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Quand les communautés microbiennes entrent en collision :
L'impact de la dynamique des communautés microbiennes des eaux usées après un traitement conventionnel et une phytofiltration

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Résumé

L'eau est une ressource essentielle et sa gestion devient de plus en plus importante à mesure que la population mondiale augmente. L'utilisation des ressources en eau génère des eaux usées qui peuvent être dangereuses pour la santé humaine et l'environnement si elles ne sont pas correctement traitées. Le traitement conventionnel des eaux usées utilise l'aération pour stimuler les microbes qui diminuent les contaminants ; cependant, il est coûteux financièrement et énergétiquement, ce qui peut entraîner un traitement limité, voire inexistant, dans les régions ne disposant pas de ressources financières suffisantes. Les progrès de la phytotechnologie ont permis de découvrir que les arbres à haut pouvoir filtrant tels que Salix miyabeana (saule), peuvent être utilisés pour traiter les eaux usées avec l'aide de leurs communautés microbiennes rhizosphériques. Cette dissertation vise à évaluer l'utilisation de la phytofiltration comme méthode de traitement des eaux usées d'un point de vue microbien en comprenant d'abord les communautés microbiennes du traitement conventionnel des eaux usées et en évaluant ensuite les effets de l'irrigation des eaux usées sur une communauté microbienne de la rhizosphère. La compréhension des similitudes ou des différences de ces communautés microbiennes ouvre la voie à l'utilisation de différentes communautés microbiennes dans différents environnements pour atteindre des objectifs similaires (par exemple, l'élimination des déchets des eaux usées). La compréhension des communautés microbiennes présentes permettra de déduire et de comprendre leurs processus de remédiation potentiels, ce qui contribuera à ouvrir l'utilisation des communautés microbiennes (méthodes biologiques) dans diverses applications.

Dans le **chapitre 1**, la communauté microbienne et les constituants des eaux usées provenant des eaux usées primaires, des boues activées et des effluents d'une petite station d'épuration ont été caractérisés et comparés tout au long des étapes du traitement. Les résultats montrent que les communautés microbiennes de chaque étape de traitement sont contrastées les unes par rapport aux autres, et que le processus d'aération est un facteur majeur de changement de la communauté en sélectionnant des bactéries capables de fonctions telles que l'oxydation de l'ammoniac et des nitrites et contre des fonctions telles que la dégradation des polymères végétaux. Les résultats suggèrent également que la concentration de différents constituants des eaux usées (c'est-à-dire le réservoir de ressources), tels que les protéines ou les nitrates et nitrites, joue également un rôle dans la composition de la communauté microbienne. Enfin, les données suggèrent que le traitement des eaux usées ne suffit pas à éliminer de nombreuses bactéries de

l'eau, car de nombreuses espèces anaérobies sont présentes en abondance relative élevée dans les effluents. De plus, certaines espèces potentiellement dangereuses présentes dans les effluents sont rejetées dans les eaux de surface.

Dans le **chapitre 2**, la communauté microbienne, ainsi que la composition du sol, d'une rhizosphère de saule témoin, d'une rhizosphère irriguée par de l'eau potable et d'une rhizosphère irriguée par des eaux usées, ont été évaluées et comparées. Les résultats ont montré que la rhizosphère témoin contient de nombreuses espèces contribuant à la promotion de la croissance des plantes et que l'irrigation à l'eau potable a peu d'effet sur la communauté microbienne de la rhizosphère. Vingt-sept pour cent des ESV de la rhizosphère sont différentiellement abondants dans le sol irrigué par les eaux usées par rapport au contrôle, avec une augmentation significative de 95 %. L'irrigation par les eaux usées enrichit de nombreuses espèces avec des caractéristiques favorisant la croissance des plantes et ajoute de nouvelles bactéries à la rhizosphère qui ne sont pas présentes dans le sol témoin et présentent des caractéristiques bénéfiques telles que la fixation de l'azote et l'oxydation du soufre. Cette étude est un regard nouveau sur la réaction de la communauté microbienne de la rhizosphère d'un saule naturel à la phytofiltration des eaux usées au niveau de la taxonomie des espèces.

Cette thèse caractérise les communautés microbiennes des eaux usées et de la rhizosphère des saules dans le contexte du traitement des eaux usées et de la phytofiltration. D'un point de vue microbien, la phytofiltration des eaux usées semble être une alternative viable au traitement conventionnel des eaux usées car elle diminue les bactéries des eaux usées, augmente les bactéries de la rhizosphère favorisant la croissance des plantes et fournit des nutriments aux arbres.

Mots-clés

Boues activées, métagénomique, communauté microbienne, phytoremédiation, phytofiltration, eaux usées primaires, taillis de saule à courte rotation, rhizosphère, eaux usées, traitement des eaux usées, ARNr 16S.

Abstract

Water is an essential resource, and its management is becoming increasingly important as global populations rise. The use of water resources generates wastewater which can be hazardous to human and environmental health if not properly treated. Conventional wastewater treatment uses aeration to stimulate microbes which decrease contaminants; however, it is financially and energetically costly, which can result in little to no treatment in areas without sufficient financial resources. Advancements in phytotechnology have discovered that high filtering trees such as *Salix miyabeana* (willow), can be employed to treat wastewater with the help of their rhizosphere microbial communities. This dissertation aims to evaluate the use of phytofiltration as a method of wastewater treatment from a microbial perspective by first understanding the microbial communities of conventional wastewater treatment and second evaluating the effects of wastewater irrigation on a rhizosphere microbial community. Understanding the similarities or differences of these microbial communities opens the potential for different microbial communities in different environments to achieve similar endpoints (i.e., removal of wastes from wastewater). Understanding the microbial communities present will help to infer and understand their potential remediation processes which will help to open the use of (biological methods) microbial communities across various applications.

In **Chapter 1**, the microbial community and wastewater constituents from the primary wastewater, activated sludge and effluent of a small-scale wastewater treatment plant were characterized and compared throughout the treatment steps. The results show that the microbial communities of each treatment step are unique from one another, and that the aeration process is a major driver of community change selecting for bacteria capable of functions like ammonia and nitrite oxidation and against functions like plant polymer degradation. Results also suggest the concentration of different wastewater constituents (i.e., the resource pool) such as proteins or nitrates and nitrites also play a role in shaping the composition of the microbial community. Lastly, data suggests that wastewater treatment is not sufficient in removing many bacteria from water, as many anaerobic species are present in high relative abundances in effluent. Additionally, some potentially harmful species present in effluent are released into surface waters.

In **Chapter 2**, the microbial community, along with soil composition, of a control willow rhizosphere, a potable water irrigated rhizosphere, and a wastewater irrigated rhizosphere, were evaluated, and compared. The results showed that the control rhizosphere contains many species

contributing to plant growth promotion and that irrigation with potable water has little effect on the rhizosphere microbial community. Twenty-seven percent of rhizosphere ESVs are differentially abundant in wastewater irrigated soil compared to the control, with 95% significantly increasing. Wastewater irrigation enriches many species with plant growth promoting traits and adds novel bacteria to the rhizosphere that are not present in control soil and display beneficial traits such as nitrogen fixation and sulfur oxidation. This study is a novel look at the reaction of a natural willow rhizosphere microbial community to phytofiltration of wastewater at species-level taxonomy.

This dissertation characterizes both the wastewater and willow rhizosphere microbial communities in the context of wastewater treatment and phytofiltration. From a microbial perspective, wastewater phytofiltration appears to be a viable alternative to conventional wastewater treatment as it decreases wastewater bacteria, enhances plant growth promoting rhizosphere bacteria and provides nutrients for trees.

Keywords

Activated sludge, metagenomics, microbial community, phytoremediation, phytofiltration, primary wastewater, short rotation willow coppice, rhizosphere, wastewater, wastewater treatment, 16S rRNA.

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List of Acronyms and Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
ACCd	1-aminocyclopropane-1-carboxylate deaminase
alkB	Alkane monooxygenase
AMF	Arbuscular mycorrhizal fungi
BLASTn	Nucleotide Basic Local Alignment Search Tool
CBOD	Carbonaceous biochemical oxygen demand
COD	Chemical oxygen demand
cpn60	The 60 kDa chaperonin protein gene
DESeq2	A tool for differential gene expression analysis of RNA-seq data, DESeq2 is a new version of DESeq and can detect more differentially expressed genes (DEGs) than DESeq2
DOC	Dissolved organic carbon
ECM	Ectomycorrhizal
EDTA	Ethylenediaminetetraacetic acid
ESV	Exact sequence variants
IAA	Indole-1-acetic acid
ICP-MS	Inductively coupled plasma mass spectrometry
ITS	Internal transcribed spacer
KCl	Potassium chloride
matK	MaturaseK gene
MHB	Mycorrhiza helper bacteria
MS	Multiple species
NCBI	National Center for Biotechnology Information
OM	Organic matter
OP	Orthophosphate
OTU	Operational taxonomic unit
PAH	Polycyclic aromatic hydrocarbon
PAO	Phosphorus accumulating organism
PCB	Polychlorinated biphenyls

PCoA	Principle coordinates analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multtivariate analysis of variance
PGPB	Plant growth promoting bacteria
pH	Potential of hydrogen
PW	Potable water (chapter 2)
PW	Primary wastewater (chapter 1)
rbcL	Ribulose-biphosphate carboxylase gene
RDP	Rhibosomal Database Project
rRNA	Ribosomal ribosenucleic acid
SILVA	Nucleotide database
TAE	TAE buffer (Tris base, acetic acid and EDTA)
TKN	Total Kjeldahl nitrogen
TOC	Total organic carbon
TP	Total phosphorus
UN	United Nations
USEPA	United States Environmental Protection Agency
WHO	World Health Organization
WW	Wastewater
WWTP	Wastewater treatment plant

Introduction

Research Overview

Freshwater is essential to human life which makes water resource management more and more important as global populations rise[1]. Wastewater is an increasing part of the problem when it comes to water management[2]. Due to various contaminants within wastewater, it can be hazardous to human and environmental health[2,3]. This necessitates proper treatment and contaminant removal before rerelease into the environment. Wastewater treatment infrastructure and conventional wastewater treatment can be costly[4,5] therefore implementation of low-cost wastewater treatment alternatives may help to protect water resources. My dissertation aims to gain a deeper understanding of the microbial community associated with successful phytofiltration of primary wastewater and to see if implementation of low-cost green technology can treat and protect Canada's water resources. To reach this aim my research focuses on two areas: (a) the wastewater microbial community and its changes through conventional activated sludge treatment and (b) the willow rhizosphere microbial community and its changes through irrigation with wastewater. These projects will also evaluate the outcome of the interaction between the wastewater microbial community and the rhizosphere microbial community as well as infer how the microbial communities may interact with the constituents and characteristics of their surrounding environments based on previously published knowledge.

The problem with wastewater

The importance of water

Water is an irreplaceable resource that is vital for all living organisms. Humans and animals alike depend on water for physiological processes in the body such as biochemical reactions within cells, material transport through the body, thermoregulation, and homeostasis[6]. Without water the bodily systems and organs could not function. Higher life forms are also dependant on water for growth of food such as agriculture or wild vegetation[7]. Water can make land productive and habitable. Water, along with vegetation, can stabilize regional climates as the evapotranspiration of trees keep local environments [8,9] It is also important for continued and increasing health and hygiene as the simple act of washing hands can help stop the spread of disease[2]. Water is an indispensable resource for all life on planet earth.

Current and future problems with water

Approximately 71% of the Earth's surface is covered by water, however, 96.5% of all Earth's water is contained in oceans[10]. Freshwater only makes up approximately 2.5% of all Earth's water, with the remaining 1% being other saline waters. Very little of Earth's freshwater is easily accessed, only 1.2% is surface water. The majority, 68.7%, is trapped in glaciers and ice caps. The rest, 30.1%, is groundwater. The distribution of freshwater between the surface, underground and glacial freeze contributes to some of the current problems facing humanity. Not everyone has access to fresh, clean, potable water.

Numerous factors affect the availability and accessibility of fresh clean water including weather patterns, water infrastructure, agriculture, population growth, increasing standards of living, contamination, and climate change[1,11,12]. The abundance of water in some areas and scarcity in others follows patterns dictated by the movement of air from heating and cooling of air masses resulting evaporation and precipitation[13,14]. These phenomena are called the orographic effect and atmospheric convection and lead to inequal distribution of water over the globe resulting in rainforests in some areas and deserts in others.

Inadequate water infrastructure adds to the current problems with access to potable water and sanitation. Currently, 2.2 billion people around the world do not have safely managed drinking water services, 4.2 billion people do not have safely managed sanitation services and 3 billion lack basic handwashing facilities[15]. Conventional water infrastructure can be costly and is a major obstacle in the United Nations' (UN) goal of water and sanitation for all. The cost of water supply infrastructure alone (not including energy, flood, or irrigation) is projected to be $6.7 trillion by 2030 and $22.6 trillion by 2050, significantly more than funding to the sector[16].

Agriculture and food production also adds significant pressure on water resources. Producing 1 kg of rice takes between 3,000 and 5,000 litres of water, 1kg of soya takes 2,000 litres, 1kg of wheat takes 900 litres and 1kg of potatoes takes 500 litres[17]. Overall, 72% of all water withdrawals are used for agriculture[18]

Population growth and higher living standards are contributing to increasing water demand. The UN projects the global population to reach 8.5 billion people by 2030. The population growth along with the UN's sustainable development goal of ending poverty by 2030 will put ever increasing pressure on freshwater supplies. Global water demand is projected to increase by 20 to 30 per cent per year by 2050[19].

Climate change is also a major contributor to unstable water resources. Approximately 74% of natural disasters between 2001 and 2018 were water related, including droughts and floods[11]. The frequency and intensity of these events are expected to increase due to climate change[20]. When natural disasters occur, they can partially or fully destroy or contaminate water and sanitation infrastructure. Changes in rainfall patterns and river flows, as well as increased demand, can contribute to increased frequency and severity of droughts. Furthermore, when rain does occur in drought-stricken areas, the soil cannot absorb the much-needed water, leading to floods, reduced aquifer recharge and contaminated water resources[21].

Contamination of freshwater resources can also contribute to water scarcity or waterborne illness. Freshwater resources can be contaminated through various means, having untreated or undertreated sewage entering surface water, agricultural runoff, industrial release, or stormwater. According to the World Health Organization (WHO), 3.4 million people, the majority being children, die from water related disease each year[22].

These factors come together to affect the accessibility to clean freshwater. According to the UN, currently 2.3 billion people live in water-stressed countries, 733 million of which live in high and critically water stressed countries[23]. A study by Burek et al. (2016) estimated that approximately half the global population lives in potential water scarce areas at least one month per year[24]. Whereas Mekonnen and Hoekstra (2016) estimate four billion people (two-thirds the global population) experience severe water scarcity during at least one month of the year[25]. While the estimates from these studies are not in exact agreement, there is no doubt that a large portion of the population already experiences water stress and scarcity at least part of the year. Furthermore, climate change is expected to worsen these effects due to higher temperatures and more extreme weather conditions affecting availability and distribution of rainfall, snowmelt, river flows and groundwater and further deteriorate water quality[26]. In the mid-2010s, 1.9 billion people, or 27% of the global population lived in potential severely water-scarce areas. In 2050 this is projected to increase to 2.7 to 3.2 billion people. Although the uneven distribution of water has always affected life on the planet, the recent onset of climate change may exacerbate the unequal distribution and adversely affect communities and ecosystems reliant on local water supplies. Due to the importance and value of water and the current stressors on water resources, sustainable use and management of water is becoming crucial to ensure water access to the current and growing global population.

Wastewater creation and potential hazards

Wastewater treatment is a large piece of sustainable water management. Most human activities that use water create wastewater[12], which has been defined as "water that has been used and contains dissolved or suspended waste materials"[27]. Wastewater can be created by a variety of activities such as domestic wastewater, municipal wastewater, urban runoff (stormwater), agricultural runoff, livestock production, land-based aquaculture, industrial wastewater, mining activities, energy generation and landfill leachate and carries with it many varying contaminants[12]. For example, domestic wastewater, often called sewage, contains human excreta (including microorganisms), which contains nutrients and organic matter and may also contain several emerging contaminants, such as pharmaceuticals, personal care products, cleaning products, illicit drugs, and endocrine disrupting compounds. Municipal wastewater is similar to domestic wastewater but can contain additional contaminants from industrial practices within the municipality. Urban runoff or stormwater can contain a wide range of contaminants from roads and other hard surfaces such as, polycyclic aromatic hydrocarbons and black carbon from fossil fuel combustion, rubber, motor oil, heavy metals, non-degradable trash such as plastics, organic waste, suspended particulate and fertilizers and pesticides from lawns. Agricultural runoff may contain soil microorganisms, nutrients from fertilizers, and pesticides. Livestock production effluents can contain large faecal loads and veterinary medicine (antibiotics and artificial growth hormones). Land-based aquaculture produces effluents typically rich in organic matter, suspended solids, dissolved nutrients, heavy metals, and emerging contaminants. Industrial wastewater contaminants vary depending on industry. Mining activities include drainage from tailings, possibly suspended solids, alkalinity or acidity, dissolved salts, cyanide, and heavy metals and sometimes may include radioactive elements depending on mine activity. Energy generation can produce thermal pollution (heated water) and usually contains nitrogen (ammonia and nitrate), dissolved solids, sulphate, and heavy metals. Landfill leachate contains organic and inorganic contaminants, with potentially high concentrations of metals and hazardous organic chemicals. Because of these various contaminants in wastewater, it can cause problems if released without treatment.

The main concern regarding wastewater is the release of these contaminants into the environment where they can affect environmental and human health[2,28]. Release of untreated or undertreated wastewater into the environment results in the pollution of surface water, soil, and

groundwater. The release of untreated or undertreated wastewater can occur due to inadequate or non-existent treatment facilities or with combined sewage overflow systems during extreme weather and may contaminate receiving surface waters. This method of contamination of water resources can lead to serious human illness. According to the UN 1.8 billion people use a source of drinking water contaminated with faeces, putting them at risk of contracting cholera, dysentery, typhoid and polio[28]. Sanitation and wastewater-related diseases are still widespread in countries with low coverage of sanitation services, where wastewater is used to irrigate food crops, and where reliance on contaminated surface water for drinking and recreational use is common. It is estimated that 842,000 deaths in middle- and low-income countries in 2012 were caused by contaminated drinking water, lack of adequate handwashing facilities, and inadequate sanitation services[29].

Not only is there risk from faecal microorganisms but concentrations of emerging contaminants in water sources may also adversely affect human beings in contact with contaminated water sources. Emerging contaminants are, by definition, unregulated contaminants[30]. For example, estrogens, both synthetic and natural, widely prescribed as birth control and hormone therapy, adversely affect human male reproductive health[31]. Another example, plasticizers, such as BPA and phthalates, have been found in many environmental samples including surface water[32–38]. Exposure to high BPA levels may impact sex hormone levels in men[39]. Studies conducted on mice and rat models have shown BPA exposure to result in changes to salivary glands[40] and to be neurotoxic[41]. Phthalates also seem to affect male reproductive health, as exposure to phthalates prenatally resulted in incomplete virilization in infant boys and perinatal exposure may affect human Leydig cell development and function in boys[42]. Phthalate metabolite concentrations in urine samples showed an inverse relationship with intelligence[43] as well as increased attention deficit hyperactivity disorder symptoms[44] in children.

Human beings are not the only species affected by contaminated water sources. Wildlife can also be adversely affected by different emerging contaminants within surface water through contamination such as pharmaceuticals and endocrine disruptors which may be released both with un- and undertreated wastewater as well as in effluents reaching regulation treatment. These substances can be hard to remove with typical wastewater treatment. Aquatic species are experiencing serious effects from exposure to some of these chemicals. Environmental estrogens alter sexual development and function in fish[45,46] and effect other physiological processes

including growth, development, osmoregulation, stress response and immune response[47]. Anti-depressants such as fluoxetine can disrupt anxiety-related behaviour in fish which may have consequences of less optimal responses to potentially threatening stimuli[48]

Contamination from both untreated or undertreated domestic wastewaters as well as agricultural runoff can lead to the serious and specific environmental problems of toxic algal blooms, direct toxicity to aquatic life and eutrophication. Both domestic wastewater and agricultural runoff contain large amounts of nitrogen and phosphorus, domestic wastewater from human excreta and agricultural runoff from fertilizers or manure. This influx of nutrients into freshwater systems leads to excessive growth of autotrophic species such as algae and aquatic plants which lead to both the increase of toxic algal blooms and sedimentation resulting in eutrophication of waterbodies[49]. Nitrogenous compounds can also be directly toxic to aquatic life[50,51].

Stormwater or road runoff can contain a vast array of contaminants. Heavy metals produced by vehicle exhaust and road, tire and brake abrasion can be deposited in road dust[52], and can be swept into road runoff during storm events. A study assessing the heavy metal and polycyclic aromatic hydrocarbon (PAH) concentrations in the suspended sediment component of runoff from two stormwater catchments in Dunedin, New Zealand found concentrations up to 527 µg/g of lead, 464 µg/g of copper, 1325 µg/g of zinc and 11.6 µg/g of 16 united states environmental protection agency (USEPA) priority listed PAHs summed together[53]. Heavy metals, while some are essential to living beings in small amounts, they can cause harm in higher concentrations. Lead is particularly toxic and exposure to lead has been associated with behavioural abnormalities, hearing deficits, neuromuscular weakness and impaired cognitive functions[54]. Copper and zinc, however, are essential nutrients for humans. Excess intake of copper may be toxic depending on individual factors[55]. Toxic effects of copper can be a result of its role in oxygen free radical generation and include increased lipid peroxidation in cell membranes and DNA damage[55]. Zinc toxicity may also occur at high or chronic concentrations. Overt toxicity from high intake of zinc result in nausea, vomiting, epigastric pain, lethargy and fatigue whereas lower levels may trigger copper deficiencies and symptoms of anemia and neutropenia and impaired immune function[56]. In addition to human toxicity many studies have shown the adverse effects of heavy metal toxicity to aquatic life[57,58]. PAHs, a chemically diverse class of pollutants, are derived from incomplete

combustion of natural and anthropogenic sources, such as forests fires and fossil fuel combustion[59]. Depending on the species of PAH, they can be classified as human carcinogens[60].

Although wastewater can be hazardous, it also contains different nutrients, that while hazardous to some life forms are nutrients to others such as plants. There are various contaminants that come from different sources of wastewater, however, the research in this dissertation will concentrate on domestic wastewater.

Conventional Wastewater Treatment

Conventional wastewater treatment plants generally employ two to three steps of treatment and combine physical, chemical, and biological processes and operations to remove solids, organic matter and sometimes nutrients from wastewater[61]. Conventional treatment typically begins with a preliminary treatment to remove larger solids through course screening, grit removal or the reduction of large particles. Primary treatment targets the removal of the settleable solids through sedimentation as well as the removal of scum such as oils and greases by skimming[61] and includes at least one of the following processes: chemical flocculation, primary sedimentation/clarification or skimming[62]. During the primary treatment phase 25 to 50% of the wastewater biochemical oxygen demand (BOD_5) is removed, 50 to 70% of the total suspended solids and 65% of the oil and grease are removed[61]. Additionally, some organic nitrogen, organic phosphorus and heavy metals associated with solids are also removed during sedimentation. Secondary treatment targets the residual organics and suspended solids[61] and usually includes a community of microorganisms[63] whose growth is encouraged through systems such as activated sludges, lagoons, storage ponds, trickling filters or oxidation ditches[62]. It involves the removal of biodegradable dissolved and colloidal organic matter using aerobic biological treatment processes[61]. Aerobic biological treatment is conducted by microorganisms that metabolize organic matter in an aerobic environment, producing more microorganisms and inorganic end products (namely CO_2, NH_3, and H_2O). There are a few different aerobic biological processes that are employed for secondary treatment differing primarily in the way oxygen is supplied to the microorganisms and in the rate at which organisms metabolize the organic matter. These can be categorized in two ways: high-rate and low-rate biological processes. High-rate are characterized by relatively small reactor volumes and high concentration of microorganisms compared with low rate processes. The growth of new microorganisms is much greater in high-rate systems because of the well-controlled

environment. Common high-rate processes include the activated sludge process, trickling filters or biofilters, oxidation ditches and rotating biological contractors. A combination of two of these processes in series (e.g., biofilter followed by activated sludge) can be employed to treat municipal wastewater containing a high concentration of organic material from industrial sources. Together with primary treatment high-rate biological treatment typically removes 85% of the BOD_5 and SS originally present in raw wastewater and some of the heavy metals. When coupled with a disinfection step, these processes can provide substantial but not complete removal of bacteria and virus. However, they remove very little phosphorus, nitrogen, non-biodegradable organics, or dissolved minerals. Tertiary treatment or advanced wastewater treatment is generally considered a polishing step to remove further impurities in the water and involves treatments such as biofiltration, biological nutrient removal, filtration or peat filters[62]. It is employed when specific wastewater constituents which cannot be removed by secondary treatment must be removed[61].

Microbiology of conventional wastewater treatment

The microbiology of wastewater treatment has been extensively studied as the efficiency and robustness of a WWTP mainly depend on the composition and activity of its microbial community[64]. Although biological wastewater treatment has been used for over a century, in depth knowledge of the microbial community was limited until recently[65]. The advent of new methodology, such as next-generation sequencing, opened up the complex microbial world[66]. An early review from 2002 of microbial diversity in wastewater treatment plants and laboratory scale reactors had already identified 13 bacterial divisions including Proteobacteria, Bacteroidetes, Chloroflexi and Planctomycetes, present in significant numbers[64]. Additionally, a reactor designed for enhanced biological phosphorus removal was shown to be dominated by Actinobacteria[67]. Other phyla present in these studies included Acidobacteria, Firmicutes, Nitrospira, Verrucomicrobia, Chlorobi, Fibrobacteres and Fusobacteria, although these were present in lower relative abundances[64]. More recently, a study evaluating a full-scale wastewater treatment plant in Warsaw, Poland found Proteobacteria, Actinobacteria and Chloroflexi were the most abundant phyla in activated sludge, Bacteroidetes, Nitrospira and Firmicutes were also detected but in lower abundances[68]. A 2012 study investigating the microbial structures of different wastewater treatment plants sampled from the aeration tanks of 12 full-scale wastewater treatment plants by 454-pyrosequencing found that in membrane bioreactors the microbial community structures at the phyla level were different between distinct membrane bioreactors[69]. In four samples the most

predominant phylum was Proteobacteria, followed by Bacteroidetes and Acidobacteria. For the other three membrane bioreactor samples Bacteroidetes was the most abundant phylum. Chloroflexi and Planctomycetes were also relatively abundant in the majority of bioreactors. In the oxidation ditch processes most samples were dominated by relatively equal amounts of Proteobacteria and Bacteroidetes. Other predominant phyla included Acidobacteria, Chloroflexi, Planctomycetes and Verrucomicrobia, which were similar to membrane bioreactor samples. In the anoxic/anoxic/oxic (A/A/O (A/O)) system Proteobacteria was the dominant phyla in all samples, Bacteroidetes, Acidobacteria, Chloroflexi and Verrucomicrobia were also highly abundant in most samples. A 2015 study sampling full-scale anaerobic digestion sludge in Beijing, China, found At the phyla level the most abundant bacteria belonged to Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria[70]. At the genera level, there were over 2900 different classifiable taxa, demonstrating the vast microbial diversity of anaerobic digestion sludge. Ten genera were abundant in percentages of 1% or higher including *Candidatus Cloacamonas,* the most dominant taxon, *Bacteroides, Clostridium, Anaerolinea, Rhodobacter, Acidovorax, Syntrophus, Geobacter, Methanosaeta* and *Rhodopseudomonas*. A study investigating the microbial communities of wastewater treatment plants in high-altitude plateau regions in comparison to lowland regions in China and Tibet found, again Proteobacteria the most abundant phylum across all samples, the other dominant phylum was Bacteroidetes, however Bacteroidetes was more abundant in the control group (lowland) than in the plateau group[71]. Chloroflexi and Firmicutes were also abundant. Other dominant phyla included Acidobacteria, Chlorobi, Saccharibacteria, Actinobacteria and Spirochaetae. At a genera level, the most representative genera in the plateau were *Haliangium*, *Roseiflexus, Smithella* and *Lachnospiraceae*. In control (lowland) wastewater treatment plants *Dokdonella, Nitrospira, Terrimonas*, unculture *Chitinophagaceae*, uncultured *Saprospiraceae* and *Haliangium* were the dominant genera. Although the dominant genera of the plateau and lowland samples were different 26 of the top 50 genera were shared by all activated sludge samples, including members of *Bacteroides, Flavobacterium*, uncultured *Anaerolineaceae*, *Thauera,* uncultured *Xanthomonadaceae, Terrimonas, Arcobacter* etc. A study of three Russian wastewater treatment plants in 2012-2013, treating different incoming wastewaters (municipal wastewater, refinery sewage and slaughterhouse wastewater) and their respective activated sludges found a total of 771 and 913 operational taxonomic units (OTUs) for activated sludges and incoming wastewaters, respectively[63]. *Actinobacter* was the most abundant genus in all three

incoming wastewaters. The incoming wastewaters shared other genera such as *Trichococcus*, *Cloacibacterium* and *Methanobrevibacter* however some genera were predominantly found in only one type of sewage. *Akkermansia* and *Prevotella* were mostly encountered in domestic wastewaters; *Acidaminococcus*, *Cloacibacterim* and *Megasphaera* in slaughterhouse wastewaters; and high levels of *Arcobacter* were observed in municipal wastewater with petroleum products. The microbial community of the municipal wastewater activated sludge was dominated by the genus *Caldilinea, Prosthecobacter, Planctomyces, Thiothrix, Opitutus, Pasteuria* and *Halea*; whereas the municipal wastewater with petroleum products had *Opitutus* as the dominant genus, followed by *Caldilinea* and *Prosthecobacter*. Slaughterhouse activated sludge was dominated by *Flavobacterium* and *Luteolibacter*. Overall Shchegolkova et al. (2016)[63] found that the metabolic potential of the three bacterial communities clearly reflected the substrate composition and chemical, rather than bacterial composition of the incoming wastewater was the main factor in activated sludge structure formation. As seen above at the phyla level, studies investigating wastewater and wastewater treatment plants seem to share many of the same phyla but may differ in relative abundances, for example, Proteobacteria and Bacteroidetes seem to dominate many wastewater treatment plants. As sequencing and culturing techniques continue to improve and more of the microbial world is studied and named, studies will be able to resolve more of the bacteria present to genera and species-level taxonomy. Although studies with lower-resolved taxonomy were an important foundation of microbial studies, studies resolving taxonomy to higher levels will help deepen the understanding of microbial communities and may reveal more complex systems.

Effluent release regulations

In Canada, effluent from wastewater systems is the largest source of pollution, by volume, in surface waters[72]. According to the *Wastewater Systems Effluent Regulations* the main requirements state that the effluent deposited cannot be acutely lethal and the effluent concentrations of carbonaceous biochemical oxygen demand (CBOD), suspended solids, total residual chlorine and un-ionized ammonia have limitations[73]. These regulations dictate that CBOD have an average of less than or equal to 25 mg/L, suspended solids have an average of less than or equal to 25 mg/L, total residual chlorine should be on average less than or equal to 0.02 mg/L, un-ionized ammonia should have a maximum of less than 1.25 mg/L (expressed as nitrogen at 15ºC). The *Guideline for Release of Ammonia in Wastewater Effluents* also specifies that the release of

unionized ammonia in effluents entering surface waters should not exceed 0.019 mg/L in the aquatic environment[74].

While strict effluent regulations are limited to those four parameters by the government of Canada, the *Guideline for Release of Ammonia in Wastewater Effluents* also specifies that owners of wastewater systems should consider actions that reduce or eliminate risks posed by other substances that may be found in municipal wastewater effluent, in particular the following substances which are specified in *Schedule 1* of the *Canadian Environmental Protection Act (1999)*[74–76]. These substances include chlorinated wastewater effluents, inorganic chloramines, inorganic arsenic compounds, inorganic cadmium compounds, hexavalent chromium compounds, lead, mercury, effluents from textile mills that use wet processing, and nonylphenol and its ethoxylates. The *Guideline* also specifies owners of wastewater systems should consider that nitrogen in ammonia, along with phosphorus, is a nutrient responsible for stimulating plant and algal growth in the aquatic environment and excessive amounts of ammonia and phosphorus can cause over-fertilization or eutrophication, resulting in excessive growth of algae. Eutrophication reduces available dissolved oxygen, can have toxic effects on aquatic organisms, harm spawning grounds, alter habitat, lead to a decline in certain species, and impair the aesthetic enjoyment of water. Municipal wastewater is the largest point source of nitrogen and phosphorus released to the Canadian environment[74].

Nitrogen in wastewater can exist in a few different forms and come from different sources. Human faeces contain organic nitrogen (proteins) that can be broken down into ammonia, additionally urine also contains ammonia[77]. This produces a problem as releasing large amounts of ammonia into the environment can be hazardous[50,51]. Certain microorganisms can oxidize ammonia and thereby start the process of degradation to atmospheric nitrogen. Classically the ammonia oxidizing bacteria belong to genera such as *Nitrosomonas, Nitrosococcus* and *Nitrosospira*[78,79]. These bacteria transform ammonia into nitrite. Nitrite is then transformed by another set of bacteria *Nitrobacter*, *Nitrospina*, *Nitrococcus* and *Nitrospira* into nitrate[80–83]. Another group of bacteria capable of complete ammonia oxidation from ammonia to nitrate have also recently been discovered[84]. These bacteria that oxidize ammonia are typically aerobic bacteria and are therefore dependant on the oxidation stages within wastewater treatment. After the oxidation steps other bacteria then start reducing nitrate to atmospheric nitrogen depending on the bacteria, they transform nitrate to various nitrogenous species along the way. For example,

Dechloromonas hortensis, *Azonexus hydrophilus* and *Variovorax paradoxus* all reduce nitrate to nitrite[85–87], *Ottowia thiooxydans* reduces nitrate to nitrous oxide[88] and species such as *Comamonas denitrificans*, *Bilophila wadsworthia*, *Bacillus azotoformans* and *Dechloromonas aromatica* can all completely reduce nitrate to atmospheric nitrogen[89–92]. This bacterial mediated process helps reduce the nitrogen and more specifically the ammonia in wastewater. Phosphorus is another contaminant in wastewater originating largely from human excrements and phosphorus containing soaps and detergents, just like nitrogen, phosphorus is another nutrient that can lead to overproduction by primary producers depleting freshwater environments, it is also important to limit during the wastewater treatment process[93–96]. There are bacteria that can accumulate phosphate in their cells thereby removing it from the liquid portion of effluent and increasing the phosphorus content of the solids[97]. These are species such as *Accumulibacter phosphatis* and *Halomonas phosphatis*[98,99].

As far as can be found for the Canadian governance, there are no regulations that dictates concentration or screening for microbial life in effluents being released. Effluent wastewaters need to be disinfected to protect downstream municipal water supplies, recreational waters and shellfish-growing areas from bacterial contamination and other agents causing waterborne disease. Many treatment plants do use a chlorination or disinfection step to disinfect effluent prior to its discharge into the receiving environment but it was discovered that certain levels of chlorinated wastewater effluents from wastewater treatment plants cause acute lethality to fish and invertebrate species. Chlorinated wastewater effluents were added to the *List of Toxic Substances* in *Schedule 1* of the *Canadian Environmental Protection Act* in November of 1999[100]. An archived report of Chlorinated Wastewater Effluents from 1993 estimated that 400 municipal wastewater treatment plants[101] out of approximately 2800[102] discharged chlorinated effluents into aquatic systems. Therefore, alternative methods of wastewater treatment are important to investigate because of the necessity to improve wastewater treatment and protect freshwater resources.

Phytoremediation

Phytoremediation is the use of higher plants for the cost-effective, environmentally friendly rehabilitation of soil and groundwater contaminated by toxic metals and organic compounds[103]. The term phytoremediation really is an all-encompassing term for a multitude of processes that can occur facilitated by certain plants and their microbiomes. Phytoremediation technologies can

include the process of phytoextraction which utilizes pollutant accumulating plants that can take up metals or organics from soil. Phytodegradation refers to plants and associated microorganisms that can degrade organic pollutants and phytostabilization that uses plants to reduce the bioavaliabiliy of pollutants in the environment. One important process, that can be implemented in the treatment of wastewater is rhizofiltration, which uses plant species with high water filtering abilities to absorb and adsorb pollutants from aqueous wastes[104]. Phytoremediation in general has been shown to clean up both organic and inorganic pollutants. Organic pollutants that have been successfully remediated using plants solvents such as trichloroethylene[105,106], herbicides[107], explosives[108], petroleum hydrocarbons[109,110], fuel additives[111–113], and polychlorinated biphenyls[114]. Inorganic pollutants need to be sequestered or stabilized as they cannot be degraded. These include macronutrients like nitrogen and phosphorus[115], trace and nonessential elements[115,116], and radioactive isotopes[117,118].

In the rhizosphere, the region of soil in the vicinity of plant roots in which the chemistry and microbiology is influenced by their growth, respiration, and nutrient exchange, high selectivity of microorganisms exists. While the rhizosphere effect (the enhancement of soil microorganisms growth resulting from physical and chemical alterations of the soils by root excretions and debris) is known to increase the abundance of microorganisms[119,120], it is now known that the rhizosphere also exhibits a high selectivity of particular types of microorganism[121]. Therefore, while density and biomass of microorganisms increase substantially in the rhizosphere compared to bulk soil, microbial diversity decreases. As the selection of microbes can be attributed to both plant species traits and microbial genetic capabilities, this results in a microbial community with highly specific abilities. One of which can be considered the use of plant-specific exudates[122–125]. These plant specific exudates, however, do not only benefit microbes but are also highly useful to the plants themselves. Organic acid exudates are known to play a role in nutrient acquisition[126], stress alleviation[127] and metal detoxification[128]. In fact, increased root exudation has been frequently observed for plants under stress and may be a well-developed coping mechanism which involves the increase of these highly selected microbes[129–132].

The plant's role in the rhizosphere

Root architecture shapes the rhizosphere; however, root architecture is malleable. Resources in soil are unevenly distributed in space and time (daily, seasonally and larger time frames)[133] due to how nutrients are recycled (leaf litter decomposition and dead animal decay) and

their mobility in soil[134]. Plants must be able to adapt to this heterogeneous matrix, to perceive and respond to nutrients by directing root growth, in essence, they need to seek out nutrients. Spatial deployment of the root system determines the ability of plants to exploit heterogeneous soil resources[135]. Nutrients may act as signalling molecules to trigger root growth[136]. Some specific examples of how plants do this is by increasing root depth in drought tolerant plants (such as beans, wheat and maize) and increasing dense shallow root systems for topsoil foraging for phosphorus[137]. The response of plants to environmental, climate, biological, and soil conditions in search or nutrients forms roots system[138].

Plants also release a wide range of chemicals called exudates into the rhizosphere[139]. Exudates can include active excretions, passive diffusates, and whole-cells or lysates of the epidermis and cortex[140,141]. Exudates are generally classified into two groups: high molecular weight or low molecular weight compounds. High molecular weight compounds, for example polysaccharides (mucilage or cellulose) or proteins, are not easily used by microbes. Low molecular weight exudates can include molecules such as organic acids, amino acids, sugars, phenols (e.g. flavonoids), fatty acids, sterols, enzymes, plant growth regulators and other secondary metabolites used by microbes[139,142,143]. The chemicals released by the plants are influenced by many factors, including but not limited to plant species, as well as surrounding plant species, soil and climate conditions, and the microbial community[144]. Low molecular weight molecules may aid in nutrient acquisition, allelopathy, or attraction of symbionts and beneficial microbial colonization of root surfaces[142]. Root exudates can help with nutrient acquisition by making them more accessible to uptake by plants through changing the pH or redox conditions within the rhizosphere, directly chelating nutrients for absorption, dissolving insoluble minerals, and desorbing nutrients from clay or organic matter[140]. For example, oxalic acid, a common root exudate, promotes soil carbon loss by liberating organic compounds from protective associations with minerals, which are then susceptible to microbial use[145]. Exudates can also change the pH of the soil, plants can raise or lower the pH of the surrounding soil through exudates, which helps in avoiding aluminum toxicity when soils are too acidic or iron and phosphorus deficiencies when soils are too alkaline[146–148]. Exudates not only provide carbon to symbiotic microorganisms, they can also recruit them by signalling molecules meant for communication[149]. Exudates also help to protect roots for both biotic and abiotic stress. For example, root cells can be released into the soil and can continue to function for a while to act as bait cells for surrounding pathogens[150,151].

Mucilage is also released around the roots to help protect from the damage roots might incur while pushing their way through soil[140]. Root exudates can be sloughed off cap and border cells, released dead and lysed epidermal cells, secreted insoluble mucilage, volatile organic compounds, and soluble exudates such as: sugars, amino acids, organic acids, phenolic compounds, and other secondary metabolites.

Bacterial life in the rhizosphere

Hiltner, the man who coined the phrase rhizosphere, discovered that the rhizosphere is much higher in bacteria abundance than in bulk soil[152]. This happens because plants secrete metabolites that can be used as nutrients by the bacteria. In fact, plants are known to secrete 5 to 21% of the carbon they fix as root exudates[143]. While it has been established that the bacterial concentration in the rhizosphere is 10 to 1000 times higher than bulk soil due to root exudates, it is still considered a malnourished lifestyle and to exert their beneficial properties for the plant community, bacteria need to be able to compete for nutrients secreted by plants. Competitive colonization is necessary for many of the plant-reaped benefits[153].

Beneficial bacteria in the rhizosphere are now typically categorized into plant growth promoting bacteria (PGPB) and mycorrhiza helper bacteria (MHB). PGPB can also be divided into mechanisms of direct plant growth promotion (such as biofertilizers, rhizoremediators, phytostimulators and stress controllers), and indirect plant growth promotion involving biocontrol (such as antagonism, signal interference, predation, and parasitism, induced systemic resistance, and interference with pathogen activity[153]. Biofertilizers are bacteria that can supply plants with nutrients. The most well-known of these types of bacteria are Rhizobium, these bacteria form nodules in roots of legumes and fix atmospheric nitrogen into ammonia which can be used as a nitrogen source by the plant[154]. Additionally, low soluble phosphate levels can limit plant growth, some PGPB can turn bound phosphates (organic or inorganic) into soluble phosphates for uptake[155,156]. Rhizoremediators can protect plants against soil pollutants by degrading them. Naphthalene, a polycyclic aromatic hydrocarbon can be used as a pesticide in agriculture. A strain of *P. putida* was found to effectively use root exudates, degraded naphthalene around the root and protected seeds from being killed by the pesticide, allowing the plant to grow normally[157]. Phytostimulators tend to do what the name implies; these bacteria can produce substances that stimulate plant growth. These substances include hormones (like auxin)[158,159], certain types of volatiles[160], and the cofactor pyrrolquinoline quinone[161]. Finally, of the categories in direct plant

growth promotion, stress controllers contain an enzyme called 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Bacteria with this enzyme can help lower ethylene levels by taking up the ethylene precursor ACC and turning it into 2-oxobutanoate and NH_3. This ability promotes plant growth by relieving stress from phytopathic bacteria, polyaromatic hydrocarbons, heavy metals, salt and draught[162].

Indirect methods of promoting plant growth, that is biocontrol, includes antagonism. This happens either when bacteria can synthesize and release antibiotics and successfully outcompete other organisms for nutrients, and niches on the root[163]. Another method of indirect growth promotion is signal interference. Signal interference occurs with the degradation of quorum-sensing molecules such as homoserine lactones (a molecule required for the synthesis of cell-wall-degrading enzymes of certain pathogens[164]. Predation and parasitism are used by some fungal species (i.e. *Trichoderma*) and are based on enzymatic destruction of fungal cell walls[165]. Induced systemic resistance (ISR), yet another mechanism, results from the interaction of plant roots with some bacteria. Examples of ISR include induced resistance in carnations against Fusarium wilt by the rhizobacterium *Pseudomonas* sp. Strain WCS417r[166], and in cucumber against *Colletotrichum orbiculare* by selected rhizobacteria[167]. Finally, one of the last types of indirect plant growth promotion: interference with pathogen activity. An example of this is the bacterium *P. fluorescens*, which react to fusaric acid released by *Forl* hyphae, it is a chemoattractant, during biocontrol the bacteria colonize the hyphae of the pathogen *Forl,* it is believed they colonize it to use it as a food source[168–170].

The other major classification of rhizosphere bacteria is mycorrhizae helper bacteria (MHB). MHB, exclusively form associations with mycorrhizae, however, they are known to associate with both ECM and AMF[171,172]. Garbaye (1994) suggested that MBH should be defined as bacteria associated with roots and mycorrhizal fungi that selectively promote the establishment of the mycorrhizal symbiosis[173]. As Rigamonte et al. (2010) points out, the lineages of MHB belong to many bacterial taxa groups, such as *Proteobacteria* (*Agrobacterium, Azospirillum, Azotobacter, Burkholderia, Bradyrhizobium, Enterobacter, Pseudomonas, Klebsiella* and *Rhizobium*), *Firmicutes* (*Bacillus, Brevibacillus,* and *Paenibacillus*) and *Actinomycetes* (*Rhodococcus, Streptomyces,* and *Arthrobacter*)[172]. According to Garbaye (1994) MHB are selective in associations with fungal species, or in other words they are fungus-specific, whereas plant associations show no specificity[173]. Of the ectomycorrhizal fungi, basidiomycetes were the

only ones to have been observed to interact with MHB[171]. Frey-Klett et al. (2007) demonstrated that ectomycorrhizal fungi has an indirect positive effect on the selection of bacterial communities[171]. Their study demonstrated that the ectomycorrhizal symbiosis determined the *Pseudomonas fluorescens* population and selects beneficial strains to the symbiosis and the plant. MHB seem to help mycorrhizae with five main activities. First, they help with the reception of the root by the mycobiont, they aid in root-fungus recognition, stimulate fungal growth, they modify rhizosphere soil, and they help in the germination of fungal propagules[173].

Wastewater as a potential resource

Although wastewater represents a hazard to human and environmental health if released into surface waters untreated, it is also a potential source of fertilizer for agriculture as faeces provide nitrogen and phosphorus, two nutrients that are growth limiting factors[12]. Treated and untreated wastewater has been used to irrigate agricultural fields. Irrigation with reclaimed wastewater increased the organic matter, total carbon and total nitrogen contents in the top 10-cm soil layers in long term treatment (8 and 20 years), it also decreased pH in soil over 20 years and increased both the total and extractable metals in the fields[174]. A study investigating the effects of reclaimed wastewater versus canal water and fertilizer on the growth, yield and fruit quality of grapefruit trees found that trees receiving low and moderate levels of reclaimed wastewater had the largest canopies, trunk diameters and highest yields[175]. Another study found that the use of domestic wastewater with fertilizer improved the physicochemical properties of soil, crop yield and also the nutrient status in comparison with irrigation with groundwater with fertilizer[176]. Yet another study found that under abattoir wastewater irrigation *Pennisetum purpureum*, *Helianthus annuus, Sinapis alba* and *Medicago sativa* showed about 70% higher yields than tap water irrigated plants[177]. The use of wastewaters in agriculture has clearly demonstrated benefits in terms of plant growth however there are potential negative impacts from the use of wastewater for irrigation.

The potential problems with the use of wastewater for irrigation include first and foremost the farmers. Farmers and their families are exposed to health risks from parasitic worms, protozoa, viruses, and bacteria[178,179]. These farmers also report skin and nail problems[180–182]. Contamination of food crops and therefore potential exposure for consumers, is another problem, although less frequent[179]. Heavy metal contamination from wastewater is another major concern of wastewater use for irrigation. Heavy metals can adversely affect agricultural and human health. From the application of wastewater, they can be retained in soil such as silver, chromium, and arsenic. They

can be phytotoxic such as copper, nickel, and zinc. They can be translocated in plants tissue at levels that pose human and animal health risks and bioaccumulate through the soil-plant-animal chain[183]. Other organic and inorganic compounds from wastewater irrigation may also pose problems. Various kinds of salts, pesticides, pharmaceutically active compounds, and endocrine-disrupting chemicals[179]. Salinization of agricultural lands is perhaps the most critical negative effect of wastewater irrigation. Salinization and sodicity of soils are caused by inorganic salts and affect soil productivity. Salts can also lead to toxicity within the plants by the uptake of certain ions.

Phytoremediation of wastewater

Phytoremediation methods have been well studied over the last few decades. Studies have investigated the efficacies of different plants to remediate varying contaminants in the environment, from heavy metal rich mine tailings to hydrocarbon contamination and wastewater remediation. These studies have found the use of different plants such as *Limnocharis flava, Thalia geniculate and Typha latifolia*[184]*, Phragmites australis*[185–187]*, Typha domingensis*[188]*, Eichhornia crassipes*[189]*, Carex cuprina*[190]*, Alisma lanceolatum*[190]*, Iris pseudacorus*[190]*, Zea mays*[191]*, Festuca arundinacea*[191]*, Bassia indica*[192] can help with the remediation of different contaminants such as heavy metals[184–189], hydrocarbons[190,191], anionic detergents[190], salinity[192]. Willow specifically has demonstrated phytoremediation potential on numerous occasions[193–196]. The use of willow trees to remediate wastewater has also been developed and employed in countries such as Sweden as a low-cost eco-friendly alternative for wastewater treatment and energy production[197,198]. Short-rotation willow coppice is a non-edible plant and has many of the requirements to make it suitable as a vegetation filter[199]. The filtering capacity is very high, the crop promotes denitrification in the root zone. It has a highly selective uptake of heavy metals, especially cadmium, which enables remediation of contaminated soils. Willows also have a high evapotranspiration rate facilitating high loads. In an evaluation of willow filtration systems filtering wastewater it was estimated that willows were able to remove nearly 90% of nitrogen and 85% of the phosphorus found in wastewater[200]. Short-rotation willow coppice has also been evaluated for biomass for potential energy production[197] and extractable phytochemicals[201]. The demonstrated potential of short-rotation willow coppice as a usable wastewater remediation strategy with resulting potential economic products illuminates the need for further study and understanding of the mechanisms of wastewater phytoremediation using willow.

Metagenomics as a method of studying wastewater and rhizosphere microbial communities

Microbial communities are involved in many important processes such as fermentation, organic matter decomposition, digestion, plant growth, pedogenesis and wastewater treatment to name a few. These processes are often integral to human life and natural processes, however, many of them are still very poorly understood as the discovery of microorganisms themselves only occurred in 1676 by Antoine van Leeuwenhoek. The field of microbiology has also progressed relatively slowly as inoculation and culturing techniques had to be developed. Although culturing provided a valuable tool for the early study of microbes and allowed for complex understanding of certain organisms[202], purely culturing microbes can be difficult and timely and is challenging when the desired microorganism has unique metabolic needs[203]. Moreover, most microorganisms are uncultivable[204–206]. Typical testing only identifies a small fraction of the microbiome[207,208]. Unfortunately, the routine use of an artificial homogeneous growth medium is biasedly selective. Artificial media does not provide the ecological niches and symbiotic relationships required to maintain and support the vast microbial diversity found in natural ecosystems[209]. For example, it is well known that many viruses, including enteric viruses cannot be cultivated using standard techniques[210]. A 2019 study attempted to census the bulk of Earth's bacterial and archaeal clades and to estimate overall global richness[211]. They concluded there are 2.2 to 4.3 million full-length operational taxonomic units (OTUs; used as a proxy for species). Currently there are approximately 30,000 formally named species that are in pure culture and for which the physiology has been investigated[212]. Given the current number of known species, the difficulty with culturing as a primary means of microbial identification, and the estimated number of possible species (OTUs) globally, the introduction of genome sequencing has helped decrease the limitations of old culturing techniques. While genomics has allowed higher identification of bacterial species and knowledge of individual organisms, metagenomics has established knowledge of microorganism communities. Metagenomics refers to the study of genetic material not from a unique species but from an environmental sample. This field has advanced microbiology rapidly and has helped to identify many previously unidentifiable species because it eliminates the need for *in vitro* cultivation of microorganisms[213]. It can be used as a powerful tool to have a more encompassing look at what organisms are present in each system.

Considering the need for an intensified effort in species identification and sequencing, one technique that has become quite popular, due to relatively low cost and faster processing time, is

isolating, amplifying up and sequencing one gene and assigning taxonomy based on its sequence. This process is known as barcoding, its goal is to identify individual species based on specific genetic sequences. Identifying species relies on the ability to match sequences with reference barcodes for taxonomic identification[214]. DNA barcoding uses short genetic sequences as markers to identify an organism as a particular species[215]. These marker genes, which vary between the different kingdoms, contain highly conserved and highly variable regions of the DNA allowing for the unique identification of different species. While theoretically any gene could be used as a marker, some of the more common markers include: 16S rRNA[216–218], 18S rRNA[219], ITS (internal transcribed spacer)[220], cytochrome oxidase 1[215,221,222], rbcL (ribulose-biphosphate carboxylase gene)[223,224], matK (maturaseK gene)[225,226], and alkB (alkane monooxygenase)[227]. DNA barcoding uses universal primers that bind to highly conserved regions and allow the sequencing of the highly variable regions to attain a unique barcode for each species. While these techniques have led to advances in molecular biology and phylogeny and a better understanding of relatedness and evolution, they are limited by the presence of variable copy numbers in bacterial genomes and sequence variation within closely related taxa or within a genome[228]. These methods are also not full proof at detecting organisms. The gene chosen as a barcode can introduce some biases. For example, Uyaguari-Diaz et al. (2016)[229] observed clear differences among taxa within the same fraction when using 16S rRNA as their barcode compared to the use of the cpn60 gene as a barcode. 16S rRNA gene amplification has become quite a popular method for investigating prokaryotic communities. This gene selection is based on the 16S ribosomal RNA gene from the model organism *Escherichia coli*, and uses primers designed to amplify one of the variable regions (based off *E.coli* structure). Amplifying only one region of the 16S rRNA gene leads to some possible flaws. As this technology is based around *E.coli*, selected primers may only identify portions of the microbial community that share similar regions with *E.coli*, as this technology is based on the idea that all highly conserved regions are highly conserved, and all highly variable regions are highly variable, whereas evaluation of different sets of primers covering the same regions do not result in the exact same microbial community[230]. By illuminating as much of the microbial community as possible to the highest taxonomic resolution possible the different patterns of bacteria in wastewater and phytoremediation can be studied.

Project introduction

The potential of using wastewater to irrigate agriculture has a dual purpose, first as a low-cost treatment of wastewater and second as source of water (and nutrients) for crops. While the use of wastewater to irrigate crops has been common practice for many years in certain areas, the understanding of the microbial mechanisms of both the treatment of wastewater and the promotion of agricultural growth has not been well studied. This project will track contaminant and nutrient fate from wastewater while looking intently at the complex interactions of the microbial community as well as the changes that occur in the microbiome of the rhizosphere during wastewater phytofiltration. The research presented in this dissertation will evaluate if the use of phytofiltration with its associated microbial community can offer a low-cost green infrastructure alternative to conventional wastewater treatment and if phytofiltration is a superior technology for removing potential hazardous bacteria and constituents present in wastewater, which will help mitigate their potential release and spread in Canada's waterways.

To disentangle the highly complex process of natural phytofiltration, this doctoral project will address three objectives: First, expose the changes to the microbial community through wastewater treatment in a small scale activated sludge wastewater treatment plant. Second, reveal the complex structure and infer functionality of the microbial community in the willow rhizosphere. Third, observe the changes in the willow rhizosphere microbial community after wastewater irrigation/phytofiltration of wastewater. To understand the microbial structure throughout wastewater treatment and compare it to the microbial structure of phytoremediation of wastewater, this research will take a two-fold approach to understand the role microorganisms in the rhizosphere play in phytoremediation of wastewater. First, this research will investigate the changes in the microbial community in wastewater through a conventional activated sludge wastewater treatment plant. Second, it will investigate the microbial community changes in a rhizosphere that has been employed in the task of wastewater phytoremediation. The comparison and contrast of these two studies will help to understand the microbial community structure of conventional wastewater treatment, the microbial community structure of wastewater phytofiltration, infer potential mechanisms of wastewater treatment due to these community structures and what may be different between the two.

This research aims to answer the overarching question: can a deeper understanding of the microbial community associated with successful phytofiltration of primary wastewater facilitate implementation of low-cost green technology to treat and protect Canada's water resources? To answer this overarching question two hypotheses have been developed to guide this research.

Hypothesis 1 – Changes in the phenotype of wastewater will cause changes in the composition and individual species relative abundances of the wastewater microbial community.

Primary wastewater will have a specific constituent profile (1) that will change from one wastewater treatment step to the next based on the stimulation of bacteria growth and constituent breakdown through the aeration process. Constituents will transform from larger to smaller molecules, for example, nitrogen will be released from proteins and urea and become oxidized in active sludge and then reduced to atmospheric nitrogen. Phosphorus will shift to the soluble orthophosphate and carbon will decrease from primary wastewater to effluent. (2) The shifts in environment (i.e., the change in dissolved oxygen through aeration and the changing constituent profile) will change the microbial community to consist of bacteria based on their endemic aerobic/anaerobic needs as well as their ability to utilize the forms of the constituents present in each treatment step.

Hypothesis 2 – Irrigation of willow trees with primary wastewater will alter the rhizosphere's unique microbial community to better utilize wastewater nutrients and contamination. Any rhizosphere may (1) have a unique microbial community composition (which may vary according to genotype and soil constituents) due to selective pressures. (2) This selective pressure may decrease microbial variety, possibly due to microbe specialization of resources utilization (however, multiple species of microbes may have similar genes to metabolize a certain resource). While the addition of wastewater to the willow rhizosphere will increase nutrients and resources, it will also cause the mix of microbial communities. Additionally, (3) the influx of nutrients from wastewater may shift the microbial community structure favouring species better able to utilize the new resources.

Chapter 1

Microbial community origin and fate through a rural wastewater treatment plant

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Microbial community origin and fate through a rural wastewater treatment plant

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Originality-Significance Statement

Effective microbial treatment of municipal wastewater is essential to protecting human and ecosystem health. This research identifies microbiota at species-level, allowing for accurate statistical comparison of the microbial community throughout wastewater treatment and providing an unprecedented perspective of treatment-associated species dynamics. The 860 Exact Sequence Variants (ESVs) identified here included putative species involved in proteolysis, nitrification and denitrification, phosphorus accumulation and plant polymer deconstruction, should serve as an important resource for future research in the field. Species-level community profiling also allowed the potential origin of wastewater associated bacteria to be explored and the fate of species to be tracked throughout wastewater treatment, revealing the extent of human and land-animal associated species within primary wastewater to be estimated as well as the depletion, persistence or enrichment of species in effluent, including those with the potential to negatively impact human and ecosystem health.

Summary

Conventional wastewater treatment relies on a complex microbiota; however, much of this community is still to be characterised. To better understand the origin, dynamics and fate of bacteria within a wastewater treatment plant: untreated primary wastewater, activated sludge, and

post-treatment effluent were characterised. From 3,163 Exact Sequence Variants (ESVs), 860 were annotated to species-level. In primary wastewater, 28% of ESVs were putative bacterial species previously associated with humans, 14% with animals and 5% as common to the environment. Differential abundance analysis revealed significant relative reductions in ESVs from potentially humans-associated species from primary wastewater to activated sludge, and significant increases in ESVs from species associated with nutrient cycling. Between primary wastewater and effluent, 51% of ESVs from human-associated species did not significantly differ, and species such as *Bacteroides massiliensis* and *Bacteroides dorei* increased. These findings illustrate that activated sludge increased extracellular protease and urease-producing species, ammonia and nitrite oxidizers, denitrifiers and specific phosphorus accumulators. Although many human-associated species declined, some persisted in effluent, including strains of potential health or environmental concern. Species-level microbial assessment may be useful for understanding variation in wastewater treatment efficiency as well as for monitoring the release of microbes into surface water and the wider ecosystem.

Keywords: 16S rRNA gene, activate sludge, metagenomics, microbiome, wastewater, wastewater treatment

Introduction

The World Health Organization estimated 271 trillion litres of household wastewater was generated globally in 2020 and only 150 litres, or 55.5%, was safely treated before release into the environment[231]. Conventional wastewater treatment plants generally employ two to three steps of treatment. Primary treatment includes at least one of the following processes: chemical flocculation, primary sedimentation/clarification or skimming[62]. Secondary treatment typically includes a community of microorganisms[63] whose growth is encouraged through systems such as activated sludges, lagoons, storage ponds, trickling filters or oxidation ditches[62]. Tertiary treatment is generally considered a polishing step to remove further impurities in the water and involves treatments such as biofiltration, biological nutrient removal, filtration or peat filters[62]. This extensive wastewater treatment process can be costly[232] which can lead to release of undertreated wastewater into surface waters. In Canada, an average of 5.8 trillion litres of municipal wastewater was discharged into Canadian surface waters every year between 2013 and 2017, 1.5 trillion litres of which have only received primary treatment, 2.7 trillion litres which received some form of

secondary treatment and 1.4 trillion litres which received tertiary treatment[62]. The release of wastewater into surface waters, especially untreated or undertreated, can be problematic for both environmental and human health[233].

Microorganisms are a key part of wastewater treatment, and 95% of those found in wastewater treatment are bacteria[234]. Bacteria found in municipal wastewater are often also found in association with human skin, respiratory tract, oral cavity, gastrointestinal tract and urogenital tract[235]. Studies by Shanks et al. (2013)[236] and Newton et al. (2015)[237] found that between 12% and 15% of municipal wastewater taxa were likely attributed to human faeces and the most common microbes in faeces matched the most common and abundant in the wastewater microbial community. While newer sewer systems tend to separate domestic wastewater from stormwater and runoff, older systems often combined them. A study investigating the microbial community from combined sewer systems found that both the infiltration of rainwater and stormwater inputs modulated the community composition and that microbial sewage communities represented a combination of human faecal microbes and an enrichment of environmental microbes to form a unique population structure[238]. Determining the origin of bacteria throughout wastewater treatment may help to improve understanding of the interactions within this microbial community and how they might impact treatment.

For the majority of municipal wastewater treatment plants in Canada, secondary treatment is the main biological treatment step. During secondary treatment in an activated sludge system, air is pumped through the sludge to encourage growth of aerobic bacteria playing key roles in contaminant removal, such as protein and urea degradation and nitrification in nitrogen cycling, phosphorus accumulation and organic matter degradation. Protein or urea degradation is the first step in breaking down nitrogenous compounds in wastewater and is facilitated through proteases and ureases produced by bacteria, such as *Bacteroides*[239] and *Pseudomonas*[240]. Further steps in nitrogen cycling involve ammonia and nitrite oxidation carried out by species from genera such as *Nitrosomonas, Nitrosococcus* and *Nitrosospira*[78,79] and *Nitrospira, Nitrospina, Nitrococcus* and *Nitrobacter*[80–83], respectively. Nitrogen cycling also involves species that can partially reduce nitrate to the major end-products nitrite, nitric oxide or nitrous oxide, or completely reduce nitrate to atmospheric nitrogen. There are species capable of complete denitrification, such as *Comamonas denitrificans*[89]; however, the process can also be carried out sequentially by different bacteria such as *Aeromonas dhakensis*[241], *Alcaligens xylosoxidans*[242] and *Pseudomonas*

fluorescens[243]. Phosphorus removal is also an important part of wastewater treatment for environment health[94] and can be performed by phosphorus accumulating organisms, such as species within the genera *Accumulibacter, Halomonas* and *Gemmatimonas*[98,244,245]. These organisms take up phosphorus and store it as polyphosphate, thereby removing it from the wastewater[97]. Besides protein degradation, major organic matter degradation and removal often involves degradation of recalcitrant carbonaceous material such as plant cell wall polymers and resistant starches by species in genera such as *Clostridium, Cellulomonas, Cytophaga and Streptomycetes*[246]. These taxa use extracellular enzyme suites, which can include diverse cellulases, pectinase, xylanase and/or amylase activities, to depolymerize plant polysaccharides to release soluble sugars[247,248], which can then be used as an energy source.

These microbially mediated processes of nitrogen cycling, phosphorus removal and plant polymer degradation are critical to wastewater treatment, as is tracking the fate of human-associated bacteria throughout the process. Studies assessing these important bacterial taxa through wastewater systems have largely explored these community dynamics at genus-level. Here, the wastewater microbial community was assessed with species-level identification where possible and significant changes in microbial community dynamics were determined.

Experimental Procedures

Sample collection

Wastewater samples were collected from a small rural municipal wastewater (combined sewer system) treatment plant (WWTP) of St. Roch de l'Achigan, Quebec, from the influent wastewater (primary), the aeration treatment stage (activated sludge) and the plant effluent (effluent) entering the river on July 19th, 2018. The wastewater treatment plant services the township of St. Roch de l'Achigan and combines stormwater with the sewage system. As indicated by the treatment plant technician the primary wastewater sampled may have been diluted due to rains the evening before sampling. No animal processing plants directly feed into the sewage system, however, due to stormwater collection, agricultural runoff may feed into the system.

Five replicate samples of 10 ml were collected from each treatment step by submerging a 500ml collection vessel ~1 m into treatment stage tank at approximate time intervals of seconds to minutes between samplings and approximately 10 to 15 minutes between samplings of treatment steps. Samples for DNA extraction and chemical characterization were immediately flash frozen

in liquid nitrogen before transport in dry ice and storage at -80ºC. Additional samples for chemical characterization were immediately acidified to a pH of <2 with sulfuric acid following procedures of *Standard Methods for the Examination of Water and Wastewater:* 1060 Collection and Preservation of Samples[249].

Determining wastewater constituents and characteristics

Wastewater analyses for total organic carbon (TOC) and dissolved organic carbon (DOC) were performed according to the *Méthode d'Analyse* from the *Centre d'Expertise en Analyse Environnementale du Québec* 300-C 1.0 Infrared Detection Method[250]. Analyses of total Kjeldahl nitrogen (TKN) used *Méthode d'Analyse* 300-NTPT 2.0 Acid Digestion and Automated Colorimetric Method[251]. Ammonia, nitrates and nitrites, orthophosphate and total phosphorus were analysed using *Standard Methods for the Examination of Water and Wastewater:* 4500-NH3 G Automated Phenate Method, 4500-NO3-G Automated Hydrazine Reduction Method, 4500-P F Automated Ascorbic Acid Reduction Method without Persulfate Autoclave Digestion, 4500-P F Automated Ascorbic Acid Reduction Method with Persulfate Autoclave Digestion, respectively[249]. Chemical Oxygen Demand (COD) was analysed according to Hach method 8000 based on the closed reflux colorimetric method using the Hach low range (3 to 150 mg/L; for the effluent) and high range (20 to 1500 mg/L; for the activated sludge and the primary wastewater) COD kits[252].

DNA extraction, 16S rRNA gene amplification, sequencing and amplicon processing

DNA was extracted from 10 mL wastewater samples using a 0.2 µm pore size filter and QIAGEN DNeasy PowerWater DNA Extraction kit. The QIAGEN DNeasy PowerWater kit utilises 5 mL QIAGEN bead tubes for bead beating and an additional lysis step of heating for 65ºC for 10 mins after elution. DNA was eluted in 1 mL QIAGEN DNeasy PowerWater Solution PW1. Extractions were checked and nucleic acid content was determined using a Nanodrop 2000 spectrophotometer and running a 1% electrophoresis gel in TAE buffer. Primers for 16S rRNA gene amplification targeted the V5-V6 region: P609D 5'-GGMTTAGATACCCBDGTA-3'and P699R 5'-GGGTYKCGCTCGTTR-3'[230]. PCR amplification and sequencing were performed by Genome Quebec at McGill University using the MiSeq250 platform was used for 2 x 250 bp paired-end sequencing of PCR products. PCR conditions were an initial denaturation step of 98°C, for 30 secs, before 20 cycles of 98°C, for 10 secs, 58°C, for 15 secs and 72°C, for 30 secs, with the final extension at 72°C, for 2 min. Reagent controls were below the detection limit used by

Genomic Quebec Innovation Centre for quality assurance. Sequence counts were processed and annotated using the ANCHOR pipeline[253]. Briefly, sequences were aligned and dereplicated using Mothur[254] before selection of exact sequence variants (ESVs) using a count threshold of 5 across all samples. Annotation queried four sequence repositories with strict BLASTn criteria (>99% identity and coverage): NCBI curated bacterial and Archaea RefSeq, NCBI nr/nt, SILVA, Ribosomal Database Project (RDP). Database versions were from May 2019; all annotation is considered putative and subject to improvement as database errors are resolved and new species are characterized. When the highest sequence identity is shared amongst multiple species, all are retained and reported. Published functional evidence from strains within species identified here is provided in Supplementary file 1, but no biological function of species was directly assessed here. Potential association of bacterial species to human, animal or environmental sources reflects previous reported associations in the current literature (Supplementary file 1) but should be considered as speculative given all species identified could, in this case, derive from this wastewater environment. Those species very commonly found in multiple categories including humans here were assumed to be human-associated.

Diversity and differential abundance analysis

Alpha diversity calculated using ESVs was measured using both the Shannon and Inverse Simpson diversity indices within the Phyloseq package[255], was tested for normal distribution using Shapiro-Wilk test and was compared between treatment steps using a t-test (parametric) or Wilcoxon test (non-parametric) with a false discovery rate (Benjamini-Hochberg) corrected p-value of <0.05 (see Supplementary file 1). Analysis of principle coordinates (PCoA) ordination was performed based on Bray-Curtis ecological distances using the Phyloseq package[255] and dispersion ellipses were drawn using the veganCovEllispse function from the Vegan package[256] in R[257]. To evaluate significant differences between the treatment communities, Permutational Multivariate Analysis of Variance (PERMANOVA) was applied on bray distance matrices using the adonis function in Vegan R-package (see Supplementary file 1). Differential abundance analysis on 16S rRNA gene amplicons was performed using DESeq2[258,259], which can perform well with uneven library sizes and sparsity common to 16S rRNA gene data[253,260]. Sparsity and low-count cut-offs were applied whereby an ESV count in a single sample is < 90% of the count in all samples, and ESV counts must be > 2 in 40% of the samples[253,261]. A false discovery rate (Benjamini-Hochberg) corrected $p < 0.1$ was applied[258,262].

Results

Wastewater composition

Untreated primary wastewater, activated sludge and effluent samples were characterized by measuring pH, chemical, total and dissolved oxygen demand (COD, TOC, DOC), total phosphorus, orthophosphates, total kjeldahl nitrogen (TKN), ammonia, and nitrates-nitrites (Table 1.1). The primary wastewater pH of 7.79 ± 0.04 was significantly higher than both the activated sludge pH of 7.08 ± 0.01 and the effluent pH of 7.21 ± 0.07. The COD for primary wastewater was significantly lower at 446.57 ± 5.59 mg L^{-1}, than the COD for activated sludge 2302.47 ± 36.10 mg L^{-1}, both were significantly higher than effluent at 36.7 ± 2.72 mg L^{-1}. The TOC and DOC showed similar patterns, significantly increasing from 91.66 ± 4.52 mg L^{-1} and 65.60 ± 3.19 mg L^{-1} in primary wastewater to 287.80 ± 14.40 mg L^{-1} and 186.40 ± 3.74 mg L^{-1} in activated sludge before a significant and substantial decrease below initial primary wastewater levels to 15.14 ± 1.30 mg L^{-1} and 13.30 ± 0.61 mg L^{-1} in effluent, respectively. Total phosphorus significantly varied in each step, increasing from 4.92 ± 0.21 mg L^{-1} in primary wastewater to 57.40 ± 0.40 mg L^{-1} in activated sludge and decreasing to 0.39 ± 0.01 mg L^{-1} in effluent. However, orthophosphate, a measure of biologically available phosphorus, showed a significant increase from 3.17 ± 0.09 mg L^{-1} in primary wastewater to 27.70 ± 0.44 mg L^{-1} in activated sludge before dropping to 0.13 ± 0.003 mg L^{-1} in effluent. TKN was significantly higher in activated sludge at 213.20 ± 3.22 mg L^{-1} compared to either primary wastewater at 38.00 ± 1.14 mg L^{-1} or effluent at 23.00 ± 0.00 mg L^{-1}. The concentration of ammonia, however, was highest in primary wastewater at 46.00 ± 0.00 mg L^{-1}, and subsequently dropped significantly to 31.40 ± 0.51 mg L^{-1} in activated sludge and to 21.00 ± 0.00 mg L^{-1} in effluent. Similarly, nitrates and nitrites had the highest levels in primary wastewater at 3.17 ± 0.09 mg L^{-1} but dropped below detection (<0.04 ± 0.00 mg L^{-1}) in activated sludge and increased to 0.13 ± 0.003 mg L^{-1} in effluent.

Microbial community overview

The average extracted DNA concentrations from the five replicate samples taken from each wastewater treatment step were 9.11 ± 0.57 ng/µl for primary wastewater, 430.00 ± 22.46 ng/µl for activated sludge and 9.20 ± 0.48 ng/µL for effluent (Figure 1.2 B). 16S rRNA gene amplification and sequencing generated 1,278,781 counts, with an average of 85,252 ± 12,135 counts per sample. Rarefaction curves were produced to display the high sampling depth and Good's coverage was >99 for all samples (Table 1.2). A total 3,163 ESVs were identified across

all 15 samples with an average of 2,150 ESVs per sample. These could be annotated at various taxonomic levels, including 860 ESVs to species level, 858 ESVs only to genus level, 215 ESVs only to family level, 134 ESVs to higher taxonomic levels and 1,096 ESVs were not similar (<99% identity) to any characterized taxa (Figure 1.2 A, E and F). From the ESVs which could be annotated at the species level, 577 could be assigned to a single putative species and 283 could be annotated as multiple putative species which share identical rRNA gene sequences at the V5-V6 region of the 16S rRNA gene. Overall, 762,867 out of the 1,278,781 (59.66%) total sequence counts and 860 of the 3164 (27.18%) ESVs from all wastewater samples were identified at species level. In primary wastewater, 807 ESVs were annotated as putative species, capturing 328,346 sequences counts (78.22% of total counts in primary wastewater). In activated sludge, 807 ESVs were annotated as putative species capturing 231,894 sequence counts (48.09% of total activated sludge sequence counts). In effluent, 809 ESVs were annotated as species capturing 202,627 sequence counts (53.81% of total effluent sequence counts).

The Shannon diversity index was significantly different between all three steps of wastewater treatment, with activated sludge having the highest and primary the lowest score (Figure 1.2 C; $p < 0.05$ Benjamini-Hochberg corrected t-test, see Supplementary file 1). The Inverse Simpson diversity index was also significantly higher in activated sludge than both primary wastewater and effluent, however primary wastewater and effluent were not significantly different from one another. Principal Coordinates Analysis (PCoA) showed the samples segregated by treatment and multivariate analysis confirmed significant variation between treatment groups (Figure 1.2 D; PERMANOVA $p < 0.001$, see Supplementary file 1).

Primary wastewater microbial community

Primary wastewater ESVs came from eight phyla, including: Proteobacteria (369), Firmicutes (196), Bacteroidetes (161), Actinobacteria (73), Fusobacteria (3), Euryarchaeota (3), Verrucomicrobia (1) and Gemmatimonadetes (1). Thirty-four ESVs had high relative abundance of >0.5% of total sequence counts and accounted for 62% of all sequence counts in primary wastewater (Figure 1.3). These were from seven different classes: Bacteroidia (8), Betaproteobacteria (8), Clostridia (7), Gammaproteobacteria (5), Bacilli (2), Epsilonproteobacteria (2) and Coriobacteriia (1). Five hundred and thirty-four ESVs were annotated as species associated with either humans, other animals or the environment (Supplementary file 1), in addition to wastewater.

A total of 322 primary wastewater ESVs were annotated as species associated with humans (Supplementary file 1) in addition to wastewater systems, accounting for ~28% of total primary counts (Figure 1.3) and coming from six phyla: Firmicutes (146), Bacteroidetes (101), Proteobacteria (44), Actinobacteria (29), Euryarchaeota (1) and Verrucomicrobia (1). Eleven human-associated ESVs had high relative abundance of > 0.5% counts within primary wastewater from the three classes including Bacteroidia (7), Clostridia (3), and Gammaproteobacteria (1). Differential abundance analysis revealed 274 human-associated ESVs significantly differed between primary wastewater and activated sludge. Three human-associated ESVs significantly increased in activated sludge, including Bacteroides_fluxus_1, Pseudomonas_MS_2 and Ruminococcus_bicirculans_2. Two hundred and seventy-one human-associated ESVs were significantly lower in relative abundance in activated sludge compared to primary wastewater, 20 human-associated ESVs decreased beyond detection limits in activated sludge (Figure 1.3). Forty human-associated ESVs did not differ significantly between primary wastewater and activated sludge.

Twenty-six ESVs were annotated as bacterial species most commonly associated with land animals and/or fish (Supplementary file 1) in addition to wastewater systems. These came from four different phyla, including: Firmicutes (10), Proteobacteria (8), Bacteroidetes (7) and Actinobacteria (1). Nineteen were associated with land animals (other than humans) and accounted for 12.55% of total primary counts, whereas 7 ESVs were associated with fish (only 5 of which were detected in primary wastewater) and accounted for 0.04% of total primary counts (Figure 1.3). Of the 19 ESVs associated with land animals, three had high relative abundance in primary wastewater including Arcobacter_MS_3, Uruburuella_suis_1 and Vitreoscilla_stercoraria_1 at 9.43%, 1.58% and 0.75% of all sequences counts in primary wastewater. No animal-associated ESVs increased in relative abundance activated sludge, 17/19 animal-associated ESVs significantly decreased from primary wastewater to activated sludge, an additional 1 animal-associated ESVs were below detection limits and 1 animal-associated ESVs were not significantly different. Of the 5 ESVs associated with fish present in primary wastewater, 4 significantly increased from primary wastewater to activated sludge and one did not significantly change. In addition, 2 ESVs associated with fish which were not detected in primary wastewater were present in activated sludge (Figure 1.3).

One hundred and eighty-three ESVs were commonly associated with environmental sources such as soil and water, in addition to wastewater systems. These ESVs belonged to seven phyla, including: Proteobacteria (131), Bacteroidetes (26), Actinobacteria (14), Firmicutes (7), Gemmatimonadetes (1), Chlorobi (1), Euryarchaeota (1), Fusobacteria (1) and Verrucomicrobia (1). One hundred and sixty of these 183 environment-associated ESVs were present in primary wastewater and accounted for 4.14% of total primary counts (Figure 1.3). Sixty-two environment-associated ESVs were significantly higher in relative abundance in activated sludge compared to primary wastewater while 49 ESVs were significantly lower. Twenty-three ESVs that were present in activated sludge were not detected in primary wastewater whereas 5 ESVs were not detected in activated sludge but were present in primary wastewater (Figure 1.3). Forty-four environment-associated ESVs were not significantly different between primary wastewater and activated sludge.

Activated sludge microbial community

Activated sludge ESVs were comprised of 10 phyla, including: Proteobacteria (375), Firmicutes (177), Bacteroidetes (166), Actinobacteria (72), Euryarchaeota (3), Fusobacteria (3), Gemmatimonadetes (2), Nitrospirae (2), Verrucomicrobia (2) and Chlorobi (1). Eighteen ESVs had high relative abundance of >0.5% sequence counts (27% of activated sludge counts in total) and belonged to five classes including Betaproteobacteria (11), Alphaproteobacteria (3), Flavobacteriia (2), Bacilli (1) and Actinobacteria (1). Forty-eight ESVs were detected in activated sludge but not in primary wastewater and belonged to six phyla: Proteobacteria (30), Bacteroides (12), Nitrospirae (2), Chlorobi (1), Gemmatimonadetes (1), and Verrucomicrobia (1). One hundred and sixty-three ESVs could be putatively associated with nitrogen cycling function, including extracellular protease and/or urease production, ammonia oxidation, nitrite oxidation, nitrate reduction, and denitrification, 8 to phosphorus accumulating organisms, and 91 to breakdown of cellulose, pectin, resistant starch, or xylan (Figure 1.4; Supplementary file 1).

Sixty-six ESVs were annotated as species associated with extracellular protease and/or urease production (Supplementary file 1) and came from four different phyla, including Proteobacteria (37), Bacteroidetes (24), Firmicutes (4) and Actinobacteria (1) (Figure 1.4). From these, 26 significantly increased from primary wastewater to activated sludge and 7 were not present in primary wastewater. Twenty-nine ESVs significantly decreased in activated sludge when compared to primary wastewater. Three ESVs were annotated as taxa associated with

ammonia oxidation (Figure 1.4, Supplementary file 1), all of which were absent in primary wastewater and only one of which could be identified at species-level, Nitrospira_nitrosa_1, from the phyla Nitrospirae. Six ESVs were annotated as species associated with nitrite oxidation (Figure 1.4, Supplementary file 1) and belonged to three phyla including Nitrospirae (4), Proteobacteria (1) and Chloroflexi (1). Five of these were significantly higher in activated sludge (4 were not detected in primary wastewater) and one was not significantly different between primary wastewater and activated sludge. Eighty-six ESVs were annotated as species associated with nitrate to nitrite reduction (Figure 1.4, Supplementary file 1) and belonged to four phyla including Proteobacteria (73), Firmicutes (6), Actinobacteria (5) and Bacteroidetes (2). Twenty of these ESVs significantly increased in activated sludge whereas 54 ESVs significantly decreased in activated sludge (4 did not significantly differ). In addition to the 86 ESVs capable of nitrate reduction, 22 ESVs were capable of complete denitrification (Figure 1.4, Supplementary file 1), 8 of which significantly increased in activated sludge and 10 which significantly decreased. Across the different treatment stages, ESVs annotated as species potentially capable of complete denitrification and in high relative abundance included Comamonas_denitrificans_1 in primary wastewater, Simplicispira_MS_1 in activated sludge and effluent, and Zoogloea_caeni_1 in effluent alone.

From 8 ESVs annotated as species associated with phosphorus accumulating organisms (Supplementary file 1), four significantly increased in activated sludge compared to primary wastewater, including Accumulibacter_phosphatis_1 and 2, and Halomonas_phosphatis_1 (with highest relative abundance), two significantly decreased in activated sludge and two were not statistically different between primary wastewater and activated sludge.

Ninety-one ESVs in primary wastewater were annotated as species associated with the degradation of cellulose, pectin, resistant starch or xylan (plant polymers; Figure 1.5, Supplementary file 1) and belonged to four phyla, including Firmicutes (49), Bacteroidetes (31), Actinobacteria (6) and Proteobacteria (5). Seven-eight ESVs associated with species potentially capable of plant polymer degradation significantly decreased from primary wastewater to activated sludge, while none significantly increased. Fifty-four ESVs significantly increased from activated sludge to effluent (Figure 1.5) while 32 ESVs did not significantly differ.

Effluent microbial community

The 809 ESVs identified within effluent were composed of 10 phyla, including Proteobacteria (374), Firmicutes (184), Bacteroidetes (167), Actinobacteria (71), Euryarchaeota (3), Fusobacteria (3), Gemmatimonadetes (2), Nitrospirae (2), Verrucomicrobia (2) and Chlorobi (1). Twenty ESVs had high relative abundance of >0.5% of total sequences in effluent (29% of effluent sequence counts in total). These ESVs came from seven classes, including Betaproteobacteria (9), Bacteroidia (5), Gammaproteobacteria (2), Flavobacteriia (1), Bacilli (1), Alphaproteobacteria (1) and Epsilonproteobacteria (1), and included Prevotella_copri_2, Bacteroides_uniformis_1 and Bacteroides_vulgatus_1, as well as Arcobacter_MS_3, which had the highest relative abundance in effluent of 10.75% of all effluent counts. A total of 520 ESVs in effluent were annotated as species associated with either humans, other animals or the environment in addition to wastewater systems.

The three hundred and twenty-two ESVs in primary wastewater annotated as species associated with humans were from six phyla, including Firmicutes (146), Bacteroidetes (101), Proteobacteria (44), Actinobacteria (29), Euryarchaeota (1) and Verrucomicrobia (1) (Figure 1.6, Supplementary file 1). Of these, 27 significantly increased, 130 significantly decreased, 8 were below detection limits and 157 were not significantly different from primary wastewater to effluent, such as Escherichia_coli_1 and Roseburia_faecis_1. All 27 significantly increasing ESVs were annotated as species associated with the human gut, such as Bacteroides_dorei_1, Bacteroides_massiliensis_1 and Prevotella_copri_1.

Twenty-six ESVs within wastewater were annotated as species associated with land animals and/or fish (Supplementary file 1), and were from four different phyla, including: Firmicutes (10), Proteobacteria (8), Bacteroidetes (7) and Actinobacteria (1). Of these, 19 ESVs were associated with land animals (other than humans) and 7 were associated with fish (Figure 1.6). Three ESVs annotated as species associated with land animals significantly increased from primary wastewater to effluent, including Arcobacter_MS_3, Arcobacter_suis_1, and Selenomonas_bovis_1, while 11 decreased significantly and 5 did not significantly differ in relative abundance. Two ESVs annotated as species associated with fish were not detected in primary wastewater but were present in effluent, Flavobacterium_tructae_1 and Flavobacterium_succinicans_1, while 5 did not significantly differ in relative abundance between primary wastewater and effluent.

One hundred and eighty-three ESVs present throughout wastewater treatment were annotated as species associated with environmental sources (Supplementary file 1), such as soil and water, in addition to wastewater systems. These belonged to 9 phyla, including Proteobacteria (131), Bacteroidetes (26), Actinobacteria (14), Firmicutes (7), Gemmatimonadetes (1), Chlorobi (1), Euryarchaeota (1), Fusobacteria (1), and Verrucomicrobia (1). One-hundred and sixty-nine of these ESVs were present in effluent. Fifty-nine ESVs significantly increased in relative abundance with an additional 20 ESVs not detected in primary wastewater but present in effluent, 27 ESVs significantly decreased with an additional 11 ESVs present in primary wastewater but beyond detection limit in effluent, and 63 ESVs did not significantly differ from primary wastewater to effluent (Figure 1.6).

From 850 ESVs annotated as putative species and compared from primary wastewater to effluent, 316 ESVs significantly decreased (including 38 below detection limits), 194 significantly increased in relative abundance (including 43 only present in effluent), and 340 did not differ significantly. Thirty-five ESVs were annotated as species containing strains potentially pathogenic to humans, land animals or fish (Figure 1.7, Table 1.2, Supplementary file 1), and came from 6 different classes: Gammaproteobacteria (18), Bacteroidia (7), Clostridia (3), Betaproteobacteria (3), Flavobacteriia (3) and Epsilonproteobacteria (1). One potentially pathogenic ESV, Arcobacter_butzleri_1, increased from primary wastewater to effluent and one potentially pathogenic ESV, Flavobacterium_succinicans_1, was absent in primary wastewater but present in effluent. Eleven significantly different ESVs decreased from primary wastewater to effluent, 3 ESVs were present in primary wastewater but reduced beyond detection limits in effluent and 19 ESVs were not significantly different between primary wastewater and effluent.

Discussion

Distinct composition and microbial community throughout wastewater treatment

This study assessed wastewater transformation through an activated sludge treatment process, investigating the putative origin, association with nutrient metabolism and fate of the microbial community at each of three steps of wastewater treatment (untreated primary wastewater, activated sludge and effluent; Figure 1.1) at a single timepoint. The activated sludge process significantly lowered the pH of wastewater which can impact microbial community composition in soil, water and wastewater in addition to changes in oxygen[263–265]. The relatively

small changes in pH from 7.79 in primary wastewater to 7.08 in activated sludge, however, may not be large enough to drive the substantial change in the microbial community indicated by increases in COD, TOC, and DOC in activated sludge[266]. Total phosphorus concentrations substantially increased by 1067% from primary wastewater, which was accompanied by a 774% increase in orthophosphate, indicating a general concentration of wastewater in activated sludge but also a shift away from larger phosphorus-containing molecules. Nitrogen concentration increased by 416% from primary wastewater to activated sludge. However, ammonia sequentially decreased through the steps and nitrates-nitrites were effectively removed by the activated sludge step, suggesting transformation into other nitrogenous compounds. Although total ammonia decreased throughout the steps, the concentration was 21.00 ± 0.00 mg L^{-1} in effluent. The calculated concentration[73] of unionized ammonia was 0.093 mg L^{-1} and therefore below Canadian regulations for acute toxicity of 1.25 mg L^{-1} but above the maximum of 0.019 mg L^{-1} for chronic toxicity in an aquatic environment[74] prior to dilution of effluent in receiving waters. Based on these substantial differences in the wastewater composition between the three treatment steps, the microbial communities would be expected to vary extensively in each step.

DNA concentrations increased by 4,620% from primary wastewater to activated sludge but returned to a similar DNA concentration as primary wastewater in effluent. Diversity indices showed species diversity in activated sludge was higher than primary wastewater and effluent. While the Shannon diversity index also indicated a significant difference between primary wastewater and effluent (effluent being more diverse than primary wastewater), the Inverse Simpson index did not. The substantial increase in bacterial diversity in activated sludge (Figure 1.2), may be driven by the aeration of wastewater in the activated sludge step[267] proving an aerobic environment in addition to an anaerobic environment (mixed). The disparity in diversity indices between primary wastewater and effluent may indicate that effluent retains species from both the primary wastewater and activated sludge microbial communities. Previous metagenomic 16S rRNA analysis of wastewater treatment plants identified 527 bacterial genera[265]. Here 860 ESVs could be annotated with >99% similarity to at least one strain of a species or multiple species, with 577 putative species identified as unique throughout the wastewater treatment process. Despite similar diversity of primary and effluent, ordination indicates that the microbial communities of all three steps were highly distinct from one another (Figure 1.2).

Human, other animal and environmental microbes dominate primary wastewater

In addition to previous associations to wastewater, bacteria in primary wastewater could also be associated to humans, other (non-human) animals (including fish) and the environment (Figure 1.3, Supplementary file 1). Potentially human-associated bacteria contributed the largest proportion of primary wastewater, representing 37% of identified species. Three human-associated species, *Prevotella copri*, *Bacteroides uniformis* and *Bacteroides vulgatus,* were highly abundant in primary wastewater, suggesting prevalent human gut bacteria are adaptable to water environments. However, the ESV Arcobacter_MS_3 (which could represent *Arcobacter cibarius* and/or *Arcobacter cryaerophilus*) had the highest relative abundance in primary wastewater and could potentially be associated with other animals. *Arcobacter cibarius,* first isolated from the skin of broiler chicken carcasses[268], and *Arcobacter cryaerophilus*, first isolated from pig and bovine fetuses[269], are commonly found in livestock and particularly domestic poultry[270,271], in addition to wastewater. Similarly, *Uruburuella suis*, only isolated from diseased pig organs to date[272], was also highly prevalent. *Arcobacter* have been associated with livestock animals and their meat[273–275] and although no animal processing plants fed into the St. Roch de l'Achigan wastewater treatment plant, the presence of *Arcobacter* could derive from agriculture or household meat consumption in the municipality feeding into this wastewater treatment plant.

The overall microbial community of primary wastewater showed similarities to other studies describing the microbial community at genera level. The *Arcobacter* genus has been previously identified as the most abundant in municipal wastewater in the USA and China[276,277]. Additionally, high relative abundance of the genera *Bacteroides, Trichococcus, Acinetobacter* and *Aeromonas* are also common in other municipal wastewater[276,277], suggesting the samples from St. Roch de l'Achigan follow the general microbial fingerprint of primary municipal wastewater.

Most human-associated species were significantly reduced by the activated sludge step (Figure 1.3, Supplementary file 1). The air pumped through the wastewater in the activated sludge step increases dissolved oxygen content[278] which can encourage growth of aerobic bacteria proficient in nutrient breakdown[279,280]. The substantial reduction in human-associated species could therefore be expected as a large proportion of gut-related species are considered to be anaerobic (45%) or microaerophilic (11%)[281], such as the obligate anaerobes *B. vulgatus* and *P. copri*[282,283] (prevalent here). Although oxidation in the activated sludge step is likely a major driver shaping the microbial community, a significant reduction in relative abundance of the strict aerobe

Acinetobacter schindleri[284] indicates other factors also influence the microbial community. Surprisingly, two human-associated species significantly increased from primary wastewater to activated sludge, *Ruminococcus bicirculans* and *Bacteroides fluxus,* both of which are considered anaerobes[285,286] although identification here suggests oxygen tolerance in some strains.

Animal-associated species such as *A. cibarius/cryaerophilus, U. suis* and *Vitreoscilla stercoraris* had the highest relative abundance in primary wastewater. Other animal-associated species such as *Megamonas rupellensis, Phascolarctobacterium faecium and Clostridium perfringens* were significantly reduced from primary wastewater to the activated sludge step and reduced below-detection in the case of *Lactobacillus animalis* (Figure 1.4, Supplementary file 1). As these species are considered anaerobic[287–291], this microbial community change could also be driven by aeration. Certain species associated with fish also decreased in activated sludge, however, four species increased, *Chryseobacterium chaponense, Flavobacterium succinicans, Simplicispira piscis*, and *Flavobacterium branchiophilum*. The genera *Chryseobacterium, Flavobacterium* and *Simplicispira,* while associated with fish, have also been identified in wastewaters[292–294] and may aid in breakdown of nitrogenous constituents in activated sludge. *Chryseobacterium chaponense* possesses extracellular ureases[295], *Flavobacterium succinicans* and *Flavobacterium branchiophilum* possess extracellular proteases[296,297], and *Simplicispira piscis* is capable of complete denitrification[298]. The increase in these fish-associated species could be due to the increase in nitrogenous resources in wastewater as concentrations of constituents such as proteins (as shown by the proxy TKN measurement) increased from primary wastewater to activated sludge.

The decline in 31% of putative environment-associated bacteria (Figure 1.3, Supplementary file 1) from primary wastewater to activated sludge could not be simply explained through aerobic or anaerobic metabolism alone. Although reductions of anaerobic species such as *Alistipes putredinis, C. perfringens, Clostridium beijerinckii, Comamonas guangdongensis,* and *Prevotella paludivivens*[299–303] occurred, species commonly reported as aerobic also declined, such as *Acinetobacter soli, Bacillus azotoformans, Zoogloea oryzae*, *Acinetobacter baumannii*, *Pseudomonas flexibilis*[91,304–307]. These species can use nitrates and ammonia[91,305,308,309], which were also reduced in activated sludge, indicating resource availability may have driven these significant changes in the microbial community. The resource pool, including nitrogenous molecules like proteins, as well as carbohydrates such as cellulose and resistant starches,

significantly changed from primary wastewater to activated sludge as larger molecules are degraded. This aligned with increases in 42% of putative soil and water-associated species in activated sludge (11% being absent from primary wastewater), such as *Uliginosibacterium gangwonense, Flavobacterium ardleyense, Leucobacter zeae, Ferruginibacter alkalilentus*, and *Curvibacter fontana*, characterized as extracellular proteases producers[310–315]. Similarly, increases in starch utilizing *Flavobacerium aquicola* and *Flavobacterium cheonanense*[314,316] and lipid degrading *Agitococcus lubricus*[317] were observed in activated sludge. This suggests that the microbial community change is associated with important compositional change of proteins, carbohydrates and lipids, commonly observed in gut microbiome studies[318].

Activated sludge encourages specific nutrient metabolizing microbial species

The diverse activated sludge microbial community was comparatively enriched in species involved in the important wastewater treatment functions of nitrogen cycling and phosphorus accumulation, whereas species associated with the degradation of recalcitrant carbohydrates were reduced. Ninety-seven putative species were identified as associated with nitrogen cycling, six as phosphorus accumulating species and 48 as species capable of degradation of cellulose, pectin, resistant starch or xylan (Figure 1.4, Figure 1.5, Supplementary file 1).

Nitrogen cycling in wastewater systems starts with proteins and urea and can progress to the production of atmospheric nitrogen by ammonia oxidizing bacteria, nitrogen oxidizing bacteria and denitrifying bacteria (Figure 1.4, Supplementary file 1). Around half (46%) of the species producing extracellular protease and/or urease decreased in activated sludge from primary wastewater, whereas other extracellular protease and/or urease producing species (37%) increased, including seven species that were not detected in primary wastewater. Protein degrading species were present in each treatment step, indicating the function may be preserved but the significant changes suggest anaerobes are replaced by aerobes. The species potentially contributing to ammonia oxidation in activated sludge were all absent from primary wastewater (Figure 1.4, Supplementary file 1). These included the nitrite and ammonia oxidizer *Nitrospira nitrosa*[84] as well as uncharacterized species within *Nitrosomonadaceae*, a taxon which contains species capable of contributing to ammonia oxidation. Typical ammonia oxidizing bacteria within the genera *Nitrosomonas, Nitrosococcus* and *Nitrosospira*[78,79] were not detected and addition of ammonia oxidizers such as these within activated sludge could potentially help reduce the ammonia levels in the resulting effluent. The presence of nitrite oxidizers likely contributed to the

removal of nitrites through oxidation to nitrates, which may then undergo denitrification. Nitrite oxidizers present in activated sludge included *N. nitrosa* as well as *Nitrospira japonica* and uncharacterized species from the genera *Nitrospira*, *Nitrolancea* and *Nitrobacter*, the majority of which were not detected in primary wastewater. As aerobic autotrophs, ammonia and nitrite oxidizing bacteria are expected to prefer an aerobic environment[319], however previous studies have illustrated that ammonia and nitrite oxidizing bacteria could have also contributed to nitrification at low dissolved oxygen levels[320]. The increase of ammonia and nitrite oxidizing species only in activated sludge therefore highlights the importance of this process step and these species for effective nitrogen cycling.

The final steps in the reduction of nitrogenous compounds from wastewater is the process of denitrification. Although species able to reduce nitrate to nitrite were more numerous in primary wastewater, nitrate reduction was a common trait to species present in all treatment steps. Similarly, while denitrification is thought to occur in anoxic or low oxygen conditions[319,321], species capable of complete denitrification were present in each step (Figure 1.4, Supplementary file 1). The denitrification microbial community did, however, shift with the distinct environment present in each wastewater treatment step. For example, *Comamonas denitrificans*[89] had high relative abundance in primary wastewater, *Simplicispira sp.*[322] had high relative abundance in both activated sludge and effluent and *Zoogloea caeni*[323] had high relative abundance in effluent.

Seven species of bacteria were classified as phosphorus accumulating organisms (PAOs) across wastewater samples. Phosphorus is an important nutrient for all ecosystems; however, it can also contribute to eutrophication when in excess[94], therefore it is essential to monitor and remove excess phosphorus from wastewater before effluent enters surface water. While two PAOs decreased in relative abundance from primary wastewater to activated sludge, *Tetrasphaera australiensis* and *Pseudomonas aeruginosa,* three PAOs were significantly higher in relative abundance in activated sludge when compared to primary wastewater, *Accumulibacter phosphatis, Gemmatimonas aurantiaca* and *Halomonas phosphatis*, and two PAOs showed no significant differences between primary wastewater and activated sludge (Supplementary file 1). This suggests some PAOs are present and able to accumulate phosphorus throughout wastewater treatment, consistent with findings of enhanced biological phosphorus removal when PAOs are exposed to alternating carbon rich anaerobic and carbon deficient aerobic conditions[324,325]. Alternatively, different species of PAOs could be interchanged between wastewater treatment

steps within the niche. For example, *Tetrasphaera* bacteria have been shown to actively uptake orthophosphate and form polyphosphate under anaerobic conditions[326], whereas *Halomonas phosphatis* is specialized in aerobic phosphate accumulation[98].

All plant polymer degrading species (capable of cellulose, pectin, resistant starch or xylan degradation) decreased from primary wastewater to activated sludge (Figure 1.5, Supplementary file 1). The majority of these species were anaerobic and associated with the human gut. These included the pectin-degrading butyrate producer *Faecalibacterium prausnitzii*[327], the xylan-degrading *Bacteroides fragilis*[328] and the cellulolytic *Bacteroides cellulosilyticus*[329]. Although not measured, biological degradation of plant polymers may be diminished in activated sludge in line with this shift in the microbial community. However, degradation of up to 60% of cellulose has been shown to occur in activated sludge[330]. Surprisingly, many plant polymer degrading species increased from activated sludge to effluent, suggesting the potential continued availability of some plant polymers in effluent, such as cellulose.

Substantial microbial community proportion persists in wastewater effluent

The effluent microbial community retained species of both the primary wastewater and activated sludge; however, species diversity was closer to that of primary wastewater. Three human associated species that had high relative abundance in primary wastewater also had high relative abundance in effluent, *B. vulgatus*, *B. uniformis* and *P. copri*, suggesting prevalent human gut bacteria survive the wastewater treatment process. Other species remained in high abundance in effluent after their increase in activated sludge, such as *Simplicispira sp*. and *U. gangwonense*. Approximately half of the bacterial species associated with humans (49%) significantly differed in relative abundance between primary wastewater and effluent, indicating that at least part was affected by wastewater treatment (Figure 1.6). However, approximately half of human-associated species (51%) did not significantly differ in relative abundance between primary wastewater and effluent, including prominent gut bacteria such as *Escherichia coli,* and *Roseburia faecis*. Some human-associated species, such as *Bacteroides dorei, Bacteroides massiliensis* and *P. copri*, increased in relative abundance between primary wastewater and effluent, suggesting this common wastewater treatment process may provide an environment in which specific human associated species can live. Similarly, a quarter of the species (26%) associated with land animals showed no significant difference between primary wastewater and effluent, indicating no universal reduction of these species during wastewater treatment. The animal-associated species with the highest

relative abundance in primary wastewater, *A. cibarius/cryaerophilus*, also had the highest relative abundance of any species in effluent. Additionally, while some species decreased, other animal-associated species such as *Arcobacter butzleri*, *C. beijerinckii*, *Arcobacter suis*, and *Bacteroidetes graminisolvens* significantly increased in relative abundance. A similar pattern was observed in species associated with fish, where approximately two-thirds of the species (71%) did not significantly differ in relative abundance between primary wastewater and effluent.

Although the culture-independent approaches used here did not test bacterial viability (which can be challenging in high complexity systems where sometimes <1% of species are culturable[331], amplicon data suggests that relatively few species were reduced beyond detection limits from primary wastewater to effluent and many did not significantly change or increased in relative abundance. The lack of significant difference in DNA concentrations between primary wastewater and effluent (Figure 1.2) supports this, although the possibility of lysed non-viable cells but high persistence of intact DNA cannot be discounted (although this is unlikely given the heterogenous differential abundance). A high microbial load in effluent could have deleterious environmental impact to freshwater ecosystems although Canadian government regulations do not specify requirements for microbial content in effluent, with regulation focused on limiting biological oxygen demand, suspended solids, chlorine and ammonia[73].

At least 22 species were present in primary wastewater which have been reported as including pathogenic strains (Figure 1.7, Table 1.2, Supplementary file 1). Species such as *Ruminococcus gnavus*, *B. fragilis*, *C. perfringens* and *A. butzleri* can be pathogens or opportunistic pathogens (but also commensal) in humans[332–339]. Other species have been associated with disease in animals, such as *U. suis*[272] and some species have also been associated with damage to aquatic life, such as *F. succinicans and F. branchiophilum*[340,341]. Of these 22 potentially pathogenic species, 9 decreased significantly from primary wastewater to effluent, including species such as *R. gnavus, U. suis* and *Moraxella osloensis*, and two species were reduced in relative abundance beyond detection limit, *A. baumannii* and *A. haemolyticus*. The removal of the species here before entering surface waters is important so as to not contribute to the spread of this disease; however, nine potentially pathogenic species did not change significantly from primary wastewater to effluent, including *F. branchiophilum, Pseudomonas alcaligenes, B. fragilis* and *C. perfringens*. *F. branchiophilum* is considered a causative agent of bacterial gill disease in trout and salmonids[341]. *P. alcaligenes* has been identified as a causative agent in disease of Chinese

sturgeon[342] and is also implicated in human infections, such as endocarditis[343] and blood infections[344]. Certain strains of *B. fragilis* produce a metalloprotease toxin which has been associated with inflammatory bowel disease and colorectal cancer[345,346]. *C. perfringens*, although a common gut bacterium[347], has been linked to food poisoning[348].

Two potentially pathogenic species significantly increased in relative abundance in effluent from primary wastewater, *A. butzleri* and *F. succinicans*. *F. succinicans* is also considered a causative agent of bacterial gill disease in trout and salmonids (alongside *F. branchiophilum*)[341]. Whereas *A. butzleri* has been associated with diarrhea and abdominal cramping[349–352] as well as a few cases of blood infections[353–355] in humans. Although pathogenic bacteria released into surface waters may not be viable or may not persist in the biosphere, these findings suggest the potential for environmental concern and that high resolution analysis of high complexity metagenomic wastewater samples could have some utility as an additional treatment quality assessment step.

Conclusion

Bacterial species identified in primary wastewater may have originated from the three broad categories of humans, other animals, and the environment. Communities in each treatment step varied significantly from one another. The aeration of activated sludge helped to decrease human and animal-associated bacteria and also enriched taxa with ammonia and nitrite oxidation traits but depleted taxa with traits such as degradation of plant polysaccharides. Anaerobic species persisted in effluent, including some species known to be pathogenic to humans, other animals, and fish. While wastewater treatment plants target the degradation and removal of organic matter which is hazardous to environmental health, these findings indicate there may be value in assessment of microbial communities released into surface water in effluent after treatment. Greater knowledge of the species present and persisting through treatment could help reveal how specific community members influence wastewater treatment efficiencies and any potential direct impact upon the environment.

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Supplementary Data

All raw sequence data is available at NCBI: Bioproject – http://www.ncbi.nlm.nih.gov/bioproject/773401. ESV and differential abundance tables including relative abundance, annotation, count distribution, blast statistics, alternative database hits, and sequences are provided in Supplementary file 1. Evidence supporting species association with humans, other animals or the environment, as well as tables with alpha and beta diversity analysis tables is also provided in supplementary file 1.

Tables

Table 1.1. Measured wastewater characteristics at the three sampled locations within the treatment plant. pH N = 10 per treatment, COD N = 15 per treatment, all others N = 5.

Characteristics	Primary wastewater	Activated sludge	Effluent
pH	7.79 ± 0.04	7.08 ± 0.01	7.21 ± 0.07
COD (mg L^{-1})	446.57 ± 5.59	2302.47 ± 36.10	36.7 ± 2.72
Constituents			
TOC (mg L^{-1})	91.66 ± 4.52	287.80 ± 14.40	15.14 ± 1.30
DOC (mg L^{-1})	65.60 ± 3.19	186.40 ± 3.74	13.30 ± 0.61
Total Phosphorus (mg L^{-1})	4.92 ± 0.21	57.40 ± 0.40	0.39 ± 0.01
Orthophosphate (mg L^{-1})	3.17 ± 0.09	27.70 ± 0.44	0.13 ± 0.003
TKN (mg L^{-1})	38.00 ± 1.14	213.20 ± 3.22	23.00 ± 0.00
Ammonia (mg L^{-1})	46.00 ± 0.00	31.40 ± 0.51	21.00 ± 0.00*
Nitrates-nitrites (mg L^{-1})	3.17 ± 0.09	<0.04 ± 0.00	0.13 ± 0.003

*Ammonia concentrations in effluent exceeded regulatory guidelines

Table 1.2. Potentially pathogenic species present in effluent from a conventional activated sludge wastewater treatment plant. Prescence in effluent and relative change from primary wastewater is provided (presence denotes at least one ESV is present). Fold change from primary wastewater to effluent and differential abundance statistics are available in Supplementary file 1.

Species	Associated hosts	Effluent release	Potential Pathogen To:	Evidence of Potential Pathogenesis
Acinetobacter baumannii	Human (Bouvet and Grimont, 1986), Environment (Bouvet and Grimont, 1986)	Below detection	Humans	Hospital acquired pneumonia (Peleg *et al.*, 2008)
Acinetobacter haemolyticus	Human (Stenzel and Mannheim, 1963; Friederichs *et al.*, 1967; Bouvet and Grimont, 1986), Environment (Bouvet and Grimont, 1986)	Below detection	Humans	Bloody diarrhea (Grotiuz *et al.*, 2006)

Neisseria animaloris	Human (Vandamme *et al.*, 2006)	Present	Humans	Human wound infections (Heydecke *et al.*, 2013)
Ruminococcus gnavus	Human (Henke *et al.*, 2019)	Present – decreased	Humans	Septic arthritis in immunocompromised patient (Titécat *et al.*, 2014), associated with Crohn's disease and produces an inflammatory polysaccharide (Henke *et al.*, 2019)
Stenotrophomonas maltophilia	Environment (Brooke, 2012)	Present – decreased	Humans	Blood stream infections and pneumonia (Looney *et al.*, 2009)
Clostridium tertium	Human (Minerbi *et al.*, 2019)	Present – decreased	Humans	Septicemia (King *et al.*, 1963; Speirs *et al.*, 1988; Valtonen *et al.*, 1990), bacteremia (Miller *et al.*, 2001), meningitis in a 12-year-old child (Kourtis *et al.*, 1997), septic arthritis (Gredlein *et al.*, 2000), enterocolitis (Coleman *et al.*, 1993), peritonitis (Butler and Pitt, 1982), a post traumatic brain abscess (Lew *et al.*, 1990), flesh eating disease and gangrene (Ray *et al.*, 2003)
Pseudomonas aeruginosa	Environment (Hardalo and Edberg, 1997)	Present – decreased	Humans	Bacteremia in severe burn victims, chronic lung infections in patients with cystic fibrosis and acute ulcerative keratitis in contact-lens wearers (Lyczak *et al.*, 2000)
Haemophilus parainfluenzae	Human (Smith *et al.*, 1976; Minerbi *et al.*, 2019)	Present	Humans	Opportunistic urogenital pathogen (Sierra *et al.*, 2020), digestive and biliary tract infections (Frankard *et al.*, 2004)
Bacteroidetes fragilis	Human (Huang *et al.*, 2011; Minerbi *et al.*, 2019)	Present – no change	Humans	Diarrhea (Sack *et al.*, 1992), appendicitis (Elhag *et al.*, 1986), inflammatory bowel disease (Prindiville *et al.*, 2000)
Butyricimonas virosa	Human (Toprak *et al.*, 2015; Minerbi *et al.*, 2019), Animal (Sakamoto *et al.*, 2009)	Present – no change	Humans	Bacteremia (Toprak *et al.*, 2015), bacteremia and bowel disease (Enemchukwu *et al.*, 2016), necrotizing fasciitis (De Donder *et al.*, 2019)
Escherichia coli	Human (Tenaillon *et al.*, 2010), Animal (Wasyl *et al.*, 2013), Environment (Walk *et al.*, 2007)	Present – no change	Humans	Diarrhea (Kaper *et al.*, 2004)
Arcobacter butzleri	Human (P. Vandamme *et al.*, 1992), Animal(P. Vandamme *et al.*, 1992; Rivas *et al.*, 2004), Environment(Rice *et al.*, 1999)	Present - increased	Humans	Foodborne pathogen causing diarrhea (Taylor *et al.*, 1991; Lerner *et al.*, 1994), bacteremia (On *et al.*, 1995; Yan *et al.*, 2000; Lau *et al.*, 2002), recurrent abdominal cramps (P Vandamme *et al.*, 1992)
Acinetobacter lwoffii	Animal (Debarry *et al.*, 2007; Kozińska *et al.*, 2014), Fish (Kozińska *et al.*, 2014)	Present	Fish	Emerging opportunistic pathogens in farmed fish (common carp) (Kozińska *et al.*, 2014)
Flavobacterium branchiophilum	Fish (Wakabayashi *et al.*, 1989)	Present – no change	Fish	May be the cause of bacterial gill disease in rainbow trout (Wakabayashi *et al.*, 1989; Good *et al.*, 2015)
Flavobacterium succinicans	Fish (R. L. Anderson and Ordal, 1961)	Present	Fish	May contribute to bacterial gill disease in trout (Good *et al.*, 2015), associated with furunculosis disease in Chinook salmon (Richard L Anderson and Ordal, 1961)
Uruburuella suis	Animal (Vela *et al.*, 2005)	Present – decreased	Animals	Isolated from lungs and hearts of pigs with pneumonia and pericarditis (Vela *et al.*, 2005), and produces a lipopolysaacharide endotoxin (Silipo *et al.*, 2012)
Moraxella osloensis	Environment (Tan and Grewal, 2001), Human (Adapa *et al.*, 2018)	Present - decreased	Humans and Slugs	Endocarditis (Gagnard *et al.*, 2015), Peritonitis (Adapa *et al.*, 2018), Septicemia (Fritsche *et al.*, 1976) and ocular infections (LaCroce *et al.*, 2019) in humans, fatal pathogen to slugs (Tan and Grewal, 2001)

Aeromonas caviae	Human (Altwegg, 1985), Environment (Callister and Agger, 1987)	Present – no change	Humans and Fish	Watery diarrhea in infants (Namdari and Bottone, 1990b), cytotoxic activities (Namdari and Bottone, 1990a), cystitis (Al-Benwan *et al.*, 2007), bacteremia (Kimura *et al.*, 2013), hepatic and renal lesions in fish (Baldissera *et al.*, 2018)
Aeromonas dhakensis	Human (Esteve *et al.*, 2012; Beaz-Hidalgo *et al.*, 2013), Fish(Beaz-Hidalgo *et al.*, 2013), Environment (Beaz-Hidalgo *et al.*, 2013)	Present – no change	Humans and Fish	Wound infections and necrotizing fasciitis (Chen *et al.*, 2014), bacteremia (Wu *et al.*, 2015), diarrhea (Huys *et al.*, 2002), acute haemorrhagic septicaemia in farmed fish (Carriero *et al.*, 2016), causative agent of fish disease (Soto-Rodriguez *et al.*, 2018)
Pseudomonas alcaligenes	Environment (Marty *et al.*, 1986; Oliveira *et al.*, 2009)	Present – no change	Humans and Fish	Blood stream infections (Suzuki *et al.*, 2013), endocarditis (Valenstein *et al.*, 1983; Martino *et al.*, 1990), bullous keratitis in a thoroughbred mare (Utter and Wotman, 2009), fatal bacterial disease in farmed Chinese sturgeon (Xu *et al.*, 2015), hemorrhagic disease of silver carp (He *et al.*, 1993) and caverned disease in soft-shelled turtles (Qingman *et al.*, 1998)
Acinetobacter schindleri	Human (Nemec *et al.*, 2001), Animal (Reddy and Mastan, 2013)	Present – decreased	Humans and Animals	Nosocomial infections in humans (Forster and Daschner, 1998; Dortet *et al.*, 2006), red eye infections in fish (Reddy and Mastan, 2013)
Clostridium perfringens	Human (Pruteanu and Shanahan, 2013), Animal (Labbe and Juneja, 2017), Environment (Smith, 1975)	Present – no change	Humans and Animals	Food poisoning and necrotic enteritis (García and Heredia, 2011; Sim *et al.*, 2015; Heida *et al.*, 2016), necrotic enteritis in broiler chickens (Olkowski *et al.*, 2008), infection in horses (Gohari *et al.*, 2014), non-haemorrhagic enteric clostridiosis in piglets (Songer and Uzal, 2005)

Figures

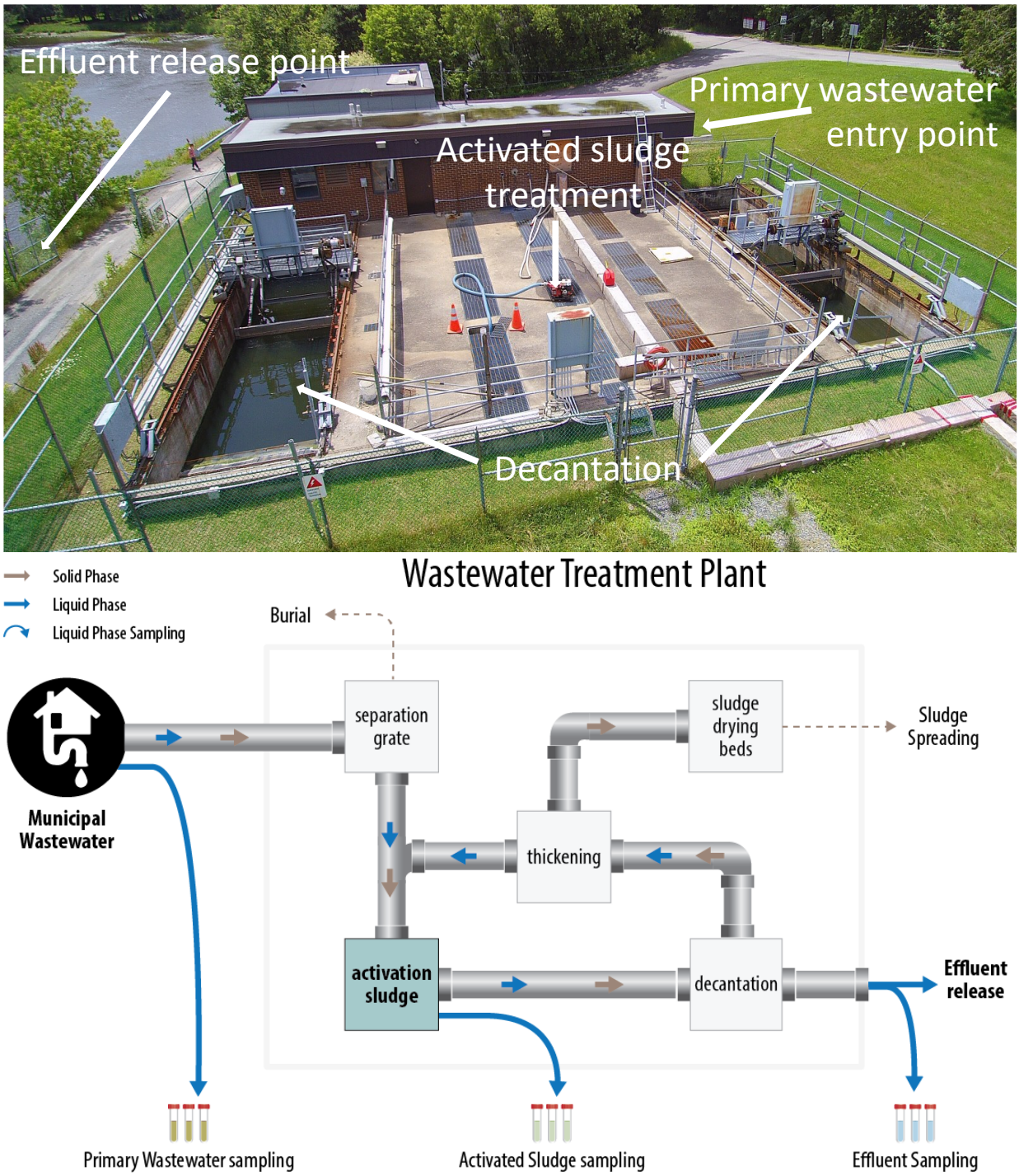

Figure 1.1. Arial photograph of the sampled activated sludge wastewater treatment plant in St. Roch de l'Achigan, Quebec, Canada, and a process schematic highlighting the wastewater sampling points. Liquid phase and solid phase flow during wastewater treatment process is presented in blue and brown.

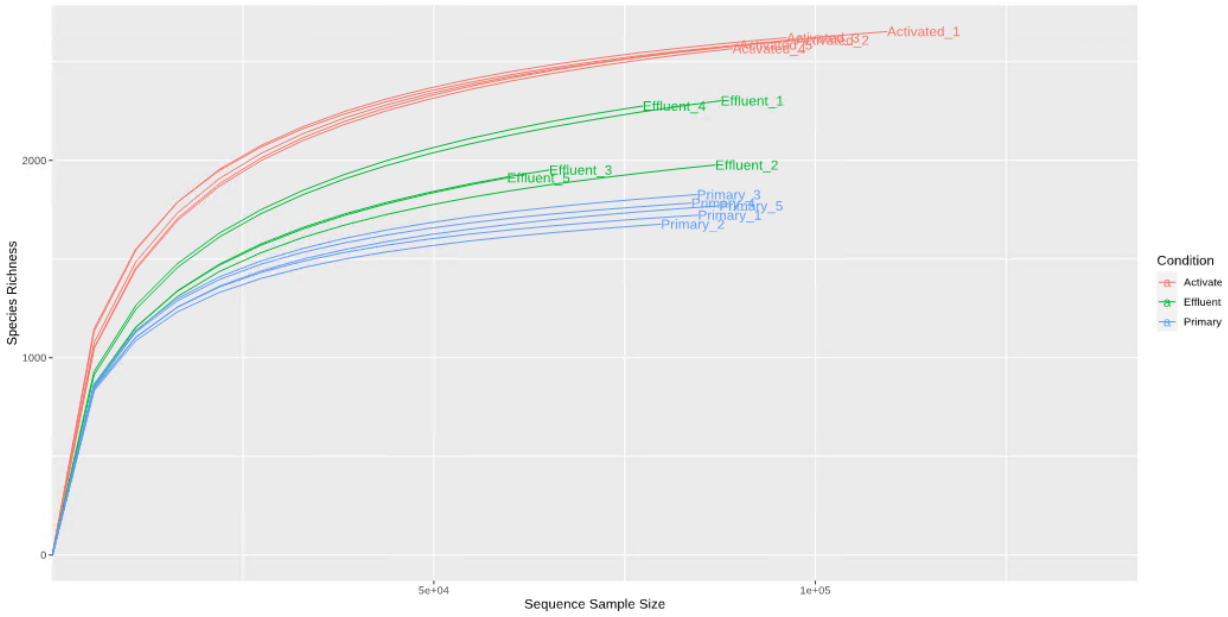

Figure 1.2. Rarefaction curve of ESVs by wastewater treatment plant samples indicating sampling depth captured most of the microbial community.

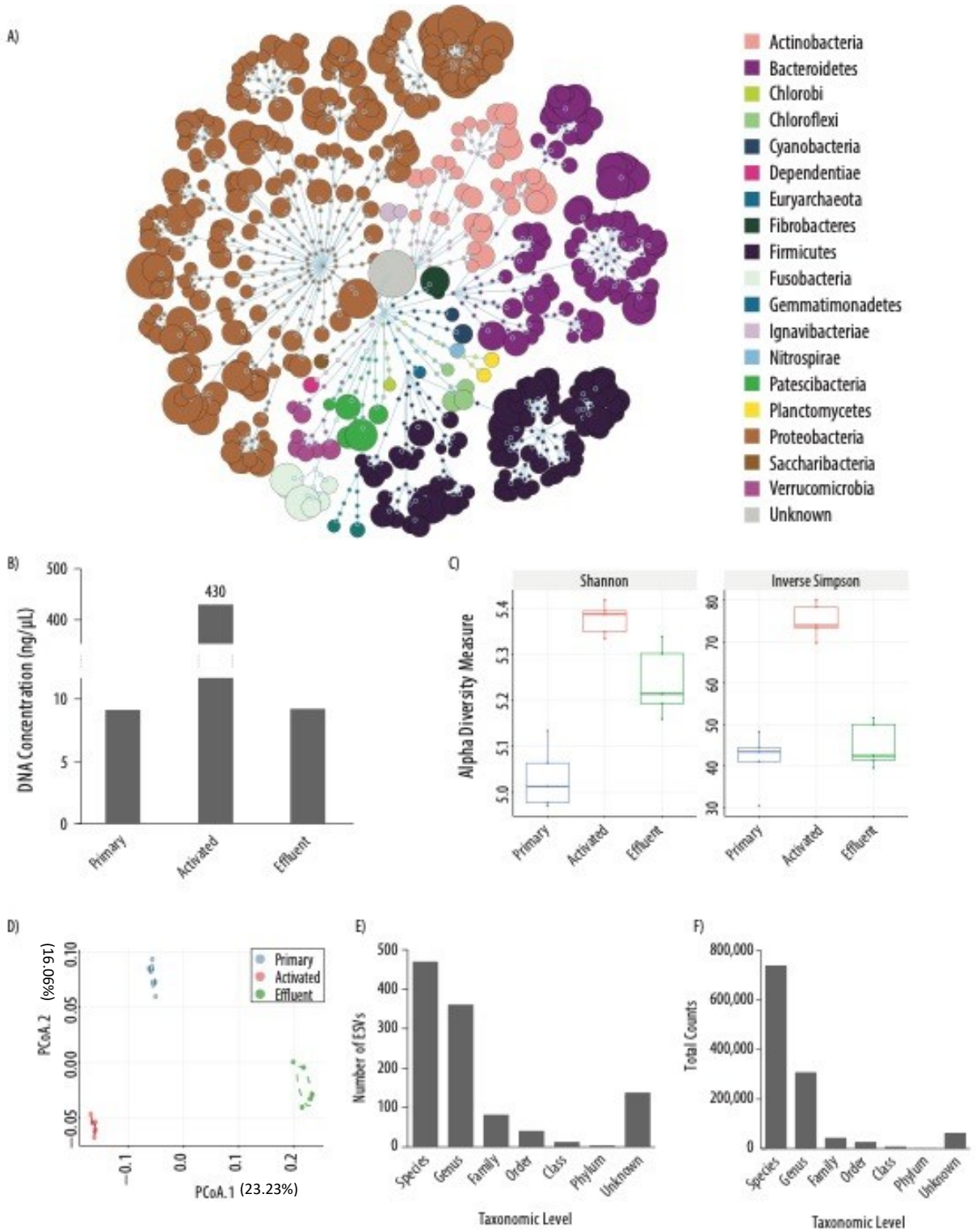

Figure 1.3. Wastewater treatment plant microbial community overview of a) the total microbial community throughout wastewater treatment (*n*=15), b) DNA concentration extracted from primary wastewater (*n*=5), activated sludge (*n*=5) and wastewater treatment (*n*=5), c) Shannon and Inverse Simpson diversity indices of the microbial communities in each treatment step, d) Principal Coordinates Analysis of microbial communities present in each treatment step, e) Number of ESVs per taxonomic category and f) Number of sequence counts per taxonomic category or unknown sequences.

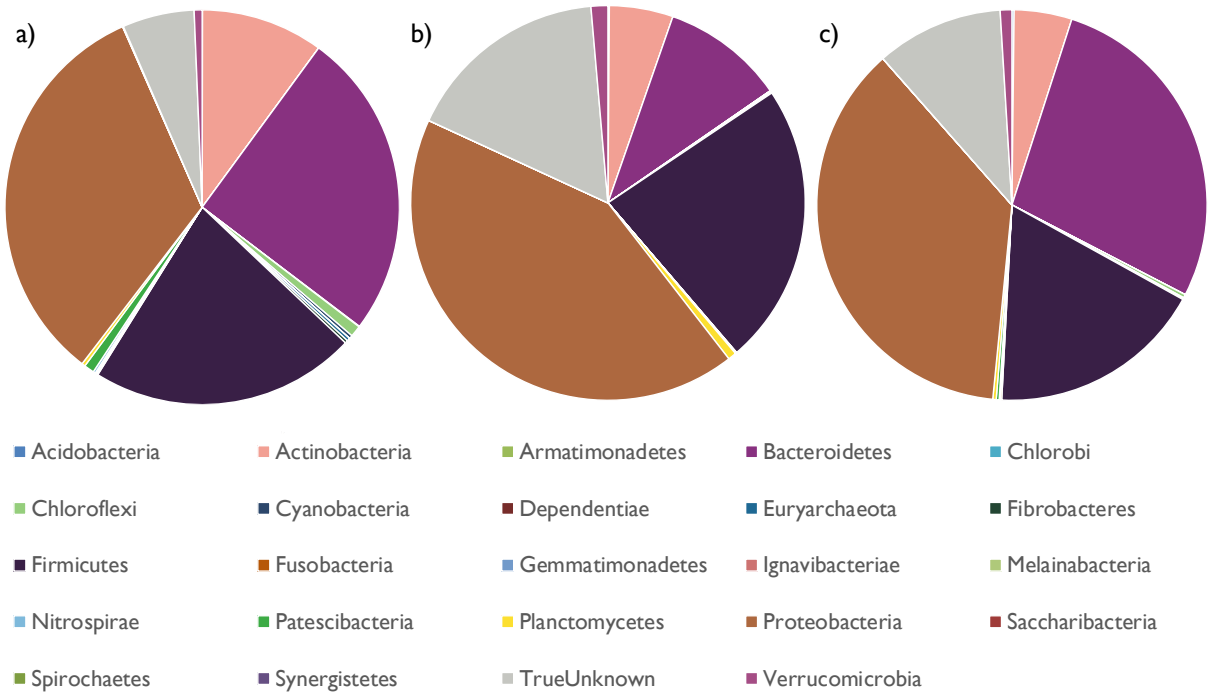

Figure 1.4. Microbial community structure at the Phyla level of the microbial community in a) primary wastewater, b) activated sludge and c) effluent.

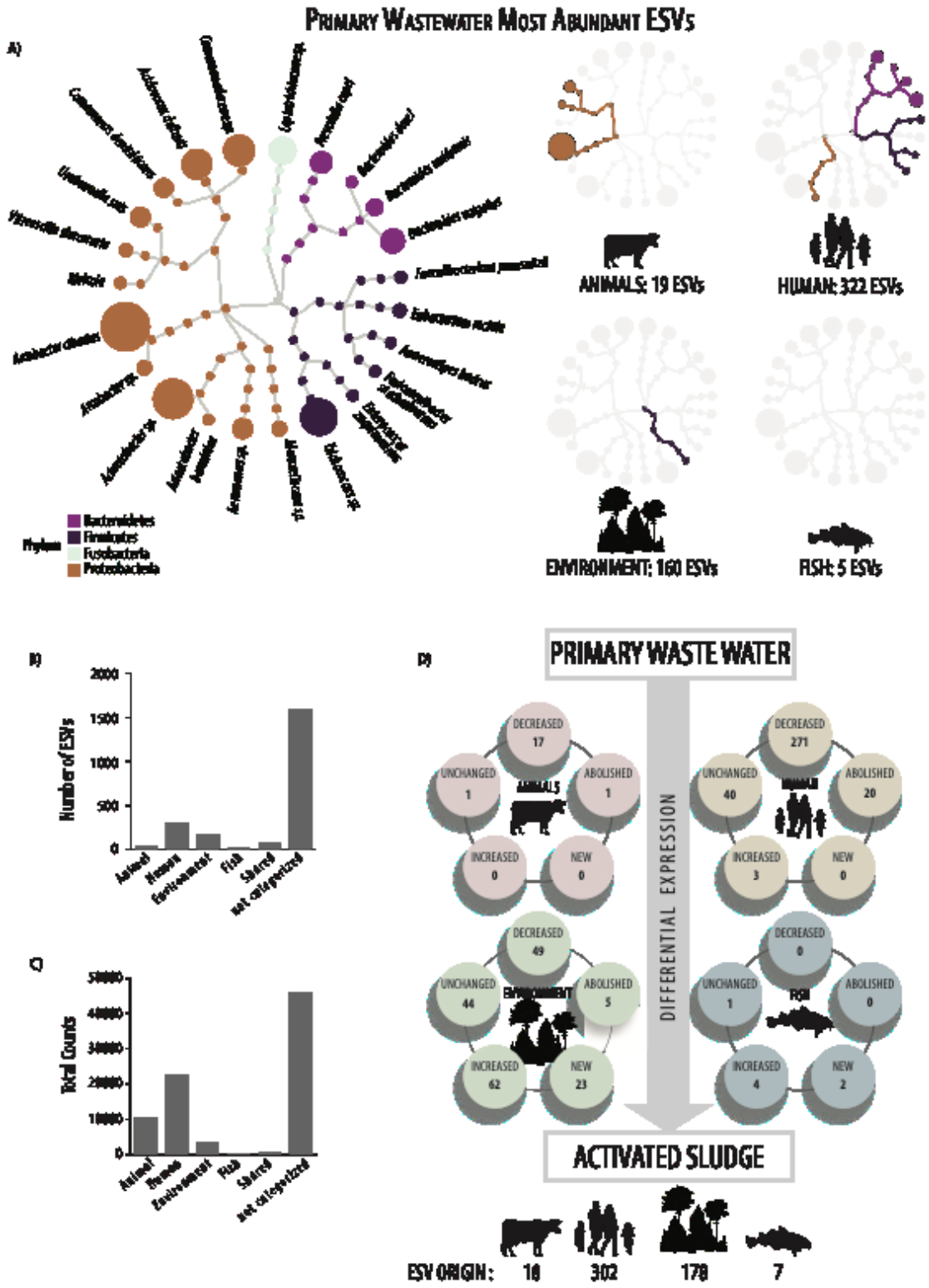

Figure 1.5. Microbial community origin and significant change from primary wastewater to activated sludge. a) Primary wastewater ESVs of highest relative abundance (>0.5% of all primary sequence counts) and their association category, b) Number of ESVs in primary wastewater based on association category with each node shared node representing shared taxonomy, c) Total counts of ESV sequences

in primary wastewater based on association category, d) Number of differentially abundant ESVs between primary wastewater and activated sludge (and unchanged) by association category, as well as the number of ESVs in activated sludge based on association category. Evidence supporting species putative association (in addition to wastewater) with humans, other animals or the environment, as well as ESV relative abundance, annotation, count distribution, blast statistics, alternative database hits, and sequences are provided in Supplementary file 1.

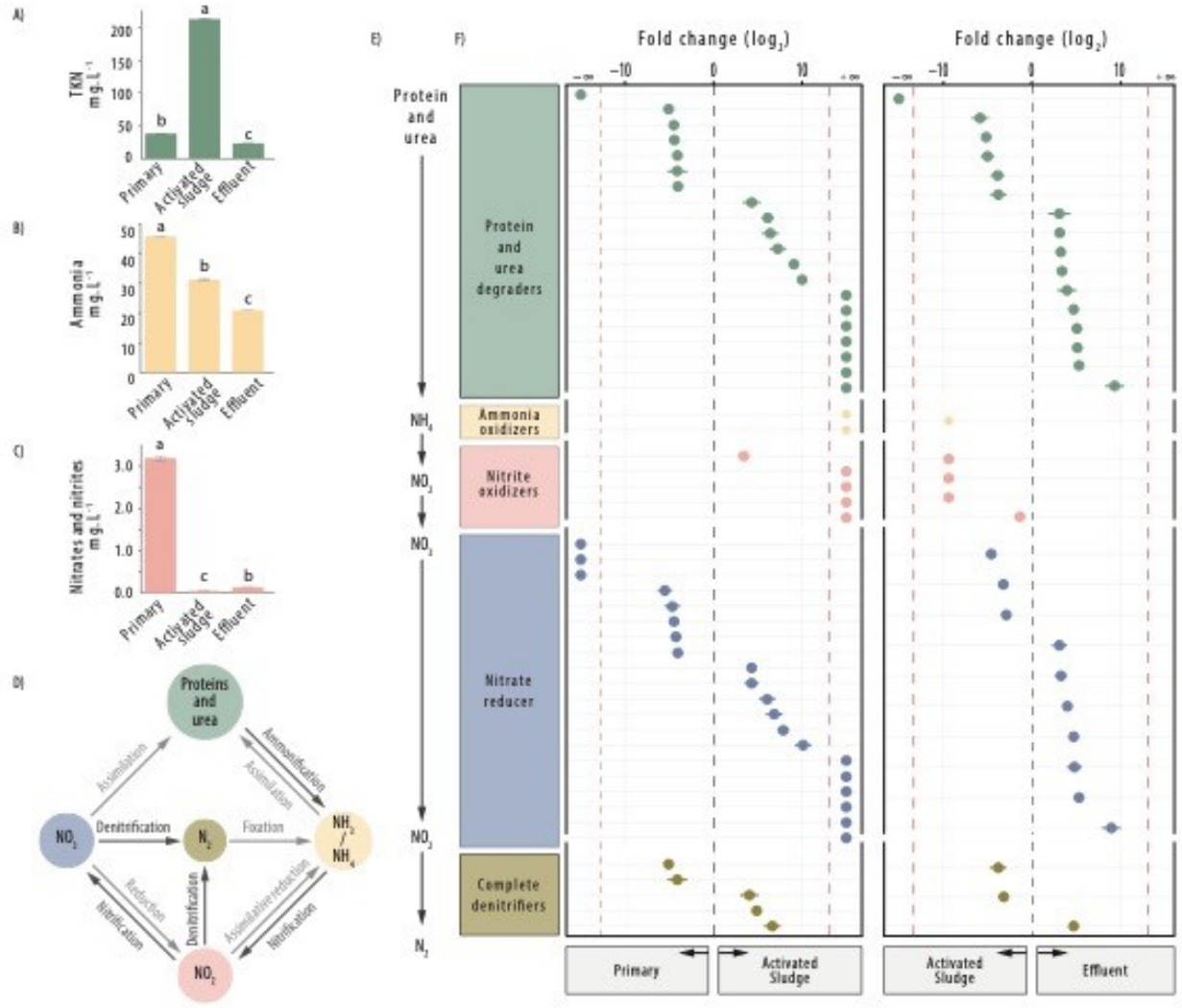

Figure 1.6. Microbial community dynamics associated with nitrogen. a) Concentrations of total kjeldahl nitrogen (TKN, used as a proxy for protein content), b) Concentrations of ammonia and c) Concentrations of nitrates-nitrites throughout the three treatment steps (n = 5 per treatment step), d) Illustration of nitrogen cycle through wastewater treatment, flow of nitrogen in wastewater outlined in dark grey, e) Simplified nitrogen transitions throughout the wastewater treatment process, f) Differential abundance of ESVs associated with nitrogen dynamics from primary wastewater to activated sludge, and activated sludge to effluent. ESVs past the dashed line represent detection in one condition only. Evidence supporting species functional categorisation, ESV relative abundance, annotation, count distribution, blast statistics, alternative database hits, and sequences are provided in Supplementary file 1.

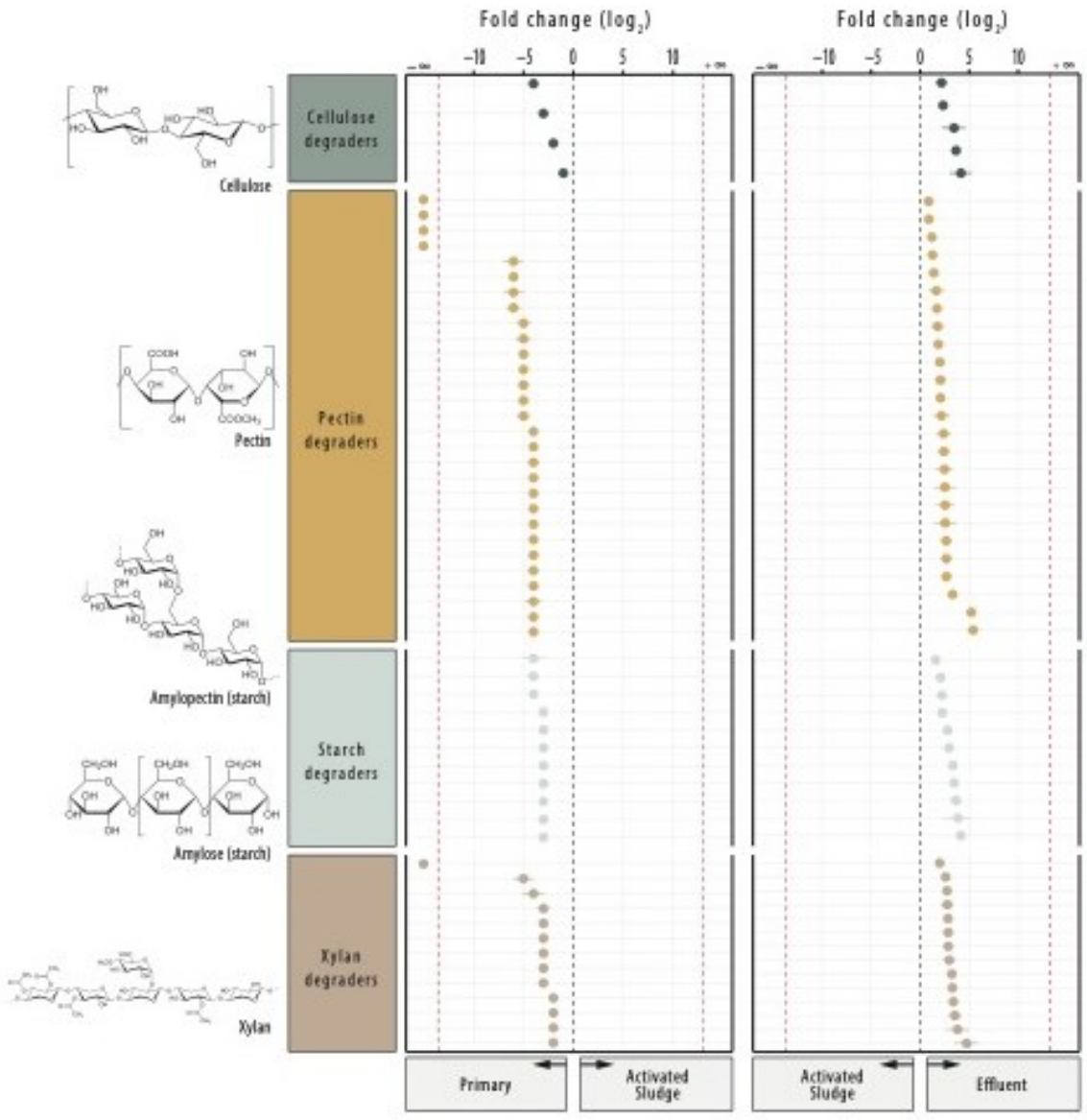

Figure 1.7. Microbial community dynamics associated with plant polymer degradation. Differential abundance of ESVs associated with plant polymer degradation between primary wastewater and activated sludge and activated sludge and effluent. ESVs past the dashed line represent detection in one condition only. ESV relative abundance, annotation, count distribution, blast statistics, alternative database hits, and sequences are provided in Supplementary file 1.

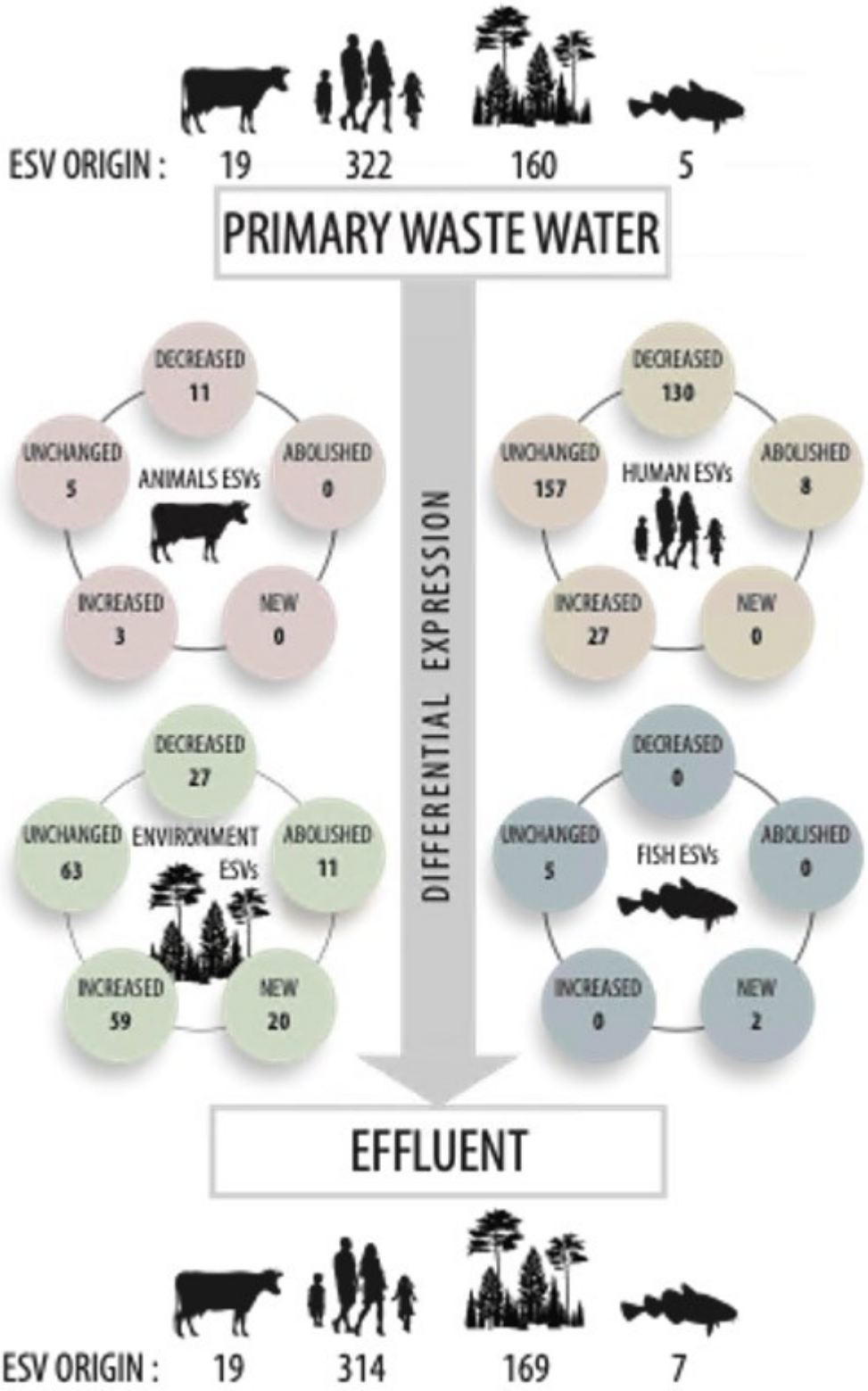

Figure 1.8. Number of differentially abundant ESVs between primary wastewater and effluent (and unchanged) by association category, as well as the number of ESVs in primary wastewater and effluent based on association category. Evidence supporting species putative association (in

addition to wastewater) with humans, other animals or the environment, as well as ESV relative abundance, annotation, count distribution, blast statistics, alternative database hits, and sequences are provided in Supplementary file 1.

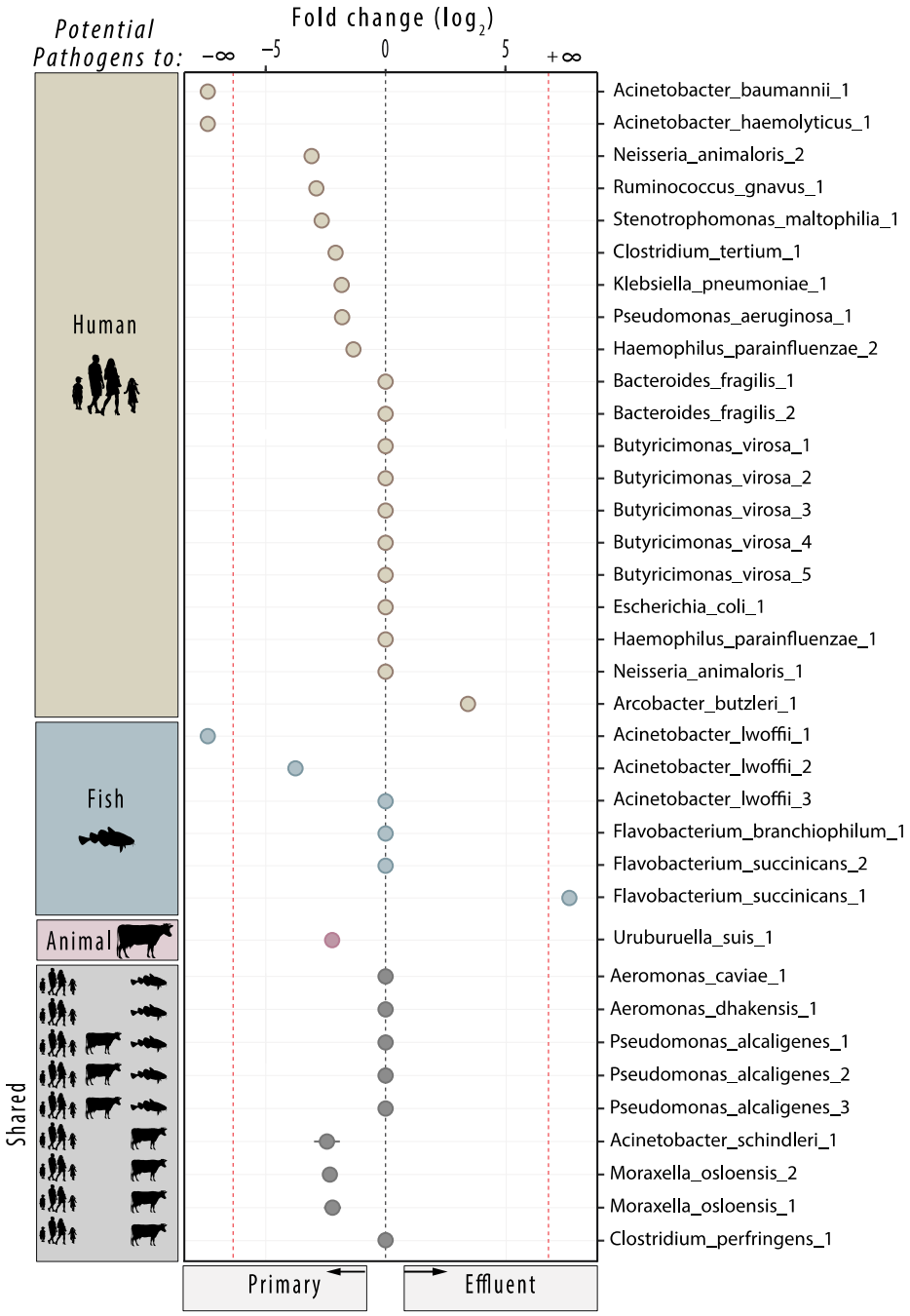

Figure 1.9. Differential abundance of ESVs annotated to potentially pathogenic species between primary wastewater to effluent. ESVs with a fold change of zero represent no significant difference (persistence) between primary wastewater and effluent. ESVs past the dashed line represent detection in one condition only. ESV relative abundance, annotation, count

distribution, blast statistics, alternative database hits, and sequences are provided in Supplementary file 1.

Chapter 2

Willow rhizosphere microbial community reaction to crop irrigation with municipal wastewater

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Nicholas Brereton – conducted editing

Emmanuel Gonzalez – bioinformatic processing

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Frederic Pitre – supervision

Willow rhizosphere microbial community reaction to crop irrigation with municipal Wastewater

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Abstract

Wastewater (WW) is a problem for societies as conventional infrastructure and treatment can be costly and damage the environment. Phytoremediation of WW with short rotation willow coppice and its associated rhizosphere microbial community offers an alternative technology to remediate WW while simultaneously producing plant biomass for potential extractable phytocompounds and biofuels. The willow rhizosphere microbial community can help nutrient acquisition, pathogen control and contaminant degradation; however, the impact of WW irrigation upon this microbial community has yet to be explored. This study aimed to determine the effects of WW irrigation on the willow rhizosphere microbial community to establish if WW irrigation increases or decreases beneficial bacteria such as plant growth promoting or pollutant degrading bacteria to evaluate phytofiltration as a sustainable method for WW treatment. Rhizosphere soil samples from a field trial of control (unirrigated), potable water (PW) irrigated, and WW irrigated willows were assessed. From a total 3,707 identified Exact Sequence Variants (ESVs), 598 were annotated to species-level. From the control rhizosphere microbial community 18% were resolved to species level, many of these ESVs were annotated as species associated with plant growth promoting abilities. Thirty-four specie-level ESVs significantly differed between control soil and PW irrigated soil, 31 of which decreased in PW irrigated soil compared to the control with 83.87% being from Actinobacteria. One-hundred and sixteen ESVs significantly differed between control soil and WW irrigated soil, 110 of which increased in WW irrigated soil compared to the control with 51.81% being from Proteobacteria, 34.55% from Actinobacteria and 9.09% from Bacteroidetes. Additionally, 40 ESVs not present in control soil were present in WW irrigated soil

90% being from Proteobacteria. Many of the species which increased in relative abundance were associated with plant growth promoting traits, such as nitrogen fixation, and stress tolerant traits, such as hydrocarbon degradation. Additionally, plant growth promoting bacteria were detected in the WW irrigated rhizosphere, such as the nitrogen-fixing *Azoarcus communis* and the sulfur-oxidizing *Thiobacillus denitrificans* and *Thiobacillus sajanensis*, which were not detected in control or PW irrigated rhizospheres. These findings suggest WW irrigation could lead to an increase in beneficial bacteria in the rhizosphere and illustrate the large influence WW has on the abundance and trait selection of the rhizosphere community, which may facilitate the increase in biomass of willow trees.

Introduction

Wastewater (WW) generated from human activities can cause negative environmental impacts as well as cause and spread disease if not properly treated. Globally, it is estimated that 271 trillion litres of household WW are generated each year[231]. In Canada that number is approximately 5.8 trillion litres per year[62]. Conventional WW treatment is costly and in many smaller and rural areas the cost of WW treatment infrastructure can be a large burden or may dictate only minimal treatment infrastructure exists[16]. Untreated or undertreated WW released into the environment, specifically surface waters, can lead to eutrophication as well as direct toxicity to aquatic life[49–51]. When looking through a different lens, however, WW can be seen as a resource as it is a source of nutrients such as nitrogen and phosphorus as well as organic matter. These constituents in WW can be used as fertilizers for plants. For example, nitrogen and phosphorus are growth limiting elements that are required for all life to flourish[356] and in WW they often exist in forms readily available for plant uptake[357].

Phytoremediation, that is, the use of plants and their associated microorganisms to clean contaminants in the environment, could offer a low-cost alternative to conventional WW treatment[358] and allow for the use of WW as a resource[12]. Phytoremediation is the culmination of a series of scientific discoveries over the past few centuries[359–361] consisting of mechanisms inherent to plants such as phytoextraction, phytodegradation, rhizofiltration, phytostabilization and phytovolatilization[362], and can be employed to remediate both organic and inorganic contaminants from the environment. Nitrogen and phosphorus, considered inorganic contaminants, are also necessary for growth. In the correct forms these can be absorbed through roots and contribute to

plant growth. Heavy metals, also considered inorganic contaminants, are non-degradable by any biological or physical process and are persistent in soil for long periods[363]. Metals can be essential to organisms or non-essential. Essential metals, like copper, iron, manganese, nickel and zinc, which are necessary for physiological and biochemical processes for plants[364], may become toxic when in excess[365]. Non-essential metals such as lead, cadmium, arsenic and mercury, are highly toxic with no known function in plants[366] and may negatively affect plant growth[367]. Certain plants and their associated microorganisms, such as *Arabidopsis halleri* are capable of phytoextraction of heavy metals from soil or can lower their bioavailability in soil[368]. In the case of organic contaminants, such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and more recently emerging contaminants such as pharmaceuticals, can be extracted, degraded, volatilized, or complexed by species such as *Medicago sativia*[369], *Panicum virgatum*[369], *Zea mays*[370], *Triticum aestivum*[370]. These processes can occur directly by uptake into plants or within the rhizosphere. Direct uptake of organic contaminants is dependent on the availability and mechanism of transport involved[362]. Contaminants can remain in plant tissue or may be degraded enzymatically. In the rhizosphere organic chemical contaminants can be degraded or complexed in the soil by plant exudates[362,371]. Plant exudates can also recruit members of the rhizosphere microbial community capable of contaminant degradation or plant growth promotion.

The diversity of the microbial community provides the rhizosphere with a multitude of genes capable of many different functions. Different species of microorganisms can produce different molecules that are used as plant hormones such as auxins (e.g. indole acetic acid (IAA)), gibberelin, cytokinin, ethylene and abscisic acid as well as enzymes such as 1-aminocyclopropane-1-carboxylate deaminase (ACCd). These molecules are or help create different phytohormones. For example, IAA helps in the production of longer roots with increased number of root hairs and root laterals which are involved in nutrient uptake[372] as well as helps increase plant height, the number of leaves per plant, and the fruit size resulting in higher seed yield[373–375]. ACCd degrades ACC, the immediate precursor of ethylene in higher plants[376], and can help with plant growth in stressed environments such as in salt[377] and hydrocarbon pollution[378]. Rhizosphere species can also help with the acquisition of nutrients. Nitrogen is one of the essential nutrients for all life[379] and is often a limiting factor for growth[380]. Diazotrophic bacteria such as *Frankia alni*[381], turn atmospheric nitrogen into ammonium in the rhizosphere for use by plants and other microorganisms[382]. Phosphorus is another growth limiting nutrient that greatly affects the growth

of both bacteria and plants[383]. Phosphorus is generally abundant in soils and WW as both inorganic and organic phosphorus, however, only approximately 0.1% of the total phosphorus in soils exists in a soluble form for plant uptake[384]. Inorganic phosphorus exists in soil in forms such as insoluble mineral complexes[385] which cannot be absorbed by plants[386]. Inorganic phosphorus can be released from these complexes and converted into plant soluble forms by different organic acids (e.g. citric or oxalic acid), inorganic acids (e.g. sulfuric acid) or by soil acidification (i.e. the release of protons)[387]. Organic phosphorus mineralization occurs by way of phosphatase enzymes that break down organic phosphorus therefore making phosphorus available for uptake[386]. In rhizosphere soils different microorganisms, such as *Pseudomonas aeruginosa* or *Aeromicrobium ginsengisoli*, can release phosphorus through solubilization and mineralization[388–390]. Iron is also an essential nutrient for organisms[391]. Most iron existing in the environment exists in an insoluble form for organism uptake. Siderophores, iron-chelating molecules secreted by bacteria, such as *Pseudomonas fluorescens* can solubilize iron (transport iron across cell membranes)[392,393] Although plants can also produce siderophores, bacteria siderophores can efficiently provide iron to plants[394]. Sulfur, another nutrient necessary for plant life, is available for plant uptake as sulfate[395]. Certain bacteria are capable of sulfur oxidation to sulfate, such as *Mesorhizobium thiogangeticum*[396]. Other bacteria produce various antibiotics, like *Streptomyces*[397], or can degrade polymers in fungal cell walls, like *Bacillus licheniformis*, and thereby offer protection against phytopathogens[398]. Furthermore, bacteria exists in the rhizosphere that are capable of degrading organic chemicals such as *Bacillus subtilis*[399] or emerging contaminants like acetaminophen such as *Pseudomonas moorei*[400], making the rhizosphere a potential tool to remediate contaminated environments and water resources.

According to the UN, WW is used to irrigate crops in some developing countries, with great success for increased crop productivity[12]. There are some considerations and risks that arise with WW irrigation of crops such as health concerns for farmers and consumers. Farmers in developing nations using WW to irrigate crops are exposed to potentially hazardous bacteria present in WW. Skin conditions and illnesses are often reported by farmers and their families that use WW for irrigation. There is also the risk of food crops contaminated with hazardous bacteria, with the potential to spread or cause disease in the consumer.

As outlined in the previous chapter, the bacteria in the municipal WW microbial community came from different origins and were associated with varying functions. A large

portion of the bacteria was from humans, especially the human gut such as *Prevotella copri*[283], *Bacteroides uniformis*[401] and *Bacteroides vulgatus*[402], which were relatively high in abundance in primary WW samples. *Arcobacter cibarius*, was the most abundant species in primary WW samples and was associated with animals[270,271], along with other relatively abundant species such as *Uruburuella suis*[272] and *Vitreoscilla stercoraris*[403]. Species facilitating nitrogen cycling in WW were present such as extracellular protease producers, nitrite and ammonia oxidizers such as *Nitrospira nitrosa*[84], and denitrifiers such as *Comamonas denitrificans*[89]. Phosphorus accumulating organisms were present such as *Halomonas phosphatis*[98], as well as plant polymer degrading species like *Bacteroides fragilis*[328]. Other species were present that were linked with disease in humans and animals such as *Arcobacter butzleri*[350,351,353–355,404,405]. Although bacteria in WW were associated with various functions, it is still unknown how the species in the WW microbial community will interact with the rhizosphere.

This current study was a part of a larger overarching project assessing various aspects of using phytofiltration to treat WW while simultaneously increasing biomass of a short rotation willow coppice for biorefinery potential (Figure 2.1). Concurrent studies of the same field trial found that short rotation willow coppice allowed efficient removal of organic matter in WW irrigated rhizospheres (91 ± 6% of COD) with average soil pore water COD concentrations of 18 ± 6 mg/L and 21 ± 9 mg/L for PW irrigated and WW irrigated rhizospheres, respectively[406]. Short rotation willow coppice also removed nitrogen at 98 ± 1% removal of TKN with average soil pore water TKN concentration of 0.6 ± 0.3 mg/L and 0.7 ± 0.2 mg/L in PW irrigated and WW irrigated rhizospheres, respectively as well as 94 ± 11% removal of TN with average soil pore water concentrations of 0.7 ± 0.3 mg/L and 2.0 ± 2.7 mg/L in PW irrigated and WW irrigated rhizospheres, respectively, and almost complete removal of total phosphorus (98 ± 1%) with average soil pore water TP concentrations of 0.08 ± 0.03 and 0.07 ± 0.02 mg P/L, additionally o-PO^4 (orthophosphate) concentrations in soil pore water measured 0.02 ± 0.02 and 0.02 ± 0.02 mg P/L in PW irrigated and WW irrigated rhizospheres, respectively, however with higher irrigation rates, available phosphorus in soil significantly increased suggesting an eventual phosphorus soil profile saturation with continued WW irrigation. Lachapelle et al. (2019)[406] also observed an imbalance between irrigation and willows needs with a constant hydraulic loading rate indicating a need for modulation of hydraulic loading following the seasonal transpiration trends of willow. Jerbi et al. (2020)[407] found an increased leaf area, leaf nitrogen content, chlorophyll a + b content,

higher stomatal sizes and higher stomatal pore index but lower stomatal density, resulting in increased stomatal conductance and ultimately a substantial increase in biomass yield. Finally, Sas et al. (2021)[201] found that the persistent extractable phytochemical profile shifted with WW irrigation and biomass increased up to 200%. WW irrigation subjected trees to high loads of water, macronutrients, and salts (29.5 million L of water ha^{-1} yr^{-1}, 1245 kg nitrogen ha^{-1} $yr^{-1}$and 121 kg phosphorus ha^{-1} yr^{-1}, and 4.31 t sodium ha^{-1} $yr^{-1}$and 5.63 t chlorine ha^{-1} yr^{-1}). Sas et al. (2021)[201] also found that metal concentrations for chromium, nickel, arsenic, cadmium, lead, zinc and copper in biomass did not vary between control and WW irrigated trees, however iron was significantly higher in WW irrigated trees, increasing from 13.14 ± 0.35 mg iron kg^{-1} to 25.72 ± 1.00 mg iron kg^{-1} dry matter. Overall, these concurrent studies showed that WW phytofiltration using short rotation willow coppice offers a viable WW treatment method able to reduce environmental burdens of WW treatment by allowing for the reduction of WW contaminants such as organic matter, nitrogen and phosphorus while increasing production of biomass with the caveat of modulation of hydraulic loading rate to match seasonal transpiration trends of willow as well as soil nutrient and salt loading over time. While these studies have demonstrated the successful treatment of primary WW using phytofiltration, the microbial mechanisms that help facilitate WW phytofiltration have yet to be explored.

The use of phytoremediation to address the increasing problem of WW treatment has the potential to solve pollution issues associated with WW while simultaneously using it as a resource. Phytoremediation shows the potential to remediate WW and provide nutrients to help crop growth, although the use of WW to irrigate crops for consumption has the potential to spread disease to consumers and amongst farmers. Agricultural crops not for consumption, but for biofuel and extractable phytochemical production such as short rotation willow coppices may provide a way of employing phytofiltration to treat WW while simultaneously fertilizing a crop for biomass production and decreasing potential human exposure to WW pathogens. Although phytoremediation of WW has been well studied, the underlying mechanisms, that is the microbial community, of WW irrigation has yet to be explored. This study aimed to uncover how a willow rhizosphere bacterial community changes when irrigated with primary WW to infer potential roles of different bacteria in the remediation process.

Methods

Experimental design

A short rotation willow coppice was established as a vegetation filter at a site in Saint-Roch-de-l'Achigan to test the efficacy of irrigation with WW. The site consisted of a two-hectare willow plantation (*Salix miyabeana* 'SX67' at 16000 plants/ha). The site was originally established in 2008 and harvested in 2011 and 2015. The willow coppice field was located near the municipal wastewater treatment plant (WWTP) of St-Roch-de-l'Achigan, Quebec, Canada (Figure 2.1). For detailed experimental design see Amiot et al. (2020)[408], Lachapelle-T. et al. (2019)[406] and Jerbi et al. (2020)[407].

For the current study, the established willow coppice (three-year-old stems on ten-year-old rootstock) was subjected to one of three treatments: no irrigation (control), irrigation with potable water (PW) and irrigation with wastewater (WW). Each treatment consisted of three plot replicates (each plot measured 108 m^2 or 10 m x 10.8 m) receiving the treatment for a total of nine experimental plots. Within each plot four rows of six were irrigated (72 m^2 irrigated out of 108 m^2).

WW composition contained a pH of 7.79 ± 0.04, a COD of 446.57 ± 5.59 mg L^{-1}, a total organic carbon (TOC) concentration of 91.66 ± 4.52 mg L^{-1}, a dissolved organic carbon (DOC) concentration of 65.60 ± 3.19 mg L^{-1}, a total phosphorus (TP) concentration of 4.92 ± 0.21 mg L^{-1}, an orthophosphate (OP) concentration of 3.17 ± 0.09 mg L^{-1}, a total kjeldahl nitrogen TKN concentration of 38.00 ± 1.14 mg L^{-1}, an ammonia concentration of 46.00 ± 0.00 mg L^{-1}, a nitrates and nitrites concentration of 3.17 ± 0.09 mg L^{-1} as stated in the previous chapter. Both PW and WW were applied at a rate of 10 mL per day from May 5th 2018 to July 8th 2018 and at a rate of 16 mL per day from July 9th until August 26th 2018 to the 72m^2 irrigated portion of the 108m^2 plots.

Sample collection

For information of WW sample collection please refer to the previous chapter. Rhizosphere soil samples were collected on August 27th, 2018, from a willow field in St. Roch de l'Achigan (Figure 2.1). Allotted plots in this field had been subjected to three treatments, a control (un-watered), PW (total volume over growing season: 1872 mm) and WW (total volume over growing season: 1872 mm). Irrigation with each given treatment was turned off the day before sampling. Rhizosphere samples were collected from the surface of the soil (due to majority of roots being at

the surface). The willow rhizosphere was a thick intertwined mat of roots, samples were taken from breaking off sections of the thick mat of roots to access the soil along with the roots. Thirty-six soil samples were taken (four from each plot) from a distance between 10-30 cm from the base of the willow trees from opposite sides of the tree. Samples were homogenized, and composite samples were put into 50 mL falcon tubes for DNA extractions. These were immediately flash frozen in liquid nitrogen and then stored on dry ice until they could be stored in a -80ºC freezer. Samples to conduct soil phenotype analyses were taken and stored on ice for transport.

Soil constituent and characteristic determination

Soil analyses were performed in-house or outsourced to EnvironeX labs or AgroEnvironLab. Soil analyses measured pH, total nitrogen, ammonia, nitrate-nitrite, and total phosphorus, orthophosphate, total organic carbon, and total carbon. Ammonia and nitrates-nitrites analyses were conducted in house. Soil was sifted to remove root and wood pieces, ammonia and nitrates-nitrites were extracted from damp soil using a 2M KCl extraction. These solutions were analysed for ammonia and nitrates-nitrites using Lachat flow injection analysis and the resulting ammonia and nitrates-nitrites concentrations in solution were calculated to amounts in soil using the formula:

$$C = \frac{AVF}{P} \times \frac{100}{100 - H}$$

Where C is the concentration of ammonia or nitrates-nitrites in soil (mg/kg), A is the ammonia or nitrates-nitrites in solution (mg L^{-1}), V is the volume of extraction (mL), F is the dilution factor if necessary, P is the weight of the soil for extraction (g), $\frac{100}{100-H}$ is the conversion factor to express the result on a dry basis taking into account the percentage of humidity H (%) of the sample[409,410]. Wet soil samples were sent to EnvironeX labs for analysis of phosphorus and orthophosphate. Phosphorus was determined using organic solid digestion and ICP-MS[411] whereas orthophosphate was determined using ionic chromatography[412]. Remaining portions of samples were then air dried at room temperature and sent to AgroEnvironLab for pH, total nitrogen, organic materials, and total carbon (C:N ratio). Total nitrogen, organic materials and pH analyses were performed according to the *Méthode d'Analyse des Sols, des Fumiers et des Tissus Végétaux* from the *Conseils des Productions Végétales de Quebec*[413]. Total nitrogen analysis used the Mehlich method (ME-1) with ICP-OES (Agilent brand, model 725-OES). Organic material content was

determined by the incineration method (MA-2) and pH was determined using the PH-1 method. Total carbon (C:N ratio) was measured by combustion method using an elemental analyzer (LECO brand, TruMac CNS model).

Additional WW characteristics

WW samples collected July 2018 were analysed for content of emerging contaminants using HPLC by an environmental chemistry laboratory at the University of Montreal.

DNA extraction, 16S rRNA gene amplification, sequencing, and amplicon processing

Soil samples were first ground using a mortar and pestle under liquid nitrogen to safeguard as much genetic material as possible then DNA was extracted from ground soil samples using a QIAGEN DNeasy PowerSoil DNA Extraction kit. Extractions were checked and nucleic acid content was determined using a Nanodrop 2000 spectrophotometer and running a 1% electrophoresis gel in TAE buffer. Primers for 16S rRNA gene amplification targeted the V5-V6 region: P609D 5'-GGMTTAGATACCCBDGTA-3'and P699R 5'-GGGTYKCGCTCGTTR-3'[230]. PCR amplification and sequencing were performed by Genome Quebec at McGill University using the MiSeq250 platform was used for 2 x 250 bp paired-end sequencing of PCR products. PCR conditions were an initial denaturation step of 98°C, for 30 secs, before 23 cycles of 98°C, for 10 secs, 58°C, for 15 secs and 72°C, for 30 secs, with the final extension at 72°C, for 2 min. Reagent controls were below the detection limit used by Genomic Quebec Innovation Centre for quality assurance. Sequence counts were processed and annotated using the ANCHOR pipeline[253]. Briefly, sequences were aligned and dereplicated using Mothur[254] before selection of exact sequence variants (ESVs) using a count threshold of 5 across all samples. Annotation queried four sequence repositories with strict BLASTn criteria (>99% identity and coverage): NCBI curated bacterial and Archaea RefSeq, NCBI nr/nt, SILVA, Ribosomal Database Project (RDP). Database versions were from January 2020; all annotation is considered putative and subject to improvement as database errors are resolved and new species are characterized. When the highest sequence identity is shared amongst multiple species, all are retained and reported (ESVs including the suffix MS for multiple species).

Diversity and differential abundance analysis

Alpha diversity was measured using Shannon and Inverse Simpson, Observed and Chao1 diversity indices within the Phyloseq package[255], and was compared between treatment steps of

samples with t-tests. Analysis of principle coordinates (PCoA) ordination was performed based on Bray-Curtis ecological distances using the Phyloseq package[255] and dispersion ellipses were drawn using the veganCovEllispse function from the Vegan package[256] in R[257]. Differential abundance analysis on 16S rRNA gene amplicons was performed using DESeq2[258,259], which can perform well with uneven library sizes and sparsity common to 16S rRNA gene data[253,260]. Sparsity and low-count cut-offs were applied whereby an ESV count in a single sample is <90% of the count in all samples, and ESV counts must be >2 in 40% of the samples[253,261]. A false discovery rate (Benjamini-Hochberg procedure) < 0.1 was applied[258,262].

Results

Soil composition

Soil samples from a naturally irrigated (rainwater) willow rhizosphere (control), a PW irrigated willow rhizosphere and a WW irrigated rhizosphere were characterized by measuring pH, total nitrogen, ammonia, nitrate-nitrite, total phosphorus, orthophosphate, total organic carbon, and total carbon and iron. The control soil pH of 5.96 ± 0.04 was significantly lower than the PW irrigated soil pH of 6.59 ± 0.07 and the WW irrigated soil pH of 6.17 ± 0.09, the pH of WW irrigated soil was also significantly lower than irrigated soil (Table 2.1; Figure 2.2). Total nitrogen was not significantly different between control soil at 1900 ± 100 mg kg^{-1} and PW irrigated soil 1800 ± 100 mg kg^{-1}, however it was significantly different between control soil and WW irrigated soil at 2300 ± 100 mg kg^{-1} as well as between PW irrigated soil and WW irrigated soils. Ammonia in control soil at 3.30 ± 0.65 mg kg^{-1} was significantly lower than in PW irrigated soil at 20.74 ± 2.96 mg kg^{-1} and in WW irrigated soil at 9.58 ± 2.92 mg kg^{-1}. Nitrates-nitrates in control soil were 7.32 ± 0.62 mg kg^{-1} and were not significantly lower than in PW irrigated soil at 27.09 ± 7.23 mg kg^{-1} which were both significantly lower than in WW irrigated soil at 173.90 ± 34.44 mg kg^{-1}. Total phosphorus was 934.00 ± 16.80 mg kg^{-1} in control soil and was not significantly lower than PW irrigated soil at 980.33 ± 115.23 mg kg^{-1} which was not significantly lower than WW irrigated soil at 1001.33 ± 29.70 mg kg^{-1}. Orthophosphate was 2.33 ± 0.33 mg kg^{-1} in control soil but was not significantly lower in PW irrigated soil at 2.00 ± 0.58 mg kg^{-1} or in WW irrigated soil at 2.00 mg kg^{-1} ± 0.00 mg kg^{-1}. Total carbon made up 26800 ± 300 mg kg^{-1} of control soil and was significantly lower in PW irrigated soil at 24300 ± 600 mg kg^{-1} but was significantly higher in WW

irrigated soil at 28200 ± 1400 mg kg^{-1}. Organic matter made up 49400 ± 600 mg kg^{-1} of control soil was significantly lower in PW irrigated soil at 45400 ± 1300 mg kg^{-1} and significantly higher in WW irrigated soil at 52100 ± 2400 mg kg^{-1}. Soil iron content significantly decreased from control soil at 247.22 ± 4.78 mg kg^{-1} to PW irrigated soil at 232.33 ± 10.99 mg kg^{-1} and decreased again but not significantly to 226.67 ± 4.65 mg kg^{-1} in WW irrigated soil.

Additional WW characteristics

WW samples contained an average of 86176 ± 1098 ng L^{-1} caffeine, 1240 ± 10 ng L^{-1} ethinyl estradiol, 155 ± 6 ng L^{-1} estriol and 84 ± 2 ng L^{-1} estrone (Table 2.2).

Microbial community overview

16S rRNA gene amplification and sequencing generated 1,489,921 counts, with an average of 1,655,547 ± 22,503 counts per sample. Rarefaction curves were produced to display the high sampling depth and Good's coverage was >99 for all samples (Table 2.3). A total 3,707 ESVs were identified across all 9 samples with an average of 3,355 ESVs per sample. These could be annotated at various taxonomic levels, including 598 ESVs to species level, 1690 ESVs to genus level, 549 ESVs to family level, and 807 ESVs were not similar (<99% identity) to any characterized taxa (Figure 2.3). Overall, 370,049 out of the 1,489,921 (24.83%) total sequence counts were captured by ESVs annotated at species level. In control soil 529 ESVs were annotated as putative species, capturing 58,043 sequences counts (13.64% of total counts in control soil; Figure 2.4). In PW irrigated soil, 527 ESVs were annotated as putative species capturing 50,213 sequence counts (16.48% of total PW irrigated soil sequence counts). In WW irrigated soil, 569 ESVs were annotated as species capturing 227,169 sequence counts (30.73% of total WW irrigated soil sequence counts).

The Shannon diversity and Inverse Simpson diversity indices were not significantly different between the three different treatments (t-test $p < 0.05$; Figure 2.3). Principal Coordinates Analysis (PCoA) showed the samples segregated by treatment and multivariate analysis confirmed significant variation between treatment groups (PERMANOVA $p < 0.005$; Figure 2.3).

Control rhizosphere microbial community

Control rhizosphere soil contained 3,090 ESVs in total, 540 of which were classified to species level taxonomy (Figure 2.3). The species-level ESVs present in control soil belonged to ten different phyla Proteobacteria (286), Actinobacteria (195), Bacteroidetes (47), Firmicutes (40),

Acidobacteria (7), Verrucomicrobia (5), Planctomycetes (2), Streptophyta (2), Nitrospirae (1), Spirochaetes (1) and Thaumarchaeota (1). After applying the sparsity filter to the data control soil contained 2,956 ESVs, 529 of which were identified to species level taxonomy. The species-level ESVs present in control soil belonged to ten different phyla Proteobacteria (238), Actinobacteria (190), Bacteroidetes (44), Firmicutes (39), Acidobacteria (7), Verrucomicrobia (5), Planctomycetes (2), Streptophyta (2), Nitrospirae (1), and Thaumarchaeota (1).

From the sequence counts identified to species-level taxonomy, forty-one ESVs representing 53.45% of the species-level sequence counts had high relative abundance (here defined as greater than 0.5% of species-level control soil sequence counts). Twenty-two species-level ESVs had sequence counts of 1% or greater. Ten of these 22 ESVs could be annotated as a single species. Bradyrhizobium_valentium_1 had the highest relative abundance in control soil, contributing 5.75% of species-level sequence counts. Pseudolabrys_taiwanensis_2 contributed 4.4% of species-level sequence counts. Variovorax_ginsengisoli_1 1.5%, Nakamurella_panacisegetis_1 1.4%, Nocardioides_agariphilus_1, Ferruginibacter_lapsinanis_1, Flavobacterium_ardleyense_1 and Terrimonas_soli_1 each contributed 1.3%, Reyranella_soli_1 and Dactylosporangium_aurantiacum_1 each contributed 1.0%. The remaining twelve were resolved to species level but could not be distinguished between multiple species. These were Streptomycetes_MS_10 which contributed 4.2% of species-level sequence counts, Bradyrhizobium_MS_2 which contributed 2.7%, Microbacterium_MS_4 which contributed 2.2%, Friedmanniella_MS_1 and Mycobacterium_MS_6 which each contributed 1.4%, Pedomicrobium_MS_1, Microbacterium_MS_1 and Burkholderiales_MS_1 which each contributed 1.3%, Magnoliopsida_MS_1 and Nakamurella_MS_1 which each contributed 1.2%, Mycobacterium_MS_8 which contributed 1.1% and Mycobacterium_MS_5 which contributed 1.0%.

PW irrigated rhizosphere community

The PW irrigated rhizosphere soil community consisted of 460 species-level ESVs from 11 different phyla, including Proteobacteria (201), Actinobacteria (168), Firmicutes (38), Bacteroidetes (33), Acidobacteria (7), Verrucomicrobia (4), Streptophyta (2), Nitrospirae (1), Planctomycetes (1), Spirochaetes (1) and Thaumarchaeota (1).

Thirty-four out of 460 species-level ESVs (7.39%) present in control soil significantly differed between control soil and PW irrigated soil. Four-hundred and twenty-six (92.60%)

species-level ESVs present in control soil did not significantly differ between control soil and PW irrigated soil. Three species-level ESVs increased in PW irrigated soil and no species-level ESVs are only in PW irrigated soil. Thirty species-level ESVs decreased in PW irrigated soil, and one significantly different species-level ESVs decrease beyond detection limit in PW irrigated soil (Figure 2.5).

WW treated rhizosphere community

The WW microbial community from the previous chapter identified 860 species-level ESVs in WW samples. Of the 860 WW ESVs from the previous chapter 112 ESVs in WW remain in WW irrigated rhizosphere. These 112 ESVs belonged to six different phyla, Proteobacteria (95), Actinobacteria (15), Bacteroidetes (10), Firmicutes (5), Nitrospirae (1) and Verrucomicrobia (1). Eighty-seven of the 112 surviving WW ESVs, such as Nitrospira_japonica_1, were present in the control rhizosphere before WW irrigation as well as in WW samples. Twenty-five ESVs unique to WW (not present in control soil samples) remained in the rhizosphere after WW irrigation such as Arcobacter_cibarius_1, Acidovorax_defluvii_1, Trichococcus_MS_1, Ferribacterium_limneticum_1, Lysobacter_lycopersici_1, Novosphingobium_tardaugens_1, Rhodobacter_blasticus_1 and 2, Thiobacillus_thiophilus_1 and Zoogloea_caeni_1.

The WW irrigated rhizosphere soil community consisted of 569 species-level ESVs from 10 different phyla, including Proteobacteria (274), Actinobacteria (192), Bacteroidetes (45), Firmicutes (40), Acidobacteria (7), Verrucomicrobia (5), Planctomycetes (2), Streptophyta (2), Nitrospirae (1) and Thaumarchaeota (1). 156 species-level ESVs (27.40%) significantly differed between control soil and WW irrigated soil. Four-hundred and thirteen species-level ESVs (72.60%) did not significantly differ between control soil and WW treated soil.

Six species-level ESVs significantly decrease from control soil to WW irrigated soil including Rhodococcus_maanshanensis_1, Micromonospora_MS_1, Sporosarcina_soli_1, Bacillus_shackletonii_1, Bacillus_MS_14 and Solibacter_usitatus_1 and no species-level ESVs were present in control soil and not present in WW irrigated soil. One-hundred and ten species-level ESVs significantly increased in WW irrigated soil. Forty ESVs were present in the WW irrigated rhizosphere soil but not detected in the control rhizosphere.

Twenty-five of these 40 species-level ESVs were annotated as species from WW samples of the previous chapter including Ferribacterium_limneticum_1, Bacteroides_graminisolvens_1, Arcobacter_cibarius_1, Hydrogenophaga_taeniospiralis_1, Rhodobacter_blasticus_1 and 2,

Novosphingobium_tardaugens_1, Thiobacillus_thiophilus_1, Lysobacter_lycopersici_1 and Zoogloea_caeni_1. The remaining 15 ESVs such as Actinoplanes_ferrugineus_1, Azoarcus_communi_1, Blastochloris_sulfoviridis_1, Deviosa_insulae_1, Mesorhizobium_denitrificans_1, Novosphingobium_subterraneum_1, Thiobacillus_denitrificans_1 and 2, Thiobacillus_MS_1 and Thiobacillus_sajanensis_1 were not detected in either WW samples from the previous chapter or control rhizosphere soil but were present in WW irrigated rhizosphere soil.

Two of the 110 increasing species-level ESVs, Variovorax_ginsengisoli_1 and Variovorax_paradoxus_1 were annotated as species capable of producing plant hormones (Figure 2.6). At least 39 other ESVs were annotated as species beloning to genera exhibiting some plant hormone production including Bacillus (2), Mesorhizobium (3), Microbacterium (2), Micromonospora (1), Nocardioides (13), Rhizobium (2), Sphingomonas (3), Sphingopyxis (2), Sporosarcina (1), Streptomyces (1) and Variovorax (1) (Figure 2.7).

Five of the 110 increasing species-level ESVs were annotated as nitrogen fixing bacteria including Mesorhizobium_opportunistum_1, Mesorhizobium_tianshanense_1, Mesorhizobium_MS_8, Rhizobium_tubonense_1 and Rhizobium_MS_1 (Figure 2.6). Three of the six decreasing species-level ESVs were annotated to a genus exhibiting some nitrogen fixing bacteria including Bacillus_shackletonii_1, Bacillus_MS_14 and Micromonospora_MS_1. Four species-level ESVs annotated as nitrogen fixing bacteria were added to the WW irrigated rhizosphere from WW including Bacteroides_graminisolvens_1, Rhodobacter_blasticus_1 and 2, and Zoogloea_caeni_1. Two species-level ESVs annotated as nitrogen fixing bacteria were not present in WW samples or in control rhizosphere soil samples but were present in WW irrigated soil samples including Azoarcus_communis_1 and Mesorhizobium_denitrificans_1. One species-level ESV, Nitrospira_japonica_1, from the family Nitrospiraceae was annotated as species capable of nitrite oxidation.

No differentially abundant species-level ESVs could be confirmed as inorganic phosphorus solubilizing species. At least 25 ESVs were annotated as species belonging to genera exhibiting some inorganic phosphorus solubilization and were differentially abundant including Bacillus (2), such as Bacillus_shackletonii_1, Flavobacterium (2), such as Flavobacterium_saccarophilum, Mesorhizobium (4), such as Mesorhizobium_denitrificans_1 and 2 and Mesorhizobium_tianshanense_1, Novosphingobium (5), such as Novosphingobium_hassicum_1,

Novosphingobium_subterraneum_1 and Novosphingobium_tardaugens_1, Pseudomonas (2), such as Pseudomonas_MS_1, Rhizobium (2), such as Rhizobium_tubonense_1, Rhodococcus (2), such as Rhodococcus_psychrotolerans_1 and Rhodococcus_maanshanensis_1, Sporosarcina (1) and Thiobacillus (5), such as Thiobacillus_denitrificans_1 and 2, Thiobacillus_sajanensis_1 and Thiobacillus_thiophilus_1 (Figure 2.8).

Sixteen species-level ESVs were annotated as species capable of producing organic phosphorus hydrolyzing phosphatases (Figure 2.6). These came from nine genera including Aeromicrobium (3), such as Aeromicrobium_ginsengisoli_1 and 2 and Aeromicrobium_panaciterrae_1, Dokdonella (1), such as Dokdonella_immobilis_1, Dyadobacter (1), such as Dyadobacter_endophyticus_1, Halioglobus (1), such as Halioglobus_pacificus_1, Mesorhizobium (4), such as Mesorhizobium_tianshanense_1, Phenylobacterium (2), such as Phenylobacterium_conjunctum_1, Rhizobium (2), such as Rhizobium , such as Rhizobium_tubonense_1, Roseomonas (1), such as Roseomonas_lacus_1 and Woodsholea (1), such as Woodsholea_maritima_1. Two of the 110 increasing species-level ESVs were annotated as species capable of phosphorus accumulation, Accumulibacter_phosphatis_1 and Tetrasphaera_duodecadis_1 (Figure 2.8).

Three of the 110 increasing species-level ESVs were annotated as siderophore producing species, that is, Variovorax_paradoxus_1, Nordella_oligomobilis_1 and Sphingopyxis_bauzanensis_1 (Figure 2.6, Figure 2.8). One ESV, Ferribacter_limneticum_1, was annotated as a species capable of reducing ferric iron to ferrous iron. At least 30 other species-level ESVs were annotated as species belonging to genera exhibiting some inorganic phosphorus solubilization and were differentially abundant including Bacillus (2), Blastochloris (1), Bosea (1), Brevundimonas (1), Lysobacter (3), Mesorhizobium (3), Microbacterium (2), Novosphingobium (3), Pseudomonas (2), Rhizobium (2), Rhodococcus (2), Roseomonas (1), Sphingopyxis (2), Streptomyces (1) and Variovorax (2). Bacillus_shackletonii_1, an ESV annotated as a species capable of siderophore production decreased in the WW irrigated rhizosphere compared to the control.

One increasing species-level ESV, Variovorax_paradoxus_1, was annotated as a species capable of sulfur oxidation (Figure 2.6, Figure 2.7). One species-level ESV, Thiobacillus_thiophilus_1, annotated as a species capable of sulfur oxidation was added to the WW irrigated rhizosphere from WW. Five species-level ESVs, Blastochloris_sulfoviridis_1,

Thiobacillus_denitrificans_1 and 2, Thiobacillus_MS_1 and Thiobacillus_sajanensis_1, were annotated as species capable of sulfur oxidation and were not detected in either WW samples or control rhizosphere soil samples but were present in WW irrigated rhizosphere soil samples.

Three species-level ESVs, Actinoplanes_philippinensis_1, Janthinobacterium_lividum_1 and Variovorax_paradoxus_1, annotated as species capable of biocontrol mechanisms increased from control rhizosphere soil to WW irrigated rhizosphere soil (Figure 2.6, Figure 2.9). One ESV, Devosia_insulae_1, annotated as a species capable of biocontrol mechanisms was present in WW irrigated rhizosphere soil but not present in WW samples from the previous chapter or control rhizosphere soil samples. At least 26 other species-level ESVs were annotated as species belonging to genera exhibiting some biocontrol mechanisms and were differentially abundant including Actinoplanes (3), Lysobacter (3), Micromonospora (1), Nocardiodes (13), Pseudomonas (2), Streptomyces (1) and Variovorax (2).

Five increasing species-level ESVs, Dokdonella_immobilis_1, Hydrocarboniphaga_daqingensis_1, Novosphingobium_aromaticivorans_1, Pedobacter_steynii_1 and Sphingopyxis_bauzanensis_1, were annotated as species capable of organic pollutant degradation (Figure 2.6, Figure 2.9). One species-level ESV, Hydrogenophaga_taeniospiralis_1, annotated as a species capable of organic pollutant degradation was added to the WW irrigated rhizosphere from WW. One species-level ESV, Blastochloris_sulfoviridis_1, annotated as a species capable of organic pollutant degradation, was present in the WW irrigated rhizosphere but not present in WW samples or control rhizosphere samples. At least 40 other species-level ESVs were annotated as species belonging to genera exhibiting some organic pollutant degradation and were differentially abundant including Azoarcus (1), Blastochloris (1), Brevundimonas (1), Hydrogenophaga (2) Lysobacter (3), Microbacterium (2), Mycobacterium (1), Nocardiodes (13), Novosphingobium (4), Phenylobacterium (2), Pseudomonas (2), Pseudoxanthomonas (1), Rhodobacter (2), Sphingomonas (3), Sphingopyxis (1), Streptomyces (1) and Thiobacillus (5).

Four species-level ESVs, Dokdonella_immobilis_1, Novosphingobium_tardaugens_1 and Rhodobacter_blasticus_1 and 2, were annotated as species capable of degrading different emerging contaminants (estrogens and acetaminophen; Figure 2.6, Figure 2.9). Dokdonella_immobilis_1, increased in WW irrigated rhizosphere soil,

Novosphingobium_tardaugens_1 and Rhodobacter_blasticus_1 and 2 were added to the rhizosphere from WW.

Discussion

Soil composition

This current study assessed the effects of irrigation and WW irrigation on a natural willow rhizosphere microbial community. Soil composition of each treated rhizosphere was examined to observe effects of WW irrigation on different constituents in soil as well as on the rhizosphere microbial community. Both irrigation with PW and WW significantly increased the pH of soil, however the increase with PW was higher (Table 2.1; Figure 2.2). Changes in pH have been shown to affect the composition of microbial communities of soil as bacteria tend to be sensitive to pH range[264]. The change in pH from control to PW irrigated soil may have affected the decreasing ESVs, however, the 0.63 difference may not be enough of a change to cause an effect as most bacteria species can growth within a range of pH. WW irrigation significantly increased total nitrogen in soil, whereas irrigation with PW did not have an effect. Unexpectedly, ammonia concentration was highest in PW irrigated soil in comparison to both control soil and WW treated rhizosphere soil. This decrease in ammonia concentration from PW to WW irrigated rhizopsheres may be due to stimulation of bacteria capable of ammonia oxidation to nitrate, another nitrogen form accessible for plants[414]. Whereas nitrates-nitrites sequentially increased from control soil to PW irrigated soil to WW irrigated soil. Nitrate is one of the preferred nitrogen forms for uptake by plants for growth and development[414]. The 2275% increase in nitrates-nitrites in WW irrigated soil may have influenced the 200% increased willow[415] biomass resulting from WW irrigation. Ammonium is also a usable nitrogen form by plants, and perhaps results in a lower concentration of Ammonia in WW irrigated soil due to uptake by plants. Total phosphorus on average increases from control soil to PW irrigated soil to WW irrigated soil, however surprisingly these increases are not statistically significantly different. Likewise, orthophosphate does not significantly differ from one treatment to the next, however it is highest in control soil and decreases slightly in PW irrigated soil but remains at the same level in WW irrigated soil. The slight decrease in orthophosphate could represent its uptake by willow trees, as it does not significantly differ from PW irrigated soil to WW irrigated soil, although WW does contain additional phosphate concentrations, the addition of water may facilitate mobility to tree roots[416]. Organic matter and

total carbon followed a similar pattern to each other. Each decrease significantly from control soil to PW irrigated soil and increase significantly from PW irrigated soil to WW irrigated soil. With WW irrigated soil retaining the highest percentages of both organic matter and total carbon. Organic matter and the carbon in it help soil with water-retention capacity, structure and reduces risks of erosion and nutrient leaching[417–420]. Iron content sequentially decreased from control soil to PW irrigated soil to WW irrigated soil, however only significantly decreased from control soil to PW irrigated soil. The addition of WW may stimulate the solubility and uptake of iron from soil, this would also indicate that iron concentrations in WW are minimal and do not replace levels lost from soil. Alternatively, this could indicate greater use of iron by microbes and willows in PW irrigated and WW irrigated rhizospheres. The changes in soil constituent concentrations indicate that WW irrigation favourably changes nitrogen (most notably nitrates-nitrites) and carbon (also organic matter) of soil which may in-turn have affected both the rhizosphere microbial community and finally willow growth. In summation, PW irrigation does affect some soil characteristics and constituent concentrations, however most effects on soil are more pronounced with WW irrigation. More precisely, PW increased pH, ammonia and nitrates-nitrites concentrations while decreasing total carbon, organic matter and iron, whereas, WW increased pH, total nitrogen, ammonia, nitrates-nitrites, total carbon, organic matter while decreasing iron. Surprisingly, phosphorus concentrations were not significantly affected by either irrigation.

Microbial community

Only 16.13% of ESVs, capturing 24.83% counts, were able to be annotated to species-level taxonomy (Figure 2.3). This percentage of count capture at species-level taxonomy was in line with a recent study investigating phytoremediation using three different crops, which found 24.60% of counts captured at the species level[421]. In control soil 14.22% of ESVs, capturing 13.64% counts, were resolved to species level, in PW irrigated soil 14.22% of ESVs capturing 16.48% sequence counts were resolved to species level, whereas in WW irrigated soil 15.35% of ESVs capturing 30.73% sequences counts were resolved to species level (Figure 2.4). WW irrigated ESVs capture nearly double the sequences of control soil and PW irrigated soil without the proportional rise in percent of ESVs resolve to species level, indicating a greater presence of characterized species in the WW irrigated rhizosphere, which is not surprising as more studies investigate the human gut microbiome in comparison to soil microbial communities. The results

capturing only 14 to 15% of ESVs at a species level annotation suggest this current study is only looking at a small proportion of the bacteria present and active in the rhizosphere.

The alpha diversity as measured by Shannon diversity and Inverse Simpson diversity indices was not significantly different indicating the diversity was stable between the three different treatments (Figure 2.3). These results were in line with a study by Zolti et al. (2019)[422] that found no significant differences in microbial alpha diversity in soil or roots with WW irrigation. A study by Dang et al. (2019)[423], however, investigating the response of soil microbial community structure to long-term WW irrigation and soil depth found alpha diversity to be significantly higher in treated WW irrigated rhizospheres. These varying reports suggest WW irrigation of rhizospheres may affect microbial communities differently depending on the experimental conditions. Principal Coordinates Analysis (PCoA) showed the samples segregated by treatment and multivariate analysis confirmed significant variation between treatment groups indicating that although microbial communities in the three rhizospheres are similar, they also differ (Figure 2.3). In essence, only a small proportion of the rhizosphere microbial community is resolved to species level. WW irrigation facilitates a greater presence of characterized species in the rhizosphere. Alpha diversity indices confirm that rhizosphere microbial communities are largely stable, while multivariate analyses indicate there are some differences between the treatments.

Characteristics of a control rhizosphere microbial community

From the identified control rhizosphere community only an approximate 18% (17.48%) was resolved to species-level taxonomy, illustrating the gap in knowledge of soil microbes and the rhizosphere microbial community. Many species discovered in control rhizosphere soil may possess direct and indirect plant growth promoting traits such as nitrogen fixation, phosphorus solubilization and pathogen control. The most abundant species-level ESV was *Bradyrhizobium valentinum*, a nitrogen fixing species that establishes a diazotrophic root nodule with the legume *Lupinus mariae-josephae*[424]. *Pseudolabrys taiwanensis,* also highly abundant belongs to the *Rhizobiales*[425]*,* an order containing many nitrogen fixers and plant-symbionts[426]. *Nocardioides agariphilus*, members of the *Nocardioides* genus have demonstrated positive plant growth promotion[427], *Nocardioides agariphilus* exhibitied good growth on agar without addition of nitrogen[428] suggesting an ability to fix nitrogen. The genus *Reyranella*, from the species *Reyranella soli*, the was found to be a core endophyte of Agave and in community with diazotrophs and could

potentially play a role in drought tolerance[429], however the genus was also found to be positively correlated with the disease index of bacterial wilt[430]. *Variovorax ginsengisoli*, along with *Flavobacterium ardleyense*, also produces acid phosphatase enzymes[431,432] which play a role in the mineralization of organic phosphorus in soil[433]. The *Terrimonas* genus was found to be enriched in phosphorus amended soils[434] and sewage[435], which may suggest phosphorus solubilization capabilities of *Terrimonas soli*. *Ferruginibacter lapsinanis*, a member of the family Chitinophagaceae[311], may aid in biocontrol of fungi as fungal infection of plant roots has been observed to result in an increase of members of Chitinophagaceae in root endospheres resulting in stimulation of enzymatic fungal cell-wall degradation[436]. *Dactylosporangium aurantiacum*, a producer of the antibiotic tiacumicin B[437], may provide biocontrol against undesirable phytopathogens in the willow rhizosphere. Apart from ESVs resolved to single species, some of the highly abundant ESVs resolved to multiple species may exhibit plant growth promoting abilities such as nitrogen fixation by *Bradyrhizobium*[438], polyphosphate accumulation by *Friedmanniella*[439], IAA production by *Microbacterium*[440] and *Mycobacterium*[441], and control of plant disease *Streptomycetes*[442]. In sum, only 18% of the control rhizosphere microbial community was resolved to species level, however, of the proportion resolved to species level many ESVs belong to species capable of plant growth promotion or to genera associated with plant growth promotion.

Changes from a control rhizosphere to a PW irrigated rhizosphere

The rhizosphere microbial community remained largely the same from control soil to PW irrigated soil as only 7.29% of ESVs changed significantly from one to the other (Figure 2.5). Although the rhizospheres' microbial communities did not differ greatly from control soil to PW irrigated soil, 91.18% of the differentially abundant ESVs decreased from control soil to PW irrigated soil. This decrease indicates that irrigation of a rhizosphere with PW may disperse some bacteria to different areas by way of water flow. While no individual ESVs displaying significant change between the control rhizosphere and the PW rhizosphere were associated with plant growth promoting functions, several ESVs belonged to genera that exhibited some plant growth promoting abilities such as *Actinoplanes, Chitinophaga, Microbacterium, Mycobacterium, Nocardioides, Pseudomonas, Sphingomonas*, *Sporosarcina* and *Streptomyces*, which may be shared by some of these ESVs. In conclusion, the microbial community only exhibited a small amount of significant

change between the control and PW irrigated rhizosphere, however, the change that did occur indicated irrigation with PW may disperse a small proportion of the microbial community.

Changes from a control rhizosphere to a WW irrigated rhizosphere

The previous chapter identified 860 species-level ESVs in WW samples. Of these WW ESVs 112 remained in the WW irrigated rhizosphere until sampling indicating that the majority (86.98%) of WW ESVs do not remain in the rhizosphere. As many primary WW bacteria are anaerobic species (refer to last chapter), this may indicate that these soils are aerobic environments. Eighty-seven of the 112 surviving WW ESVs, such as *Nitrospira japonica*, however, were not unique to WW but were also already present in the control rhizosphere before WW irrigation meaning that only 25 ESVs unique to WW survived in the rhizosphere suggesting that the majority of the benefit of WW irrigation is from the nutrients in WW, however, some of the added species may still have an effect. Some of these 25 were high in relative abundance in primary WW including *Arcobacter cibarius, Acidovorax defluvii* and *Trichococcus sp.* (Trichococcus_MS_1) and may only be established in the rhizosphere due to the initial abundance in WW, whereas others such as *Ferribacterium limneticum, Lysobacter lycopersici, Novosphingobium tardaugens, Rhodobacter blasticus, Thiobacillus thiophilus* and *Zoogloea caeni* were relatively low in abundance in primary WW which may imply these species offer a benefit to the rhizosphere and thereby become established.

The addition of nutrients and contaminants in WW does not necessarily have a large effect on the microbial community composition in terms of relative abundance, however, it may affect total abundance, which should be assessed in future studies. Differential abundance analysis revealed a difference in 27.40% of species-level ESVs between control soil and WW irrigated soil suggesting that although WW irrigation causes a greater change in the rhizosphere microbial community compared to irrigation with PW, nearly three-quarters of the rhizosphere species-level ESVs does not differ from the control rhizosphere microbial community. This indicates that the rhizosphere microbial community is largely resilient to change. Furthermore, the 27.40% of species-level ESVs displaying differential abundance from the control rhizosphere to the WW irrigated rhizosphere mostly increased. To be exact, 110 ESVs increased (94.83%) whereas 6 ESVs decreased (3.82%) from control rhizosphere soil to WW irrigated rhizosphere soil suggesting a mostly positive effect on rhizosphere bacteria from WW irrigation.

Although the effects of WW irrigation on rhizosphere bacteria are mostly positive, WW irrigation of the willow rhizosphere may decrease some beneficial species for plant growth. Six ESVs (3.82% significantly differentially abundant species level ESVs) decreased from control soil to WW irrigated soil. *Micromonospora sp*. (Micromonospora_MS_1), *Sporosarcina soli*, *Bacillus shackletonii* and *Bacillus sp.* (Bacillus_MS_14), were from genera that are associated with plant growth promotion. Species and strains within the genus *Micromonospora* produce some of the best known antibiotics[443] which play a role in biocontrol[444–447], the genus is also known for potent chitinases[448,449] which may help with fungal control. Additionally, species and strains from the genus *Micromonospora* have displayed direct promotion on shoot growth supposedly through bioactive metabolites[450], nitrogen fixation *ex planta*[451], and while do not display roots nodulation on their own help stimulate nodulation from nodulating species[452]. Although not well studied itself, *Sporosarcina soli*, belongs to the genus *Sporosarcina*. This genus has species with different plant growth promoting traits such as nitrogen fixation, IAA production as well as phosphorus and zinc solubilization[453,454], which may suggest similar traits in *Sporosarcina soli*. *Bacillus* is also a genus containing many species exhibiting plant growth promotion, with traits such as nitrogen fixation, phosphorus, potassium, and zinc solubilization as well as IAA, gibberellic acid, and ACC production[453,455]. *Bacillus shackletonii* and *Bacillus sp.* (Bacillus_MS_14) may also share some plant growth promoting traits. While the majority of differentially abundant ESVs increased from control soil to WW soil indicating positive effects of WW irrigation for rhizosphere bacteria, the decrease of these species that may have plant growth promoting abilities indicates that positive effects from WW irrigation are not universal for rhizosphere bacteria, there is potential for WW irrigation to negatively affect some species in general and also some that may have plant growth promoting effects. The decrease in these ESVs may be caused in part by the presence of other chemicals such as emerging contaminants in WW. Caffeine, a ubiquitous emerging contaminant in WW[456], measured approximately 86µg L^{-1} in primary WW samples (Table 2.2), as caffeine has been shown to inhibit growth of *Bacillus subtilis,* other *Bacillus* species such as *Bacillus shackletonii* and *Bacillus sp.* (Bacillus_MS_14) may be susceptible to inhibitory effects of caffeine, assuming similar susceptibilities across the genus[457]. *Bacillus shackletonii*, however, also decreased in PW irrigated soil, suggesting exposure to caffeine may not be the causative agent. No ESVs were detected only in control soil in comparison to WW irrigated soil suggesting that,

although a few species' abundances may be negatively affected, the addition of WW to rhizosphere soil does not have deleterious effects on the rhizosphere microbial community.

WW irrigation increased 110 ESVs (20.79% of the rhizosphere community) and added 40 ESVs to the rhizosphere suggesting (as mentioned previously) that some WW bacteria are able to establish themselves in the rhizosphere microbial community. Furthermore, only 25 of the 40 newly added ESVs were present in primary WW indicating that WW irrigation of the willow rhizosphere stimulated the growth of the remaining 15 ESVs (such as *Actinoplanes ferrugineus, Azoarcus communis, Blastochloris sulfoviridis, Deviosa insulae, Mesorhizobium denitrificans, Novosphingobium subterraneum, Thiobacillus denitrificans* and *Thiobacillus sajanensis*) not present in either rhizosphere soil or WW (most likely these species were present in either soil or WW below detection limits and were able to use the newly provided resources).

Two increasing species in the WW irrigated rhizopshere, *Variovorax ginsengisoli* and *Variovorax paradoxus,* were confirmed to produce phytohormones[87,458] (Figure 2.6). The increase in these two species suggests that WW irrigation positively impacts the growth of some phytohormone synthesizing rhizosphere bacteria. This is not surprising as the resulting willow trees irrigated by WW displayed greater biomass[201]. In addition to *Variovorax ginsengisoli* and *Variovorax paradoxus*, forty-one of the increasing or newly added ESVs to the WW irrigated rhizosphere belonged to twelve genera (*Bacillus*[453,455], *Lysobacter*[459–461], *Mesorhizobium*[462–466], *Microbacterium*[467,468], *Micromonospora*[450], *Nocardiodes*[469], *Novosphingobium*[470–472], *Pseudomonas*[473], *Rhizobium*[474–476], *Sphingomonas*[477–479], *Sphingopyxis*[480], *Streptomyces*[481–483] *and Variovorax*[484–486] (Figure 2.7)) that included species displaying phytohormone synthesis. Phytohormone synthesis could not be confirmed for these particular species, however, as species from these genera have the ability to synthesize phytohormones, this may be a trait shared by the increasing or added species to the WW irrigated rhizosphere. This lends support to the idea that WW irrigation of the rhizosphere increases synthesis of plant hormones. Not many studies have assessed the impact of WW irrigation on plant hormone synthesis by rhizosphere bacteria, however, a study investigating the effects of inoculation with plant growth promoting bacteria, silver nanoparticles and untreated municipal WW on maize, found the highest levels of IAA production in plant leaves were in treatments including municipal WW irrigation[487]. The observed increases in IAA production with the addition of WW irrigation lends support to the idea that WW

irrigation may increase phytohormone synthesis by rhizosphere bacteria, however, as little research has investigated this effect, more studies are necessary to confirm this hypothesis.

WW irrigation increased nitrogen fixing bacteria already present in soil such as *Mesorhizobium opportunistum*[488,489], *Mesorhizobium tianshanense*[488,489] and *Rhizobium tubonense*[490], as well as added species from WW capable of nitrogen fixation such as *Zoogloea caeni*[323], *Rhodobacter blasticus*[491] and *Bacteroides graminisolvens*[492] (Figure 2.6, Figure 2.7). Furthermore, WW irrigation stimulates the growth of nitrogen fixing species, such as *Azoarcus communis*[493] and *Mesorhizobium denitrificans*[488,489], not detected in either control soil or WW samples (most likely below detection limit in soils or WW). The increase, survival and appearance of nitrogen fixing bacteria in the WW irrigated rhizosphere indicates WW irrigation enriches nitrogen fixing species. WW enrichment of nitrogen fixing bacteria has also been observed in previous studies. A study by Kannan et al. (1990)[494] found increased abundances of nitrogen fixing bacteria *Rhizobium* and *Azotobacter* in paper mill effluent treated sugarcane rhizospheres. Likewise, a study by Ibekwe et al. (2018)[495] found higher numbers of OTUs (operational taxonomic units) for nitrogen fixing bacteria such as *Bradyrhizobia* and *Agrobacterium* in WW treated soil. Although nitrogen fixing species were enriched in the WW irrigated rhizosphere, nitrogen fixation may not have been. Transcriptomics of rhizosphere soil was not conducted during this present study, however a study on the influence of different fertilizers on nitrogen fixation in a model rice paddy mesocosm found that nitrogen fixing activity decreased with increasing urea concentrations[496]. Another study examining the *nifH* gene expression (i.e. a gene for nitrogenase) in rice paddy soil amended with rice straw over the long-term found that long-term rice straw addition significantly increased diazotroph abundance, but in contrast, sharply reduced *nifH* gene expression and nitrogen fixation activity[497]. These studies indicate that although nitrogen rich fertilization of soils may increase nitrogen fixing bacteria species, it may have the opposite effect on actual nitrogen fixation. Additionally, *Nitrospira japonica*, a nitrite oxidizer[498], also increased with WW irrigation, possibly helping to increase the conversion of nitrite to nitrate, one form of nitrogen that is soluble to plants[414]. Nitrites-nitrates measured in rhizosphere soil samples increased 2275%, and while nitrite and nitrate were not measured separately, an increase in nitrite may have allowed the increase of this species.

Although no differentially abundant species could be confirmed as inorganic phosphorus solubilizers, species such as *Flavobacterium saccharophilum*, *Mesorhizobium tianshanense*,

Novosphingobium hassicum, *Rhizobium tubonense* and *Rhodococcus psychrotolerans* that are from genera associated with inorganic phosphorus solubilization[466,499–508] increased from control soil to WW irrigated soil, species added from WW such as *Novosphingobium tardaugens*, *Pseudomonas sp.* (Pseudomonas_MS_1) and *Thiobacillus thiophilus* were also from genera associated with inorganic phosphorus solubilization[504–506,509–511] (Figure 2.8). Novel species not present in either WW or control soil samples such as *Mesorhizobia denitrificans*, *Novosphingobium subterraneum*, *Thiobacillus denitrificans*, and *Thiobacillus sajanensis* were also from genera associated with inorganic phosphorus solubilization[466,502–506,510,511]. Although inorganic phosphorus solubilization could not be confirmed for these unique species, they may share these traits with other species in the genera. The increase, survival and appearance of species potentially contributing to inorganic phosphorus solubilization suggests WW irrigation may enrich species capable of inorganic phosphorus solubilization however species decreasing in WW irrigated soil, such as *Rhodococcus maanshanensis* and *Bacillus shackletonii* were also from genera associated with inorganic phosphorus solubilization[500,504,506,508]. These decreasing species suggest the potential enriching effects of WW irrigation are not universal for all inorganic phosphorus solubilizing species. Koo et al. (2005)[512] tested the effects of biosolid amendments (solid wastes taken out during the WW treatment process) on organic acid production in a corn rhizosphere and found that biosolids enhanced organic acid production and influenced the composition of the organic acid mixtures. The increase in organic acids in the corn rhizosphere with biosolid soil amendments observed by Koo et al. (2005)[512] and the increase, survival, and appearance of phosphorus solubilizing bacteria species in the WW treated willow rhizosphere observed in this present study may indicate that WW irrigation (or indeed biosolid amendments) not only enriches inorganic phosphorus solubilizing species but also may have an effect on organic acid production and therefore may increase inorganic phosphorus solubilization in the willow rhizosphere.

Increasing species in the WW irrigated rhizosphere such as *Aeromicrobium ginsengisoli, Aeromicrobium panaciterrae, Dokdonella immobilis, Dyadobacter endophyticus, Halioglobus pacificus, Mesorhizobium tianshanense, Phenylobacterium conjunctum, Rhizobium tubonense, Roseomonas lacus,* and *Woodsholea maritima* all produce phosphatase enzymes[388,466,488,513–520] and may contribute to organic phosphorus mineralization in the rhizosphere (Figure 2.6, Figure 2.8). Although many studies exist evaluating how plant growth promoting rhizobacteria, including

organic phosphorus mineralizers, can help in a contaminated rhizosphere[521–523], no studies have evaluated how WW irrigation affects phosphatase producing bacteria. One study, however, assessing rhizosphere dynamics during olive mill WW (containing 820 mg phosphorus L^{-1}) phytoremediation did assess phosphatase production and in general found higher production of phosphatase in soils treated with olive mill WW[524]. As would be expected, irrigation with phosphorus rich WW increased phosphorus solubilizing species.

Additionally, *Accumulibacter phosphatis* and *Tetrasphaera duodecadis* are phosphorus accumulating bacteria[245,525] which may indicate, in addition to phosphorus solubilizing organisms, phosphorus accumulating organisms may exist in symbiosis with plants and participate in soil phosphorus cycling (Figure 2.6, Figure 2.8). Although little research exists on phosphorus accumulating organisms in plant rhizospheres, Li et al. (2013)[526] identified a phosphorus accumulating strain of *Arthrobacter* in the rhizosphere of maize. A recent review by Akbari et al. (2021)[527] also refers to an unpublished study observing an enrichment of phosphorus accumulating organisms in maize rhizospheres in comparison to bulk soil and suggests a potential role of phosphorus accumulating organisms in soil phosphorus cycling and plant symbioses. Together with the enhancement of *Accumulibacter phosphatis* and *Tetrasphaera duodecadis* in the WW irrigated willow rhizosphere, these may be the first indications that phosphorus accumulating organisms may act in symbioses with plants. Alternatively, as both species were also detected in control rhizosphere soils, their enhancement by WW irrigation may be opportunism. They may use the increased levels of phosphorus from WW to their advantage with no notable symbioses with the plants.

Only three species, *Variovorax paradoxus*, *Nordella oligomobilis* and *Sphingopyxis bauzanensis*, increasing in WW irrigated soil were found to produce siderophores[528,529] (Figure 2.6). Interestingly, iron content of soil significantly decreased from control soil to WW irrigated soil. Although iron concentration of WW samples was not measured, the decrease in concentration of iron from control soil to WW irrigated soil may indicate two things. First, that the primary WW used for irrigation does not have a high concentration of iron, and second, that WW irrigation may facilitate siderophore production in *Variovorax paradoxus, Nordella oligomobilis* and *Sphingopyxis bauzanensis* therefore allowing for the willow to use the iron present in soil. *Ferribacterium limneticum*, a species added to the rhizosphere from WW, is capable of reducing ferric iron to ferrous iron[530], which also provides a soluble form of iron for plant uptake[531].

Together the increase of siderophore producing species and the addition of a ferric iron reducing species may have contributed to the decrease in iron concentration of WW irrigated soil. Several other ESVs belonged to genera with species displaying siderophore activity including *Bosea*[529], *Brevundimonas*[532], *Lysobacter*[533–535], *Mesorhizobium*[465,536], *Microbacterium*[532], *Novosphingobium*[470,537,538], *Pseudomonas*[528,532,539], *Rhizobium*[536,540], *Rhodococcus*[528,541], *Roseomonas*[542,543], *Sphingopyxis*[528], *Streptomyces*[541] and *Variovorax*[532,544] (Figure 2.8). Although siderophore production cannot be confirmed for these specific ESVs, it is possible some ESVs share the genes for siderophore production. Additionally, *Bacillus shackletonii*, which decreased in WW irrigated soil, can also produce siderophores[545]. The increase and decrease of bacteria with genes for siderophore production suggests that WW irrigation does not have a universally beneficial impact on siderophore producing species however it may stimulate the growth of certain siderophore producing species and allow for higher iron uptake in willows. Interestingly, as measured by Sas et al. (2021)[415], for the same field trial, willow biomass contained significantly higher iron concentrations in trees irrigated with WW. Along the significantly decreasing iron concentration in WW irrigated soil, higher iron concentration in willow biomass lends further support to the conclusion that WW irrigation not only causes an increase of siderophore producing species, such as *Variovorax paradoxus*, *Nordella oligomobilis* and *Sphingopyxis bauzanensis*, but also stimulates the production of siderophores from these species. A study by Tripathi et al. (2011)[546] also found a 179% increase in siderophore producing species in a WW effluent irrigated field in comparison to a well-water irrigated field. Yadav et al. (2011)[547] investigating the functional diversity of *Bacillus* strains in soils irrigated with pulp and paper mill effluent over the long term found that siderophore activity was relatively higher in effluent irrigated fields in comparison to water irrigated fields. These two studies help to support the finding that WW irrigation not only enriches siderophore producing species but also increases siderophore production, thereby facilitating the increased uptake of iron by WW irrigated trees.

Sulfur, another nutrient necessary for plant life, is available for plant uptake as sulfate[395]. Surprisingly, WW irrigation enriched sulfur oxidizing bacteria species. *Variovorax paradoxus*[87] increased in WW irrigated soil, *Thiobacillus thiophilus*[548] was provided by WW and *Blastochloris sulfoviridis*[549], *Thiobacillus denitrificans*[548], *Thiobacillus sp.* (Thiobacillus_MS_1)[511] and *Thiobacillus sajanensis*[550] were all present in WW irrigated soil but not in WW samples or rhizosphere control soil (Figure 2.6, Figure 2.7). The peculiarity of these newly appearing species

is most likely due to undetected sequence counts in either rhizosphere soil or WW samples. The enrichment of sulfur oxidizing species in the WW irrigated rhizosphere suggests that WW may not only contain higher sulfur concentrations than found in the control rhizosphere but also that it adds or greatly stimulates the growth of species capable of sulfur oxidation which may establish in the rhizosphere and therefore increase availability to willows. The enrichment of sulfur oxidizing bacteria in WW irrigated soil is in line with a study by Ashraf et al. (2018)[551] that investigated sulfur oxidizing bacteria and found they were enriched in both sewage water and sewage sludge in comparison to control soil. As sulfur oxidizing bacteria are enriched in different WWs and sludges, high levels of sulfur may be common in WWs and result in the growth of sulfur oxidizing bacteria. WW irrigation may, therefore, provide the function of sulfur oxidation to the rhizosphere.

Biocontrol of phytopathogens is an indirect plant growth promoting effect of some bacteria. WW irrigation increased species such as *Actinoplanes philippinesis, Janthinobacterium lividum* and *Variovorax paradoxus* with different biocontrol functions[87,552,553] (Figure 2.6). Other ESVs in the genera *Actinoplanes*, *Lysobacter, Nocardiodes*, *Pseudomonas*, *Streptomyces* and *Variovorax* also increased in WW irrigated soil, these genera contain species with biocontrol abilities[554–566] (Figure 2.6), which may suggest these ESVs may have similar abilities, although not confirmed. In addition, *Devosia insulae* was present in WW irrigated soil but absent in both WW samples and the control rhizosphere. These species and genera have displayed various biocontrol functions, for example, *Actinoplanes philippinensis* caused the lysis of *Pythium aphanidermatum* hyphae an oomycete plant pathogen[552]. *Janthinobacterium lividum* produces an antifungal metabolite[567] and *Variovorax paradoxus* is capable of degrading chitin[87] a key polymer in fungal cell walls[568]. Species in the genus *Lysobacter* use a diverse arsenal of weapons to prey on other microorganisms, including fungi and oomycetes[559]. *Lysobacter* secrete small molecular toxins against filamentous fungi, hydrolytic enzymes against non-filamentous fungi and can kill by lysing cells on contact[559]. *Streptomyces* are well known for the variety of antibiotics they secrete which can act against various microorganisms[397]. *Devosia insulae* can degrade the mycotoxin deoxynivalenol[569]. The enrichment of these species contributing to biocontrol in the rhizosphere by WW irrigation may suggest that WW irrigation increases protection of plants. A study investigating if bacteria from the aeration chamber of a WW treatment plant could be used as biocontrol agents against *Fusarium* in wheat cultivation, found that *Pseudomonas helleri* was capable of reducing the disease index[570].

This evidence, in addition to the appearance of *Devosia insulae* in WW irrigated soil suggests that WW irrigation could potentially add species capable of varying biocontrol functions to the rhizosphere, in addition to enrichment of species capable of biocontrol already present in the rhizosphere.

Another function provided by bacteria in the rhizosphere is bioremediation of environmental contaminants. Bacteria capable of organic contaminant remediation also increased, survived, and appeared in the WW irrigated rhizosphere. *Dokdonella immobilis*[520,571], *Hydrocarboniphaga daqingensis*[572], *Novosphongobium aromaticivorans*[573,574], *Pedobacter steynii*[575], *Sphingopyxis bauzanensis*[576], and increased in WW irrigated soil (Figure 2.6). *Hydrogenophaga taeniospiralis* was added to the rhizosphere from WW and *Blastochloris sulfoviridia* appeared newly in WW irrigated rhizosphere soil. These species are all capable of degradation of organic pollutants such as crude oil[575], aliphatic[572] and polycyclic aromatic hydrocarbons[573,574,577,578]. Along with these species other ESVs belonging to genera containing some species with bioremediation abilities also increased, survived, and appeared in WW irrigated rhizosphere soil, these genera included *Azoarcus*[579–581], *Brevundimonas*[582–585], *Dokdonella*[586], *Hydrocarboniphaga*[572], *Lysobacter*[587–589], *Microbacterium*[590], *Mycobacterium*[591–595], *Nocardiodes*[596–601], *Novosphingobium*[602], *Phenylobacterium*[421,603], *Pseudomonas*[604–607], *Pseudoxanthomonas*[608–611], *Sphingomonas*[612–615], *Sphingopyxis*[616], *Streptomyces*[617,618], and *Thiobacillus*[619–621] (Figure 2.9). The increase and presence of organic chemical degrading species in the WW irrigated rhizosphere may indicate that WW enriches the growth of species and may add new species that aid in bioremediation but also it may indicate that WW increases the concentration of pollutants such as hydrocarbons in the soil. While many studies exist examining the effects of WW irrigation on the remediation of heavy metals, the effects of WW irrigation on bioremediation of organic pollutants such as PAHs have yet to be examined.

Interestingly, two species were added to the rhizosphere from WW *Novosphingobium tardaugens* and *Rhodobacter blasticus*, these species can both degrade estrogens[622,623] (Figure 2.6; Figure 2.7). Estrone, estriol and ethinyl-estradiol measured 84.00 ± 2.00 ng L^{-1}, 155.00 ± 6.00 ng L^{-1}, and 1240.00 ± 10.00 ng L^{-1}, respectively, in primary WW samples. As many WW species (86.98%) from primary WW did not survive in the rhizosphere the survival of these two species suggests that the estrogens in WW may remain in soil and be used as a resource by these species. Furthermore, *Dokonella immobilis* (increased in WW rhizosphere) is capable of degradation of

acetaminophen[624], and although not measured in these primary WW samples, is often detected in WWs[625]. The increase and addition of these species may indicate that phytoremediation of WW enriches and supports species capable of certain emerging contaminant degradation. If soil can retain the emerging contaminants from WW and support the growth of species capable of degrading these substances, thereby degrading them, and stopping the spread in surface waters, short rotation willow coppice may be a superior form of WW treatment to the small-scale two-step activated sludge treatment plant.

In summary, the control rhizosphere microbial community may be largely resilient to change as the addition of WW did not have a large effect on relative abundances in the rhizosphere, 72.60% of ESVs did not significantly differ in relative abundances. Twenty-five ESVs endemic to WW sample resolved to species-level taxonomy remained in the rhizosphere after irrigation, possibly indicating these ESVs may offer a benefit to the rhizosphere and potentially may be able to establish within the microbial community. The majority of the significant change exhibited between control rhizosphere and the WW irrigated rhizosphere increased relative abundancies, however this effect was not universal as six ESVs decreased, indicating the enriching effects of WW were not universal. Interestingly, 15 ESVs not present in either WW or the control rhizosphere, were present in the WW irrigated rhizosphere, suggesting nutrients in WW may stimulate growth of species below detection limits. WW irrigation of a willow rhizosphere enriched many bacteria species with plant growth promoting abilities including those involved in phytohormone production, nitrogen fixation, phosphorus solubilization, iron uptake in plants, sulfur oxidation, biocontrol agents and remediation of organic pollutants. The relative enrichment of these species may not necessarily signify that these functions are occurring in the rhizosphere as a result of the WW irrigation, for example, species capable of nitrogen fixation were enriched, however, as nitrogen fixation is energetically costly, the easy acquisition of nitrogen supplied in WW may have decreased the need for nitrogen fixation[497]. However, other bacterial functions in the rhizosphere were enriched, such as facilitation of iron uptake by willows. Not only were siderophore producing and iron reducing species enriched or added to the rhizosphere, but iron concentration in soil significantly decreased and iron concentration in willow trees significantly increased[201] indicating a WW-mediated effect on iron uptake in willows. Furthermore, while one increasing species present in rhizosphere soil was capable of sulfur oxidation, WW added four additional species (six ESVs) capable of sulfur oxidation to a plant soluble form indicating that

WW irrigation may also supply additional plant growth promoting bacterial functions to the rhizosphere. Finally, WW irrigation also enriched and added species capable of organic pollutant breakdown (including some emerging contaminants) which may suggest that not only does municipal WW contains some organic pollutants (such as hydrocarbons), and emerging contaminants (such as ethinyl estradiol and acetaminophen) which are added to the rhizosphere, but it also supplies and enriches bacteria capable of remediating these pollutants. These findings suggest that (a) WW not only transfer nutrients such as nitrogen and phosphorus to the rhizosphere but possibly also sulfur and varying organic pollutants, and (b) more importantly that WW transfers new bacterial species capable of new plant growth promoting and pollutant remediating functions.

Conclusion

Bacterial species identified in an unirrigated control willow rhizosphere included those associated with plant growth promoting abilities. Irrigation of the rhizosphere with PW had little effect on the rhizosphere microbial community, very few ESVs showed differential abundance indicating the control rhizosphere and the PW irrigated rhizosphere remained similar to one another, however the significant change between these treatments may indicate PW irrigation has a slight diluting or dispersing effect on a portion of the microbial community. Irrigation with WW significantly altered approximately a quarter of the species-level rhizosphere microbial community, leaving approximately three-quarters unaltered, suggesting that the rhizosphere microbial community is relatively stable and resilient to change. The changes in relative abundances that did occur between the control and WW irrigated rhizospheres suggested a positive effect on the relative abundances of some existing species with plant growth promoting abilities such as phytohormone synthesis, nitrogen fixation, nitrite oxidation, organic phosphorus mineralization, phosphorus accumulation, siderophore production, iron reduction and sulfur oxidation. Additionally, species were added to the rhizosphere from WW that may contribute to functions such as sulfur oxidation. This analysis, however, cannot infer functionality, further studies investigating RNA expression should be conducted to confirm effects of WW irrigation on the functionality of the rhizosphere microbial community. The microbial community reaction to WW irrigation of short rotation willow coppice supports the use of this technology to promote growth for greater biomass production. Further research should also examine total microbial

abundances, how microbial biomass changes in a WW irrigated rhizosphere and how a WW irrigated rhizosphere microbial community changes over time to further understand the effects of WW irrigation.

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Figures and Tables

Table 2.1. Measured soil characteristics of control rhizosphere, PW irrigated rhizosphere and WW irrigated rhizosphere.

Soil characteristic	Control soil	PW irrigated soil	WW irrigated soil
pH	5.96 ± 0.04^{c}	6.59 ± 0.07^{a}	6.17 ± 0.09^{b}
Total Nitrogen (mg/kg)	1900 ± 100^{b}	1800 ± 100^{b}	2300 ± 100^{a}
Ammonia (mg/kg)	3.30 ± 0.65^{c}	20.74 ± 2.96^{a}	9.58 ± 2.92^{b}
Nitrates-nitrites (mg/kg)	7.32 ± 0.62^{c}	27.09 ± 7.23^{b}	173.90 ± 34.44^{a}
Total phosphorus (mg/kg)	934.00 ± 16.80^{a}	980.33 ± 115.23^{a}	1001.33 ± 29.70^{a}
Orthophosphate (mg/kg)	2.33 ± 0.33^{a}	2.00 ± 0.58^{a}	2.00 ± 0.00^{a}
Total carbon (mg/kg)	26800 ± 300^{b}	24300 ± 600^{c}	28200 ± 1400^{a}
Organic matter (mg/kg)	49400 ± 600^{b}	45400 ± 1300^{c}	52100 ± 2400^{a}
Iron (mg/kg)	247.22 ± 4.78^{a}	232.33 ± 10.99^{b}	226.67 ± 4.65^{b}

Table 2.2. Additional WW characteristics capturing certain emerging contaminants measured by HPLC

Contaminant in primary WW	Concentration in primary WW (ng L^{-1})
Caffeine	86176 ± 1098
Ethinyl estradiol	1240 ± 10
Estriol	155 ± 6
Estrone	84 ± 2

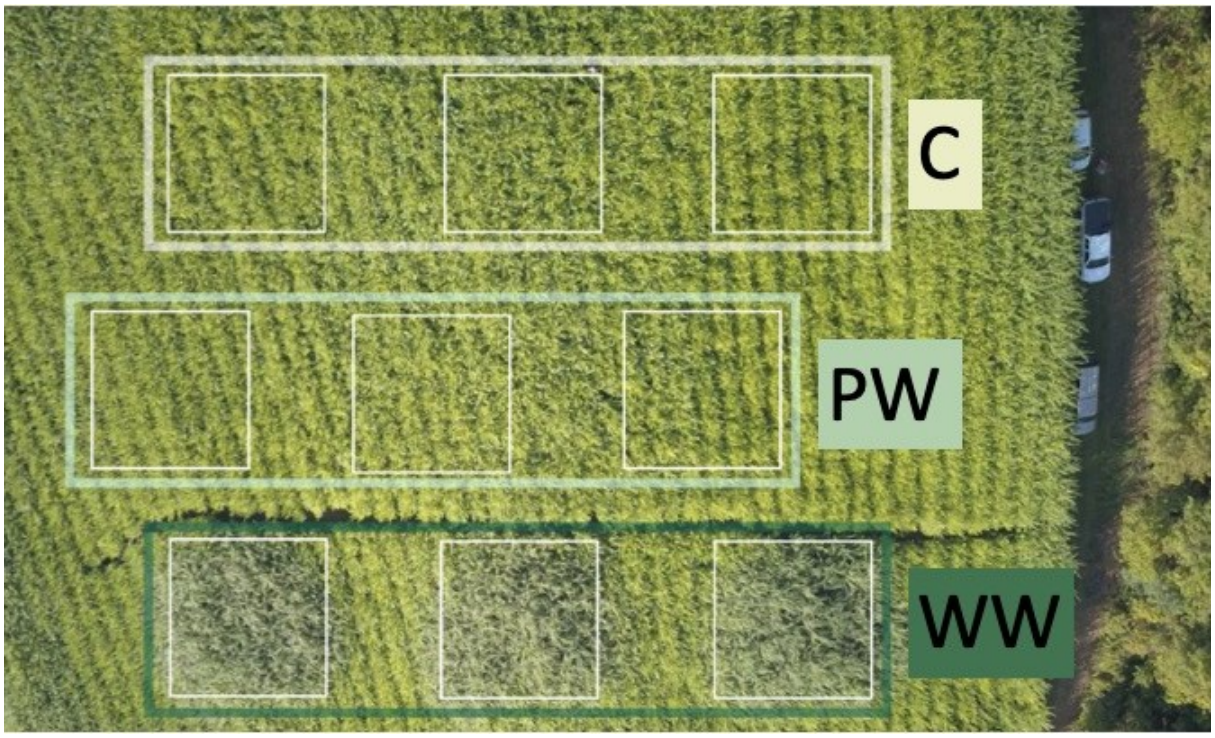

Figure 2.1. Aerial photograph of the St. Roch de l'Achigan field site and experimental design courtesy of Ahmed Jerbi. The Salix miyabeana 'SX67' plantation was established at a density of 16,000 trees ha^{-1} across four hectares northeast of Montreal, Canada. Nine experimental square plots of 100 m^2 (10 m × 10 m), each containing six rows of trees, were treated with one of three treatments (three plots per treatment): unirrigated control (C), potable water (PW) and primary wastewater (WW) irrigated. Photograph taken in 2017 before harvest.

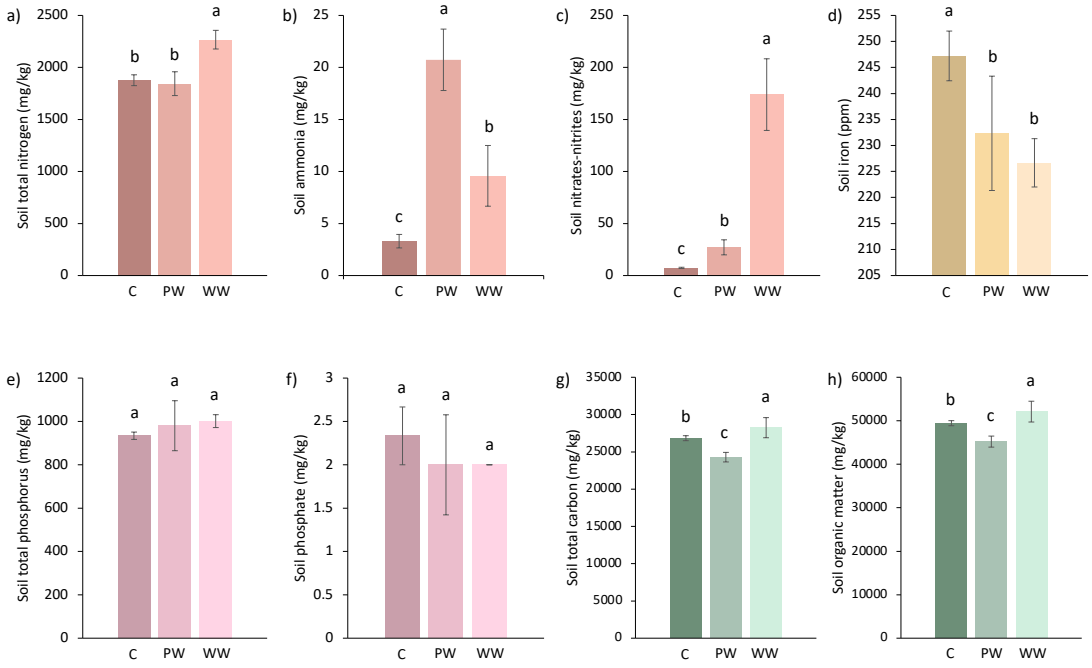

Figure 2.2. Soil constituent concentrations for a) total nitrogen, b) ammonia, c) nitrates-nitrites, d) iron, e) total phosphorus, f) phosphate, g) total carbon and h) organic matter for control rhizosphere soil, potable water (PW) irrigated rhizosphere soil and wastewater (WW) irrigated rhizosphere soil. Significant differences displayed using a, b and c lettering.

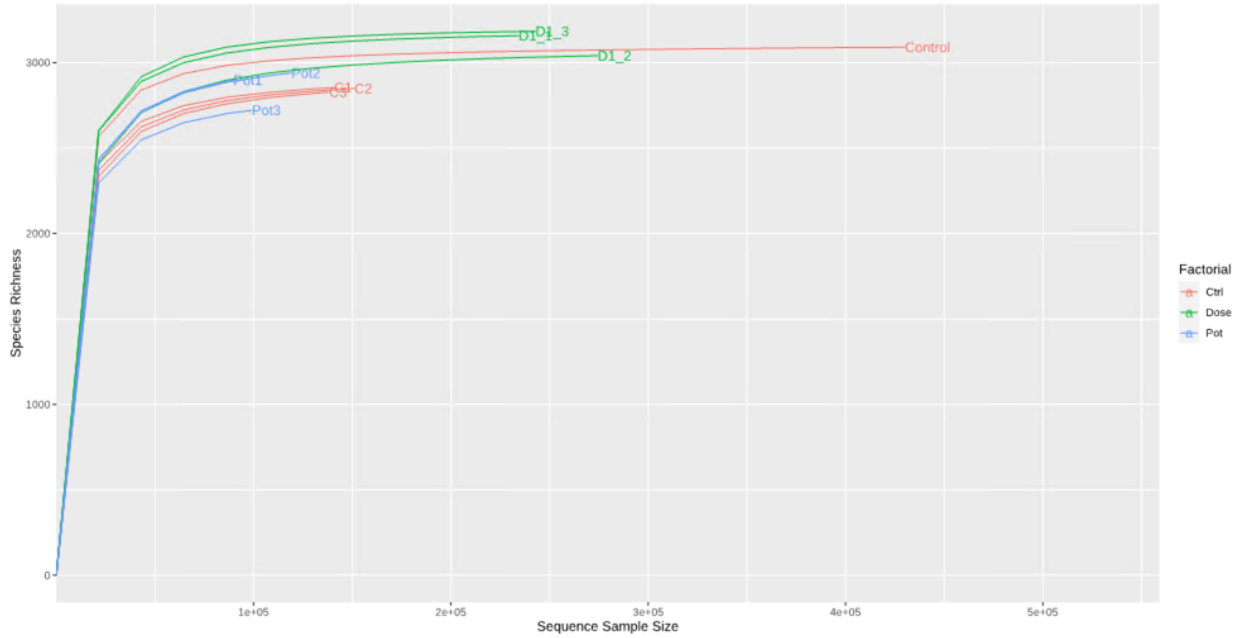

Figure 2.3. Rarefaction curve of ESVs by wastewater treatment plant samples indicating sampling depth captured most of the microbial community. Ctrl = control rhizosphere, Pot = PW irrigated rhizosphere and Dose1 = WW irrigated rhizosphere

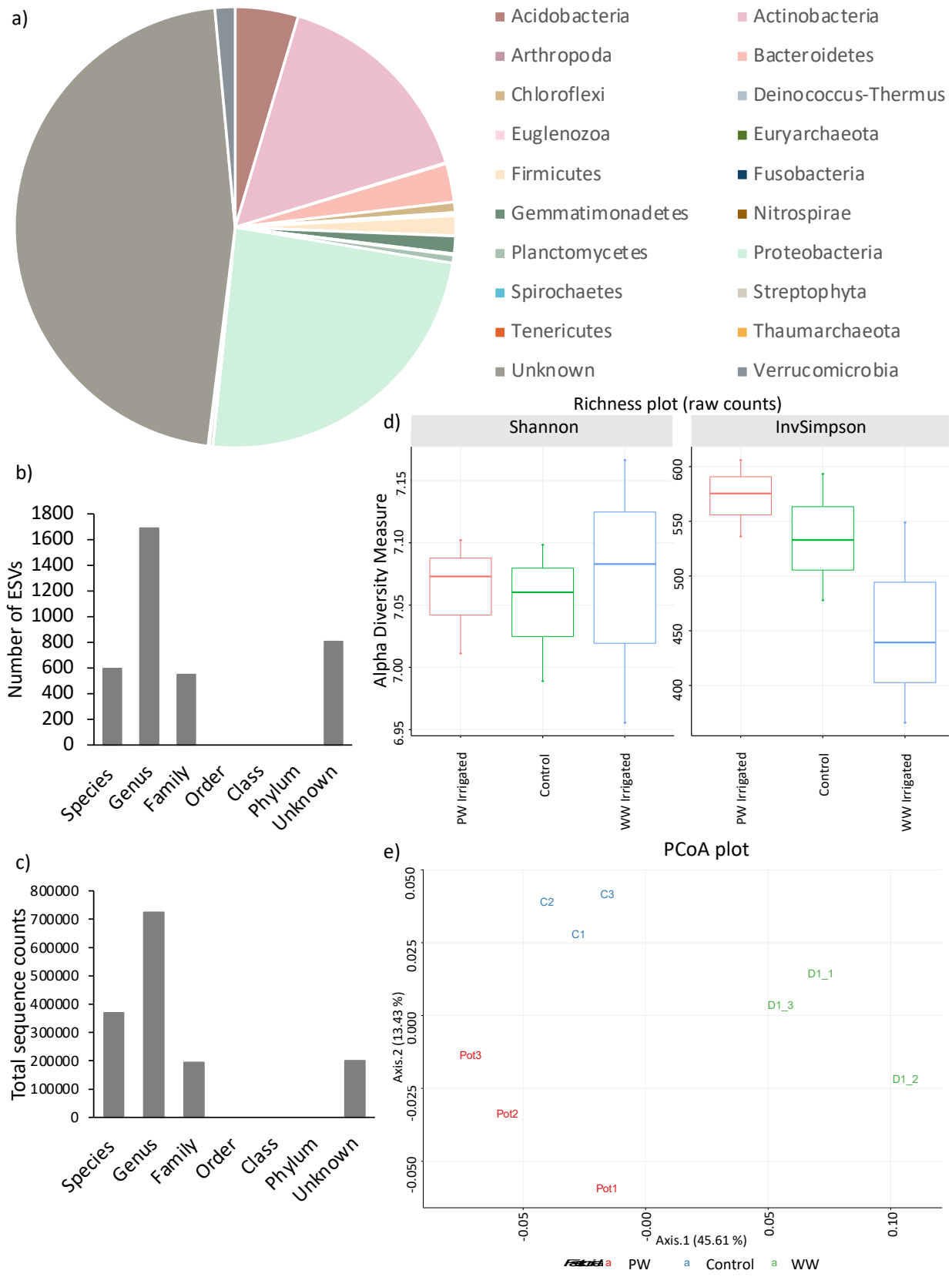
a)
Acidobacteria
Actinobacteria
Arthropoda
Bacteroidetes
Chloroflexi
Deinococcus-Thermus
Euglenozoa
Euryarchaeota
Firmicutes
Fusobacteria
Gemmatimonadetes
Nitrospirae
Planctomycetes
Proteobacteria
Spirochaetes
Streptophyta
Tenericutes
Thaumarchaeota
Unknown
Verrucomicrobia
b)
Number of ESVs
1800
1600
1400
1200
1000
800
600
400
200
0
Species
Genus
Family
Order
Class
Phylum
Unknown
c)
Total sequence counts
800000
700000
600000
500000
400000
300000
200000
100000
0
Species
Genus
Family
Order
Class
Phylum
Unknown
d)
Richness plot (raw counts)
Shannon
InvSimpson
Alpha Diversity Measure
7.15
7.10
7.05
7.00
6.95
600
550
500
450
400
PW Irrigated
Control
WW Irrigated
PW Irrigated
Control
WW Irrigated
e)
PCoA plot
C2
C3
C1
D1_1
D1_3
Pot3
D1_2
Pot2
Pot1
Axis.2 (13.43 %)
0.050
0.025
0.000
-0.025
-0.050
-0.05
-0.00
0.05
0.10
Axis.1 (45.61 %)
a PW
a Control
a WW

Figure 2.4. a) Control rhizosphere microbial community composition by relative abundance based on phyla (including all taxonomic levels), b) Number of ESVs per taxonomic category, c) Number of sequence counts per taxonomic category and unknown sequence counts, d) Shannon and Inverse Simpson diversity indices of the microbial communities in control rhizosphere, potable water (PW) irrigated rhizosphere and wastewater (WW) irrigated rhizosphere, e) Principal Coordinates Analysis of microbial communities present in control, PW irrigated and WW irrigated rhizospheres (n = 3 each treatment).

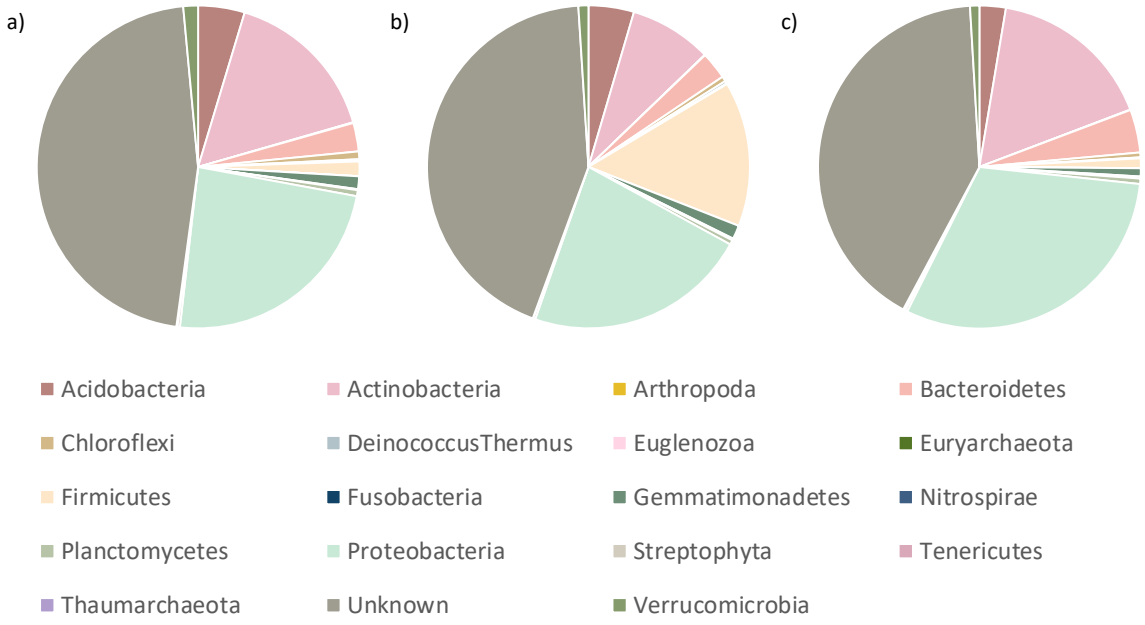

Figure 2.5. Microbial community structure at the Phyla level of the microbial community in a) the control rhizosphere, b) the PW irrigated rhizosphere and c) the WW irrigated rhizosphere.

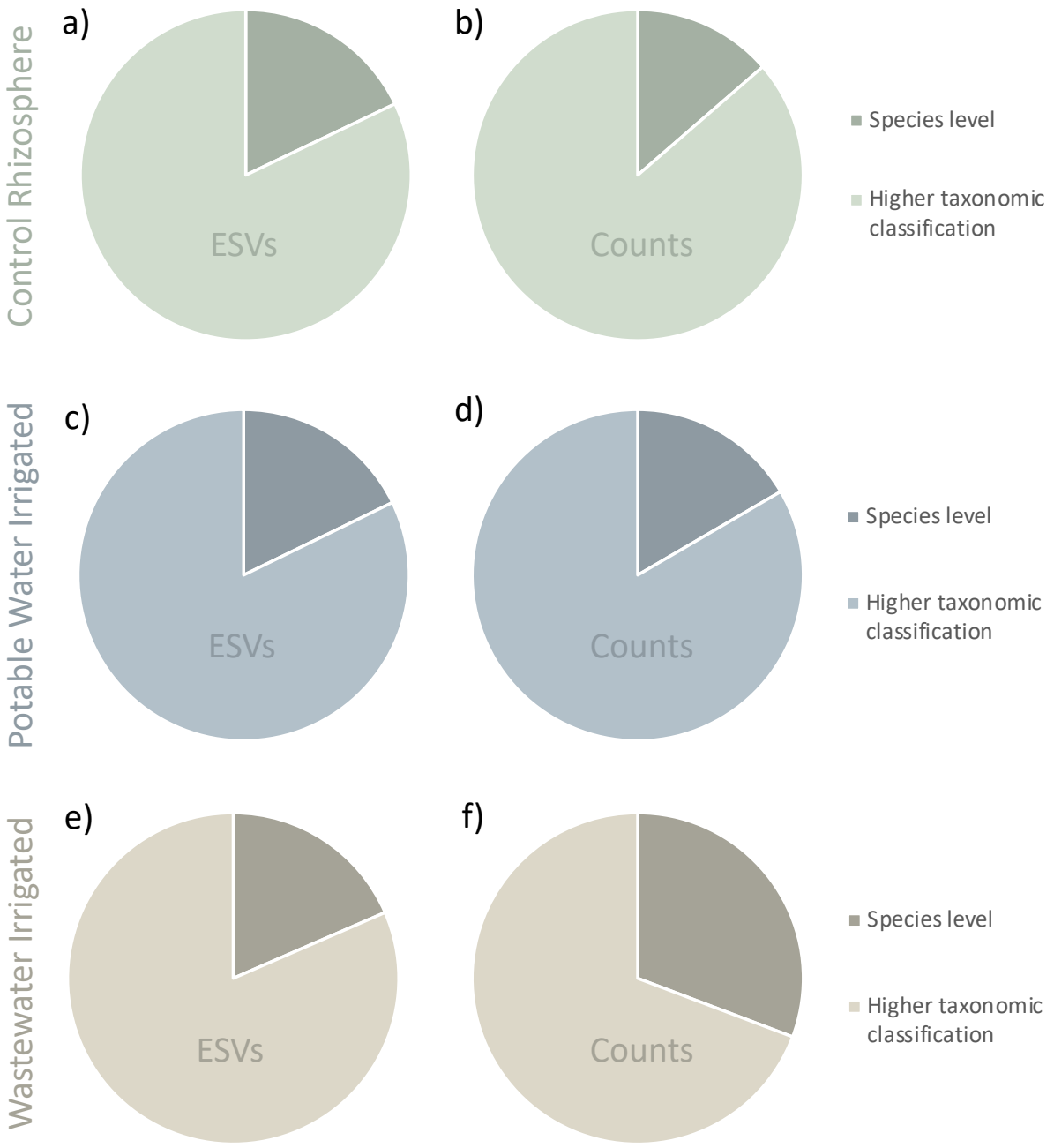

Figure 2.6. a) The proportion of control soil ESVs resolved to species-level taxonomy, b) the proportion of control soil counts captured by species-level ESVs, c) The proportion of potable water (PW) irrigated ESVs resolved to species-level taxonomy, d) the proportion of PW irrigated counts captured by species-level ESVs, e) The proportion of wastewater (WW) irrigated ESVs resolved to species-level taxonomy, f) the proportion of WW irrigated counts captured by species-level ESVs.

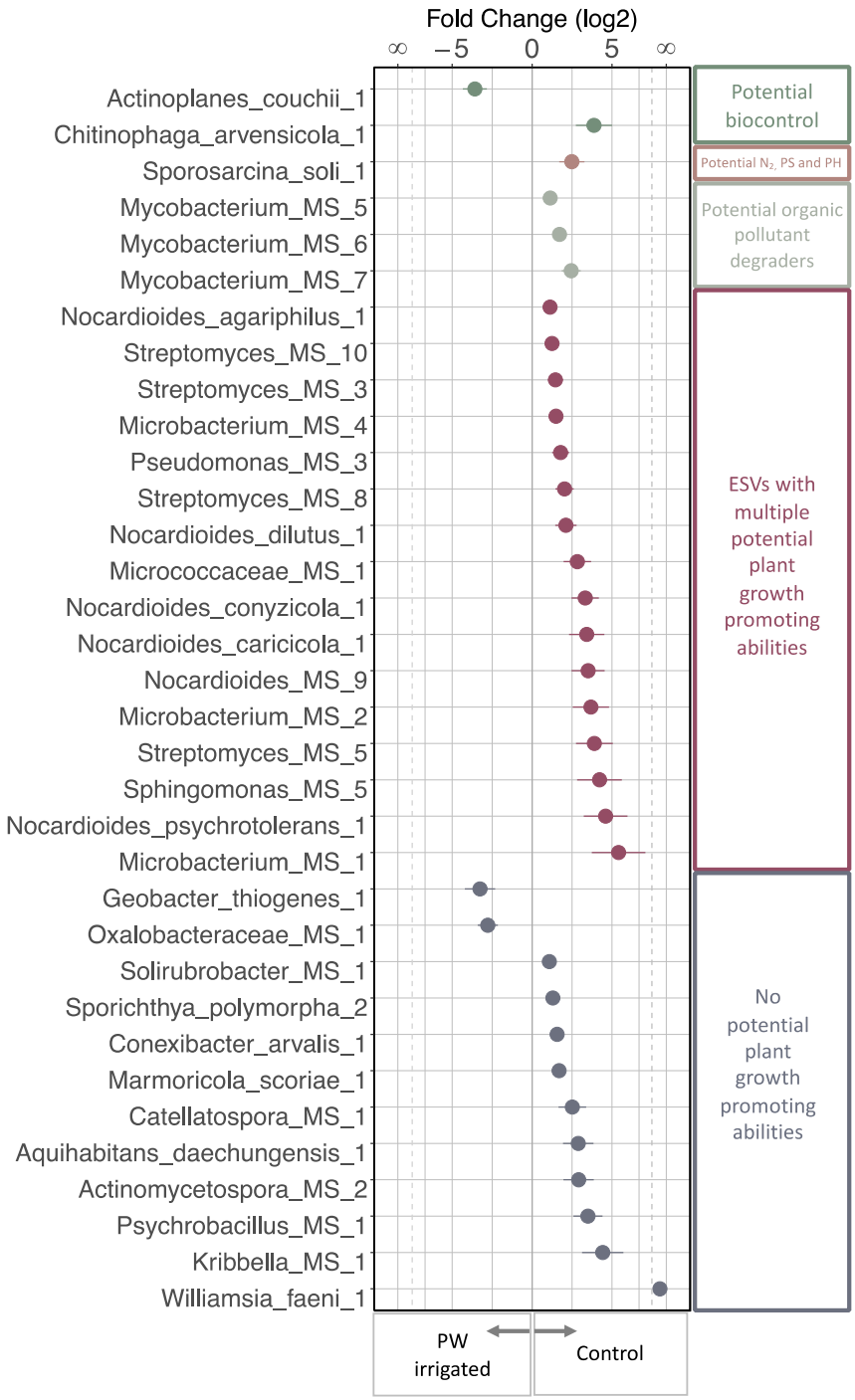

Figure 2.7. Differentially abundant ESVs from control rhizosphere soil to potable water irrigated rhizosphere soil grouped by their potential plant growth promoting abilities based on presence of plant growth promoting traits within their genus.

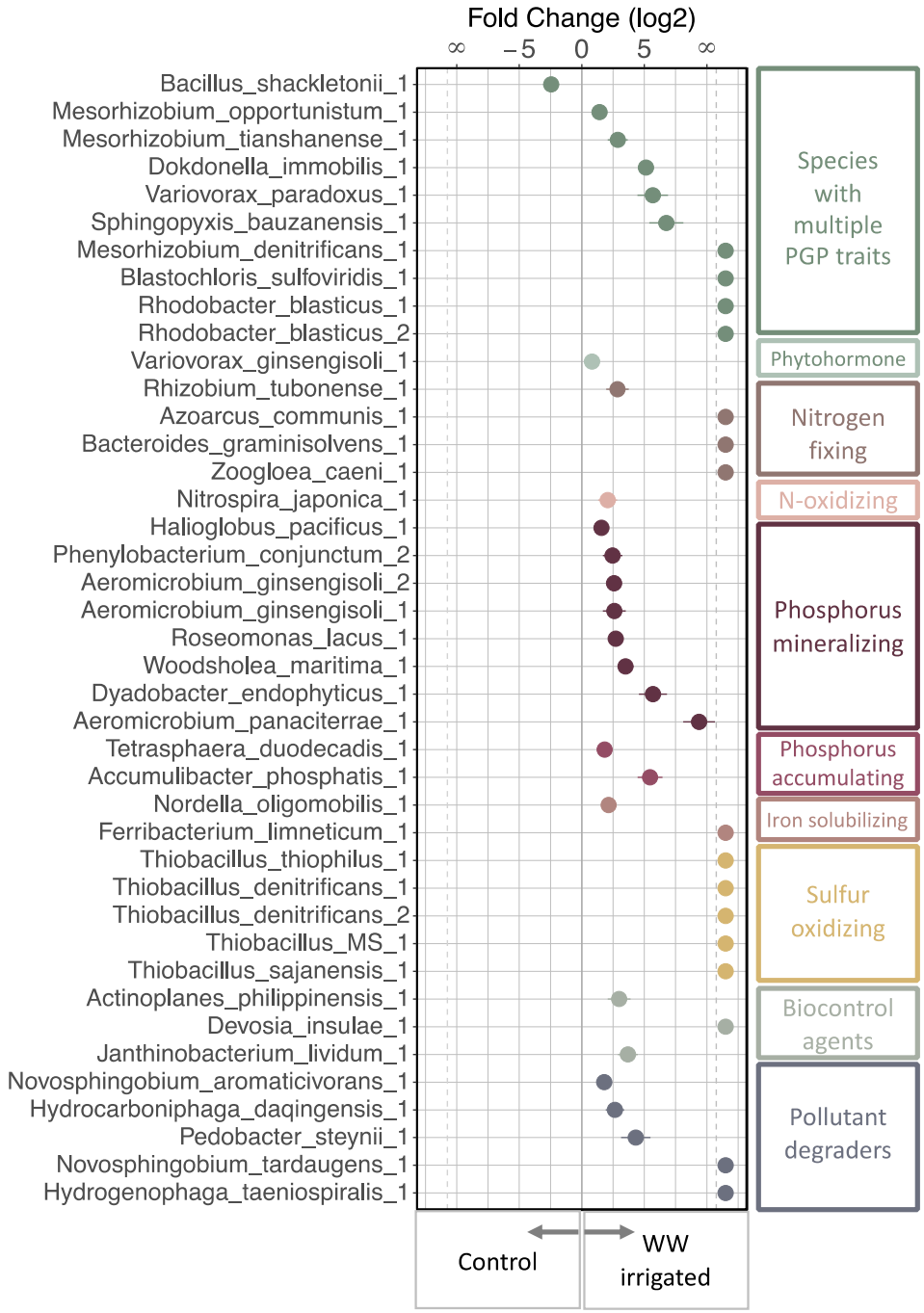

Figure 2.8. Selected differentially abundant ESVs from control rhizosphere soil to wastewater (WW) irrigated rhizosphere soil confirmed to have specific plant growth promoting and pollution remediating functions.

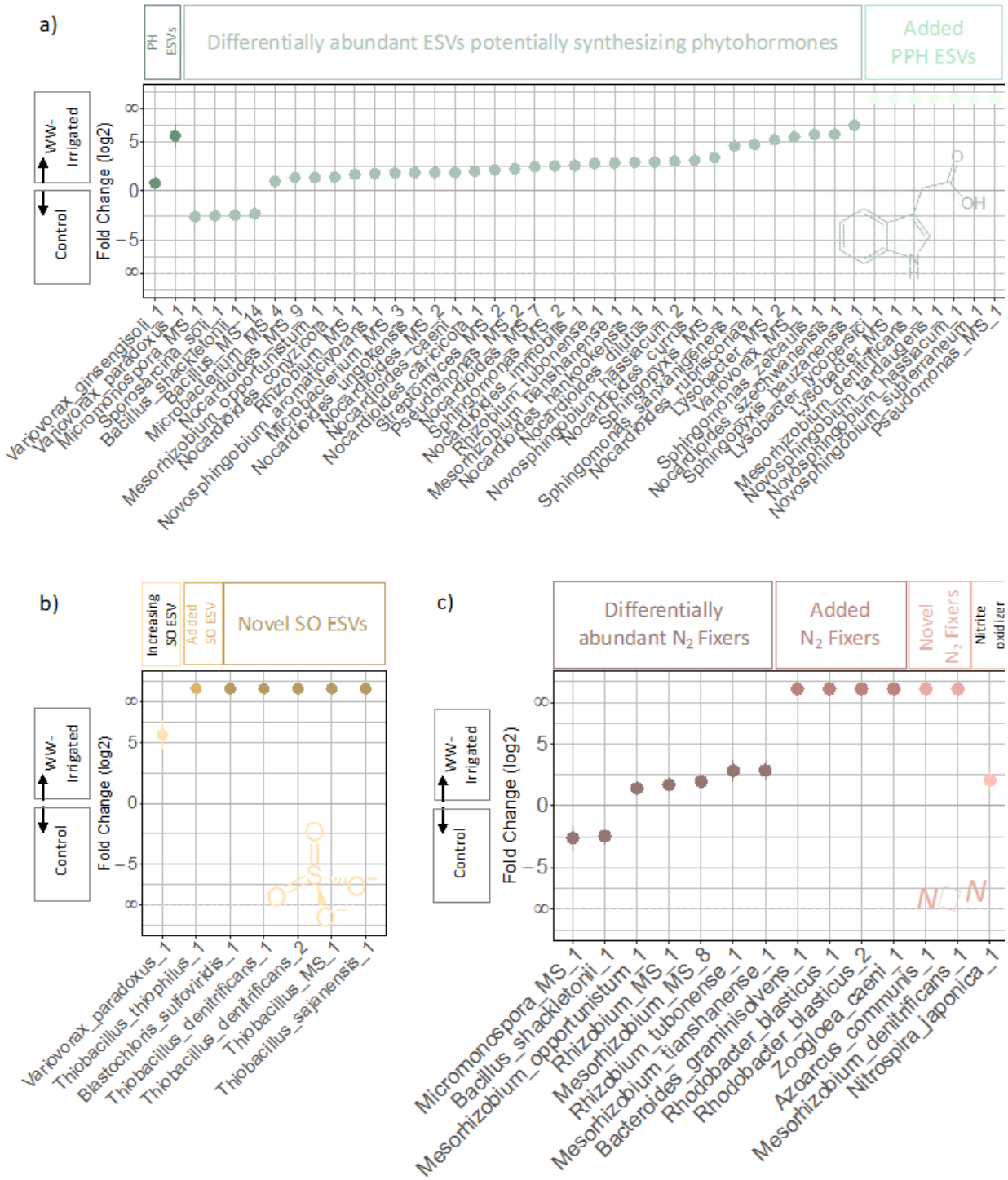

Figure 2.9. a) Differentially abundant and wastewater-added phytohormone synthesizing (PH) and potential phytohormone synthesizing (PPH) ESVs from control rhizosphere soil to wastewater irrigated rhizosphere soil, b) Differentially abundant, wastewater-added and completely novel* sulfur-oxidizing (SO) ESVs from control rhizosphere soil to wastewater irrigated rhizosphere soil, c) Differentially abundant, wastewater-added and completely novel* nitrogen fixing ESVs as well as the differentially abundant nitrite-oxidizing ESV.

*Completely novel ESVs were not present in wastewater samples or in rhizosphere soil samples before wastewater irrigation.

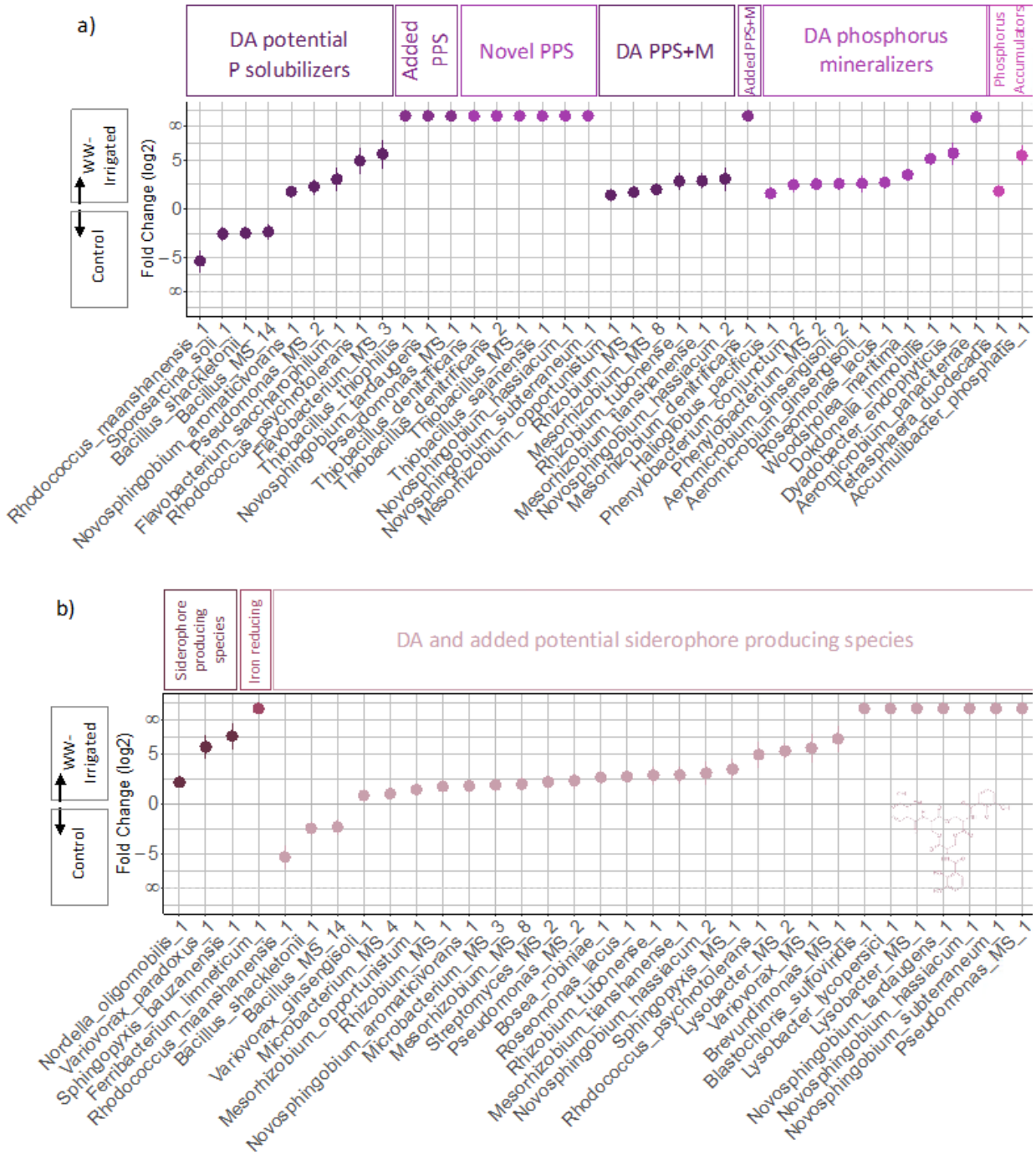

Figure 2.10. a) Differentially abundant (DA), added and novel* potential inorganic phosphorus (P) solubilizers and organic phosphorus mineralizers (M), ESVs potentially capable of both phosphorus solubilization and mineralization (PPS+M) and differentially abundant phosphorus accumulators from control rhizosphere soil to wastewater irrigated rhizosphere soil, b) Differentially abundant siderophore producing species, the novel iron reducing species and the differentially abundant and added potential siderophore producing species.
*novel species were not present in wastewater samples or rhizosphere samples before irrigation

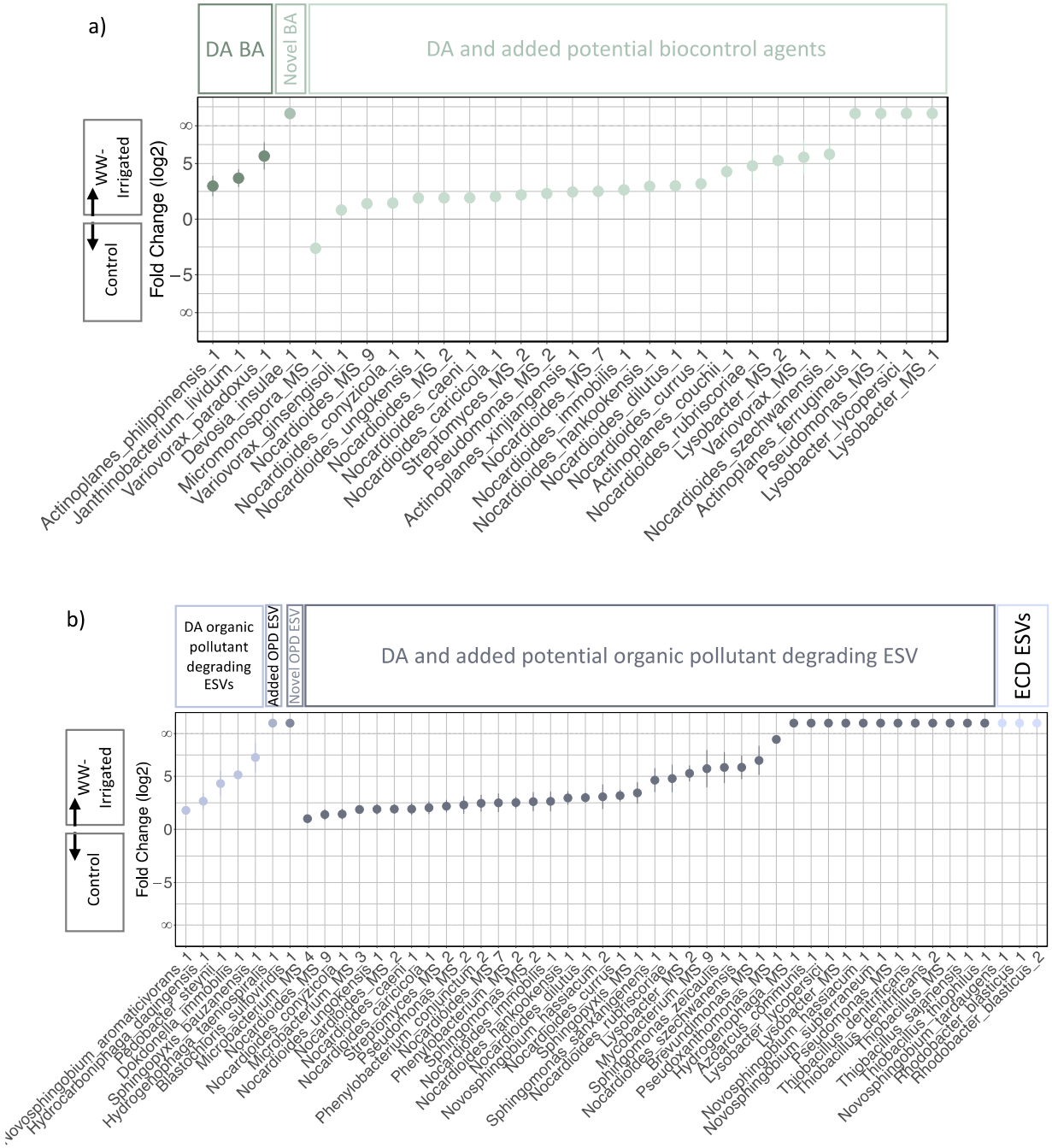

Figure 2.11. a) Differentially abundant (DA) biocontrol agent ESVs, novel biocontrol ESV as well as differentially abundant and wastewater-added potential biocontrol agent ESVs from control rhizosphere soil to wastewater irrigated rhizosphere soil, b) Differentially abundant, added and novel organic pollutant degrading (OPD) ESVs, differentially abundant and added potential organic pollutant degrading ESVs as well as emerging contaminant degrading (ECD) ESVs.

*novel species were not present in wastewater samples or rhizosphere samples before irrigation

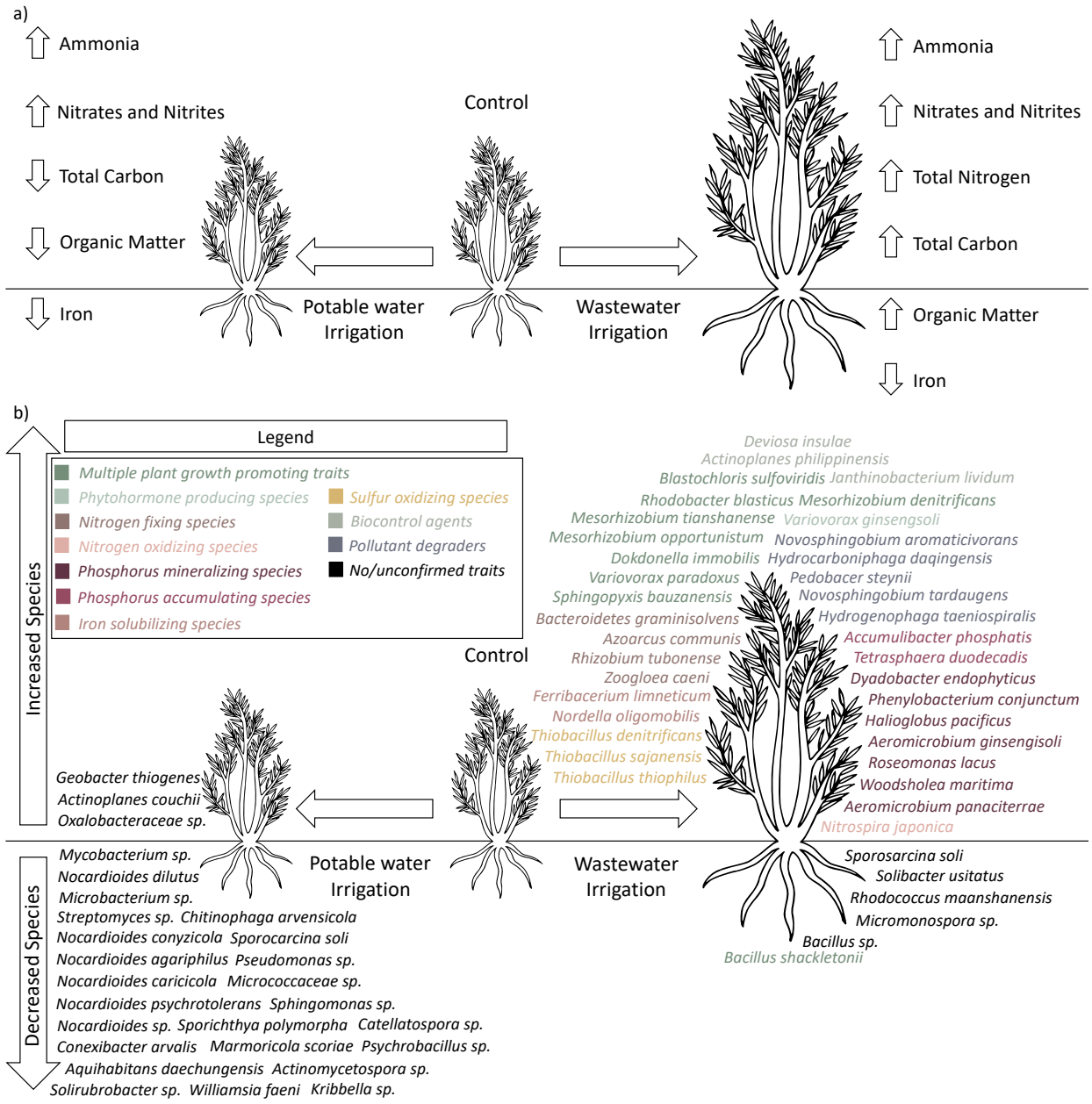

Figure 2.12. Graphical representation of a) the changes in soil constituents and b) the changes in the microbial community from the control rhizosphere to a PW irrigated rhizosphere and from the control rhizosphere to a WW irrigated rhizosphere. Note: not all significantly different species between the control and the WW irrigated are displayed, only those with plant growth promoting traits and decreasing species.

General Conclusions

Overarching Conclusions and Context

The first chapter of this dissertation found that the origins of the primary wastewater microbial community came largely from the broad categories of human, animal and environment associated species. While other studies have not assessed origins of wastewater bacteria other than from humans Newton et al. (2015)[237] and Shanks et al. (2013)[236] found lower proportions of the wastewater microbial community originating from humans. Newton et al. (2015) found that only 15% of sewage sample sequence reads were attributed to human faecal origin[237]. Shanks et al. (2013) estimated bacteria in wastewater originating from faeces to be 12.1% [236]. In chapter one, the 28% of counts attributed to human associated bacteria is most likely higher than these comparative studies due to the species level resolution employed in this dissertation and the continued advancements in species discovery as well as sequencing technologies. The aeration process of activated sludge largely contributed to shaping the microbial community. A study investigating the effects of dissolved oxygen on ammonia oxidizing bacterial communities in activated sludge found that different bacteria were enriched in high and low dissolved oxygen content[626]. Another study investigating the shifts in microbial community in response to dissolved oxygen levels in activated sludge found that dissolve oxygen content affected the diversity of bacteria[627]. These studies lend support to the idea that the dissolved oxygen content due to aeration of activated sludge shapes the composition of the microbial community. The changing resource pool (e.g., different proteins, carbohydrates, fatty acids and their degradation residues) may have also contributed to shaping species composition within the wastewater microbial community through treatment. This suggests that the microbial community change is associated with important compositional change of proteins, carbohydrates and lipids, commonly observed in gut microbiome studies[318]. Furthermore, bacteria capable of desirable functions within wastewater treatment, such as nitrification of ammonia, are selected by the aeration process, whereas bacteria capable of other functions, such as plant polymer degradation are diminished by aeration, but return in effluent once aeration has subsided. Conventional wastewater treatment does not remove all microbial life from wastewater before rerelease into surface waters. Furthermore, many human gut bacteria are present in effluent as well as some species identified as being potentially pathogenic to humans and animals, especially fish. The presence of human associated and

potentially pathogenic species present in wastewater effluents has been well documented[628–632] which indicates along with this dissertation, metagenomic analysis of wastewater treatment plants and their effluents could help as an additional treatment quality assurance step to guard waterways.

The second chapter of this dissertation found that the rhizosphere microbial community was relatively stable. Potable water irrigation had little effect on the rhizosphere microbial community, however the majority of species showing differential abundance between control soil and potable water irrigated soil decreased. Krause et al. (2020) found with irrigation, regardless of water quality, rhizosphere bacteria diversity declined[633]. These decreases could be a result of simple dispersion of bacteria through soil or the change to the environment (e.g., many soils are aerobic, oxygen may decrease during irrigation) that may provide a less habitable environment for certain species. Wastewater irrigation significantly changed 27.40% of rhizosphere ESVs. Wastewater added 40 ESVs to the rhizosphere, however the majority of wastewater species present in primary wastewater samples from the first chapter were not detected in the wastewater irrigated rhizosphere. To the best of this researcher's knowledge this is the first study assessing remaining wastewater species in the rhizosphere after phytofiltration of wastewater however one study identified bacteria in wastewater with plant growth promoting abilities and used these plant growth promoting bacteria to inoculate maize[634]. These findings suggest wastewater may contain different species capable of plant growth promotion that may or may not be able to establish in the rhizosphere with wastewater irrigation. Wastewater irrigation had a mostly enriching effect with only a few ESVs negatively impacted. A study by Guo et al. (2017) found Proteobacteria, Gemmatimonadetes and Bacteroidetes were more abundant in soils irrigated with reclaimed water[635], however Krause et al. (2020)[633] and Cui et al. (2019)[636] found decreased diversity and decreased abundance in rhizosphere bacteria watered with different waste and reclaimed waters. The varying results of these studies suggests that effects of wastewater irrigation on rhizosphere microbial communities are not straight forward. Different wastewater compositions as well as different hydraulic loadings of irrigation regimens may affect the rhizosphere microbial community in different ways. Wastewater irrigation enriched and added species capable of plant growth promoting function such as phytohormone synthesis, nitrogen fixation, phosphorus solubilization and mineralization, siderophore production and iron reduction, sulfur oxidation, biocontrol agents and as well as organic pollutant metabolization. Kannan et al. (1990)[494] found increased abundances of nitrogen fixing bacteria *Rhizobium* and *Azotobacter* in paper mill effluent

treated sugarcane rhizospheres and Ibekwe et al. (2018)[495] found higher numbers of OTUs (operational taxonomic units) for nitrogen fixing bacteria such as *Bradyrhizobia* and *Agrobacterium* in WW treated soil. Tripathi et al. (2011)[546] also found a 179% increase in siderophore producing species in a WW effluent irrigated field in comparison to a well-water irrigated field. Ashraf et al. (2018)[551] that investigated sulfur oxidizing bacteria and found they were enriched in both sewage water and sewage sludge in comparison to control soil. These studies confirm that different species of bacteria with plant growth promoting effects can be enriched by wastewater irrigation of rhizospheres. However, some of the functions provided by plant growth promoting bacteria may have been enriched such as siderophore production facilitating iron uptake in willows while others, such as nitrogen fixation may not have been although nitrogen fixing bacteria were enriched. A study examining the *nifH* gene expression (i.e. a gene for nitrogenase) in rice paddy soil amended with rice straw over the long-term found that long-term rice straw addition significantly increased diazotroph abundance, but in contrast, sharply reduced *nifH* gene expression and nitrogen fixation activity[497]. Koo et al. (2005)[512] tested the effects of biosolid amendments (solid wastes taken out during the WW treatment process) on organic acid production in a corn rhizosphere and found that biosolids enhanced organic acid production and influenced the composition of the organic acid mixtures, indicating the amendments may increase inorganic phosphorus solubilization. Another study, assessing rhizosphere dynamics during olive mill WW (containing 820 mg phosphorus L^{-1}) phytoremediation assessed phosphatase production and found higher production of phosphatase in soils treated with olive mill WW[524]. These studies suggest that while wastewater may enrich different plant growth promoting bacterial, the expression of plant growth promoting functionality may be dependent on the particular plant growth promoting function. Overall, wastewater irrigation had the effect of enriching and adding species capable of plant growth promoting functions to the rhizosphere.

The research in this dissertation illustrates from a microbial perspective that short rotation willow coppice offers a viable option of an alternative wastewater treatment technology that uses contaminants as nutrients for growth as well as transfers some plant growth promoting functionality from species that survive and establish in the rhizosphere. This phytofiltration technology may offer a superior treatment option for wastewater as contaminants in wastewater are used as a resource to help willow growth. Additionally, human faecal bacteria as well as potentially pathogenic species present in wastewater are not detected in wastewater irrigated

rhizosphere soil after phytofiltration. Use of phytofiltration by short rotation willow coppice for wastewater treatment may help prevent spread of disease and pollution of Canadian surface waters. Further investigation, however, is necessary to assess whether use of phytofiltration successfully metabolizes various added contaminants from wastewater, such as organic pollutants including emerging contaminants, whether addition of wastewater facilitates growth of endemic soil species that may be pathogenic to humans as well as whether any contaminants or bacteria from wastewater are filtered through to ground water.

General synthesis

Main findings from chapter 1

Wastewater treatment significantly changes wastewater constituents present in primary wastewater from one treatment step to the next. In addition to previous associations to wastewater bacteria in primary wastewater were potentially associated to humans, other (non-human) animals and the environment. The bacteria identified as potentially being human-associated contributed the largest proportion of primary wastewater species (37% of identified species) and indicate human gut bacteria are adaptable to water environments. Most potentially human associated species were significantly reduced by activate sludge, which may be explained by a large proportion of gut bacteria being anaerobic or microaerophilic as oxidation in activated sludge is likely a major driver shaping the microbial community. Functionality, such as nitrogen cycling, of species in activated sludge may be another driver shaping the microbial community as the relative abundance of many species with specific functions changed with important compositional changes of proteins, carbohydrates and lipids from one treatment step to the next. Activated sludge enriched species involved in important wastewater functions of nitrogen cycling and phosphorus accumulation, whereas species involved in degradation of recalcitrant plant polymers were reduced. Species capable of protein degradation, denitrification and phosphorus accumulation were present in each step indicating the function may be preserved (functional redundancy) but the significant changes indicate anaerobic species are replaced by aerobic species. Ammonia and nitrite oxidizing species were mostly absent from primary wastewater and enriched in activated sludge. Plant polymer degrading species decreased from primary wastewater to activated sludge, which is not surprising as most were anaerobic and human-gut associated species. This reduction of plant polymer degrading species in activated sludge may also diminish biodegradation of plant

polymers in activated sludge. Surprisingly, many plant polymer degrading species increased from activated sludge to effluent suggesting the potential continued availability of some plant polymers in effluent (e.g., cellulose). A substantial proportion of the microbial community in wastewater persists in effluent and retains species from both primary wastewater and activated sludge. Three highly relatively abundant human gut associated species in primary wastewater were also highly abundant in effluent suggesting prevalent human gut bacteria survive the wastewater treatment process (although viability was not tested). Approximately half the potentially human associated species differed between primary wastewater and effluent indicating at least partial successful treatment. Some human and animal associated species increased in relative abundance between primary wastewater and effluent suggesting this common wastewater treatment process may provide an environment in which specific human associated species can live. Although cell viability was not tested, amplicon data suggests relatively few species are reduced beyond detection limits. The high microbial load in effluent may negatively affect the receiving waters, however, regulations do not specify criteria for wastewater effluents regarding microbial load. In addition to a human associated species remaining in effluent, at least 22 species present in primary wastewater have been reported as including pathogenic strains to humans, animals and aquatic life. Of these 22 ESVs only two were effectively removed (below detection limit), nine decreased from primary wastewater to effluent, nine did not significantly differ between treatments and two significantly increased. Although potentially pathogenic bacteria released into surface waters may not be viable or may not persist in the biosphere these findings suggest the potential for environmental concern and that high resolution metagenomic analysis of wastewater samples could have some utility as an additional treatment quality assessment step.

Main findings from chapter 2

PW irrigation does affect some soil characteristics and constituent concentrations, however most effects on soil are more pronounced with WW irrigation. More precisely, PW increased pH, ammonia and nitrates-nitrites concentrations while decreasing total carbon, organic matter, and iron, whereas, WW increased pH, total nitrogen, ammonia, nitrates-nitrites, total carbon, organic matter while decreasing iron. Surprisingly, phosphorus concentrations were not significantly affected by either irrigation. Only a small proportion of the rhizosphere microbial community is resolved to species level. WW irrigation facilitates a greater presence of characterized species in the rhizosphere. Alpha diversity indices confirm that rhizosphere microbial communities are

largely stable, while multivariate analyses indicate there are some differences between the treatments. Only 18% of the control rhizosphere microbial community was resolved to species level, however, of the proportion resolved to species level many ESVs belong to species capable of plant growth promotion or to genera associated with plant growth promotion. The microbial community only exhibited a small amount of significant change between the control and PW irrigated rhizosphere, however, the change that did occur indicated irrigation with PW may disperse or decrease a small proportion of the microbial community. In summary, the control rhizosphere microbial community may be largely resilient to change as the addition of WW did not have a large effect on relative abundances in the rhizosphere, 72.60% of ESVs did not significantly differ in relative abundances. Twenty-five ESVs endemic to WW sample resolved to species-level taxonomy remained in the rhizosphere after irrigation, possibly indicating these ESVs may offer a benefit to the rhizosphere and potentially may be able to establish within the microbial community. The majority of the significant change exhibited between control rhizosphere and the WW irrigated rhizosphere increased relative abundancies, however this effect was not universal as six ESVs decreased, indicating the enriching effects of WW were not universal. Interestingly, 15 ESVs not present in either WW or the control rhizosphere, were present in the WW irrigated rhizosphere, suggesting nutrients in WW may stimulate growth of species below detection limits. WW irrigation of a willow rhizosphere enriched many bacteria species with plant growth promoting abilities including those involved in phytohormone production, nitrogen fixation, phosphorus solubilization, iron uptake in plants, sulfur oxidation, biocontrol agents and remediation of organic pollutants. The relative enrichment of these species may not necessarily signify that these functions are occurring in the rhizosphere as a result of the WW irrigation, for example, species capable of nitrogen fixation were enriched, however, as nitrogen fixation is energetically costly, the easy acquisition of nitrogen supplied in WW may have decreased the need for nitrogen fixation[497]. However, other bacterial functions in the rhizosphere were enriched, such as facilitation of iron uptake by willows. Not only were siderophore producing and iron reducing species enriched or added to the rhizosphere, but iron concentration in soil significantly decreased and iron concentration in willow trees significantly increased[201] indicating a WW-mediated effect on iron uptake in willows. Furthermore, while one increasing species present in rhizosphere soil was capable of sulfur oxidation, WW added four additional species (six ESVs) capable of sulfur oxidation to a plant soluble form indicating that WW irrigation may also supply additional plant

growth promoting bacterial functions to the rhizosphere. Finally, WW irrigation also enriched and added species capable of organic pollutant breakdown (including some emerging contaminants) which may suggest that not only does municipal WW contains some organic pollutants (such as hydrocarbons), and emerging contaminants (such as ethinyl estradiol and acetaminophen) which are added to the rhizosphere, but it also supplies and enriches bacteria capable of remediating these pollutants. These findings suggest that (a) WW not only transfer nutrients such as nitrogen and phosphorus to the rhizosphere but possibly also sulfur and varying organic pollutants, and (b) more importantly that WW transfers new bacterial species capable of new plant growth promoting and pollutant remediating functions.

Novel findings of this research

Chapter one found that approximately 27% of ESVs capturing approximately 60% of counts could be resolved to species level taxonomy. The implication of this finding is that a large portion of ESVs throughout wastewater remain unknown and therefore the depth of knowledge of microbial communities in wastewater treatment remains superficial. While superior methodology exists to examine microbial communities such as whole genome sequencing (WGS), the costs of these superior methodologies are often prohibitive. Reducing the costs of WGS should help to close the gap of microbial community identification. Additionally, many species exist that have yet to be named and studied, a greater effort in microbial research should also help to bridge this gap. Both oxidation and the changing constituent resource pool contributed to shaping the structure of the microbial community throughout wastewater treatment. Although it has been demonstrated before that oxidation can impact bacterial community structure, this finding demonstrates that oxidation may be a contributing factor but not the sole driver of microbial structure. This current research was only able to identify oxidation and changing resource pool as factors contributing to community structure, however, other factors may also contribute to microbial community structure. Further research should try to identify what other possible factors may affect the structure of microbial communities. Many species, including many human-associated species survive the wastewater treatment process. Wastewater treatment is a complex science, different parameters can have different effects on the treatment. Perhaps, given the presence of many bacteria in effluent, greater attention and care needs to be paid to adjustable factors affecting the treatment process. Additional treatment steps could also be employed to further reduce microbial presence in wastewater effluent. Protein degradation, denitrification and phosphorus accumulation

functionality seem to be present in all treatment steps however the species responsible for them shift from one treatment step to the next, whereas species capable of ammonia and nitrite oxidation are not present in primary wastewater but are present in activated sludge. Species with some desired functions in wastewater treatment are present in all treatments indicating functional redundancy for capabilities such as protein degradation, denitrification, and phosphorus accumulation, however, species capable of ammonia and nitrite oxidation are less resilient and are more dependent on specific environments. For an activated sludge system, the oxidation stage is important for the oxidation steps of nitrogen cycling. Species potentially pathogenic to humans, land animals and fish are present throughout wastewater treatment and are released into receiving surface waters. The presence of potentially pathogenic species, particularly those harmful to fish, gives cause for concern as the effluent directly enters freshwater ecosystems. This reiterates the need for complex analyses of the effluent microbial community as an additional step for wastewater treatment quality standards and potentially the required implementation of a tertiary treatment step.

Chapter two found that only approximately 16% of rhizosphere ESVs capturing 25% of counts are identifiable at species level taxonomy. The rhizosphere microbial community is under-characterized in comparison to the wastewater microbial community of conventional treatment. This is not surprising as the human gut microbiome has been the subject of greater study than soil microbial communities. Additionally, microbial diversity of human gut, while possibly very complex may not be as complex as soil and rhizosphere microbial communities. The species-level resolution of the rhizosphere microbial community reveals the gap in characterized species in soils. As mentioned above, trying to employ state-of-the-art sequencing techniques such as WGS may help to bridge some of the gap in knowledge. Also, continued efforts to identify and characterize more microbial species will help to illuminate a greater proportion of microbial communities. The undisturbed control rhizosphere contained many species with plant growth promoting capabilities. The species identified in the control rhizosphere microbial community already possessed many plant growth promoting capabilities, this finding is in line with previous research identifying the recruitment of beneficial bacteria in the rhizosphere[149,637]. Irrigation with PW had very little effect on the willow rhizosphere microbial community, only approximately 7% showed significant differential abundance between the control and PW irrigated rhizospheres. The slight effect shown by PW irrigation mostly decreased the relative abundance of significantly different species which

may be due to dispersal by water. While no overall biomass measurements were taken for this present study, a study by Qi et al. (2022)[638] found long term irrigation decreased fungal biomass in the rhizosphere, however, it did not affect bacteria biomass. The finding that PW irrigation had a slight decreasing effect on the rhizosphere microbial community may indicate that more care needs to be taken with irrigation of crops in general. This rests on the assumption that higher relative abundance of species is more beneficial, which at this point cannot be confirmed and may vary by species. As water is integral to life this finding is slightly surprising but may be due to oversaturation. Indeed, previous studies have found waterlogged samples to have the lowest microbial population[639]. Therefore, it may be prudent to closely monitor irrigation levels to ensure necessary hydration is achieved without oversaturation of crop soils. The rhizosphere microbial community is relatively stable and resilient to change by irrigation as even with WW irrigation only approximately a quarter showed significant change between the control and WW irrigated rhizospheres. The constituents and other potential contaminants in WW supply the willow rhizosphere with an influx of new resources, therefore it is surprizing that only approximately a quarter of the microbial community showed significant change, however, rhizosphere microbial communities may be relatively stable due to the high selectivity of the rhizosphere microbial community[119–121]. The new resources supplied by the wastewater may then contribute to growth of well-established species of the rhizosphere. WW irrigation seems to have a mostly increasing effect on relative abundance however these increasing effects are not universal as six species decreased. While the influx of nutrients from wastewater may facilitate growth of many species, the application of water, as demonstrated by PW irrigation, may also lead to decreases in rhizosphere species. Additionally, other mechanisms may play a role in the decrease of these species, such as exposure to certain emerging contaminants. WW irrigation does increase relative species with plant growth promoting abilities such as phytohormone synthesis, nitrogen fixation, nitrogen oxidation, organic phosphorus solubilization, siderophore production, sulfur oxidation, biocontrol and bioremediation. As mentioned above, the control rhizosphere microbial community naturally contained many species with plant growth promotion functionality, the addition of nutrients from wastewater increasing relative abundance of some of these species might have been expected as these species may be well established and well positioned in the rhizosphere to utilize the influx of nutrients. Interestingly, PW and WW irrigation seems to facilitate iron uptake from soil by willow, with the effect from WW irrigation being more pronounced. This discovery has

interesting real-world application potential, if phytoremediation is employed to remediate iron rich soils, the knowledge that both PW and WW irrigation helps facilitate the uptake of iron from soils into willow biomass, may help to speed the process of soil remediation. WW stimulates the growth of species absent in WW or the control rhizosphere. While most of the rhizosphere microbial community is well-established and some species benefit from the addition of WW, the introduction of WW to a willow rhizosphere also stimulates the growth of species absent from both WW and the control rhizosphere. These species included the most abundant species in WW irrigated rhizosphere, *Azoarcus communis*, a nitrogen fixing bacterium, and other known plant growth promoting species such as *Mesorhizobium denitrificans*. The novel species also included known sulfur oxidizing bacteria. Most likely, these novel bacteria were present in soils or WW below detection limits and the influx of WW stimulated their growth. The presence of these species may be in response to the large increase of nitrogen in the soil from WW. Likewise, the increase in sulfur oxidizing species may indicate high sulfur content in WWs. However, sulfur content in WW or soils were not measured. This unique result suggests that microbial communities can respond to different inputs. This may have an encouraging implication in application of phytoremediation for different remediation strategies. Soil communities may have some flexibility to respond to different contaminants. Perhaps the diversity of soil microbial communities are vast with many species capable of different functionality sparsely present and able to take advantage of different inputs. WW irrigation may add species capable of novel functions in the rhizosphere such as sulfur oxidation and emerging contaminant degradation (ethinyl estradiol and acetaminophen degradation). In addition to the possibility of species with specific functionality being present in low but undetectable abundancies, WW may also transfer species with specific functionality to the rhizosphere such as *Novosphingobium tardaugens* and *Rhodobacter blasticus*, which can degrade estrogens[622,623] or *Dokonella immobilis*, able to degrade acetaminophen[624]. This could have implications for real world application as the addition of wastewater to contaminated sites employing phytoremediation may help supply or increase additional functionality and capabilities useful for remediation projects.

Return to initial research aims and hypotheses

Chapter 1 hypothesis

Chapter one aimed to test the hypothesis: changes in the abiotic physicochemical characteristics and constituents of wastewater will cause changes in the composition and individual species abundances of the wastewater microbial community.

The research presented in this chapter suggests that the abiotic physicochemical characteristics and constituents of wastewater, more specifically the dissolved oxygen content from aeration during activated sludge as well as nutrient resource availability contribute to the changes in differential abundance of the microbial community throughout the wastewater treatment process. Aeration in activated sludge probably selects for aerobic species and encourages certain wastewater treatment functions from aerobic bacteria, such as ammonia and nitrite oxidation. Aeration also selects against other functions such as plant polymer breakdown. Nutrient resource availability also seemed to affect species composition as decreasing nutrient forms were associated with decreasing species abundances.

Chapter 2 hypothesis

Chapter two aimed to test the hypothesis: irrigation of willow trees with primary wastewater will alter the rhizosphere's unique microbial community to better utilize wastewater nutrients and contamination.

The research presented in this chapter gives a more nuanced answer to this hypothesis. Irrigation of willow trees with primary wastewater does alter a portion of the microbial community significantly, but only 27% of the rhizosphere ESVs. As the majority of these significantly different ESVs increase from control soil to wastewater irrigated soil, this research suggests that most of the altered microbial community is better able to utilize wastewater nutrients and contaminants. The remaining 73% of rhizosphere ESVs, however, do not change significantly, indicating that the species composition and relative abundances within the rhizosphere microbial community maintain a certain amount of stability and that wastewater irrigation does not alter a substantial proportion of the rhizosphere community. Many of the 27% of rhizosphere ESVs that were altered were annotated as species associated with plant growth promoting abilities such as phytohormone production, nitrogen fixation, phosphorus solubilization, siderophore production, biocontrol agents and pollutant remediation. Wastewater irrigation also increased iron uptake by

willow and established new species in the rhizosphere capable of plant growth promoting traits but most noticeably added species capable of sulfur oxidation into plant soluble forms.

Overarching research aims of this dissertation

The main goal of this dissertation was to answer the question: can a deeper understanding of the microbial community associated with successful phytofiltration of primary wastewater facilitate implementation of low-cost green technology to treat and protect Canada's water resources?

The research presented in this dissertation affirms that the use of phytofiltration with its associated microbial community can offer a low-cost green infrastructure alternative to conventional wastewater treatment. This research also suggests that the use of phytofiltration for wastewater treatment may be more substantial at removing potential hazardous species present in wastewater, such as those identified as pathogens as well as the human gut bacteria. It may also help mitigate their potential release and spread in Canada's waterways. Furthermore, phytofiltration not only removes contaminants from wastewater but also uses these contaminants as a resource for both microbial and willow growth.

Limitations of research

There are various limitations to this research. Most notably the current depth of species-level microbial knowledge as well as the limitations of the current technology using single gene barcodes to identify the microbial community. As displayed by this research, only small proportions of the microbial communities were identified down to species-level taxonomy. This can be due to novel sequences unknown and unnamed in the current microbial databased or it can be due to undifferentiated genes common to many bacteria. This research also failed to test the viability of the microbial community which could give better insight and understanding to functioning and impacts of the microbial communities. This research also failed to assess the actions in both the wastewater and rhizosphere microbial communities as it did not assess RNA expression (i.e. gene expression) of the members of the communities. This research also did not test a dynamic understanding of the microbial communities over time. Perhaps if samples were taken periodically, they would have shown the complete dissolution of wastewater bacteria in the soil rhizosphere. Additionally, temporal samples could have shed light on how the microbial community changes as soil salinity increases and if this affects plant growth promotion. This

research also did not assess how dose-dependent the changes in the rhizosphere microbial community are, that is, would higher doses cause a greater proportion of the microbial community to change. Finally, this research did not explore the greater environmental context. That is, it did not assess effects of effluent constituent and microbial community release into surface waters, and it did not assess the possible leaching of contaminants and bacteria through soil into groundwater holdings.

Recommendations for future research

The research in this dissertation has highlighted the need for additional research to better understand microbial communities and their functioning in wastewater treatment and phytofiltration. This research was a first look at a species-level understanding of microbial actions in wastewater treatment and phytofiltration, however as sequencing technologies improve and more of the microbial life on earth is identified, research will be able to gain a deeper and more encompassing understanding of microbial functioning during wastewater treatment and phytofiltration. Wastewater treatment facilities could benefit from a more in depth look at how changing different treatment process parameters, such as hydraulic retention time, may impact microbial species composition and therefore nutrient and contaminant metabolization. This research highlights the need to understand how conventional wastewater treatment effluent release, specifically microbial community release, may impact surface waters and could be a vector of transmission of disease (most specifically in fish). This research also highlights the need for more research investigating the microbial function during wastewater phytofiltration. The indication that most wastewater bacteria are diminished in the willow rhizosphere warrants investigating how long wastewater bacteria remain in the rhizosphere. Given that the rhizosphere was sampled less than 24 hours after stopping irrigation, wastewater bacteria may be completely diminished after a slightly longer time span. Additionally, this research did not investigate if the changes in the microbial community of the rhizosphere are dose dependent. Perhaps higher doses of wastewater during irrigation would cause significant differential abundance in a greater proportion of the rhizosphere microbial community. Continuing research should assess the relation between wastewater irrigation dose and microbial change. The salt load of wastewater irrigation is one of the main concerns with this phytotechnology. As the microbial community of the wastewater irrigated rhizosphere displayed both salt intolerant species as well as salt-reliant species and

halophiles, further investigation of how the microbial community adapts to the concentration of salts in the soil over time is necessary for proper management of this technology. Understanding if the microbial community can adapt to increasing salt concentrations while simultaneously retaining plant growth promoting traits would help in understanding the life span of a given phytofiltration field. This research also would benefit from investigating the RNA expression in the rhizosphere to see what genes are expressed by these microbial communities. Lastly, research investigating the possibility of wastewater constituent, contaminant and bacteria leaching into ground water from the use of wastewater irrigation is necessary to fully evaluate the potential use and environmental impact of wastewater phytofiltration.

Research contributions to science

The research in this dissertation has contributed to the greater body of knowledge by giving a more in depth (species-level) characterization of the microbial communities involved in conventional wastewater treatment and phytofiltration as well as a better understanding of what is being released in Canadian waterways. It has given an idea of where the bacteria in wastewater may originate and a better understanding of the relationship between wastewater characteristics and constituents shape the wastewater microbial community. This research has contributed a better understanding of how the microbial community responds to the introduction and removal of the aeration mechanism of activated sludge. Additionally, it illuminates what microbial functions aeration selects for and against. This research has discovered that most wastewater bacteria do not persist in the rhizosphere and that the rhizosphere microbial community is relatively stable in species composition and abundance as approximately three-quarters of the rhizosphere microbial community is unchanged from wastewater irrigation. The changes in the remaining quarter of rhizosphere bacteria demonstrate that wastewater irrigation enhances plant growth promoting bacteria. It also confirms that wastewater irrigation enhances overall abundance of bacteria in the rhizosphere. Ultimately, the research in this dissertation supports the hypothesis that phytofiltration can be used to decontaminate wastewater. Further research is, however, needed on the mechanisms by which wastewater affects the rhizosphere microbial community and the impact of willow wastewater phytofiltration on the surrounding ecosystem, which could demonstrate that this technique can be used to treat wastewater.

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