Simultaneous wastewater treatment and poplar biomass production: treatment performance and microbial-mediated nitrogen cycling

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Abstract

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As demand for clean water increases, it is important to employ strategies to preserve both water quality and quantity. One strategy is the use of treated wastewater for irrigation, which can also serve as a wastewater treatment mechanism. Poplar trees and associated soil microorganisms remove pollutants from the irrigation water and the nutrient inputs increase crop growth. While treatment performance in wastewater infiltration systems has been studied, the contribution of the soil microbial community is not well-understood. We aimed to study nitrogen removal performance and biomass production in a poplar-planted wastewater infiltration system and assess impacts on microbial community structure and function. Reactors, along with bare-soil controls, were irrigated with synthetic wastewater effluent with increasing nitrogen concentrations for 18 months. Experiments were conducted each season, and soil samples were collected after each experiment. Soil DNA extracts were sequenced with 16S third-generation nanopore sequencing to examine community composition and droplet digital PCR was used to quantify key nitrogen cycling genes. Across all seasons, significant nitrate and total nitrogen removal was observed in planted reactors, while performance of unplanted reactors decreased in the winter months and under high nitrogen loads, highlighting the importance of poplars in taking up nitrogen and providing organic carbon. Microbial community diversity was not impacted by exposure to low-strength wastewater, but statistical visualization suggested differences in key microbial groups between reactors with and without trees. Nitrogen cycling in planted and unplanted soils was altered by wastewater. Nitrification was impacted at the gene level, with wastewater irrigated reactors having higher abundance of *amoA*, while denitrification was impacted at the level of activity, with increased denitrification potential in soils treated with higher nitrogen and organic matter loads. Compared to control reactors receiving tap water, poplars irrigated with wastewater produced 3 times more aboveground biomass, and an economic evaluation showed that establishing tertiary treatment wastewater infiltration systems for bioenergy production and water recovery (WISER) could produce substantial profits for wastewater treatment facilities via the sale of recovered water. These results supported the potential for year-round operation of infiltration systems for treatment and biomass production and highlighted the importance of vegetation for optimal treatment performance. Impacts to the soil community were minimal and served to enhance treatment without impacting overall community structure and function. This work will help

inform the design and operation of WISER, providing opportunities for inexpensive tertiary treatment and the expansion of the bioeconomy.

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1. Background and Literature Review

Our world currently faces challenges in maintaining both the quality and quantity of water resources, and it is important to focus on solutions that can address both aspects of this challenge. One method is through the reuse of treated wastewater (TWW), which has been designated as a valuable resource due to its potential for crop irrigation and nutrient recovery (Programme 2017). TWW can take the place of clean water in various applications, including agricultural irrigation. Biomass crops, such as hybrid poplar (*Populus*) have been irrigated with TWW in wastewater infiltration systems. The impacts on the soil microbial community are not well understood. Plant-microbe relationships may improve the practice.

Here, I tested the capacity of poplar trees and associated soil microorganisms to recover nutrients from synthetic wastewater effluent applied at high volumes and assessed associated changes to the composition and function of the soil microbial community. Experiments were conducted in simulated wastewater infiltration systems which were planted with poplar trees and drip irrigated with synthetic TWW year-round. Soil properties and water recovery were monitored, and water recovered from the system was tested for nitrogen species to quantify removal. I assessed changes to the microbial community structure in TWW-irrigated soils via nanopore 16S sequencing. Finally, I explored nitrification and denitrification, quantifying key nitrogen cycling genes and microbial activity in the soil.

1.1. Wastewater treatment

Nitrogen is a key pollutant in wastewater. Recognition of the importance of limiting nitrogen discharge is increasing in many regions, including the Puget Sound. In 2022, a new permit went into effect that more tightly regulates the discharge of total inorganic nitrogen (TIN) into the Puget Sound. Treatment plants are required under the permit to keep their TIN discharge under 10mg/L yearly average and 3mg/L summer seasonal average (Ecology 2021). To meet these goals, treatment plants will need to improve the effectiveness of their current technologies or implement new tertiary treatment technologies. Tertiary treatment can be expensive and may present a barrier to some of these facilities in meeting their goals. To achieve these goals, affordable solutions are needed.

1.1.1. Traditional primary and secondary wastewater treatment

Wastewater treatment removes nutrients and pollutants from wastewater before returning it to the environment. Required treatment typically consists of primary and secondary steps. Primary treatment is physical settling of solids out of the water. Secondary treatment is biological, utilizing microorganisms to remove organic matter and other pollutants (Tchobanoglous, Stensel et al. 2014). Despite the effectiveness of these processes, most wastewater effluents contain low levels of residual nutrients, including nitrogen and phosphorus, which are not targeted in primary and secondary treatment. These nutrients may be released to local waters in effluent and accumulate over time.

Tertiary treatment methods can increase nutrient removal, including nitrogen and phosphorus. Examples include additional filtering (reverse osmosis, ultrafiltration) or disinfection (UV, ozonation) steps. These treatments can be expensive and energy intensive (Tchobanoglous, Stensel et al. 2014). Nature-based solutions can provide treatment at lower costs than many other tertiary methods while also providing ecosystem services and other potential benefits.

1.1.2. Land treatment and wastewater infiltration systems

Land treatment is a nature-based solution that applies treated wastewater to the soil surface (Crites and Tchobanoglous 1998) to take advantage of the natural pollutant attenuation mechanisms present in the soil, including filtration by the soil matrix and activity by soil microorganisms (de Bustamante 1990). Land treatment systems are also called wastewater infiltration systems (Li, Su et al. 2023), and this term has been used to discuss studies focused on nutrient fate in addition to water treatment (Lu, Gao et al. 2022). Vegetation filters additionally refer to planted soils, which are beneficial over bare-soil systems because the root structure of the plants harbors a larger and more diverse population of microorganisms (Sylvia, Hartel et al. 2005, de Miguel, Meffe et al. 2014), which are a major driver in pollutant mineralization (Champagne and Bhandari 2007). When soils are planted with specific types of crops, still other names may be used, such as short-rotation coppice (SRC) in the biofuels industry for biofuel crops. Land treatment systems are useful as treatment in rural communities with small populations (Amiot, Jerbi et al. 2020), and as end of treatment-train steps to existing wastewater treatment processes.

In addition to the wastewater treatment benefits provided by land treatment, the process of applying wastewater can be paired with agricultural irrigation. This is particularly beneficial in water-stressed areas where fresh water for irrigation is not readily available.

1.1.3. Nutrient removal in land treatment systems

Examples of nutrient removal performance in land treatment systems of multiple types are shown in Table 1. Studies have documented treatment performance under all levels of development from column studies (Pang, Pan et al. 2020, Chen, Jiang et al. 2021, Prodanovic, Zhang et al. 2023) up to small-scale wastewater treatment systems (de Miguel, Meffe et al. 2014, Lachapelle-T, Labrecque et al. 2019). While a small fraction of test systems accumulate nitrate and convey it to groundwater (Pradana, Hernandez-Martin et al. 2021), most successfully remove pollutants in wastewater to below regulatory limits. When nitrate breakthrough does occur, it is often in systems treating raw or primary wastewater, or industrial wastewater with no pretreatment (Pradana, Hernandez-Martin et al. 2021). At the secondary effluent stage, nutrients are already greatly reduced to concentrations amenable to land treatment.

Table 1: Nutrient removal in land treatment systems with biomass crops

Crop	Water loading (mm/day)	Total N removal	NO_3 removal	COD removal	Ref
Willow		35%-37%		63-68%	(Khurelbaatar, van Afferden et al. 2021)
Poplar, willow	4.9	80%		86%	(Khurelbaatar, Sullivan et al. 2017)
Willow	10 16	94% 87%	96-98%		(Lachapelle-T, Labrecque et al. 2019)
Willow	11		11-100%		(Elowson 1999)
Poplar	0.7-6	73%		85%	(de Miguel, Meffe et al. 2014)
Willow		93%		96%	(Amiot, Jerbi et al. 2020)
Willow, poplar	7.0	Willow 95% Poplar 30-50%		Willow 35% Poplar 25%	(Dimitriou and Aronsson 2011)

1.2. Impacts of reclaimed wastewater irrigation

1.2.1. Soil properties and crops

Impacts of TWW irrigation on soil properties are summarized in Table 2. TWW irrigation generally increases soil organic carbon (de Miguel, Meffe et al. 2014, Becerra-Castro, Lopes et al. 2015, Chen, Lu et al. 2015), which is correlated with COD removal from influent (de Miguel, Meffe et al. 2014, Mojid, Hossain et al. 2019). Impacts on nutrient content are mixed, with nitrogen and phosphorus increasing in some studies, again due to inputs from the TWW (Becerra-Castro, Lopes et al. 2015, Chen, Lu et al. 2015, Zolti, Green et al. 2019) and remaining unchanged in others (Obayomi, Edelstein et al. 2020, Lavi, Bar-Massada et al. 2024). The forms of nitrogen in the soil may also be impacted, such as a shift from ammonium-N to nitrate-N (Liu, Xue et al. 2022). Soil moisture in long-term irrigated soils (13-30+ years) was shown in at least two cases to be higher than unirrigated controls (Wafula, White et al. 2015, Kargol, Cao et al. 2022), but this is likely related to the consistent water application, rather than the quality of the water.

Salinity almost universally increases in soils irrigated with TWW (Biswas and Mojid 2018, Lavi, Bar-Massada et al. 2024), as residual salts accumulate in soil over time (Haj-Amor, Araya et al. 2022). Soil salinity can influence other soil properties including organic matter and greenhouse gas emission potential (Haj-Amor, Araya et al. 2022). Soil structure can also be impacted by salinity. A transition from mixed mono- and di-valent salts to high sodium levels can disrupt associations between clay particles and cause dispersion of soil particles, disrupting soil structure (Sparks 1995). Changes to salinity may also alter the structure and function of the microbial community (Li, Wang et al. 2021), in extreme cases selecting for salt-tolerant microbial communities under long-term salt stress (Rath, Fierer et al. 2019, Zhang, Shi et al. 2019). Fortunately, salinity buildup can be moderated by applying irrigation water in excess of crop requirements to leach salts from the soil (EPA 2006, Chen, Lu et al. 2015).

Changes to soil pH in response to TWW irrigation have also been observed. Soil pH can impact nutrient and metal availability in soils (Becerra-Castro, Lopes et al. 2015). Low pH (below 6) can even lead to increased heavy metal uptake by crop species (EPA 2012) while high pH may limit the availability of nutrients in the soil (Sparks 1995). Frequently, soil pH increases in response to TWW (Wafula, White et al. 2015, Siggins, Burton et al. 2016, Zolti, Green et al.

2019). A decrease in pH observed in one study was attributed to the production of organic acids from the components in the wastewater, which acidified the soil environment (Mojid, Hossain et al. 2019).

Microbial activity in soils is often characterized by the activity of extracellular enzymes (Thiele-Bruhn, Schloter et al. 2020), and increases in the activity of key enzymes can indicate higher overall activity in a system (Burns, DeForest et al. 2013). The activity of enzymes including alkaline phosphatase, dehydrogenase, and B-glucosidase have been shown to increase in soils treated with TWW (Adrover, Farrus et al. 2012, Chen, Lu et al. 2015, Siggins, Burton et al. 2016). The magnitude of changes to soil characteristics under TWW irrigation varies greatly between systems. This suggests that other environmental factors, such as soil properties, climate, and crop species, may be as or more important than irrigation water quality in shaping soil response to TWW.

The impact of TWW on crops is varied. Biomass crop response depends on tree properties such as age and water use efficiency (Kwon, Law et al. 2018). Many studies have found that nutrient inputs from TWW increase poplar growth (Justin, Pajk et al. 2010, Dimitriou and Aronsson 2011, Holm and Heinsoo 2013, Jerbi, Nissim et al. 2014, Houda, Bejaoui et al. 2016, Khurelbaatar, Sullivan et al. 2017). While some food crops also experience this benefit (Leonel and Tonetti 2021), many exhibit decreased biomass (Zolti, Green et al. 2019) due to factors such as the negative impact of salinity on plant growth in many species (Levy and Tai 2013, dos Santos, Ribas et al. 2022, Haj-Amor, Araya et al. 2022). Trees may be more resilient than food crops to salinity and other potential shifts in the soil environment.

Table 2: Impact of irrigation with reclaimed wastewater on soil properties

Wastewater characteristics	Crop	Soil type	SOC	pH	Salinity	Nitrogen	Phosphorus	Microbial activity	Microbial biomass	Other	Ref
Secondary municipal	alfalfa										(Adrover, Farrus et al. 2012)
Secondary	potato									soil bulk density	(Biswas and Mojid 2018)
Tertiary municipal	bare soil	various									(Chen, Lu et al. 2015)
Primary or secondary	olive trees										(De las Heras and Mañas 2020)
Tertiary municipal	tomatoes	clay soil									(Guedes, Martins et al. 2022)
Secondary municipal, 30-year history	pine trees or pasture	sandy								micronutrients	(Gutiérrez-Ginés, Robinson et al. 2023)
Primary, generated on-site	pasture (winter)	clay soil								EC, Cl, Ca, Mg	(Lavi, Bar-Massada et al. 2024)
Rural domestic sewage											(Lu 2017)
Primary municipal	soil columns	ag soil								bulk density	(Mojid, Hossain et al. 2019)
Secondary municipal	melon and cucumber										(Obayomi, Edelstein et al. 2020)
Secondary and tertiary	carrot	loam							*E. coli*	crop yield	(Ofori, Abebrese et al. 2024)
graywater	bare soil										(Siggins, Burton et al. 2016)
Primary	poplar and eucalyptus	low OM clay									(Tzanakakis, Paranychianakis et al. 2011)
Tertiary, mixed municipal and industrial	tomato and lettuce	sandy loam								crop yield	(Zolti, Green et al. 2019)

Green – increase; Red – decrease; Gray – no change; White – not tested; TWW – treated wastewater

1.2.2. Concerns with TWW irrigation

There are several concerns associated with long-term TWW irrigation. The first is that changes to soil properties induced by wastewater application could be detrimental to ecosystem health. Maintaining soil health and soil microbial communities is vital for retaining ecosystem services (Wagg, Bender et al. 2014), and thus the impacts of wastewater on the system could have implications for ecosystem function. Additionally, there is potential for accumulation of heavy metals, trace organic compounds, and emerging contaminants in the soil over time (Chen, Lu et al. 2015).

Another concern with TWW is the accumulation of antibiotic resistance genes (ARGs) in the soil. ARGs of several antibiotic classes are found in effluent from wastewater treatment plants around the world (Wang, Chu et al. 2020). When distributed on soils in a wastewater infiltration system, there are concerns that the genes could persist and proliferate in the community, contributing to the antibiotic resistance epidemic. The potential for this to occur on a widespread scale is not fully understood. A comprehensive review of wastewater-irrigated soils found that irrigation did not change the abundance of antibiotic resistant organisms in soils in a number of studies (Gatica and Cytryn 2013). Conversely, Kampouris et al. (Kampouris, Agrawal et al. 2021) studied an agricultural field irrigated with TWW and found significant differences in several ARGs between irrigated and unirrigated soils, while Seyoum observed minimal but quantifiable impacts (Seyoum, Obayomi et al. 2021). The connection between TWW irrigation and antibiotic resistance gene proliferation requires additional investigation.

An additional concern with TWW irrigation is the potential for release of pathogenic organisms in wastewater and their subsequent proliferation in soils (EPA 2006). Others studying the microbial community in TWW-irrigated soils have examined the presence of target organisms like *E. coli* and Giardia and found little risk when wastewater is properly treated prior to reuse (Leonel and Tonetti 2021). Even when these organisms are detected in the reused wastewater prior to application, they do not appear in significant proportions in the soil community (Obayomi, Edelstein et al. 2020), suggesting that they are unable to establish in the soil due to the presence of a strong native community.

1.3. Soil microbiology

1.3.1. The Nitrogen Cycle

Controlling the nitrogen cycle is one of the Engineering Grand Challenges of the 21st century (National Academies of Sciences 2016). In the context of wastewater treatment, the important steps of the nitrogen cycle are nitrification and denitrification (Figure 1). During nitrification, ammonium is converted to nitrite then nitrate in an aerobic environment. Denitrification involves the conversion of nitrate to nitrite, nitrous oxide, N_2O, and finally nitrogen gas under anoxic conditions. The nitrogen cycle in the soil is also mediated by interactions with other components of the environment including soil particles and plants, with immobilization and uptake as additional potential fates (Singer and Munns 2002).

Nitrification is also carried out by comammox bacteria, which are capable of both steps of nitrification (van Kessel, Speth et al. 2015). A study comparing the prevalence of traditional ammonia oxidizing bacteria (AOB) and comammox showed that at ammonium concentrations below 0.2 mg/L, comammox dominate over AOB (He, Li et al. 2021). All currently identified comammox bacteria are members of the genus *Nitrospira*.

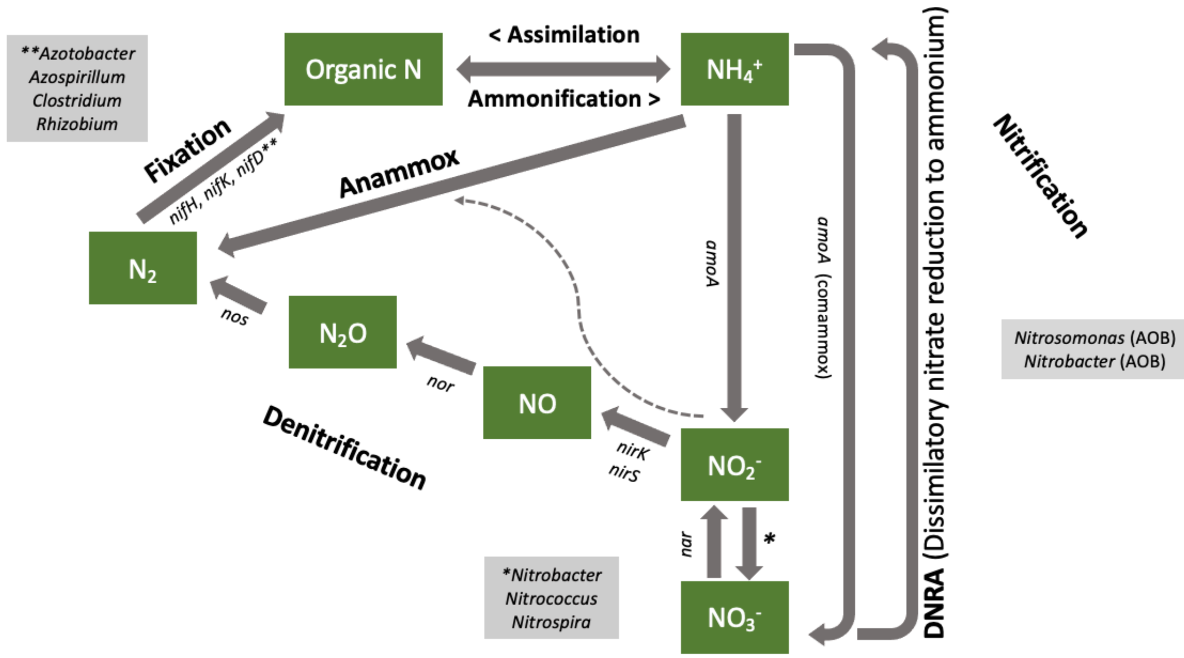

Figure 1: The nitrogen cycle, including major steps, key enzymes (italics) and microbial groups responsible for key steps (gray boxes). Based on Kirchman et al (Kirchman 2012).

Several additional pathways are active in the microbial nitrogen cycle. Bacteria capable of the anammox process oxidize ammonium with nitrite as a terminal electron acceptor under anoxic conditions, producing N_2. Dissimilatory reduction of nitrate to ammonium (DRNA) converts nitrate to ammonium for a lower energetic yield than denitrification. DRNA is favored under high organic matter, low nitrogen conditions (Kirchman 2012).

Each step of the nitrogen cycle is carried out by a complex of several enzymes. A few key genes are targeted to assess abundance and diversity of nitrogen cycling organisms in the soil. The *amoA* gene is used to assess population and diversity of nitrifiers (Khanal and Lee 2020, Guo, Bayu et al. 2021). Diversity of denitrifiers is assessed by targeting *nir* and *nos* genes (Braker, Zhou et al. 2000, Zhang, Jing et al. 2021). Nitrogen fixation is studied via *nifH* (Marusina, Boulygina et al. 2001, Gaby and Buckley 2012, Shu, Pablo et al. 2012).

1.3.2. Contaminant fate in soils

Soils can act as a filter and attenuate pollutants as they move through the soil profile (Champagne and Bhandari 2007). The pollutants may be removed by two mechanisms: sorption and degradation. Sorption is the interaction between charged dissolved molecules and charged surfaces of soil particles (Pradana, Hernandez-Martin et al. 2021). In the context of contaminant degradation, sorption is important because it slows the movement of contaminants through the soil, providing the necessary time for microorganisms to degrade them. In other words, sorption increases pollutant retention time without altering water retention time.

While sorption is an effective short-term solution, without accompanying degradation, there is a risk of contaminant build-up in the soil, which could be released in a storm event (EPA 2006). Therefore, only degradation can provide a complete solution to pollutant removal. This degradation is mediated by soil microorganisms.

1.3.3. The rhizosphere

The rhizosphere is defined as the area of soil directly surrounding and influenced by plant roots (Philippot, Raaijmakers et al. 2013, Ren, Wang et al. 2020). The rhizosphere is a diverse and dynamic environment. Plants release carbon compounds into the soil which recruit and sustain microorganisms, while rhizosphere organisms in turn provide many services to plants including nitrogen fixation, micronutrient provision, and protection from pathogens (Lambers, Mougel et al. 2009).

The rhizosphere differs significantly from the bulk soil in both physical and microbial characteristics (Tian, Qiu et al. 2022), with increased microbial diversity and up to two orders of magnitude more biomass (Sylvia, Hartel et al. 2005). Nutrient characteristics also differ, with increased organic carbon, higher rates of nitrogen fixation, and a nitrogen concentration about

10 percent higher in rhizosphere soils compared to bulk soils (Liu, He et al. 2022). The elevated nutrients favor the selection of fast-growing organisms like *Pseudomonas* while suppressing slow growers like *Azotobacter* species (Atlas and Bartha 1987).

1.3.4. Impact of reclaimed wastewater on microbial communities

Nutrient inputs into soils, including nutrients in the form of reclaimed wastewater, can alter microbial community composition (Ren, Wang et al. 2020). Irrigation water quality (fresh or reclaimed) is a major factor in shaping the soil microbial community (Zolti, Green et al. 2019, Xu, Liu et al. 2020).

The impacts of reclaimed wastewater on specific microbial groups are varied. Consistently, Acidobacteria populations increase in TWW-irrigated soils, while Actinobacteria decrease (Wafula, White et al. 2015, Guo, Qi et al. 2018, Ren, Wang et al. 2020, Cui, Li et al. 2022, Kargol, Cao et al. 2022). Other groups that may be altered include Firmicutes, Gemmatimonadetes (Guo, Qi et al. 2018) and Nitrospirae (Hernandez-Guzman, Perez-Hernandez et al. 2022).

In addition to composition, TWW irrigation can also change which organisms in the system are active. One study found that when irrigated with treated wastewater, the community composition (16S rRNA gene) did not change. However, the active community (rRNA) was significantly different, indicating differences in nutrient cycling capacity (Frenk, Dag et al. 2015). TWW irrigation may also alter which organisms are present and active at specific soil depths, without changing the overall community composition (Dang, Tan et al. 2019, Li, Cao et al. 2019). There may also be changes to the abundance of functional genes (Wafula, White et al. 2015, Vallejos, Marcos et al. 2022, Moulia, Ait-Mouheb et al. 2023).

Finally, community shifts induced by TWW irrigation are not always permanent. For example, changes in composition of the microbial community in an orchard were observed in response to TWW irrigation. However, when the irrigation source was changed from TWW to rainwater, the community returned to a baseline state (Frenk, Hadar et al. 2014)

Despite the thorough characterization of the impacts of TWW on soil communities, particularly in food crops and orchards, limited attention has been given to the impacts on the rhizosphere of biomass crops. Truu et al (Truu, Truu et al. 2009) studied willow trees in pots and concluded that the microbial community changed when irrigated with TWW. Liu et al examined microbial biomass and activity of key enzymes, again in SRC willows, and found fewer genes for stress response in TWW-irrigated soils compared to grass field control (Liu, Xue et al. 2022). Studies of poplar plantations fertilized with nitrogen suggest that communities become specialized and stabilized (Wang, Chen et al. 2021), which may lead to a decrease in alpha diversity (Wang, Li et al. 2021).

1.3.5. Clustering methods impact understanding of microbial communities

16S sequencing data has historically been processed by clustering similar reads into operational taxonomic units (OTUs) and assigning taxonomy. Sequences are clustered based on a similarity threshold, usually 97%, (Blaxter, Mann et al. 2005) and the consensus sequence is run through a database to determine identity (Chiarello, McCauley et al. 2022). Recently, a new method for 16S classification has emerged. Amplicon sequence variants (ASVs) are corrected sequences obtained using an error model to determine the most likely “true” sequence of each read (Callahan, McMurdie et al. 2016). Corrected sequences differing by even one nucleotide are classified as different ASVs, so this method can result in millions of unique ASVs obtained from a dataset (Jeske and Gallert 2022).

Comparisons between OTU and ASV clustering have been conducted and found mixed results in their similarity. When VSEARCH for OTUs and DADA2 for ASVs were used to classify the same dataset, differences in alpha and beta diversity (Chiarello, McCauley et al. 2022) and composition (Jeske and Gallert 2022) were observed between methods. Results from the VSEARCH and DADA2 pipelines were similar enough that they would lead to the same general interpretations, but small and potentially important differences were also observed (Jeske and Gallert 2022). Notably, these comparative studies all conducted short-read sequencing of the 16S V3 or V4 region, which are less than 300 base pairs in length. The effect of identification method on long-read sequencing data (>1500 base pairs), which has more errors and thus may lead to fewer exact matches between sequences, has not been evaluated.

1.4. Integration of wastewater infiltration systems into the biomass industry

Emphasizing the benefits of wastewater infiltration systems for biomass production will help expand the circular economy. Also important is having a full understanding of the costs associated with establishing and operating a biomass production site using treated wastewater. Capital and operating costs of such operations are evaluated through economic analysis (Chai, Phang et al. 2022). Careful evaluation of the benefits and costs is needed when considering the construction of a new wastewater infiltration system at a wastewater treatment plant.

1.4.1. Economic and environmental benefits of poplar short-rotation coppice

There are many economic benefits associated with reusing treated wastewater for biomass irrigation. Biomass growers generate increased revenue relative to irrigation with clean water, because the nutrients in the irrigation water increase biomass production. Wastewater treatment plants experience cost savings from the natural treatment of nitrogen and phosphorus, which is significantly cheaper than conventional tertiary treatment (Rosenqvist, Aronsson et al. 1997, Tobin, Gustafson et al. 2020). Ecosystem services such as carbon sequestration are also provided. Biomass crops grow well on marginal land and can be harvested on a more flexible schedule as needed, allowing biomass galleries to serve as "living storage" for trees (Dou, Marcondes et al. 2017). Finally, communities experience socioeconomic benefits from growing biomass crops, including the creation of local jobs and economic resilience against the failure of food crops (Licht and Isebrands 2005).

When lignocellulosic biomass was first investigated for the bioproducts industry, most research was done using short-rotation forestry (SRF), which operates in 12-year harvesting cycles (Limayem and Ricke 2012). More recent research has shown that short-rotation coppice (SRC) is comparable to forestry in sugar yields and is economically viable if leaves are removed before processing (Dou, Marcondes et al. 2017). Compared to SRF, additional benefits of SRC include reduced infiltration into forests and greater economic benefits for rural communities (Chudy, Busby et al. 2019).

1.4.2. Addressing costs and barriers

SRC has shown potential for use in small communities while remaining economically viable (Rosenqvist, Aronsson et al. 1997), but most studies conducted in the past 5 years regarding expansion SRC to larger scales have found that scale-up would require significant improvements to processes or yields (Hart, Townsend et al. 2018, Chudy, Busby et al. 2019, Chowyuk, El-Husseini et al. 2021). One method for increasing economic viability is the creation of valuable co-products (Devappa, Rakshit et al. 2015). Recent examples include glacial acetic acid (Morales-Vera, Crawford et al. 2020) and carboxylic acid (Klein, Scheidemantle et al. 2024), both of which can be produced at competitive prices as value-added products from biorefineries. Another potential solution to increase economic viability is the incorporation of water recovery and reuse (Chowyuk, El-Husseini et al. 2021, Pascoli, Suko et al. 2021).

Major costs associated with biorefineries include pretreatment (Limayem and Ricke 2012, Seufitelli, El-Husseini et al. 2022), biomass transportation (Cozzi, Viccaro et al. 2015), harvesting (Dou, Marcondes et al. 2017), heat/electricity, and wastewater treatment (Viccaro, Cozzi et al. 2017, Seufitelli, El-Husseini et al. 2022). Attempts to improve pretreatment over recent years have led to developments such as new acid-based preprocessing steps to increase fermentability of sugars produced from biomass (Pascoli, Suko et al. 2021), and treatment using white rot fungi which reduced sugar production cost by 14.5%, although the process is not yet economically viable due to capital costs (Wittner, Vasilakou et al. 2023).

To address transportation issues, GIS-based tools have been developed which can identify land suitable for SRC and also located reasonably close to a wastewater treatment plant (Cozzi, Viccaro et al. 2015, El-Husseini, Chowyuk et al. 2023). A stakeholder survey found that 8.1 km was the maximum distance the effluent could be pumped from the main facility to the biomass field without significantly increasing costs (Hart, Townsend et al. 2018).

1.5. Summary

The key aspects of wastewater infiltration systems for bioenergy and water recovery– nutrient removal performance, microbial community composition and function, and biomass production – are typically studied individually. Performance and biomass production are assessed, or genes are quantified alongside microbial community composition, but studies linking macro-scale properties with micro-scale trends in the same system are limited.

Here, I studied lab-scale wastewater infiltration systems with or without poplar trees under long-term wastewater application, quantifying carbon and nitrogen removal, shifts in microbial community structure, and community nitrogen cycling potential and function. I connected observed nutrient removal trends with soil microbial community properties to better understand the contributions of vegetation and soil microorganisms in land treatment of wastewater.

2. System and study design

2.1. Background

Short-rotation coppice (SRC) is a strategy for large-scale biomass production. In SRC, biomass crops are planted, often on marginal land that cannot support food crops, and then harvested every 3-5 years. Harvest occurs by coppice, where trees are cut to stumps and new branches sprout directly from the stump, allowing biomass harvest without disturbing the root structure. Hybrid poplar (*Populus)* and willow (*Salix)* are the preferred trees due to their prevalence as biomass sources, their vigorous regrowth after coppice, and their ability to grow with minimal water and nutrient inputs (Isebrands, Aronsson et al. 2014). Based on growing interest in poplar biomass production for biofuels in the Pacific Northwest (Hart, Townsend et al. 2018), hybrid poplar was selected for the study system. I constructed lab-scale reactors to simulate a poplar biomass production system designed to treat wastewater and recover nutrients. Wastewater irrigation occurred for 18 months, and system properties were monitored including physical, chemical, and biological characteristics of soil and effluent, and soil microbial community composition and nitrogen cycling potential.

2.2. Site characteristics and system construction

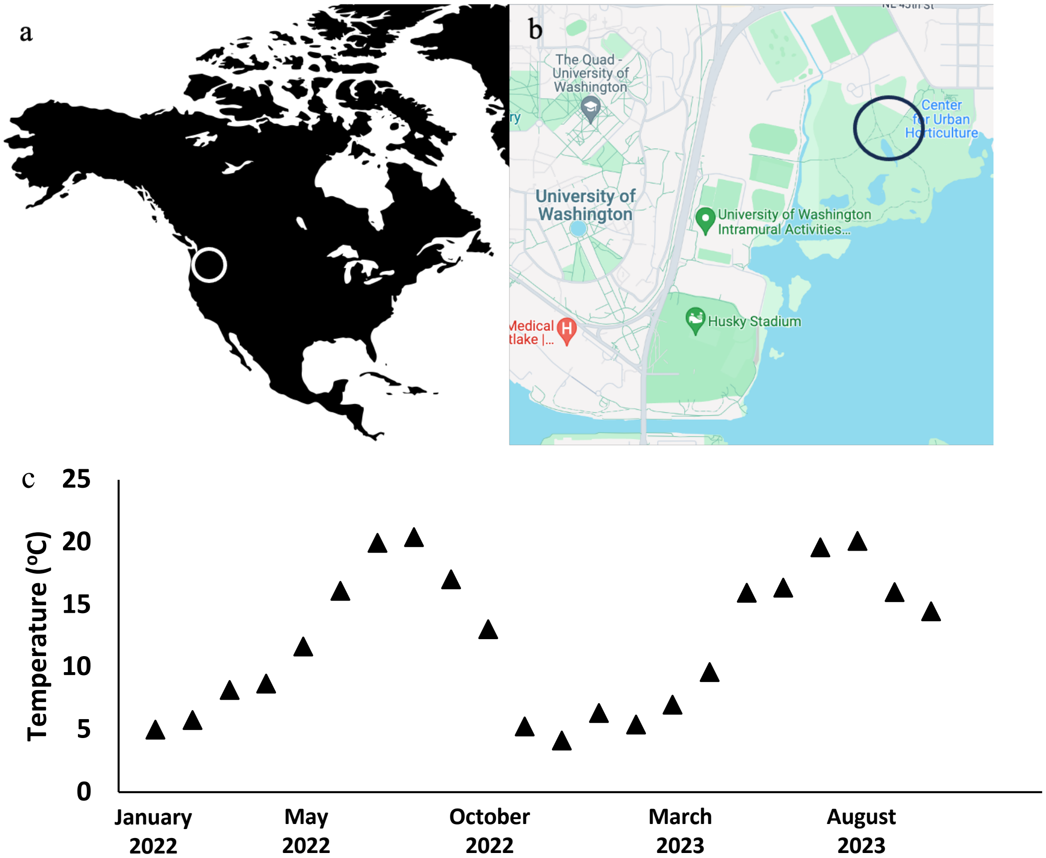

Figure 2: Site characteristics for poplar infiltration reactors. a. Location of reactors - Seattle, Washington, USA (white circle); b. Location of reactors on the University of Washington campus (black circle); c. Average monthly temperature for the experimental period, January 2022-October 2023

Simulated wastewater infiltration galleries were constructed at the University of Washington Douglas Research Conservatory outdoor greenhouse (Figure 2a, 2b). Daily average, minimum, and maximum temperatures (Figure 2c) were obtained from the Washington State University AgWeatherNet Seattle station (https://weather.wsu.edu/), which is located approximately 200 m from the site of the reactors.

Reactors were constructed from 640 L galvanized steel tanks (Behlen Country Farm and Ranch Equipment item 50130048; Columbus, Nebraska, USA) measuring 0.6 × 0.6 × 1.8 m (*w/h/l*, sold as 2 × 2 × 6 ft). Four side ports were cut into the tanks, two each at 0.2 m and 0.4 m from the soil surface, and bottom ports for water outflow were added. Reactors were filled with 2 inches of mixed pea gravel and small stones, and 0.7 m of sandy loam soil (Sawdust Supply Company, Seattle, Washington, USA or Washington Rock Quarries, Orting, Washington, USA). Hybrid poplar trees (clone 5077*,* provided by Greenwood Resources, Inc; Portland, Oregon, USA) were grown from cuttings established in one-gallon containers in summer of 2018. Three trees were planted in each reactor, with three replicates per treatment. The unplanted reactors did not have planted trees. Drip irrigation lines (PTFE chemical resistant tubing 3/16 in inner diameter, 1/4 in outer diameter; Grainger, Lake Forest, Illinois, USA) were used to evenly apply across the soil surface. Reactors were covered with tarps to minimize the influence of precipitation and evaporation.

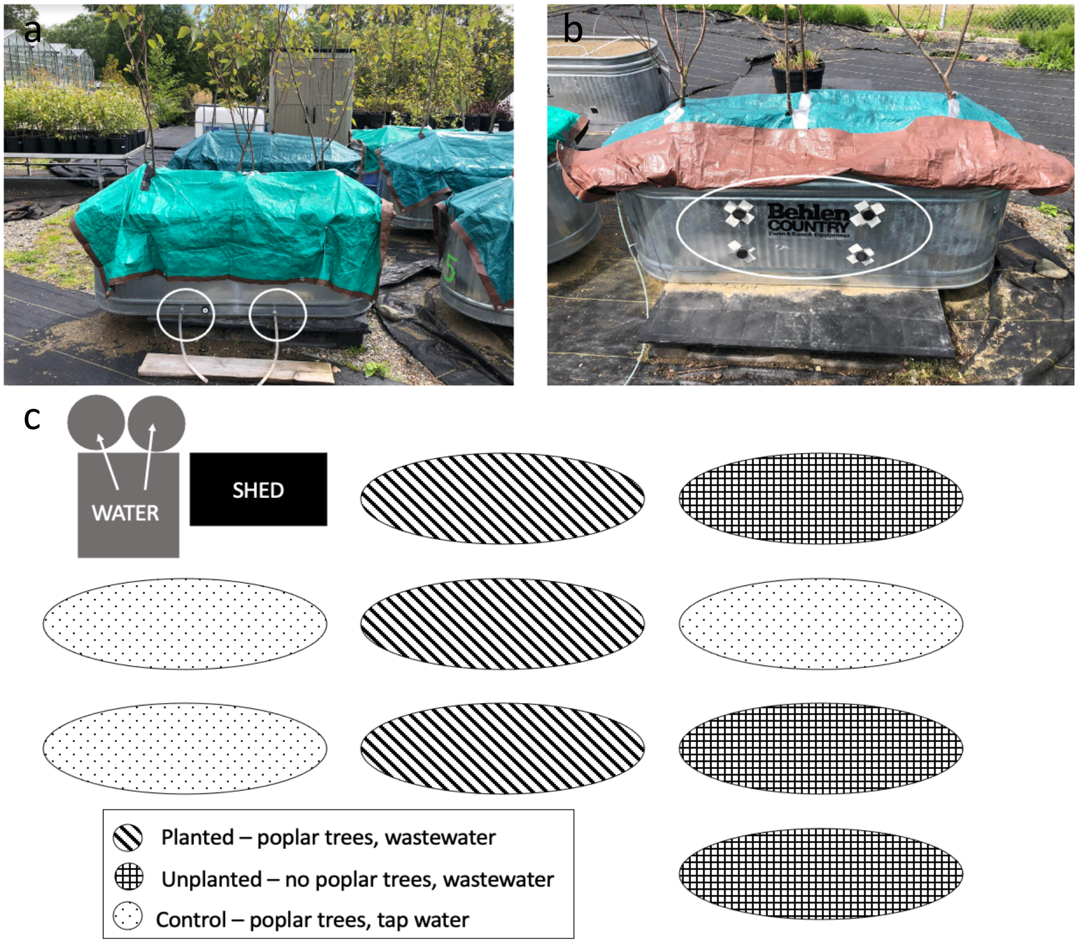

Figure 3: Poplar reactors for studying wastewater treatment by planted infiltration gallery in a controlled setting. a. Effluent collection ports (circled) allow for water recovery and performance testing; b. soil sampling ports (circled) allow for the characterization of physical, chemical, and microbial components of the system at two depths. c. site layout.

2.3. Synthetic wastewater formulation

Due to the costs associated with synthesizing large volumes of synthetic wastewater from pure chemicals, an inexpensive recipe was developed for preparing wastewater from dog food. Dog food was soaked in water for 24 hours and strained to remove large particles. The target compounds were nitrogen pollutants ammonium and nitrate, and organic matter, measured as chemical oxygen demand (COD), a common measure of organic carbon in the wastewater treatment industry. The dog food-synthetic wastewater (DSW) base medium had COD and nitrogen concentrations in similar proportions to those found in secondary wastewater effluent (Tchobanoglous, Stensel et al. 2014). The concentrated DSW was then transported to the experimental site and diluted 1:100. The recipe was supplemented with $NaNO_3$ and NH_4Cl to increase concentration of target nitrogen species in the forms in which they are found in secondary wastewater effluent (Table 3). This medium reduced costs by 83% compared to synthesizing medium with equivalent COD content from pure chemicals. The full manuscript documenting the formula was published in PLOS water in 2023 (Kargol, Burrell et al. 2023) and can be found in Appendix A.

Table 3: Characteristics of synthetic secondary effluent

	COD (mg/L)	**Total nitrogen (mg/L-N)**	**Nitrate (mg/L-N)**	**Ammonium (mg/L-N)**	**pH**
Synthetic WW, concentrated	5580	283	18.5	8.9	6.4
Synthetic WW, diluted on site	40.5 ± 10.8	2.0 ± 0.5	0.8 ± 0.1	0.15 ± 0.04	7.7 ± 0.1
Synthetic WW, supplemented	41.1 ± 5.1	4.8 ± 0.9	1.3 ± 0.2	1.2 ± 0.5	7.9 ± 0.2
Typical secondary effluent*	20-80	2-35	1-30	0.1-10	N/A

* Tchobanoglous, G., Stensel, H. D., Tsuchihashi, R., Burton, F., Abu-Orf, M., Bowden, G., & Pfrang, W. (2014). *Wastewater Engineering, Treatment and Resource Recovery* (5th ed.): McGraw Hill Education.

2.4. Experimental design

Reactors were irrigated with DSW delivered via drip irrigation lines and peristaltic pumps. Experiments were conducted in three-week blocks to allow the system to adapt to the irrigation water. To limit water pooling and avoid saturation of the soils, a block irrigation schedule was used. Pumps cycled between on and off every 3 hours continuously. Irrigation volumes varied by season and experimental goal.

After each experiment, soil and effluent were sampled. Soils were collected from all four ports on the final day of the experiment using a soil coring device. Samples were transported to the lab on dry ice and processed immediately for soil moisture, pH, and organic carbon, or archived at -80^{O}C for DNA extraction. Effluent was collected from effluent ports on the final two days of the experiment and was tested for chemical oxygen demand, nitrate, ammonium, and total nitrogen and concentrations were compared to influent to quantify nutrient removal. Water balance was quantified by collecting effluent near the end of the experiment for a period of 6 hours to reflect the duration of one irrigation cycle.

Table 4: Irrigation and nutrient regime for poplar reactors over 2 years

Season	Irrigation L/day	Irrigation mm/day	Wastewater supplementation*	Notes
		Phase 0 – Initial measurements, initial irrigation		
Winter 2022	varied		none	Retention time tests
Spring 2022	42.6 ± 1.0	34.2 ± 0.8	none	Effluent recovery data not collected
		Phase 1 – Low nutrients/secondary effluent		
Summer 2022	69.7 ± 0.3	60.0 ± 0.2	5 mg/L NH_4Cl and $NaNO_3$	
Fall 2022	72.3 ± 2.6	58.1 ± 2.0	5 mg/L NH_4Cl and $NaNO_3$	
Winter 2023	76.5 ± 9.6	61.4 ± 7.7	5 mg/L NH_4Cl and $NaNO_3$	
Spring 2023	71.8 ± 0.6	57.6 ± 0.5	5 mg/L NH_4-N and NO_3-N	
		Phase 2 – Increased nutrient loading/primary effluent		
Summer 2023 (High nitrogen)	74.7 ± 5.62	60.0 ± 4.5	20.0 mg/L NO_3-N 9.75 mg/L NH_4-N	Nitrate time-series samples collected
Autumn 2023 (High COD)	83.9 ± 0.6	67.4 ± 0.5	615 mg/L COD 33.6 mg/L total N	

*All seasons used DSW base except Winter 2022, which used salt water

3. Populus species mitigate nitrate accumulation in wastewater infiltration systems for year-round treatment and recovery

Publication: This work is being prepared for submission to the Journal of Environmental Engineering.
Co-authors: Heidi L. Gough, Daniel Montes

Abstract

Wastewater infiltration systems offer a method for coupling wastewater treatment with reuse for crop irrigation to conserve water resources. Here, we tested the use of wastewater infiltration systems to remove nitrogen and organic matter from synthetic secondary and primary effluents. Reactors were drip-irrigated along with bare soil controls, and clean water controls were also maintained. Water was collected after passing through the soil and tested for total nitrogen, nitrate, ammonium, and chemical oxygen demand (COD). Soil properties and the impact of intermittent irrigation were also assessed. Nitrogen attenuation in planted reactors was always greater than unplanted reactors, especially in the dormant season and with the increase to primary effluent concentrations. Leaf nutrient content also differed; treated leaves had significantly higher content of phosphorus, potassium, and total nitrogen than controls, suggesting leaf uptake as a potential fate for influent. Performance of unadapted reactors was significantly below that of treated reactors, highlighting that both vegetation and system adaptation are important components of achieving high nitrogen removal. This work suggested the potential for irrigation of planted infiltration galleries with high volumes of primary or secondary effluent and presented a noninvasive method for testing wastewater infiltration under local conditions.

Keywords: wastewater infiltration system, poplar, nitrate, nutrient recovery

3.1. Introduction

Nature-based approaches for wastewater treatment have long been valued for their ability to clean water while requiring minimal oversight or energy for operation (United Nations Environment Programme 2021). This type of treatment is particularly beneficial in smaller communities (Amiot, Jerbi et al. 2020, Martinez-Hernandez, Meffe et al. 2020), where other options are less economically feasible. Among the approaches, land treatment by infiltration uses the natural attenuation capacity of soil to remove pollutants (de Bustamante 1990, EPA 2003).

Land treatment has a long history as a treatment approach for wastewater and continues to be a recognized option (EPA 2021). A challenge is that nitrogen species can leach into near-by surface or groundwater causing contamination of freshwater resources. To mitigate this concern, either the rate of water application is regulated or underdrains collect partially treated water for additional nutrient removal (tertiary treatment) before reuse or return to the environment (EPA 2003). Another solution for mitigating nitrogen leaching is planting vegetation in the land treatment system, which can significantly improve nutrient removal (Dimitriou and Aronsson 2011, Khurelbaatar, Sullivan et al. 2017, Sheng, Zhang et al. 2021). This presents an opportunity to couple land application systems with treated wastewater reuse for irrigation, which helps meet water needs for a growing population (EPA 2012).

The use of partially-treated wastewater for irrigation of non-edible bioenergy crops, such as short-rotation hybrid poplar, has been proposed as a viable approach for improving the economic feasibility of the bioproducts industry (Chowyuk, El-Husseini et al. 2021). Quantifying water quality improvements during irrigation of short-rotation poplar is critical both for understanding design implications and for minimizing potential for impacts on nearby water ways.

A challenge in studying land treatment is the variety of systems available, each with slightly different characteristics and associated terminology. Land treatment systems, the most broad term, apply treated wastewater to the soil surface (Crites and Tchobanoglous 1998). Vegetation filters refer specifically to planted soils, which enhances the treatment capacity of the system (de Miguel, Meffe et al. 2014). When soils are planted with specific types of crops, still other names may be used, such as short-rotation coppice (SRC) in the biofuels industry for woody biofuel crops. Wastewater infiltration systems focus on treatment with less attention given to plants, and often utilize subsurface application (Zhang, Huang et al. 2005, Pan, Yuan et al. 2016). Our system has properties of wastewater infiltration and land treatment but incorporates unique aspects as well. To encompass the scope and goals, including wastewater treatment, biomass production, and water recovery, we used the term Wastewater Infiltration System for (bio)Energy and Recovery, or WISER. We refer to other systems as land treatment regardless of characteristics.

Studies have documented the performance of land treatment systems under conditions including soil column studies (Pang, Pan et al. 2020, Chen, Jiang et al. 2021, Prodanovic, Zhang et al. 2023), lysimeters (Dimitriou and Aronsson 2011) and small-scale wastewater treatment (de Miguel, Meffe et al. 2014, Lachapelle-T, Labrecque et al. 2019). Willow trees have been studied extensively for use in land application, with different clones showing optimal performance under specific sets of conditions (Holm and Heinsoo 2013, Shi, Sun et al. 2016, Moreno, Lara-Borrero et al. 2019, Pradana, González et al. 2023). Few studies have focused on poplar performance alone, but mixed stands of poplar and willow have suggested similar performance between the two crops (Khurelbaatar, Sullivan et al. 2017). Land application has been tested in a variety of climates (Grebenshchykova, Brisson et al. 2020, Postila and Heiderscheidt 2020, Pradana, Hernandez-Martin et al. 2021), but studies focused on performance in a modified Mediterranean climate have not been undertaken.

Evapotranspiration (ET) rates vary by climate, tree age, and species. Values for hybrid poplar are shown in Table 5. Typical values range from 1-4 mm/day (Afas, Marron et al. 2008, Jassal, Black et al. 2013, Bloemen, Fichot et al. 2017) with increased rates sometimes observed with tree aging (Jassal, Black et al. 2013). Maximum values are more varied, from 6.0 mm/day (Petzold, Schwärzel et al. 2010), up to 11.6 mm/day (Pistocchi, Guidi et al. 2009). In western Washington state, USA, the location of the study, estimated ET for hybrid poplar during the growing season ranges from 1.5 to 5.7 mm/day for 3+ year-old stands (Peters, Nelson et al. 2005, Washington State University 2024). ET values for willow exhibit similar averages but higher maximum values (Guidi, Piccioni et al. 2008), up to 34 mm/day in one study (Amiot, Jerbi et al. 2020), which may be one of the reasons willow is more commonly used for wastewater infiltration treatment than poplar.

Table 5: Evapotranspiration rates for hybrid poplar

Average (mm/day)	Maximum (mm/day)	Annual (mm/yr)	Ref
2 (year 1) 3 (year 2)	-	725 (year 1) 1100 (year 2)	(Guidi, Piccioni et al. 2008)
2.4	11.6	-	(Pistocchi, Guidi et al. 2009)
3.6	4.8	-	(Hinckley, Brooks et al. 1994)
2.2	6.7	463-486	(Petzold, Schwärzel et al. 2010)
3.4	-	364 (year 1) 398 (year 2)	(Jassal, Black et al. 2013)
1.9	5	320 (Apr 1 – Oct 31)	(Meiresonne, Nadezhdin et al. 1999)
1.1-1.3	-	-	(Bloemen, Fichot et al. 2017)
1.8		670	(Afas, Marron et al. 2008)
2.75		495 (6 months)	(Parish, Kendall et al. 2019)

This study builds on the work of several other groups testing wastewater infiltration systems with poplar and willow trees under a variety of conditions and loading rates (Tzanakakis, Paranychianakis et al. 2009, Khurelbaatar, Sullivan et al. 2017, Lachapelle-T, Labrecque et al. 2019). Knowledge gaps include year-round water application, maximizing application rates, and water recovery for process reuse. The aims of this study were to 1) quantify nutrient removal in wastewater infiltration galleries at high application rates, 2) test year-round system performance in an oceanic climate, and 3) assess the feasibility of water recovery for reuse.

3.2. Methods

3.2.1. Reactor design and experimental location

Reactor construction is detailed in Chapter 2. Briefly, reactors were constructed from 640 L (sold as 169 gallons) galvanized steel tanks (Behlen Country Farm and Ranch Equipment item 50130048; Columbus, Nebraska, USA) measuring 0.6 × 0.6 × 1.8 m (*w/h/l*, sold as 2 × 2 × 6 ft). To build the reactors, the tanks were modified to include 4 side sampling ports, two each at

0.2 m and 0.4 m, and two bottom water drains. The troughs were filled with 2 inches of mixed pea gravel and small stones, and 0.7m of sandy loam soil (Sawdust Supply Company, Seattle, Washington, USA or Washington Rock Quarries, Orting, Washington, USA). Hybrid poplar trees (clone 5077; hybrid of *Populus deltoides* x *P. trichocarpa,* provided by Greenwood Resources, Inc; Portland, Oregon, USA) were grown from cuttings established in one-gallon containers with potting soil in summer of 2018. Three (3) trees were planted in each treatment and control reactors (triplicate reactors) in summer 2019 or summer 2020. The unplanted reactors did not have poplar trees. Drip irrigation lines (PTFE chemical resistant tubing 3/16 in inner diameter, ¼ in outer diameter; Grainger, Lake Forest, Illinois, USA) on the soil surface were distributed to evenly apply across the soil surface. Reactors were covered with tarps to minimize the influence of precipitation and evaporation on experimental comparison.

Reactors were located at the University of Washington Douglas Research Conservatory outdoor research area in Seattle, Washington. Site layout and weather are shown in Chapter 2. Daily average, minimum, and maximum temperatures per month based on weather data from the Washington State University AgWeatherNet Seattle station (https://weather.wsu.edu/), which is located approximately 200 m from the site of the reactors. Seattle's climate is defined by dry summers, characteristic of a Mediterranean climate, with wetter winters, leading to classification as a modified Mediterranean climate (Goble and Hirt 2012). The weather patterns in Seattle allowed for experimental testing in winter months following leaf loss when the trees were dormant.

Experiments were conducted from spring 2022 through autumn 2023. Data collection was split into four seasons (spring, SP; summer, SU; autumn, AU; and winter, WI) and two years (2022 – year 1; 2023 – year 2).

3.2.2. Water application and recovery

The infiltration rate was measured using the USDA field method for infiltration (USDA 2008) to ensure applied water would not pool on the soil surface. The mean residence time in the reactors was estimated by measuring the transport time of a sodium chloride solution through the reactor. Briefly, 0.025M NaCl solution was applied to the soils at a rate of 70 mm/day. The effluent was monitored using an Orion 2-Cell conductivity probe (013010MD; Thermo Fisher Scientific, Waltham, Massachusetts, USA). Data were graphed and the best fit was identified using the equation for one-dimensional flow in unsaturated soils (Bedient, Rifai et al. 1994):

$$\frac{C(x,t)}{C_0} = \frac{1}{2}(erfc\left[\frac{L - v_x t}{2\sqrt{D_x t}}\right] + \exp\left(\frac{v_x L}{D_x}\right) erfc[\frac{L + v_x t}{2\sqrt{D_x t}}]$$

where C is chemical concentration, C_0 is initial concentration, L is depth of the reactor, v_x is velocity in one dimension, t is time, and erfc and D_x are parameters defined by Bedient et al.

The treated and unplanted reactors were irrigated with dog food synthetic wastewater (DSW) diluted 1:100 in municipal tap water, which has been previously described (Kargol, Burrell et al. 2023). Control reactors were irrigated with tap water. Influent water was dosed to the reactors using peristaltic pumps calibrated on-site for accurate control of volume application. After 3 weeks of equilibration, effluent was collected from reactors to quantify water recovery. Carboys were positioned under each effluent port and collected water for a period of 6 hours, reflecting one complete cycle of the irrigation system (3 hours on, 3 hours off). Volume of effluent was determined by mass.

3.2.3. Wastewater characteristics and experimental applications

Synthetic effluent was created using dog food synthetic wastewater (DSW) (Kargol, Burrell et al. 2023) supplemented with nitrogen and organic carbon sources. Nutrients were measured periodically to confirm a chemically consistent wastewater composition and average values are reported in Table 6. Data for individual batches is shown in Table S1. Calculated nutrient loading, in kg/ha day^{-1} is shown in Table S2.

In the second year of operation, nutrient concentrations were increased. First, nitrogen was increased in the form of ammonium and nitrate in SU2. Then, COD was increased by adding dextrose to match characteristics of primary effluent in AU2 (Tchobanoglous, Stensel et al. 2014). Additionally, time-series effluent and soil samples were collected and tested to examine the impact of intermittent irrigation on reactor performance and soil properties.

3.2.4. Water analysis

Nutrients in influent and effluent were quantified using Hach water testing kits (Hach company, Loveland, USA). Total nitrogen was measured using Hach TNT Plus Total Nitrogen kit (Method 826, 1-16 mg/L). Ammonium-N was measured using the Hach TNT Plus ultra-low range Ammonia kit (Method 830, 0.015-2.0 mg/L), which measures both ammonia and ammonium. Nitrate-N was quantified using Hach TNT Plus Nitrate kit (Method 835, 0.23-13.5 mg/L). Chemical oxygen demand (COD) was measured using the Hach low-range COD kit (Cat numbers 2125815, 3-150 mg/L). pH was measured with an Orion Dual Star pH/ISE meter (Thermo Scientific, Waltham, USA). Hach tests were conducted according to manufacturer protocol. In place of a Hach brand spectrophotometer with built-in standard curves, a Genysis50 spectrophotometer (Thermo Scientific) was used to read tests. Standard curves were generated for each kit.

Water samples were collected from effluent ports for 15 minutes and subsampled for analysis. Samples were collected on two subsequent days at the end of the experiment, between 9 am and 10 am. In SU2 trials, samples were collected on two days at the end of the experiment, with time-series samples collected hourly on one of the two sampling days. Effluent samples were tested for nitrate, ammonium, total nitrogen, and COD.

3.2.5. Soil sampling and water recovery

Soils were sampled at 0.2 m and 0.4 m using a soil coring device. Samples were collected in 50 ml tubes, transported to the lab on ice, and stored at -20^{O}C. Moisture, pH, and organic carbon analysis were conducted within 48 hours. Moisture was measured by oven drying and organic carbon by loss on ignition (ASTM 2007). Soil pH was determined by EPA method 9045D for soil and waste pH (EPA). Soil texture was measured using the hydrometer method, Standard Test Method for Particle-Size Distribution (ASTM 2017). Bulk density was calculated using USDA Method 3.3 for in-field bulk density (USDA Natural Resources Conservation Service 2004).

3.2.6. Adaptation testing

Along with treated and unplanted reactors, control reactors were maintained throughout the duration of the experiment and irrigated with tap water. To test the impact of adapting the community to wastewater, two of the control reactors were treated with wastewater in the final trial, AU2. Influent concentrations of COD and total nitrogen were 125 mg/L and 40 mg/L respectively. Samples were collected to evaluate wastewater treatment performance and soil properties compared to adapted controls.

3.2.7. Leaf nutrient analysis

Leaves were sampled in AU2, both from the tree (harvested) and from the ground after falling (fallen) to assess nutrient fate. Ten leaves per tree were randomly selected for harvesting. Harvested leaves were weighed and dried at 60^{o}C to determine leaf biomass and moisture content (Babu, Kumaresan et al. 2018). Dry mass only was recorded for fallen leaves because their exact fall-time and thus their initial mass could not be accurately measured. Nutrient analysis was performed by Kansas State Soil Testing Laboratory (Manhattan, Kansas, USA). The leaves were tested for total nitrogen, nitrate, phosphorus, and potassium. NPK were analyzed by the sulfuric peroxide digest method (Linder and Harley 1942) and nitrate was analyzed using a 1M KCl extraction (EPA 1993). Nitrogen, phosphorus, and potassium (NPK) values were reported as percentages while nitrate values were reported as ppm due to their very low contribution to leaf composition.

3.2.8. Statistical analysis

Statistical analysis were conducted in RStudio version 2023.12.0+369 (RStudio Team 2020) using the vegan package (Oksanen, Blanchet et al. 2013). Analysis of variance (ANOVA) was used to compare nutrient between treatments as well as differences in soil properties. When applicable, Tukey's Honest Significant Difference test (HSD) was used to evaluate specific differences based on treatment and season. T-tests were used to compare leaf nutrient content between treated and control reactors.

3.3. Results

3.3.1. Water recovery

Average effluent recoveries are shown in Figure 4. One recovery value of 32% observed in SP2 was considered a potential outlier and not included in the statistical analysis. A two-way ANOVA was conducted to test the effects of treatment and season on water recovery.

Treatment resulted in a significant difference in water recovery (p = 0.03). Further analysis revealed that the difference was between control and treated reactors (p = 0.02), while unplanted reactors did not differ from either group.

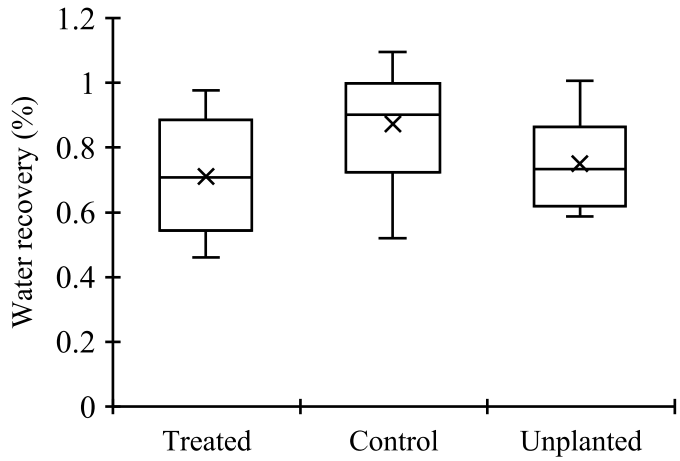

Figure 4: Water recovered from poplar wastewater infiltration reactors.

Season did not significantly impact water recovery (p = 0.64). This differs from other studies that have observed seasonal variation in transpiration rates in poplar trees (Guidi, Piccioni et al. 2008, Petzold, Schwärzel et al. 2010), which impacts soil moisture. It is possible that evapotranspiration effects were masked due to the high application rates and young tree age, even during the growing season. Other work has demonstrated that, at high volumes and in certain climates, water recoveries close to 100% can be obtained (Benoist, Parrott et al. 2023).

3.3.2. Soil properties

Soil texture and bulk density are shown in Table S3. Soil texture was on average 85.5% sand, 11.9% silt, and 2.6% clay and did not vary by treatment or depth (p > 0.05). Bulk density differed significantly between reactors (p = 0.001). Further investigation revealed significantly higher bulk density in unplanted reactors compared to control reactors (Tukey's HSD, p =

0.0007), and also to planted reactors, although this difference was not statistically significant ($p = 0.06$).

Average residence time in the reactors was 23 ± 4 hours with a range of 14-29 hours (Table S3). The notably shorter residence time of 14 hours was observed in unplanted reactor 3. The variation in residence time can be attributed to development of different patterns of dispersion that develop in vertical-flow systems (Bedient, Rifai et al. 1994). However, treatment performance did not differ between reactors regardless of residence time, suggesting that even 14 hours is sufficient for treatment of wastewater at the tested concentrations.

Average values of soil moisture and organic carbon are shown in Table S4. Time-series measurements showed fluctuations in soil moisture of < 5 percent throughout the irrigation cycle, and fluctuations were similar in all reactors. Moisture content during water application ranged from 14% to 21%, with most samples falling between 15% and 19%. Seasonal differences in moisture were not observed except for SP2 and SU2 (t-tests, $p = 0.01$ and 1.0e-7), where the higher values were seen in control reactors.

Soil organic matter differed significantly by treatment, with unplanted reactors having significantly less organic matter in 7 of 9 seasons (Table S4). This difference was more pronounced in SU2 and AU2. Conversely, organic matter did not differ by season, suggesting that it did not accumulate in any of the treatments over time.

3.3.3. Water Treatment Performance

Effluent nutrient concentrations are shown in Table 7, along with average nutrient removal values for planted and unplanted reactors. The tap water used to irrigate control trees had concentrations of total nitrogen, nitrate, ammonium, and COD below our limits of detection (0.5, 0.25, 0.015, and 3 mg/L, respectively). The presence of those compounds in control effluent was assumed to be a product of natural processes within the reactor.

Ammonium concentration was below the detection limit of 0.015 mg/L in 64% of samples and below 0.05 mg/L in all but 3 samples.

Table 6: Synthetic wastewater characteristics by season (mg/L)

Season	COD	Total N	NO3	NH4	pH
SP1	47 ± 11	2.9 ± 1.5	0.8 ± 0.1	0.2 ± 0.04	7.7 ± 0.1
		Synthetic secondary effluent			
SU1	41 ± 5	4.8 ± 0.9	1.4 ± 0.2	1.2 ± 0.5	7.6 ± 0.2
AU1 – SP2	74 ± 3.0	11.4 ± 2.2	2.9 ± 0.2	1.8 ± 0.9	7.6 ± 0.3
		Synthetic primary effluent			
SU2	102 ± 41	32 ± 3.3	20 ± 1.1	9.8 ± 0.3	7.6 ± 0.5
AU2	615 ± 120	34 ± 10	< 0.5	< 0.015	7.0 ± 0.2

Table 7: Effluent nutrient concentrations and removal performance in wastewater infiltration systems

		COD (mg/L)	Removal (%)	Total nitrogen (mg/L-N)	Removal (%)	Nitrate (mg/L-N)	pH
SP1	Planted	12.7 ± 18.6		6.3 ± 3.8		0.8 ± 0.5	n.a.
	Unplanted	< LOD		5.5 ± 5.0		1.6 ± 1.2	n.a.
	Control			3.2 ± 2.2		0.7 ± 0.1	n.a.
			Influent = Synthetic Secondary Effluent				
SU1	Planted	12.7 ± 18.6	68.8 ± 45.2	4.0 ± 4.6	11.6 ± 100.6	0.3 ± 0.1	6.8 ± 0.2
	Unplanted	<LOD	96.5 ± 0.3	8.4 ± 1.1	-74.5 ± 19.6	5.8 ± 2.4	7.2 ± 0.1
	Control	<LOD	-	1.2 ± 0.7	-	<LOD	6.9 ± 02
	p-value*	0.68		0.30		0.001	
AU1	Planted	6.6 ± 9.1	92.5 ± 10.6	1.7 ± 1.9	85.9 ± 16.0	<LOD	n.a.
	Unplanted	<LOD	98.9 ± 0.0	3.4 ± 0.5	81.2 ± 2.5	3.7 ± 0.6	n.a.
	Control	7.7 ± 10.3	-	2.2 ± 2.8	-	<LOD	n.a.
	p-value*	0.87		0.95		6.4e-11	
WI2	Planted	25.5 ± 12.4	78.6 ± 9.1	1.2 ± 1.3	92.9 ± 8.4	<LOD	n.a.
	Unplanted	14.4 ± 5.0	84.2 ± 6.7	10.4 ± 5.3	47.5 ± 25.5	1.8 ± 1.5	n.a.
	Control	15.2 ± 7.3	-	1.7 ± 2.0	-	<LOD	n.a.
	p-value*	0.96		0.006*		0.02	
SP2	Planted	4.6 ± 4.0	96.1 ± 3.8	3.7 ± 3.3	89.8 ± 8.9	< LOD	6.7 ± 0.1
	Unplanted	< LOD	99.0 ± 0.4	5.2 ± 1.6	87.1 ± 5.6	3.4 ± 0.5	6.7 ± 0.1
	Control	< LOD	-	< LOD	-	< LOD	6.5 ± 0.1
	p-value*	0.54		0.27		7.4e-5	
			Influent = Synthetic Primary Effluent				
SU2	Planted	< LOD	98.9 ± 0.2	5.9 ± 5.4	86.7 ± 11.6	6.4 ± 3.4	6.0 ± 0.4
	Unplanted	< LOD	98.9 ± 0.2	32.6 ± 2.6	31.3 ± 12.5	28.2 ± 4.1	6.2 ± 0.2
	Control	4.1 ± 3.9	-	3.7 ± 4.8	-	3.5 ± 4.9	6.5 ± 0.5
	p-value*	1.3e-4*		7.6e-5*		3.9e-14	
SU2-A+	Control+	< LOD	98.6 ± 0.1	25.5 ± 14.7	25.1 ± 41.4	14.3 ± 1.5	6.4 ± 0.1
	p-value**			0.001		1.5e-8	

AU2	Planted	103.8 ± 112.5	90.8 ± 9.9	3.2 ± 3.7	93.0 ± 8.2	0.3 ± 0.1	5.8 ± 0.5
	Unplanted	12.4 ± 7.6	98.2± 1.2	13.4 ± 6.6	75.2 ± 20.7	7.6 ± 3.5	6.5 ± 0.5
	p-value*	0.01		0.003		7.5e-6	

COD – Chemical oxygen demand
<LOD – below limit of detection of Hach nutrient test kit. Limits of detection for COD, Total nitrogen-N, and Nitrate-N are 3mg/L, 1mg/L, and 0.5 mg/L, respectively.
*Analysis of variance (ANOVA)
**t-test
+SU2-A = adaptation trial; control reactors received wastewater and were compared to SU2 planted reactors.

Nitrate

Nitrate concentration was near or below the detection limit of 0.25 mg/L nitrate-N in planted reactor effluent across all seasons except for SU2, when higher doses of nitrogen were introduced (Figure 5). In contrast, nitrate was always detected in effluent from unplanted reactors, at between 2 and 20 times the concentration of planted reactors. Differences were more pronounced in high-nitrogen trials. In control reactors, effluent nitrate was below detection in four of six seasons and peaked at a concentration of 3.5 mg/L in SU2, suggesting limited but detectable background nitrate production by poplar trees and associated microorganisms.

Nitrate effluent concentrations differed significantly by treatment and season (p < 2e-16 for both variables) with significant interaction effects. Tukey's HSD revealed treatment differences between unplanted reactors, and both treated and control reactors (p < 2e-16 for both treatments) while the treatments with trees did not differ in effluent concentration (p = 0.17), despite the substantial difference in nitrogen loading to the reactors. This suggested that effluent concentrations in planted reactors receiving wastewater were indistinguishable from concentrations in planted reactors receiving only tap water. Regarding seasonal differences, SU2 differed from all other seasons (p < 2e-16), while no other seasons differed from each other (p > 0.05). The interaction effects can be attributed to the variation in nitrate loading rate across seasons, which induced different responses in performance based on treatment. ANOVA results for individual seasons are shown in Table 7.

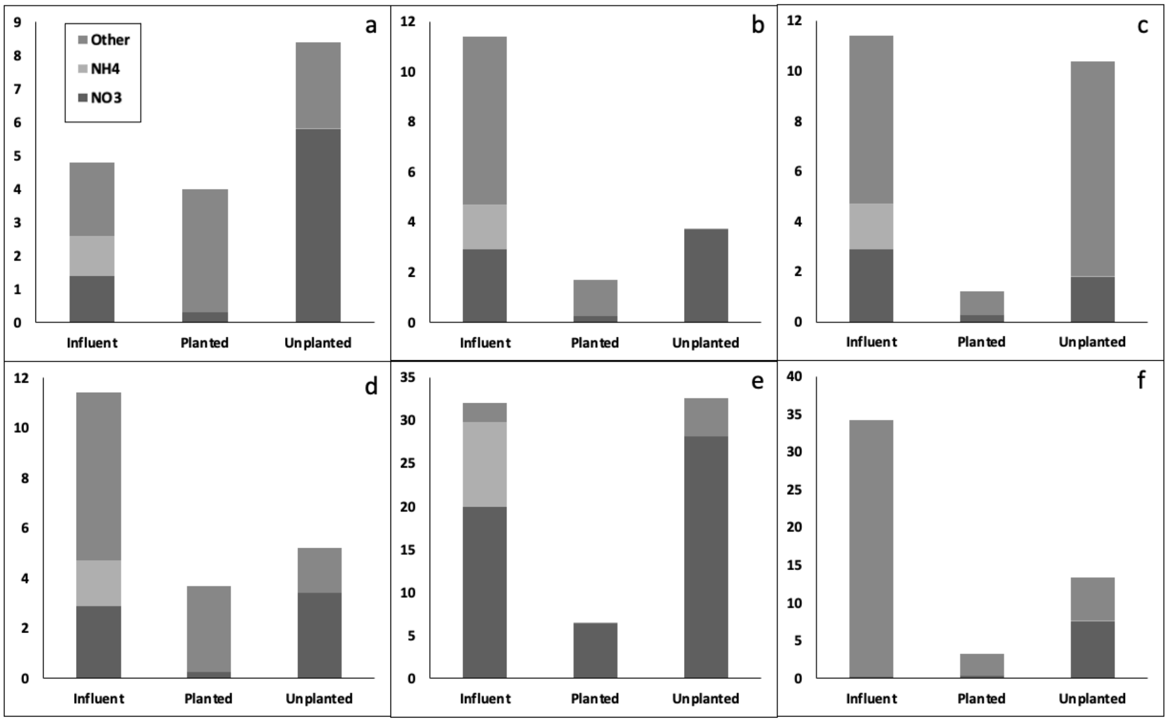

Figure 5: Influent and effluent nutrient composition across seasons. a) SU1 (June-Aug 2022); b) AU1 (Sept-Nov 2022); c) WI2 (Dec 2022-Feb 2023); d) SP2 (Mar-May 2023); e) SU2 (June-Aug 2023); f) AU2 (Sept-Oct 2023). All seasons received secondary effluent except SU2 and AU2, which received primary effluent.

Figure 6 shows nitrate content in effluent throughout the 6-hour intermittent irrigation cycle. Nitrate was higher during the dry period and lower in the irrigation period. Despite the observed variation throughout the irrigation cycle, timepoint did not significantly influence effluent nitrate concentration (p = 0.10).

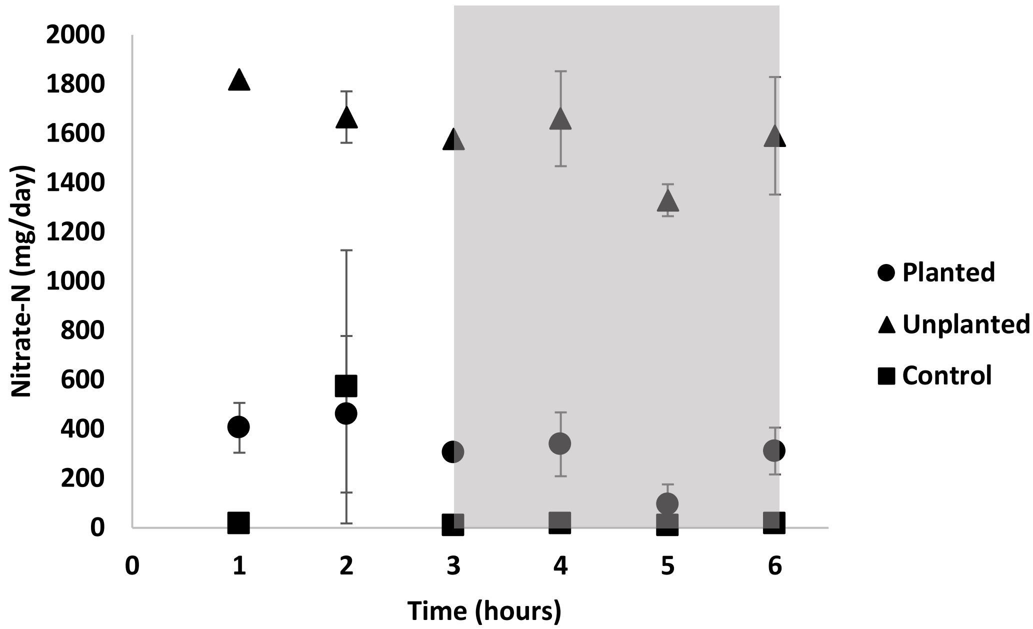

Figure 6: Effluent nitrate concentration throughout one 6-hour irrigation cycle. Gray shaded region represents irrigation period.

Total nitrogen

Effluent total nitrogen (TN) concentrations fluctuated throughout the experiment with variation across seasons. Nitrogen removal in planted reactors was consistently greater than 85% from AU1 through the end of the experiment, while it fluctuated with season in unplanted reactors. Effluent TN was always higher in unplanted reactors than planted or control reactors, and this difference was statistically significant in WI2, SU2, and AU2 (Table 7).

Total nitrogen varied by treatment and season (p= 8.1e-7 and 2.1e-13). Tukey's HSD revealed that unplanted reactors differed from both planted and controls, while both treatments with trees were not significantly different (p = 0.41). Effluent total nitrogen did not vary with time throughout the irrigation cycle (p = 0.36).

Some background release of total nitrogen was observed in control reactors in all seasons except spring 2023, where TN was below detection. Effluent total nitrogen concentration in control reactors was similar to planted reactors, although the forms of nitrogen may have been different.

Chemical oxygen demand

COD was reduced by 69-99 percent in all reactors and to below detection (< 3 mg/L) in 74 percent of samples (Table 7). Effluent COD was lower in unplanted than planted reactors in all seasons, but the difference was only statistically significant in AU2 (p = 0.01). The high COD removal in all reactors may be due in part to the low-organic matter soil used in this experiment, resulting in a system that was carbon-starved.

COD concentration was higher in treated effluent than control and unplanted effluent, and this difference was statistically significant (ANOVA, p = 0.007). Post-hoc analysis revealed that treated reactors differed from both unplanted (p = 0.02) and control (p = 0.05) reactors. Effluent COD also differed by season (ANOVA, p = 0.001), specifically in SU2 and AU2 (Table 5). In SU2 this was due not to wastewater effects, but to the detection of COD in the effluent of control reactors. The source of this organic matter was likely root exudates and other compounds produced in the rhizosphere (Angers and Caron 1998, Singer and Munns 2002).

Phosphorus and pH

Effluent phosphorus was measured in SU2 and AU2 and did not differ based on treatment or season (ANOVA, p = 0.55 and 0.88 respectively). Effluent pH differed significantly by season (ANOVA, p = 7.6e-9), with average values decreasing over the course of the experiment. The pH also varied by treatment (p = 0.002), specifically between unplanted and treated reactors (Tukey's HSD, p =0.002).

3.3.4. Adaptation testing

Control reactors received wastewater in AU2 and were compared to performance of planted reactors in SU2, which received the same nutrient concentrations. COD removal by unadapted reactors was > 98%, which is on par with the performance of treated reactors. However, nitrogen removal performance was significantly below that of treated reactors (t-test, p = 1.5e-8 for nitrate and p = 0.001 for total nitrogen). Effluent concentrations of nitrate and total nitrogen were 14 ± 1.5 and 28 ± 17 mg/L respectively, which represents removal rates of 25% and 29%. This is compared to >99% nitrate removal and 93% total nitrogen removal in adapted reactors. This suggested that system adaptation was a key component of successful nitrogen treatment in the system.

3.3.5. Leaf analysis and nutrient fate

Harvested treated leaves had higher average nitrogen, phosphorus, and potassium concentrations than control leaves, while nitrate content was not significantly different between treated and control trees (Table 8). Additionally, the average dry mass of treated leaves was greater than that of control leaves. This indicated a significant difference in total leaf biomass and nutrient content between treatments. Based on dry mass of the harvested leaves, treated leaves had 9.9 times greater leaf nitrogen mass than control leaves (0.021 g/leaf and 0.002 g/leaf, respectively). Total phosphorus and potassium mass were 4.7 times and 3.6 times greater, respectively, in treated leaves.

Nitrate concentrations in fallen leaves were 2.7 times greater for treated leaves and 5.3 times greater for control leaves. While nitrate did not differ between treatments in harvested leaves, fallen control leaves had noticeably higher nitrate content than fallen treated leaves, suggesting differences in winter nitrate storage strategies between treated and control trees.

NPK were all lower in fallen leaves from treated trees compared to harvested leaves. Control fallen leaves had similar NPK composition to harvested counterparts. Due to low sample size (n=2), the fallen leaves could not be compared statistically.

Qualitative comparison showed clear differences in leaf coloration. Leaves from treated trees were dark green in color, thick, and had a glossy sheen. Leaves from control trees were light green to yellow-green in color and were notably thinner than treated leaves.

Table 8: Leaf nutrient content in leaves from treated and control trees

	Leaf dry mass	N (%)	P (%)	K (%)	NO_3 (ppm)
			Collected leaves		
Treated	0.74 ± 0.29	2.78 ± 0.26	0.21 ± 0.02	1.40 ± 0.14	10.3 ± 3.7
Control	0.34 ± 0.04	0.61 ± 0.07	0.1 ± 0.003	0.86 ± 0.08	12.8 ± 3.0
p-value	1.7e-10	1.3e-11	2.9e-8	1.9e-9	0.14
			Fallen leaves		
Treated	-	0.59 ± 4.7e-3	0.05 ± 1.3e-4	0.83 ± 0.02	27 ± 0.9
Control	-	0.86 ± 1e-4	0.08 0.01	0.60 ± 0.04	68 ± 3.2

3.4. Discussion

3.4.1. Nitrogen removal was enhanced by poplar trees

This study demonstrated the importance of plants to wastewater treatment in WISER. Consistently across seasons, planted reactors outperformed bare-soil reactors in nitrate and total nitrogen removal. While studies directly comparing planted and unplanted systems are rare, those that do have observed significantly lower effluent nitrogen concentrations in the presence of vegetation (Sun, Chen et al. 2018). In one study, performance of mixed poplar-willow stands was almost double that of bare soil beds, with 80% and 43% TN removal, respectively (Khurelbaatar, Sullivan et al. 2017). Another study found that in sandy soils, effluent nitrate concentration was always higher in bare soil beds than poplar or willow beds (Dimitriou and Aronsson 2011).

The nitrogen removal in planted reactors was as good as or better than that observed in other planted land treatment systems. In studies using poplar and willow to treat primary effluent, total nitrogen removal rates greater than 75 percent (de Miguel, Meffe et al. 2014, Chen, Jiang et al. 2021, Khurelbaatar, van Afferden et al. 2021) and even 90 percent (Duan and Fedler 2010, Amiot, Jerbi et al. 2020) were observed. Lower removal rates of 55% to 58% were observed in studies treating secondary effluent in Estonia, but concentrations were still below national discharge standards (Truu, Truu et al. 2009, Holm and Heinsoo 2013).

Treated reactors outperformed unadapted reactors in nitrogen removal performance, suggesting that system adaptation is a key component of treatment at higher nitrogen concentrations. In a series of studies conducted in Canada, willow trees near a wastewater treatment plant were used to treat first secondary effluent, then primary effluent. Tertiary treatment performance was 90%, and subsequent secondary treatment was highly effective, with 98% total N removal over approximately 3 months (Lachapelle-T, Labrecque et al. 2019). This adaptation occurred over a period of four years, compared to 1.5 years for this study. Understanding system adaptation is important when establishing a new treatment WISER.

3.4.2. Nitrification-Denitrification Tradeoffs

Total nitrogen removal in land treatment systems is typically attributed to a combination of plant uptake and soil nitrification-denitrification processes (de Miguel, Meffe et al. 2014). In the soil, influent ammonium is converted to nitrate aerobically and nitrate is converted to nitrogen gas in anoxic conditions. When anoxic conditions are not provided, nitrate accumulation can occur (Prodanovic, Zhang et al. 2023). The intermittent irrigation of the reactors provided an environment with a balance of aerobic and anoxic niches to carry out both nitrification and denitrification continuously.

Land treatment systems achieve near-complete nitrification, regardless of the irrigation regime (Zhang, Huang et al. 2005) which was also reflected in this study. This process typically occurs quickly, in the upper layer of the soil (Dimitriou and Aronsson 2011, Prodanovic, Zhang et al. 2023). A study by Chen et al (Chen, Jiang et al. 2021) quantified oxygen concentrations in the upper (aerated) zone of a soil column during nitrification. They documented a linear decline in oxygen concentrations in the soil matrix over the 5 hours following an irrigation period. This cycle corresponded with changes to nitrogen processing in the soil columns. A similar pattern was observed by Pang et al (Pang, Pan et al. 2020). Notably, the oxygen concentration in the soil never reached zero in either experiment, suggesting that these systems also retained partial capacity for nitrification during the irrigated period.

Denitrification can be a limiting factor in land treatment systems (Duan and Fedler 2016, Lachapelle-T, Labrecque et al. 2019). Complete nitrification and incomplete denitrification is observed and expected (Grebenshchykova, Brisson et al. 2020), and the current state of land treatment assumes some level of residual nitrate in the treated effluent. For example, a study in Mongolia saw a peak of 31 mg/L nitrate in the middle depths of their reactors, but a reduction to 19 mg/L by the bottom of the 60 cm column (Khurelbaatar, van Afferden et al. 2021), which corresponds to a 38.7 percent nitrate reduction. Systems often observe a tradeoff, optimizing either ammonium or nitrate removal based on irrigation regime (Jia, Zhang et al. 2010). Our results suggested that in unplanted reactors, intermittent irrigation allowed the soil environment to maintain a balance between aerobic and anoxic spaces, reducing the need for the tradeoff.

3.4.3. Nitrate fate in trees

Trees can be a sink for nitrogen compounds (Lachapelle-T, Labrecque et al. 2019). In addition to transformation in the soil, plants in a WISER may be directly responsible for some of the nitrogen removal. Benoist et al attributed their almost complete ammonium removal to uptake by plants (Benoist, Parrott et al. 2023), while Mohsin et al. quantified increased nitrogen accumulation in willows under wastewater irrigation (Mohsin, Kaipiainen et al. 2021).

Trees may take up nutrients from wastewater and store them in leaves (Elowson 1999). The increase in leaf size and nutrient content in treated leaves supported that much of the nitrogen from the wastewater was taken up in leaves. Increased leaf area was observed in other wastewater studies (Jerbi, Brereton et al. 2020, Salehi, Zalesny Jr et al. 2023). Jerbi et al. attributed increased leaf and total biomass production in part to changes in leaf morphology, including increases in stomatal conductance and chlorophyl content, induced by nitrogen inputs in treated wastewater (Jerbi, Brereton et al. 2020). The significantly larger leaves on treated trees may have reflected these physiological changes. In addition, the dark green coloring of the treated leaves suggested higher chlorophyl content.

3.4.4. Fate of organic matter

Organic carbon removal was higher in unplanted reactors than planted reactors across all seasons. Poplar trees are known to act as a source of organic carbon in the soils (Isebrands, Aronsson et al. 2014). The COD found in the effluent may have been organic compounds produced by the trees or may have been excess organic matter from the influent that was not used by soil microorganisms. This would also explain the occasional COD found in effluent from control reactors as being produced by the tree and released into the soil, then washed out in the applied tap water.

The findings regarding COD removal from wastewater are mixed. Most studies observed removal rates greater than 90 percent (Jia, Zhang et al. 2010, Lachapelle-T, Labrecque et al. 2019, Amiot, Jerbi et al. 2020, Chen, Jiang et al. 2021) or between 60 percent and 90 percent (de Miguel, Meffe et al. 2014, Martinez-Hernandez, Meffe et al. 2020, Benoist, Parrott et al. 2023). COD removal in reactors, both planted and unplanted, was on par with many of these studies.

Nitrogen conversion depends on organic carbon for the process. The C:N ratio in land application systems should be at least 3:1 (Crites and Tchobanoglous 1998), which was approximately met in the SU2 synthetic primary effluent. However, incomplete denitrification in unplanted reactors despite complete COD removal suggested that this ratio was insufficient in unplanted reactors. In AU2, sufficient COD was provided in influent for denitrification in unplanted reactors. COD removal to below detection suggested that a ratio as high as 18:1 may be needed for complete denitrification in low-nutrient soils, particularly in unplanted systems. A much lower ratio may be sufficient in systems with additional carbon inputs supplied by plants. COD attenuation can decrease in soil with more organic material (Martinez-Hernandez, Meffe et al. 2020), suggesting that land treatment systems may be limited by treatment capacity for organic matter, rather than nitrogen.

Carbon limitation is also suggested by the lack of organic matter accumulation throughout the experiment. There were carbon pools in both reactors, with significantly larger pools in planted reactors, but the size of the pools was relatively stable. Most land treatment systems exhibit some accumulation of organic matter in soils (Tzanakakis, Paranychianakis et al. 2011, Becerra-Castro, Lopes et al. 2015, Chen, Lu et al. 2015), corresponding to removal of COD from influent wastewater (de Miguel, Meffe et al. 2014, García-Orenes, Caravaca et al. 2015). The lack of accumulation in this study serves to highlight the carbon limitation, suggesting that even in planted reactors, most of the carbon entering the system was used by the trees or soil. Soil organic carbon in planted soils increased in AU2 (Table S3) but that difference was not statistically significant. The results suggest the possibility that the system reached a stable carbon pool that was not impacted by additional COD addition.

3.4.5. Leaf nutrients and nutrient mass flow

Two possible scenarios arose for mass flow of C and N from wastewater into the system. In a carbon-driven nutrient removal scenario, sufficient carbon is available in the wastewater for complete denitrification, and excess is taken up and stored in trees. This situation would occur in the presence of excess organic carbon. The second scenario of nitrogen-driven nutrient removal assumes that the carbon in the wastewater is first used for denitrification but is not sufficient to remove all nitrogen. Some of the remaining nitrogen is taken up by the trees and may be incorporated into chlorophyl in leaves (Evans 1989), increasing photosynthetic potential (Tsvetkov, Tzvetkova et al. 2021), or into other compounds needed for plant growth. If needed, the resulting carbon can then return to the soil for additional denitrification. The observed nutrient removal patterns and differences in leaf properties suggested that the nitrogen-driven scenario occurred in SU2, and carbon-driven scenario occurred in AU2.

The significantly lower nitrate concentration in treated fallen leaves was promising for nitrogen removal from the system. It suggested that, compared to poplar trees without wastewater, those receiving wastewater pull more nitrate from the leaf back into the tree before abscission. This is good for the system because fallen leaves will not be returning nitrogen to the soil at the end of the season, which could negate some of the positive effects of the treatment. However, the leaves would serve as an additional organic matter input as they decomposed, returning carbon to the system. Leaf removal may be needed to prevent organic matter accumulation that would limit system performance.

3.4.6. Water recovery and winter performance suggested potential for expanded applications of WISER

Other work showed a significant decrease in nitrogen removal performance when trees entered dormancy (Amiot, Jerbi et al. 2020), but in this work performance was maintained above 90 percent in WI2 in the presence of poplar trees. This suggested that current trends of applying secondary treated wastewater only in the growing season (Khurelbaatar, Sullivan et al. 2017) may be unnecessarily limiting the application of land application in certain climates. Notably, winter performance was tested with secondary effluent only. Future work should include testing winter treatment of primary effluent.

Effluent recovery rates above 50% observed in all seasons suggested the potential for water recovery and reuse for processes. Other studies applying water at higher rates have observed similar levels of recovery (Benoist, Parrott et al. 2023). Poplar evapotranspiration rates in the study area are typically below 6 mm/day (Peters, Nelson et al. 2005). At the application rates in this study, this represented less than 10% of the total water applied, meaning water recoveries up to 90% were not unexpected. Water recovery was also enhanced by covering the reactors to limit evaporation from the soil surface. In a full-scale system, soils would be exposed to the air, increasing surface evaporation and thus decreasing water recovery. Conversely, higher evaporation from the soil surface could further increase the treatment capacity of the system.

Many studies of land application recommend application at only crop evapotranspiration rates (Tzanakakis, Paranychianakis et al. 2009), even with the goal of wastewater treatment (Lachapelle-T, Labrecque et al. 2019), in order to maximize nutrient usage and limit potential for nitrogen leaching. This study suggested that nutrients were used by the trees and soil microbial community even at higher nutrient loading rates, although this may not be true for all systems.

One limitation of this study was that while the artificial environment of the reactors improved the ease of testing, it prevented interactions between the tree root structure and the surrounding ecosystem. Reactors were isolated and could not exchange nutrients or microorganisms between trees, which would happen in a real WISER.

3.5. Conclusion

In this study, a wastewater infiltration system for bioenergy and recovery exhibited nitrogen removal from high volumes of synthetic secondary and primary effluent to below discharge standards. Trees served as a nitrogen sink and a carbon source, depending on loading rate of both carbon and nitrogen. By testing bare-soil controls under identical conditions, we also verified the importance of the plants for nitrogen removal performance. The test system described here, using metal troughs to simulate wastewater infiltration treatment aboveground and easily collect water that has passed through the system, could be adapted for onsite testing of performance before implementing a full-scale system. Better understanding of system performance and resulting expansion of the use of WISER for tertiary wastewater treatment offers an effective solution for decreasing nitrate runoff.

4. Economic evaluation of a poplar-planted wastewater treatment gallery

Publication: This chapter is being prepared for submission to the Journal of Environmental Engineering as a research note, accompanying the manuscript described in Chapter 3.
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Abstract

Production of a reliable biomass supply is a vital part of expanding the bioeconomy. One method is to pair biomass production with wastewater treatment, which increases biomass yields. Wastewater infiltration systems planted with hybrid poplar trees were irrigated with secondary and then primary effluent. Biomass production was quantified and compared to trees irrigated with clean water. An economic evaluation was also conducted to quantify the costs and revenue associated with establishing a wastewater infiltration system for biomass production at a 1MGD wastewater treatment facility. After 19 months of irrigation, trees receiving wastewater produced 3x as much dry biomass as control trees. In addition, economic analysis indicated a potential annual revenue of $200,000 - $600,000 from sale of recovered treated wastewater. This work demonstrated the potential economic value to wastewater treatment plants of establishing a wastewater infiltration system for biomass production.

4.1. Introduction

Nature-based wastewater treatment approaches are often valued for their cost-savings for small facilities (i.e., < 1MGD). Established approaches include constructed wetlands and infiltration galleries. Previous work has indicated that growing poplar trees using reclaimed wastewater for irrigation has potential to improve the economics of poplar-based biorefineries

(Chowyuk, El-Husseini et al. 2021). However, the economic value for the wastewater industry has not yet been evaluated.

There are several economic benefits associated with using treated wastewater (TWW) for biomass irrigation and tertiary treatment. Biomass growth is increased significantly with TWW application for both short rotation poplar (Houda, Bejaoui et al. 2016, Salehi, Zalesny Jr et al. 2023) and willow species (Khurelbaatar, van Afferden et al. 2021, Mohsin, Kaipiainen et al. 2021). This translates to increased revenue for the biomass growers. Additionally, nature-based treatment of nitrogen and phosphorus is significantly cheaper than conventional treatment (Rosenqvist, Aronsson et al. 1997, Tobin, Gustafson et al. 2020) while also providing ecosystem services such as water purification and carbon sequestration. Biomass crops can be harvested flexibly as needed, allowing biomass galleries to serve as "living storage" for trees (Dou, Marcondes et al. 2017). Finally, communities may experience socioeconomic benefits from growing biomass crops, including the creation of local jobs and economic resilience against the failure of food crops (Licht and Isebrands 2005).

Emphasizing the benefits of wastewater reuse for biomass production is vital for the expansion of the circular economy. Also important is having a full understanding of the costs and potential revenue associated with establishing and operating a biomass production site with TWW irrigation. Capital and operating costs of such operations are evaluated through economic analysis, which utilize existing price values to estimate the costs of industrial processes (Chai, Phang et al. 2022).

Here, we quantified the differences in poplar biomass production in a wastewater infiltration gallery receiving wastewater or tap water. Additionally, we evaluated the projected costs and revenues associated with operating a wastewater infiltration system, using the yields obtained in the study to estimate biomass production. The goal was to identify incentives toward incorporating a nature-based treatment solution into the wastewater treatment process for nutrient removal, thereby incorporating wastewater treatment into the circular economy.

4.2. Methods

4.2.1. Experimental Setup and Performance.

The wastewater infiltration system used for the experiments is described in detail in Chapter 2. Briefly, reactors were constructed from galvanized steel troughs filled with sandy loam soil and three hybrid poplar trees (clone 5077; hybrid of *Populus deltoides* and *P. trichocarpa,* provided by Greenwood Resources, Inc; Portland, Oregon, USA) were planted in each reactor. Reactors were treated for 1.5 years with synthetic wastewater (Kargol, Burrell et al. 2023) or tap water. Water purification performance is described in Chapter 3.

4.2.2. Poplar Biomass characterization

Poplar trees were harvested in winter of 2024 after complete abscission (i.e., natural loss of leaves). The trees were coppiced 3 inches above soil level. Trunk diameters and tree heights were recorded. The mass of each tree was measured following ASTM E871-82 for Moisture Analysis of Particulate Wood Fuels (ASTM 2019). Briefly, each tree, including branches, was sectioned to fit into paper bags and weighed. Trees were dried at 60^{o}C until mass changes were below 1% per hour (approximately 7-10 days of drying time).

4.2.3. Economic Evaluation

An economic evaluation was conducted to estimate the potential costs and savings associated with utilizing land application for tertiary wastewater treatment and biomass production. The system was defined as a theoretical 1 million gallon per day (1MGD) wastewater treatment plant (WWTP) located in Centralia, Washington, USA and collocated with a biorefinery to which biomass would be sold. The potential for constructing a biorefinery in Centralia was previously explored in a collaboration between the University of Washington and Advanced Hardwood Biofuels (Chowyuk, El-Husseini et al. 2021).

The costs considered were land, purchase of trees, and capital costs for constructing the wastewater infiltration system. The amount of land needed for treatment at the target application rate was calculated in previous work to be 14.3 acres (Chapter 3). Suitable land parcels and costs were identified using the Advanced Hardwood Biofuels Parcel Viewer (https://nrsig.org/apps/ahbnw/) created by the Natural Resources Spatial Informatics Group (Rogers, Cooke et al. 2016). Maximum distance of the land parcel from the treatment plant was 8.1 km, which was identified from a stakeholder survey as the furthest reasonable distance for pumping (Hart, Townsend et al. 2018). Values for capital and operating costs were collected from the literature. We did not consider values related to construction and operating of the wastewater treatment plant through the secondary stage. The analysis assumed that the treatment plant had already been constructed and was deciding to install a wastewater infiltration system for tertiary treatment and biomass production.

The revenue sources considered were sale of biomass to the biorefinery and recovered water sale to industry and the community for reuse. Biomass revenue for a similar scenario was previously estimated (Chowyuk, El-Husseini et al. 2021). Water recovery profits were estimated based on municipal reports of recovered water pricing structures in several counties in California, USA. Construction costs were updated to January 2024 values using the Chemical Engineering Plant Cost Index (Chemical Engineering Magazine 2024) and other costs were updated using the Bureau of Labor Statistics Consumer Price Index inflation calculator (Bureau of Labor Statistics 2024).

4.3. Results

4.3.1. Biomass production

Table 9 shows characteristics of treated and control poplar trees. The average mass of treated trees was approximately 3.1 times the mass of control trees (Figure 7). This indicated a clear difference between the treatment types, with benefits conveyed to the trees receiving wastewater. Production by treated trees equated to 30.5 tons/ha during the experimental period based on planting density in the reactors. This was within the typical range for hybrid poplar grown on marginal land (Ghezehei, Ewald et al. 2021). The difference in biomass observed between the two treatment types was highly significant (t-test, $p = 8.34 \times 10^{-5}$), as were differences in height and trunk diameter.

Table 9: Characteristics of treated and control poplar biomass

	Dry biomass (g)	Trunk height (cm)	Trunk diameter (cm)
Treated	1040 ± 100	320 ± 26	4.1 ± 0.3
Control	340 ± 24	240 ± 34	2.8 ± 0.3
p-value*	8.34e-5	6.0e-4	6.8e-7

Average deviation is shown; *t-test

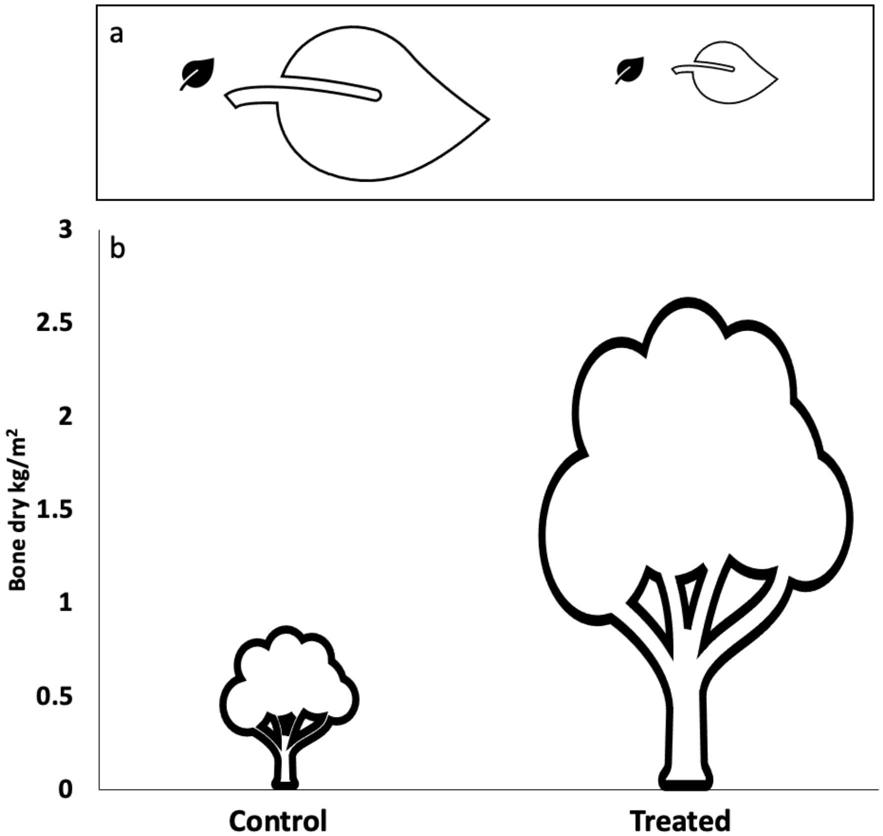

Figure 7: Biomass production and leaf nitrate concentrations in a wastewater infiltration system. a) leaf nitrogen content (Chapter 3); filled shapes represent leaves harvested from trees, unfilled shapes represent fallen leaves collected on the ground; size of leaves is proportional to nitrogen content. b) biomass yields are represented by tree height.

4.3.3. Economic evaluation

Costs and revenues considered for the analysis are shown in Table 10. Land cost was obtained from the AHB Parcel Viewer set with a radius of 8.1 km from the location of the theoretical WWTP. Only undeveloped rural parcels were considered. The tool identified 73 parcels of land in that radius that were moderately suitable or better to hybrid poplar production. Of those, 19 were between 15 and 25 acres, a reasonable size to accommodate the 15-acre biomass growing site and allow the potential for expansion without incurring unnecessary costs. An additional 5 parcels were eliminated due to significantly higher cost per acre ($85,000-$1,900,000). In total, 14 suitable land parcels were identified, with an average adjusted cost of $5,710/acre, median of $4,800, and a range of $1,400-$14,400 per acre. At the average price, land cost for a 15-acre growing site would be $85,650.

Construction cost for the pumps, conveyance system, and site fencing was based on estimates from the EPA Manual of Constructed Wetlands. Cost in 1997 was $17,297/ha (EPA 1999), which was adjusted using the CEPCI to $35,840/ha, or $216,000 for a 15-acre biomass growing site.

Biomass revenue was calculated using $57.80/bone dry Mg as an estimated selling price for biomass only (i.e. no transportation costs due to collocation with the biorefinery) (Chowyuk, El-Husseini et al. 2021). Based on yields from a 14.3-acre site, this would equate to $23 for the harvest of the first rotation. Selling prices for reclaimed water were highly variable, ranging from $300-$500 per acre-foot in northern California (Clumpner 2016) to as high as $1,530 in Los Angeles County (LA County 2022). Average selling price was $1,120/acre-foot, or a daily revenue of $1,720 for 500,000 gallons (assuming 50% water recovery from a 1MGD facility). A more conservative scenario assuming $400/acre-foot would equate to a daily revenue of $610.

Table 10: Major costs associated with biomass production

Component	Cost/revenue	Ref
Land	-$85,650	(Rogers, Cooke et al. 2016)
Distribution system	- $216,000	(EPA 1999)
Biomass	+$23*	(Chowyuk, El-Husseini et al. 2021)
Reclaimed water	+$222,650 - 627,800/year	(Clumpner 2016, LA County 2022)

*Biomass harvest from first rotation

4.4. Discussion

Increased biomass production by 300 percent in treated trees suggested that wastewater irrigation produced significant improvements in biomass yield in a wastewater infiltration system. The increase in biomass production was likely due to the nitrogen and organic carbon in the wastewater, which was taken up by trees (Benoist, Parrott et al. 2023). The yellow-green appearance of control leaves suggested nutrient limitation in control reactors (Justin, Pajk et al. 2010) and further supported this conclusion.

Total biomass produced depends on factors such as age and planting density (Kwon, Law et al. 2018), as well as clone selection. Some clones perform better in certain regions or conditions (Holm and Heinsoo 2013) and careful selection is vital to the success of the wastewater infiltration system (Postila and Heiderscheidt 2020). Our results supported that this hybrid of *P. deltoides* and *P. trichocarpa* performs well in the Pacific Northwest.

Many studies have found that nutrient inputs from TWW increase biomass growth (Dimitriou and Aronsson 2011, Houda, Bejaoui et al. 2016, Mohsin, Kaipiainen et al. 2021). While one study noted a 6500 percent increase (Mohsin, Kaipiainen et al. 2021), most experiments observed more modest increases of 40-300 percent (Justin, Pajk et al. 2010, Holm and Heinsoo 2013, Jerbi, Nissim et al. 2014). The yield increase in this study falls at the higher end of the typical range. One study noted that while aboveground biomass increased under TWW irrigation due to nutrient inputs, belowground biomass decreased due to a reduced need for roots to scavenge nutrients (Jerbi, Nissim et al. 2014). It is important to consider that impact in a real system and how it might affect trees in the long-term. Conversely, this could also have the positive impact of limiting the infiltration of tree roots into the water collection system.

The poplars in this study were in their establishment rotation and had never been coppiced. The first rotation of a short-rotation coppice crop typically has a significantly lower yield than subsequent rotations (Chowyuk, El-Husseini et al. 2021). When irrigated with TWW, others have observed an increased number of resprouted shoots relative to biomass not receiving additional nutrient inputs (Tsvetkov, Tzvetkova et al. 2021). This suggests the possibility that differences in yield between treated and control trees would be even more drastic in subsequent rotations.

Economic analysis revealed that for a 1MGD wastewater treatment facility in western Washington, most revenue could come from selling recovered water to local municipalities. With a predicted construction cost of $301,000, this would allow treatment plants to recover installation costs for the WIS within just a few years. With the system focused on maximizing wastewater treatment potential in a small area, biomass revenue was negligible. This suggested that reduced tertiary treatment costs, reclaimed water revenue, and intangible benefits of ecosystem services would be the main incentives for wastewater treatment plants to install wastewater infiltration systems.

This study investigated biomass production in a wastewater infiltration system and utilized the biomass yield values obtained in an economic analysis to quantify cost savings associated with reusing treated wastewater. We found that hybrid poplar biomass increased 300 percent due to inputs of nitrogen, demonstrating the effectiveness of treated wastewater as an irrigation water and nutrient source to increase biomass production. Wastewater treatment plants can generate significant revenue and quickly recover costs by choosing nature-based tertiary treatment coupled with biomass production, which will in turn benefit the circular economy by expanding the biomass market.

5. Soil community genetic adaptation to increased nitrogen in a poplar-vegetated wastewater infiltration system

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Abstract

Understanding nitrogen cycling in land treatment systems is important for predicting the fate of nitrogen compounds. We investigated the role of soil microorganisms in nitrogen cycling in a poplar wastewater treatment system. Soil samples were collected from synthetic wastewater-irrigated planted (hybrid poplar, treated) and unplanted (control) infiltration galleries, as well as planted controls irrigated with clean water. DNA was extracted and key nitrogen cycling functional genes *amoA* and *nirK*, as well as bacterial 16S, were quantified. Ex-situ nitrate activity tests with soil slurries were also conducted to quantify denitrification potential of the soil community. It was found that total biomass varied by season but not by treatment, and the *nirK* gene abundance followed a similar pattern. However, the *amoA* gene abundance was impacted by treatment and increased in abundance with nitrogen inputs. Nitrate dynamics tests showed similar denitrification rates in planted and unplanted communities, revealing that the soil communities had similar denitrification potentials. These results suggested that wastewater irrigation impacted nitrification on the level of gene abundance and denitrification on the level of activity. This in turn suggested that differences in nitrogen removal at a system level were influenced by the presence of trees, and their ability to take up nitrogen from the influent, rather than by differences in microbial community denitrification potential.

5.1. Background

Soil microorganisms are a critical component of pollutant attenuation in wastewater infiltration systems (WIS). These complex microbial communities transform residual nutrients in the wastewater. Understanding nitrogen cycling dynamics in WIS is important for predicting the final fate of the compounds (EPA 2006) and for optimizing treatment.

Lab-scale wastewater infiltration systems, consisting of metal troughs filled with sandy loam soil and planted with poplar trees, or left without trees as unplanted controls, were irrigated with synthetic secondary effluent (Chapter 2). Differences in nitrate removal performance were observed in the first six months of irrigation (3-year-old trees, planted summer 2018). Planted wastewater infiltration systems performed significantly better than unplanted systems in removing nitrate from the synthetic wastewater (Chapter 3). This suggested differences in nitrogen cycling in planted and unplanted systems which could be further explored. Direct comparisons of planted and unplanted systems are rare, with a few studies finding improved nitrogen removal performance in the presence of vegetation (Dimitriou and Aronsson 2011, Khurelbaatar, Sullivan et al. 2017, Sun, Chen et al. 2018).

Quantifying nitrogen cycling genes can be a method to correlate genetics with observed biogeochemical cycles (Voegel, Larrabee et al. 2021). Total nitrogen in wastewater is a combination of ammonium (free and sorbed), nitrate, and organic nitrogen. During processing, the organic nitrogen (typically present as proteins) is released as ammonium. Under aerobic and microaerobic conditions, ammonium is oxidized (nitrification) into nitrite (which is short-lived) and nitrate. In planted systems, nitrate is the form most commonly taken up by plants (Zayed, Hewedy et al. 2023). In the absence of plant uptake, nitrate is reduced (denitrification) to nitrogen gas by microorganisms under anoxic conditions. Each of these processes requires specialized enzymes to catalyze the transformation. Key marker genes for bacterial nitrification and denitrification are *amoA* (Keeley, Rodriguez-Gonzalez et al. 2020, Guo, Bayu et al. 2021) and *nirK* (Bowen, Maul et al. 2020, Khanal and Lee 2020), respectively. Both genes have been quantified in wastewater-irrigated systems with mixed results.

An emerging technology that can be applied to quantification of genes in whole sample DNA extracts is droplet digital PCR (ddPCR). ddPCR is a method for absolute DNA quantification without the need for the standards used in qPCR. PCR reagents are partitioned into individual droplets for amplification, and the fluorescence of the droplets corresponds to the concentration of DNA in the original sample (Hindson, Ness et al. 2011). ddPCR reactions are prepared by combining extracted DNA with a master mix containing, along with enzymes and other required reagents, fluorescent indicators associated with quencher molecules. Reactions are then partitioned via a droplet generator into thousands of small oil droplets, each of which may contain zero, one, or multiple copies of the target gene. Amplification is carried out using a traditional thermocycler. As amplification occurs, the fluorescent dye molecules intercalate between the strands of synthesized DNA, dissociating from the quencher and releasing the previously masked florescence. A droplet reader then classifies the droplets as positive (florescence) or negative (no fluorescence). It uses a Poisson distribution to account for the probability of 2 or more copies of the target gene in each positive droplet, and then adjusts the count of positive droplets to a final value of copies of the target gene per microliter.

ddPCR has several advantages over qPCR. First, it has equal or greater precision and reproducibility, which can be attributed to the partitioning of the reactants into droplets, which decreases variability (Hindson, Chevillet et al. 2013, Wang, Wang et al. 2022). ddPCR is also more robust to the inhibitors commonly found in samples from complex environments like soil (Hindson, Chevillet et al. 2013, Voegel, Larrabee et al. 2021, Wang, Wang et al. 2022) because inhibitors are diluted by the partitioning. Assays have been developed to quantify total microbial biomass, as well as key nitrification and denitrification genes via ddPCR (Voegel, Larrabee et al. 2021).

In this study, the biologic potential for nitrification and denitrification were compared between soils from unplanted and planted experimental wastewater infiltration systems and controlled planted reactors that received clean tap water. Ex situ nitrate reduction activity was measured in suspended soil slurries, and the concentration 16S (bacterial domain), *amoA,* and *nirK* genes were quantified using ddPCR.

5.2. Methods

5.2.1. System design and sample collection and DNA extraction

Simulated wastewater infiltration systems were established at the University of Washington (Seattle, Washington, USA) for controlled testing. Reactor design is described in detail in Chapter 2. Briefly, metal troughs were filled with sandy loam, low-organic matter soil and planted with hybrid poplar trees or left bare as unplanted controls. Reactors were irrigated with synthetic wastewater (Kargol, Burrell et al. 2023) for 18 months. Control reactors, irrigated with tap water, were also maintained. Irrigation occurred in 3-week blocks to allow the soil environment and microbial community time to adapt to synthetic wastewater.

Soil samples were collected from depths of 0.2 m and 0.4 m using a soil coring device and transported to the lab on dry ice, where they were stored at -80^{O}C until DNA extraction. Samples from three seasons were tested: summer 2022 (SU1 – baseline community with low-nutrient wastewater simulating secondary effluent, 3 months of irrigation), summer 2023 (SU2

– increased nitrogen concentrations to primary effluent, 15 months of irrigation) and autumn 2023 (AU2 – increased organic matter to primary effluent concentrations, 18 months of irrigation).

5.2.2. DNA extraction

DNA was extracted using the Qiagen DNEasy Powermax Soil DNA extraction kit (Qiagen Sciences, Germantown, Maryland, USA) according to manufacturer instructions. Briefly, cells were lysed using a FastPrep-24 bead beater and lysis system (MP Biomedicals, Irvine, California, USA) with BigPrep 50 mL tube adapter (Product number 116002525) and washed to remove RNA and proteins. DNA was eluted into DNase-free water and checked for quality using a Nanodrop One-C (Thermo Scientific, Waltham, Maryland, USA). DNA was extracted quantitatively by recording volume transfers at relevant steps, to allow for calculation of gene copies per unit of soil.

5.2.3. ddPCR for bacterial biomass and nitrogen cycling genes

Primers used for ddPCR are described in Table 12. Bacterial 16S primers 1114F and 1492R were used to quantify soil bacterial populations as copies of the 16S gene per gram of soil. In addition, key nitrogen cycling genes *amoA* and *nirK* were quantified via ddPCR. Primers for *amoA* were developed by Keeley et al. (Keeley, Rodriguez-Gonzalez et al. 2020) and primers for *nirK* were developed by Braker et al. (Braker, Fesefeldt et al. 1998). These primers were identified as having the best coverage of *nirK* in samples from soil environments (Gaby and Buckley 2012). ddPCR was first optimized for use with these primers by conducting a temperature gradient PCR, with annealing temperatures reported in the original papers as a starting point.

ddPCR reactions were assembled according to the protocol provided by BioRad (BioRad Inc, Hercules, California, USA) using EvaGreen chemistry. Reactions were prepared with 11 μl of EvaGreen 2x Mastermix, 1.1 μl of 20x primer stock (final primer concentration of 0.125 μM), 4.9 μl DNase-free water, and 5 μl of target DNA. Reactions were partitioned into droplets using the QX200 Droplet Generator (BioRad Inc).

The protocol used for 16S and *amoA* amplification was as follows: 5 minutes at 95^{O}C for enzyme activation, 40 cycles of 96^{O}C for 30 seconds for denaturation and 1 minute at select annealing temperature (Table 13) for annealing and extension, a final 5-minute extension step at the designated annealing temperature, 95^{O}C for 5 minutes for enzyme deactivation, and a hold at 4^{O}C.

For *nirK* amplification, conditions from Braker et al. were used (Braker, Zhou et al. 2000). Program was as follows: 5 minutes at 95^{O}C, denaturation at 94^{O}C for 30 seconds, annealing for 30 seconds with touchdown PCR for the first 10 cycles (56^{O}C - 51^{O}C, decreasing in 0.5 ^{O}C increments), followed by 19 additional cycles at 54^{O}C, extension at 72^{O}C for 30 seconds, a final extension step of 5 minutes at 72^{O}C, and a hold at 4^{O}C.

In addition to experimental samples, each run contained 3 positive control samples (*Sphingobium* BiD32 (Zhou, Lutovsky et al. 2013) for 16S, plasmid containing *amoA* gene for *amoA*, and *Paracoccus denitrificans* (DSMZ 2024) pure culture DNA for *nirK*), 3 negative controls (*Sphingobium* BiD32 for *amoA* and *amoA* plasmid DNA for 16S and *nirK*), and 3 no-template controls (DNA-free water).

Droplet fluorescence was read using a QX200 Droplet Reader (BioRad Inc). Positive droplet thresholds in samples were determined using positive, negative, and NTC controls (Kokkoris, Vukicevich et al. 2021, Kargol, Cao et al. 2022).

Table 11: Primers for quantifying key nitrogen cycling genes via ddPCR

Gene	Primers	Annealing temp (^{O}C)	Forward primer	Reverse primer	Ref
16S - bacteria	1114F 1492R	56	5'-CGG CAA CGA CGC CAA CCC-3'	5'- CCA TTG TAG CAG CAC GTG TGT AGC C-3'	(Lane 1991)
amoA	amoA F amoA R	55	5'-GAC TGG GAY TTC TGG MTK GAY TGG AA-3'	5'-TGY GAC CAC CAG TAR AAW CCC CAG-3'	(Keeley, Rodriguez-Gonzalez et al. 2020)
nirK	nirK 1F nirK 3R	54	5'-ATG GCG CCA TCA TGG TNY TNC C-3'	5'-TCG AAG GCC TCG ATN ARR TTR TG-3'	(Braker, Fesefeldt et al. 1998)

5.2.4. Nitrate ex-situ activity assay

Ex-situ activity assays were conducted in a series of steps, each of which provided new insights into the activity of microbes in the reactors. First, ammonium removal and nitrate production were tested. A large initial loss of ammonium was observed which did not correspond to an increase in nitrate. This was attributed to sorption of ammonium to soil particles. To minimize sorption effects, I tested the fate of nitrate additions, but still observed limited denitrification. Incubating flasks without shaking to create an anoxic environment also did not increase transformation of nitrate.

Two potential hypotheses could explain the limited nitrate transformation in the lab. The trees could be contributing to nitrate removal with limited contributions by the microbes, or the lab soil tests lacked sufficient organic carbon to sustain denitrification. For the latter, trees are a source of organic carbon that could lead to the differences observed in nitrate removal in planted vs unplanted reactors in the system.

When organic carbon was added to the batch test media at 1g/L, denitrification was observed within 24 hours. Organic carbon is used as an electron donor during biologic denitrification. This suggested that carbon limitation in the nitrate-amended soil slurries was the cause of the lack of degradation in previous trials.

Ex-situ activity tests were conducted according to the OECD protocol for aerobic and anaerobic transformation in soils (OECD 2002). Briefly, soil samples from SU and AU were collected in the same day as the samples used for DNA extraction (n = 4 for each treatment). Slurries consisted of 25 g of soil in flasks of 150 ml of medium with 10 mg/L nitrate and 1 g/L dextrose. Flasks were incubated in the dark at room temperature for 120 hours. Samples for nitrate analysis were collected daily and filtered through a 0.45μm cellulose acetate filter (Thermo Fisher Scientific) to remove soil particles. Nitrate-N was quantified using the Hach TNT Plus low-range kit, Method 835 (Hach Company, Loveland, Colorado, USA).

5.2.5. Statistical analysis

Shapiro-Wilks tests were used to check values for normality of ddPCR data. Analysis of variance (ANOVA) was conducted to test for effects of treatment, season, and depth on abundance of nitrogen cycling genes. When normality assumptions were not met, Kruskal-Wallace tests were also utilized.

Ex-situ activity test results were fitted assuming first-order degradation of nitrate. Results were fitted according to the equation:

$$C = C_0 e^{-kt}$$

where C is the concentration, C_0 is the initial concentration, k is degradation rate constant, and t is time. To determine the k-values, the equation was linearized to:

$$\ln(C) = -kt + \ln(C_0)$$

and solved graphically for the degradation slope (k). ANOVA was then used to test the impact of treatment and season on k.

5.3. Results

5.3.1. Functional gene abundance

Table 14 shows the abundance of 16S, *amoA,* and *nirK* in samples. Values were corrected for initial soil added and volume loss in DNA extraction to obtain results in copies/g dry soil.

Table 12: Abundance of functional genes by season and treatment

	16S*	***amoA****	***nirK****
Summer 2022			
Planted	2.2 ± 1.0	4.8 ± 2.6	5.2 ± 2.8
Unplanted	2.0 ± 0.8	9.1 ± 8.1	6.1 ± 3.7
Control	2.1 ± 0.7	1.2 ± 0.3	4.3 ± 1.9
Summer 2023			
Planted	3.9 ± 1.1	7.6 ± 4.3	9.0 ± 4.0
Unplanted	4.5 ± 0.9	8.7 ± 5.8	9.5 ± 2.2
Control	4.6 ± 1.4	2.4 ± 0.8	9.5 ± 3.3
Autumn 2023			
Planted	2.7 ± 0.7	10.1 ± 5.5	12.2 ± 3.7
Unplanted	3.0 ± 0.5	11.1 ± 6.9	8.7 ± 1.5
Control	2.2 ± 0.2	5.3 ± 1.2	11.9 ± 3.3

*all genes are reported as copies/g dry soil; 16S is reported as copies/10^7 and *amoA* and *nirK* abundance are reported as copies/10^4

Microbial biomass/16S

Abundance of 16S varied over the course of the study, reaching a peak in SU2 (Figure 8). For all treatments, abundance followed the pattern SU2 > AU2 > SU1. This suggests that soil microbial biomass generally increased over the course of the study while also exhibiting seasonal trends. There was no clear pattern of abundance by treatment; in fact, a different treatment had the highest 16S abundance in each season (SU1- planted, SU2- control, AU2- unplanted).

Abundance of 16S in soils did not very by treatment (p = 0.37), or by depth (p = 0.98). However, biomass abundance differed significantly by season (p = 2.41e-9). Tukey HDS revealed differences between SU2 biomass abundance and both SU1 and AU2 (p = 16e-8 and 2.46e-4, respectively), supporting a seasonal spike in SU2. This spike was not present in SU1, suggesting that it was a function of both the season and the increased development of the soil community in the second year of the study.

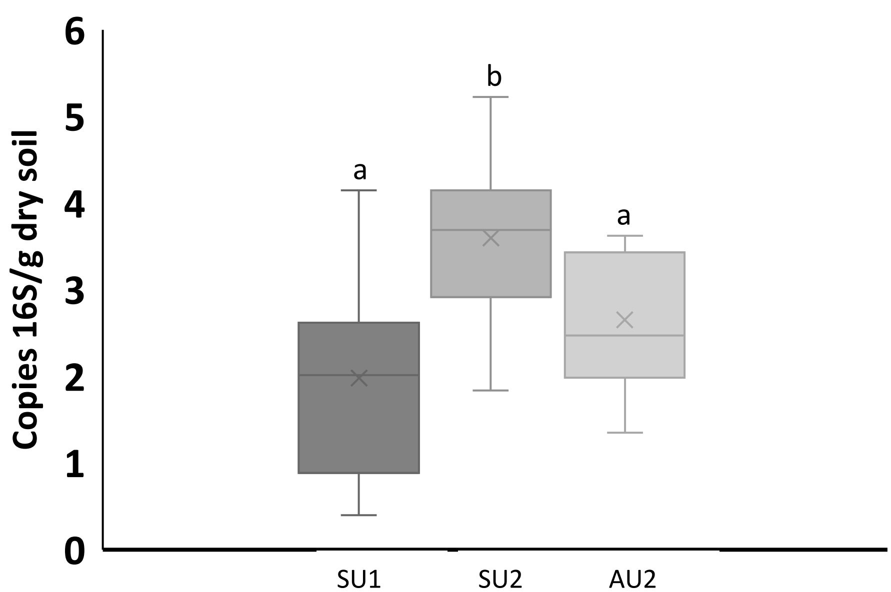

Figure 8: Abundance of bacterial 16S by season. Values are shown as copies/10^7. Seasons with different letters were significantly different. SU1 – summer 2022 (3 months operation), SU2 – summer 2023 (15 months operation), AU2 – autumn 2023 (18 months operation).

Ammonium oxidation/*amoA*

Across all seasons, *amoA* abundance was always higher in reactors receiving wastewater than controls (Figure 9). In SU1 and SU2, abundance was highest in planted reactors, while it was slightly higher in unplanted reactors in AU2. Average values increased each season and were notably highest in AU2, when planted and unplanted reactors received increased doses of both nitrogen and organic carbon.

Two values were removed as outliers. Data was then checked for normality using the Shapiro-Wilk test, which revealed that samples were not normally distributed (p = 6.78e-9). As a result, Kruskal-Wallace tests were used to test for differences based on treatment, season, and depth. These tests revealed significant differences in *amoA* abundance by treatment (p = 0.01) and season (p = 0.03) but not by depth (p = 0.23). The clear difference in abundance between reactors receiving wastewater and control reactors demonstrates the influence of treatment, and the increasing abundance of *amoA* in all seasons supports seasonal differences (Figure 9).

Analysis of variance revealed similar patterns in depth and treatment to the Kruskal-Wallace test but did not show seasonal variation (p = 0.30). However, there were interaction effects observed between season and depth (p = 0.007). Overall, wastewater irrigation, and the duration of the irrigation, influenced the abundance of *amoA* in soil communities.

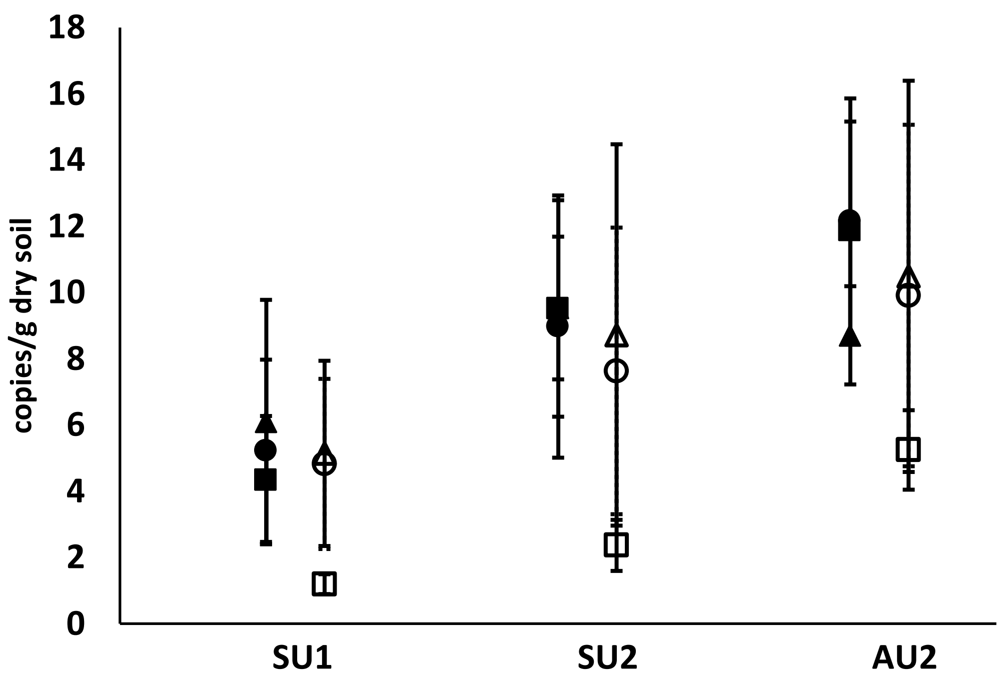

Figure 9: Abundance of functional genes nirK *(filled shapes) and* amoA *(unfilled shapes) in soils from poplar soil reactors. Values are shown a copies/10^4. Circles – planted reactors; triangles – unplanted reactors; squares – control reactors.*

Denitrification/*nirK*

Average *nirK* abundance in planted and control reactors increased each season, following the pattern AU2 > SU2 > SU1 (Figure 9). Values for unplanted reactors peaked in SU2 and decreased in AU2. A Shapiro test revealed that data was not normally distributed (p = 0.02). Kruskal-Wallace tests then revealed that abundance differed by season (p = 4.3e-4) but not by treatment (p = 0.73) or depth (p = 0.90).

Analysis of variance considering all three variables agreed with Kruskal-Wallace results. Abundance differed by season only (p = 0.001) and no interaction effects were noted. Tukey's HSD analysis revealed that *nirK* abundance in SU1 was significantly lower than SU2 (p = 0.03) and AU2 (p = 0.001), while SU2 and AU2 did not differ significantly (p = 0.54). This suggested that differences in abundance were related to microbial community succession with time rather than impacts of wastewater irrigation, and that the community composition stabilized between SU1 and SU2.

5.3.2. Denitrification ex-situ activity assay

Denitrification was observed in soils from planted and unplanted reactors in two trials (Figure 10). A lag phase was observed from Day 0 to Day 1, so degradation rates were calculated starting on Day 1. Based on linearized degradation curves (Figure 10b and 10c), degradation followed first-order kinetics. Planted reactors had degradation rates (k) of 1.7 ± 0.1 for SU2 and 1.9 ± 0.1 AU2. Unplanted reactor values were 1.6 ± 0.1 for SU2 and 2.0 ± 0.01 for AU2.

Degradation rate varied based on season (ANOVA, p = 0.003) but not by treatment (p = 0.85). This could reflect a response to seasonal changes, or the change in rate may have been influenced by the change in influent COD concentrations from SU2 to AU2.

The autoclaved control showed limited sorption of nitrate to the soil, as evidenced by the lack of change in nitrate concentration over 70 hours. The presence of organic carbon was important for degradation, as shown by the control with no COD, which exhibited no degradation.

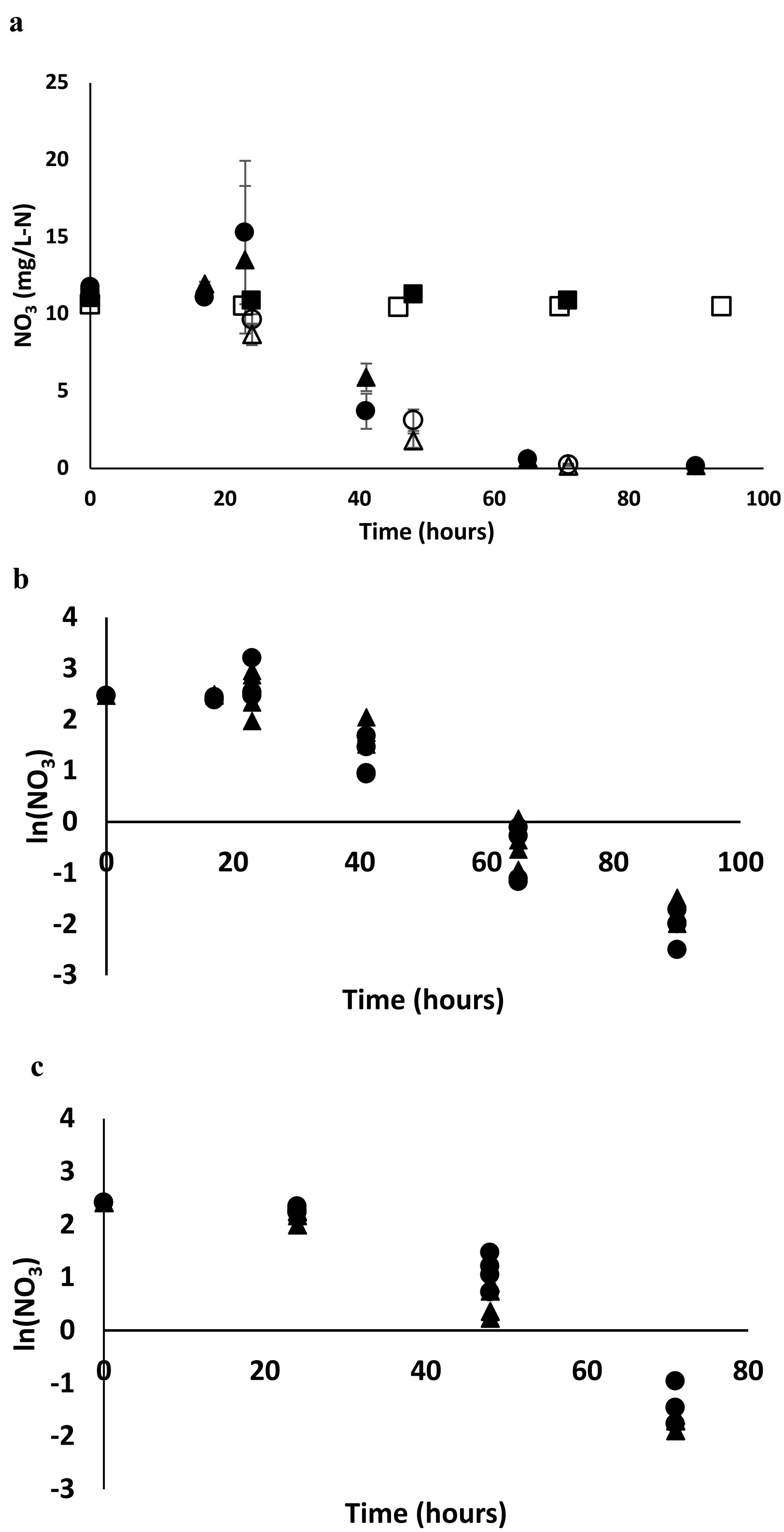

Figure 10: Denitrification kinetics in planted and unplanted reactors; a) nitrate degradation curve exhibiting first-order nitrate degradation kinetics. Circles represent planted reactors and triangles represent unplanted reactors .Closed shapes are SU2 and open shapes are AU2. Open squares represent controls without COD and closed squares represent autoclaved controls; b) linearized degradation curve for SU2; c) linearized degradation curve for AU2.

5.4. Discussion

5.4.1. Wastewater irrigation impacted microbial nitrification

Wastewater irrigation was the most important factor influencing *amoA* abundance, as demonstrated by differences between reactors receiving wastewater and controls. This suggested that the microbial communities in the system were involved in the nitrification process. Similar responses have been observed in the soils of other systems receiving wastewater. Abundance of AOB increased in bare soils with exposure to ammonium inputs in olive mill wastewater (Tsiknia, Tzanakakis et al. 2014, Frenk, Dag et al. 2015) and fish-processing wastewater (Marcos, González et al. 2021, Vallejos, Marcos et al. 2022), which were attributed to ammonium inputs (Moulia, Ait-Mouheb et al. 2023). Studies of the poplar rhizosphere in this context are rare, but in one study, wastewater slurry applied to a poplar plantation induced increases in *amoA* abundance (Ren, Yu et al. 2020).

It was unexpected that *amoA* abundance did not increase further in response to higher ammonium concentrations. Instead, the differences between wastewater and clean water treatments were evident in SU1 under secondary effluent nitrogen concentrations and did not increase significantly with the transition to primary effluent. This suggested a rapid adaptation by the system to ammonium inputs, which was then maintained throughout the experiment. This is similar to the adaptation period of a study testing land application treatment of olive mill wastewater, in which *amoA* abundance significantly increased in soils by 78 days (Tsiknia, Tzanakakis et al. 2014).

5.4.2. Nitrate removal was dominated by poplar trees

The abundance of *nirK* did not vary by treatment, suggesting that wastewater irrigation was not a significant influence on denitrification. One potential explanation for the lack of observed differences is that trees took up nitrate, one of the most common nitrogen sources for trees (Zayed, Hewedy et al. 2023). Thus the poplars, rather than microbes, were the key drivers of nitrate removal in planted systems. In unplanted reactors, soil microorganisms were not able to compensate with denitrification processes, resulting in nitrate accumulation.

Our results differed from other research showing that organic matter inputs from wastewater impacted denitrification gene abundance (Guo, Deng et al. 2013). *nirK* has been observed to be less abundant at lower organic loading (Chen, Jiang et al. 2021) and increase with OM load (Zhang, Cao et al. 2023). Notably, differential responses of denitrification genes have been observed in different systems (Sun, Guo et al. 2015, Chen, Jiang et al. 2021), suggesting that responses may be system specific. Future work could include quantifying additional denitrification genes, such as *nirS,* which has been shown to have differential responses to organic matter than *nirK* (Chen, Ma et al. 2023).

Despite no differences in gene abundance between SU2 and AU2, nitrate dynamics tests revealed increased degradation rate constant for both planted and unplanted reactors after treatment with higher-organic matter wastewater. This suggested that denitrification gene activity, rather than abundance, may have been impacted by nitrogen application.

5.4.3. Gene abundance shifted with time

nirK abundance was not influenced by treatment, meaning that even control trees experienced an increase in *nirK* abundance over time, without additional nitrogen inputs. This points to time-mediated microbial community shifts as a key driver of *nirK* abundance in the reactors. Season was also a significant factor influencing *amoA* and bacterial 16S, suggesting that observed differences in gene abundance may have been influenced by the general process of microbial community assembly and succession in the soil ecosystem.

Microbial biomass generally increases with time in soils (García-Orenes, Caravaca et al. 2015, Zhou, Wang et al. 2017). This pattern was observed in this study until AU2, where biomass decreased after peaking in SU2, possibly due to seasonal variation and a seasonal decrease in soil microbial biomass. This decrease was unexpected, considering the increased inputs of organic matter into the system in AU2, which has led to increased microbial biomass accumulation in other studies (Adrover, Farrus et al. 2012, Siggins, Burton et al. 2016). However, other studies have shown that microbial biomass can remain unchanged with wastewater irrigation (Li, Cao et al. 2019), particularly after a threshold of soil COD content has been reached (Martinez-Hernandez, Meffe et al. 2020). This suggested other fates in our system for organic matter besides microbial biomass accumulation, one of which may be accumulation in plants (Chapter 4).

Another unexpected trend was the abundance of 16S in unplanted reactors, which did not differ from planted and control reactors with a rhizosphere. Typically, the rhizosphere supports up to 100x more bacteria than the bulk soil (Sylvia, Hartel et al. 2005). Applying wastewater to the unplanted reactors may have provided a source of nutrients to support an increased population size.

In addition to succession, initial community assembly may have played a role in the observed patterns. All reactors were assembled from soil with the same properties and allowed to establish for the same amount of time and watered with tap water, even unplanted reactors without trees, for a year before treatment. This may have allowed the establishment of a stable initial community, including populations of denitrifying bacteria, and then maintained that population through the course of the experiment, instead adapting with shifts in gene expression to facilitate activity changes (Frenk, Dag et al. 2015). Future experiments could include quantifying RNA in the wastewater infiltration system to better understand the activity of *nirK* and other denitrification genes.

5.4.4. Macro-scale homogeneity in reactors limited depth effects

No impacts of depth were observed for any of the target genes, which contrasts with patterns observed in other studies. In the natural soil environment, conditions can differ drastically by depth (Singer and Munns 2002). Lack of depth effects may be attributed to homogeneity of the reactor environment. Differences in organic matter (Silveira, Filho et al. 2021) and oxygen levels (Chen, Jiang et al. 2021, Zhang, Cao et al. 2023) at different soil depths were important factors in influencing gene abundance in other studies. Previous work (Chapter 3) suggested that neither of these selection pressures were present in the reactors. There was no difference in organic matter by depth, and aerobic pockets were maintained in the soil, so oxygen limitation at lower depths was also not a selection pressure.

One explanation for potential homogeneity in the reactors compared to natural soils was the method of system construction. Reactors were assembled from homogeneous quarry soil, then isolated from the greater environment with no opportunity for nutrient exchange as would occur in a natural system. Though some compaction and shifts in soil moisture were observed (Chapter 3), generally the soil environment did not change with time. This may have resulted in the observed even distribution of functional genes in the soil.

Despite macro-scale homogeneity in reactors, it is important to consider micro-scale variation which may have contributed to masking large-scale trends. Microbial “hotspots” and “hot moments” are locations and timepoints, respectively, in the soil with temporarily increased activity relative to the surrounding environment (Kuzyakov and Blagodatskaya 2015).

In this system, hotspots could be caused by nutrient inputs from wastewater flowing into microaerobic or microanoxic pockets in the soil, where they experienced rapid degradation. Despite efforts capture community variation by sampling multiple points at multiple depths, and attempts to limit fluctuations by standardizing sampling procedures, the transient nature of microbial communities means that activity in specific hotspots may have been missed. These pockets of elevated denitrification activity could also play a role in the differences observed in planted and unplanted systems, as the unplanted reactors had higher bulk density (Chapter 3) and therefore fewer soil pores for development of such environments.

5.5. Conclusion

This study of nitrogen cycling in planted and unplanted WIS suggested that wastewater irrigation may influence nitrification gene abundance. Increased *amoA* abundance under wastewater irrigation suggested that nutrient inputs stimulated the abundance of the gene. Similarities in *nirK* abundance and activity between microbial communities in planted and unplanted soils suggested similar roles of the soil community in denitrification for both treatments. This in turn confirmed that another element, namely the poplar trees, was responsible for the previously recorded differences in nutrient removal performance. The study highlighted the importance of both soil microorganisms and vegetation in nitrogen cycling and removal in wastewater infiltration systems.

6. Microbial community dynamics in poplar tree wastewater infiltration system with recovery

Publication: This work is being prepared for publication as a manuscript in Environmental Engineering Science
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Abstract

Treatment with soil microorganisms in a wastewater infiltration system is a method for reducing nitrogen pollution in secondary effluent. To understand the nitrogen removal potential of the system, it is important to understand the microbial communities and the changes to community structure induced by wastewater. Wastewater infiltration systems were irrigated with synthetic wastewater or clean water and soil communities were characterized via 16S community sequencing. Sequencing was conducted using the Oxford Nanopore MinION third-generation system and data was analyzed using the Ribosomal Database Project classifier. Community composition and alpha diversity did not differ with treatment, suggesting limited impacts of wastewater on general community structure. However, statistical visualization revealed composition differences at finer phylogenetic resolution. Reactors without trees had significantly lower abundance of Acidobacteria and instead had increased abundances of Nitrospirae, and Firmicutes, both of which have potential roles in nitrogen cycling, and Gemmatimonadetes. These findings suggested that microbial community structure was robust to wastewater irrigation, with some specialization occurring in response to nutrient additions. Future work examining communities with long-term wastewater exposure may reveal differences in the community structure correlating with higher nutrient inputs.

Keywords: 16S, MinION, nanopore sequencing, nitrogen, wastewater infiltration system

6.1. Background

Soil microbial communities have long been recognized as essential components of treatment in wastewater infiltration systems (WIS) (EPA 1987). Microorganisms are major drivers of biogeochemical cycling in soils (Atlas and Bartha 1987) and are important for maintaining ecosystem function and performing ecosystem services (Wagg, Bender et al. 2014). Residual nutrients, such as nitrogen, associated with wastewater impact soil microbial communities (Guo, Qi et al. 2018, Zolti, Green et al. 2019) and the influence of wastewater exposure on soil community composition in the rhizosphere of hybrid poplar (*Populus*) is not fully understood.

Wastewater infiltration systems apply secondary or tertiary effluent to soils and allow the soil microbial community to provide natural wastewater treatment. Engineering controls, such as addition of vegetation (Khurelbaatar, Sullivan et al. 2017), system adaptation, and application above evapotranspiration rate, allow for enhanced secondary and tertiary wastewater treatment (Chapter 3).

The impact of wastewater on soil microbial communities has been studied in systems irrigating crops including lettuce, tomato, cucumber (Guo, Qi et al. 2018, Li, Cao et al. 2019, Zolti, Green et al. 2019, Obayomi, Edelstein et al. 2020, Moulia, Ait-Mouheb et al. 2023), and orchard crops (Frenk, Dag et al. 2015, García-Orenes, Caravaca et al. 2015). Impacts to microbial community structure and function depend on wastewater characteristics such as treatment stage and nutrient content (Moulia, Ait-Mouheb et al. 2023). Studies using secondary or tertiary effluent (i.e., tertiary or quaternary treatment) largely find no impacts on community structure and diversity (Ibekwe, Gonzalez-Rubio et al. 2018, Li, Cao et al. 2019, Obayomi, Edelstein et al. 2020). At higher nitrogen concentrations, above those found in primary effluent, decreases in community diversity have been documented (Wafula, White et al. 2015, Vallejos, Marcos et al. 2022). Microbial phyla that commonly experience shifts in abundance with wastewater irrigation include Protobacteria, Actinobacteria, Acidobacteria, and Bacteroidetes (Frenk, Hadar et al. 2014, Wafula, White et al. 2015, Vallejos, Marcos et al. 2022, Moulia, Ait-Mouheb et al. 2023).

Multiple technologies are available to evaluate microbial community structure using 16S rRNA sequencing. The most prevalent methods use sequencing by synthesis (termed second-generation sequencing) such as the Illumina technologies (Kargol, Cao et al. 2022). Third-generation sequencing using nanopore technologies has recently emerged and is employed by Oxford Nanopore Technologies (ONT) and Pacific Biosciences. Nanopore sequencing directly reads shifts in the electrical potential as a DNA strand moves through a nanopore; the shift is associated with the identity of nucleotides in the DNA strand. DNA molecules are attracted to nanopores by adaptors ligated to the strands during amplification (Jain, Olsen et al. 2016, Braley, Jewell et al. 2023). The raw signals are then interpreted by a base-calling software (Wick, Judd et al. 2019) and nucleotide sequences are recorded in fastq format for downstream analysis.

Nanopore sequencing accuracy has improved rapidly in the past decade. When the technology was first introduced in 2015, the accuracy was estimated at about 60% (Laver, Harrison et al. 2015), but it has now increased to 97% for general sequencing and >99% for certain applications (Kerkhof 2021). Early-release Kit 14 chemistry has even shown potential for accuracy >99% (Zhang, Li et al. 2023) making it a viable option for microbial community characterization. Advantages to nanopore sequencing include long read lengths up to full genome length (Chalupowicz, Dombrovsky et al. 2018, Braley, Jewell et al. 2023), low cost, rapid results (Ciuffreda, Rodriguez-Perez et al. 2021), and ability to sequence in remote locations (Burton, Stahl et al. 2020).

Applications of nanopore sequencing for 16S environmental microbial community analysis is an emerging field. Applications with single-organism detection are well-validated. For example, nanopore sequencing is frequently used in medical applications including clinical diagnostics (Kai, Matsuo et al. 2019, Sheka, Alabi et al. 2021) and tracking antibiotic resistant bacteria (Břinda, Callendrello et al. 2020, Wu, Che et al. 2022). In recent years, the technology has also been employed in a limited number of environmental studies, characterizing newly isolated organisms from contaminated environments (Vasconcelos, Andreote et al. 2022, Mahbub, Chenard et al. 2023) and for natural product discovery (Rajwani, Ohlemacher et al. 2021). The microbial communities of river water ecosystems (Reddington, Eccles et al. 2020), anaerobic digesters (Hardegen, Latorre-Perez et al. 2018), and thermal hot springs (Akaçin, Ersoy et al. 2023) have been studied with nanopore sequencing. The technology has been employed to detect pathogens in agricultural systems (Braley, Jewell et al. 2023, Theologidis, Karamitros et al. 2023) as well as beneficial organisms (Sarao, Boothe et al. 2024). Relevant to this study, Nanopore technology has been used to study microbial communities in soils (Li, Kong et al. 2022, Vasconcelos, Andreote et al. 2022, Braley, Jewell et al. 2023), the rhizosphere (Srivastava, Srivastava et al. 2020), and wastewater (Klair, Dobhal et al. 2023).

In this study, soil microbial community composition was examined in samples collected from outdoor experimental wastewater infiltration systems which were previously described for their wastewater treatment efficiency (Chapter 3). Our aim was to understand soil microbial communities in planted and unplanted wastewater infiltration systems irrigated with synthetic secondary effluent.

6.2. Methods

6.2.1. Study system and soil sample collection

Lab-scale reactors simulating a wastewater infiltration system with recovery were located at the University of Washington in Seattle, USA. The reactor construction and experimental design are described in Chapter 2, treatment capacity is described in Chapter 3, and impact on biomass production is discussed in Chapter 4. Briefly, metal troughs filled with 0.7 m of sandy loam soil were planted with hybrid poplar trees (3 years old at the start of the experimental period). Reactors had sampling ports at depths of 0.2 m and 0.4 m below the soil surface. Three planted reactors were drip irrigated with synthetic wastewater (Kargol, Burrell et al. 2023) and three received tap water. Three unplanted controls were also maintained and received wastewater. Synthetic wastewater was applied via surface drip irrigation for three weeks to allow the soil moisture to equilibrate and the microbial community to adjust. Reactors were operated for 18 months and samples were collected at three months (SU1), 15 months (SU2), and 18 months (AU2) of operation. Nutrient concentrations for the duration of the study are described in Table 11. Samples from SU1 were sequenced in this study.

Table 13: Nutrient concentrations and hydraulic loading for the study period

	Wastewater description	Influent (L/day)	Total nitrogen (mg/L-N)	NO_3-N (mg/L)	NH_4-N (mg/L)	COD (mg/L)
SU1	Secondary effluent	42.6 ± 1.0	4.8 ± 0.9	1.4 ± 0.2	1.2 ± 0.5	41 ± 5.0
SU2	Primary effluent	69.7 ± 0.3	32 ± 3.3	20 ± 1.1	9.8 ± 0.3	102 ± 41
AU2	Primary effluent with high COD	72.3 ± 2.6	34 ± 10	< 0.25	< 0.015	615 ± 120

6.2.2. DNA extraction

At the end of the three-week irrigation period, soil samples were collected, transported to the lab on dry ice, and stored at -80^{o}C for DNA extraction. DNA was extracted from soil samples using the Qiagen DNEasy Powermax Soil DNA extraction kit with 50 mL tubes (Qiagen Sciences, Germantown MD, USA) according to manufacturer protocol. Briefly, soil was added to a tube with beads and homogenized using a FastPrep-24 bead beater and lysis system (MP Biomedicals, Irvine, CA, USA) with BigPrep 50 mL tube adapter (Product number 116002525) for 20 seconds at 4.0m/s. DNA was precipitated, purified, and eluted in 5 μl of DI water. Extractions were conducted quantitatively by recording volume transferred at each relevant step. DNA was quantified using a Nanodrop One-C (Thermo Scientific, Waltham, MA, USA). A260/280 and A260/230 ratios were used to determine purity with regards to RNA and proteins.

6.2.3. 16S amplification and purification

Sequencing was conducted following the 16S Barcoding Protocol provided by Oxford Nanopore (http://nanopore.com/community). Library preparation was conducted using the MinION 16S Barcoding Kit which allows for the downstream multiplexing of multiple samples in a single run. The 16S Barcoding Kit 1-12 (SQT016) was used for SU1 samples. This kit was updated mid-2023 to the 16S Barcoding Kit 1-24 (SQT024), which was used for SU2 and AU2 samples. The kits were identical in chemistry and differed only in how barcodes were prepared (concentrated in 1 μl of buffer or dilute in 10 μl of buffer) and how many samples could be pooled (12 or 24).

Extracted DNA was diluted to 1 ng/μl. DNA was prepared as following for the 1-12 barcoding kit: 10 μl of prepared DNA was mixed with 25 μl of LongAmp Mastermix (New England Biolabs, Ipswich, Massachusetts, USA), 14 μl of water, and 1 μL of primer. For the 1-24

Barcoding Kit: 10 μl prepared DNA was mixed with 25 μl LongAmp Mastermix, 5 μl of water, and 10 μl of primer. Primers were modified 27F/1492R (Lane 1991), with barcodes added for multiplexing (Forward primer: 5' - ATC GCC TAC CGT GAC - barcode - AGA GTT TGA TCM TGG CTC AG - 3'; Reverse primer: 5' - ATC GCC TAC CGT GAC - barcode - CGG TTA CCT TGT TAC GAC TT - 3'). PCR amplification conditions were as follows: Initial denaturation at 95 ^{O}C for 1 minute, then 25 cycles of denaturation at 95 ^{O}C for 20 seconds, annealing at 55^{O}C for 30 seconds, and extension at 65 ^{O}C for 2 minutes, a final extension at 65 ^{O}C for 5 minutes and a hold at 4 ^{O}C.

Amplified DNA was purified using AMPure XP beads (Beckman Coulter, Brea, California, USA). PCR product was mixed with magnetic beads, incubated, and pelleted on a magnet. Pellet was washed twice with 70% ethanol and then resuspended in 10 mM Tris-HCl pH 8.0 with 50 mM NaCl (Fisher Scientific, Waltham, Massachusetts, USA). Beads were removed by pelleting on a magnet. Double-stranded PCR product was quantified using a Qbit fluorometer (Fisher Scientific).

6.2.4. MinION sequencing and data processing

DNA was sequenced using the Oxford Nanopore MinION device with R9.4.1 flow cell chemistry (Oxford Nanopore Technologies, Oxford, UK). DNA was pooled in equal ratios for a total of 100 ng per run. Flow cell was prepared using the Flow Cell Priming protocol (http://nanopore.com/community); priming mix was loaded onto the flow cell via the Priming Port, then the library was prepared by mixing pooled DNA with 34 μl sequence tether, 25.5 μl sequencing beads (vortexed immediately before adding), 11 μl of pooled library, and 4.5 μl water. The mixture was loaded dropwise onto the flow cell via the Sample Port.

Sequencing was conducted using the MinKNOW desktop software version 22.10.10 (SU1) or version 23.11.4 (SU2 and AU2). Runtime was set to 48 hours with guppy real-time base-calling enabled. Barcoding was enabled to automatically split reads by sample.

Raw fastq files were first preprocessed using chopper (De Coster, D'Hert et al. 2018) to trim low-quality bases. Reads were then classified via the ribosomal database project (RDP) classifier (Wang, Garrity et al. 2007), which utilizes a probabilistic model to assign taxonomy to sequences. A cutoff score of 0.85 confidence was used.

6.2.5. Statistical analysis

Species richness was calculated by summing total ASVs in each sample and Shannon index were calculated in RStudio (RStudio Team 2020). Analysis of variance was used to test for differences between community diversity statistics based on treatment. Differential abundance of microbial groups was assessed with one-way ANOVA. Principal component analysis (PCA) was conducted in RStudio using the vegan package (Oksanen, Blanchet et al. 2013). The arguments coordinates and rotation were extracted to identify ASVs that contributed most strongly to the principal coordinates.

6.3. Results

6.3.1. Sequencing statistics

The number of reads per sample ranged from 158,522 to 756,814. Variation in reads was distributed across treatment, suggesting that treatment did not impact efficiency of DNA sequencing. Using a cutoff score of 0.85, 932 amplicon sequence variants (ASVs) were obtained from the RDP classifier.

Communities were dominated by 9 phyla which made up 86.2- 93.6% of the community in all reactors. In the ANOVA tests, only the abundance of Chloroflexi differed by treatment (p =

0.04). Notably, a large proportion of the microbial community in the samples was uncharacterized, meaning the sequences have never been submitted to the RDP database. Between 5.6% and 9.3% of reads in each sample were classified only to the domain level. Another portion of the community was classified to the phylum level only, suggesting additional unexplored diversity in the reactors within dominant groups. The percentage of sequences classified only to the phylum level varied across phyla. Proteobacteria were unclassified at rates from 1.5%-2.4%. Firmicutes were similarly well-classified. On the other hand, both Acidobacteria and Actinobacteria had between 5.5% and 6.5% of sequences unclassified.

6.3.2. Community diversity

Community diversity statistics are shown in Table 12. Species richness ranged from 507 to 723 ASVs. Planted reactors had the highest richness, while controls had highest evenness. A one-way ANOVA revealed no differences in either metric. Shannon diversity was lower in unplanted reactors than reactors with trees, but the difference was also not statistically significant (p = 0.07).

Table 14: Community diversity statistics

	No. reads	Richness	Shannon	Evenness	% unclassified
Planted	392,900 ± 108,000	664 ± 32	4.1 ± 0.2	0.63 ± 0.01	7.6 ± 1.4
Unplanted	623,700 ± 121,600	629 ± 64	3.8 ± 0.1	0.60 ± 0.02	6.9 ± 0.8
Control	579,100 ± 94,400	603 ± 46	4.2 ± 0.1	0.65 ± 0.01	8.5 ± 1.1

Principal coordinate analysis (Figure 11) showed clustering of unplanted communities, which were separated from communities with trees along PC1. Control and planted reactors were distributed along PC1 and were difficult to resolve from each other. PC2 mostly separated control reactors and somewhat separated planted communities as well. Most of the top ASVs contributing to PC2 were Firmicutes present at low abundance (.001%-.01%).

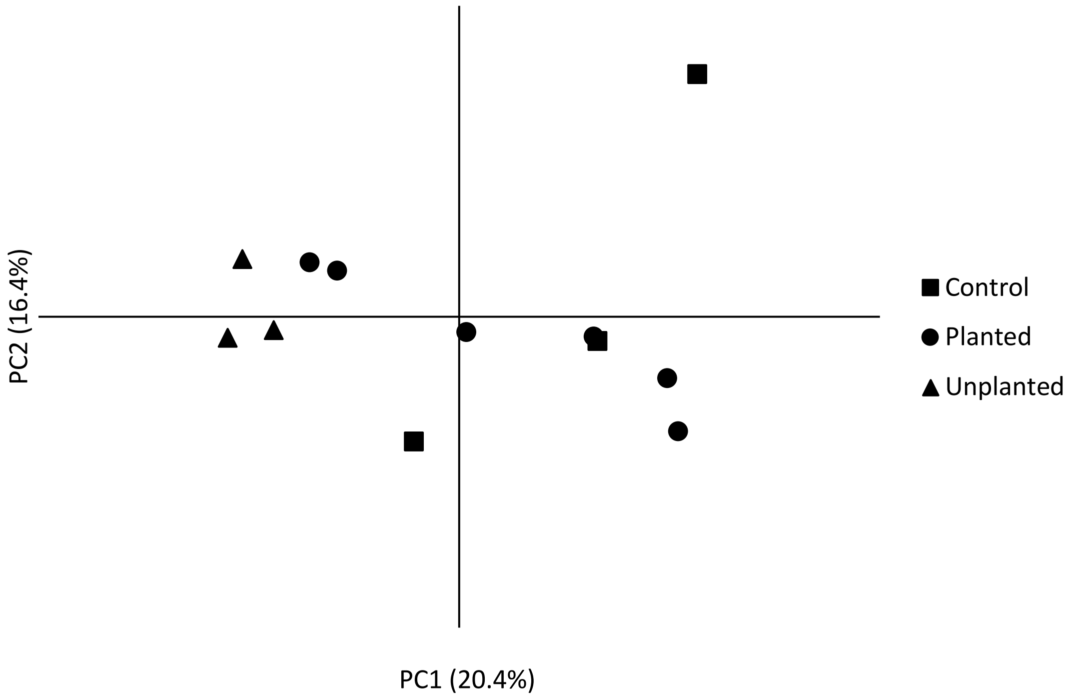
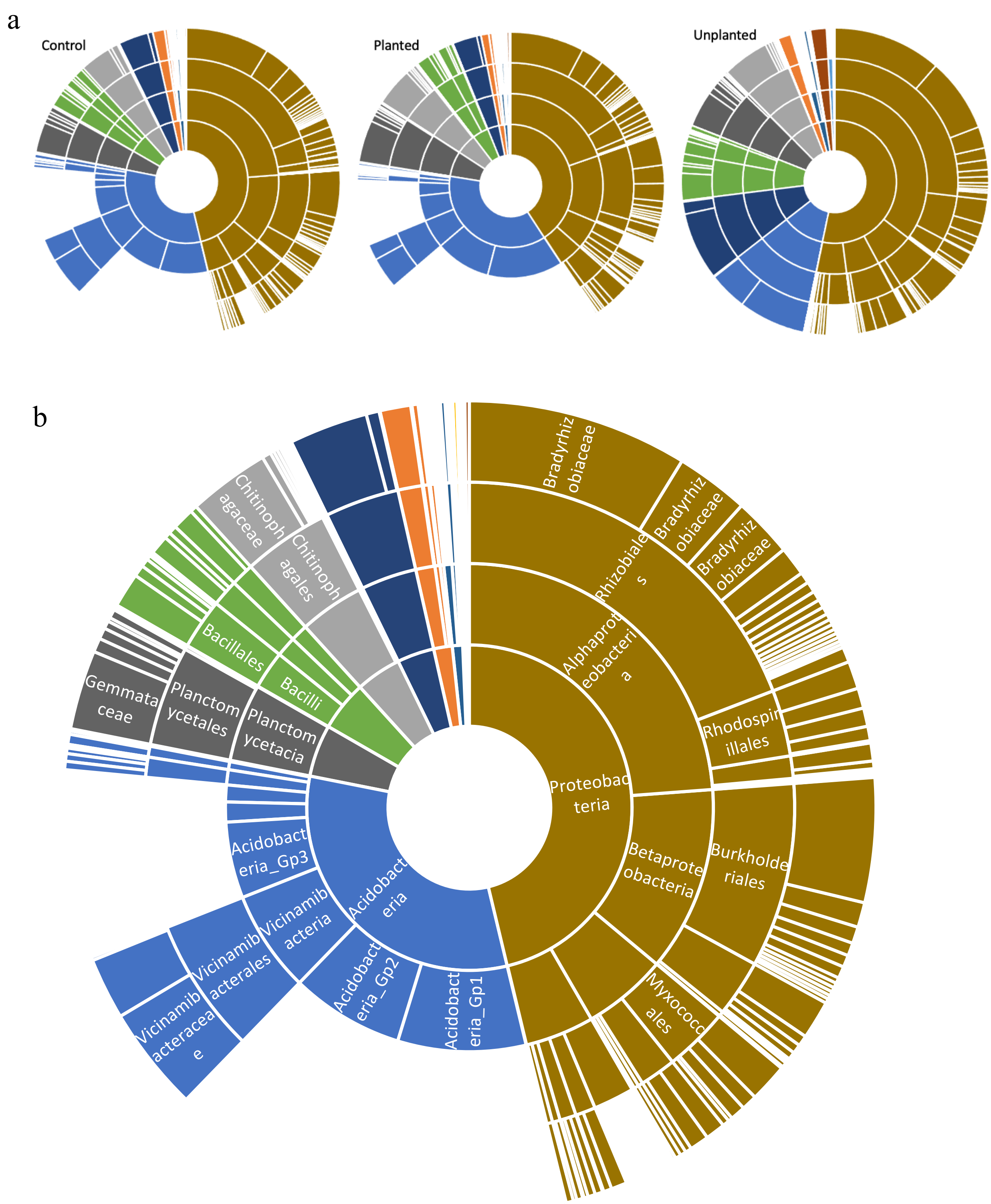


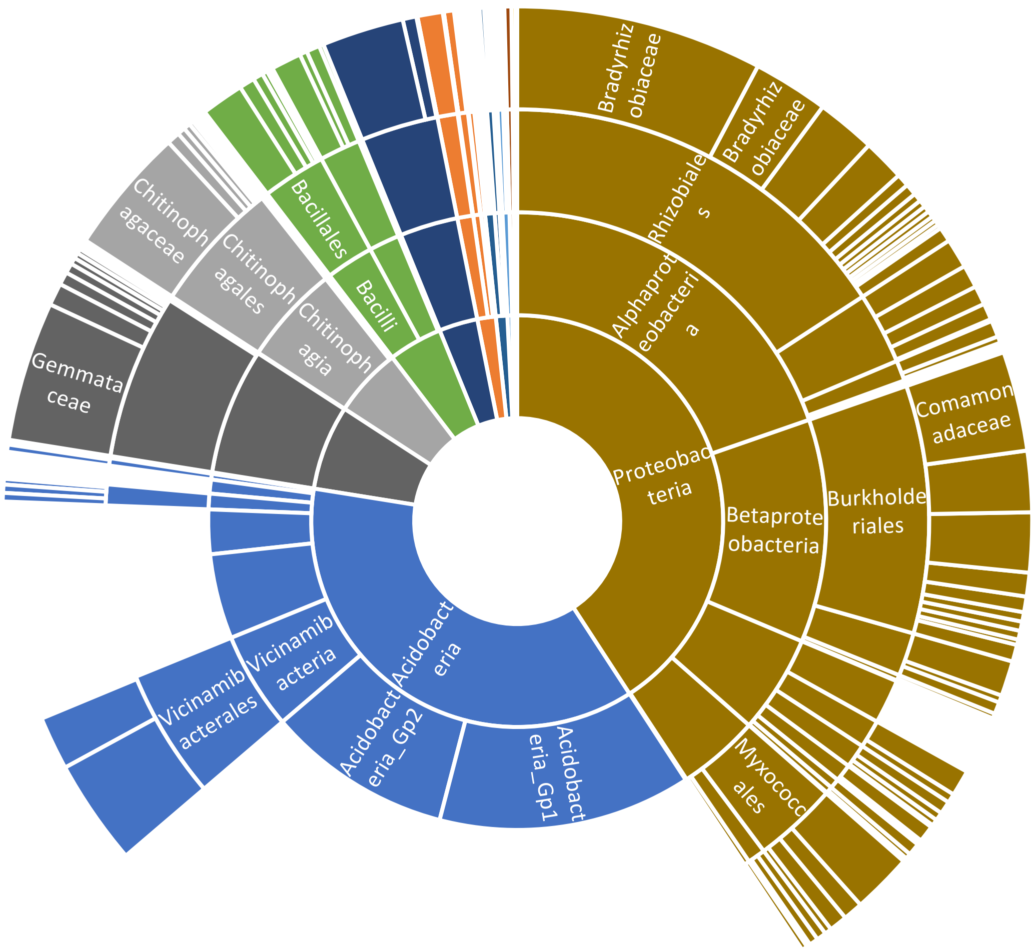
Figure 11: Principal component analysis of soil bacterial communities from planted and unplanted reactors and clean water controls.

Sunburst plots showing ASVs from dominant phyla that were present at 0.01% or greater in the communities demonstrated differences in community composition between treatments (Figure 12). Control and planted reactors showed clear differences from unplanted reactors. Communities with trees had higher abundance of Acidobacteria, a large portion of which was unclassified beyond the phylum level. Unplanted reactors had more Nitrospirae, Gemmatimonadetes, and Firmicutes than planted counterparts.



a
Control
Planted
Unplanted
b
Bradyrhiz obiaceae
Bradyrhiz obiaceae
Bradyrhiz obiaceae
Rhizobiale s
Alphaprot eobacteri a
Rhodospir illales
Proteobac teria
Betaprote obacteria
Burkholde riales
Myxococc ales
Chitinoph agaceae
Chitinoph agales
Bacillales
Bacilli
Gemmata ceae
Planctom ycetales
Planctom ycetacia
Acidobact eria_Gp3
Acidobact eria
Vicinamib acteria
Vicinamib acterales
Vicinamib acteracea e
Acidobact eria_Gp2
Acidobact eria_Gp1

c



Bradyrhiz obiaceae
Bradyrhiz obiaceae
Rhizobiale s
Alphaprot eobacteri a
Proteobac teria
Comamon adaceae
Burkholde riales
Betaprote obacteria
Myxococc ales
Acidobact eria_Gp1
Acidobact eria_Gp2
Acidobact eria
Vicinamib acteria
Vicinamib acterales
Gemmata ceae
Chitinoph agaceae
Chitinoph agales
Chitinoph agia
Bacillales
Bacilli

d

Figure 12: Sunburst plots comparing microbial community composition between treatments. Brown – Proteobacteria, light blue – Acidobacteria, dark gray – Planctomycetes, green – Firmicutes, light gray – Bacteroidetes, dark blue – Gemmatimonadetes, orange – Actinobacteria, red – Nitrospirae; a) Side-by-side comparison of reactor composition; b) Control; c) Planted; d) Unplanted

6.4. Discussion

Phyla that differed with treatment included Acidobacteria, Nitrospirae, Gemmatimonadetes, Firmicutes, and Chloroflexi. Differences were largely between reactors with and without trees and were observed at finer taxonomic resolution (order and family level). This was also reflected in the clustering of unplanted communities separately from those with trees in the PCA.

Due to the design of the wastewater infiltration reactors, the microbial communities in this study were isolated from the environment. The only sources of microbial populations into the system were the low-organic matter quarry soil and the synthetic wastewater. Reactors with trees were also seeded by the rhizosphere, which was originally established in nutrient-rich potting soil as the poplar cuttings rooted. Community similarity may partially be explained by these limited inoculum sources. For example, the Acidobacteria shared by all treatments may have been seeded from the quarry soil, while the additional diversity observed in planted and control reactors came from the potting soil. A full-scale wastewater infiltration system would have opportunities for nutrient and microorganism exchange with the environment, leading to the establishment of more diverse communities.

One of the major goals of this study was to understand nitrogen cycling patterns observed in the system on a macro scale (Chapter 3, Chapter 4). The phylum Nitrospirae has a role in the nitrogen cycle as a nitrite oxidizer (Madigan and Martinko 2006). This phylum, and specifically the genus *Nitrospira*, was much more prevalent in unplanted reactors compared to planted, suggesting a key role in bare soils. Abundance was much lower in reactors with trees, suggesting that different organisms, possibly associated with trees, carried out nitrite oxidation. There was no obvious difference in abundance of common denitrifying bacteria, despite observed differences in effluent nitrate (Chapter 3). This agrees with results from gene studies (Chapter 5) showing no difference in the abundance of a key denitrification gene, *nirK,* by treatment. It points to the presence of trees as being responsible for observed trend of nitrogen removal, with accumulation observed in unplanted reactors because denitrification organisms in the soil could not fully match the activity of the trees.

Abundance of Firmicutes was also elevated in bare soils. Several members of the phylum Firmicutes are capable of nitrogen fixation and may have been the main drivers of nitrogen inputs into unplanted reactors aside from the wastewater. Conversely, in planted reactors nitrogen fixation may have been driven by endophytes associated with the poplar trees (Doty, Sher et al. 2016). The relatively higher Firmicutes population in bare-soil reactors may partially explain an unexpected trend observed in treatment performance. In unplanted reactors in SU1, the season in which these communities were sampled, effluent nitrogen concentration was higher than influent concentration (Chapter 3, Figure 5). This suggested the potential for free-living nitrogen fixation (FLNF) in the reactors. The role of FLNF in the global nitrogen cycle is not fully understood, but it is thought to be responsible for 1/3 of all nitrogen inputs into terrestrial ecosystems (Davies-Barnard and Friedlingstein 2020). The potential mechanism of wastewater exposure for triggering increased FLNF is an area for future study.

Lower Acidobacteria abundance was observed in unplanted reactors, which agrees with one study that found more Acidobacteria in forest soils than in pasture soils (Navarrete, Venturini et al. 2015). Other studies reported Acidobacteria shifts based on wastewater irrigation (Guo, Qi et al. 2018, Hernandez-Guzman, Perez-Hernandez et al. 2022, Kargol, Cao et al. 2022). The Acidobacteria are a highly diverse group of soil and marine organisms with a variety of metabolic roles. One hypothesized role is in degradation of cellulose and other complex polymers (Madigan and Martinko 2006), which may be more abundant in reactors with trees compared to those without.

The function of Gemmatimonadetes, which was more abundant in unplanted reactors, is not well understood despite their prevalence in soils (Mujakic, Piwosz et al. 2022). In the context of the nitrogen cycle, several members possess the genes for N_2O reduction (Park, Kim et al. 2017).

The only phylum significantly impacted by wastewater irrigation was Chloroflexi. Abundance was different in controls relative to planted and unplanted reactors. Chloroflexi exhibit an oligotrophic metabolism (Fu, Yan et al. 2022), and thus the addition of nutrients in wastewater may have selected against this group.

6.5. Conclusion

In this study, microbial communities from wastewater infiltration systems with and without trees were characterized. Principal component analysis suggested differences between planted and unplanted communities that were not present with controls, suggesting a key role of trees in influencing nitrogen removal from the influent. Visualization revealed fine-scale compositional differences between communities with and without trees, which may have been related to nitrogen cycling dynamics. Increased understanding of the microbial community impacts of wastewater can help us plan, design and predict performance of wastewater infiltration systems incorporating bioenergy and water recovery.

7. Conclusions and future directions

This work demonstrated that wastewater infiltration systems for bioenergy and recovery (WISER) was an effective combined method for wastewater treatment and biomass production. The system removed nitrogen pollutants through microbial- and vegetation-mediated mechanisms and utilized the nitrogen to facilitate increased biomass production. The combined treatment strategy showed promise for treating high volumes of wastewater with limited field space, becoming economically appealing when water recovery was incorporated into the treatment process. WISER successfully treated secondary, then primary wastewater effluent, removing nitrate to below discharge limits even during the first rotation. Unplanted reactor performance was less successful and left residual nitrogen above discharge limits in higher-nutrient treatment, highlighting the importance of the vegetation.

Performance findings correlated with genetic findings, with both nitrification and denitrification pathways impacted by wastewater exposure to different degrees. Community composition at the phylum level was largely unaltered by wastewater but differences in abundance of two key microbial groups supported differences in nitrogen cycling pathways between reactors with and without a rhizosphere. Control and treated planted reactors had similar composition, suggesting that trees may serve as a buffer against changes induced by wastewater exposure. Future work including bare-soil controls irrigated with tap water could help resolve which differences in the community were induced by wastewater and which were related to vegetation. By directly pairing performance and microbial community studies, we definitively linked observed nitrogen removal performance with genetic and microbial characteristics.

In the context of the bioeconomy, the clear differences in treatment performance may promote the planting of trees on land treatment sites over bare-soil infiltration systems. Financial returns on biomass crops in WISER are limited, but for treatment facilities establishing new tertiary treatment to meet stricter discharge standards, wastewater infiltration systems are among the cheaper options (Tchobanoglous, Stensel et al. 2014). Financial incentives for ecosystem service provision could serve as another mechanism for increasing WISER implementation.

Future work with wastewater infiltration systems as tertiary treatment will involve investigations at the macro scale and the microbial community scale. One important step is understanding the fate of emerging wastewater contaminants, such as pharmaceuticals and personal care products, and their potential impacts on microbial community structure. Some research suggests the possibility of buildup of these compounds in the soil environment (Chefetz, Mualem et al. 2008). Others have observed the potential for transformation of some contaminants in planted systems (Kargol, Cao et al. 2022). The interplay of factors including soil and vegetation properties and local conditions will result in unique responses for each system.

Further study of functional genes in poplar treatment systems would help us better understand nitrogen cycling patterns. This could include quantifying nitrogen fixation genes for both poplar endophytes and free-living nitrogen fixers, which may provide insight into the potential upregulation of nitrogen fixation in unplanted reactors. Another option would be to explore nitrogen cycling gene regulation at the level of RNA instead of DNA. This would provide additional insight into the active fraction of the community, which other work suggests may change under wastewater irrigation even if composition does not (Frenk, Dag et al. 2015).

Evidence suggests that microbial communities fluctuate with time and in response to wastewater. Samples from the system at 15 months and 18 months of operation are available for 16S community sequencing. Study of additional seasons will provide information on long-term responses and adaptation of the soil microbial community and may reveal additional differences between planted and unplanted reactors.

Wastewater infiltration systems are established based on regional and industry knowledge. This work added to the regional knowledge of land treatment in the Pacific Northwest and the industry knowledge of specific nitrogen cycling trends. Increasing the use of wastewater infiltration as a tertiary treatment and biomass production method will allow us to meet growing water demands without threatening water quality or quantity for future generations.

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9. Appendices

Appendix A: Synthetic wastewater prepared from readily available materials: characteristics and economics

Kargol AK, Burrell SR, Chakraborty I, Gough HL. Synthetic wastewater prepared from readily available materials: Characteristics and economics. PLOS Water. 2023; 2 (9). 10.1371/journal.pwat.0000178

Abstract

The wastewater used for experimental research is typically collected from a wastewater treatment plant or prepared as a synthetic solution in the lab. These options represent transportation and cost challenges, respectively, particularly for experiments requiring large volumes of wastewater. Here, we describe a method for creating inexpensive synthetic wastewater from readily available household products. The base solution, synthesized by soaking dog food pellets for 24 hours and straining the solution, had average nutrient values of 9.7 mg/L ammonia as N, 12.2 mg/L nitrate as N, 227 mg/L total nitrogen, and 4870 mg/L chemical oxygen demand (COD). Degradation tests demonstrated that soluble COD was biodegradable. The base solution was then used to prepare synthetic wastewater that met the requirements for two experimental applications: (1) anaerobic treatment of primary effluent and (2) land-application treatment of secondary effluent. Cost analysis indicated that the single-ingredient synthetic wastewater cost 92% less to produce than synthetic wastewater recipes that used laboratory chemicals, and reduced preparation time. These results demonstrated that use of commercial products can simplify the wastewater synthesis process and reduce experimental costs for large-volume research applications while still maintaining consistent wastewater characterization.

Keywords: synthetic wastewater, pilot scale, cost analysis, chemical oxygen demand, nitrogen

9.1. Introduction

Synthetic wastewater (SWW) has long been used in wastewater research (Cokgor 1998). It is used when a predictable wastewater composition is required (VanderGheynst, Gossett et al. 1997, de Kreuk, Heijnen et al. 2005, Wu, Fan et al. 2015). SWW is also used when access to a wastewater treatment facility is limited (O'Flaherty and Gray 2013) or year-round access and transport are not realistic (Prieto, Criddle et al. 2019). Most SWW recipes include many chemical components and can be time-consuming and costly to prepare (Boeije, Corstanje et al. 1999, Khurelbaatar, Sullivan et al. 2017). Recipes commonly include peptone, meat and yeast extracts, cellulose, casamino acids, urea, and various trace elements to simulate the complex mixture of carbon and nutrients found in real wastewater (OECD 2001, Prieto, Criddle et al. 2019). For studies that require large volumes of wastewater (Guidi Nissim, Jerbi et al. 2015, Khurelbaatar, Sullivan et al. 2017, Martinez-Hernandez, Leal et al. 2018, Amiot, Jerbi et al. 2020), producing a steady supply of synthetic wastewater could become a challenge.

Published SWW recipes vary in their characteristics and limitations. For example, the Organization for Economic Co-operation and Development (OECD) (OECD 2001) has published a standard method for preparation of SWW that includes peptone, meat extract, urea, and trace elements (OECD 2001), which they recognize to contain higher nitrogen, and lower carbon (as chemical oxygen demand, COD) content than typical wastewater. The recipe for another SWW (“SYNTHO”) uses similar components but in different proportions, resulting in a COD to total N ratio of 7.3:1 (Boeije, Corstanje et al. 1999). A review by Prieto et al. (Prieto, Criddle et al. 2019) of 24 other SWW recipes found that most are either based on one of the recipes above or are unique to individual studies. These recipes are generally prepared with a large number of ingredients to create well-defined synthetic wastewater. The use of unique recipes or significant modification of existing formulas suggests that the standard recipes are not able to meet the experimental needs for all wastewater studies.

Preparation cost is also a barrier, which is stated in the ASTM SWW recipe protocol (ASTM 2018). Some recipes replace laboratory chemicals with less expensive commercial products such as whey, milk powder, soybean oil (de Sousa and Foresti 1996, Prieto, Criddle et al. 2019) or molasses (Duran and Speece 1999). Others have utilized waste products, including watermelon peels (Hasanin and Hashem 2020), cheese whey water, and sweet potato extract (Ibrahim 2021) to reduce costs. Additionally, when organic material is added to synthetic wastewater as simple carbon sources such as glucose, sucrose, or acetate (O'Flaherty and Gray 2013), it does not replicate the complex mixture of compounds in real wastewater. Components such as trace organics and metals may also be omitted (Krismastuti and Hamim 2019). In all cases, the SWW requires multiple chemical components, which additionally introduces a preparation time barrier.

In this paper, we describe the development of a SWW recipe using dry dog food as the base ingredient. Commercially available dog food has previously been explored as a way to simulate biosolids (Duran and Speece 1999) and food waste (VanderGheynst, Gossett et al. 1997), and to increase particulate concentration in colloidal wastewater (Langenhoff, Intrachandra et al. 2000), but has not been used as the main carbon and nitrogen source in a synthetic wastewater. The formulation of dog food combining protein, fats, and simple carbohydrates makes this an ideal candidate for SWW. Using the dog food SWW (DSW) base, we evaluated the addition of supplemental household and laboratory chemicals to achieve the characteristics needed to satisfy different experimental requirements. Cost analysis was conducted to compare the new DSW formula with the OECD standard method for synthetic wastewater preparation. This simplified synthetic wastewater recipe offers a novel approach for cost and preparation time challenges that will advance research capabilities for large volume bioreactor testing and for easier transportation to remote field-scale biological treatment testing sites.

9.1. Methods

Selection of dog food brand

Four brands of dry dog food were compared. The formulations tested were Pedigree Small Dog Roasted Chicken, Rice, and Vegetable Flavor (Pedigree), Rachel Ray Super Medleys Superfoods and Beef Recipe (Rachel Ray), Nature's Recipe Prime Blends Salmon, Barley, and Chicken recipe (Nature's Recipe), and Blue Wilderness Adult Small Breed Mix with Chicken (Blue Wilderness). Brands were selected based on price, nutritional information on the packaging for protein and fat composition (Table A1), and their routine availability within the United States. Costs for each brand were obtained in June 2023 from chewy.com. Tests were conducted within four weeks of purchase date and within two weeks after opening the package.

Wastewater characterization

The following methods were used to determine the characteristics of SWW. pH was measured with an Orion Dual Star pH/ISE meter (Thermo Scientific, Waltham, USA). Total nitrogen was measured using Total Nitrogen Acid and Hydroxide (Hach Company Reagent sets 2672145 and 2714045, Loveland, USA) reagents or Hach Test-n-Tube (TNT) Plus Total Nitrogen kits (Method: TNT 826). Ammonia-N was measured using the Hach AmVer High Range Ammonia (Reagent set 2606945) and TNT Plus Ammonia (Method 830) kits. Nitrate-N was quantified using Hach NitraVer X Test-N-Tube kits (Reagent set 2605345). Chemical oxygen demand (COD) was measured using Hach high-range and low-range COD digestion vials (Hach product numbers 2125915 and 2125815, respectively). Total suspended solids (TSS) and volatile suspended solids (VSS) were determined using Standard Method 2540D/E (AWWA 1998) using a 0.45 μm glass fiber filter (VWR International, Radnor, USA).

Synthetic wastewater preparation

Fig 1 illustrates the steps used to develop the preparation protocol. Deionized (DI) water was obtained from a Millipore System (MilliPak 0.22μm filter). Pedigree dog food was added to DI water at 60 g/L dry and either autoclaved at 121^{o}C for 20 minutes or incubated at room temperature for 24 or 72 hours.

The impact of filtering on nutrient concentration was also tested. Dog food was soaked for 24 hours and filtered through 0.2 μm cellulose acetate filters (VWR International, Radnor, USA) to remove particulate matter or strained (~2mm mesh bag) to remove only large particles. Filtered and strained wastewater were tested for ammonia, nitrate, and total nitrogen.

For comparison of dog food brands, the following preparation was used: 60 g/L of dog food in DI water, incubated at room temperature for 24 hours. Liquid was strained and spent pellets were discarded. For final characterization of the DSW base solution, Pedigree brand dog food was used and 3 replicate batches were compared.

Dog food synthetic wastewater biodegradability

To test the biodegradability of DSW, COD was measured in batch inoculations over time following OECD standard method #307 (OECD 2002). Briefly, 25g dry equivalent of soil from a wastewater discharge infiltration gallery was used as a microbial inoculum. The inoculum was suspended in 200 mL of DSW base solution. The solutions were incubated at room temperature and aerated with filtered ambient air (Rezist 0.2μm PTFE filter). Samples were collected over 48 hours, filtered through 0.2 μm cellulose acetate filters, and tested for soluble COD. A sample of the initial feed was also filtered.

Preparation of DSW base for application

DSW base was used to prepare SWW with consistent characteristics of (1) synthetic primary effluent (DSPE), and (2) synthetic secondary effluent (DSSE), each for a different laboratory study.

The DSPE was prepared for use in rapid-upflow anaerobic reactors (rapid UASB) designed to simulate an operation being tested by partners in another region. The DSW base was diluted 1:10 to reach the targeted influent COD concentration. The base was supplemented with whey powder (Nature's Best Isopure whey protein isolate, unflavored, 3 g/L) to decrease the C:N ratio and food-grade baking soda ($NaHCO_3$, 5 g/L) to increase alkalinity to match the characteristics measured by the collaborative partner.

For DSSE, the base DSW was supplemented with NH_4Cl and $NaNO_3$ in lab to introduce the non-organic forms of nitrogen typical for secondary effluent. The supplemented DSW base was transported to a field site for experimental use in vegetated infiltration galleries. On site, the DSW was diluted 1:100 using municipal tap water. Following on-site dilution, the DSSE was characterized for ammonia, nitrate, total nitrogen, and COD. To test reproducibility, nine preparations were compared over three months.

Cost analysis

The cost to synthesize the DSW base was compared to the price of preparing SWW following the OECD protocol (OECD 2001). Costs were normalized to the COD concentration. Chemical costs were obtained from the Fisher Scientific on-line catalog, using the least expensive formulation of each product. COD of the OECD SWW was calculated based on COD values of individual ingredients (peptone, meat extract, and urea).

9.2. Results and discussion

Comparison of preparation methods

Figure A1 illustrates the decision tree used to compare the preparation methods. In the autoclaved preparation, dog food particulates dissolved into the solution, which was bright orange in color. Total nitrogen was 3150 mg/l, likely due to the complete dissolution of the particulates and release of large quantities of organic nitrogen into the solution. The dissolved dog food made the SWW difficult to work with, and particulates could not be removed. Autoclaving was eliminated as an option because the solids content in the SWW could not be used for pump-fed reactors, as it would clog the tubing. The DSW soaked for 72 hours had a strong odor and was eliminated from consideration.

Pellets soaked for 24 hours absorbed significant water volume and released particulate matter into the solution. Water volume decreased by approximately 10 percent, so to recover 1L of solution, 1.1 L should be prepared. Ammonia concentration in the filtered 24-hour batch was too low for wastewater applications (<0.015 mg/L); filtering large volumes was also considered impractical for producing large volumes of DSW. Therefore, filtration was eliminated. When strained to remove large food pieces, the final SWW had an ammonia concentration of 9.7 mg/L, which falls within the typical secondary effluent range of 0.1-10 mg/l (Tchobanoglous, Stensel et al. 2014). The finalized approach selected for DSW preparation was soaking for 24 hours and straining through a mesh bag. The prepared DSW was frozen within a day of preparation for storage until use.

Protocol for DSW preparation

The following protocol can be used to prepare DSW:

1. Weigh out 60g of dog food (opened within one month)
2. Add dog food to 2L container
3. Add 1L of water and swirl container several times
4. Soak pellets at room temperature overnight or for at least 12 hours
5. Strain pellets out of water by pouring liquid into secondary container through mesh bag
6. Discard dog food pellets
7. Use wastewater within 1 day or store at -20 ^{O}C for up to one month

Wastewater characteristics

To evaluate if manufacturer-reported nutrient content would influence the final DSW characteristics, four brands were compared. Table A1 shows the manufacturer-reported nutrient content and the resulting DSW characteristics. pH was consistent among brands while total nitrogen varied by 20%. Ammonia and nitrate content had the highest variability among the measured parameters and were not predicted by the manufacturer protein content; Pedigree and Rachel Ray had higher nutrient values. Three brands had similar COD values while Nature's Recipe was 2,000 mg/L higher than the others. Three brands had no more than a 35 mg/L difference between TSS and VSS, while Rachel Ray had a nearly 500 mg/L difference.

Pedigree and Rachel Ray were found to produce DSW with typical wastewater characteristics, although both fall near the lower end of the range for ammonia and nitrate concentrations. Pedigree brand was chosen for use in the synthetic wastewater recipe. The results suggested that a variety of dog food brands could be suitable for DSW synthesis. Suitability should be confirmed by testing key characteristics.

Dry dog food stored longer than 12 weeks produced wastewater with reduced carbon content, with COD decreasing by 67%. The food was stored in a covered but not airtight container. This storage method may have caused moisture losses over time, compacting particle structure and decreasing the available surface area for nutrient release. Therefore, it is recommended to purchase dog food in smaller bags to reduce moisture loss over time. Storage in an airtight container was not tested but may increase the stability.

Comparison to other wastewater recipes

Table A3 shows the characteristics of DSW and two other synthetic wastewater recipes. Concentrated DSW had a COD content about 10 times higher than most synthetic wastewater recipes (Prieto, Criddle et al. 2019), 6 times higher than SYNTHO (Aiyuk and Verstraete 2004) and 8 times higher than OECD SWW (OECD 2001). DSW was advantageous over the other recipes because the dog food base added complex COD, nitrogen, and trace elements to the SWW in a single step. Total nitrogen in other wastewater recipes ranged from 5.2 mg/l to 165 mg/l with supplementation, while the DSW base reached the higher end of that range without the addition of supplemental nitrogen. The testing method used in this study measures free ammonia. Thus, it is likely that most nitrogen in the dog food exists as organo-complexes (e.g., proteins).

Biodegradability of DSW

Figure A2 shows the decrease in COD over time. Over two days, COD decreased on average by 69% ± 7.2%. This showed that the carbon in the SWW was readily available for biodegradation by microorganisms. Therefore, it can be used as feed for wastewater bioreactors to provide an organic carbon and nitrogen source.

Preparation of SWW for experimental application

DSW prepared from Pedigree brand was used as the base ingredient for preparation of synthetic wastewater for two different research needs, one requiring primary effluent and one requiring secondary effluent. For each, we demonstrated how addition of small amounts of supplemented materials were used to match the COD, nutrient, and alkalinity experimental needs.

DSSE was analyzed 9 times over 3 months of experiments in a planted infiltration gallery (Table AS1). Average COD content aligned with typical values for secondary effluent (Tchobanoglous, Stensel et al. 2014). Total nitrogen and ammonia were similar to typical secondary effluent, while nitrate was slightly below typical. When supplemented with nitrogen sources, nitrate-N and ammonia-N concentrations increased to 1.3 mg/L and 1.2 mg/L, respectively. Consistency was observed over 9 separate preparations, which suggested that, once methods for synthesis and baseline nutrient concentrations have been established, consistent concentrations can be expected. Thus, the recipe can be used long-term with only occasional quality control testing needed, which simplifies its use at field sites.

These two examples show how the base recipe is readily modifiable through addition of other simple products (whey powder, baking soda) or small masses of lab chemicals (nitrate). This allows the recipe to be adapted to match the characteristics of primary or secondary wastewater from both strong and dilute sources.

Cost analysis for DSW preparation

The costs to prepare DSW and OECD wastewater were compared in Table A4. Prices were obtained in June 2023 and reflect the costs to obtain materials at the study location. Costs might vary in other locations or by other factors such as institutional discount pricing. Costs were normalized to COD concentration. OECD wastewater was calculated to have a COD of 555 mg/L (Table AS2); thus, 10.7 L of OECD SWW was needed to achieve the COD mass equivalent of 1L of DSW. For the same COD equivalent, DSW can be synthesized for 8% of the cost of the OECD SWW. For an experiment requiring 20L of SWW base per week, this would result in a monthly cost of $10.40 compared to $126.40 for the OECD recipe. Note that this cost savings does not include the additional savings associated with reduced person work hours required to prepare a single-ingredient synthetic wastewater.

9.3. Conclusion

We have demonstrated the preparation of a single-ingredient synthetic wastewater starting with commercial dog food. The resulting COD and nitrogen content were similar to wastewater and (with dilution) treated wastewater effluent. Straining enabled removal of large particles that might clog tubing used with lab- or pilot-scale reactors. Cost comparisons showed that the DSW cost was 8% of the multi-component synthetic wastewater recipe published by OECD. With minor supplementation, the DSW was adapted to meet study needs for two different research applications. The difference in nutrient content among brands highlighted the importance of characterizing DSW before use. This approach can be expanded to add other nitrogen components or targeted compounds such as emerging contaminant chemicals to the DSW. Having cost effective means to produce large volumes of synthetic wastewater with reliable characteristics advances the ability to conduct reproducible research when access to materials from a wastewater treatment facility is not feasible. This is important as climate change and other factors require the development of creative new wastewater treatment solutions, all of which must be scaled up and tested before implementation.

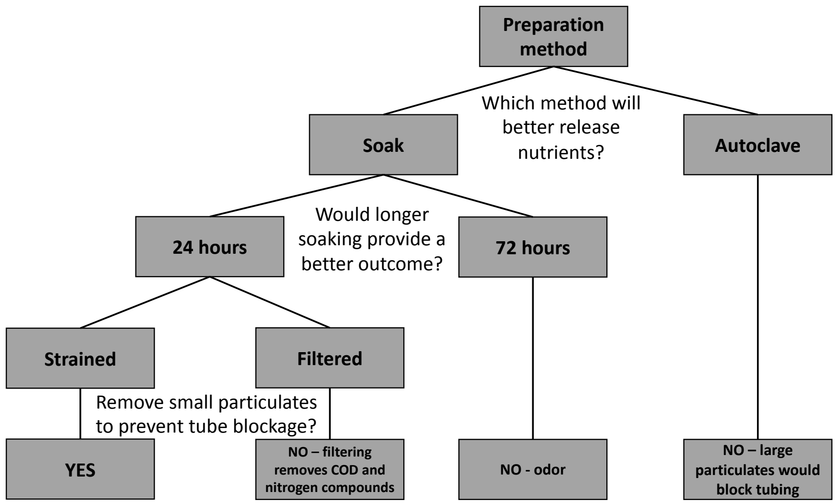

Figure 1: Decision tree for selecting the method for synthetic wastewater preparation. Wastewater was prepared with Pedigree brand dog food.

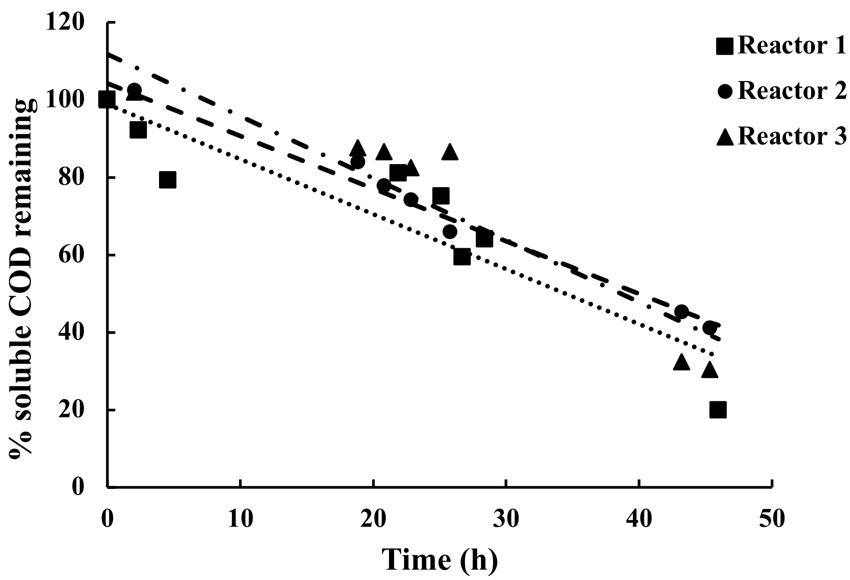

Fig 2: Reduction of soluble chemical oxygen demand in synthetic wastewater in batch reactors seeded with soil microbes collected from a wastewater vegetated infiltration gallery. All samples were filtered through 0.2 μm cellulose acetate filters. Dotted line – Reactor 1; Dashed line – Reactor 2; Combined dot-dash line – Reactor 3

Table A1: Comparison of synthetic wastewater characteristics prepared with different brands of dry dog food

Brand	Protein (%)*	Fat (%)*	Moisture (%)*	Cost/kg (USD)	pH	Conductivity (µS/cm)	Total N (mg/L)	Ammonia (mg/L as N)	Nitrate (mg/L as N)	COD (mg/L)	TSS (mg/L)	VSS (mg/L)	Fixed solids (mg/L)
Pedigree	21	10	12	$2.36	6.4	3090	283	8.9	18.5	5580	1200	1170	30
Rachael Ray	26	14	10	$3.71	5.9	3480	149	15.6	6.7	5440	1400	880	520
Nature's Recipe	30	13	13	$5.51	5.9	2930	220	0.9	0.2	7610	1540	1500	40
Blue Wilderness	36	16	10	$8.81	6.2	2640	171	0.1	4.1	5200	830	790	40
Average				$5.10	6.1 ± 0.2	3040 ± 250	206 ± 46	6.4 ± 5.9	7.4 ± 5.6	5960 ± 830	1240 ± 230	1090 ± 250	160 ± 180

COD, chemical oxygen demand; TSS, total suspended solids; VSS, volatile suspended solids; *values from commercial product label; error shows the average deviation from the mean. Cost/kg reflects cost of dry mass

Table A2: Applications of synthetic wastewater using the dog food synthetic wastewater base prepared using Pedigree brand

Recipe	Replication	Application	Supplements	Preparation	COD (mg/L)	Total nitrogen (mg/L)	Nitrate (mg/L as N)	Ammonia (mg/L as N)	TSS (mg/L)	pH
Base	n=3		None	24-hour soak	4870 ± 474	227 ± 37.4	12.2 ± 4.2	9.7 ± 0.6	1030 ± 250.0	6.4
Synthetic Primary effluent	n=3	Anaerobic secondary treatment	Whey protein (0.3 g/L) $NaHCO_3$ (0.5g/L)	Dilute 1:10	1210 ± 35.2	98.6 ± 12.4	2.24 ± 0.25	2.25 ± 0.01	23.3 ± 0.3	7.5 ± 0.5
Synthetic Secondary effluent	n=9	Soil-based tertiary treatment	$NaNO_3$ (5 mg/L) NH_4Cl (5mg/L)	Dilute 1:100 at field site	44.7 ± 9.5	2.0 ± 0.5	1.3 ± 0.2	1.2 ± 0.5	n.d.	7.6 ± 0.2
Secondary effluent[+]					5-80	2-35	1-30	0.1-10	5-25	N/A

COD, Chemical oxygen demand; TSS, total suspended solids; n.d., no data; replicates compared batches of synthetic wastewater prepared separately. Error shows average deviation from the mean.

[+]Tchobanoglous, G., Stensel, H. D., Tsuchihashi, R., Burton, F., Abu-Orf, M., Bowden, G., & Pfrang, W. (2014). *Wastewater Engineering, Treatment and Resource Recovery* (5th ed.): McGraw Hill Education

Table A3: Characteristics of DSW compared to two other wastewater recipes

Recipe	Total nitrogen (mg/L)	Nitrate (mg/L as N)	Ammonia (mg/L as N)	COD (mg/L)	Ref
DSW base	227	12.1	9.7	4870	This study
OECD	27	0	0	100 (DOC)	(OECD 2001)
SYNTHO	40	0	11	570	(Boeije, Corstanje et al. 1999)

Note: Nutrient contents for OECD and SYNTHO were calculated based on provided recipes

Table A4: Cost of ingredients for synthetic wastewater

	Source	CAS	cost/g	g / L	g / EQ*	cost / EQ
OECD						
Peptone	Fisher	73049-73-7	\$0.34	0.16	1.7	\$0.58
Meat extract	Fisher	68990-09-0	\$0.72	0.11	1.2	\$0.86
Urea	Fisher	57-13-6	\$0.07	0.03	0.32	\$0.02
K_2HPO_4	Fisher	7758-11-4	\$0.35	0.028	0.30	\$0.10
NaCl	Fisher	7647-14-5	\$0.09	0.007	0.08	\$0.007
$CaCl_2\ 2H_2O$	Fisher	10035-04-8	\$0.14	0.004	0.04	\$0.006
$MgSO_4\ 7H_2O$	Fisher	10034-99-8	\$0.08	0.002	0.02	\$0.002
						\$1.58
Dog Food Synthetic wastewater (DSW)						
Dog food	Pedigree		\$0.002	60	66	\$0.13

OECD = Organization for Economic Co-operation and Development; OECD Guideline for the Testing of Chemicals No. 307. Aerobic and Anaerobic Transformation in Soil. US EPA; 2002.*1 EQ = 1L recovered DSW = 10.7 L OECD SWW (equivalent COD to 1L DSW).

S1 Table: Nutrient characteristics of synthetic secondary wastewater effluent prepared from Pedigree brand dog food including chemical oxygen demand, total nitrogen, ammonia, and nitrate.

	COD*	**Total N***	**Ammonia-N***	**Nitrate-N***	**pH**
Typical^{+}	*20-80*	*2-35*	*0.1-10*	*1-30*	*N/A*
1	37.0	1.3	0.1	0.96	7.9
2	53.9	2.6	0.13	n.d.	7.6
3	56.2	2.4	0.19	n.d.	7.7
4	57.0	2.1	0.22	n.d.	7.8
5	44.4	0.73	0.1	0.86	7.7
6	36.8	2.4	0.1	0.52	7.7
7	28.1	2.3	0.14	0.76	7.8
8	54.2	n.d.	0.19	0.89	n.d.
9	34.6	2.4	0.19	0.8	7.5
Average	44.7 ± 9.5	2.0 ± 0.5	0.15 ± 0.04	0.80 ± 0.1	7.7 ± 0.1

*mg/L; $^{+}$Secondary effluent: Tchobanoglous, G., Stensel, H. D., Tsuchihashi, R., Burton, F., Abu-Orf, M., Bowden, G., & Pfrang, W. (2014). *Wastewater Engineering, Treatment and Resource Recovery* (5th ed.): McGraw Hill Education.

Table AS2: Chemical oxygen demand of standard synthetic wastewater recipe provided by the Organization for Economic Co-Operation and Development (OECD), calculated for comparison to dog food synthetic wastewater

Component	**mg COD⁺ / g**	**g / L**	**mg COD⁺ / L**
OECD			
Peptone	2230*	0.160	357
Meat extract	1150**	0.110	126
Urea	2400*	0.030	72
K_2HPO_4	-	0.028	-
NaCl	-	0.007	-
$CaCl_2$ $2H_2O$	-	0.004	-
$MgSO_4$ $7H_2O$	-	0.002	-
			555

*Calculated
**Quantified in lab
$^{+}$COD- chemical oxygen demand

Appendix B: Supplemental Data for Chapter 3

Table S1: Characteristics of synthetic wastewater used in wastewater infiltration systems

Date	COD	TN	NO_3	NH_4	pH
Spring 2022					
3/15/22	37.0	1.3	1.0	0.1	7.9
3/21/22	53.9	2.6	n.a.	0.1	7.6
3/25/22	56.2	2.4	n.a.	0.2	7.7
4/2/22	57.0	2.1	n.a.	0.2	7.8
4/5/22	44.4	0.7	0.9	0.1	7.7
5/8/22	36.8	2.4	0.5	0.1	7.6
5/11/22	28.1	2.3	0.8	0.1	7.7
5/14/22	54.2	n.a.	0.9	0.2	n.a.
5/17/22	34.6	2.4	0.8	0.2	7.7
5/23/22	68.4	9.5	n.a.	n.a.	7.6
Summer 2022					
7/5/22	39.0	3.4	1.6	1.7	7.9
7/7/22	43.3	6	1.4	0.6	7.4
7/11/22	47.6	6.3	1.4	n.a.	7.4
8/3/22	n.a.	3.8	0.9	n.a.	7.5
8/5/22	45.2	4.5	1.4	n.a.	7.7
8/7/22	30.3	4.5	1.4	n.a.	7.6
Autumn 2022					
9/30/22	82.9	11.8	2.4	n.a.	6.7
10/4/22	61.3	n.a.	2.9	n.a.	7.3
10/24/22	91.9	10.3	3.9	n.a.	7
10/25/22	n.a.	8.6	2.9	n.a.	7.3
11/23/22	n.a.	10.5	3.8	n.a.	7.9
11/23/22	n.a.	7.9	3	n.a.	7.8
Winter 2023					
1/27/23	176	13.6	5.1	0.5	6.4
3/2/23	90.7	15.9	1.5	1.4	n.a.
3/21/23	66.0	n.a.	1.5	n.a.	7.9
3/20/23	60.9	n.a.	n.a.	n.a.	7
Spring 2023					
4/26/23	80.9	10.1	3	2.7	7.8
5/29/23	48.4	3.2	1.43	1.4	7.9
6/1/23	84.8	15.7	3.74	3.9	8.4
*Summer 2023**					

6/30/23	67.6	33.2	22.3	10.2	7.8
7/5/23	53.5	30.1	19.5	9.3	7.6
7/12/23	n.a.	30.5	19.3	9.7	8.9
10/19/23	162.5	40.0	20.4	n.a.	6.4
10/24/23	123.7	28.3	18.5	n.a.	7.4
Autumn 2023					
8/22/23	716	33.9	n.a.	n.a.	n.a.
9/1/23	825	47.9	n.a.	n.a.	7.4
9/8/23	444.7	22.5	n.a.	n.a.	7.2
9/12/23	617.1	43.0	n.a.	n.a.	7.1
10/16/23	782.2	20.1	n.a.	n.a.	6.4
10/19/23	473.6	n.a.	n.a.	n.a.	6.7
10/24/23	517.1	45.4	n.a.	n.a.	7
10/30/23	541	22.2	n.a.	n.a.	7

*Includes wastewater from control reactors in autumn 2023

Table S2: Nutrient loading (kg/ha day^{-1} equivalent)

	COD*	Total nitrogen	Nitrate	Ammonium	TKN
Spring 2022	18.8 ± 4.3	1.1 ± 0.6	0.32 ± 0.04	0.06 ± 0.02	0.8 ± 0.5
Summer 2022	24.7 ± 3.1	2.9 ± 0.6	0.8 ± 0.1	0.7 ± 0.3	2.0 ± 0.5
Autumn 2022	39.0 ± 4.3	5.3 ± 0.7	1.6 ± 0.2	n.a.	3.71 ± 0.5
Winter 2023	42.2 ± 22.6	8.6 ± 0.7	1.6 ± 0.9	0.5 ± 0.3	7.0 ± 3.5
Spring 2023	46.0 ± 9.9	12.4 ± 5.5	1.8 ± 0.6	1.7 ± 0.5	10.7 ± 6.0
Summer-Fall 2023	68.2 ± 27.7	21.7 ± 2.2	13.4 ± 0.7	6.5 ± 0.2	5.5 ± 0.4
High-COD	470.3 ± 96.3	26.7 ± 7.5	<LOD	<LOD	n.a.

*COD – chemical oxygen demand; **LOD (limit of detection) = 0.5mg/L for nitrate and 0.015mg/L for ammonium; n.a. – not analyzed

Table 3: Soil properties

Reactor	Soil texture (% sand/silt/clay)	Bulk density (g/cm^3)	Breakthrough time (hours)
1	85.9/10.0/4.0	1.31 ± 0.11	26
2	86.7/11.0/2.3	1.11 ± 0.14	19
3	86.5/10.8/2.7	1.11 ± 0.13	27
4	80.6/15.8/3.7	1.45 ± 0.32	21
5	84.2/11.9/4.0	1.56 ± 0.12	26
6	86.2/10.6/3.2	1.67 ± 0.10	25
7	81.9/15.5/2.6	1.27 ± 0.15	28
8	89.3/9.7/1.0	1.55 ± 0.13	19
9	90.1/7.9/2.0	1.49 ± 0.13	14

Table S4: Soil moisture and organic carbon

Season	Treatment	Soil moisture (%)	p-value	Organic carbon (%)	p-value	pH
Spring 2022	Planted	18 ± 2	0.23	3.3 ± 0.3[a]	0.01*	n.a.
	Unplanted	17 ± 1		2.6 ± 0.1[b]		
	Control	15 ± 4		3.3 ± 0.3[a]		
Summer 2022	Planted	19 ± 2	0.07	3.4 ± 0.3[a]	0.01*	n.a.
	Unplanted	17 ± 1		3.0 ± 0.1[b]		
	Control	1 ± 1		3.4 ± 0.1[a]		
Fall 2022	Planted	18 ± 2	0.39	3.4 ± 0.3[a]	0.02*	n.a.
	Unplanted	16 ± 2		2.9 ± 0.2[b]		
	Control	17 ± 1		3.2 ± 0.3[ab]		
Winter 2023	Planted	17 ± 1	0.21	3.2 ± 0.3	0.19	6.6 ± 0.1
	Unplanted	17 ± 2		3.3 ± 0.6		6.8 ± 0.1
	Control	19 ± 2		3.9 ± 0.8		6.5 ± 0.2
Spring 2023	Planted	14 ± 1[a]	0.01*	3.3 ± 0.4	0.66	n.a.
	Unplanted	15 ± 1[ab]		3.2 ± 0.6		
	Control	16 ± 2[b]		3.0 ± 0.2		
Summer/Fall 2023	Planted	16 ± 2[a]	1.0e-7*	3.4 ± 0.3[a]	1.7e-5*	5.8 ± 0.1
	Unplanted	17 ± 1[a]		3.0 ± 0.2[b]		5.9 ± 0.2
	Control	21 ± 4[b]		3.6 ± 0.3[a]		6.0 ± 0.2
High COD	Planted	20 ± 2	0.193	3.6 ± 0.3[a]	8.6e-6*	5.9 ± 0.2
	Unplanted	19 ± 3		3.1 ± 0.3[b]		5.9 ± 0.1

*significantly different (ANOVA for treatment). Data were subset by season. Different letters indicate significantly different values.