnerable to leaching losses. Western forest ecosystems are naturally low in NO_3^- (Shan et al., 2014) and elevated levels in soil may increase the risk of NO_3^- leaching (Polglase et al., 1995) and ground water contamination (Bond, 1998).

Organic pools of N, dissolved organic nitrogen (DON) is also one of the major forms of N lost from soil to freshwater (Jones & Willett, 2006). Organic N is the most dominant form of N in most soils but is largely unavailable to vegetation due to high molecular weight and typically is taken up once it is broken down by exoenzymes into smaller soluble units (Jones et al., 2005). Proteolytic enzyme activity produced by microbes, mycorrhizal fungi and plant roots sustains the high concentration of organic N availability for plant uptake in the form of amino acids, amino sugars and other low molecular weight N compounds (Näsholm et al., 2009). While NO_3^- leaching is the dominant pathway of N loss in northern temperate forests, DON has been found to be particularly important for N loss under N-saturated conditions where studies show an increase in DON efflux with increased N input (Compton et al., 2003; Fang et al., 2009; McDowell et al., 2004).

P availability is limited by slow rock weathering and adsorption into Fe and Al oxides in acidic forest soils (Binkley & Fisher, 2013). With increased contact time during matrix-flow, most of

the P is fixed in top and sub-soil. Long-term application and elevated loading rates of P may exceed the maximum P sorption capacity of soil, resulting in deep percolation of P through the soil profile. High accumulation of P due to prolonged application has been reported to cause downward movement of P to deeper layers particularly in coarse-textured sandy loam soils (Aulakh et al., 2007). While soils are considered to have high P sorption capacity in the subsoil, P leaching is highly dependent on site-specific factors and occurs due to preferential-flow when water and P bypass soil P sorption capacity (Beven & Germann, 1982; Bol et al., 2016; Djodjic et al., 2004).

The goal of this study is to examine long-term effects of reclaimed water application on drainage, soil water nutrient concentrations and leaching potential in native coniferous forests of northern Idaho. The permitted reclaimed water loading rates are untested over the long term. Limited research has been conducted on characterization of site hydrology, seasonal trends on soil nutrient concentrations, and leaching potential in western forests at forest water reclamation facilities. This is particularly concerning in areas such as Idaho, where many forest water reclamation facilities have been in operation for prolonged time periods and are located near the regional lakes which hold important cultural, historical, recreational, and other socio-economic values. Proper metering of hydraulic and constituent loading rates at forest water reclamation facilities is thought to protect regional freshwater from nutrient leaching losses. We hypothesized that, regardless of operation time, forest water reclamation facilities will minimize nutrient leaching losses. We also hypothesize that N losses occur through both matrix and preferential-flow paths, while P losses predominantly occur through preferential-flow regardless of facility age.

3.3 Material and methods

3.3.1 Study Sites

The study was conducted along a time-series of five water reuse facilities situated adjacent to Lake Coeur d'Alene and Lake Pend Oreille in northern Idaho, United States (Figure 2.1). Facilities established operations between 1978 and 2013 (Table 3.1). Five tenth-acre measurement plots were installed at each of the five study facilities along with five adjacent control plots for comparison of the effect of wastewater treatment ($n=50$). Where possible, the control plots selected had comparable soil, stand composition and structure as the treatment plots. The treatment and control plots were established on locations with ≤ 5% slope.

Soils varied between facilities (Table 3.2). Soils at the Pend Oreille facilities were characterized by parent material of volcanic ash and/or loess over till derived from granite and/or metamorphic rock. The soils were well drained, and the ecological sites were ashy over loamy, glassy over mixed, superactive, frigid Alfic Udivitrands (Soil Survey Staff, 2019). Heyburn State Park soils

were moderately well drained, and the ecological site was warm-frigid, xeric, unglaciated, loamy and fragipans. Soils at Heyburn facility were Fine-silty, mixed, superactive, frigid Vitrandic Fragixeralfs (Soil Survey Staff, 2019). Cave Bay soil was well drained, and the ecological site was warm-mesic, xeric, and unglaciated (Soil Survey Staff, 2019).

Table 3.1: Study site information, including location, elevation, establishment date, mean annual precipitation, mean annual temperature, average maximum temperature, and average minimum temperature.

Facility	Coordinates	Location	Elevation (m)	Estd. date	MAP[1] (mm)	MAT[1] (°C)	T_{max}[1] (°C)	T_{min}[1] (°C)
Cave Bay	47.4703 ° N 116.8803° W	Worley, ID	711	2013	534.4	8.4	19.6	-5.1
Heyburn State Park	47.3462° N 116.7821° W	Plummer	769	2010	662.6	8.2	19.4	-5.2
Ellisport Bay	48.2159° N 116.2696° W	Hope, ID	659	2000	633.1	7.7	18.8	-5.6
Bottle Bay	48.2018 ° N 116.4207° W	Sagle, ID	696	1989	752.4	7.4	27.4	-5.7
Garfield Bay	48.2287° N 116.4384° W	Sagle, ID	707	1978	708.9	7.4	27.4	-5.8

[1]30-year average data from (PRISM Climate Group). MAP, mean annual precipitation; MAT, mean annual temperature; T_{max}, average maximum temperature; T_{min}, average minimum temperature.

Table 3.2: Study site vegetation information including dominant tree species and initial soil properties for the top 15 cm of mineral including soil texture, pH and bulk density.

Reuse Facility	Dominant tree species[1]	Soil Series[2]	Soil texture[3]	Soil pH	Soil bulk density (gm^{-3})
Cave Bay	DF, PP	Lacy	Gravelly loam	6.9±0.09	0.8±0.04
Heyburn SP	PP, DF	Carlinton	Ashy Silt loam	6.5±0.09	0.99±0.05
Ellisport Bay	WRC, WH, GF	Pend Oreille	Silt loam	6.4±0.07	0.85±0.04
Bottle Bay	WRC, WL, DF, PB, GF	Pend Oreille	Silt loam	6.2±0.08	0.68±0.04
Garfield Bay	WRC, WL, DF, PB	Pend Oreille	Silt loam	6.7±0.09	0.71±0.04

[1]PP=ponderosa pine (*Pinus ponderosa* Douglas ex C. Lawson), DF=Douglas-fir (*Pseudotsuga menziesii* var. *glauca* (Mirb.) Franco), GF=grand fir (*Abies grandis* (Douglas ex D. Don) Lindl.), WRC= western redcedar (*Thuja plicata* Donn ex D. Don), WL=western larch (*Larix occidentalis* Nutt.), WH= western hemlock (*Tsuga heterophylla* (Raf.) Sarg.), PB= paper birch (*Betula papyrifera* Marsh.)

[2,3]Soil Survey Staff, 2019.

3.3.2 Class C Reclaimed Water Amendment

The water reuse facilities in the study have low-cost lagoon primary treatment system and generate Class C municipal reclaimed water which undergoes aeration and disinfection before being

land applied at the forest water reclamation facilities. All five facilities have been applying reclaimed water during the growing season approximately April 1 to October 31 (IDEQ, 2014) over their respective times of operation. Estimated cumulative loading at the oldest facility is more than ten times greater than that applied at the youngest facility (Figure 3.1).

Table 3.3. Average annual hydraulic and nutrient loading rates.

Parameter	Average loading rate[1]
Hydraulic	30 ± 4.3 cm yr^{-1}
Nitrogen	37 ± 5.9 kg ha^{-1} N
Phosphorus	14 ± 1.5 kg ha^{-1} P

[1]The values reported are average (mean ± SE) of all facilities based on the annual reports submitted to IDEQ. The hydraulic loading rate is based on an average of four sites (Heyburn SP, Ellisport Bay, Bottle Bay and Garfield Bay) during 2021.

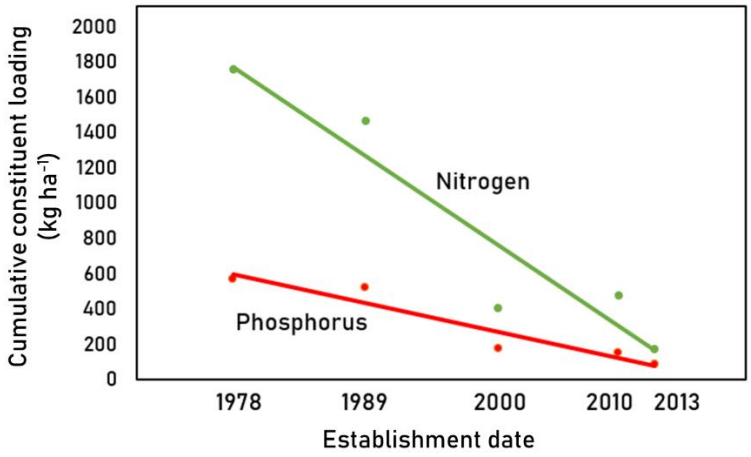

Figure 3.1: Cumulative constituent N and P loading rates (kg ha^{-1}) at facilities established during different time periods. Cumulated nutrient loading rates calculated from loading rates in Idaho Department of Environmental Quality annual reports.

3.3.3. Tension lysimeters installation and sample collection

A tension lysimeter (Soil Moisture Equipment Corp., Santa Barbara, CA, USA) was installed in each study plot at all five facilities (*n = 50*) to assess nutrient loss by matrix flow. Nutrient concentrations in soil water collected with tension lysimeters measure the water held under tension through capillary action (Singh et al., 2018). The tension lysimeters were installed below the rooting zone at 80 cm based on the predicted depth above which 80% of roots occur (Jackson et al., 1996). At each plot, soil was cored using a 5.3 cm (2 ¼ in) auger (AMS, Inc.) to 80 cm and was sieved through a 0.64 mm (1/4 in) mesh screen to remove pebbles and rocks and to ensure uniform backfill soil. The tension lysimeters were inserted using manufacturer recommended procedures including submerging the porous cup in a slurry of silica flour to assure a matrix connection with soil moisture and a

bentonite plug to avoid edge drainage. The lysimeters were evacuated the day before the sampling event to ensure fresh samples for nutrient composition analysis. Lysimeter samples were collected seasonally. During the wet seasons, the lysimeters were purged to remove stagnant water before charging with a vacuum suction of 60-80 kPa for 24 h to collect the samples. During the dry seasons, the lysimeters were wetted for 15 mins with distilled water and purged to establish a hydraulic connection between the cup and soil. Lysimeter soil pore water samples were transported on ice to the laboratory after collection and frozen until analysis.

3.3.4. Drain Gauges installation and sample collection

Drain Gauge G3s (METER Group, Pullman, WA) (Gee et al., 2009) and handmade drain gauges (Hall, 2018) were installed at two treatment and one control plot at each facility (Clark, 2022). Three drain gauges were installed at each facility, one G3 Drain Gauge and one handmade drain gauge were installed in two respective effluent plots and one handmade drain gauge was installed in a control plot (*n=15*). Both types of drain gauges contained a passive wick to create capillary connection that facilitates drainage from the suspended soil column. Each drain gauge reservoir was installed with a Hydros 21 (METER Group, Pullman, WA) water level sensor that measured hourly drainage. Two tensiometers (METER Group) soil water potential sensors were installed at 10 cm (Terros 21) and 50 cm (MPS-2) soil depth at each drain gauge plot to measure hourly soil temperature and soil water potential. The drainage was measured with a water level sensor placed in the reservoir of the drain gauge. Soil moisture, temperature and water level were logged hourly (EM50, Decagon Devices, Pullman, WA). The drain gauge samples were collected monthly using a pressure vacuum pump (Soil Moisture Equipment Corp., Santa Barbara, CA, USA), and the soil water potential, temperature and water level readings were extracted from the data loggers using ECH20 Utility Software (ICT International). Drain gauge soil water samples were transported to the laboratory and frozen until analysis.

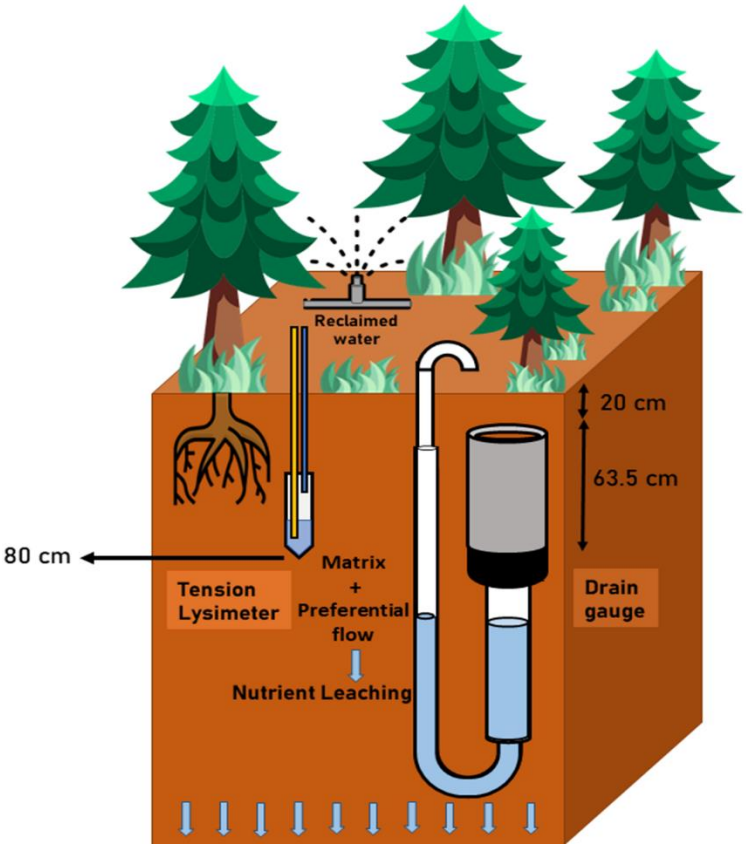

Figure 3.2: Conceptual diagram of field site instrumentation: Tension lysimeter and drain gauge installation.

3.3.5. Modeling Drainage

The Watershed Erosion Prediction Project (WEPP) was calibrated with drainage estimated in drain gauge plots and the calibrated model used to predict drainage from plots without direct drainage measurements. WEPP is a hydrology and erosion model used to assess the impacts of various land management practices on soil, hydrologic, and vegetative watershed components (Brooks et al., 2016). WEPP was parameterized using drainage information collected from the drain gauge plots and facility-specific soil physical input parameters including field capacity, wilting point, soil texture (Soil Survey Staff, 2019), daily climate (WEPP Cloud program), and irrigation rates (Annual reports, IDEQ). The drainage from remaining plots were estimated using WEPP and facility specific parameters based on the three drain gauge plots. Prior to the simulation model runs for each plot, plot-specific soil data and facility-specific climate and irrigation data were gathered for the model input parameters. Soil storage was quantified in the model by applying the gathered input data to a daily forest water balance equation. Daily drainage model output for days within each season (between solar equinox and solstice) were summed to provide seasonal drainage for each plot. Clark (2022) describes the details of hydraulic modeling within this time-series study.

3.3.6. Sample Chemistry using Microplate Colorimetric Assay

The lysimeter and drain gauge sample chemistry was analyzed using a suite of microplate reader-based colorimetric assays. The samples were filtered using 0.45 µm membrane filter for analysis of dissolved ortho-phosphate and with Whatman-42 filter paper for analysis of ammonium, nitrate, total dissolved N and dissolved organic N. The filtered samples were analyzed using a suite of rapid microplate reader-based colorimetric assays using standard 96-well microplates (Hood-Nowotny et al., 2010; Ringuet et al., 2011).

3.3.6.1. Ammonium (NH_4^+)

Ammonium concentrations were analyzed using the sodium salicylate method (Baethgen & Alley, 1989; DeForest, 2011), which is based on the reaction between ammonium and a weakly alkaline mixture of sodium salicylate and sodium hypochlorite. The reagent develops a green color with ammonium. Triplicates of standards and samples (80 µl) were added to a 96-well clear microplate followed by 60 µl of salicylate cocktail and 60 µl of sodium hypochlorite. The plates were incubated for 45 minutes and read at absorbance of 650 nm using a multimode microplate reader (Synergy 4 Biotek®). A standard curve was prepared using the standard solution of 100 ppm $(NH_4)_2SO_4$ (Ammonium sulphate). The unknown sample concentrations were fitted on the standard curve between concentration and absorbance with R-squared of 0.999.

3.3.6.2. Nitrate (NO_3^-)

Nitrate determination was based on Vanadium (III) chloride reduction method (Chidester, 2010; Doane & Horwáth, 2003),where nitrate was quantitatively reduced to nitrite, which was detected by using Griess reagent. The reagent included sulfanilamide and N-(1-naphthyl) ethylenediamine which developed a pink coloration with nitrite. The nitrite ions initially react with sulfanilamide to form a diazonium salt which reacts with N-(1-naphthyl) ethylenediamine in an azo coupling reaction to form the pink azo dye (Moorcroft & Compton, 2001). The standards and samples (30 µl) were added to a microplate in triplicates followed by 300 µl of Vanadium reagent solution. The plates were incubated overnight and read at absorbance of 610 nm. Solution of 100 ppm KNO_3 (Potassium nitrate) was used as a standard. The unknown sample concentrations were fitted on the standard curve between concentration and absorbance with R-squared of 0.995.

3.3.6.3. Dissolved organic nitrogen (DON)

DON values were determined by subtraction of inorganic N (NH^{4+} and NO^{3-}) from total dissolved nitrogen concentrations. Total dissolved nitrogen was determined using alkaline persulfate digestion method (Cabrera & Beare, 1993; Hood-Nowotny et al., 2010). Alkaline persulfate reagent oxidizes NH_4^+ and organic N to NO_3^- which was quantified using the VCl_3/Griess method for NO_3^- (Hood-Nowotny et al., 2010). The persulfate reagent was prepared fresh daily. 2.5 ml of soil water samples and digest check standards (alanine and urease, 1 and 5 ppm) were mixed with 0.5 ml of persulfate reagent. Sample to persulfate reagent ratio of 5:1 was always maintained (Studt et al., 2020) and the solution was autoclaved for 40 minutes at 120 °C. Digestion check standards were used to check the test digestion efficiency to ensure complete digestion of organic components. A 100 ppm KNO_3 solution was used as a standard solution. The unknown sample concentrations were fitted on the standard curve between concentration and absorbance with R-squared of 0.999.

3.3.6.4. Dissolved reactive phosphate or orthophosphate (PO_4^{-3})

Dissolved orthophosphate was determined using the molybdenum blue method (Murphy & Riley, 1962). The blue coloration in the molybdate test developed from reaction of orthophosphate in reagent solution consisting of ammonium molybdate, sulfuric acid, ascorbic acid, and antimony potassium tartrate in an acid medium (4.9 N sulfuric acid) to form an antimony-phospho-molybdate complex. The complex is reduced by ascorbic acid to generate a blue coloration, the intensity of which is directly proportional to the orthophosphate concentration in the samples (Pote & Daniel, 2009). Triplicates of standard and samples (200 µl) were added to 96-well clear microplates followed by 50 µl reagent solution. The microplates were incubated for 30 minutes and read at an absorbance of 880 nm. A standard curve was prepared using the standard solution of 100 ppm KH_2PO_4 (Potassium phosphate monobasic). The unknown sample concentrations were fitted on the standard curve between concentration and absorbance with R-squared of 0.999.

3.3.7. Soil nutrient flux

N and P flux in soil then can be calculated as a product of soil pore water nutrient concentration and drainage rate or water flux (Gaskin et al., 1989). The nutrient concentrations (mg L^{-1}) were used to calculate nutrient flux (kg ha^{-1}) using drainage volumes simulated by WEPP. The nutrient flux was used to determine seasonal and annual leaching loss of the soil nutrients.

3.4. Statistical analysis

The effect of reclaimed water treatment, facility (or date of establishment), seasons, and their interactions on soil water nutrient concentrations and leaching were tested. A three-way analysis of variance included facility as a categorical variable while a three-way analysis of covariance tested facility establishment date as a continuous variable. Missing nutrient concentration data needed to produce a complete annual nutrient flux dataset were calculated by averaging nutrient concentration data by facility, treatment, and season. Missing seasonal data was replaced with the averages of a different season if no variance was observed using analysis of variance. The assumption for normality for Shapiro-Wilk's for the transformed data was met at $\alpha = 0.05$. If normality and homoscedasticity assumptions for analysis of variance were not met, the data was transformed (Box & Cox, 1964) and used for statistical analysis. Tests of fixed effects were used to examine the main effects and their interactions for each variable. Differences were considered significant at $P \leq 0.05$. All analyses were performed in R version 4.1.2 (Core, 2021). Package rstatix provided the functions used to execute ANOVA tests and check for violations of ANOVA assumptions on the data. MASS package contained the boxcox function used to apply a box-cox transformation on the data and packages ggplot2, emmeans, and multcomp were used to create the figures illustrating the analyzed data.

3.5.Results

3.5.1 WEPP modelled drainage

Drainage is critical to the calculation of leaching. Drainage has a strong seasonal pattern and therefore, directly affects the magnitude of seasonal leaching. Monthly drainage results are presented in detail by Clark et al. (In preparation). Here, we briefly present seasonal drainage corresponding with seasonal soil water sampling.

Analysis of WEPP predicted drainage showed effect of season, facility and treatment (Table 3.4, Figure 3.3). Treatment had an important effect on drainage which depended on facility and season (Table 3.4, TxF, TxS, $P < 0.01$). Greatest differences in drainage were observed at the long-established facilities Bottle Bay and Garfield Bay. Average drainage in effluent plots at Bottle Bay was 120% greater than the control plots in Fall 2020 and 12% greater in Fall 2021, while the difference was negligible at Garfield Bay for Fall 2020 but was greater by 42% in effluent plots in Fall 2021 compared to the unamended controls.

Season has a pronounced effect on drainage (Table 3.4, S, $F = 3502.54$). Drainage was significantly higher during the wet seasons with highest drainage observed during Winters of both 2021 and 2022 (Figure 3.3). Drainage in Fall 2020 was approximately 38% of that in Winter of 2021, while drainage wasn't significantly different between Fall 2021 and Winter 2022. Drainage for Spring

2022 was notably higher compared to the preceeding year while drainage during summer was minimal during both sampling years. The seasonal variation also explained differences in drainage among the facilities (Table 3.4, FxS, $P < 0.01$). During wet seasons, higher drainage occurred at Garfield Bay, Bottle Bay and Ellisport Bay, but during dry seasons, drainage was greatest at Garfield Bay and Cave Bay (Figure 3.3).

Table 3.4: Three-way analysis of variance results for drainage generated in WEPP. *F* statistic (*F*) and *P*-values (*P*) are presented for measured effects of reclaimed water treatment (T), Facility (F), season (S), and their interactions. Boldface indicates significance at $P \leq 0.05$.

Effect	Drainage (cm)	
	***F*[1]**	***P*[1]**
T	90.51	**<0.01**
F	235.07	**<0.01**
S	3502.54	**<0.01**
TxF	17.92	**<0.01**
TxS	23.94	**<0.01**
FxS	76.53	**<0.01**
TxFxS	12.53	**<0.01**

[1]F statistics (F) and P-values (P).

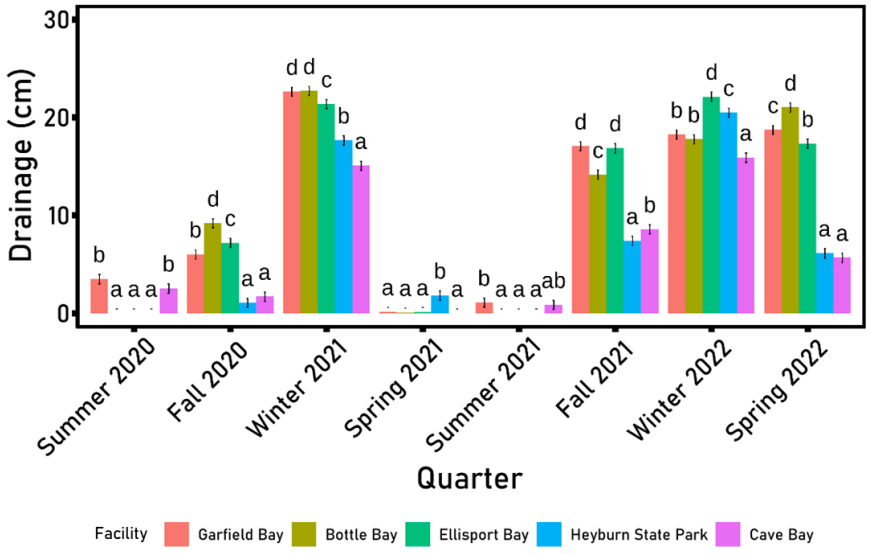

Figure 3.3: Facility by season variation in drainage (cm) across forest water reclamation facilities. Each column represents the mean of all plots per facility (n = 10). Means within each season having the same letter are not statistically different ($P \leq 0.10$).

3.5.2 Tension lysimeter nutrient concentrations and leaching

The effect of effluent treatment on lysimeter nutrient concentrations depended on facility or season. The greatest effect of treatment was observed for NO_3^-, which significantly varied among facilities (Table 3.5 & Table B1, T, $F > 44.147$). NO_3^- concentrations for treated effluent plots at the long-established facilities (Garfield Bay, Bottle Bay and Ellisport Bay) were an order of magnitude higher compared to the unamended control plots at the recently established facilities (TxF, $P < 0.01$, Table 3.5, Figure 3.4a). In contrast, the effect of treatment differences in PO_4^{3-} concentrations between control and effluent plots were only observed for the longest established facility Garfield Bay and the most recently established facility Cave Bay and were not statistically different at the other facilities (Figure 3.4d). While effect of treatment by facility was significant for NH_4^+ concentrations (Table 3.5, TxF, $P = 0.044$), DON did not significantly vary among treatments for long-established facilities. However, it did vary inconsistently at the most recently established facility (Table 3.5, TxF, $P = 0.027$, Figure 3.4d).

Table 3.5: Three-way analysis of variance results for nutrient concentrations of samples collected from lysimeters. *F* statistic (*F*) and *P*-values (*P*) are presented for measured effects of reclaimed water treatment (T), Facility (F), season (S) and their interactions on nitrate (NO_3^-), phosphate (PO_4^{3-}), ammonium (NH_4^+), and dissolved organic nitrogen (DON) concentrations Boldface indicates significance at $P \leq 0.05$.

Effect	**NO_3^- (mg L^{-1})**		**PO_4^{3-} (mg L^{-1})**		**NH_4^+ (mg L^{-1})**		**DON (mg L^{-1})**	
	F	***P***	***F***	***P***	***F***	***P***	***F***	***P***
T	51.329	**<0.01**	1.933	0.166	1.153	0.284	0.305	0.581
F	8.334	**<0.01**	7.932	**<0.01**	3.049	**<0.01**	12.758	**<0.01**
S	1.424	0.197	4.432	**<0.01**	22.692	**<0.01**	5.954	**<0.01**
TxF	7.013	**<0.01**	10.157	**<0.01**	2.486	**0.044**	2.789	**0.027**
TxS	0.706	0.667	0.211	0.983	1.315	0.244	1.414	0.200
FxS	0.343	0.999	0.888	0.623	2.513	**<0.01**	0.434	0.992
TxFxS	0.512	0.964	0.751	0.777	0.996	0.469	0.667	0.863

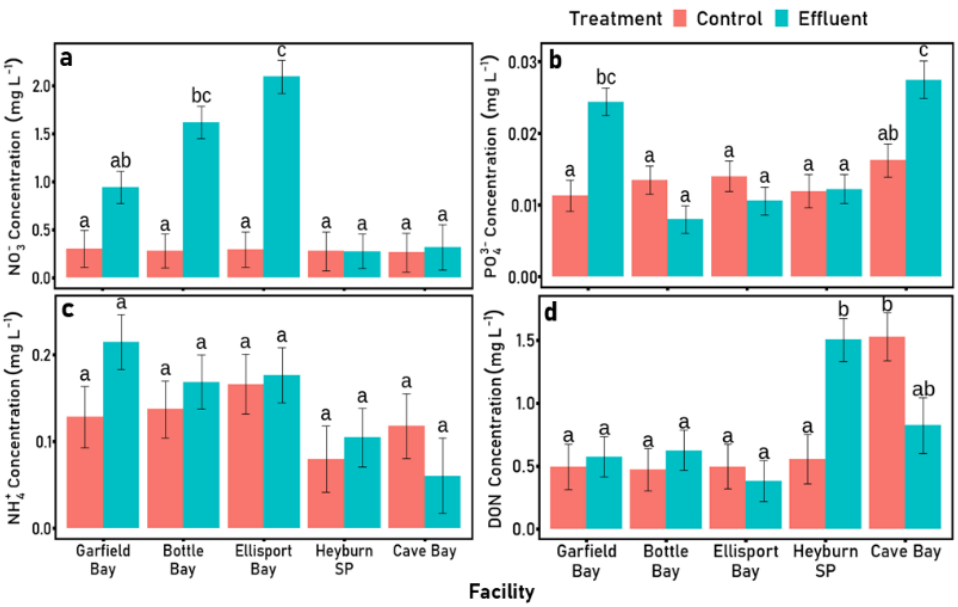

Figure 3.4: Facility by treatment variations of soil water concentrations of (a) nitrate [NO_3^-] (mg L^{-1}), (b) phosphate [PO_4^{3-}] (mg L^{-1}), (c) ammonium [NH_4^+] (mg L^{-1}), and (d) dissolved organic nitrogen [DON] (mg L^{-1}) in lysimeter samples at Garfield Bay (GB), Bottle Bay (BB), Ellisport Bay (EB), Heyburn State Park (HSP) and Cave Bay (CB) facilities. Each column represents the mean of all plots across seasons per facility (n = 40). Means within each panel having same letter are not statistically different ($P \leq 0.10$).

Season had a significant effect on all lysimeter nutrient concentrations except NO_3^- (Table 3.5, S, $P < 0.01$). Although, the trend of higher mean NO_3^- concentrations in Fall of both years will affect leaching as it is a product of both concentration and drainage. The seasonal variation in PO_4^{3-} concentration was observed for Fall 2021 concentration, which was almost half the magnitude compared to the other seasons (Table 3.5, Table B1, Figure 3.5b). While, for NH_4^+, there was a seasonal effect with the highest concentrations observed during Fall and Winter of the first sampling year. However, there was no treatment effect (Figure 3.5c). Both analysis of variance (Anova) and analysis of covariance (AnCova) were used to understand the differences between the effect of facility or establishment date. Including Facility as a categorical variable in the Anova models were more helpful for describing nutrient concentration than was including establishment date as a continuous variable in the AnCova models. The use of facility establishment date as a continuous variable in AnCova models was informative only to describe lysimeter NH_4^+ concentrations (TxE, $P = 0.014$) (Table B1). In the covariate analysis, NH_4^+ concentrations in effluent plots at Garfield Bay (est. 1978) were three times higher than those at Cave Bay (est. 2013). This gradient was shallower for control plots (Figure B3a). Consequently, the Anova models were useful for describing nutrient concentrations, while the AnCova models were used to explain nutrient leaching and testing our first hypothesis.

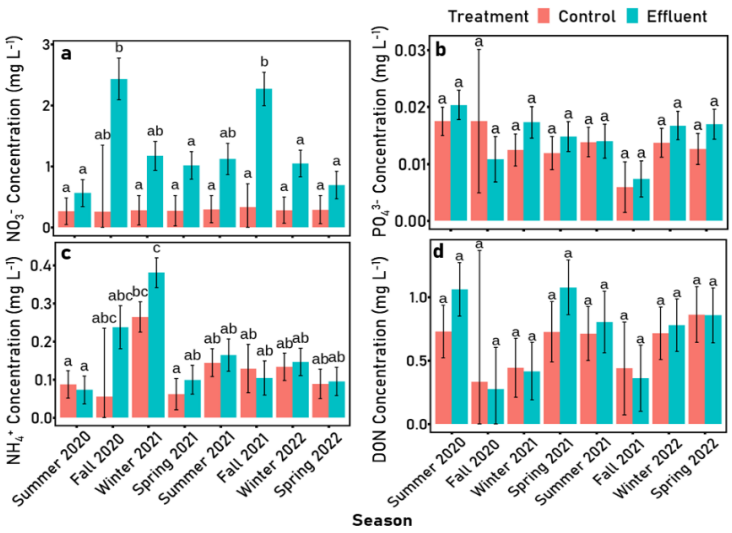

Figure 3.5: Seasonal concentrations of (a) nitrate [NO_3^-] (mg L^{-1}), (b) phosphate [PO_4^{3-}] (mg L^{-1}), (c) ammonium [NH_4^+] (mg L^{-1}), and (d) dissolved organic nitrogen [DON] (mg L^{-1}) in lysimeter samples. Each column represents the mean of all plots per facility (n = 25). Means within each panel having same letter are not statistically different ($P \leq 0.10$).

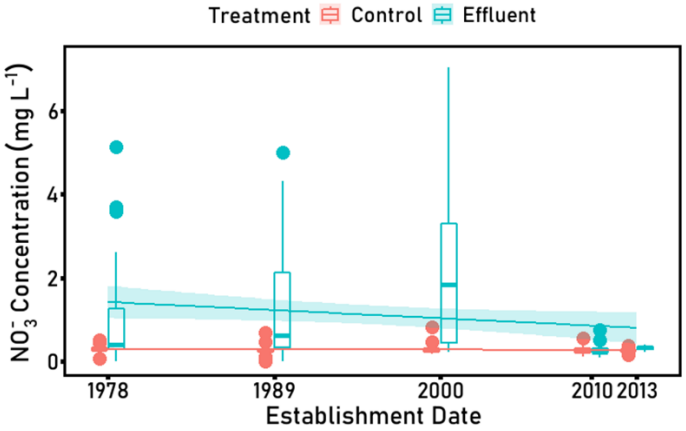

Figure 3.6: Lysimeter NO_3^- concentrations (mg L^{-1}) for facilities with different dates of establishment. Boxes delimit the 0.25 and 0.75 quantiles, horizontal lines indicate medians, whiskers extend to minimum and maximum values, and outliers are plotted as individual points. The highest outliers for the 2000 established facility were excluded for clarity. Each box and whisker represents n=40 observations.

The AnCova model revealed that response of lysimeter nutrient leaching to effluent treatment depended on facility establishment date and season (Table 3.6, TxE, TxS, $P < 0.01$). The effect of treatment on nutrient leaching varied by facility establishment date, particularly for NO_3^- (Table 3.6, TxE, $P < 0.01$). NO_3^- leaching in control plots was largely not affected by facility establishment date whereas NO_3^- leaching increased in effluent treated plots with time since facility establishment (Figure 3.7a). However, leaching responses of PO_4^{3-}, NH_4^+ and DON to treatment were not similarly influenced by facility establishment date (Figure B3).

Season explained much of the variation in nutrient leaching rates (ExS, Table 3.6, Figure 3.7b). Leaching rates were greatest during the wet season and minimal in Summer reflecting the influence of the seasonal dynamics of drainage. The seasonal treatment difference was distinct for NO_3^- leaching, with consistently higher leaching at the long-established facilities during Fall and Winter of both sampling years (Figure B1a). Similarly, PO_4^{3-}, NH_4^+ and DON leaching rates were higher during wet seasons (Table 3.6, S, $P < 0.01$). However, the leaching rates were equivalent at both effluent treated plots and unamended control plots (Figure B1b, c & d).

Table 3.6: Three-way analysis of covariance results for nutrient leaching of samples collected from lysimeters. *F* statistic (*F*) and *P*-values (*P*) are presented for measured effects of reclaimed water treatment (T), Facility (F), season (S) and their interactions on nitrate (NO_3^-), phosphate (PO_4^{3-}), ammonium (NH_4^+), and dissolved organic nitrogen (DON) leaching. Boldface indicates significance at $P \leq 0.05$.

Effect	**NO_3^- (kg ha^{-1})**		**PO_4^{3-} (kg ha^{-1})**		**NH_4^+ (kg ha^{-1})**		**DON (kg ha^{-1})**	
	F	***P***	***F***	***P***	***F***	***P***	***F***	***P***
T	12.277	**<0.01**	2.293	**0.021**	1.773	0.081	4.901	**<0.01**
E	94.700	**<0.01**	11.170	**<0.01**	89.726	**<0.01**	0.543	0.462
S	129.659	**<0.01**	30.867	**<0.01**	120.837	**<0.01**	71.605	**<0.01**
TxE	16.298	**<0.01**	1.896	0.169	2.500	0.115	0.689	0.407
TxS	3.857	**<0.01**	0.731	0.646	1.114	0.353	5.587	**<0.01**
ExS	12.257	**<0.01**	2.841	**<0.01**	5.380	**<0.01**	4.183	**<0.01**
TxExS	1.294	0.252	0.350	0.930	1.135	0.340	1.470	0.177

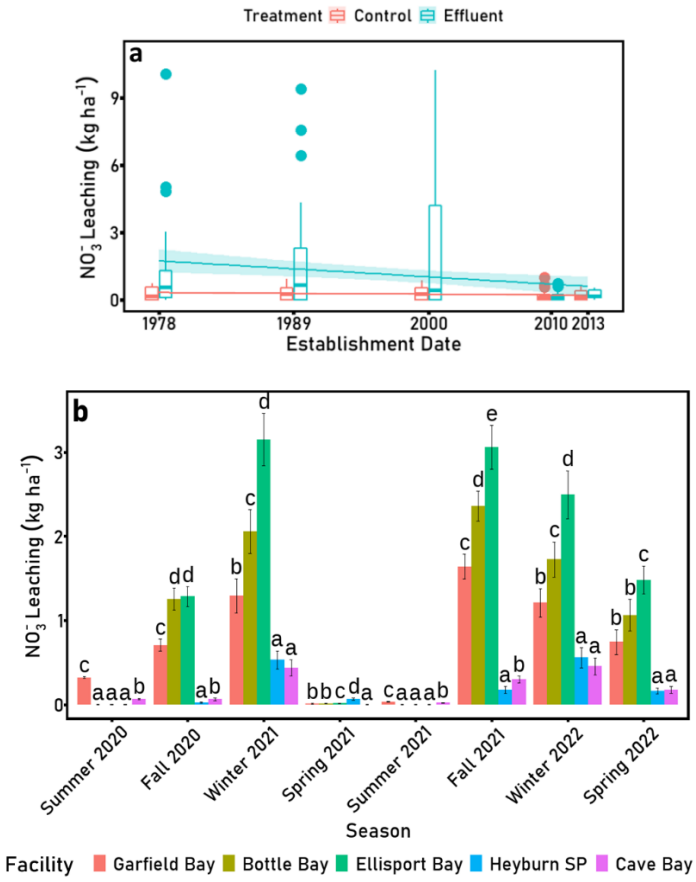

Figure 3.7: Nitrate leaching of soil pore water collected with lysimeter: (a) NO_3^- leaching (kg ha^{-1}) for facilities with different dates of establishment.Boxes delimit the 0.25 and 0.75 quantiles, horizontal lines indicate medians, whiskers extend to maximum values, and outliers are plotted as individual points. The highest outliers for the 2000 established facility were excluded for clarity. Each box and whisker represents n=40 observations. (b) NO^{3-} leaching (kg ha^{-1}) across seasons. Error bars are standard error of the mean (n=10). Means within each season having the same letter are not statistically different ($P \leq 0.10$).

3.5.3 Drain gauge nutrient concentrations and leaching

Facility and season had significant effects on drain gauge nutrient concentrations (Table 3.7, F, S, $P < 0.01$). Higher drain gauge NO_3^- concentrations consistently occurred in effluent treated plots regardless of facility (Table 3.7, TxF, $P = 0.399$). PO_4^{3-} concentrations varied by treatment but depended upon facility (Table 3.7, TxF, $P < 0.01$). While drain gauge PO_4^{3-} concentrations were significantly higher in effluent plots at recently established facilities (Heyburn State Park and Cave Bay) compared with other facilities (Figure B4a).

A consistent seasonal variation occurred for NO_3^- and PO_4^{3-}, with highest concentrations observed during Fall (Figure 3.8). Notably, PO_4^{3-} concentrations were two orders of magnitude lower than NO_3^-. While treatment did have a significant effect on all four nutrient concentrations (Table 3.7, T, $P < 0.01$), in no case was this dependent upon season (TxS, $P > 0.18$).

Table 3.7: Three-way analysis of variance results for nutrient concentrations of samples collected from drain gauges. *F* statistic (*F*) and *P*-values (*P*) are presented for measured effects of reclaimed water treatment (T), Facility (F), season (S) and their interactions on nitrate (NO_3^-), phosphate (PO_4^{3-}), ammonium (NH_4^+), and dissolved organic nitrogen (DON) concentrations. Boldface indicates significance at $P \leq 0.05$.

Effect	NO_3^- (mg L^{-1})		PO_4^{3-} (mg L^{-1})		NH_4^+ (mg L^{-1})		DON (mg L^{-1})	
	F	***P***	***F***	***P***	***F***	***P***	***F***	***P***
T	21.758	**<0.01**	22.229	**<0.01**	17.064	**<0.01**	6.472	**0.017**
F	8.583	**<0.01**	4.149	**<0.01**	1.270	0.305	5.025	**<0.01**
S	3.542	**<0.01**	5.340	**<0.01**	1.690	0.152	2.686	**0.029**
TxF	1.020	0.399	3.722	**0.023**	2.440	0.085	1.055	0.384
TxS	1.567	0.186	1.330	0.273	0.765	0.621	0.645	0.715
FxS	1.570	0.125	1.001	0.495	0.869	0.634	1.135	0.371
TxFxS	0.776	0.698	1.045	0.445	0.270	0.996	0.520	0.914

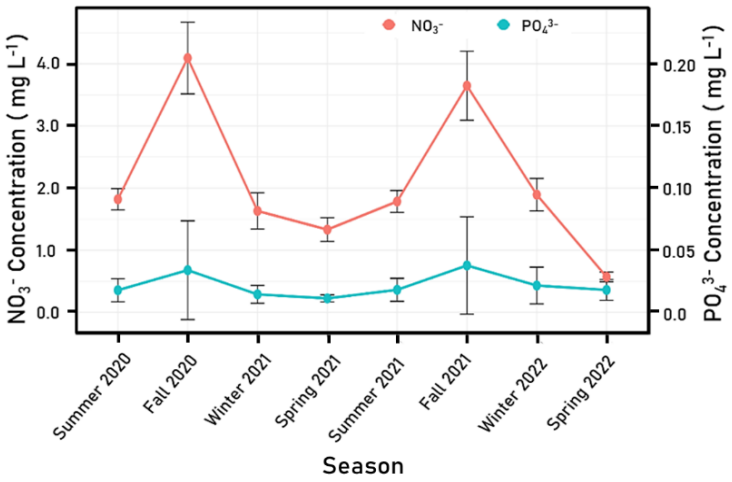

Figure 3.8: Drain gauge seasonal nitrate and phosphate concentrations (mg L^{-1}) across seasons. Bars are standard error of the mean (n=50).

The treatment effect on drain gauge leaching rates depended on facility establishment date and season (Table 3.8). Leaching rates varied between treatments for different facility establishment date (Table 3.8, TxE, $P = 0.04$), being highly significant for PO_4^{3-} (TxE, $P<0.01$), but only marginally significant for NO_3^- and DON (TxE, $P \leq 0.063$). While treatment differences were observed for all nutrients, highest leaching rates were observed for NO_3^- (Figure 3.9). NO_3^- leaching increased from recently established facilities to long-established facilities where average leaching rates approached 5 kg ha^{-1} yr^{-1}. On the other hand, absolute rates of PO_4^{3-}, NH_4^+, and DON leaching and the differences between effluent treated and untreated controls were greater at recently established facilities where average values did not exceed 1 kg ha^{-1} yr^{-1} (Figure B4 b, d & f). The dependance of facility establishment date on season (Table 3.8, ExS, $P < 0.01$) again reflects that

leaching is derived from drainage and we know that drainage was predominantly controlled by season (Table 3.4).

Table 3.8: Three-way analysis of covariance results for drain gauge nutrient leaching. *F* statistic (*F*) and *P*-values (*P*) are presented for measured effects of establishment date (E), reclaimed water treatment (T), season (S), and their interactions on nitrate (NO_3^-), phosphate (PO_4^{3-}), ammonium (NH_4^+), and dissolved organic nitrogen (DON) leaching. Boldface indicates significance at $P \leq 0.05$.

Effect	NO_3^- (kg ha^{-1})		PO_4^{3-} (kg ha^{-1})		NH_4^+ (kg ha^{-1})		DON (kg ha^{-1})	
	F	***P***	***F***	***P***	***F***	***P***	***F***	***P***
T	19.904	**<0.01**	32.555	**<0.01**	17.431	**<0.01**	8.642	**<0.01**
E	14.267	**<0.01**	1.311	0.253	5.281	**0.022**	6.664	**0.066**
S	47.391	**<0.01**	137.714	**<0.01**	13.921	**<0.01**	56.820	**<0.01**
TxE	3.476	0.063	33.032	**<0.01**	4.267	**0.04**	3.548	0.061
TxS	9.099	**<0.01**	11.559	**<0.01**	6.199	**<0.01**	4.152	**<0.01**
ExS	4.225	**<0.01**	8.876	**<0.01**	4.306	**<0.01**	16.568	**<0.01**
TxExS	0.680	0.689	2.410	**0.02**	1.056	0.392	0.785	0.600

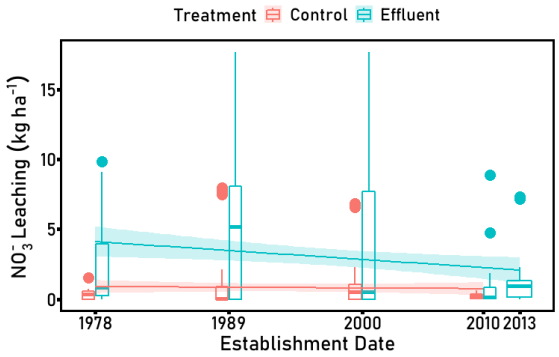

Figure 3.9: NO_3^- leaching of drainage sample collected with drain gauges: NO_3^- leaching (kg ha^{-1}) for facilities with different dates of establishment. Boxes delimit the 0.25 and 0.75 quantiles, horizontal lines indicate medians, whiskers extend to minimum and maximum values, and outliers are plotted as individual points. The highest outliers for 1989 and 2000 established facilities were excluded for clarity. Each box and whisker represents n=40 observations.

3.5.4 Comparison between lysimeter and drain gauge concentrations

Treatment and sampler type (lysimeter or drain gauge) had a significant effect on nutrient concentrations, except for DON (Table B5, T, Sp, $P < 0.043$). The inorganic nutrient concentrations (NO_3^-, PO_4^{3-} and NH_4^+) at effluent treated plots were consistently higher for drain gauge samples compared to lysimeter samples. Also, greater differences beween control and effluent treatments were observed for all nutrients in drain gauge samples. In contrast, nutrient concentrations between control and effluent treatments for lysimeter samples were only different for NO_3^- (Figure 3.10a), showing elevated levels of NO_3^- for both lysimeter and drain gauge samples at effluent treated plots. Lysimeter

DON concentrations were not significantly different between control and effluent treatments, and were comparable with drain gauge DON concentration at effluent treated plots (Figure 3.10d).

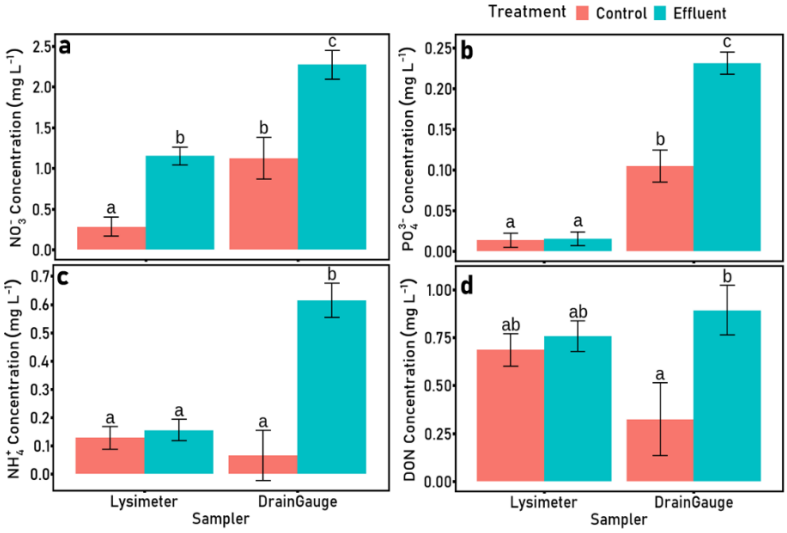

Figure 3.10: Comparison of soil water concentrations of (a) nitrate [NO_3^-] (mg L^{-1}), (b) phosphate [PO_4^{3-}] (mg L^{-1}), (c) ammonium [NH_4^+] (mg L^{-1}), and (d) dissolved organic nitrogen [DON] (mg L^{-1}) in lysimeter and drain gauge samples for control and effluent treatments. Each column represents the mean of all plots per sampler type (n = 200). Means within each panel having same letter are not statistically different ($P \leq 0.10$).

3.6. Discussion

Our results indicate that the regional forest water reclamation (FWR) facilities may be approaching nitrogen saturation, which may potentially lead to nitrate leaching with continued long-term reclaimed water application. The elevated soil water NO_3^- concentrations and leaching rates, particularly at long established facilities suggest that NO_3^-—N is most vulnerable to leaching with prolonged reclaimed water inputs (Figure 3.4a, 3.7 & 3.9). We observed significant responses of drainage, nutrient concentrations and leaching to treatment, facility, and season (Figure 3.3, 3.5, 3.7 & 3.8). The strong seasonal responses demonstrate the accuracy and precision of tests for treatment and facility. Early in operation, FWR facilities were highly effective at filtering N & P from the applied secondary treated wastewater. Effective assimilation of applied nutrients in reclaimed water at the FWR facilities is demonstrated by enhanced forest growth responses (Joshi & Coleman, 2023). However, with continuous annual application at permitted rates, nutrients increased in soil water concentration and leaching, indicating that forest ecosystems may have a finite capacity to assimilate nutrients.

3.6.1. Seasonal trends in drainage

Nutrient leaching is highly dependent on seasonal facility-specific conditions and is driven by drainage. Nutrient leaching rates were predominantly affected by seasonal drainage trends at each

facility, with treatment having a significant effect on overall drainage. Gibson et al. (2020) identified facility-specific seasonal factors such as precipitation as well as irrigation practices as the drivers of on-site drainage, with drainage remarkably increasing during the wet years with irrigation. The consistent and pronounced drainage trends across wet and dry seasons in our study also suggests a strong influence of season on drainage. Furthermore, we noted highest drainage during the wet season of both sampling years for the effluent treated plots at the long-established facilities, with drainage peaking in winter of both years (Figure 3.3). This suggests that treatment and season are important factors that significantly influence drainage which ultimately drives nutrient leaching. However, it is important to note that unexpected incidents may also affect drainage. For instance, mechanical failure caused Garfield to delay effluent application until late in season in 2022, which affected the drainage and ultimately the nutrient leaching rates.

3.6.2. Long-term Nutrient Assimilation in Forest Water Reclamation Systems

We hypothesized that regardless of operation time, forest water reclamation facilities will minimize nutrient leaching losses. Our results indicated that the soil water nutrient concentrations and leaching rates were well within the safe drinking water standards demonstrating that the forest water reclamation facilities did indeed continue to assimilate a range of water and nutrient additions up to permitted amounts. Yet, the nitrate concentrations and leaching rates were notably higher at long established facilities that have receive more than three decades of reclaimed water amendment, indicating that concentrated long-term reclaimed water application may cause nutrient saturation which may eventually lead to leaching losses from such forest land application ecosystems (Figure 3.9). Such nutrient saturation and subsequent leaching can be avoided, and the life expectancy of forest land application sites can be extended by spreading reclaimed water over larger area, establishing alternate treatment plots, and partnering with adjacent landowners to spread reclaimed water more broadly.

3.6.2.1. N saturation and leaching

N addition through reclaimed water application is known to increase forest N availability which causes N saturation, increased N cycling, nitrification and ultimately leaching (Magill et al., 2004). Increased nitrification is common in N-saturated forest soils (Isobe et al., 2018) and N addition is linked with higher ammonia monooxygenase gene (*amoA*) (Compton et al., 2004). Found in ammonia-oxidizing bacteria, *amoA* encodes for ammonia monooxygenase which is used for autotrophic nitrification involving conversion of ammonia to nitrite (Purkhold et al., 2000). NO_3^--N is especially vulnerable to leaching due to minimal anion binding sites and low reactivity within the soil

profile. Our study found NO_3^- concentrations were orders of magnitude higher than other nutrient concentrations and was greatly affected by treatment, with effluent showing significantly higher NO_3^- concentrations compared to control. While NH_4^+ dominates over NO_3^- in organic horizon and is retained by sorption complex, NO_3^- ions are mobile and easily leached into the soil profile (Bechtold et al., 2003). Further research involving quantification of nitrifying organisms and nitrification rates at the forest water reclamation facilities will provide more insight into N cycling in response to decadal time-scale of reclaimed water application.

Feyen et al. (1999) suggested that NO_3^- losses are common during periods of high water-flow which takes place in winter and snowmelt periods during the wet season. Other factors that can result in nutrient leaching are periods of low demand, enriched upper soil layer and flushing (Feyen et al., 1999). Our results agreed by showing peaking of lysimeter NO_3^- concentrations and leaching rates during fall of both sampling years. This peaking of leaching rates during the wet season and immediately following the treatment season is also likely due to the decrease in nutrient demand during non-growing season and flushing of nutrients accumulated during Summer application. Snowmelt in Spring may account for some of the leaching observed in Spring 2022. Low denitrification rates are attributed to limited soil moisture availability during summer when site conditions are hot and dry that restrict solute diffusion, and aerobic conditions which limit metabolism of denitrifying bacteria (J. Luo et al., 1999). Other studies suggest that bacterial metabolism and organic N mineralization and nitrification rates may increase in summer due to high temperature and moisture conditions which may accelerate movement of water and substrate in soil-water system (Hooda et al., 2003). Despite expected increase in nitrification rates during Summer, we assume that the reduced leaching rates are likely due to increased vegetation uptake and reduced overall drainage. On the other hand, NO_3^- utilization greatly decreases in winter season due to reduced microbial activity, mineralization and nitrification, and vegetation uptake and increases in Spring with rise in soil temperature (Sahoo, 2022). Similarly, Johnson (1992) and Strader et al. (1989) suggest that mineralization and nitrification rates are highest in spring and fall and linked with increased leaching through soil, which agrees with our findings. However, others have found NO_3^- leaching to be the lowest during the winter months in majority of the forest ecosystems, particularly in the upper soil horizons (Foster et al., 1989; Nadelhoffer et al., 1983; Shepard et al., 1990; Strader et al., 1989).

Similarly, our findings show that the drain gauge nutrient concentrations and leaching rates, particularly NO_3^-, were greatly affected by treatment and season. DON concentrations in drain gauge samples, while lower than NO_3^- concentrations, were considerably higher at younger facilities (Figure B4). This is likely due to reduced mineralization of organic N due to shorter treatment periods at the younger facilities. While our results did not show significant DON leaching losses, others suggest that

DON is susceptible to leaching from forest floor (Qualls et al., 2000). Furthermore, Harvard Forest chronic N study also showed that N addition enhances DON leaching from the forest floor (Mcdowell et al., 1998). Others suggest that DON flux with similar inorganic inputs has been found to be considerably lower for temperate forests, however, DON leaching still remains a risk under saturated conditions (Fang et al., 2009). However, NO_3^- leaching was dominant at our forest water reclamation facilities.

3.6.2.2.Phosphorus leaching

Our study found that the PO_4^{3-} concentrations to be quite low, which may be attributed to slow diffusion and high P fixation or accumulation in the topsoil (Schachtman et al., 1998; J. Shen et al., 2011). This might also be explained by the significantly lower cumulative P loading compared to N loading (Figure 3.1). Despite low inputs, vertical macropores that bypass soil matrix can drain or leach soluble reactive P from the topsoil deeper through the soil profile (Gächter et al., 2004; Gächter & Wehrli, 1998; Stamm et al., 1998). In addition, P is required by vegetation at a lower level compared to N. Results show PO_4^{3-} concentrations approaching 1 ppm (Figure 3.8), which while comparatively lower than N, is the set limit by EPA as a clean water standard (US EPA, 1986). P is considered completely removed from the soil solution by sorption reaction on soil colloidal surfaces and thus no leaching occurs. In addition, P availability is limited by slow rock weathering and adsorption into Fe and Al oxides in acidic forest soils (Binkley & Fisher, 2013). However, a ten-fold increase in PO_4^{3-} concentration was observed for drain gauge vs lysimeter in control plots and a doubling of PO_4^{3-} concentration of effluent over control, which shows an increased risk of environmental pollution even with low PO_4^{3-} concentrations. Similar to NO_3^- , PO_4^{3-} concentrations showed similar seasonal trends, slightly peaking during Fall. However, the magnitude of PO_4^{3-} concentrations were significantly lower compared to NO_3^-, which is likely due to adsorption of majority of applied PO_4^{3-} in the soil matrix. Lower PO_4^{3-} leaching rates can also also be due to lower cumulative constituent P loading rates in the applied reclaimed water which are orders of magnitude lower than N. However, elevated P concentrations in northern Idaho surface water sources has been an important environmental concern. Understanding P cycling and fate of P at forest water reclamation facilities is essential and environmental risk for P leaching losses can be quantified using indices that measure the degree of P saturation (Kovar et al., 2009).

3.6.3. Factors affecting nutrient leaching losses

Efficacy of forest ecosystems to assimilate and retain nutrients from applied wastewater is largely dependent on irrigation management as well as facility-specific soil physical, chemical and

biological attributes, vegetation type and uptake capacity, and seasonal conditions (Barton et al., 2005a; Borken & Matzner, 2004; Gibson et al., 2020; Sahoo, 2022). Forest ecosystems in well-managed land application systems are able to effectively assimilate contaminants and nutrients in applied wastewater with improved vegetation growth responses (Magesan & Wang, 2003), nonetheless many facility-specific factors cannot be controlled and hydraulic and constituent loading rates needs to be customized befitting each facility to prevent nutrient losses. Other factors include tree species or vegetation type (Borken & Matzner, 2004), rainfall, irrigation, and seasonal drainage rates (Gibson et al., 2020). Greater leaching losses have been observed in mature forests compared to young vigorously growing forests (Vitousek & Reiners, 1975; Van Miegroet, Cole & Foster, 1992). Stand age plays an important role when determining N uptake, with uptake rates declining sharply after crown closure as the nutrient-rich foliar biomass reaches a steady state (Miller, 1981; Switzer & Nelson, 1972; Turner, 1981). Because our FWR facilities have reached maturity, NO_3^- leaching losses may be due to decreased assimilation capacity that comes with stand maturity. Decline in N assimilation and continued application in mature stands have been found to potentially lead to NO_3^- leaching losses (Hook & Kardos, 1978). Hydraulic constituent loading rates may ultimately need to be adjusted with time to match the stand assimilation capacity because nutrient leaching is highly dependent on irrigation rates. While temperate forests have been found to be limited in N and able to retain low levels of N amendments (Fox et al., 2007), forest ecosystems can become saturated when application rates exceed forest soil and vegetation assimilation capacities (Aber et al., 1989). NO_3^- may be accumulated over longer time periods and leaching limited by availability of transport (Bechtold et al., 2003). Thus, length of application, particularly at the older facilities, may have increased soil nutrient concentration and subsequent leaching losses.

3.6.4. Matrix versus Preferential Flow paths

Flow pathways can include matrix flow, P sorption to soil matrix in the deeper layers or fast flow along preferential flow paths that are biogeochemically inert (Gächter et al., 2004). Present loading rates by Idaho Department of Environmental Quality (IDEQ) assume that the receiving forests can assimilate the applied water and nutrients, and we only need to avoid drainage during the growing season. However, observed elevated drainage levels and leaching in Winter, Fall and Spring suggest that drainage and nutrient leaching may occur during other seasons. Our study found that NO_3^- loss occurred through both matrix and preferential flow where NO_3^- concentration in both lysimeter and drain gauge samples were significantly higher for effluent treatment. In contrast, NH_4^+ concentrations were significantly lower than NO_3^- for both lysimeter and drain gauge samples. This is likely because soil exchangeable NH_4^+ may be elevated for a short time-period after fertilization

(Johnson et al., 1980; Morrison & Foster, 1977), but is rapidly reduced to low concentrations by vegetation and heterotrophic uptake, volatilization, non-biological reactions in soil with humus and 2:1 clays and nitrification (Johnson, 1992).

Preferential flow is the pre-dominant flow path for nutrient transport in forests and has a significant effect on increasing the risk of soil and groundwater contamination (Julich et al., 2017a; Lipsius & Mooney, 2006; Ronkanen & Kløve, 2009). Preferential flow also decreases the duration of contact or the residence time of the water in the soil (Addiscott & Thomas, 2000). While PO_4^{3-} moves along pathways bypassing a fraction of porous matrix, NO_3^- can move downwards via matrix flow or preferential flow pathways, indicating a risk of non-point source pollution with N and P (Salazar et al., 2018). The amount of nutrients leached is also controlled by the leached water volume (Duan, Fedler, et al., 2010). Thus, site hydrologic processes combined with rapid movement through preferential flow path may accelerate leaching and affect the amount of nutrients lost from soil. Rapid preferential flow of water and solutes occur through macropores, root channels, cracks and fissures which allows for little interaction with the soil matrix, plant roots and microbes (Feyen et al., 1999; Hornberger et al., 1991; Mulholland et al., 1990; Turton et al., 1995). Reduced demand for nutrient uptake by vegetation during the dormant seasons (Fall and Winter) may be attributed to the observed losses through preferential flow paths.

Our study found that the treatment differences for PO_4^{3-} concentrations in lysimeters samples show minimal P loss through matrix flow. In contrast, large treatment differences were observed with drain gauges, indicating P loss to occur through both matrix and preferential flow paths. While WEPP model revealed that the P loss through runoff is minimal at forested sites (Clark, 2022), there is still a risk of P loss through preferential flow. This is consistent with other studies that have shown that P can also be lost through preferential flow paths in forest ecosystems (Bol et al., 2016; Julich et al., 2017b; Makowski et al., 2020). However, it is important to note that the drain gauges may create preferential flow paths at the edge of the soil column along the diversion tube. Therefore, our results maybe overestimates of nutrient losses through preferenital flow.

3.6.5. Time series platform for understanding nutrient leaching

The time-series nature of this study provides a valuable opportunity to study the long-term effects of reclaimed water on nutrient saturation and leaching potential in forested land application systems, particularly NO_3^-, which is vulnerable to leaching. More detail on time series studies can be found in Chapter 2. Our results indicate that facilities are approaching N saturation with elevated NO_3^- leaching within three decades of reclaimed water application. Such long-term leaching rates enable facility managers and regulators to identify hydraulic and nutrient assimilation limits of

receiving forests and formulate management strategies that prevent nutrient saturation and environmental degradation.

3.7. Conclusions

While reclaimed water addresses water and nutrient limitations in western forests, there are potential environmental implications when hydraulic and constituent loading rates exceed forest requirements. Prolonged continuous input of reclaimed water amendment may saturate the forest system and increase the risk of nutrient leaching. The time-series of facilities presents a unique opportunity to investigate long-term effects of reclaimed water amendment on forest soil water nutrient concentrations and leaching rates. Proper metering of hydraulic and constituent loading rates is important to prevent saturation and leaching and to protect surface and ground water resources. While forests are effective at nutrient assimilation through uptake and storage, continued artificial long-term inputs may elevate nitrification and potentially lead to NO_3^- leaching, particularly through preferential flow pathways. Western forests are inherently limited in N and P, and long-term reclaimed water application may exceed the forest nutrient assimilation capacity and result in soil nutrient saturation and leaching. Results suggest that forest water reclamation systems may start leaching nitrate within 22 years of application with NO_3^- leaching reaching as high as 5 kg ha^{-1}. While the nutrient concentrations in leachates are well within drinking water quality standards, older facilities are at greater risk of increased nutrient losses and impacts on environmental quality. It is therefore important to implement management practices that minimize preferential flow and increase reclaimed water contact time in soil that allows plant uptake and soil storage.

Chapter 4: Soil Biological Responses at Forest Water Reclamation Facilities in Northern Idaho

4.1 Abstract

Forest water reclamation is a well-established approach to repurpose reclaimed water using forested land application systems. However, addition of nutrients such as nitrogen and phosphorus in reclaimed water can alter soil CO_2 efflux, exoenzyme activities, and amino compounds (alanine, leucine, and N-acetyl-D-glucosamine), and potentially impact soil quality and forest productivity. The objective of this study was to characterize how soil quality biological indicators such as soil CO_2 efflux, exoenzyme activities and amino compound concentrations respond to decades of soil resource amendments in comparison to adjacent non-amended stands. Soil CO_2 efflux, exoenzyme activities and amino compounds were studied at a time-series of five forest water reclamation facilities in northern Idaho. We investigated the treatment effects on soil CO_2 efflux, evaluated activities of five exoenzymes that represent C-cycling, N-release, and P-release, and quantified the concentrations of amino compounds at the forested systems. Reclaimed water amendment during the growing season had little effect on soil CO_2 efflux. Though there were no treatment effects, we found a seasonal response, with lowest soil CO_2 efflux observed during winter. N-releasing chitinase activity and P-releasing acid phosphatase activities were suppressed by effluent treatment especially at long-established facilities due to a possible shift in microbial composition. In addition, higher amino acid uptake for the reclaimed water treated plots at the recently established facilities may indicate N-limitation and reliance on a broader range of organic and inorganic sources for N acquisition from the ecosystem.

Keywords: reclaimed water, soil CO_2 efflux, exoenzyme activity, amino compounds, soil quality

4.2 Introduction

Forest water reclamation addresses nutrient and water limitations in western forests and has the potential to improve tree growth responses through increased resource availability (Joshi & Coleman, 2023). Reclaimed water has often been used to improve soil quality due to the nitrogen (N), phosphorus (P) and micronutrient content which can be beneficial for soil productivity (Brzezińska et al., 2001; Brzezinska et al., 2006). Soil biological characters are considered sensible indicators of soil quality, and are more sensitive than physical or chemical soil properties (Friedel et al., 2000). Long-term, continuous amendments of N and P may alter biological processes such as nutrient cycling, and other microbiological processes of forest ecosystems. Reclaimed water application has been known to both increase and decrease soil biological activity (Brzezińska et al., 2001; Jian et al., 2016; Meli et

al., 2002). While biological processes are sensitive indicators, they also depend on many environmental factors, making it necessary to also understand the interactions with these factors.

Soil CO_2 efflux is sensitive to biotic and abiotic environmental conditions such as temperature, moisture, soil, vegetation, substrate availability, composition and activity of microbial community (Y. Luo & Zhou, 2006; Schlesinger & Andrews, 2000; Fér et al., 2022). Although irrigation in arid environments typically increases soil CO_2 efflux (Y. Luo & Zhou, 2006), additions of inorganic N (NH_4^+ and NO_3^-) has been found to have variable effects on soil CO_2 efflux and other measures of microbial activity (Micks et al., 2004). Soil respiration increases (Hopkins et al., 2013) or decreases (Giardina et al., 2003; Sun et al., 2014; Zhou et al., 2014; Olsson et al., 2005; Phillips & Fahey, 2007; Zhang et al., 2016; Janssens et al., 2010) in response to fertilization. N directly stimulates primary production (Vitousek & Howarth, 1991) resulting in more substrate for soil respiration (Y. Luo & Zhou, 2006). However, in environments with surplus of N, fertilization could cause N leaching and have little effect on soil respiraiton (Y. Luo & Zhou, 2006). In reclaimed water or chronic N addition studies, soil CO_2 efflux does not increase (Micks et al., 2004; Schipper et al., 1996a) and in some cases decreases over time (Bowden et al., 2004). Soil CO_2 efflux is an important indicator of biological response, but its response to reclaimed water or chronic N amendment suggests that such integrating measures of biological response can be neutral or negative despite accumulations in soil organic matter.

Nutrient cycling is mediated by soil microbiological processes on which nutrients added with reclaimed water could have substantial impacts. Soil enzymes are produced by soil microbes to catalyze biogeochemical cycling of nutrients such as C, N and P (Alkorta et al., 2003). These extracellular enzymes mediate organic matter decomposition and nutrient cycling in soil and breakdown of biological macromolecules present in litter and soil such as cellulose, hemicellulose, chitin and protein (Allison et al., 2007; DeForest, 2009a; Fog, 1988; Saiya-Cork et al., 2002a). N mineralization is mediated by microbes through the regulation of activities of cellulase (β-glucosidase), protease (aminopeptidase), and chitinase (β -N-acetyl-glucosaminidase) (Ekenler & Tabatabai, 2004; Tabatabai et al., 2010). Phosphatase enzyme transforms P from unavailable organic form into PO_4^{3-} ions that are easily available for uptake for plants and microbes (Eivazi & Tabatabai, 1977), and can be an indicator of potential P mineralization and biological activity in soils (Dick & Tabatabai, 1993). C and N releasing exoenzyme activities help maintain balance between C and N availability in soil and are correlated with phosphatase activities (Bowles et al., 2014; Fatemi et al., 2016; Shan et al., 2014; R. L. Sinsabaugh & Shah, 2012). This is particularly important in western forests limited by N (Edmonds et al., 1989; Shan et al., 2014). N addition has been shown to increase (Brzezinska et al., 2006; W. Chen et al., 2008; Dong et al., 2015; Filip et al., 2000) and decrease

(Fatemi et al., 2016) exoenzyme activity or have both positive and negative effects (Allison & Vitousek, 2005; H. Chen et al., 2018; Jian et al., 2016). Reclaimed water application may change microbial dynamics, community composition and enhance biological activities due to added nutrients (W. Chen et al., 2008).

Decomposition of organic matter by soil enzymes and release of available nutrients are profoundly important soil biological processes that can respond to wastewater amendments. Assessment of product pools of amino compounds relates to the importance of released compounds relative to their demand. Measuring nutrient demand allows us to understand forest productivity and soil quality as nutrient uptake and forest productivity are tightly linked (Aubrey et al., 2012). Soil amino acids organic N are integral for plant nutrition (Werdin-Pfisterer et al., 2009). Plants are able to take up free amino acids and do not have to completely rely on inorganic N for nutrient acquisition when mineralization is limited (Näsholm et al., 1998; Nordin et al., 2001; Schimel & Stuart Chapin, 1996; Werdin-Pfisterer et al., 2009). However, free amino acids are present in low concentrations in bulk soil likely due to rapid turnover by soil microbes (Jones, Shannon, et al., 2005). An increase in amino acid uptake during plant and microbial growth cycles can cause decline in soil amino acid concentrations (Jones, Healey, et al., 2005). Seasonal dynamics may result in decomposition of soil organic matter in litter with microbial, mycorrhizal and root tissue lysis followed by flushing of amino acids into soil (Ivarson & Sowden, 1966; Lipson & Monson, 1998). Therefore, amino compounds availability are largely dependent on facility-specific and environmental factors.

Many land application systems have been in operation for decades. Yet, soil nutrient cycling responses to long-term reclaimed water land application and effects of N enrichment on enzyme activities is poorly understood (H. Chen et al., 2018; Schnecker et al., 2015). The goal of this study is to characterize some soil biological responses to decades of resource amendments in comparison to adjacent non-amended stands. We are not aware of existing literature on the long-term impacts of land application of reclaimed water on the combination of soil CO_2 efflux, exoenzyme activities and available product pools. Evaluation of enzyme product pools provides a measure of nutrient turnover which is indicative of nutrient availability and improved soil quality and is thus an important tool for understanding biological processes. Regular inputs of water and nutrients are expected to significantly alter nutrient cycling processes. I hypothesize that increased nutrient inputs over decadal time scales will not greatly enhance soil CO_2 efflux. With higher inorganic ion concentrations and resulting negative feedback, I also hypothesize that N additions from reclaimed water uptake will cause a shift in exoenzyme activity away from N limitation to P limitation, resulting in suppressed N-releasing exoenzyme activities and enhanced P-releasing exoenzyme activity. Enzyme activities will be higher

in litter due to greater substrate availability. Amino compound concentrations will not be affected by reclaimed water amendments, also due to readily available inorganic nutrient pools.

4.3 Material and methods

4.3.1 Study Facilities

The study was conducted at five water reuse facilities situated along Lake Coeur d'Alene and Lake Pend Oreille in northern Idaho, United States (Figure 2.1). All facilities were established between 1978 and 2013 to create a four-decade time series. To determine reclaimed water treatment effects on forest responses, five one-tenth-acre measurement plots were established in management units at each of the five study facilities along with five adjacent control plots (n=50, Figure A1 and Figure A2). Where possible, the control plots selected had comparable soil, stand composition and structure as the treatment plots.

The Cave Bay reuse facility was established in 2013 and had Lacy gravelly loam soils with parent material of loess and/or colluvium over bedrock derived from basalt. The Heyburn State Park reuse facility was established in 2010 and the soils were Carlinton ashy silt loam derived from volcanic ash over loess and were moderately well drained, and the ecological site was warm-frigid, xeric, unglaciated, loamy and fragipans (Soil Survey Staff, 2019). The Ellisport Bay facility was established in 2000. The Bottle Bay and Garfield Bay facilities were established in 1989 and 1978. Soils at the Pend Oreille facilities (Ellisport Bay, Bottle Bay and Garfield Bay) were Pend Oreille silt loam characterized by parent material of volcanic ash and/or loess over till derived from granite and/or metamorphic rock (Table 4.1) (Soil Survey Staff, 2019). More details on location, vegetation and soil information can be found in Chapter 1.

Table 4.1. Study facilities tree and soil information, including dominant tree species, soil texture, soil depth, soil pH and soil bulk density.

Reuse Facility	Soil texture[1]	Soil depth (cm)	Soil pH	Soil bulk density (gm^{-3})
Cave Bay	Gravelly loam	0-15	6.9±0.09	0.8±0.04
Heyburn SP	Silt loam	0-15	6.5±0.09	0.99±0.05
Ellisport Bay	Silt loam	0-15	6.4±0.07	0.85±0.04
Bottle Bay	Silt loam	0-15	6.2±0.08	0.68±0.04
Garfield Bay	Silt loam	0-15	6.7±0.09	0.71±0.04

[1]Soil Survey Staff, 2019

4.3.2 Class C Reclaimed Water Amendment

The water reuse facilities in the study have low-cost lagoon primary treatment system and generate Class C municipal reclaimed water which undergoes aeration and disinfection before being applied on regional forests. All five water reuse facilities have been annually applying reclaimed water during the growing season between April 1 to October 31 (IDEQ) since their establishment.

The average annual hydraulic loading rate for reclaimed water was 30 cm yr^{-1} and nutrient loading rates of 37 kg ha^{-1} N and 14 kg ha^{-1} P were applied at the effluent amended facilities. Thus, the facility established in 1978 has received almost ten times more N and almost seven times more P than the facility established in 2013 (Chapter 2).

4.3.3. Soil Respiration

Soil CO_2 efflux was measured quarterly (Summer 2020-Spring 2022) using a Portable CO_2 Gas Analyzer (EGM-5 with an SRC-2 Soil Respiration Chamber, PP-Systems). Three soil CO_2 efflux measurements were collected in each of the 50 experimental plots and were averaged for analysis. The soil respiration chamber (SRC) was inserted directly into the soil surface with a volume of 1093 ml and soil surface area of 78 cm^2. The SRC termination settings were set at a ΔT of 60 seconds, ΔC of 600 ppm, where T is the time and C is the concentration of gas (g CO_2 cm^{-3} air). A manual zero was initiated before each reading before taking a measurement at each plot to ensure calibration accuracy.

4.3.4. Soil pH, moisture, and temperature

Soil pH was measured (1:1, v:v, soil to water) on composite samples from each plot with ion electrodes (Accumet pH meter) in summer 2020 before performing the seasonal exoenzyme assays. Field soil temperature and moisture measurements were collected seasonally with each soil CO_2 efflux measurement at 15 cm soil depth with a soil temperature probe (HydroSense II, Campbell Scientific). A soil temperature probe (6000-09TC Soil Probe Thermocouple, Li-Cor) was also used for concurrent measurement of field temperature during collection of soil samples for enzyme assay.

4.3.5 Exoenzyme Assay

Activities of six exoenzymes were measured. N-releasing exoenzymes which catalyze the end reaction of protein depolymerization and control the release of the amino compounds included alanine aminopeptidase (AM) which represents the aminopeptidases that free single amino acids during protein degradation, leucine aminopeptidase (LAP) which catalyzes the hydrolysis of leucine and other hydrophobic amino acids at the N-terminus of polypeptides, and N-acetyl-β-D-glucosaminidase or chitinase (CH) which releases glucosamine from chitin were measured. The C exoenzyme was measured as β-glucosidase (BG) which frees glucose, and the P exoenzyme as acid phosphatase (PH) which frees phosphate from organic matter (Table 4.2) (Wang et al., 2020; Shan & Coleman, 2020).

Table 4.2. Substrates and standards used in enzyme assay.

Enzyme	Substrate	Standard
Alanine Aminopeptidase (AM, EC [Enzyme Commission] 3.4.11.2)	L-Alanine 7-amido-4-methylcoumarin (AMC) trifluoroacetate salt (A4302, Sigma)	7-Amino-4 Methylcoumarin (257370, Sigma)
Leucine Aminopeptidase (LAP) (EC 3.4.11.1)	L-leucine-7-amido-4-methylcoumarin hydrochloride (L2145)	7-Amino-4 Methylcoumarin
β-glucosidase (BG, EC 3.2.1.21)	4-Methylumbelliferyl (MUB) β-D-glucopyranoside (M3633, Sigma)	4-Methylumbelliferone (M1381, Sigma)
N-acetyl-β-D-glucosaminidase or Chitinase (CH, EC 3.1.6.1)	4-MUB-N-acetyl-β-D-glucosaminide (M2133, Sigma)	4-Methylumbelliferone
Acid Phosphatase (PH, EC 3.1.3.2)	4-MUB-phosphate (M8883, Sigma)	4-Methylumbelliferone

4.3.5.1. Sample Collection and Exoenzyme Assay

Soil samples were collected seasonally from three random locations in each plot by removing the litter and excavating soil from A and B_w horizon to a 10 cm depth. Soil temperatures were collected at each plot during the time of sampling. The composite soil samples from the three random locations were transported on ice to the lab. In lab, the composite samples were homogenized and passed through a 42-mm sieve to remove roots, coarse fragments, and rocks, and refrigerated at 4˚C. The litter and soil samples were collected for Spring 2022. The litter samples were cut into 1 cm^2 pieces using a food chopper (Cuisinart).

Due to the time-sensitive nature of enzyme assay, degradation of enzymes occur and measured activity is greater with less time in refrigeration prior to analysis (DeForest, 2009b). The buffer, substrate stock solutions and standard solutions were prepared prior to analysis. Sodium citrate buffer (500 mM) was prepared by mixing sodium citrate and citric acid (Sigma) with milli Q water. The pH was adjusted using 6M NaOH and stored at 4˚C between analysis for no longer than 7 days and remade every week of analysis. Due to the long-term acclimation of soil enzyme activity to pH (Puissant et al., 2019), the pH of the buffer was adjusted to the mean soil pH of each facility. Substrate stock solutions of 200 µM (Table 4.2) were prepared in advance except peroxidase which was freshly prepared the day of analysis. Extracellular or exoenzyme assays were carried out within 24 hours of sample collection. Soil moisture content of soil enzyme samples was measured in the laboratory using a moisture balance (HB43-S Mettler-Toledo).

Soil slurries were prepared by adding 1 g dry equivalent soil with 125 ml of 50 mM citrate buffer (adjusted with facility pH) in 200-ml polypropylene bottles and homogenized for 1 min using a handheld blender (Cuisinart Smart Stick). The suspensions were transferred to Pyrex bowls (Corning, NY, USA) and continuously stirred using a magnetic stir plate. Aliquots of 200 µl of suspensions were dispensed into 96-well microplates containing buffer, substrates, and standards. The microplates were incubated with temperature set to seasonal facility conditions, for 6 h (AM), 6 h (LAP), 3 h (BG), 2 h (CH), and 4 h (PH). A 10 µl aliquot of 0.1 M (AMC Assays) and 1.0 M (MUB Assays) NaOH was added to each well to stop the reaction. Fluorescence was measured using a microplate reader (Synergy HT, Bio Tek) with a 360-nm excitation and 450-nm emission filters. Enzyme activities were corrected for negative controls and quenching and expressed in units of nmol h^{-1} g^{-1} (DeForest, 2009a; Saiya-Cork et al., 2002b).

4.3.6 Amino Compounds

Amino compounds, measured as concentrations of alanine, leucine and N-acetyl-D-glucosamine (NAG), were extracted in 0.5 M HCl (hydrochloric acid) and measured using GCMS (Wei et al., 2015; Zampolli et al., 2007) and HPLC (Glaser et al., 2004; Huber & Bonn, 1995) respectively. A soil mass of 7 g dry equivalent was mixed with 35 ml of DI water. The samples were shaken (60 minutes), centrifuged (2600 rpm for 20 minutes at 4 ˚C) and the supernatant was filtered through a vacuum filter using Whatman 42 filter paper. The filtered sample was lyophilized until dehydrated and stored in freezer until analysis.

For amino acid analysis, the lyophilized samples were reconstituted with 1 ml 0.05 HCl, sonicated and centrifuged. Alanine and leucine in the supernatant were determined using GC-MS method based on a derivatization reaction which uses a mixture of alkyl chloroformate-alcohol-pyridine as reagents (Zampolli et al., 2007). The reagents used for derivatization reaction were pyridine, chloroform, methyl chloroformate (MCF), methanol, and acetonitrile (Sigma-Aldrich). A standard solution of methyl laurate in acetonitrile (0.012 M) was prepared as internal standard. An aliquot of 25 µl of the standard stock solution (0.01 M of L-alanine and L-leucine) was pipetted into a 0.6 ml plastic tube. A dilution series of the standard stock solutions of 0.001 M and 0.0001 M were prepared for calibration. 100 µl of soil supernatant was pipetted into the tube. To the amino acid standards and soil solutions, 10 µl of internal standard, 50 µl of methanol and 15 µl of pyridine were added and the tube was closed and shaken. 15 µl of MCF was added to each sample and standard in 5 µl increments slowly and the tube was vortexed at first for 30 seconds, allowed to rest for 5-10 minutes and then re-vortexed for the second time for 10 seconds. Saturated NaCl (sodium chloride) aqueous solution (20 µl) was added to the vortexed tubes and the suspension was vortexed again for

15 seconds. MCF (200 µl) was added to the tube and the suspension was further mixed and let stand for 2-5 minutes followed by centrifugation for 1 minute for phase separation. The bottom organic layer was removed in two 90 µl aliquots and transferred to a GC vial and analyzed using Thermo ISQ7000-Trace GCMS with autosampler (Thermo Fischer Scientific) (Analysis Protocols, Appendix D6).

For NAG analysis, 900 µl of supernatant extracted in 0.05 M HCl was sonicated, centrifuged, and filtered through 0.45µm syringe filter into a HPLC vial. The samples were analyzed using Thermo Scientific SpectraSYSTEM AS3000 autosampler (Thermo Scientific) along with N-acetyl glucosamine standard (1 mg in 100 ml DI water) and a series of dilutions of the stock standard solution (1/10, 1/20 and 1/50) (Analysis Protocols, Appendix D5).

4.4 Statistical Analysis

The effect of reclaimed water treatment, facility, season, date of establishment and their interactions on soil CO_2 efflux, soil volumetric content, soil temperature, exoenzyme activities and amino compounds were tested with SAS Software version 9.4 (SAS Institute Inc, Cary, NC). Analysis of variance was performed on the above-mentioned dependent response variables in a two-way factorial model that included treatment, facility, and season as class variables. Analysis of covariance was performed on the same dependent variables with treatment and season as independent class variables and establishment date of facility as the continuous covariate in the models. Type III test of fixed effects were used to examine the main effects and interactions. Differences were considered significant at $P \leq 0.05$. If a significant effect was found, Tukey-Kramer tests were performed for multiple comparisons. Where necessary, the data was transformed to meet the normality and homoscedasticity assumptions for analysis of variance.

4.5 Results:

4.5.1 Soil Environment and CO_2 Efflux

The effect of effluent treatment on soil temperature, moisture and soil CO_2 efflux depended on facility and season (Table 4.3, TxFxS, $P < 0.01$). Differences in temperature between control and effluent treatments were only observed at Heyburn State Park (Figure 4.1 a). The greatest effect of treatment was observed on volumetric water content, which was consistently higher at effluent treated plots for all five reuse facilities (Table 4.3, T, TxF, $P < 0.01$, Figure 4.1 b). Soil CO_2 efflux also varied by facility with lowest soil CO_2 efflux observed for Ellisport Bay effluent treatment (Figure 4.1 c).

Season had a significant effect on soil temperature, moisture, and soil CO_2 efflux (Table 4.3, S, $P < 0.01$). The greatest seasonal effect was observed for soil temperature (Table 4.3, S, F = 4432.03), with highest temperature measured during Spring and Summer and lowest temperature during Winter, as expected (Figure 4.2 a). However, soil temperature was not different between control and effluent treated plots. Similarly, volumetric water content was highest during Fall and Winter and was comparatively higher at effluent treated plots (Table 4.3, TxS= 0.078, Figure 4.2b). Facility-specific differences between treatment in soil volumetric water content is more clearly observed in Figure 4.1 b. Soil CO_2 efflux was lowest during Winter of first year of sampling (Table 4.3, TxS, $P = 0.047$, Figure 4.2c). We were unable to measure soil CO_2 efflux during Winter of second year of sampling due to snow accumulation.

Facility establishment date and season also accounted for treatment effect on soil volumetric water content and CO_2 efflux (Table 4.3, TxE, ExS, TxExS, $P < 0.01$). Highest soil CO_2 efflux was observed during Spring of the second sampling year at effluent treated long-established facilities (Figure C1f), while soil CO_2 efflux during Summer of 2020 was greatest at the effluent treated plots at the recently-established facilities (Figure C1a).

Table 4.3. Three-way analysis of variance and covariance results for Tsoil, VWC% and soil CO_2 efflux. *F* statistic (*F*) and *P*-values (*P*) are presented for measured effects of reclaimed water treatment (T), Facility (F), season (S), establishment date (E), and their interactions on soil temperature (Tsoil), volumetric water content (VWC%) and soil CO_2 efflux. Boldface indicates significance at $P \leq 0.05$.

Effect	**Tsoil**		**VWC%**		**soil CO_2 efflux**	
	F	***P***	***F***	***P***	***F***	***P***
ANOVA						
T	4.23	0.109	60.24	**<0.01**	0	0.970
F	491.88	**<0.01**	12.26	**<0.01**	15.78	**<0.01**
S	4432.03	**<0.01**	270.54	**<0.01**	34.25	**<0.01**
TxF	4.57	**0.012**	5.13	**<0.01**	3.53	**0.030**
TxS	3.24	0.138	4.7	0.078	6.41	**0.047**
FxS	531.37	**<0.01**	7.61	**<0.1**	14.03	**<0.01**
TxFxS	1.64	0.170	2.28	0.056	5.08	**<0.01**
ANCOVA						
T	0.02	0.891	7.38	**<0.01**	1.34	0.248
E	0.65	0.421	0.06	0.809	2.55	0.111
S	7.83	**<0.01**	6.45	**<0.01**	11.45	**<0.01**
TxE	0.02	0.889	7.15	**<0.01**	1.34	0.249
TxS	0.02	1	1.7	0.120	6.34	**<0.01**
ExS	7.99	**<0.01**	6.51	**<0.01**	11.45	**<0.01**
TxExS	0.02	1	1.69	0.123	6.35	**<0.01**

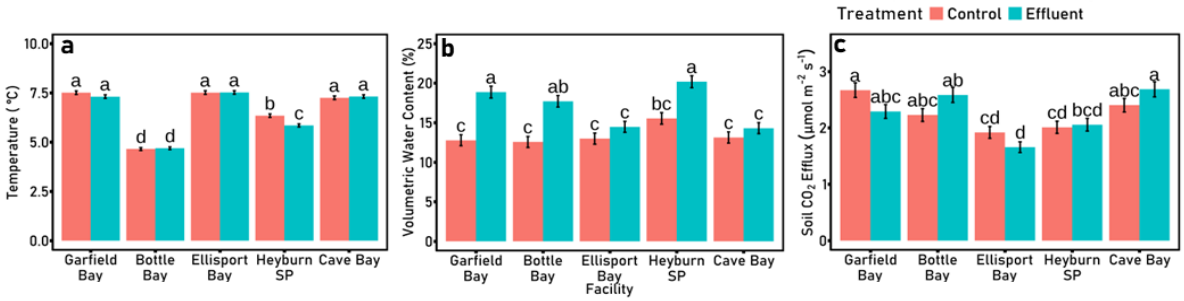

Figure 4.1. Facility by treatment variations of soil (a) temperature (˚C), (b) volumetric water content (%), (c) CO_2 efflux (µmol m^{-2} s^{-1}) at the five water reuse facilities. Each column represents the mean of all plots per facility (n = 35). Means within each panel having same letter are not statistically different ($P \leq 0.10$).

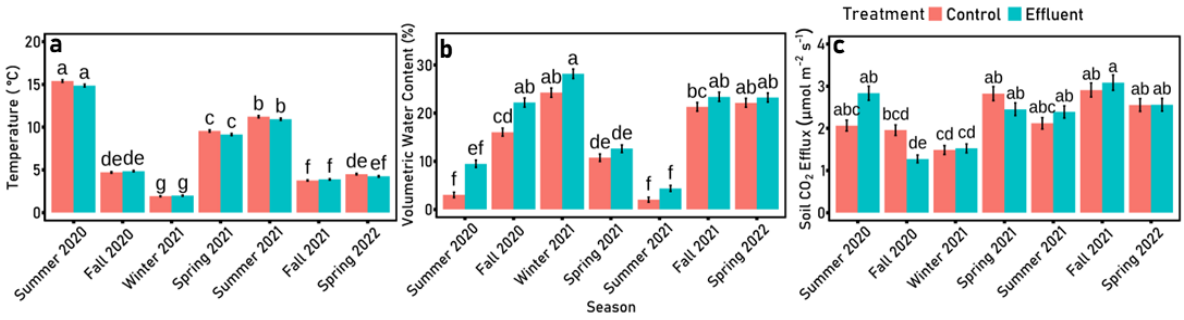

Figure 4.2. Season by treatment variations of soil (a) temperature (˚C), (b) volumetric water content (%), (c) CO_2 efflux (µmol m^{-2} s^{-1}) at the five water reuse facilities. Each column represents the mean of all plots per facility (n = 25). Means within each panel having same letter are not statistically different ($P \leq 0.10$).

4.5.2. Exoenzyme Activities

4.5.2.1 Mineral Soil Exoenzyme Activities

The effect of treatment on the mineral soil enzyme activities that release N and P from soil depended on facility and season (Table 4.4, TxF, TxFxS, $P < 0.01$). The greatest effect of treatment was observed for chitinase activity (Table 4.4, T, TxF, $P < 0.01$, $F > 5.3$). Effluent treatment greatly suppressed chitinase activities (CH) in the effluent plots at the long-established facilities (Garfield Bay, Bottle Bay and Ellisport Bay) while the differences between treatments were not significant at the recently established facilities (Heyburn SP and Cave Bay) (Figure 4.3). β-glucosidase activity (BG) was not significantly affected by treatment but was comparatively higher at long established facilities (Figure C2a). Treatment had a significant effect on acid phosphatase activity (PH). Overall, PH activity was an order of magnitude higher than other enzyme activities and did not vary significantly across facilities. However, PH was significantly suppressed at the longest-established Garfield Bay facility and was lowest at the most recently established Cave Bay facility (Figure C2e). Furthermore, PH activity was higher in control plots at the longest established Garfield Bay facility compared to effluent plots (Table 4.4, TxF, $P<0.01$).

Season also had a significant effect on all enzyme activities (Table 4.4, S, $F > 17.54$, FxS, $P < 0.01$). Greatest enzyme activities were observed during Summer of first sampling year for all enzyme activities except for BG (Figure C3). Highest seasonal effect was observed for CH activity at Garfield Bay and Cave Bay, which were highest during Summer of both years (Figure C3d). N-releasing AM, LAP and CH activities were consistently highest in Summer at Cave Bay.

Table 4.4. Three-way analysis of variance and covariance results for C-release, N-release, and P-release enzyme activities for soil samples. *F* statistic (*F*) and *P*-values (*P*) are presented for measured effects of reclaimed water treatment (T), Facility (F), season (S), establishment date (E), and their interactions on BG (β-glucosidase), AM (alanine aminopeptidase), LAP (leucine aminopeptidase), CH (β-N-acetyl-glycosaminidase), and PH (acid phosphatase). Boldface indicates significance at $P \leq 0.05$.

Effect	**C-release**		**N-release**						**P-release**	
	BG		**AM**		**LAP**		**CH**		**PH**	
	F	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***
ANOVA										
T	0.29	0.59	1.09	0.303	6.51	**0.01**	26.79	**<0.01**	3.39	0.073
F	10.23	**<0.01**	6.53	**<0.01**	5.18	**<0.01**	2.52	0.06	11.26	**<0.01**
S	41.96	**<0.01**	71.66	**<0.01**	47.64	**<0.01**	33.9	**<0.01**	17.54	**<0.01**
TxF	2.29	0.08	1	0.421	4.12	**<0.01**	5.38	**<0.01**	4.26	**<0.01**
TxS	1.43	0.21	0.51	0.799	0.67	0.68	1.78	0.10	1.68	0.127
FxS	2.16	**<0.01**	5.73	**<0.01**	3.2	**<0.01**	2.39	**<0.01**	1.91	**<0.01**
TxFxS	1.34	0.14	0.94	0.550	1	0.47	1.56	**0.05**	0.96	0.518
ANCOVA										
T	0.94	0.33	0.8	0.372	2.74	0.099	13.87	**<0.01**	5.1	**0.025**
E	7.97	**<0.01**	6.46	**0.012**	0.82	0.37	2.69	0.102	3.57	0.060
S	1.25	0.28	7.5	**<0.01**	4.37	**<0.01**	1.83	0.094	1.6	0.148
TxE	0.93	0.34	0.79	0.375	2.71	0.10	13.64	**<0.01**	5.05	**0.025**
TxS	0.75	0.61	1.15	0.332	0.83	0.55	1.04	0.399	1.18	0.320
ExS	1.25	0.28	7.47	**<0.01**	4.33	**<0.01**	1.81	0.097	1.58	0.152
TxExS	0.75	0.61	1.16	0.329	0.83	0.59	1.04	0.401	1.17	0.325

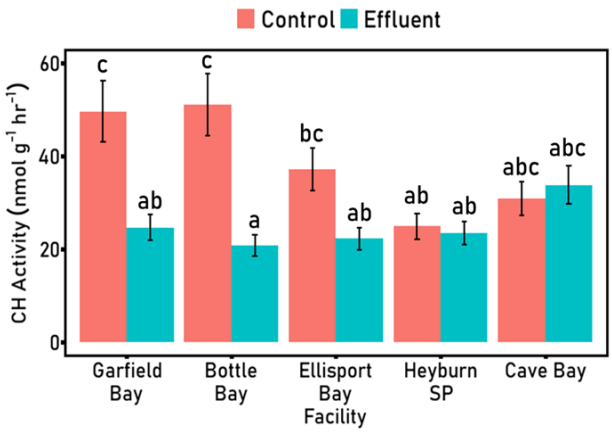

Figure 4.3. Facility by treatment variations of mineral soil Chitinase Activity (β-N-acetyl-glycosaminidase) (nmol g^{-1} hr^{-1}) at the five water reuse facilities. Each column represents the mean of all plots per facility (n = 35). Means within each panel having same letter are not statistically different ($P \leq 0.10$).

Facility date of establishment accounted for a significant treatment effect on mineral soil CH activity and marginal effect on PH activity (Table 4.4, TxE, P= 0.025, Figure 4.4a & b). The greatest effect of treatment was observed in CH activity where control plots at the long-established facilities (Garfield Bay, Bottle Bay and Ellisport Bay) consistently showed higher activity (Figure 4.4a). Similarly, PH activity was higher in control plots at the longest established facility Garfield Bay (Figure 4.4b). BG, AM, and LAP activities did not significantly vary across the different dates of establishment (Figure C6).

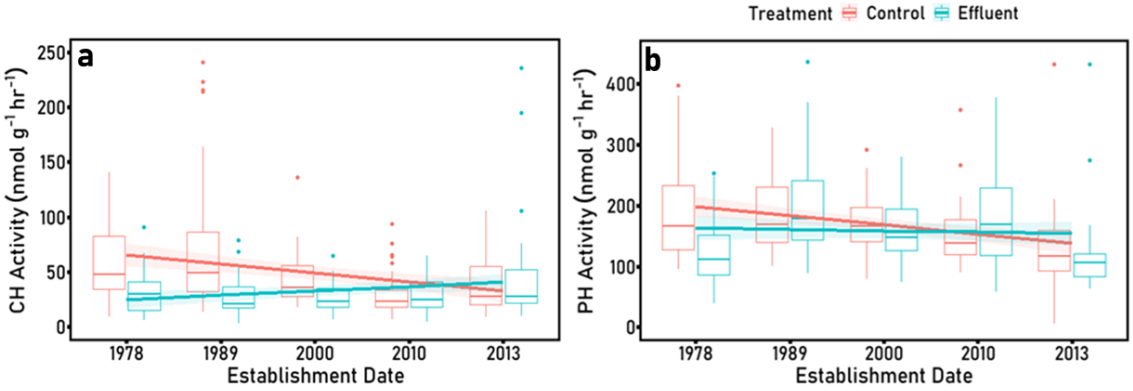

Figure 4.4. Mineral soil (a) Chitinase (CH) activity and (b) Phosphatase (PH) activity for facilities with different dates of establishment. Boxes delimit the 0.25 and 0.75 quantiles, horizontal lines indicate medians, whiskers extend to minimum and maximum values, and outliers are plotted as individual points. Each box and whisker represents n=35 observations.

4.5.2.2. Litter Exoenzyme Activities

The effect of treatment on the litter enzyme activities that release N (AM and LAP activities) and P (PH activity) from litter depended on facility and season (Table 4.5, T, F, TxF, $P \leq 0.063$). While the differences between treatments were not remarkable, effluent treatment consistently suppressed PH activities in the effluent plots at all five facilities (Figure C4e). Similarly, CH activities were suppressed at all facilities except Ellisport Bay, where effluent plots had higher CH activity, and Heyburn SP where activities were similar in magnitude. The greatest effect of facility on enzyme activity in litter was observed for CH and PH activity (Figure 4.5d & e). Bottle Bay, Ellisport Bay and Heyburn State park showed comparatively higher litter enzyme activity compared to Garfield Bay and Cave Bay for both CH and PH. Litter enzyme activities for BG, AM and LAP were not significantly different across facilities (4.5a, b & c). Facility date of establishment accounted for a marginal treatment effect on CH and PH activities (Table 4.5, E, $P \leq 0.075$). The 1989 facility had the highest PH activity while the 2013 facility had the lowest PH activity (Figure C7e).

Table 4.5. Three-way analysis of variance and covariance results for C-release, N-release, and P-release enzyme activities for litter samples. *F* statistic (*F*) and *P*-values (*P*) are presented for measured effects of reclaimed water treatment (T), Facility (F), season (S), establishment date (E), and their interactions on BG (β-glucosidase), AM (alanine aminopeptidase), LAP (leucine aminopeptidase), CH (β-N-acetyl-glycosaminidase), and PH (acid phosphatase). Boldface indicates significance at $P \leq 0.05$.

Effect	C-release		N-release						P-release	
	BG		AM		LAP		CH		PH	
	F	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***
ANOVA										
T	0.62	0.44	1.29	0.26	0.75	0.392	1.82	0.185	5.73	**0.022**
F	3.3	**0.02**	0.67	0.62	2.97	**0.031**	3.73	**0.011**	6.2	**<0.01**
TxF	1.15	0.35	3.29	**0.02**	2.43	0.063	0.83	0.517	0.23	0.920
ANCOVA										
T	1.69	0.2	0.32	0.58	0.12	0.736	0.76	0.387	0.42	0.522
E	1.55	0.22	0.68	0.41	0.72	0.401	3.33	0.075	4.03	0.051
TxE	1.7	0.198	0.31	0.58	0.11	0.739	0.75	0.391	0.43	0.5133

Note: Litter samples were only collected in Spring 2022.

4.5.2.3. Comparison of Mineral Soil and Litter Enzyme Activities

Sample type had the greatest effect on enzyme activities (Table 4.6, Ty, $F > 206$), where litter enzyme activities were substantially greater compared to mineral soil enzyme activities (Figure 4.5). The greatest difference between mineral soil and litter enzyme activities was observed for BG activity (Figure 4.5a). Facility establishment date did not have a significant effect on litter enzyme activities except for CH and PH activity (Table 4.6, E, $P \leq 0.065$). The treatment effect was observed for both

soil and litter (Table 4.6, T, P <0.01) without an interaction with sample type (TxTy). Thus, the treatment effect was consistent for both sample types. Effluent treatment suppressed both CH activity and PH activity (Figure 4.6).

Table 4.6. Three-way analysis of variance and covariance results comparing C-release, N-release, and P- release enzyme activities for mineral soil and litter samples in spring 2022. *F* statistic (*F*) and *P*-values (*P*) are presented for measured effects of reclaimed water treatment (T), Facility (F), season (S), establishment date (E), sample type (Ty), and their interactions on BG (β-glucosidase), AM (alanine aminopeptidase), LAP (leucine aminopeptidase), CH (β-N-acetyl-glycosaminidase), and PH (acid phosphatase). Boldface indicates significance at $P \leq 0.05$.

Effect	**C-release**		**N-release**						**P-release**	
	BG		**AM**		**LAP**		**CH**		**PH**	
	F	***P***	***F***	***P***	***F***	***P***	***F***	***P***	***F***	***P***
ANOVA										
T	0.25	0.62	1.61	0.21	0.03	0.86	8.24	**<0.01**	8.6	**<0.01**
F	3.46	**0.02**	1.67	0.18	1.74	0.16	4.11	**<0.01**	16.41	**<0.01**
Ty	431.2	**<0.01**	378.63	**<0.01**	375.3	**<0.01**	231.7	**<0.01**	206.6	**<0.01**
TxF	2.34	0.07	1.4	0.25	1.23	0.32	2.2	0.09	0.38	0.83
TxTy	0.03	0.87	0.11	0.74	1.48	0.23	0.31	0.58	0.69	0.41
FxTy	0.33	0.86	4.99	**<0.01**	1.84	0.14	4.08	**<0.01**	0.69	0.61
TxFxTy	1.03	0.41	2.18	0.09	3.23	**0.02**	0.93	0.46	1.54	0.21
ANCOVA										
T	0.9	0.35	0	0.99	1.29	0.26	6.29	**0.02**	0.09	0.77
E	0.85	0.36	2.15	0.15	0	0.99	3.59	0.07	5.23	**0.03**
Ty	0.06	0.80	6.33	**0.02**	1.37	0.25	1.2	0.28	0.17	0.69
TxE	0.91	0.35	0	0.99	1.29	0.26	6.2	**0.02**	0.08	0.78
TxTy	0.02	0.89	0.65	0.43	2.45	0.13	1.07	0.31	2.33	0.13
ExTy	0.02	0.90	6.93	**0.01**	1.11	0.30	1.41	0.24	0.1	0.75
TxExTy	0.02	0.89	0.64	0.43	2.43	0.13	1.06	0.31	2.35	0.13

Note: Litter samples were only collected in Spring 2022.

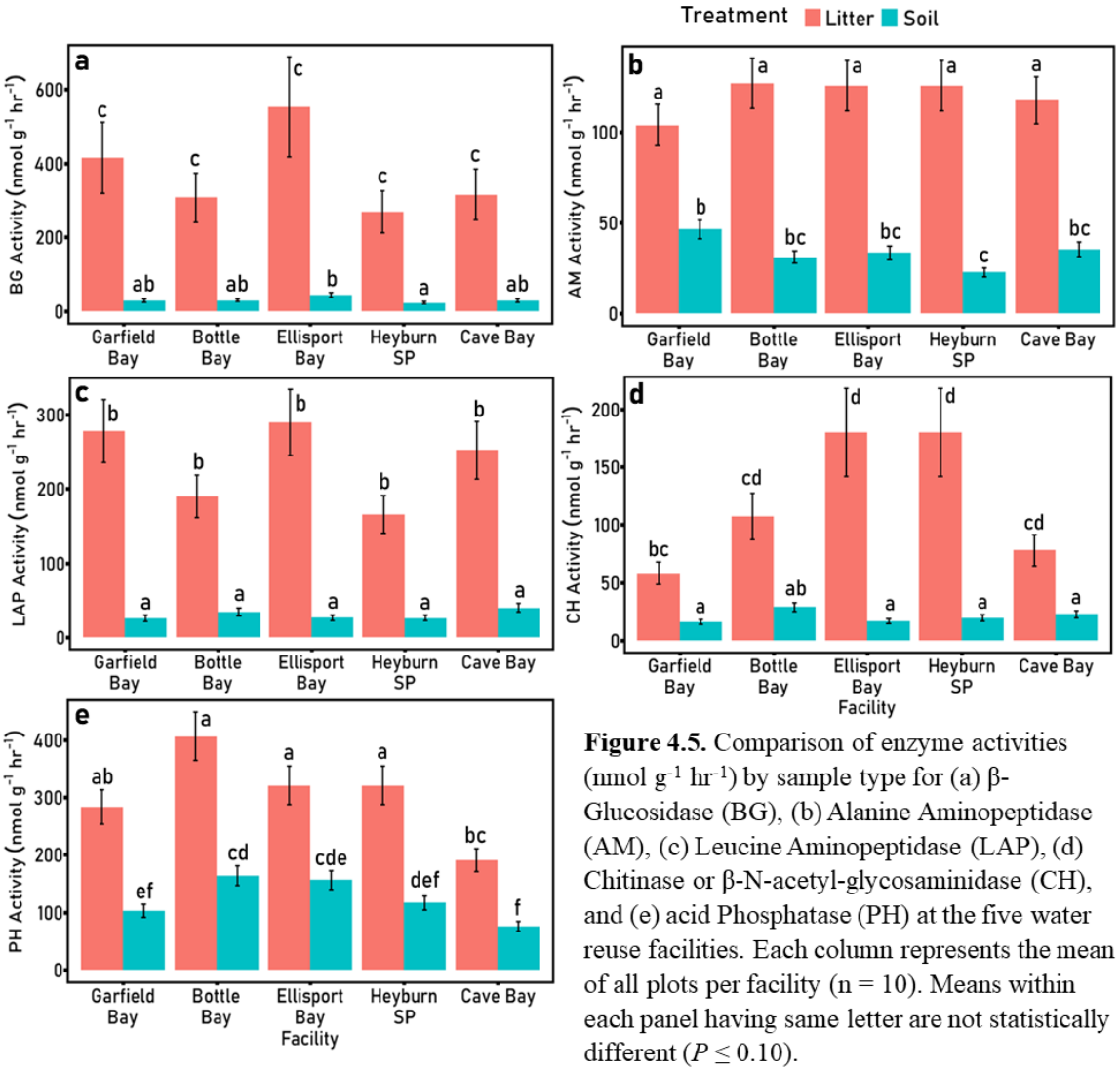

Figure 4.5. Comparison of enzyme activities (nmol g^{-1} hr^{-1}) by sample type for (a) β-Glucosidase (BG), (b) Alanine Aminopeptidase (AM), (c) Leucine Aminopeptidase (LAP), (d) Chitinase or β-N-acetyl-glycosaminidase (CH), and (e) acid Phosphatase (PH) at the five water reuse facilities. Each column represents the mean of all plots per facility (n = 10). Means within each panel having same letter are not statistically different ($P \leq 0.10$).

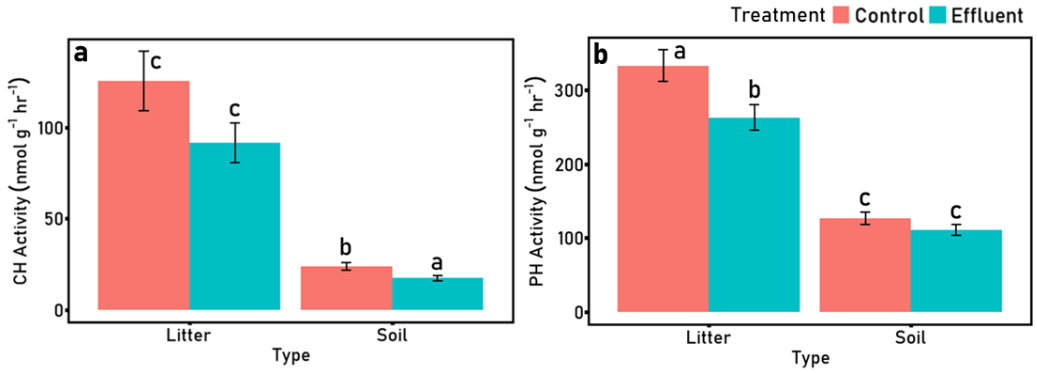

Figure 4.6. Comparison of sample type and treatment for (a) Chitinase (CH) and (b) Acid Phosphatase (PH) activity at the five water reuse faclities. Each column represents the mean of all plots per sample type (n=25). Means within each panel having same letter are not statistically different ($P \leq 0.10$).

4.5.3. Amino Compounds

4.5.3.1. Amino Compounds Soil Responses

Soil amino acids and NAG pools responded to both facility and season, but not to treatment (Table 4.7, FxS, $P < 0.01$). Bottle Bay, Garfield Bay and Ellisport Bay showed consistently lower responses for amino compounds except for Bottle Bay NAG response which was comparable with Heyburn SP and Cave Bay (Figure 4.7c). Seasonal responses across facilities during fall and spring were not significantly different but were highest in winter for Heyburn SP and Cave Bay (Figure 4.7). The treatment had only a marginally significant effect on facility establishment dates for alanine and NAG pools (Table 4.7, TxE, $P = 0.0323$ & 0.0715, Figure C9).

Table 4.7. Three-way analysis of variance and covariance results for alanine, leucine, and N-acetyl glucosamine for soil samples. *F* statistic (*F*), and *P*-values (*P*) are presented for measured effects of reclaimed water treatment (T), Facility (F), season (S), and establishment date (E), and their interactions for alanine, leucine, and NAG. Boldface indicates significance at $P \leq 0.05$.

Effect	Alanine		Leucine		NAG	
	F	***P***	***F***	***P***	***F***	***P***
ANOVA						
T	0.88	0.3551	0.14	0.713	1.03	0.3169
F	8.54	**<0.01**	5.83	**<0.01**	4.02	**<0.01**
S	254.1	**<0.01**	72.32	**<0.01**	137.02	**<0.01**
TxF	1.37	0.26	0.61	0.6562	1.7	0.1696
TxS	0.95	0.3894	1.62	0.2053	0.75	0.4753
FxS	3.55	**<0.01**	4.87	**<0.01**	2.72	**0.0106**
TxFxS	0.52	0.8401	0.4	0.9152	0.34	0.9495
ANCOVA						
T	4.7	0.0319	2.33	0.1292	3.42	0.0707
E	19.78	**<0.01**	10.64	**<0.01**	4.61	**0.0372**
S	7.54	**<0.01**	12	**<0.01**	1	0.3707
TxE	4.67	**0.0323**	2.32	0.1298	3.4	0.0715
TxS	0.44	0.6481	0.27	0.7659	0.03	0.9737
ExS	7.92	**<0.01**	11.67	**<0.01**	0.87	0.4205
TxExS	0.43	0.6533	0.26	0.7704	0.03	0.9721

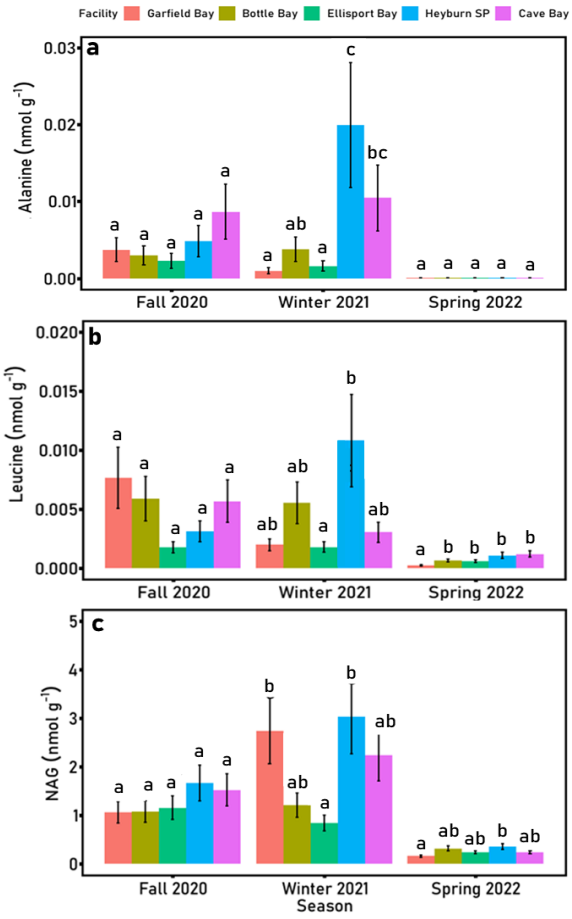

Figure 4.7. Facility by season variation in soil amino compounds (nmol g^{-1}) in soil across forest water reclamation facilities: (a) Alanine, (b) Leucine, and (c) N-acetyl glucosamine (NAG). Each column represents the mean of all plots per facility (n= 10). Means within each season having the same letter are not statistically different ($P \leq 0.10$).

4.5.3.2. Amino Compounds Litter Responses

The effect of treatment on amino compounds extracted from litter depended on facility (Table 4.8, TxF, $P < 0.05$, Figure 4.8). Sample type also had a significant effect on alanine, leucine, and NAG (Table C1, Ty, $P < 0.01$). The greatest effect of treatment for litter alanine and leucine was observed at Heyburn SP (Figure 4.8a & b). While there was no treatment effect on NAG litter concentrations (Figure 4.8c). However, NAG litter concentrations for both control and effluent plots at Bottle Bay were an order of magnitude higher compared to the other facilities.

Facility establishment date also accounted for treatment effects for both alanine and leucine extracted from litter (Table 4.8, TxE, $P \leq 0.0133$). Control plots at recently established facilities (2010 and 2013) were higher compared to the effluent treated plots (Figure 4.9). Litter responses were an order of magnitude higher than soil responses and were consistently higher in control plots for

alanine and leucine (Figure 4.10a & b), except for NAG where treatment differences in litter were not observed (Figure 4.10c).

Table 4.8. Two-way analysis of variance and covariance results for alanine, leucine, and N-acetyl glucosamine (NAG) for litter samples. *F* statistic (*F*) and *P*-values (*P*) are presented for measured effects of reclaimed water treatment (T), Facility (F), and establishment date (E), and their interactions for alanine, leucine, and NAG. Boldface indicates significance at $P \leq 0.05$.

Effect	Alanine		Leucine		NAG	
	F	***P***	***F***	***P***	***F***	***P***
ANOVA						
T	3.47	0.0699	2.93	0.0947	0.35	0.5558
F	2.1	0.0993	0.74	0.5701	6.22	**<0.01**
TxF	2.94	**0.0321**	3.84	**<0.01**	2.61	**0.0499**
ANCOVA						
T	6.57	**0.0137**	7.95	**<0.01**	2.3	0.1366
E	6.72	**0.0127**	0.06	0.8156	0.4	0.5321
TxE	6.63	**0.0133**	8.01	**<0.01**	2.29	0.1374

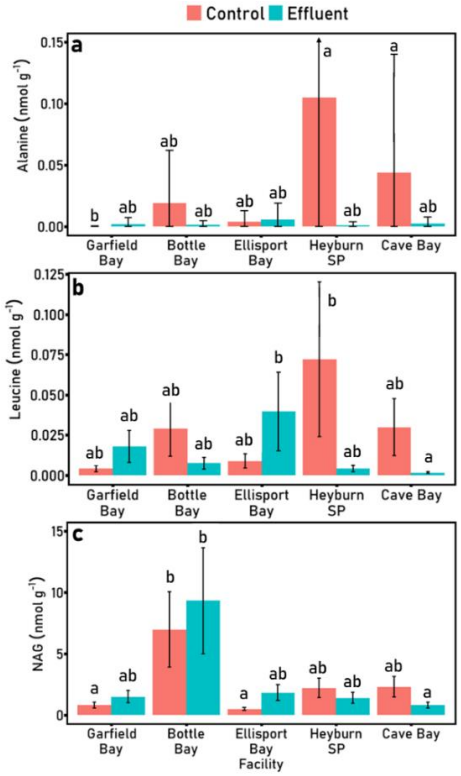

Figure 4.8. Facility by treatment variations of litter (a) Alanine, (b) Leucine, and (c) NAG (nmol g^{-1}) at the five water reuse facilities. Each column represents the mean of all plots per sample type (n=5). Means within each panel having same letter are not statistically different ($P \leq 0.10$).

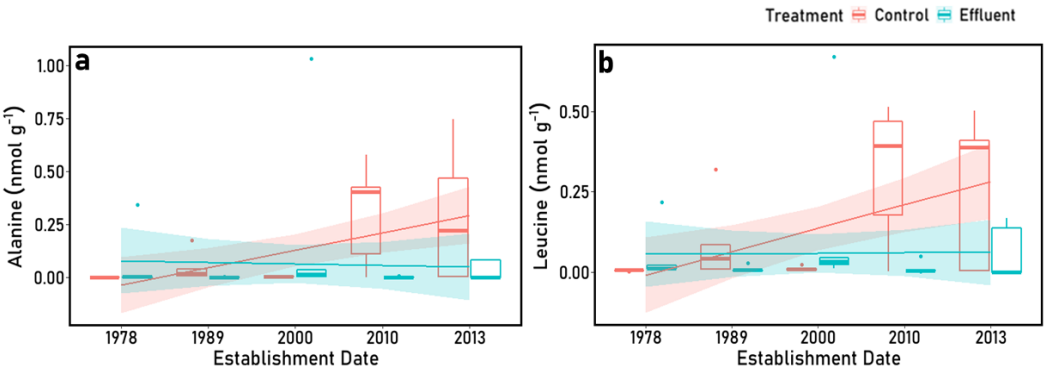

Figure 4.9. Litter amino acid pools (a) Alanine and (b) Leucine for facilities with different dates of establishment. Boxes delimit the 0.25 and 0.75 quantiles, horizontal lines indicate medians, whiskers extend to minimum and maximum values, and outliers are plotted as individual points. Each box and whisker represents n=5 observations.

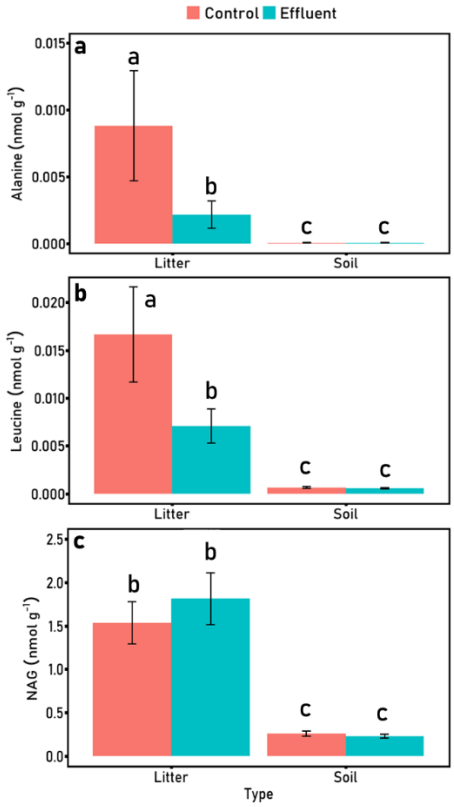

Figure 4.10. Comparison of amino compounds (nmol g^{-1}) by sample type and treatment for (a) Alanine, (b) Leucine, and (c) N-acetyl glucosamine (NAG). Each column represents the mean of all plots per sample type (n=5). Means within each panel having same letter are not statistically different ($P \leq 0.10$).

4.6. Discussion

Amending forests with reclaimed water over four decades suppressed chitinase and phosphatase exoenzyme activities. Suppression of mineral soil chitinase activity occurred specifically at the effluent treated sites of the longest-established faclities Bottle Bay and Garfield Bay (Figure 4.3) that have received more than 34 years of reclaimed water during the growing season. Such suppression of CH activity at the longest-established facilities may indicate N enrichment and a shift from N-limitation to P-limitation, or perhaps a shift in microbial functions. Litter PH activities were also consistently suppressed with reclaimed water amendment (Figure C4e), likely due to surplus of P in reclaimed water. Furthermore, litter concentrations of both alanine and leucine were greatly suppressed at the effluent treated plots of the recently established facilities Heyburn SP and Cave Bay (Figure 4.9). Thereby, suggesting a greater uptake of the amino acids with reclaimed water application at the recently established facilities. With ten times lower N loading rates compared to the long-established facilities, the recently established facilities are likely N-limited and rely on a broader range of organic and inorganic sources for N acquisition from the ecosystem. These responses suggest that N and P exoenzyme activities and amino acid pools are largely dependent on site nutrient limitations and that reclaimed water application can be a source of nutrients that can address those limitations. However, nutrient enrichment may occur with prolonged application which can repress biological processes.

4.6.1. Exoenzyme Activity

Our findings indicate that amending forests with reclaimed water suppresses mineral soil CH activity while at the same time suppressing litter PH activity. Mineral soil chitinase activities were specifically suppressed at the long-established facilities that have received more than 34 years of reclaimed water. Exoenzymes are produced to enhance nutrient availability when supply is low. Application of surplus of readily available N in reclaimed water most likely addressed the ecosystem N limitations and suppressed chitinase production (Saiya-Cork et al., 2002a; Olander & Vitousek, 2000). Furthermore, we also observed elevated nitrification rates at the long-established facities (Briggs & Coleman, Unpublished data) which increase inputs of readily available NO_3^--N. Therefore, our results show that mineral soil CH activity is suppressed mainly at long-established facilities which corresponds with increase in nitrification rates.

Irrigation with reclaimed water has been found to have a strong impact on soil microbial activities and abundance (Friedel et al., 2000; Jueschke et al., 2008), with consequent effects on enzyme activities and nutrient turnover (W. Chen et al., 2008; Heinze et al., 2014). Such enhancement in microbial activity has been linked with constant humidity from irrigation (Friedel et al., 2000). The

type of soil enzyme activities and the rates at which the substrates are broken down are also influenced by microbial community composition (Chapin et al., 2012). Findings from our study on microbial composition indicate a depression in fungal abundance with increased bacteria to fungi ratio at the effluent amended plots (Sarauer & Coleman, Unpublished data). Therefore, suggesting a shift in microbial composition from fungi to a bacteria dominated ecosystem. A meta-analysis by H. Chen et al. (2018) also suggests that N amendment can also influence soil enzyme activity by altering plant composition, microbial biomass and community structure (Cusack et al., 2011; Kjøller et al., 2012) Chitin is a natural nitrogenous biopolymer composed of N-acetyl-D-glucosamine monomers found in cell walls of fungi, insect exoskeletons and crustacean shells (Abo Elsoud & El Kady, 2019; Rathore & Gupta, 2015). Chitinases are produced by organisms to hydrolyze chitin (Skujiņš & Puķite, 1970). Reduced fungal abundance in response to reclaimed water observed at the forest water reclamation facilities corresponds with the observed suppression of chitinase activity. Decrease in fungal abundance results in lowered abundance of chitin substrate and the need for production of chitinases to hydrolyze the chitin. Furthermore, chitin is produced by microorganisms but plants can only produce proteins (Trovato et al., 2021), which may explain the decrease in soil chitinase activity but non-response for the N-releasing alanine and leucine aminopeptidases.

Litter phosphatase activity was suppressed for all five facilities (Figure C4e). Olander & Vitousek also report a repression of phosphatase activity with P application. Presence of readily available inorganic N and P species in reclaimed water may have repressed the production of phosphatase activity similar to chitinase due to negative feedback (Olander & Vitousek, 2000). Nonetheless, phosphatase activities were inherently an order of magnitude higher than other enzyme activities. The high magnitude of phosphatase activities may be due to the ability of phosphatases to persist in soil for prolonged time periods by binding to soil humics and clays (Burns, 1982; Rojo et al., 1990; Sinsabaugh, 1994). Such bound phosphatases may be released and detected during enzyme assays. P is also adsorbed onto Fe and Al (oxyhydr)oxides in soil which have high binding affinities for PO_4^{3-} (Filippelli, 2008). However, addition of P in reclaimed water can overcome this limitation and maintain the supply of P, which is likely why effluent plots for soil samples show lower phosphatase levels. In contrast, there are no artificial inputs of P in the control plots and the low P availability could have increased phosphatase production to meet the ecosystem P demand. While forest floor represents an important P pool with low adsorption capacity and high P mineralization (D. W. Cole & Rapp, 1981; Yanai, 1992), most of the P is likely adsorbed in soil which causes increase in PH activity.

Our study did not find C-releasing β-glucosidase (BG) activity to be responsive to reclaimed water treatment. However, others indicate higher BG activity in the long-term irrigated soil compared

to unirrigated soils (Filip et al., 2000). In contrast, both BG and LAP activities for litter has been found to be significantly higher in N-amended plots (Saiya-Cork et al., 2002a). Our results show that soil exoenzyme activities were significantly upregulated in litter compared to soil. Soil enzyme activities have been found to increase with litter addition due to improved substrate availability (Bandick & Dick, 1999; R. G. Burns et al., 2013; Debosz et al., 1999; Dornbush, 2007; Weintraub et al., 2013; Yao et al., 2009). Nonetheless, studies have reported both positive, negative, and neutral effects of N addition on litter decomposition. The variation in effect of N amendment on decomposition is suggested to be due to the site-specific differences in litter quality (Keeler et al., 2009).

4.6.2. Amino Compounds

Our results indicate that the amino compound concentrations are relatively high during winter and minimal during spring (Figure 4.6). But overall, the concentrations of both amino acids alanine and leucine were low which is likely due to the abundant supply of inorganic N in reclaimed water which may have limited exoenzyme activities to acquire N from organic sources, thereby resulting in reduced amino acid pools. Furthermore, free amino acids have been found to be present in low concentrations in bulk soil likely due to rapid uptake and mineralization by soil microbes (Jones, Shannon, et al., 2005). Both litter alanine and leucine concentrations were significantly higher compared to soil samples and were highest for control plots at recently established facilities, suggesting increased uptake of amino acids at the effluent treated plots for the recently established facilities. With ten times lower N loading rates compared to the long-established facilities, the recently established facilities are likely N-limited and rely on a broader range of organic and inorganic sources for N acquisition from the ecosystem.

The dissolved organic nitrogen (DON) pool is a major soluble N pool in soil and is composed of wide range of high and low molecular weight compounds including amino acids and amino sugars (Antia et al., 1991; Jones et al., 2004; Stevenson, 2015). Free amino acids have been found to only represent a small proportion of the total DON not necessarily because of a slow rate of pool recharge but rather a fast rate of removal (Jones et al., 2004). Soil microbes and plants compete for acquisition of the scarce amino acid resource (Jones et al., 2004; Owen & Jones, 2001; Streeter et al., 2000), which explains the low amino acid concentrations. While amino acid concentration in soil solution increases when NO_3^- production is low (Jones et al., 2004), the rapid uptake may have resulted in suppression of amino acids at the recently-established facilities. Furthermore, others have shown that microbes outcompete plants to acquire amino acids and that the majority of the amino acids in soil is stored in microbial biomass, with only a small fraction captured by plant roots (Owen & Jones, 2001).

DON concentrations were relatively low at both control and effluent treatment facilities (Joshi, Clark & Coleman, Unpublished data). Therefore, it is likely that a large proportion of the amino acids at the forest water reclamation sites may be stored in microbial biomass resulting in low concentrations in the soil solution. Further research on microbial biomass pools may provide a more complete insight on observed amino compound concentrations.

4.6.3. Soil CO_2 efflux

As hypothesized, we did not observe any differences among treatments for soil CO_2 efflux, which agrees with other reclaimed water and chronic N addition studies, where soil respiration did not increase with amendment (Micks et al., 2004; Schipper et al., 1996b). Fertilization has also been found to decrease soil CO_2 efflux in tropical (Giardina et al., 2003) and temperate ecosystems (Olsson et al., 2005; Phillips & Fahey, 2007). Such decline may occur due to reduced heterotrophic and autotrophic respiration (Y. Zhang et al., 2016). N addition can decrease autotrophic root respiration due to less plant allocation of C to roots and the rhizosphere (Janssens et al., 2010) and heterotrophic respiration due to decrease in enzyme activity (Olsson et al., 2005). N amendment has been known to potentially affect a number of soil respiration processes (Y. Luo & Zhou, 2006). N fertilization can reduce belowground C allocation and negatively affect root and rhizosphere microbial respiration (Franklin et al., 2003; Giardina et al., 2003, 2004; Olsson et al., 2005). In contrast, N addition can also promote dark respiration, enhance root respiration and biomass (Griffin et al., 1997; Ibrahim et al., 1997; Lutze et al., 2000; Mitchell et al., 1995). The net effects of N fertilization vary greatly with sites, soil types and vegetation cover and no clear pattern has been observed in existing literature (Y. Luo & Zhou, 2006). With soil CO_2 efflux dependent on many variables, it is not surprising that there was no effect of reclaimed water.

Soil CO_2 efflux has been found to be sensitive to abiotic environmental conditions such as temperature and moisture(Y. Luo & Zhou, 2006). We did not observe any differences in soil temperature among the control and effluent treatments (Figure 4.1). However, soil volumetric water content was higher at Bottle Bay, Garfield Bay, and Heyburn State Park and not significantly different at Ellisport Bay and Cave Bay. Soil temperature, moisture and CO_2 efflux responses were primarily driven by seasonal variation. Though there were no treatment effects, we found significant responses in soil CO_2 efflux to facility and season, with lowest soil CO_2 efflux observed during winter, most likely due to low temperature conditions. Pulses of CO_2 have been reported with increased application of water and rewetting of dry forest floor (Borken et al., 2003). Several studies suggest increase in CO_2 efflux occurs due to increased microbial activities and C mineralization(Calderón & Jackson, 2002; Curtin et al., 2000; howard & howard, 1993; Sainju et al.,

2006). Soil moisture content can influence soil respiration directly through root and microbial physiological processes and indirectly via diffusion of substrates and O_2 as the micropore spaces become water-filled (Y. Luo & Zhou, 2006). However, there are also cases where soils irrigated with treated wastewater showed no changes in soil respiration compared to the non-wastewater irrigated sites (Adrover et al., 2012), while others show drought conditions to result in decreased microbial metabolic activity followed by significant decrease in soil respiration and microbial processes (De Nobili et al., 2006).

Existing research show varied soil CO_2 efflux respones to N amendment. Additions of inorganic N may increase (Hopkins et al., 2013; Tafazoli et al., 2021) or decrease (Sun et al., 2014; Zhou et al., 2014) or cause no change in soil CO_2 efflux (Flanagan & Van Cleve, 1983; Micks et al., 2004; Salonius, 1972). N directly stimulates primary production (Vitousek & Howarth, 1991) resulting in more substrate for soil respiration (Y. Luo & Zhou, 2006). In environments with surplus of N, fertilization could cause N leaching and cause little change in soil respiraiton (Y. Luo & Zhou, 2006). Nitrification by ammonium oxidizing bacteria does not require carbon as substrate. Although, the nitrifying bacteria do fix CO_2 for their major source of cell carbon but derive their energy and reducing power from ammonia (ammonia-oxidizing bacteria) and nitrite (nitrite-oxidizing bacteria) (Katz, 2020).

Furthermore, N addition may induce carbon limitation, resulting in depression of soil respiration in N-amended forest soils (Flanagan & Van Cleve, 1983; Söderström et al., 1983), particularly with long-term additions that have resulted in microbial respiration and also root respiration due to declining productivity (Micks et al., 2004). Also, N deposition may increase carbon sequestration in forests with increasing temperature and reduce carbon loss by respiration (Sun et al., 2014). Magnitude of soil CO_2 efflux reported in some published research report values that are an order of magnitude higher than our measurements (plot mean of 205 mg CO_2-C m^{-2} hr^{-1}, i.e., 77.64 μmol m^{-2} s^{-1} (Micks et al., 2004) while others are more comparable (average of 3 μmol m^{-2} s^{-1}) (Sun et al., 2014).

4.7. Conclusion

Continuous and regular amendments with reclaimed water at regional western coniferous forests for up to four decades will not impact soil CO_2 efflux, but suppress soil chitinase and litter phosphatase activities over the long-term and suppress amino acid pools in the short-term. Suppression of soil chitinase and litter phosphatase activities suggest that artificial inputs of N and P in reclaimed water can address low site nutrient availability and suppress exoenzyme activities with reduced demand for nutrient acquisition. While increased uptake of amino acids in litter at the

recently-established facilities suggests that amino acids are important nutrient pools when readily available inorganic forms are unavailable. A follow-up study on water extractable PO_4^{3-} in mineral soil and litter would be important to understand the phosphatase product pool as an analog to the amino compound product pools. Insignificant effects on aminopeptidases despite suppression in chitinase activity at the long-establishment facilities is due to decline in fungal abundance and lower chitin substrate availability (Sarauer & Coleman, Unpublished data). Therefore, results from study on soil microbial biomass composition and abundance would provide a more complete insight on roles that microbes play on other biological responses. Elevated exoenzyme activities and amino compounds for litter compared to soil indicate litter as an important source of nutrient acquisition for both plants and microbes in coniferous ecosystems. Finally, it would be useful to determine organic N nutrient turnover using measured product fluxes (exoenzyme activities) and pools (concentration) to understand ecosystem demand for N.

Chapter 5: Conclusions

5.1 Conclusions

Forest water reclamation (FWR) presents a unique opportunity to alleviate nutrient and water limitations in western forests and convert the social liability of municipal wastewater into an asset that maximizes inherent forest growth potential (Cromer, 1980; Gessel et al., 1990a; Moffat et al., 2001; Weetman et al., 1993). Although reclaimed water offers opportunities to combine increased forest productivity with improved water quality, there are important short- and long-term precautions that must be addressed to assure sustainability. Prolonged inputs of reclaimed water have been linked to detrimental impacts including decline in forest productivity, tree mortality, altered community structure, nutrient leaching, and detrimental effects on soil physical, chemical and biological health (Aiello et al., 2007; Duan et al., 2011; Magesan et al., 2000; Oswald et al., 2009; Thomas et al., 1999; Toze, 2006; Wallach et al., 2005). In this dissertation, I investigated the effects of reclaimed water application on forest growth and vegetation diversity responses, soil water nutrient concentrations and leaching potential, and soil biological responses. This study was conducted along a four-decade time series of water reuse facilities in northern Idaho.

Tree growth and vegetation responses are integral in FWR systems. Our results show that trees respond favorably to the permitted regular low doses of growing-season reclaimed water nutrient and water amendment. Tree growth at effluent treatment plots increased from 30% to over 100% compared with the non-amended controls and there was little shift in understory for up to three decades of land application. The FWR facilities also demonstrated effective tertiary treatment of reclaimed water with an uptake of 87% of applied N and 99% of applied P. Therefore, regional FWR facilities can serve as a safe and inexpensive avenue for improving forest growth and managing reclaimed water in the regional coniferous forests. Moreover, they are well-suited for small communities with lower management, financial and technological abilities. However, continued long-term inputs without preventive strategies could modify nutrient cycling, alter ecosystem processes, and open the otherwise closed forest ecosystems to nutrient losses through leaching. Results from our tree growth and vegetation diversity study also indicates a decline in tree growth and understory vegetation diversity with increasing length of treatment, particularly after three decades of treatment, suggesting that the application rates and tree spacing may have to be corrected after a certain period of application. Similarly, our findings from the leaching study show concerning levels of NO_3^- leaching at the long-established facilities. Continued application at permitted rates may indeed exceed finite nutrient assimilation capacity of receiving forests, and lead to N saturation and greater leaching losses. Despite minimal leaching rates for PO_4^{3-}, leaching losses occurred primarily through preferential flow paths, which demonstrates that both NO_3^- and PO_4^{3-} are vulnerable to leaching from

FWR systems. The long-established FWR facilities that have been in operation for three decades may start leaching consequential amounts of nutrients within the next decade that can deteriorate regional environmental quality. Similarly, soil biological responses are used as important indicators of adverse effects of reclaimed water application on soil health (Martinez-Salgado et al., 2010; Speir, 2002). Our findings indicate that four decades of FWR has little effect on biological responses such as soil CO_2 efflux, amino compound pools, and exoenzyme activities. The exception was for soil chitinase activity and litter phosphatase activities that were suppressed with reclaimed water application. We also observed an increase in litter amino acid uptake in recently established facilities. Litter served as an important substrate for enzymatic activities and a source of amino acids for plants and microbes. Our results demonstrate that FWR can be practiced without adverse effects on soil quality. However, microbial composition shifts may occur with prolonged application, which may ultimately lead to ecosystem level changes. Nonetheless, with adequate loading rates and forest management practices, FWR can be a unique opportunity to improve soil quality and forest productivity, and simultaneously protect water quality.

FWR has important environmental, social, and economic opportunities for communities in northern Idaho. Our findings on positive effect on tree growth encourage the expansion of FWR to involve partners in protecting surface water pollution while offering opportunities for future economic and residential development. Water reuse facility managers and regional land-owners can partner to take advantage of the resources contained in reclaimed water to enhance forest growth across a wider acreage. The FWR facilities can also provide greater safeguards for surface water if applications were spread over greater area or if treatments were suspended for some number of years. Expansion of FWR across the community by partnering with adjacent landowners would help improve production, shorten forest harvest rotations, and extend facility operation time that would lower or eliminate the risk of contaminating ground and surface water sources. Of course, such expansion should follow proper forest management practices to maintain reclaimed water assimilation capacity of receiving forests and prevent adverse impacts on productivity, vegetation diversity, soil health and water quality. Sustainable forest water reclamation is greatly dependent on forest management practices. Our results indicate sustained growth responses from the regional conifers across several decades followed by a gradual decline in productivity. Such a decline is likely due to overstocked stands. Forest management practices such as periodic thinning with adjusted nutrient and hydraulic loading rates improve nutrient uptake in the residual trees, improve productivity and prevent nutrient leaching losses.

While FWR is a viable approach for recycling wastewater, there are finite limits which should be recognized. It is evident from our findings that long-term application of reclaimed water

may lead to nutrient saturation and subsequent leaching if left unaddressed. We discovered indications that concern is warranted after five decades of treatment. Nutrient saturation and leaching at FWR facilities may be prevented by adjusting the hydraulic and nutrient loading rates to match forest's assimilation capacity. Accessible, reliable and inexpensive modern tools such as qPCR (quantitative real-time polymerase chain reaction) based eDNA analysis can utilized for environmental monitoring and inventorying microbial species biodiversity (Langlois et al., 2021). Furthermore, Briggs & Coleman have unpublished data showing that ammonia-oxidizing bacterial (AOB) abundance is a critical indicator of NO_3^- abundance. Therefore, we recommend complementing the traditional approach of quantifying NO_3^- concentrations with a periodic assessment of AOB to monitor nitrification, which provides a more preventative approach of determining N saturation. An assessment of soil water nutrient concentrations, drainage and leaching rates and AOB abundance every 10 years may be adequate to track potential N saturation. Such assessments could correspond to the 10-year reuse permit renewal periods.

Our findings provide insight into the long-term effects of FWR on vegetation, nutrient leaching potential and soil biological responses. Still, further research can elucidate processes that are poorly understood. Some of the important questions in FWR are: how many years would it take for forests to recover to nutrient-limited status after saturation? What type of rest periods would be necessary to avoid N-saturation? And how broadly could the wastewater be spread to still achieve substantial year-to-year forest growth stimulation? Such questions can be addressed through continued research at FWR facilities approaching nutrient saturation where reclaimed water application has been temporarily halted to prevent further saturation and losses. Yet, such research could have economic repercussions on both the FWR facility and the community including monitoring costs, land leases for new treatment area and public sewer charges. Our results indicate that an increase in nitrification occurs after N accumulation from three decades of reclaimed water application. Repeated rest periods may allow the forest ecosystems to subside to background levels. Our findings also provide evidence that both N and P leaching losses occur primarily through preferential flow paths. There is currently no evidence that these FWR systems are approaching P saturation. Therefore, PO_4^{3-} may require less frequent monitoring compared to NO_3^-. P leaching potential could be monitored after several decades of amendment, and every five years after that, using predictive water quality risk assessment indicators such as Degree of Phosphorus Saturation (DPS) to identify threshold P that can be retained by the land treatment system without environmental loss.

Other important research areas include expanding the nitrification study on ammonia-oxidizing archaea (AOA) & AOB abundance by Briggs & Coleman (Unpublished data) to greater soil

microbiological community structure to see if there are shifts in taxa as well as functional shifts. Reclaimed water can be a source of chemicals of environmental concerns such as endocrine-disrupting chemicals (EDCs), pharmaceuticals and personal care products, organic pollutants, heavy metals, and microplastics (Kasonga et al., 2021; Nikolaou et al., 2007; Prata, 2018). Contamination of ground and surface water sources with such chemicals can have adverse effects on both environmental and public health. Soil samples from our water reuse facilities were also analyzed for Trace-level organic contaminants (TOrCs) by Kargol et al., 2022. Analysis of soil samples from effluent and control plots showed that FWR can alter rhizosphere microbial communities and increase degradation rate potential for some TOrCs, demonstrating added benefit of removal of TOrCs (Kargol et al., 2022). Therefore, forests ecosystems can also be utilized as rhizotreatment for removal of contaminants. Lastly, despite much research in current literature, soil biological responses to reclaimed water and fertilization are still ambiguous due to considerable variation related to facility-specific factors. Soil biological responses at current FWR facilities could be better understood by developing models for soil temperature and moisture effects on soil CO_2 efflux and determining turnover of soil organic nitrogen compounds from observed fluxes and pools.

There are a few limitations to this study which can be addressed in future research. All possible fates of applied nutrients are not tracked due to prioritized measurements and budget limitations. Gaseous ammonia and nitrous oxide may flux from the site, dilute, and fall downwind as wet or dry deposition. Yet, excessive reactive atmospheric N may have serious environmental implications including greenhouse effect, acidification, eutrophication and particulate matter pollution (Bai et al., 2021; Galloway et al., 2008). Such effects can alter ecosystem processes as well as adversely impact public health and wellbeing. Other concerns include site nutrient losses through runoff or erosion. However, with low slope (<5%) and infiltration rates far exceeding precipitation rates, runoff and erosion is unlikely [Clark, (2022)]. The only time of concern for runoff may be during spring snow melt. By then most summer-applied mobile nutrients are expected to be rinsed deeper in soil by abundant fall and winter rains. But these assumptions should be verified with targeted research.

There are hundreds of FWR facilities in operation across the United States. While this study attempts to capture forest responses to reclaimed water in northern Idaho, responses are largely dependent on facility-specific variables. Therefore, it is important to conduct similar research at more FWR facilities to acquire greater process level understanding as well as improve FWR management practices and ultimately help design customized loading rates, silvicultural practices, and monitoring plans that maximize inherent productivity and protect environmental quality.

Appendix A: Chapter 2 Supplementary Information

Figure A1 shows the schematic representation of the experimental design at the five water reuse facilities and Figure A2 shows the control and effluent plot locations at Heyburn State Park facility as an example. Each facility had a unique spatial distribution of control and effluent plots and the schematic diagram in Figure A1 does not represent the actual layout.

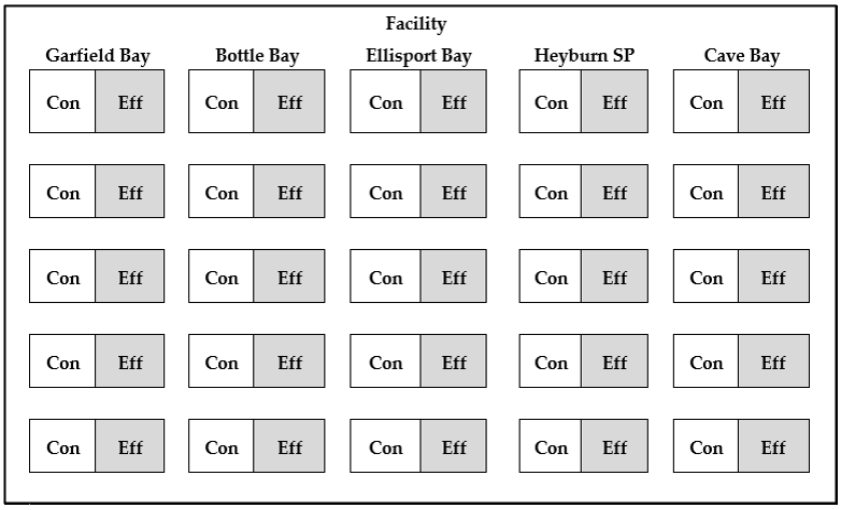

Figure A1. Schematic representation of the experimental design showing number of control (Con) and effluent (Eff) plots at each facility.

Figure A2. The location of five control plots (201HC1, 202HC2, 203HC3, 204HC4 and 205HC5) and five effluent plots (206HE1, 207HE2, 208HE3, 209HE4 and 210HE5) at Heyburn State Park facility demonstrating the unique spatial distribution of study plots. The land application site is the area enclosed by the yellow line.

Appendix B: Chapter 3 Supplementary Information

Table B1: Three-way analysis of covariance results for nutrient concentrations of samples collected from lysimeters. *F* statistic and *P*-values are presented for measured effects of reclaimed water treatment (T), Facility (F), season (S) and their interactions on nitrate (NO_3^-), phosphate (PO_4^{3-}), ammonium (NH_4^+), and dissolved organic nitrogen (DON) concentrations. Boldface indicates significance at P≤0.05.

Effect	NO_3^- (mg L^{-1})		PO_4^{3-} (mg L^{-1})		NH_4^+ (mg L^{-1})		DON (mg L^{-1})	
	F	***P***	***F***	***P***	***F***	***P***	***F***	***P***
T	44.147	**<0.01**	1.416	0.235	0.870	0.352	0.257	0.612
E	3.205	0.075	0.982	0.323	6.750	**0.01**	12.222	**<0.01**
S	2.063	**0.048**	4.129	**<0.01**	21.678	**<0.01**	6.435	**<0.01**
TxE	3.538	0.061	1.664	0.198	6.110	**0.014**	0.001	0.971
TxS	0.711	0.662	0.27	0.965	1.429	0.194	1.142	0.337
ExS	0.707	0.666	0.229	0.978	3.110	**<0.01**	0.539	0.804
TxExS	0.302	0.935	0.824	0.552	1.826	0.094	1.074	0.379

Table B2: Three-way analysis of variance results for nutrient leaching of samples collected from lysimeters. *F* statistic and *P*-values are presented for measured effects of reclaimed water treatment (T), Facility (F), season (S) and their interactions on nitrate (NO_3^-), phosphate (PO_4^{3-}), ammonium (NH_4^+), and dissolved organic nitrogen (DON) leaching. Boldface indicates significance at p≤0.05.

Effect	NH_4^+ (kg ha^{-1})		NO_3^- (kg ha^{-1})		DON (kg ha^{-1})		PO_4^{3-} (kg ha^{-1})	
	F	***P***	***F***	***P***	***F***	***P***	***F***	***P***
T	9.317	**<0.01**	156.917	**<0.01**	15.794	**<0.01**	18.101	**<0.01**
F	44.867	**<0.01**	82.289	**<0.01**	9.801	**<0.01**	10.564	**<0.01**
S	373.428	**<0.01**	760.063	**<0.01**	75.912	**<0.01**	93.725	**<0.01**
TxF	4.490	**<0.01**	18.321	**<0.01**	10.425	**<0.01**	11.575	**<0.01**
TxS	2.034	0.051	9.365	**<0.01**	0.909	0.500	1.058	0.500
FxS	7.072	**<0.01**	21.610	**<0.01**	2.367	**<0.01**	2.615	**<0.01**
TxFxS	2.499	**<0.01**	4.317	**<0.01**	1.229	0.201	1.34	0.201

Table B3: Three-way analysis of variance results for nutrient leaching of samples collected from drain gauges. *F* statistic and *P*-values are presented for measured effects of reclaimed water treatment (T), Facility (F), season (S) and their interactions on nitrate (NO_3^-), phosphate (PO_4^{3-}), ammonium (NH_4^+), and dissolved organic nitrogen (DON) leaching. Boldface indicates significance at P≤0.05.

Effect	NO_3^- (kg ha^{-1})		PO_4^{3-} (kg ha^{-1})		NH_4^+ (kg ha^{-1})		DON (kg ha^{-1})	
	F	***P***	***F***	***P***	***F***	***P***	***F***	***P***
T	704.199	**<0.01**	495.802	**<0.01**	256.800	**<0.01**	127.603	**<0.01**
F	101.383	**<0.01**	144.952	**<0.01**	113.006	**<0.01**	35.428	**<0.01**
S	671.805	**<0.01**	1630.881	**<0.01**	127.240	**<0.01**	638.466	**<0.01**
TxF	35.342	**<0.01**	7.206	**<0.01**	34.086	**<0.01**	46.100	**<0.01**
TxS	75.518	**<0.01**	43.076	**<0.01**	25.393	**<0.01**	12.447	**<0.01**
FxS	64.179	**<0.01**	43.426	**<0.01**	27.839	**<0.01**	55.237	**<0.01**
TxFxS	27.733	**<0.01**	16.852	**<0.01**	17.709	**<0.01**	20.934	**<0.01**

Table B4: Three-way analysis of covariance results for nutrient concentrations of samples collected from drain gauges. *F* statistic and *P*-values are presented for measured effects of establishment date (E), reclaimed water treatment (T), season (S), establishment date covariate (E) and their interactions on nitrate (NO_3^-), phosphate (PO_4^{3-}), ammonium (NH_4^+), and dissolved organic nitrogen (DON) concentrations. Boldface indicates significance at P≤0.05.

Effect	NO_3^- (mg L^{-1})		PO_4^{3-} (mg L^{-1})		NH_4^+ (mg L^{-1})		DON (mg L^{-1})	
	F	***P***	***F***	***P***	***F***	***P***	***F***	***P***
T	2.766	**0.011**	5.527	**<0.01**	3.308	**<0.01**	1.513	0.172
E	2.350	0.131	7.490	**<0.01**	1.454	0.233	1.894	0.174
S	1.407	0.179	3.346	**<0.01**	1.257	0.261	1.278	0.248
TxE	0.132	0.717	6.359	**0.014**	0.501	0.482	0.488	0.488
TxS	0.843	0.556	2.124	0.055	0.820	0.574	0.659	0.705
ExS	1.759	0.113	0.952	0.474	0.551	0.792	1.476	0.194
TxExS	0.080	0.999	0.784	0.603	0.108	0.998	0.638	0.722

Table B5: Three-way analysis of covariance results for nutrient leaching of samples collected from lysimeters and drain gauges. *F* statistic and *P*-values are presented for measured effects of reclaimed water treatment (T), Facility (F), season (S) and their interactions on nitrate (NO_3^-), phosphate (PO_4^{3-}), ammonium (NH_4^+), and dissolved organic nitrogen (DON) concentrations. Boldface indicates significance at p≤0.05.

Effect	NH_4^+ (kg ha^{-1})		NO_3^- (kg ha^{-1})		DON (kg ha^{-1})		PO_4^{3-} (kg ha^{-1})	
	F	***P***	***F***	***P***	***F***	***P***	***F***	***P***
T	15.344	**<0.01**	73.62	**<0.01**	0.412	0.521	8.002	**<0.01**
F	2.816	**0.026**	14.145	**<0.01**	16.124	**<0.01**	10.329	**<0.01**
S	10.946	**<0.01**	2.602	**0.013**	6.466	**<0.01**	3.422	**<0.01**
Sp	4.128	**0.043**	65.919	**<0.01**	0.003	0.959	1374.887	**<0.01**
TxF	2.315	0.058	7.587	**<0.01**	2.863	**0.024**	8.182	**<0.01**
TxS	0.717	0.657	1.603	0.135	1.646	0.123	0.396	0.904
TxSp	36.496	**<0.01**	0.995	0.319	3.34	0.069	6.694	**0.01**
FxS	2.353	**<0.01**	0.933	0.561	0.735	0.824	1.168	0.266
FxSp	3.154	**0.015**	4.189	**<0.01**	1.507	0.200	4.059	**<0.01**
SxSp	9.937	**<0.01**	2.63	**0.012**	1.462	0.181	2.408	**0.021**
TxFxS	0.956	0.524	0.805	0.724	0.723	0.821	0.79	0.743
TxFxSp	5.003	**<0.01**	0.355	0.786	1.544	0.204	4.51	**<0.01**
TxSxSp	2.035	0.061	0.717	0.636	0.359	0.905	0.603	0.728
FxSxSp	1.74	**0.023**	0.542	0.955	0.597	0.925	0.846	0.666
TxFxSxSp	0.816	0.652	0.449	0.957	0.305	0.993	0.391	0.977

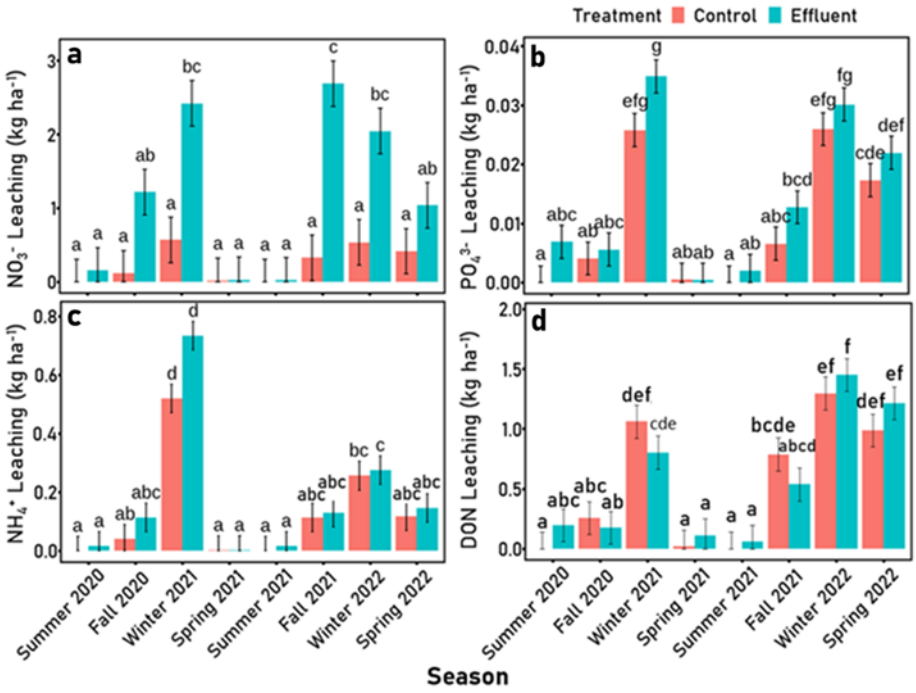

Figure B1: Seasonal leaching of (a) nitrate [NO_3^-] (kg ha^{-1}), (b) phosphate [PO43-] (kg ha^{-1}), (c) ammonium [NH_4^+] (kg ha^{-1}), and (d) dissolved organic nitrogen [DON] (kg ha^{-1}) in lysimeter samples. Each column represents the mean of all plots per sample type (n=25). Means within each panel having same letter are not statistically different ($P \leq 0.10$).

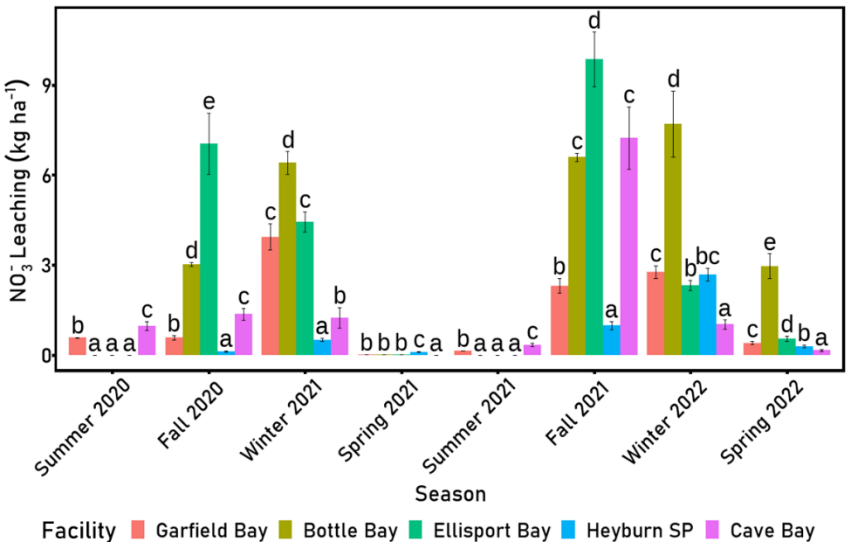

Figure B2: NO_3^- leaching of drainage sample collected with drain gauges: (a) NO^{3-} leaching (kg ha^{-1}) across seasons. Error bars are standard error of the mean (n=10).

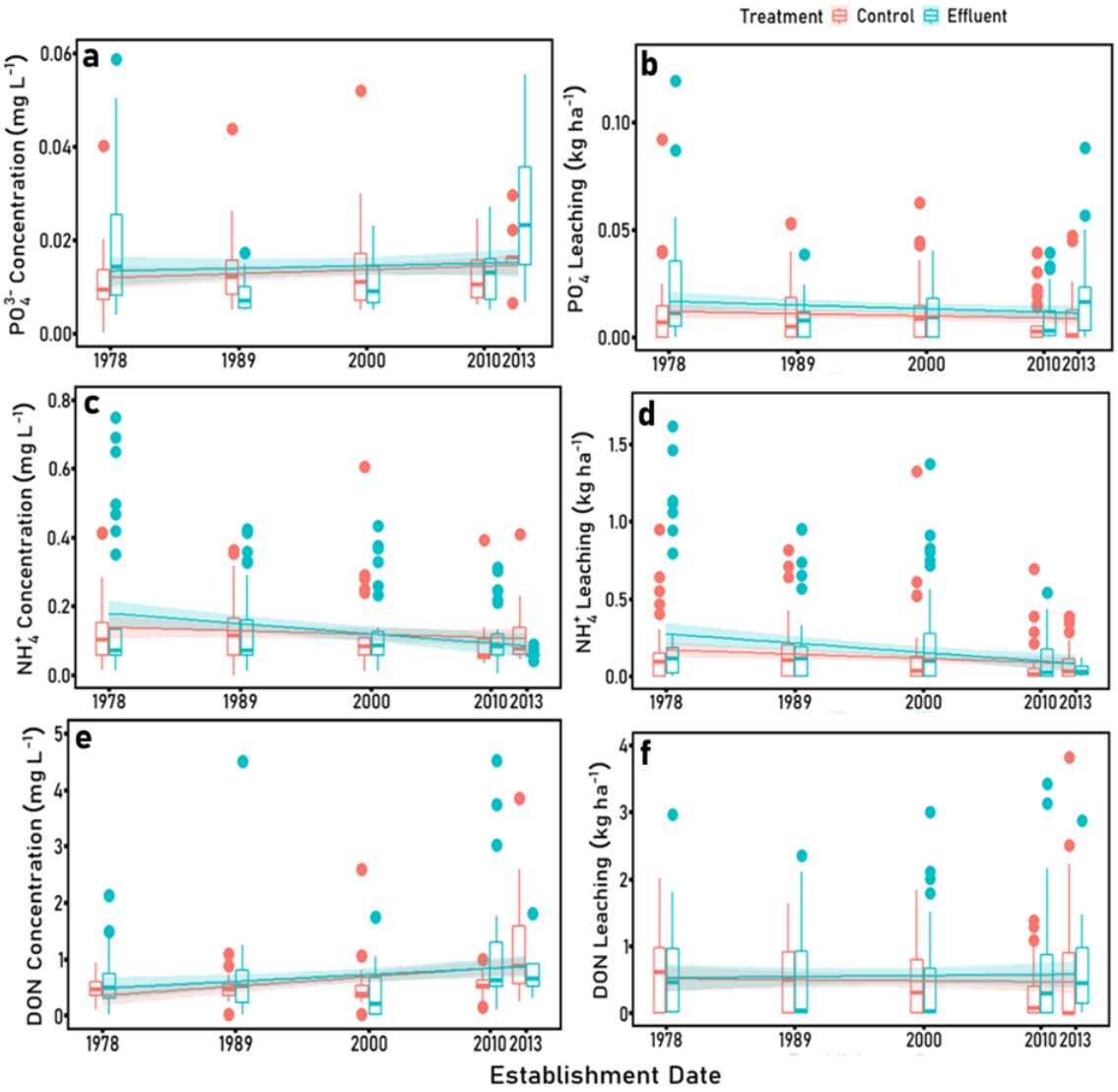

Figure B3: Lysimeter nutrient concentrations (mg L^{-1}) for facilities with different dates of establishment (a) phosphate concentration [PO_4^{3-}] (mg L^{-1}) and (b) phosphate flux [PO43-] (Kg ha^{-1}), (c) ammonium concentration [NH_4^+] (mg L^{-1}), (d) ammonium flux [NH_4^+] (Kg ha^{-1}), (e) dissolved organic nitrogen concentration [DON] (mg L^{-1}) and (f) dissolved organic nitrogen flux [DON] (Kg ha^{-1}) in lysimeter samples in response to reclaimed water amendment for facilities with different dates of establishment. Boxes delimit the 0.25 and 0.75 quantiles, horizontal lines indicate medians, whiskers extend to minimum and maximum values, and outliers are plotted as individual points. An outlier for DON concentration above 5 mg L^{-1} in 2013 for control and an outlier above 10 mg L^{-1} in 2010 for effluent, and an outlier for DON leaching above 7 kg ha^{-1} in 2010 for effluent treatment and an outlier above 4 kg ha^{-1} in 1978 for effluent treatment were excluded for clarity. Each box and whisker represents n=40 observations.

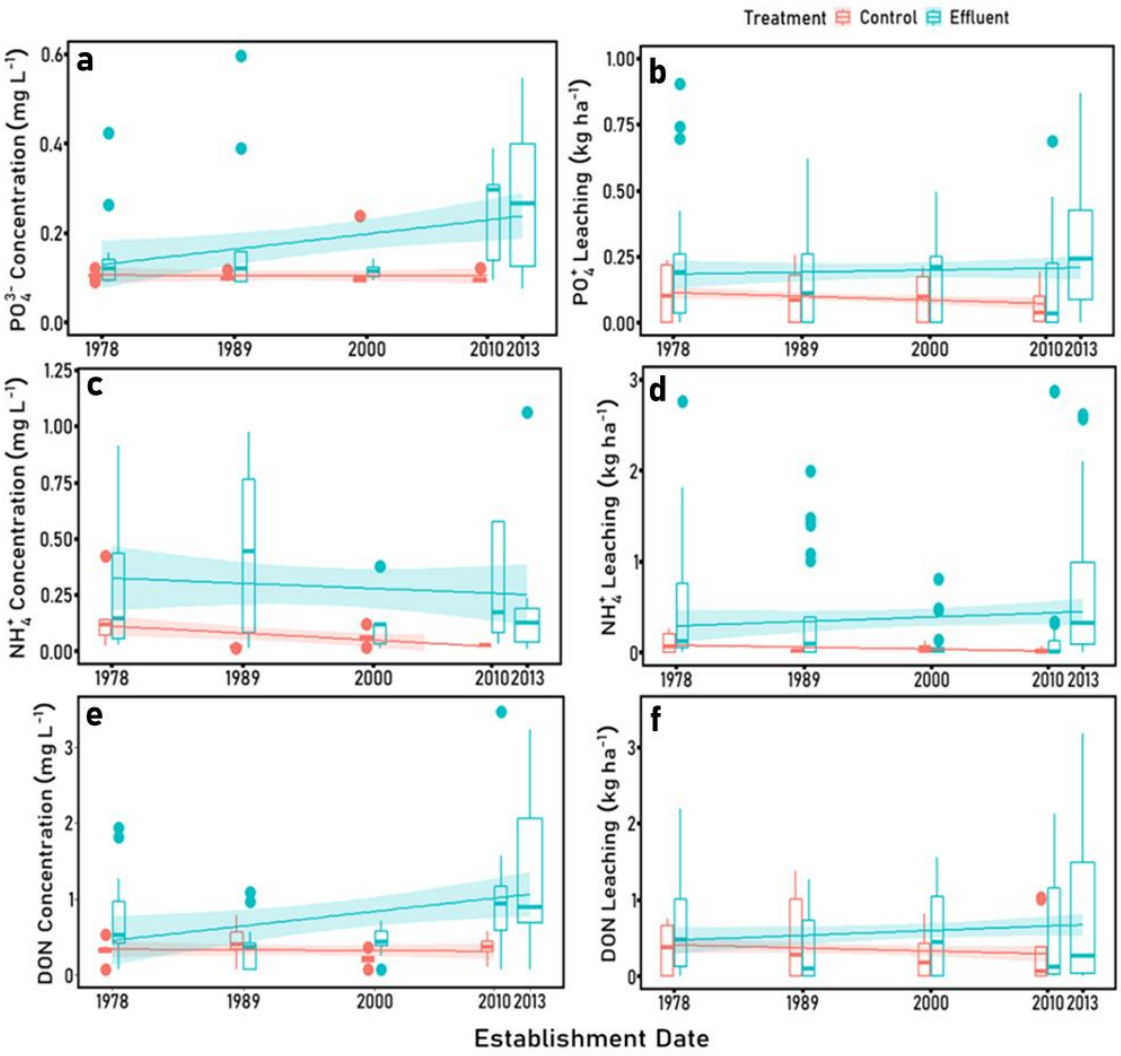

Figure B4: Drain Gauge nutrient concentrations (mg L^{-1}) for facilities with different dates of establishment (a) phosphate concentration [PO_4^{3-}] (mg L^{-1}) and (b) phosphate flux [PO43-] (Kg ha^{-1}), (c) ammonium concentration [NH_4^+] (mg L^{-1}), (d) ammonium flux [NH_4^+] (Kg ha^{-1}), (e) dissolved organic N concentration [DON] (mg L^{-1}) and (f) dissolved organic N flux [DON] (Kg ha^{-1}) in drain gauge samples in response to reclaimed water amendment for facilities with different dates of establishment. Boxes delimit the 0.25 and 0.75 quantiles, horizontal lines indicate medians, whiskers extend to minimum and maximum values, and outliers are plotted as individual points. NH_4^+ concentration had an outlier above 6 mg L^{-1} in 1989 for treatment and two outliers above 2 mg L^{-1} in 1978 for effluent. PO_4^{3-} concentration had an outlier above 1.5 mg L^{-1} in 2013 for effluent group and two outliers above 0.6 mg L^{-1} in 2010 for effluent treatment, and DON concentration had one outlier below 8 mg L^{-1} in 1978 for effluent. PO_4^{3-} leaching had three outliers above 1 kg ha^{-1} in 2010 for effluent and an outlier above 1 kg ha^{-1} in 1989 for effluent, and DON leaching had four outliers above 5 kg ha^{-1} in 1978 for effluent and an outlier above 5 kg ha^{-1} in 2013 for effluent. Each box and whisker represents n=40 observations.

Appendix C: Chapter 4 Supplemental Information

Table C1. Three-way analysis of variance and covariance results comparing soil vs samples for alanine, leucine, and N-acetyl glucosamine. *F* statistic and *P*-values are presented for measured effects of reclaimed water treatment (T), Facility (F), and establishment date (E), and their interactions for alanine, leucine, and NAG. Boldface indicates significance at $P \leq 0.05$.

Effect	Alanine		Leucine		NAG	
	F	***P***	***F***	***P***	***F***	***P***
ANOVA						
T	3.09	0.0862	3	0.0909	0	0.9542
F	2.19	0.0879	4.22	**<0.01**	8.4	**<0.01**
Ty	127.03	**<0.01**	162.46	**<0.01**	190.55	**<0.01**
TxF	2.15	0.0919	1.28	0.2938	1.71	0.1669
TxTy	3.57	0.0662	0.7	0.4069	0.95	0.3355
FxTy	1.95	0.1206	4.38	**<0.01**	2.44	0.0627
TxFxTy	3.61	**0.0132**	3.52	**0.015**	2.22	0.084
ANCOVA						
T	4.93	**0.0313**	1.55	0.2201	0.39	0.533
E	7.2	**0.0101**	11.74	**<0.01**	0.57	0.4533
Ty	5.36	**0.0252**	14.96	**<0.01**	3.44	0.0701
TxE	4.98	**0.0305**	1.57	0.216	0.39	0.5328
TxTy	7.79	**<0.01**	9.5	**<0.01**	3.35	0.0736
ExTy	5.7	**0.0211**	14.34	**<0.01**	3.16	0.082
TxExTy	7.86	<0.01	9.53	**<0.01**	3.33	0.0745

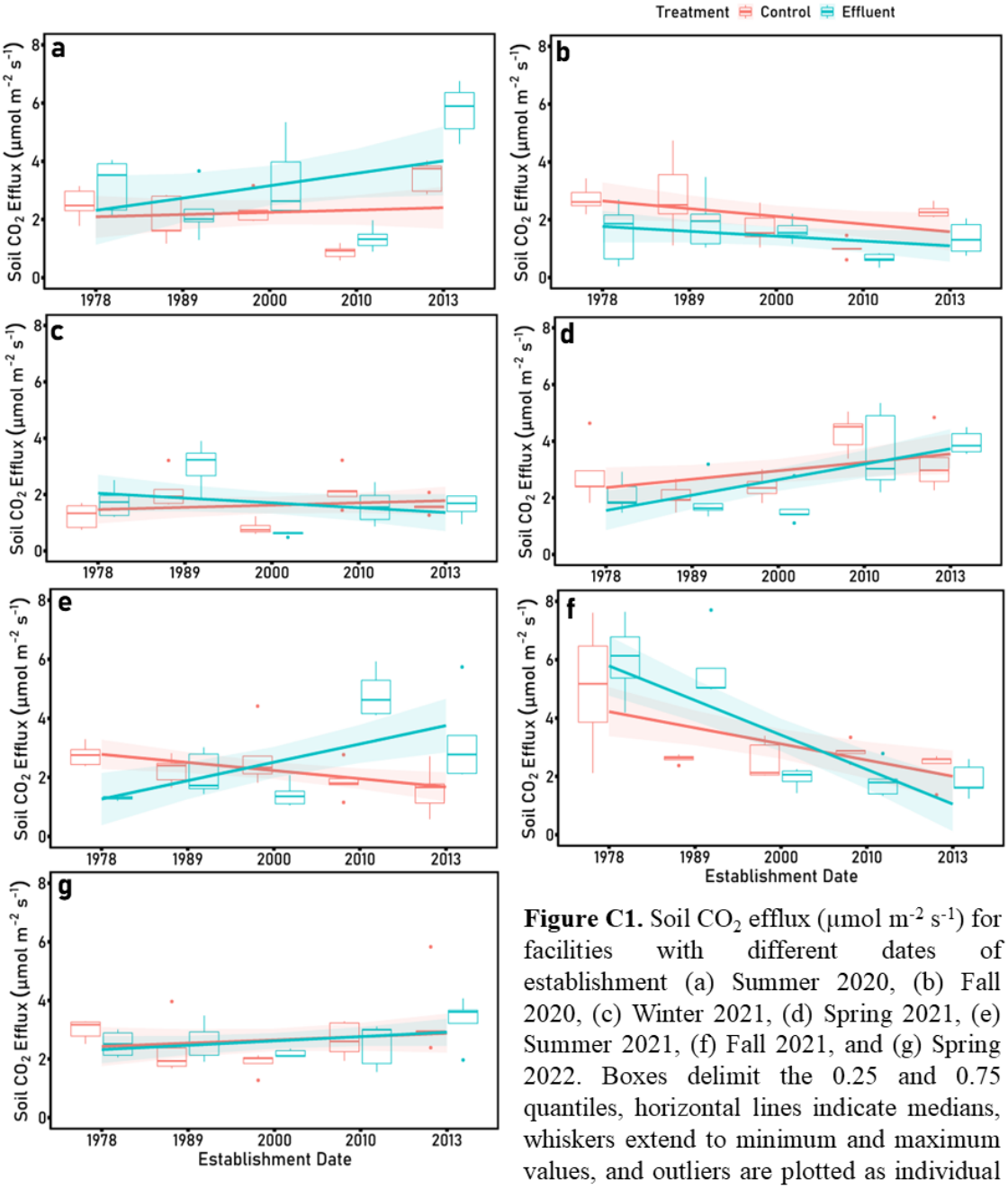

Figure C1. Soil CO_2 efflux (µmol m^{-2} s^{-1}) for facilities with different dates of establishment (a) Summer 2020, (b) Fall 2020, (c) Winter 2021, (d) Spring 2021, (e) Summer 2021, (f) Fall 2021, and (g) Spring 2022. Boxes delimit the 0.25 and 0.75 quantiles, horizontal lines indicate medians, whiskers extend to minimum and maximum values, and outliers are plotted as individual points. The highest outlier for the 2010 established facility for Summer 2021 was excluded for clarity. Each box and whisker represents n=35 observations.

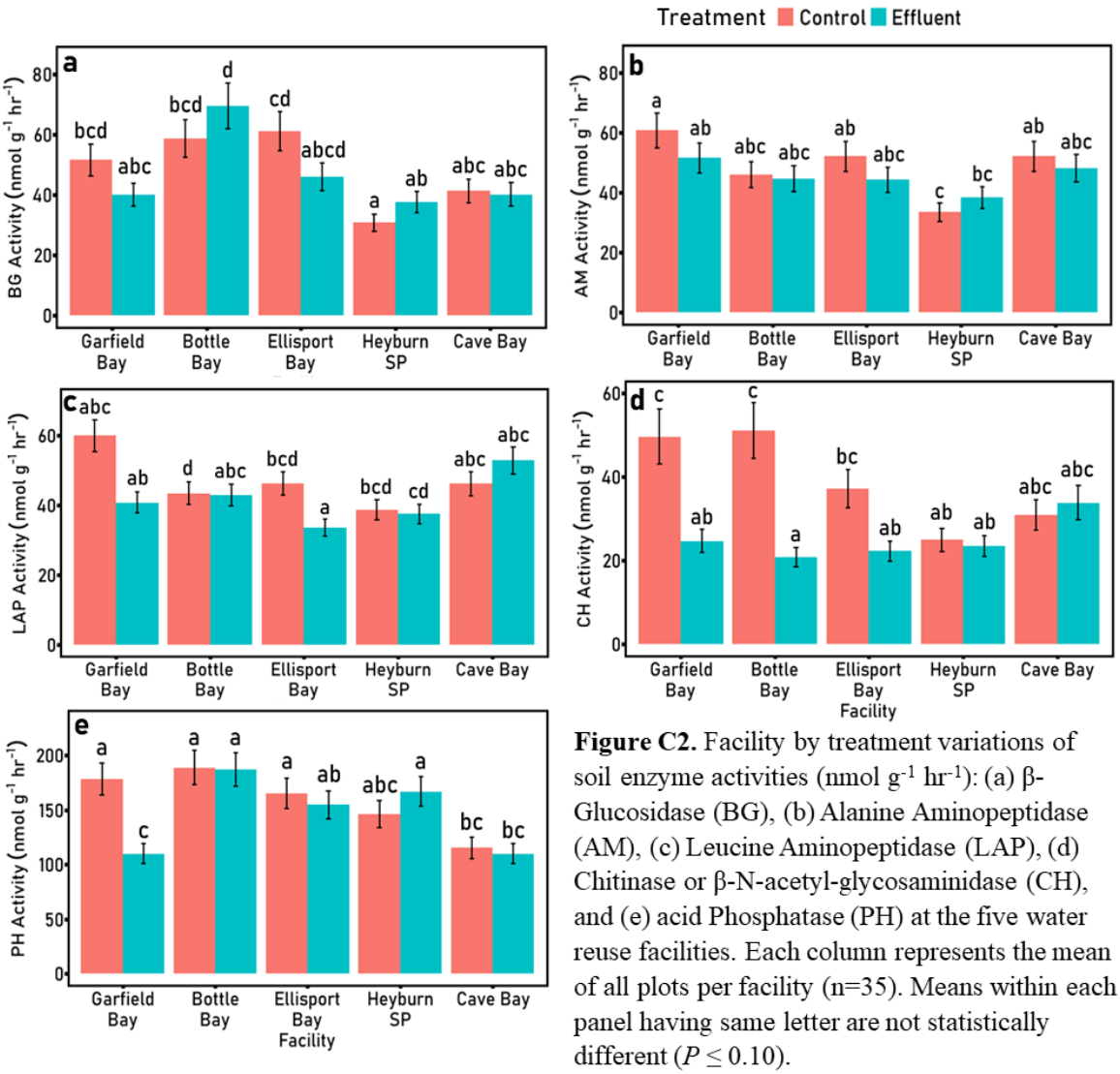

Figure C2. Facility by treatment variations of soil enzyme activities (nmol g^{-1} hr^{-1}): (a) β-Glucosidase (BG), (b) Alanine Aminopeptidase (AM), (c) Leucine Aminopeptidase (LAP), (d) Chitinase or β-N-acetyl-glycosaminidase (CH), and (e) acid Phosphatase (PH) at the five water reuse facilities. Each column represents the mean of all plots per facility (n=35). Means within each panel having same letter are not statistically different ($P \leq 0.10$).

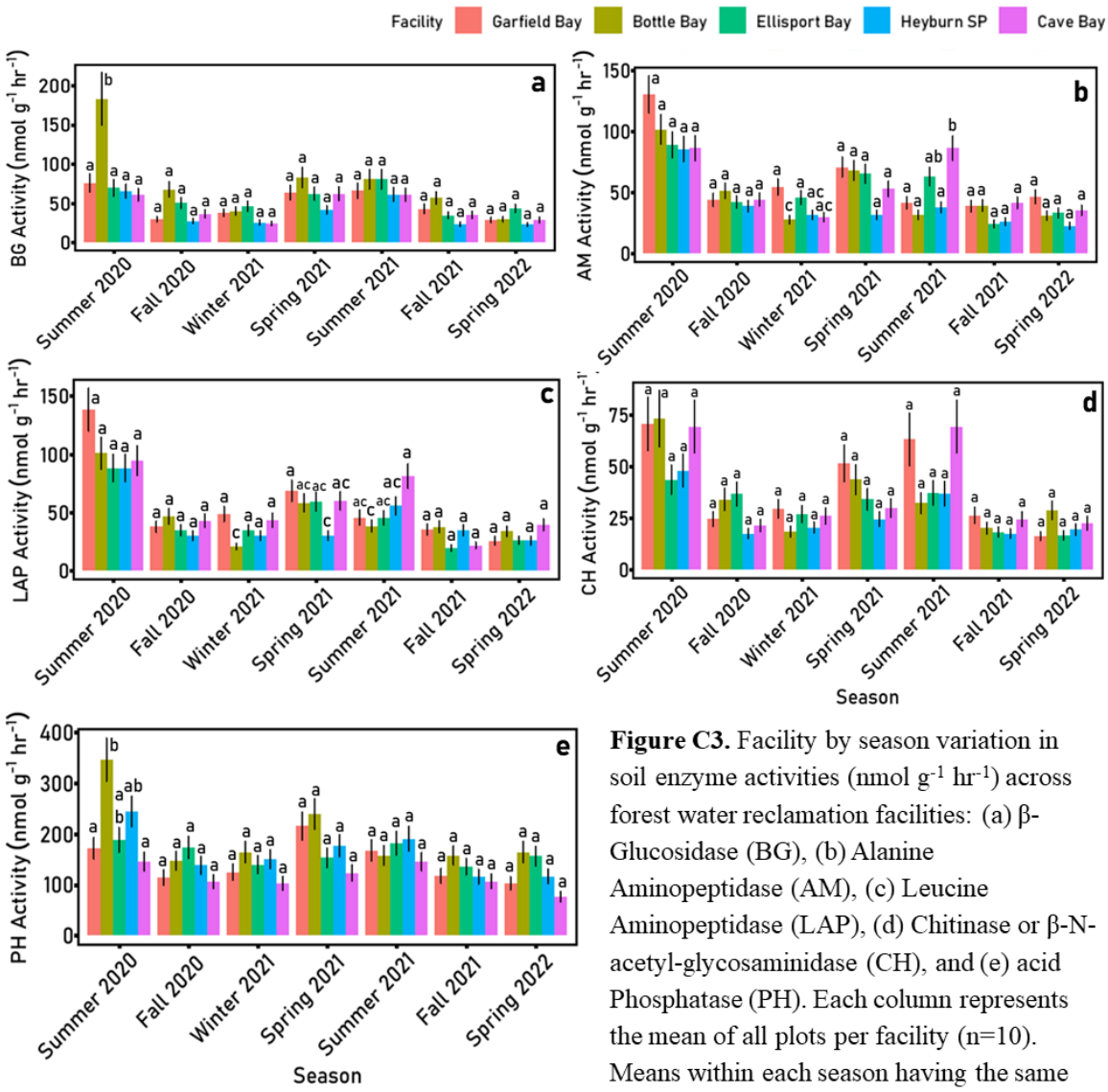

Figure C3. Facility by season variation in soil enzyme activities (nmol g^{-1} hr^{-1}) across forest water reclamation facilities: (a) β-Glucosidase (BG), (b) Alanine Aminopeptidase (AM), (c) Leucine Aminopeptidase (LAP), (d) Chitinase or β-N-acetyl-glycosaminidase (CH), and (e) acid Phosphatase (PH). Each column represents the mean of all plots per facility (n=10). Means within each season having the same letter are not statistically different ($P \leq 0.10$).

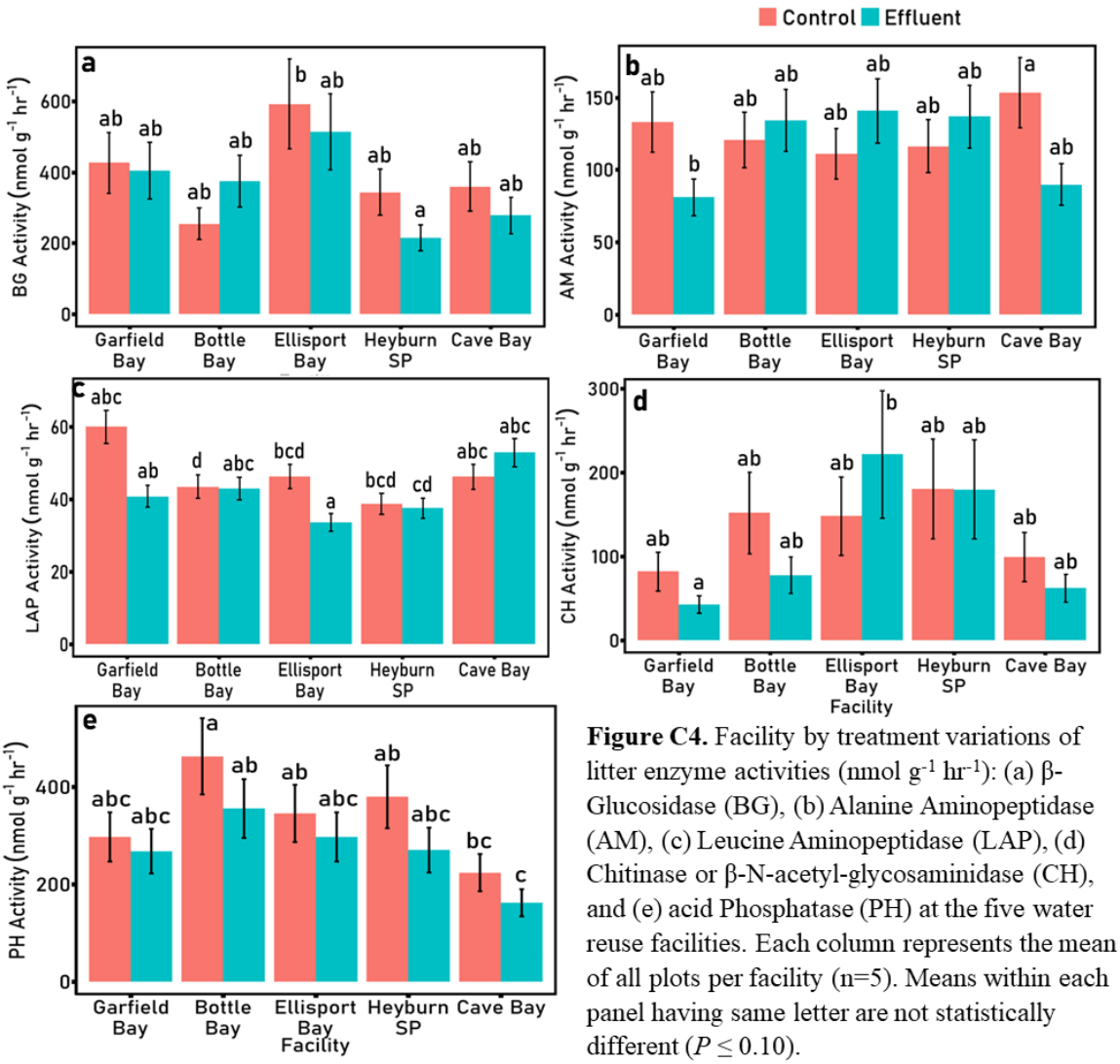

Figure C4. Facility by treatment variations of litter enzyme activities (nmol g^{-1} hr^{-1}): (a) β-Glucosidase (BG), (b) Alanine Aminopeptidase (AM), (c) Leucine Aminopeptidase (LAP), (d) Chitinase or β-N-acetyl-glycosaminidase (CH), and (e) acid Phosphatase (PH) at the five water reuse facilities. Each column represents the mean of all plots per facility (n=5). Means within each panel having same letter are not statistically different ($P \leq 0.10$).

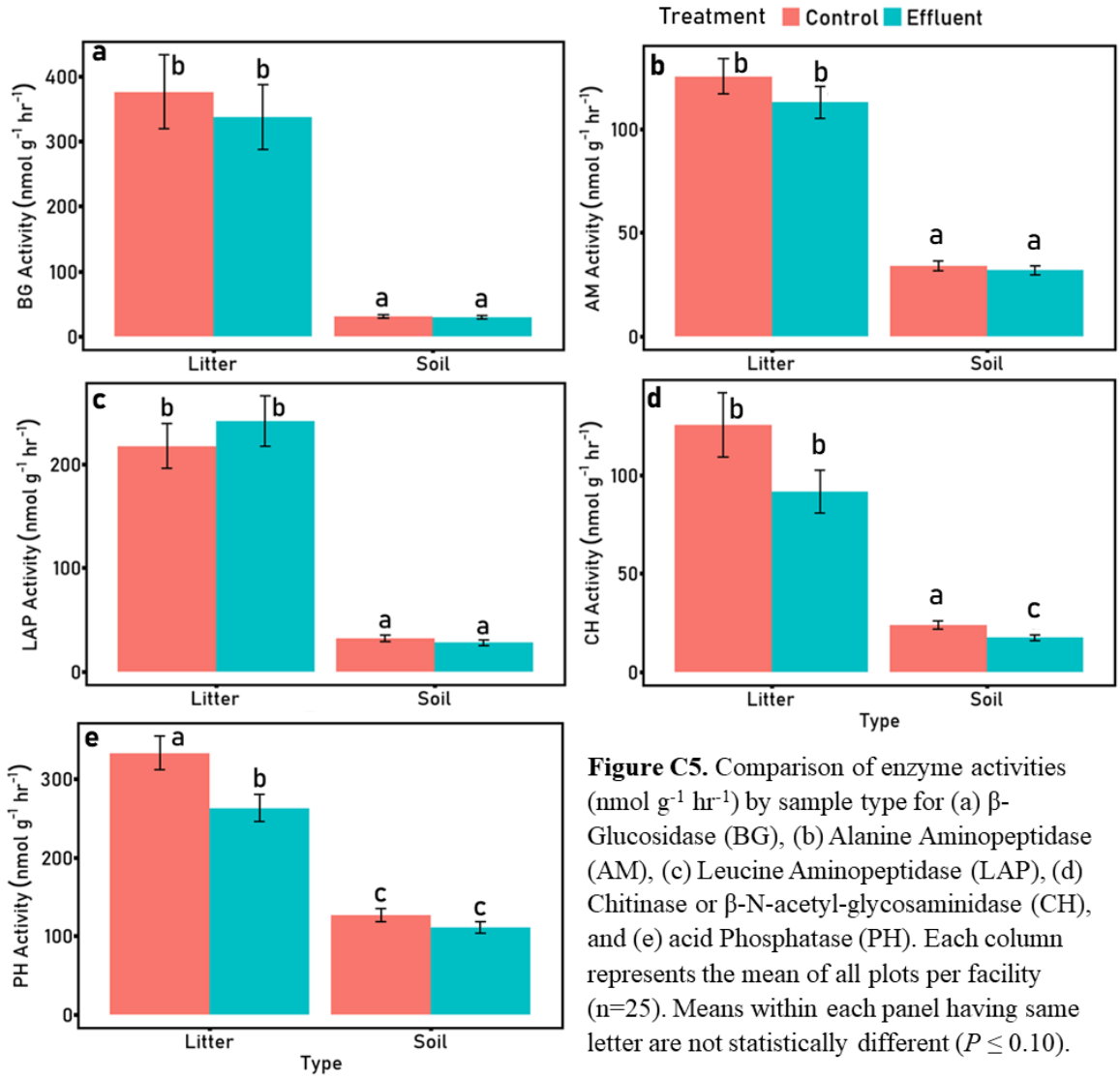

Figure C5. Comparison of enzyme activities (nmol g^{-1} hr^{-1}) by sample type for (a) β-Glucosidase (BG), (b) Alanine Aminopeptidase (AM), (c) Leucine Aminopeptidase (LAP), (d) Chitinase or β-N-acetyl-glycosaminidase (CH), and (e) acid Phosphatase (PH). Each column represents the mean of all plots per facility (n=25). Means within each panel having same letter are not statistically different ($P \leq 0.10$).

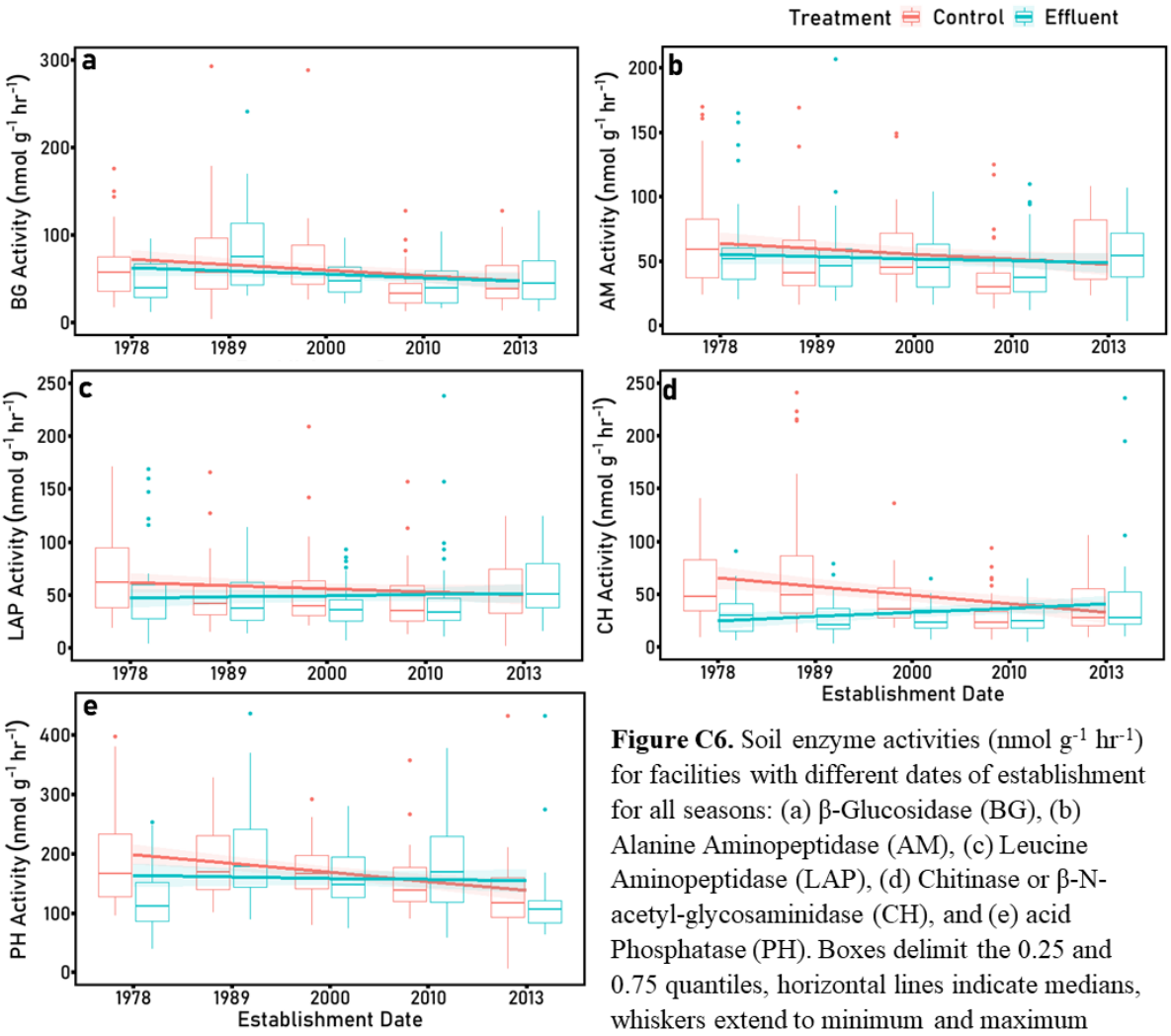

Figure C6. Soil enzyme activities (nmol g^{-1} hr^{-1}) for facilities with different dates of establishment for all seasons: (a) β-Glucosidase (BG), (b) Alanine Aminopeptidase (AM), (c) Leucine Aminopeptidase (LAP), (d) Chitinase or β-N-acetyl-glycosaminidase (CH), and (e) acid Phosphatase (PH). Boxes delimit the 0.25 and 0.75 quantiles, horizontal lines indicate medians, whiskers extend to minimum and maximum values, and outliers are plotted as individual points. Each box and whisker represents n=15 observations.

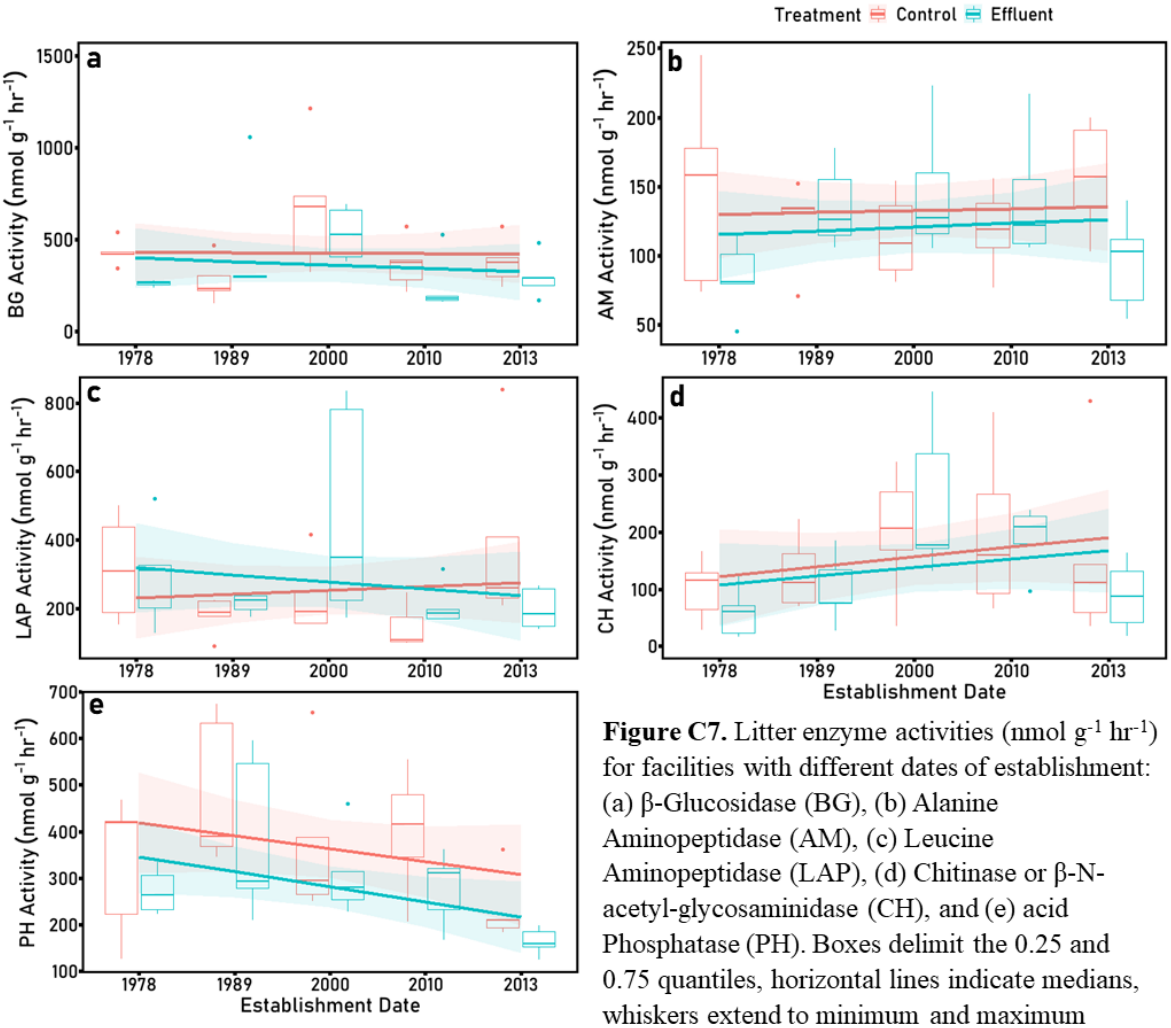

Figure C7. Litter enzyme activities (nmol g^{-1} hr^{-1}) for facilities with different dates of establishment: (a) β-Glucosidase (BG), (b) Alanine Aminopeptidase (AM), (c) Leucine Aminopeptidase (LAP), (d) Chitinase or β-N-acetyl-glycosaminidase (CH), and (e) acid Phosphatase (PH). Boxes delimit the 0.25 and 0.75 quantiles, horizontal lines indicate medians, whiskers extend to minimum and maximum values, and outliers are plotted as individual points. Each box and whisker represents n=5 observations.

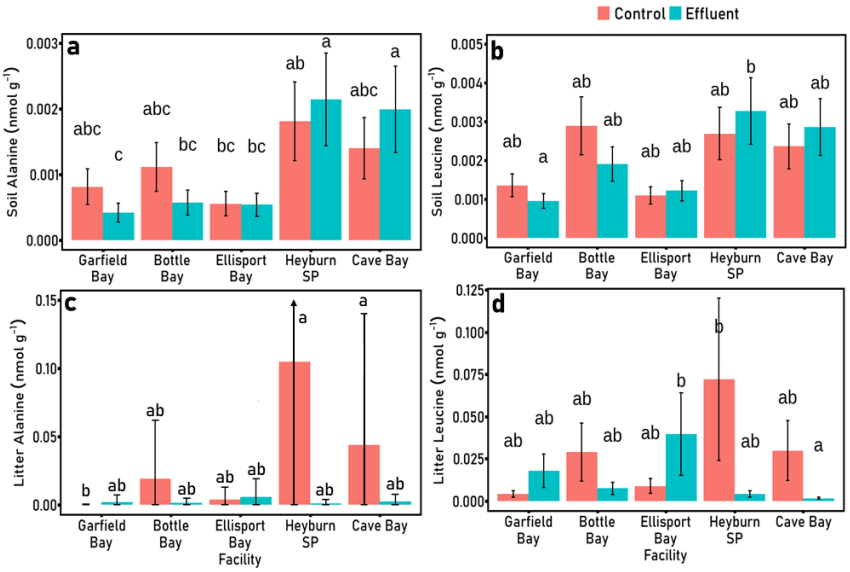

Figure C8. Facility by treatment variations of soil and litter amino acid concentrations (nmol g^{-1}) (a) soil Alanine, (b) Soil leucine, (c) litter Alanine, and (d) litter Leucine at the five water reuse facilities. Means within each panel having same letter are not statistically different ($P \leq 0.10$). Each column represents the mean of all plots per facility (n= 10).

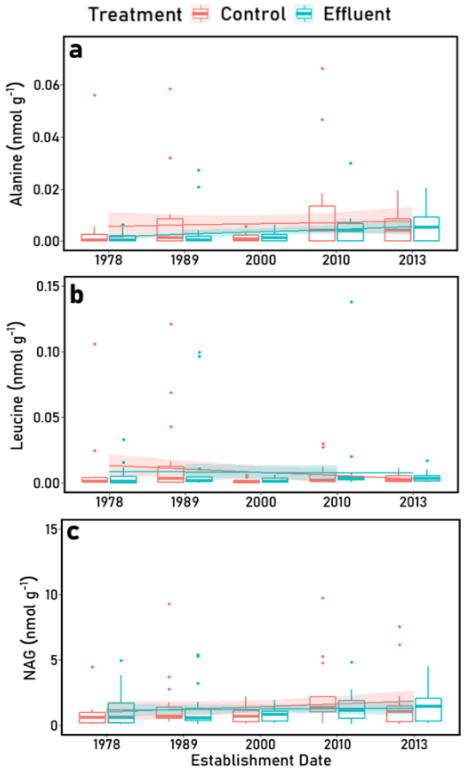

Figure C9. Soil amino acid concentrations (a) Alanine, (b) Leucine, and (c) N-acetyl glucosamine (NAG) for facilities with different dates of establishment. Boxes delimit the 0.25 and 0.75 quantiles, horizontal lines indicate medians, whiskers extend to minimum and maximum values, and outliers are plotted as individual points. Each box and whisker represents n=15 observations

Appendix D: Analysis Protocols

Microplate Nutrient Analysis for ammonium and nitrate preparation
Protocols adapted from:
Ammonium: Baethgen, W.E., Alley, M.M. (1989). A manual colorimetric procedure for measuring ammonium nitrogen in soil and plant Kjeldahl digest. Commun Soil Sci Plant Anal 20 (9&10) 961-96 and Weatherburn, M.W. (1967). Phenol-hypochlorite reaction for determination of ammonia. Analytical Chemistry 39:971-974.
Nitrate: Doane, T.A., Horwath, W.R. (2003). Spectrophotometric determination of nitrate with a single reagent. Analytical Letters 36, 2713-2722.

- Always run a standard curve of serial dilution to test for accuracy and repeated analysis of the same sample for precision.

Equipment:

- Autoclave
- Hotplate
- Stir plate, magnetic bars
- Analytical balance
- Microbalance (accurate to 0.001 mg)
- Weighing boats and sampling spatulas
- Round bottom flasks
- Beakers
- Graduated cylinders
- Reagent reservoirs
- Disposable gloves
- Amber reagent bottles, stoppers with tubings
- Refrigerator (4°C)
- Microplate fluorometer
- 96-well clear microplates with lids/sealing film
- Multichannel pipettes
- Single channel pipette
- Long volumetric pipettes (10 ml) with bulb
- Pipette tips (200 µl and 10 or 20 µl)
- Deionized and distilled water (Milli-Q or Nano-pure)

Hazardous waste: All hazardous waste needs to be disposed of properly (**Caution:** Do not pour down the sink!). Check if the chemicals can be mixed. Disposed in a hazardous waste container or separately in bottles with labels. **Caution:** Always add acid to water and wear protective gear.
Soil solution extraction for ammonium and nitrate:

- Mix composite soil samples for homogeneity and sieve (2 mm)
- Weigh 10 g eq of oven-dried field fresh sieved soil and transfer to a centrifuge tube
- Add 20 mL of 2M KCl matrix (149.1 g KCl in 1000 mL DI water)
- Shake for 1 hr and centrifuge for 10 mins at 2600 rpm
- Filter using Whatman 42 filter paper using a vacuum filtration system
- Store extract at 4°C (OR Freeze for prolonged use)

Appendix D1: Ammonium analysis

Chemicals checklist:

- Sodium salicylate
- Sodium citrate
- Sodium tartrate
- Sodium nitroprusside
- Sodium hydroxide
- Ammonium sulphate
- Potassium chloride
- 10% Hypochlorite (Bleach)

Prepare stock solutions:

Sodium salicylate cocktail:

- To a 100 mL round bottom flask, add 60 mL of water
- Add 6.8 g sodium salicylate
- Add 5 g of (tri)sodium citrate
- Add 5 g of sodium tartrate
- Add 0.025 g of sodium nitroprusside (**caution**: toxic!!)
- Dissolve and make up to 100 mL with water

NaOH solution:

- Make solution of 60 g/L NaOH (**caution:** corrosive!!) by dissolving 6g of NaOH pellets in 100 mL of water

Hypochlorite/NaOH solution (MAKE FRESH DAILY):

- This solution is unstable so only make enough solution for a day (50 mL is enough for 4 plates)
- To make 50 mL, dilute 1 mL of 10% hypochlorite (Household bleach) (**caution:** corrosive!!) to 50 mL with 60 g/L NaOH

2M KCl solution (Matrix):

- Dissolve 149.1 g KCl in DI water in a round bottom flask and bring to 1000 mL volume

100 ppm ammonium-N stock solution:

- In a 500 mL water, dissolve 0.23585 g of $(NH_4)_2SO_4$ (Ammonium sulphate)
- Store in dark at 4°C

Note: This stock solution will last a week. It is recommended that you prepare new stock solution every week. First dilute 100 ppm stock solution to 10 ppm or 1 pm depending on if you are running high concentrations or low concentrations.

10 ppm Dilution (High concentration): In a 1.5 mL centrifuge tube, mix 150 µL of stock with 1350 µL of matrix (2M KCl)

1 ppm Dilution (Low concentration): In a 50 mL flask, add 500 µL of 100 ppm stock solution and fill up to line OR make using 10 ppm in a centrifuge tube (150 µL of 10 ppm in 1350 µL matrix)

Make ammonium std curve solutions:

High Conc.	µL 10 ppm	µL matrix
0	0	1000
0.5	50	950
1.0	100	900
2.0	200	800
5.0	500	500
10.0	1000	0

Low Conc.	µL 1 ppm	µL matrix
0	0	1000
0.02	20	980
0.05	50	950
0.1	100	900
0.2	200	800
0.5	500	500
1.0	1000	0

Detection limit <0.05 ppm

Note: Make sure to run a trial assay for your samples to decide if you need to use high or low concentration standards.

For **low concentrations** (0-5 ppm) add the following to each well:

- Pipette 80 µL of sample or standard using 20-200 µL pipette
- Add 60 µL of salicylate cocktail using multichannel pipette
- Add 60 µL of hypochlorite (Bleach solution) using multichannel pipette. Gently tap the plate and pipette up and down to mix well.

For **high concentrations** (1-10 ppm) add the following to each well:

- Pipette 20 µL of sample or standard using 20 µL pipette
- Add 90 µL of salicylate cocktail using multichannel pipette
- Add 90 µL of hypochlorite (Bleach solution) using multichannel pipette. Gently tap the plate and pipette up and down to mix well.

Analysis procedure:

- Incubate for 45 mins at room temperature
- Create analysis protocol and set microplate layout in fluorometer Gen5 program
- Read absorbance at 650 nm

Notes:

- Standard curve should be run with each plate. Standard curves are added to each plate as samples.
- Samples will turn emerald green.
- However, sample will turn yellow if ammonium concentration is too high. If concentration of ammonium is too high, dilute samples or else change relative amounts of sample and reagents (e.g. 20 µL of sample, 100 µL of salicylate, 100 µL of hypochlorite)

Microplate layout:

	1 std	**2 std**	**3 std**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0	0	0	S1	S3	S5	S7	S9	S11	S13	S15	S17
B	0.5	0.5	0.5	S1	S3	S5	S7	S9	S11	S13	S15	S17
C	1.0	1.0	1.0	S1	S3	S5	S7	S9	S11	S13	S15	S17
D	2.0	2.0	2.0	S2	S4	S6	S8	S10	S12	S14	S16	S18
E	5.0	5.0	5.0	S2	S4	S6	S8	S10	S12	S14	S16	S18
F	10.0	10.0	10.0	S2	S4	S6	S8	S10	S12	S14	S16	S18
G				1.0	1.0	1.0	S19	S19	S19	S20	S20	S20
H				5.0	5.0	5.0	S21	S21	S21	S22	S22	S22

← Standards → ← Check standards →

Note: Always randomize samples (control and treated). The first three columns are horizontal triplicates of your chosen standards (high or low). The two rows (G, H → 4, 5 and 6) are check standards which are intermediate concentrations from standards. These are used to check the accuracy of the assay. I always add them as samples in the plate layout in Gen5. It is helpful to print out the above table (empty) and fill in the sample names and standards while running the assay (helps me while plating).

Appendix D2: Nitrate Analysis

Chemicals checklist:

- Sulfanilamide
- vanadium (III) chloride powder
- 1M HCl
- N-(1-naphthyl)-ethylenediamine dihydrochloride (NEED)
- Potassium nitrate (KNO_3)
- 1 M KCl

Prepare stock solutions: Stock solutions last approximately two weeks. Store in refrigerator at 4 °C.

2% w/v sulfanilamide:*(Make only for higher concentration)*

- Dissolve 2.0 g sulfanilamide in 98 mL 1 M HCl.

*Flush with N_2, may be stored in the dark (amber bottle) at 4 °C for several months, discard if discolored.

0.2% w/v N-(1-naphthyl)-ethylenediamine dihydrochloride (NEED):*(Make only for higher concentration)*

- Dissolve 0.2 g N-(1-naphthyl)-ethylenediamine dihydrochloride in 99.8 mL nanopure.
  *Flush with N_2, may be stored in the dark (amber bottle) at 4 °C for several months, discard if discolored.

  Note: See recipe below for lower concentration.

Nitrate solution (100 ppm)

- Dissolve 72.18 mg Potassium nitrate in 100 mL nanopure water.
  Keeps for a couple weeks.

1M KCl (matrix) (Use 2M KCl for soil samples, see recipe for 2M)

- Dissolve 74.59 g KCl in 1000 mL nanopure water

Prepare reagents (prepare in brown bottles)

Vanadium (III) chloride solution

(FOR **HIGHER** CONCENTRATION [>1 ppm-N])

1. Dissolve 0.35 g of vanadium (III) chloride powder in 50 mL 1M HCl. Work quickly and carefully with the vanadium (III) chloride powder; **it's very reactive with air, so work quickly! Wear a mask when weighing.**
2. Filter the vanadium solution in a 50 mL falcon tube using the vacuum apparatus. Be sure to turn filter unit on before opening valve. After filtering, transfer the vanadium chloride solution to a 500 mL brown bottle.
3. Add 3.3 mL 2% w/v sulfanilamide to the 500 mL bottle.
4. Add 3.3 mL 0.2% w/v N-(1-naphthyl)-ethylenediamine dihydrochloride to the 500 mL bottle.
5. Add 400 mL nanopure water to the 500 mL bottle.

6. Label FOR HIGHER CONCENTRATION OF NO3 – VANADIUM (III) CHLORIDE SOLUTION
7. Purge with nitrogen for at least 15 minutes. Be sure the headspace in the bottle is filled with nitrogen. Solution can be frozen and stored up to 1 year.
8. Use this reagent if your samples have a concentration of NO_3^{2-} greater than 1.0 ppm-N

(FOR **LOWER** CONCENTRATION [0 – 1 ppm-N])

1. Place 100 mL of 1.0 M HCl in a 250 mL beaker with a stir bar. Do not pour all out, so you can rinse the weighing boats.
2. Add 200 *mg* of sulfanilamide to 1.0 M HCl
3. Add 10.0 *mg* of N-(1-naphthyl)-ethylenediamine dihydrochloride to 1.0 M HCl
4. The next step needs to be done quickly so make sure its set up completely before starting. In a small clear bottle, mix 50 mL of 1.0 HCl (different HCl) with *375-450 mg* of Vanadium (III) Chloride. Gently shake intermittently until nearly all solid is dissolved (10 minutes). Do this all of this with a sense of urgency.
5. Filter the vanadium solution in a 50 mL falcon tube using the vacuum apparatus. Be sure to turn filter unit on before opening valve. After filtering, transfer the vanadium chloride solution to a 250 mL brown bottle. It should be a blueish-green color
6. Add filtered solution to beaker containing 100 mL 1.0 M HCl
7. Transfer final reagent to a 250 mL brown bottle labeled FOR LOWER CONCENTRATIONS OF NO3 – VANADIUM (III) CHLORIDE SOLUTION
8. Purge with nitrogen for at least 10 minutes. Be sure the headspace in the bottle is filled with nitrogen.
9. ***ONLY*** use this reagent if your samples have a concentration of NO_3^{2-} lower than 2.0 ppm-N

*****EXPIRED REAGENTS DISPOSED OF AS HAZ WASTE!!!**

(FOR **HIGHER** CONCENTRATION [0 – 1 ppm-N])

1. Prepare a 5 ppm-N solution by combining 5 mL of 100 ppm-N stock with 95 mL nanopure.
2. Create the following standard curves in 20 mL tubes.

Conc. (ppm-N)	mL 5 ppm N solution	mL matrix (1M KCl)
0	0	5
0.25	.25	4.75
0.5	.50	4.50
1.0	1.0	4.0
2.0	2.0	3.0
3.0	3.0	2.0
4.0	4.0	1.0
5.0	5.0	0

(FOR **LOWER** CONCENTRATION [0 – 2 ppm-N])

3. Prepare a 2 ppm-N solution by combining 2 mL of 100 ppm-N stock with 98 mL nanopure.
4. Create the following standard curves in 20 mL tubes. These only keep a couple days.

Conc. (ppm-N)	mL 2 ppm N solution	mL matrix (1M KCl)
0	0	5
0.10	0.25	4.75
0.20	0.50	4.5
0.40	1.0	4.0
0.70	1.75	3.25
1.00	2.50	2.5
1.50	3.75	1.25
2.00	5.0	0

Microplate layout:

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	ST(0)	ST(0)	ST(0)	S1	S3	S5	S7	S9	S11	S13	S15	S17
B	ST(.05)	ST(.05)	ST(.05)	S1	S3	S5	S7	S9	S11	S13	S15	S17
C	ST(.10)	ST(.10)	ST(.10)	S1	S3	S5	S7	S9	S11	S13	S15	S17
D	ST(.20)	ST(.20)	ST(.20)	S2	S4	S6	S8	S10	S12	S14	S16	S18
E	ST(.35)	ST(.35)	ST(.35)	S2	S4	S6	S8	S10	S12	S14	S16	S18
F	ST(.50)	ST(.50)	ST(.50)	S2	S4	S6	S8	S10	S12	S14	S16	S18
G	ST(.75)	ST(.75)	ST(.75)	CS(.10)	CS(.10)	CS(.10)	S19	S19	S19	S20	S20	S20
H	ST(1.0)	ST(1.0)	ST(1.0)	CS(.35)	CS(.35)	CS(.35)	S21	S21	S21	S22	S22	S22

ST= Standard S= Sample CS= Check Standard

Procedure:

- Add 30 µL of sample or standard to each well.
- Pipette triplicates of at least two (2) check standards into available wells as shown above. These check standards should be in the middle of the range of the sample nitrate concentrations if known. If this is unknown, use more check standards if space is available; otherwise use **0.1 ppm-N** and **0.35 ppm-N** (**.5 ppm-N** and **3 ppm-N** for higher concentrations) concentrations as this is the two concentrations that are in the middle of the average sample set. These check standards will be treated as "unknown samples" in order to check the accuracy of the standard curve. If the standard curve is accurate, check standards should read close to their actual concentration under the "Calculated ppm-N NO_3^-" column on the nutrient spreadsheet. Add all remaining reagents to the check standard wells as if they were normal samples.
- Add 300 µL of Vanadium solution to each well and tap plate corner to mix well, incubate for 5 hours or overnight
- Create analysis protocol and set microplate layout in Fluorometer Gen5 program
- Read plate at absorbance of **610 nm**
- Samples should turn pink in color. If the color turns yellow, the concentration of nitrate is too high, dilute the sample and run again. Standard curve should be a straight line. If it is not, try purging the Vanadium (III) Chloride Reagent for a longer period of time. Be sure to purge the vanadium solution with nitrogen for 15 minutes at a pressure of 15 psi after each use. Does not need to be purged if being used directly after it is prepared (still purge after use). Store in refrigerator. When it turns pink, it is no longer good to use.

Appendix D3: Total Dissolved Nitrogen and Dissolved Organic Nitrogen

Total Dissolved Nitrogen and Dissolved Organic Nitrogen: Total Dissolved Nitrogen (TDN) of a sample can be calculated as the sum of constituent dissolved organic and inorganic N. TDN can be determined by quantification of NO_3^- following persulfate digestion of water samples. Dissolved Organic N (DON) can be determined by subtraction of inorganic N [Ammonium (NH_4^+) and Nitrate (NO_3^-)] from TDN concentrations. Alkaline persulfate digestion involves oxidation of NH_4^+ and organic N to NO_3^-, which is subsequently quantified by the VCl_3/Griess method used for quantifying nitrate.

Chemicals:

- Potassium persulfate ($K_2S_2O_8$)
- Sodium hydroxide (NaOH)
- Boric acid (H_3BO_3)
- Glycine
- Sulfanilamide
- Vanadium (III) Chloride
- N-(1-napthyl)-ethylenediamine dihydrochloride (NEED)
- Potassium nitrate (KNO_3)

Persulfate reagent recipe:

Persulfate reagent is a combined solution of 0.185 mol L−1 $K_2S_2O_8$ (Potassium persulfate), 0.42 mol L−1 NaOH (Sodium hydroxide), and 0.485 mol L−1 H_3BO_3 (Boric acid).

- To prepare 1 L Reagent: Dissolve 50 g of $K_2S_2O_8$, 16.8 g of NaOH, and 30 g of H_3BO_3 in milli Q water with an end volume of 1 L.
- To prepare 100 ml reagent: Dissolve 5g, 1.68g and 3 g of $K_2S_2O_8$, NaOH and H_3BO_3 in 100 ml of milli Q water. (**Recommended**)

Note: Make fresh daily and store at 4 °C when not in use.

Alkaline Persulfate digestion:

1. Take 2.5-mL filtered water sample in an autoclavable 5 ml tube and mix with 0.5 mL of persulfate reagent to make a total volume of 3.0 ml. (**Note: Sample: Reagent as 5:1**)
2. **Prepare amino acid digest check solutions**: Prepare urea and alanine stock solutions (1.0 and 5.0 ppm concentrations).

   Digest Check Urea Stock Solution (100 ppm)
   Prepare 100 ppm Urea as nitrogen by dissolving 21.45mg (0.02145g) Urea in 80 mL of DI/milli Q water in a 100 mL volumetric flask. Fill to the mark with water, cap, and mix thoroughly by manual inversion. Transfer the solution to a polypropylene bottle and store at 4 °C. Prepare 1.0 and 5.0 ppm digest check solutions.
   Digest Check alanine Stock Solution (100 ppm)
   Prepare 100 ppm alanine as nitrogen by dissolving 63.64mg (0.06364g) alanine in 80 mL of DI/milli Q water in a 100 mL volumetric flask. Fill to the mark with water, cap, and mix thoroughly by manual inversion. Transfer the solution to a polypropylene bottle and store at 4

°C. Prepare 1.0 and 5.0 ppm digest check solutions.

3. Autoclave the sample tubes (sample + reagent) for Liquid 40 min cycle at 120°C along with Urea and alanine digest check standards (Amino acid + reagent).
4. Make sure the autoclaved samples and digest check solutions have cooled down to room temperature before running the nitrate assay.
5. Run nitrate assay using VCl_3/Griess method (See nitrate protocol for **high concentration**) and read plate at absorbance of 610 nm.
   *__Every sample batch must include a water blank and digestion check solutions. While using one amino acid digest check would suffice, we will use two, to check digestion efficiency after autoclaving.__

Microplate Layout:

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	ST	ST	ST	S1	S3	S5	S7	S9	S11	S13	S14	S15
B	ST	ST	ST	S1	S3	S5	S7	S9	S11	S13	S14	S15
C	ST	ST	ST	S1	S3	S5	S7	S9	S11	S13	S14	S15
D	ST	ST	ST	S2	S4	S6	S8	S10	S12	S16	S16	S16
E	ST	ST	ST	S2	S4	S6	S8	S10	S12	S17	S17	S17
F	ST	ST	ST	S2	S4	S6	S8	S10	S12	BLK	BLK	BLK
G	ST	ST	ST	CS 1	CS 1	CS 1	U1	U1	U1	A1	A1	A1
H	ST	ST	ST	CS 5	CS 5	CS 5	U5	U5	U5	A5	A5	A5

*ST: Standard (See NO3- protocol), CS: Check Standards, 1.0 and 5.0 ppm from standards (ST), U1 and U5: Digested Urea 1.0 and 5.0 ppm (Urea + Reagent), A1 and A5: Digested alanine 1.0 and 5.0 ppm (alanine + Reagent); BLK: Blank (milli Q water), and S: Digested sample (Sample + Reagent).

Calculations:

$$\boldsymbol{Amount\ in\ mol} = \frac{Amount\ in\ grams}{Molar\ mass} \qquad \boldsymbol{Molar\ concentration\ (M)} = \frac{Amount\ in\ mol}{Volume\ in\ L}$$

- **Examples using the above formula to check the quantities make 100 ppm.**

100 ppm KNO3: 72.18 mg KNO3 in 100 ml milli Q water = 7.14 mM (Used to prepare nitrate standard)

100 ppm Glycine:53.61mg (0.05361g) Glycine in 100 ml milli Q water= 7.14 mM (Had some error in digestion for glycine, **NOT recommended**)

100 ppm Urea: 21.45mg (0.02145g) Urea in 100 ml milli Q water= 7.14 mM

- **Example calculation for alanine:**

100 ppm Alanine: 63.64mg (0.06364g) Alanine in 100 ml milli Q water= 7.14 mM

$$\boldsymbol{Amount\ in\ mol} = \frac{Amount\ in\ grams}{Molar\ mass} = \frac{0.06364\ g}{89.0932\frac{g}{mol}} = 0.000714\ mol$$

$$\boldsymbol{Molar\ concentration\ (M)} = \frac{Amount\ in\ mol}{Volume\ in\ L} = \frac{0.000714\ mol}{0.1\ L} = 0.00714\ M = 7.14\ mM$$

Appendix D4: Orthophosphate Protocol (PO_4^{3-})

•Ellen Esch, May 2019, adapted from Ringuet, S., L. Sassano, and Z. I. Johnson. 2011. A suite of microplate readerbased colorimetric methods to quantify ammonium, nitrate, orthophosphate, and silicate concentrations for aquatic nutrient monitoring. Journal of Environmental Monitoring 13:370-376. **There is a correction for this paper linked on the journal website, that is where the molarity comes from, make sure to read. Also from Murphy, J., and J. P. Riley. 1986. A Modified Single Solution Method for the Determination of Phosphate in Natural-Waters. Current Contents/Agriculture Biology & Environmental Sciences:16-16.

•If you need to extract from soils, you can do that, usually you need to create resin strips and then extract P from the soil with the resin strips. Then you would strip the extracted P from the resins using a salt, and then analyze the resulting salt for P. For more information, see: Lajtha, K., C. T. Driscoll, W. M. Jarrell, and E. T. Elliott. 1999. Soil phosphorus: characterization and total element analysis. Pages 115-142 in G. P. Robertson, D. C. Coleman, C. S. Bledsoe, and P. Sollins, editors. Standard Soil Methods for Long-Term Ecological Research. Oxford University Press, New York.

Make solutions:

32 mM ammonium molybdate ($(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$)

• Look in fridge to see if any is already made

• Add 9.89 g ($(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$) into a 250 ml volumetric with nanopure.

- (1235.86 g / mol) * (32 mM / L) * (1 M / 1000 mM) * (250 ml) * (1 L / 1000 ml)
- (molecular weight) * (desired molarity) * (conversion) * (desired amount) * (conversion)

• Toxic!!!! Wear PPE, don't inhale/eat. Store, but if must dispose, it is HAZARDOUS WASTE.

4.9 N sulfuric acid (H2SO4)

• Look under hood (acid storage) to see if any is already made

- 13.6 ml 96% H_2SO_4 in 86.4 ml nanopure H_2O (**Use this instead of recipe below**).

• Add 133 ml concentrated H2SO4into 1 L volumetric with nanopore (mix acid into water!!).

- (98 g H2SO4 / 100 g acid) * (1.84 g acid / mL) * (1 mol H_2SO_4 / 98.072 g) * (1000 ml / L) = 18.39 M (or 36.8 N)
- (acid purity by weight) * (density of acid) * (molecular weight) * (unit conversion) = molarity (or convert to N)
- Normality = molar concentration of acid component; N = M * (# of hydrogen ions); 4.9 N = xM * 2 → 2.45 M

• Store, but if needed neutralize with sodium bicarbonate and dispose down drain

100 mM L-ascorbic acid (C6H8O6)

• Make fresh daily!! ascorbic acid oxidizes quickly

• Add 0.886 g ascorbic acid into a 50 mL volumetric with nanopure

- (176.12 g $C_6H_8O_6$ / mol) * (100 mM / L) * (1 M/ 1000 mM) * (50 mL) * (1 L / 1000 mL)
- (molecular weight) * (desired molarity) * (conversion) * (desired amount) * (conversion)

• Neutralize with sodium bicarbonate and dispose down drain

4.5 mM antimony potassium tartrate ($K_2Sb_2(C_4H_2O_6)_2•3H_2O$) (look in fridge to see if any is already made)

• Add 0.3005 g antimony potassium tartrate to a 100 mL volumetric with nanopure

- (667.87 g $K_2Sb_2(C_4H_2O_6)_2•3H_2O$ / mol) * (4.5 mM / L) * (1 M/ 1000 mM) * (10 mL) * (1 L / 1000 mL)

• Toxic!!!! Wear PPE, don't inhale/eat. Store, but if must dispose, it is HAZARDOUS WASTE.

Reagent solution

• make fresh daily in 100 ml beaker or volumetric (good for ~4 hrs)! and mix well after each addition, and order is important)

1. 15 ml ammonium molybdate solution

2. 50 ml sulfuric acid solution

3. 30 ml ascorbic acid solution

4. 5 ml antimony potassium tartrate solution

• HAZARDOUS WASTE (look in hood for marked container)

100 ppm stock P solution

• Add 0.2197 g oven dry potassium phosphate monobasic (KH_2PO_4) to a 100 mL volumetric with nanopure

o (0.1 g P / 1 L) * (136.084 g KH_2PO_4 / 30.974 g P) * (0.5 L) = 0.2197 g KH_2PO_4

o (desired ppm) * (percent P) * (desired volume) = g KH_2PO_4 to add

o 100 ppm P = (100 μg P / 1 mL) = (0.1 g P / 1 L)

• Add 0.2197 g oven dry potassium phosphate monobasic (KH_2PO_4) to a 100 mL volumetric with nanopore

• Store, but if must dispose, it is HAZARDOUS WASTE.

Determine how many plates you need, and create a set-up:

1. If running triplicates = each plate will have a standard curve (6 levels * 3 reps = 18 wells), so you can fit in 26 samples (78 / 3).

2. If running quadruplicates = each plate will have a standard curve (6 levels * 4 reps = 24 wells), so you can fit in 18 samples (72 / 4).

Make standard curve:

**High is crazy high for water samples, probably want to run low, but run both curves if unsure.

1. Dilute the 100 ppm stock solution to either:

•(low) 1 ppm, add 1 ml of 100 ppm stock solution to 100 ml volumetric flask

•(high) 10 ppm; add 10 ml of 100 ppm stock solution to 100 ml volumetric flask

2. Create the following standard curves in 1.5 ml centrifuge tubes.

Low			**High**		
[Std]	**µl 1 ppm**	**µl matrix**	**[Std]**	**µl 10 ppm**	**µl matrix**
0 ppm	0	1000	0 ppm	0	1000
0.05 ppm	50	950	0.5 ppm	50	950
0.10 ppm	100	900	1.0 ppm	100	900
0.20 ppm	200	800	2.0 ppm	200	800
0.50 ppm	500	500	5.0 ppm	500	500
1.00 ppm	1000	0	10.0 ppm	1000	0

Run analysis:

NOTE: run samples in triplicate (or quadruplicate!!) and add the standard curve to one plate. Add the samples to the wells first, and then use the multichannel pipette to add the reagent. Make sure to label plates and create a diagram for your sample lay-out.

Add the following to each well:

1. 200µl sample

2. 50µl reagent solution (use multichannel pipet)

Tap corner of plate to mix well, cover with foil and incubate for 30 min. Read plate at 880 nm. **Note, if your P concentration is low, the standard curve still has excellent fit for 2 hours (maybe even more??!). The standard curves are indistinguishable for 30 min, 45 min, 60 min, and 120 min. If your P concentration is high, the 30 min is important or the colors start to saturate.

Appendix D5: N-acetyl-D-glucosamine (NAG) Sample Preparation

Extraction and preservation of soil samples for NAG and Amino Acid Analysis

- For all samples [2020 Samples, 2022 Spring (qfs 1-3, 8)]:
- Weigh 7 g of dry equivalent soil mass into a 50 mL centrifuge tube and add 35 ml DI water (1:5).
- Place on end-to-end shaker for 60 minutes.
- Centrifuge at for 2600 rpm for 20 minutes at 4**°C**
- Filter the supernatant under vacuum through a Whatman 42 filter paper
- **2020 Summer, Fall & Winter (qfs 1-3)**: Split filtrate into two 50 mL centrifuge tubes in equal amount (~17.5 ml; assumed to be 3.5 g soil dry equivalent) for separate analysis of NAG (**1of2**) and amino acid (**2of2**) and lyophilize until dehydrated and store in freezer
- **2022 Spring (qfs 8)**: Place all of filtrate (~35 ml; assumed to be 7 g soil dry equivalent) into clean centrifuge tube and freeze
- Lyophilize until dehydrated and store in freezer

Solubilize and filter:

- **2020 Summer (qfs 1)**: Add 1 ml of deionized water, to **1of2** in 50 mL centrifuge tube and sonicate for 5 minutes. Transfer to an Eppendorf tube and centrifuge (small centrifuge) for approximately 5-6 minutes to remove solids. Filter supernatant through 0.45um syringe filter (13mm) into a HPLC vial.

- **2020 Samples (qfs 2-3)**: Add **1 mL** (1000 µL) 0.05 M HCl to **1of2** in 50 mL C tube and sonicate for 5 minutes. Transfer to an Eppendorf tube and centrifuge for approximately 5-6 minutes to remove solids. Filter supernatant through 0.45um syringe filter (13mm) into a HPLC vial.

- **2022 Spring (qfs 8)**: Quantitatively transfer freeze dried samples to 1.5 mL Eppendorf tube. Add 1 mL 0.05 M HCl to the Eppendorf tube, sonicate for 5 minutes, and centrifuge for approximately 5-6 minutes to remove solids. Set aside 100 µL of supernatant for AA derivatization and analysis. Filter Remaining **900 µL** of supernatant through 0.45um syringe filter (13mm) into a HPLC vial.
- Transfer to Eppendorf tube (1.5 ml or 2 ml).

Standards

- Prepare standard stock solution: Weigh 1 mg of GlcNAc/NAG and transfer into a 100 ml volumetric flask and mix well.
- Make a series of dilutions of the stock solution: 1/10, 1/20 and 1/50

1/10 dilution => 10 ml of NAG Stock in 90 ml of DI water
1/20 dilution => 5 ml of stock in 95 ml DI water
1/50 dilution => 2 ml of stock in 98 ml DI water

Appendix D6: Amino Acid Analysis Sample Preparation

Armando McDonald, January 2022

I. Materials and supplies

i. 1.5 microtubes
ii. 1000µL, 200 µL and 20 µL pipette
iii. Sonicator
iv. Centrifuge
v. GCMS
vi. 0.05 m HCl
vii. 0.01 M AA standard for Ala and Lys and dilute to 0.001 and 0.0001 M (see sec V.2-3).
viii. 0.012 M methyl laurate in acetonitrile (see sec V.1).
ix. Methanol
x. Pyridine
xi. Methyl chloroformate (MCF)
xii. 1% MCF in chloroform
xiii. Saturated NaCl

II. Soil extraction

1. Transfer freeze-dried soil powder (solids) to an Eppendorf tube (1.5 or 2 mL)
    - Dissipate static by:
        - rubbing outside with dryer cloth
        - Dip in water filled sink
2. Add 1 mL of 0.05 M HCl solution to 50 mL centrifuge tube to solubilize any remaining sample, vortex 30 sec, centrifuge on high for 1 min, and quantitatively transfer solution with suspended solids to microfuge containing the bulk of the solids.
3. Sonicate for 5 min and then centrifuge
4. Use the supernatant 100 µL (or 50 µL) for amino acid analysis

III. Amino acid derivatization

1. Pipette 25 µl of amino acid standard stock solution (0.01 M) to a 0.6 mL plastic tube. Use L-alanine and L-lysine for your standards. You can do a dilution series to get a calibration.
2. Pipette 100 µl (or 50 µL) of soil solution supernatant to a 0.6 mL plastic tube.
3. To the amino acid standards and soil solutions add (using pipettor) and **do this in the fume hood**
    a. internal standard solution (10 µl of 0.012 M methyl laurate in acetonitrile)
    b. methanol (50 µL)
    c. pyridine (15 µL)
    d. close cap and then mix by hand shaking
4. Then add 15 µL methyl chloroformate (MCF) to each sample/standard **in 5 µL increments** (I suggest that you add sequentially to all samples then go back to the first). **Slow addition of MCF is critical.**
5. Close lid and **Vortex for 30 s**, stand for 5-10 min, and **re-vortex for 10 s. This is critical**
6. Add 200 µL of 1% v/v MCF in chloroform using a pipettor and mix and let stand for 2-5 min
7. Add 20 µL of saturated NaCl aqueous solution and **vortex for 15 s**
8. Centrifuge for 1 min to get phase separation
9. Remove bottom organic layer 180 µL total (TWO 90 µL aliquots) using a pipettor and transfer to a GC vial containing an insert. Be careful to avoid getting any aqueous layer in your sample.
10. Cap the GC vial

IV. GC-MS analysis

1. Use Thermo ISQ7000-Trace GCMS with autosampler in CNR117a
2. Use method – **Amino Acids MMCF**

V. Notes:

1. Methyl laurate - weigh 0.0258 g in 10 mL acetonitrile (in hood)
2. L-Lysine - weigh 0.01462 g in 10 mL water or 0.05M HCL
3. L-alanine - weight 0.00891 g in 10 mL water or 0.05 M HCL

VI. Calculations

Multiply concentration by 5 based on sample used (III.2) and divide by 10 g sample weight

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