IDENTIFICATION OF MICROORGANISMS FOR THE BIOREMEDIATION OF NITRATE AND MANGANESE IN MINNESOTA WATER

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Satoshi Ishii

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ABSTRACT

Bioremediation is a way to safely and cost-effectively remove contaminants using living organisms. In this thesis, microorganisms capable of remediating two pollutants, nitrate and manganese, were identified using culture-dependent and –independent approaches. Nitrate in agricultural wastewater can lead to algal blooms and eutrophication. Edge-of-field woodchip bioreactors are a promising approach to prevent nitrate in wastewater from reaching surface waters by utilizing microbial denitrification to remove nitrate from the system. However, woodchip bioreactors experience low efficiency under cold temperatures, so one strategy to enhance bioreactors in the early spring involves bioaugmentation, or inoculating the bioreactors with cold-adapted denitrifying microorganisms. In order to identify a cold-adapted denitrifier for bioaugmentation, microorganisms were isolated from field woodchip bioreactors and subjected to denitrification testing under cold temperatures, measuring nitrate, nitrite, ammonium, nitrous oxide and dinitrogen gas, as well as whole genome sequencing to identify the presence of genes involved in denitrification and other important microbial processes. Based off of these results, two strains, *Microvirgula* sp. BE2.4 and *Cellulomonas* sp. WB94 were recommended for bioaugmentation. In part two, manganese was addressed. High levels of manganese in drinking water can cause health problems, and common treatment methods require cost-intensive chemicals, conditions and maintenance. In this study, a novel algae bioreactor was established to remove manganese from water. In this bioreactor, the algae provided fixed carbon for manganese-oxidizing microorganisms that oxidized the dissolved manganese, precipitating it out of solution. Using a culture-dependent approach, manganese-oxidizing bacteria and fungi were isolated from an environmental sample, including known

oxidizers *Bosea*, *Pseudomonas*, *Plectosphaerella* and *Phoma* and some not previously known to oxidize manganese such as *Aeromonas*, *Skermanella*, *Ensifer* and *Aspergillus*. A culture-independent approach was also employed to determine how abundant the isolated manganese-oxidizing bacteria are in an actively oxidizing environmental sample. Using nitrate and manganese as examples, this thesis identified useful microorganisms involved in remediation and demonstrated how microorganisms can be utilized to effectively remove pollutants from the environment.

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1. INTRODUCTION

1-1. General introduction to bioremediation

Bioremediation, or the use of living organisms to degrade, sequester or transform chemicals and compounds in a system, has emerged as a promising means for removing both pollutants and naturally-occurring hazards from our environment (Gadd et al. 2010; Wasi et al. 2013; Jan et al. 2014). It has the potential to compete economically with other conventional treatment methods, such as chemical treatments or additives and incineration, and is growing in popularity because it can replace the need for harsh chemicals while keeping costs low (Vidali 2001; Jan et al. 2014). The living organisms that are most often used and which will be the focus of this thesis are microorganisms, including bacteria and fungi. Microorganisms are natural bioremediators due to their diverse metabolic pathways and ability to survive under even the harshest conditions. In fact, microorganisms have been found in almost every environment including deep inside the Earth's crust (Lin et al. 2006), in metal-rich acid mine drainage (Baker et al. 2003) and in Antarctic ice (Bowman et al. 1997). Microorganisms also interact with and transform a diverse range of elements (Wackett et al. 2004; Gadd et al. 2010). The University of Minnesota created a Biocatalysis and Biodegradation Database to compile microbial transformation reactions from published scientific literature and has so far identified 77 elements from the periodic table that undergo a specific chemical reaction through microbial processes (Ellis et al. 2003). These range from very common elements such as carbon, nitrogen and oxygen, to less common and often toxic elements including mercury, arsenic, lead, and cadmium. Microorganisms have even been shown to transform xenobiotics, including compounds found in dyes, pesticides and solvents (Sander et al 2000; Stolz 2001).

Bioremediation can take place ex situ or in situ. For ex situ studies, contaminated soil or water is transported to another location for treatment, while in situ approaches treat the contaminated soil or water without transporting it. Reactions for both ex and in situ studies often take place in a bioreactor, which is a chamber or vessel of various designs that allow for microbial reactions to occur. One strategy to implement or enhance bioremediation involves adding living microorganisms capable of carrying out the desired bioremediation reaction to the site or contaminated substances, a technique known as bioaugmentation (Vidali et al. 2001; Gentry et al. 2004). This strategy requires identifying which microorganisms have the desired capability and understanding the ways in which they transform the substrates in question. Microorganisms selected for bioremediation purposes are often isolated from the site in question are therefore native to the specific location (Gentry et al. 2004). This approach is practical because environment-borne microorganisms can better colonize and survive in the environment than non-native microorganisms (Bouchez et al. 2000; El Fantroussi and Agathos 2005). It can be advantageous, however, to introduce a non-native microorganism to the contamination if there are no local species that are able to remove the compound or are unable to remove it rapidly or efficiently enough (Alexander 1999; Tiyagi et al. 2011). Bioaugmentation techniques have been used in agriculture since the 1800s and early techniques inoculated legumes with symbiotic, nitrogen-fixing *Rhizobium* spp. (Gentry et al. 2004).

Another method to enhance bioremediation is biostimulation, in which necessary nutrients or environmental conditions are added to the site or contaminated substances to enhance microbial activity (Atlas and Hazen 2011; Tiyagi et al. 2011). At the Exxon Valdez spill in 1989, 48,600 kg of fertilizer-N was added to the spill to promote growth of local hydrocarbon-metabolizing microorganisms (Atlas and Hazen 2011). It was estimated that 25 to 30% of the total hydrocarbon in the oil was degraded within weeks and almost 98% was removed 12 years later (Atlas and Hazen 2011). Biostimulation and bioaugmentation can be applied to more current and persistent instances of pollution that are present throughout the country and the world.

This thesis is divided into two parts that focus on two bioremedation challenges facing Minnesota: 1) nitrate pollution as a result of agricultural runoff, and 2) groundwater contamination of manganese, a naturally occurring element.

1-2: Introduction to nitrate pollution

Nitrogen is an essential element for life, but often a limiting resource in agricultural systems, which require fixed nitrogen in the form of ammonium or nitrate (Gruber and Galloway 2008). Without the use of nitrogenous fertilizers, farmers would not be able to grow enough food to support increasing human populations (Galloway et al. 2008). Consequently, from 1960 to 2000, the use of nitrogen fertilizers increased by 800% (Canfield et al. 2010). Human-induced fixed nitrogen, through the Haber-Bosch process and fossil-fuel combustion, is now double the natural rate of nitrogen fixation (Canfield et al. 2010). It is not surprising, then, that increased nitrogen use has impacted the natural nitrogen cycle and affected water quality around the world (Galloway et al.

2003; Galloway et al. 2008; Rivett et al. 2008; Schlesinger 2009). Since the development of the Haber-Bosch process, ammonia-derived fertilizers including anhydrous ammonia, ammonium nitrate (NH_4NO_3), ammonium sulfate (NH_4SO_4), and urea (NH_2CONH_2) have become the main sources of fertilizer (Canfield et al. 2010). Ammonium (NH_4^+) is commonly used because, due to its positive charge, NH_4^+ readily sorbs to soils and organic matter and is not quickly transported to groundwater. The NH_4^+ that is applied to the land, however, can be oxidized by microorganisms to nitrate (NO_3^-) in a process known as nitrification. NO_3^- is a soluble anion that is repelled from negatively-charged clay mineral surfaces, making it highly mobile and allowing it to leach into groundwater and waterways (Lin et al. 2001). Excessive nitrogen loads in waterways can cause substantial algal growth, hypoxia and unhealthy drinking water, costing the U.S. $82 million annually due to losses in the tourism and seafood industries as well as public health impacts (Hoalgand and Scatasta 2006).

The detrimental effects of nitrate pollution in the environment are best exemplified by the Dead Zone in the Gulf of Mexico, which measured 8,776 square miles June 2017 (NOAA 2017). To mitigate this, the US Environmental Protection Agency (EPA) established a multi-state Hypoxia Task Force that called on states to develop plans to reduce nitrate and phosphorus loads to the Mississippi River. As Minnesota is one of the contributing states to the Dead Zone in the Gulf of Mexico, the state has implemented a Nutrient Reduction Strategy which aims for a 45% nitrate reduction from 1980-1996 average conditions by 2040 (MPCA 2014).

Because of the current interest in mitigating nitrogen pollution, many methods are currently being researched to reduce nitrate loads. Some of these methods include controlled drainage, wetlands, crop rotations, the use of cover crops and woodchip bioreactors (Dinnes et al. 2002). Some of these methods rely on the presence of naturally occurring, heterotrophic denitrifying microorganisms which reduce nitrate through a series of steps to dinitrogen gas. Denitrification is one of the main pathways by which nitrogen reenters the atmosphere in gaseous form. These microorganisms make up a diverse group of bacteria, archaea and fungi, all of which, to date, are facultative aerobes, capable of growth under aerobic conditions, but denitrifying under anaerobic conditions.

One method known as a woodchip bioreactor has demonstrated potential in being a low-cost, low-maintenance means for reducing the flow of nitrate into surface waters. Woodchip bioreactors are designed so that nitrate-containing field effluent is diverted underground through a trench filled with organic substrate, in this case woodchips, that serve as a carbon (C) and electron source for the denitrifying microorganisms. Woodchip bioreactors do not require loss of farmland and parameters such as hydraulic retention time can be controlled to increase denitrification. In the Midwest, field-scale bioreactors have shown nitrogen load reductions of 12% to up to nearly 100%, depending on retention time, temperature and microbiology (Christianson et al. 2012b). Increasing flow rate is known to reduce nitrate removal, regardless of bioreactor substrate, due to both a lower retention time and the transport of dissolved oxygen (Greenan et al. 2009). Oxygen replaces nitrate as a terminal electron acceptor and can cause incomplete denitrification, leading to the release of N_2O instead of N_2 (Greenan et al. 2009). Lower temperatures are also known to inhibit denitrification. This is due to lower microbial activity as well as the lack of an easily available C source, which has been shown to be a limiting factor in cold temperatures. For example, Christianson et al. (2013) reported only a 9% load reduction in May 2011 at an average temperature of 9.0°C for a bioreactor in Iowa. Similarly, another study by Ghane et al. (2015) attributed low nitrate removal rates to a drop in temperature. Minnesota faces a unique challenge in that its surface water temperatures are, on average, much lower than that of the rest of the country. This is believed to decrease microbial activity and therefore denitrification. There is a need, then, to enhance woodchip bioreactor efficiency, particularly in early spring when temperature is low and runoff is high.

Woodchip bioreactors have been in use for the last couple of decades and abundant literature exists concerning their design and type of C source. Woodchips have been the most commonly used C source in the field due to their longevity and cost-effectiveness. However, it has been shown that, particularly under cold temperatures, woodchips do not provide enough microbially-available C for denitrification (Feyereisen et al. 2016). Studies have shown that more labile sources of C such as corn stalks or wheat straw can increase denitrification under cold temperatures (Greenan et al. 2006; Warneke et al. 2011; Feyereisen et al. 2016). Easily degradable C sources, however, require more frequent replacement and may create an environment favoring other processes over denitrification, such as dissimilatory nitrate reduction to ammonium (Tiedje et al. 1983; Greenan et al. 2006). Greater rates of N_2O production, an important greenhouse gas, have been reported when the bioreactor was built using wheat straw (Warneke et al. 2011). In addition, elevated amounts of total organic C and dissolved methane as well as growth of non-denitrifiers have been associated with a bioreactor filled with corn cobs (Warneke et al. 2011). Although woodchips may not provide the highest short-term NO_3^--N removal, they exhibit moderate and sustained nitrate removal and they are widely used along with the addition of a more labile C source, such as cornstalks or soybean oil, to promote denitrification (Feyereisen et al. 2016; Warneke et al. 2011; Greenan et al. 2006).

Genes encoding the nitrite reductase, *nirK* and *nirS*, are most commonly used for characterizing denitrifying communities as this is the first step in denitrification that produces a gaseous product. The use of the nitrous oxide reductase encoding gene, *nosZ*, as an indication of denitrification is also common as it denotes the final step reducing the greenhouse gas N_2O to the inert N_2 gas. The presence of denitrifiers, as represented by total *nirS*, *nirK* and *nosZ* genes, has been measured across varying C substrates with contradictory results. One study found very low denitrifier abundance in woodchips compared to other substrates (Feyereisen et al. 2016), while another found a higher proportion of denitrification genes among total bacteria in woodchips compared to corn cobs and green waste, although they had more total 16S rRNA gene copies (Warneke et al. 2011).

Despite the abundant research, there is a lack of knowledge about the microorganisms that are responsible for denitrification. Previous literature has focused solely on the presence of denitrification genes within the bioreactor to represent denitrifier abundance and confirm the occurrence of microbial denitrification. No studies to date have isolated denitrifying bacteria from a woodchip bioreactor and quantified their denitrification potential for the purpose of comparing successful bioreactors or bioaugmentation. Understanding the function and composition of denitrifying microorganisms will further enhance our capability of reducing nitrogen loads and provide insight into local environmental conditions.

Consequently, the overall goal for the first part (Chapters 2, 3, and 4) of this thesis is to understand the microbiology of woodchip bioreactors in order to enhance their performance, particularly under cold temperatures.

1-3: Introduction to manganese pollution

Manganese (Mn) is the second most abundant metal on earth and an essential element for life. Mn is frequently used as a cofactor in enzymes responsible for microbial metabolism and oxygenic photosynthesis. It is an immensely reactive element and has been referred to as an "environmental sponge" due to its ability to interact with and impact nearly all other elemental cycles (Ehrlich et al. 2015).

Due to its versatility, manganese has a number of industrial uses, including iron and steel production, dry cell batteries, adhesives, paint and fireworks (Avila et al. 2013). Manganese can exist in oxidation states ranging from 0 to +7, however in nature it is primarily found in the +2, +3 and +4 forms (Hem 1963; Ehrlich et al. 2015). Only Mn^{2+} can exist as a free ion in solution. It is thermodynamically favored in the absence of oxygen and at low pH (**Figure 1-1**) and can be present at up to millimolar concentrations in water (Tebo et al. 2004; Tebo et al. 2007; Ehrlich et al. 2015). The intermediate Mn^{3+} is unstable in solution and is commonly complexed to ligands or disproportionates into the +2 and +4 oxidation states (Tebo et al. 2007; Ehrlich et al. 2015). It may also accompany Mn^{4+} in insoluble oxides, oxyhydroxides and hydroxides (Tebo et al. 2007). These $Mn^{3/4+}$ (hydroxy)oxides have open crystal structures, large surface areas with negative charges, and exchangeable cations, rendering them highly reactive (Tebo et al. 2004).

Manganese is an important part of the human diet, with the U.S. Food and Drug Administration (U.S. FDA) suggesting a Reference Daily Intake (RDI) for Mn at 2 mg/day for adults. Despite its essentiality for a number of functions, human intake of Mn at high concentrations can lead to an accumulation in the brain, resulting in a clinical disorder known as manganism (Aschner et al. 2005; Avila et al. 2013). Elevated exposure leading to neurological problems have been known for 150 years (Avila el al. 2013). It has been shown, however, that consumption of manganese through diet is not associated with the same risk as drinking or inhaling manganese due to the route and behavior of Mn in the body (Boyes 2010; Bouchard et al. 2011; Bouchard et al. 2017). Some proposed reasons are the low efficiency of manganese uptake through consumption (believed to be 1-3%) and that manganese consumed through diet is complexed with large molecules while manganese

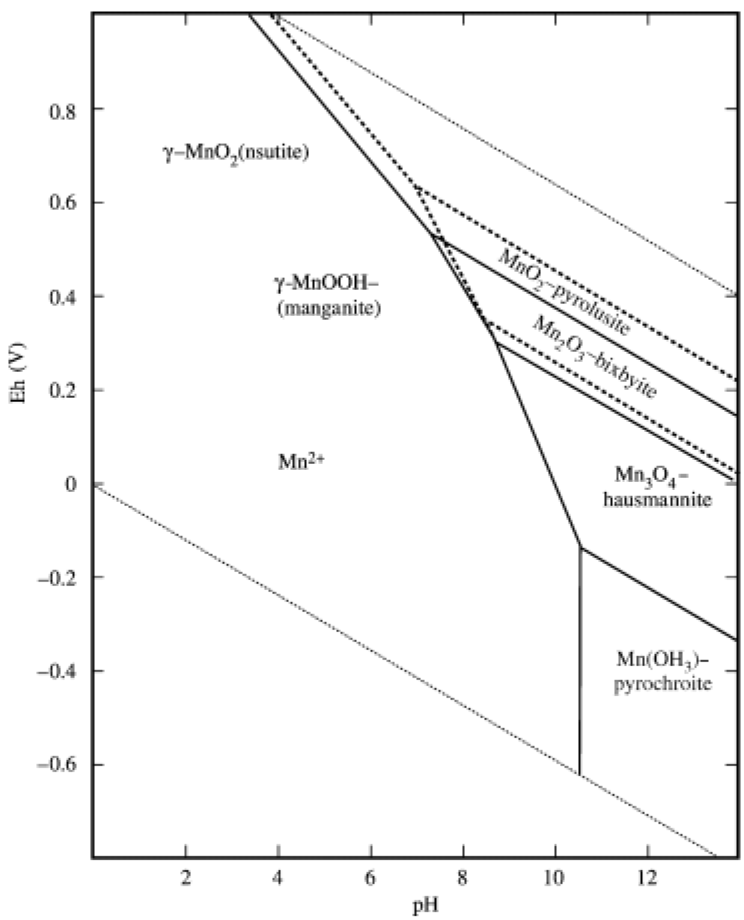


Figure 1-1: Eh-pH diagram showing the relationship between manganese oxides. Image modified from Maynard (2013).

present in drinking water exists as a free ion (Mn^{2+}) that can enter the blood stream, which is the primary route to the brain (Boyes 2010; Bouchard et al. 2017).

The most common way that Mn^{2+} enters drinking water is through the weathering and leaching of manganese-containing minerals and rocks into aquifers (Groschen et al. 2009). Manganese makes up much of Minnesota's bedrock and soil, and groundwater concentrations range from less than 50 µg/L to over 1,000 µg/L (Minnesota Groundwater Association; Groschen et al. 2009). To address this, the Minnesota Department of Health developed non-enforceable guidelines for manganese in drinking water at 300 µg/L for adults and children one year of age or older, and 100 µg/L for infants. Additionally, the U.S. Environmental Protection Agency (EPA) advises public water suppliers to treat water to less than 50 µg/L Mn. These guidelines are non-enforceable, however, as more research is needed to understand and establish biomarkers for manganese toxicity.

Common manganese treatment methods include direct oxidation, precipitation and removal of manganese oxides using strong oxidants such as chlorine dioxide, permanganate and ozone, each of which must be used carefully and appropriately as to not cause downstream water quality problems (Roccaro et al. 2007; Tobiason et al. 2016). Adsorption of dissolved manganese to a metal oxide surface, such as the natural ion exchange mineral, glauconite (greensand), is another method, however requires attention so that the adsorption capacity is not reached. Because treatment of groundwater manganese is not regulated and can require costly chemicals and equipment, some utilities do not consider manganese removal (Tobiason et al. 2016). Additionally, the use of chemicals to treat drinking water can lead to secondary impacts and by-products (Gallard and von Gunten 2002).

There is growing interest in biological manganese removal, or using manganese-oxidizing microorganisms to oxidize and remove soluble Mn^{2+} (Katsoyiannis and Zouboulis 2004; Burger et al. 2008; Hoyland et al. 2014; Tobiason et al. 2016). Manganese oxidation can be catalyzed by a diverse range of bacteria and fungi which increase the rate of reaction up to several orders of magnitude (Tebo et al. 2004; Akob et al. 2014; Bohu et al. 2015). The mechanisms by which manganese is biologically oxidized are poorly understood and many theories about how oxidation occurs currently exist. One of these includes reactive oxygen species (ROS), which were recently discovered to be involved in indirect oxidation (Learman et al. 2011; Diaz et al. 2013). ROS are by-products of aerobic metabolism and of one electron transfer reactions, such as the reduction of molecular oxygen to water, that are inevitable in oxygen redox chemistry (Hansel et al. 2016). Another proposed mechanism involves direct manganese oxidation via multicopper oxidases which are believed to have a role in manganese oxidation despite being strictly one-electron catalysts (Francis & Tebo, 2001; Soldatova et al. 2012). Manganese oxidation genes encoding this putative multicopper oxidase (MCO) have recently been characterized (Romano et al. 2017) and confirmed to carry out the two-step reaction oxidizing Mn^{2+} to Mn^{3+} and Mn^{3+} to Mn^{4+} (Butterfield et al. 2013). Despite this discovery, however, there is still debate as to how MCOs carry out the two electron transfers (Soldatova et al. 2017a,b; Wright et al. 2018). In addition to the questions remaining over the mechanisms for manganese oxidation, the advantage to the microbe, and therefore the reason for manganese oxidation, remains unclear (Tebo et al.

2004; Ehrlich et al. 2015). In fact, there is no evidence to show that microorganisms gain any energy from manganese oxidation. As such, there is much to learn about manganese oxidizing microorganisms (MOM) to gain an understanding of the mechanisms of manganese oxidation and toxicity as well as the microbes' potential use in treating drinking water.

1-4: Structure of this thesis

The main body of this thesis can be divided into two parts. The first part (Chapters 2, 3, and 4) is related to the bioremediation of nitrate, while the second part (Chapter 5) is related to the bioremediation of manganese. Chapter 6 deals with general conclusions from Chapters 2-5.

Specific objectives of each chapter are as follows:

Chapter 2

Objectives: (1) isolate low temperature-adapted denitrifying microorganisms, (2) identify the total and metabolically active microorganisms incubated under low temperature, denitrifying conditions, and (3) identify which low temperature-adapted denitrifying microorganisms are active in a woodchip bioreactor.

In this chapter, both culture-independent and –dependent approaches were used to identify and characterize low temperature-adapted denitrifying microorganisms from a woodchip bioreactor. Low temperature-adapted microorganisms were isolated from woodchips collected from a woodchip bioreactor in Willmar, Minnesota. The same woodchips were incubated in a lab-based microcosm experiment to measure denitrification and to compare the total and active microbial communities under denitrifying and non-denitrifying conditions. Common low temperature-adapted denitrifying microorganisms were detected in both the culture-dependent and -independent methods. This work is currently in review in the Environmental Microbiology Journal and I am the second author. I isolated and characterized denitrifying bacteria from the woodchip bioreactor near Willmar, MN, and wrote a draft manuscript for the corresponding parts. Jeonghwan Jang, a postdoctoral research associate in the Ishii lab, established woodchip bioreactor microcosms, conducted the culture-independent analysis, and wrote the draft manuscript. Rodney T. Venterea, research scientist at USDA, is the third author who contributed to the gas chromatography (GC) analysis for N_2O concentration measurements. Mike Sadowsky, Carl Rosen, Gary Feyereisen are the fourth, fifth, and sixth authors, respectively, and contributed to designing research, maintaining the field site, collecting samples, and reading and providing comments to the draft manuscript. My advisor, Satoshi Ishii, is the last and corresponding author, who contributed to designing research, analyzing data, and reading and revising the manuscript.

Chapter 3

Objectives: (1) isolate low temperature-adapted denitrifying microorganisms from woodchip samples and biofilm samples from four bioreactors in Minnesota, (2) compare the isolated denitrifying microorganisms between bioreactors and between sample types, and (3) determine whether any denitrifying microorganisms perform better than others under low temperatures for the purpose of bioaugmentation.

In this chapter, a culture-dependent approach was employed to isolate denitrifiying microorganisms under low temperatures from four bioreactors in Minnesota. Microorganisms were isolated from samples collected from woodchips and from clogging biofilms to understand the role these biofilms play in the nitrogen cycle. All isolated microorganisms were tested for denitrifying abilities by measuring nitrous oxide production, nitrate reduction and ammonium produced. The confirmed nitrate-reducing microorganisms were then subjected to denitrification rate testing by measuring $^{30}N_2$ gas over time to identify efficient denitrifying microorganisms to use for bioaugmentation studies in the field. This chapter is intended to be submitted to the Soil Biology and Biochemistry Journal.

Chapter 4

Objectives: low temperature-adapted denitrifying microorganisms isolated from Chapters 2 and 3 were subjected to whole genome sequencing to (1) identify genes involved in denitrification, (2) discover genes that may encode for important processes in woodchip bioreactors, such as cellulose degradation, and (3) select a microorganism(s) that could be used in bioaugmentation to enhance low temperature denitrification in woodchip bioreactors in Minnesota.

In this chapter, four bacteria that were isolated in Chapters 2 and 3 were selected for whole genome sequencing based on their denitrification or nitrate-reducing abilities, or on their potential to enhance denitrification in woodchip bioreactors through other means, such as complex polysaccharide degradation. Two of the strains were selected for bioaugmentation studies due to their denitrification abilities as described in Chapter 3 and the presence of relevant genes detected through whole genome sequencing. This chapter may be submitted to Genome Announcement.

Chapter 5

Objectives: (1) isolate manganese-oxidizing microorganisms from a waterfall known for high biogenic manganese-oxidation through a culture-dependent approach, (2) identify the abundance of these manganese-oxidizing microorganisms in an environmental sample using a culture-independent approach, and (3) establish a bioreactor for the biogenic oxidation and removal of manganese that will be applied to groundwater in Minnesota.

In this chapter, biofilm samples collected from a manganese-oxidizing waterfall in Hokkaido, Japan were used to establish a bioreactor and to identify manganese-oxidizing microorganisms. Sixty-eight manganese oxidizers were isolated from the biofilm samples and characterized. DNA and cDNA were extracted from the waterfall biofilm samples to identify the total and active microbial community. DNA was extracted from the bioreactor after a six-month incubation in order to compare the microbial communities between the bioreactor and the waterfall and identify the presence of manganese oxidizing microorganisms. This chapter will eventually be submitted to Environmental Science: Water Research and Technology Journal and Cara Santelli will be the co-author, however research is ongoing. We are continuing to enrich sulfate reduction in the bioreactor to couple sulfate reduction with manganese oxidation as manganese and sulfate often coexist in groundwater in southwestern Minnesota. The bioreactor medium will eventually be replaced with actual groundwater from a site in Minnesota. The addition of this data will improve the quality and impact of this work.

2. Cold-Adapted Denitrifying Bacteria in Woodchip Bioreactors

ABSTRACT

Woodchip bioreactor technology removes nitrate from agricultural subsurface drainage by using denitrifying microorganisms. Although woodchip bioreactors have demonstrated success in many field locations, low water temperature can significantly limit bioreactor efficiency and performance. To improve bioreactor performance, it is important to identify the microbes responsible for nitrate removal under low temperature conditions. Therefore, in this study, we identified and characterized low temperature-adapted denitrifiers by using culture-independent and -dependent approaches. By comparative 16S rRNA (gene) analysis and culture isolation technique, *Pseudomonas* spp., *Polaromonas* spp., and *Cellulomonas* spp. were identified as being important bacteria responsible for denitrification in woodchip bioreactor microcosms under low temperature conditions (15°C). Genome analysis of *Cellulomonas* sp. strain WB94 confirmed the presence of nitrite reductase gene *nirK*. Transcription levels of this *nirK* were significantly higher in the denitrifying microcosms than in the non-denitrifying microcosms. Strain WB94 was also capable of degrading cellulose and other complex polysaccharides. Taken together, our results suggest that *Cellulomonas* sp. denitrifiers could degrade woodchips to provide carbon source and electron donors to themselves and other denitrifiers in woodchip bioreactors. By inoculating these cold-adapted denitrifiers (i.e., bioaugmentation), it might be possible to increase the nitrate removal rate of woodchip bioreactors under cold temperature conditions.

INTRODUCTION

Nitrogen (N) and phosphorus (P) are the most important nutrients in fertilizers for agriculture. While some of them are taken up by plants or adsorbed to minerals or organic matter, a proportion of the nutrients can be leached from agricultural fields into rivers, lakes, and oceans, causing eutrophication (USEPA, 2008; MPCA, 2014). Agricultural runoff water from the Upper Midwest States is considered a major cause of the hypoxic zone, also known as the dead zone, in the Gulf of Mexico (USEPA, 2008).

Large amounts of nutrients are released from agricultural fields through subsurface (tile) drainage, which is installed to improve soil conditions for root growth and soil trafficability for timely planting and harvesting (Bhattarai et al., 2005). While artificial subsurface drainage has increased agricultural productivity, it has also increased the amount of nutrients, especially nitrate, released from fields into surrounding waterways (Gentry et al., 1998).

One approach to remove nitrate from subsurface drainage water is to install denitrifying bioreactors at the end of the drainpipes before water is discharged to ditches or streams (Warneke et al., 2011). A woodchip bioreactor is a subsurface trench filled with woodchips through which drainage water passes. The woodchips provide a carbon and energy source to denitrifying microorganisms (Schipper et al., 2010a; Ghane et al., 2015). Although woodchip bioreactors have demonstrated success in nitrate removal in many field locations (Christianson et al., 2012a), low water temperature during the cold seasons significantly limits bioreactor performance (Christianson et al., 2012a; David et al., 2016), which is likely related to the low metabolic activity of denitrifying microorganisms under low temperatures. In addition to cold temperatures (<5°C) in winter and early spring, water temperature usually ranges only from 10 to 20°C throughout the remainder of the year (Ghane et al., 2015), implying that microorganisms adapted to low temperatures might play important roles for nitrate removal more generally within woodchip bioreactors.

Previous woodchip bioreactor research has focused largely on the hydrology and engineering aspects of the system (Ghane et al., 2015; Lepine et al., 2016; Sharrer et al., 2016), although microorganisms play key roles in the technology. There have been a few reports on the microbial communities in woodchip bioreactors by using quantitative PCR (qPCR) or restriction fragment length polymormphism (RFLP) targeting denitrification functional genes (Warneke et al., 2011; Hathaway et al., 2015; Healy et al., 2015; Porter et al., 2015). However, it is still unclear which specific microorganisms are responsible for nitrate removal in woodchip bioreactors. This is partly due to difficulties in identifying denitrifying microorganisms. Denitrifying ability is sporadically distributed among taxonomically diverse groups of bacteria, archaea and fungi (Knowles, 1982; Zumft, 1997; Ishii et al., 2009). Both denitrifying and non-denitrifying strains can be present in the same genus; therefore, it is difficult to identify denitrifying organisms based on taxonomic information alone. In addition, denitrifiers in different taxa can have almost identical denitrification functional gene sequences (Philippot, 2002; Jones et al., 2008; Ishii et al., 2011). Therefore, it is also difficult to identify microorganisms based on the denitrification functional gene sequence information.

More recently, comparative 16S rRNA gene sequencing analyses have been successfully used to identify denitrifying microorganisms (Ishii et al., 2009). In this approach, microbial communities under different conditions (i.e., denitrification and non-denitrification conditions) are compared to identify microorganisms that increased their abundance under denitrification conditions. This is based on the assumption that microorganisms that grow or become more active under denitrification conditions are most likely denitrifiers. This assumption was proven feasible because most denitrifiers identified by the comparative 16S rRNA gene sequencing analysis (Ishii et al., 2009) were later isolated and confirmed as bona fide denitrifiers (Ishii et al., 2011).

In this study, we used the comparative 16S rRNA (gene) sequencing analysis to identify nitrate-reducing and denitrifying microorganisms active at the low temperature conditions found in a woodchip bioreactor. We used both DNA and RNA to identify total and metabolically active microorganisms, respectively (Gremion et al., 2003; Yoshida et al., 2012). In addition, we isolated nitrate-reducing and denitrifying microorganisms from the same woodchip samples. By characterizing these microorganisms, it may be possible to develop a strategy to enhance denitrification activity of woodchip bioreactors using bioaugmentation and biostimulation strategies. Consequently, the objective of this study was to (i) identify low temperature-adapted denitrifiers by comparative 16S rRNA (gene) analysis, (ii) isolate low temperature-adapted denitrifiers by culture method, and (iii) characterize these denitrifying strains.

METHODS

Woodchip bioreactor microcosms

Woodchip samples were collected from a field bioreactor near Willmar, MN, on 2 October 2014. Woodchip samples were kept at 4°C until use. Five grams of woodchips were placed in 210-mL vials, and mixed with 5 mL of synthetic tile drain water (**Table 2-1**) supplemented with or without 3.57 mM nitrate (50 ppm as N) and/or 3.95 mM acetate.

Table 2-1: Composition of the synthetic agricultural wastewater.

Chemical	Concentration (mg/L)
$CaCl_2$	220.5
$MgCl_2.6H_2O$	421.5
KH_2PO_4	1.3
Na_2SO_4	10.4
H_3BO_3	0.1
$FeSO_4.7H_2O$	0.625
$CuSO_4.5H_2O$	0.0775
$MnSO_4.H_2O$	0.025
$ZnSO_4.7H_2O$	0.1

Nitrate concentrations of 50 ppm-N have been observed in tile drain water in the field (Gamble et al., 2018). The concentration of acetate used provided a C/N molar ratio of around 2.0, which was previously reported as the minimum value needed to reduce almost all of the nitrate to dinitrogen (N_2) gas (Her and Huang, 1995). The vial headspace was replaced with N_2 and acetylene (C_2H_2), in a 90:10 ratio, for measuring denitrification via the accumulation of N_2O (Tiedje 1994), or with N_2 alone for microbial analysis. In both cases, microcosms were incubated at 15°C for up to 48 h. A total of five treatments were prepared: (i) W, woodchip without incubation, (ii) WINA, woodchip microcosm incubated with nitrate and acetate; (iii) WIN, woodchip microcosm incubated with nitrate but without acetate; (iv) WIA, woodchip microcosm incubated with acetate but without nitrate; (v) WI, woodchip microcosm incubated with neither nitrate nor acetate.

To determine the occurrence of denitrification, microcosms were incubated in triplicate at 15°C with the vial headspace containing 10% C_2H_2. The concentration of N_2O in the head space was measured at 0, 4, 8, 12, 24, 36, and 48 h after incubation, by using a gas chromatograph (GC) (Model 5890, Hewlett-Packard/Agilent Technologies) equipped with an electron capture detector and PoraPak Q column (Sigma-Aldrich) as previously described (Maharjan and Venterea, 2013).

RNA and DNA extractions

For RNA and DNA extractions, a different set of woodchip microcosms were prepared with the vial headspace filled with 100% N_2. Nine vials were prepared for each treatment (a total of 36 vials). The microcosms were incubated as described above. Three microcosms were sacrificed 24, 36, and 48 h after incubation, and the RNA and DNA were extracted from woodchip samples (2 g) collected from each of the microcosms. In addition, RNA and DNA were extracted from woodchip samples (n=3) without incubation (treatment W).

RNA and DNA were extracted by using a PowerSoil RNA Isolation kit (MOBIO, Carlsbad, CA) and RNA PowerSoil DNA Elution Accessory kit (MOBIO, Carlsbad, CA), respectively, according to the manufacturer’s instructions. For the extracted RNA samples, possible genomic DNA residue was removed using Turbo DNA free kit (Ambion, Austin, TX). No DNA contamination in the resulting RNA samples was confirmed by PCR targeting the 16S rRNA gene as described previously (Ishii et al., 2016). Complementary DNA (cDNA) was synthesized from the RNA samples (200 ng) by using PrimeScript RT Reagent kit (Takara Bio, Mountain View, CA) according to the manufacturer's instructions.

Microbial community analysis

Thirty nine DNA and cDNA samples shown in **Table 2-2** were individually used to amplify the V4 region of the 16S rRNA gene and 16S rRNA using the 515F-806R primer set, respectively, as described previously (Caporaso et al., 2012). Resulting amplicons were purified and used to prepare Illumina sequencing libraries with the TruSeq kit (Illumina, San Diego, CA). Paired-end sequencing reaction was done using a MiSeq platform (Illumina) with V3 chemistry (300-bp read length) at the University of Minnesota Genomics Center (Minneapolis, MN).

The paired-end raw read data were assembled, quality-filtered and trimmed using NINJA-SHI7 (Al-Ghalith GA, 2017), which is a fastq-to-combined-fasta processing pipeline. The assembled sequences were clustered into OTUs at 97% sequence similarity using NINJA-OPS (Al-Ghalith et al., 2016), which is a complete OTU-picking pipeline with advantage of the Burrows-Wheeler alignment using BowTie2. The resulting OTU tables, in sparse BIOM 1.0 format, were used for further statistical analyses done using QIIME 1.9.1 (Caporaso et al., 2010). Taxonomic assignment of the OTUs were done using the Greengenes 97 reference data set (McDonald et al., 2011).

Culture-independent identification of denitrifiers

Microbes responsive to the denitrification-inducing conditions (i.e., denitrifiers) were identified by comparing the microbial communities in denitrification-inducing conditions (i.e., treatments WINA and WIN) and those in non-denitrification conditions (i.e., treatments WIA and WI). The following steps were used for this analysis: 1) OTUs showing more than 1% relative abundance in at least one of the triplicate samples were chosen as major and represented microbial taxa; 2) OTUs showing a significant difference between the three sample types (i.e., microcosms incubated with nitrate [treatments WINA and WIN], microcosms incubated without nitrate [treatments WIA and WI], and no incubation control [treatment W]) were identified by analysis of variance (ANOVA) test (FDR p <0.05); and 3) OTUs that satisfied both steps 1 and 2 were visualized by heatmap analysis done with the Bray-Curtis distance indices. The OTU heatmaps were created by the heatmap.2 and vegdist subroutines within the gplots and vegan packages, respectably, for R.

Isolation and identification of denitrifiers

Denitrifying bacteria were also directly isolated from the woodchip samples collected from the same field bioreactor near Willmar, MN. In brief, 1 g of the woodchip sample was mixed with phosphate buffered saline (PBS, pH 7.4). The woodchip suspension was then spread-plated onto R2A agar plates, supplemented with 5 mM nitrate and 10 mM acetate (R2A-NA). The plates were incubated under anaerobic conditions, using AnaeroPak system (Mitsubishi Gas Chemical), at 15°C for 1 to 2 weeks. Colonies were picked and restreaked onto new R2A-NA agar plates to obtain well-isolated single colonies.

The ability of the strains to denitrify was examined using the acetylene inhibition assay (Tiedje, 1994). In brief, fresh cell cultures (300 µl) were inoculated into R2A-NA broth (10 ml) in 27 ml test tubes. After replacing the air phase with Ar:C_2H_2 (90:10) gas, the test tubes were incubated at 30°C. After 2-week incubation, gas samples were taken via a gastight syringe and analyzed for N_2O production by GC as described above. In addition, liquid samples were collected and analyzed for nitrate, nitrite and ammonium concentrations using the SEAL AA3 HR AutoAnalyzer. Strains that reduced ≥40% nitrate, converted <10% of nitrate to ammonium, and produced significant amount of N_2O (>50 ppm) were considered as denitrifiers. The GC system used in this study was too sensitive, and the upper quantification limit was often exceeded. Therefore, we could not calculate the percentage of nitrate reduced to N_2O.

Genomic DNA was isolated by heating cells in 100 µl 0.05 M NaOH at 95°C for 15 min (Ashida et al., 2010). After centrifugation, the supernatant was diluted 10 fold in MilliQ water, and used for PCR to amplify the 16S rRNA gene. The reaction mixture (50 µl) contained 1× Ex Taq buffer (Takara Bio, Otsu, Japan), 0.2 µM of each primer (m-27F and m-1492R; (Tyson et al., 2004)), 0.2 mM of each dNTP, 1 U of Ex Taq DNA polymerase (Takara Bio), and 2 µl of DNA template. PCR was carried out using a VeritiTM Thermal Cyclers (Life Technologies) and the following conditions: initial annealing at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 90 s, and one cycle of 72°C for 7 min. Amplification was confirmed by using agarose gel electrophoresis. The PCR products were purified using AccuPrep PCR

Purification Kit (Bioneer) and then quantitated using PicoGreen dsDNA quantitation assay (Thermo Scientific). The purified PCR products were sequenced by the Sanger method using a 3730*xl* DNA Analyzer (Applied Biosystems) at the University of Minnesota Genomics Center. The forward (m-27F) and reverse (m-1492R) reads were assembled using the phred, phrap, consed software (Ewing et al.). Strain identity was determined by using a Naïve Bayesian classifier (Wang et al., 2007a).

Whole genome sequencing

Cellulomonas sp. strain WB94 was selected for genome sequencing since this bacterium increased its relative abundance under denitrifying conditions based on the comparative 16S rRNA sequencing analysis. Genomic DNA was extracted from pure cell cultures using PowerSoil DNA Isolation Kit (MOBIO) according to the manufacturer's instructions. Sequencing libraries were prepared using the PacBio SMRT kit (Pacific Biosciences), and the genome was analyzed using the PacBio RS II platform (Pacific Biosciences). Extracted DNA was used to generate a SMRTbell library (20 kbp insert) which was sequenced at the Mayo Clinic's Molecular Biology Core (Rochester, MN). After quality filtering, reads were assembled de novo using the hierarchical genome assembly process (HGAP3) in the SMRT Link portal (v 2.3.0). Genome annotation was done using the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016). Average Nucleotide Identity (ANI) values were calculated using JSpecies (Richter and Rosselló-Móra, 2009).

Transcription analysis of the Cellulomonas nirK

Primers WB94_nirK_F (5′- AGACGCTGTGGACCTACAAC-3′) and WB94_nirK_R (5′-CGACGAACTGGTACGTCAAC-3′) were designed based on the genome sequence of *Cellulomonas* sp. WB94 and used to amplify *nirK* transcripts of *Cellulomonas*. The reaction mixture for qPCR (10 μL) contained 1× SYBR Premix ExTaq ROX plus (Takara Bio), 0.2 μM each primer, and 2 μL of cDNA samples. The qPCR was performed using StepOnePlus Real-Time PCR System v. 2.3 (Applied Biosystem) with the following conditions: 95°C for 30 sec., followed by 45 cycles of 95°C for 5 sec, 60°C, and 83°C for 30 sec. Melting curve analysis and agarose gel electrophoresis were done to confirm correct amplification of the PCR products. In addition to the *Cellulomonas nirK*, the quantity of 16S rRNA was measured by qPCR with Eub338 (5'-ACTCCTACGGGAGGCAGCAG-3') and Eub518 (5'-ATTACCGCGGCTGCTGG-3') primers (Muyzer et al., 1993). Levels of *nirK* transcripts were normalized using the quantity of 16S rRNA.

Statistical analyses

The PAST software was used to perform one-way ANOVA test to analyze statistical significance in the quantitative data obtained in microcosm treatments (Hammer, 2001).

Nucleotide sequence accession numbers

The 16S rRNA amplicon sequences were deposited to the Short Read Archive under accession number SRP149200. The 16S rRNA gene sequences of the isolated strains and the whole genome sequence of strain WB94 have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers MH196452–MH196472 and NZ_QEES00000000, respectively.

RESULTS

Occurrence of denitrification in the microcosms

To identify cold-adapted denitrifiers in the woodchip bioreactors, we established a series of reproducible woodchip bioreactor microcosms to evaluate the following five treatments: (i) W, woodchip without incubation, (ii) WINA, woodchip microcosm incubated with nitrate and acetate; (iii) WIN, woodchip microcosm incubated with nitrate but without acetate; (iv) WIA, woodchip microcosm incubated with acetate but without nitrate; and (v) WI, woodchip microcosm incubated without nitrate and acetate. Accumulation of N_2O was observed in the microcosms supplemented with nitrate regardless of the addition of acetate (**Figure 2-1**), suggesting that denitrification occurred in these conditions (i.e., treatments WINA and WIN). The N_2O concentrations were not significantly different ($p = 0.7084$ by ANOVA) between WINA and WIN treatments and nitrous oxide was not detected in the microcosms without addition of nitrate, indicating that denitrification did not occur in these conditions (i.e., treatments WIA, and WI). Concentrations of N_2O in the microcosms incubated ≥24 h were significantly larger ($p < 0.05$ by ANOVA) than those incubated ≤12 h, suggesting that denitrification activity greatly increased after 12 h.

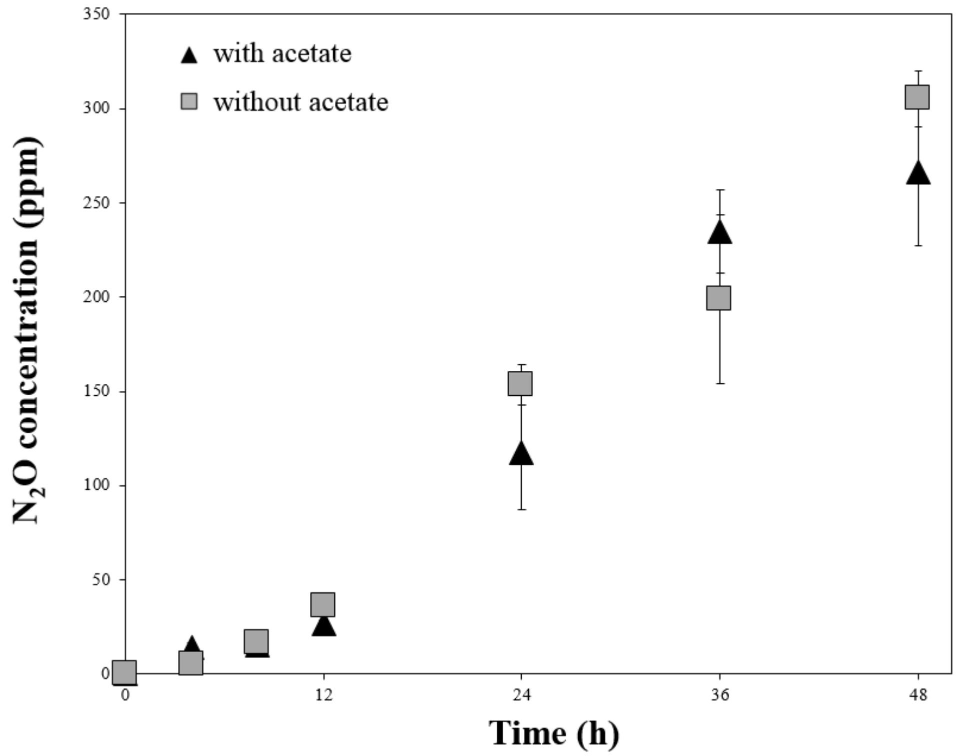


Figure 2-1. N_2O production from the microcosms supplemented with nitrate (i.e., treatments WINA and WIN) during 48-h incubation. N_2O production was not observed from the microcosms without nitrate addition (i.e., treatments WIA and WI). Legends: ▲, microcosms incubated with nitrate and acetate (i.e., treatment WINA) and ■, microcosms incubated with nitrate only (i.e., treatment WIN).

Microbial communities in the microcosms.

RNA and DNA were extracted from the microcosms after 0-, 24-, 36-, and 48-h incubations, and used for the microbial community analyses (**Table 2-2**). A total of 2,731,477 and 3,741,963 sequence reads were obtained from 39 DNA and 39 cDNA samples, respectively. The number of sequences per sample ranged from 28,609 to 115,611 and from 21,530 to 181,499 for DNA and cDNA samples, respectively. Numbers of sequences were normalized to the smallest number among the DNA and cDNA samples by random subsampling for further downstream analyses. The subsampled sequences provided sufficient resolution of the microbial communities, as indicated by Good's coverage ranging from 0.962 to 0.979 (**Table 2-3**) and by analysis of rarefaction curves (**Figure 2-2**).

Table 2-2: Samples prepared for the MiSeq 16S rRNA (gene) sequencing and nirK qPCR analyses.

Sample ID	Treatment ID	Supplement		Incubation time (h)	Sample type
		Nitrate	Acetate		
DNA01	W	−	−	0	DNA
DNA02	W	−	−	0	DNA
DNA03	W	−	−	0	DNA
DNA04	WINA	+	+	24	DNA
DNA05	WINA	+	+	24	DNA
DNA06	WINA	+	+	24	DNA
DNA07	WIN	+	−	24	DNA
DNA08	WIN	+	−	24	DNA
DNA09	WIN	+	−	24	DNA
DNA10	WINA	+	+	36	DNA
DNA11	WINA	+	+	36	DNA
DNA12	WINA	+	+	36	DNA
DNA13	WIN	+	−	36	DNA
DNA14	WIN	+	−	36	DNA
DNA15	WIN	+	−	36	DNA
DNA16	WINA	+	+	48	DNA
DNA17	WINA	+	+	48	DNA
DNA18	WINA	+	+	48	DNA
DNA19	WIN	+	−	48	DNA
DNA20	WIN	+	−	48	DNA
DNA21	WIN	+	−	48	DNA
DNA22	WIA	−	+	24	DNA
DNA23	WIA	−	+	24	DNA
DNA24	WIA	−	+	24	DNA
DNA25	WI	−	−	24	DNA
DNA26	WI	−	−	24	DNA
DNA27	WI	−	−	24	DNA
DNA28	WIA	−	+	36	DNA
DNA29	WIA	−	+	36	DNA
DNA30	WIA	−	+	36	DNA
DNA31	WI	−	−	36	DNA
DNA32	WI	−	−	36	DNA
DNA33	WI	−	−	36	DNA
DNA34	WIA	−	+	48	DNA

DNA35	WIA	–	+	48	DNA
DNA36	WIA	–	+	48	DNA
DNA37	WI	–	–	48	DNA
DNA38	WI	–	–	48	DNA
DNA39	WI	–	–	48	DNA
cDNA01	W	–	–	0	RNA (cDNA)
cDNA02	W	–	–	0	RNA (cDNA)
cDNA03	W	–	–	0	RNA (cDNA)
cDNA04	WINA	+	+	24	RNA (cDNA)
cDNA05	WINA	+	+	24	RNA (cDNA)
cDNA06	WINA	+	+	24	RNA (cDNA)
cDNA07	WIN	+	–	24	RNA (cDNA)
cDNA08	WIN	+	–	24	RNA (cDNA)
cDNA09	WIN	+	–	24	RNA (cDNA)
cDNA10	WINA	+	+	36	RNA (cDNA)
cDNA11	WINA	+	+	36	RNA (cDNA)
cDNA12	WINA	+	+	36	RNA (cDNA)
cDNA13	WIN	+	–	36	RNA (cDNA)
cDNA14	WIN	+	–	36	RNA (cDNA)
cDNA15	WIN	+	–	36	RNA (cDNA)
cDNA16	WINA	+	+	48	RNA (cDNA)
cDNA17	WINA	+	+	48	RNA (cDNA)
cDNA18	WINA	+	+	48	RNA (cDNA)
cDNA19	WIN	+	–	48	RNA (cDNA)
cDNA20	WIN	+	–	48	RNA (cDNA)
cDNA21	WIN	+	–	48	RNA (cDNA)
cDNA22	WIA	–	+	24	RNA (cDNA)
cDNA23	WIA	–	+	24	RNA (cDNA)
cDNA24	WIA	–	+	24	RNA (cDNA)
cDNA25	WI	–	–	24	RNA (cDNA)
cDNA26	WI	–	–	24	RNA (cDNA)
cDNA27	WI	–	–	24	RNA (cDNA)
cDNA28	WIA	–	+	36	RNA (cDNA)
cDNA29	WIA	–	+	36	RNA (cDNA)
cDNA30	WIA	–	+	36	RNA (cDNA)
cDNA31	WI	–	–	36	RNA (cDNA)
cDNA32	WI	–	–	36	RNA (cDNA)
cDNA33	WI	–	–	36	RNA (cDNA)
cDNA34	WIA	–	+	48	RNA (cDNA)
cDNA35	WIA	–	+	48	RNA (cDNA)
cDNA36	WIA	–	+	48	RNA (cDNA)
cDNA37	WI	–	–	48	RNA (cDNA)

cDNA38	WI	–	–	48	RNA (cDNA)
cDNA39	WI	–	–	48	RNA (cDNA)

Table 2-3 also shows species richness estimated by observed operational taxonomic units (OTUs) and Chao1 index, and species diversity represented by Shannon and Simpson indices, for microbial community in each DNA and cDNA sample. These diversity indices were significantly lower in the microbial communities from the woodchips incubated with nitrate (i.e., treatments WINA and WIN) than those from the woodchips incubated without nitrate (i.e., treatments WIA and WI) ($p < 0.05$ by ANOVA). However, no significant differences were observed between the microbial communities from the woodchips incubated with acetate (i.e., treatments WINA and WIA) and those from the woodchips incubated without acetate (i.e., treatments WIN and WI) ($p > 0.05$ by ANOVA). This suggested that α diversity in a microbial community is more influenced by the nitrate addition than by the addition of acetate.

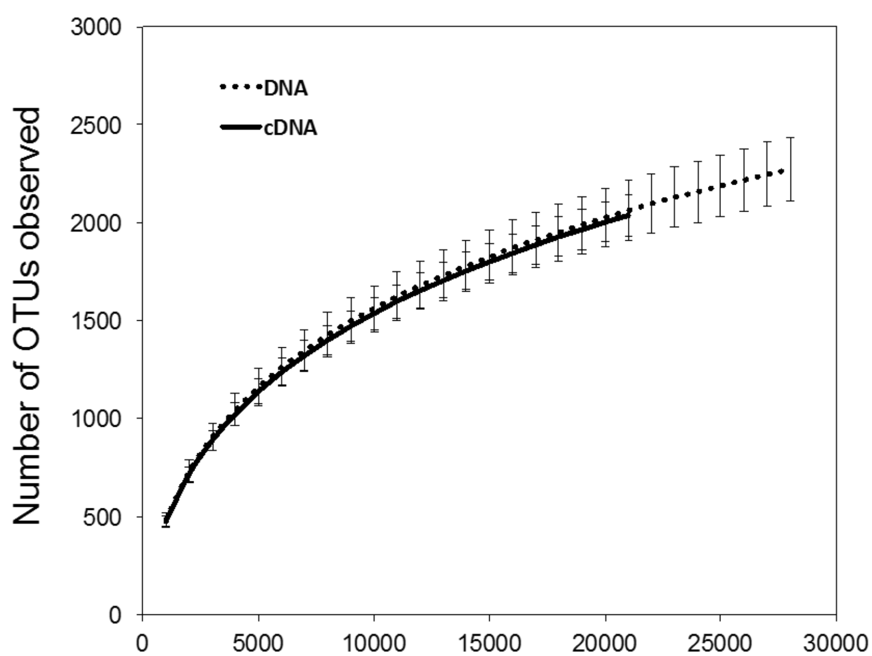


Figure 2-2. Rarefaction curve generated based on the 16S rRNA (gene) sequences obtained in this study. Total sequence reads were normalized to 28,609 and 21,530 reads per library for DNA and cDNA samples, respectively.

Table 2-3. Richness and α diversity indices of the microbial communities in the woodchip microcosms. Total sequence reads were normalized to 28,609 and 21,530 reads per library for DNA and cDNA, respectively.

Sample ID	Good's coverage	Richness		Diversity	
		Observed OTUs	Chao1	Shannon	Simpson
DNA01	0.973	2222	3171.4	9.093	0.995
DNA02	0.973	2320	3174.3	9.263	0.996
DNA03	0.973	2289	3203.8	9.257	0.996
DNA04	0.974	2162	3133.3	9.071	0.995
DNA05	0.973	2285	3126.7	9.100	0.995
DNA06	0.974	2215	3066.8	9.111	0.995
DNA07	0.973	2212	3186.4	9.232	0.996
DNA08	0.973	2265	3190.2	9.162	0.995
DNA09	0.975	2100	2954.7	9.038	0.995
DNA10	0.974	2220	3078.0	9.212	0.996
DNA11	0.977	2020	2719.1	8.865	0.992
DNA12	0.975	2167	3012.5	9.181	0.996
DNA13	0.973	2266	3169.2	9.284	0.996
DNA14	0.976	1984	2876.5	8.404	0.986
DNA15	0.973	2264	3115.8	9.160	0.995
DNA16	0.972	2264	3294.6	9.168	0.995
DNA17	0.974	2190	3055.3	8.843	0.991
DNA18	0.971	2303	3463.6	9.222	0.995
DNA19	0.979	1839	2531.2	7.988	0.974
DNA20	0.976	2030	2791.6	8.878	0.994
DNA21	0.974	2188	3066.2	9.162	0.995
DNA22	0.973	2432	3326.9	9.472	0.996
DNA23	0.974	2332	3170.4	9.384	0.996
DNA24	0.970	2594	3691.2	9.643	0.997
DNA25	0.973	2389	3323.0	9.487	0.997
DNA26	0.970	2588	3559.2	9.624	0.997
DNA27	0.972	2403	3401.2	9.495	0.997
DNA28	0.971	2488	3467.5	9.567	0.997
DNA29	0.969	2564	3716.6	9.539	0.997
DNA30	0.972	2422	3402.6	9.446	0.996
DNA31	0.974	2370	3223.7	9.488	0.997
DNA32	0.975	2262	3011.6	9.383	0.997
DNA33	0.974	2326	3170.2	9.432	0.997
DNA34	0.974	2396	3241.4	9.508	0.997
DNA35	0.975	2317	3113.7	9.457	0.997
DNA36	0.974	2357	3166.1	9.475	0.997

DNA37	0.972	2500	3336.3	9.554	0.997
DNA38	0.972	2430	3386.5	9.487	0.997
DNA39	0.975	2394	3232.1	9.583	0.997
cDNA01	0.965	2088	3101.0	9.198	0.996
cDNA02	0.965	2082	3080.0	9.152	0.995
cDNA03	0.964	2124	3088.4	9.204	0.996
cDNA04	0.971	1821	2532.3	8.576	0.990
cDNA05	0.967	1997	2817.2	8.923	0.994
cDNA06	0.967	2000	2827.2	9.051	0.995
cDNA07	0.969	1954	2698.5	9.036	0.995
cDNA08	0.966	2019	2894.3	9.045	0.995
cDNA09	0.969	1912	2663.6	9.024	0.995
cDNA10	0.967	2018	2848.3	9.124	0.996
cDNA11	0.966	1938	2922.6	8.771	0.992
cDNA12	0.967	2056	2862.6	9.199	0.996
cDNA13	0.966	1965	2954.4	8.927	0.993
cDNA14	0.970	1752	2614.0	8.534	0.990
cDNA15	0.965	1996	2920.4	9.035	0.995
cDNA16	0.967	1968	2829.5	9.036	0.995
cDNA17	0.967	1966	2896.3	8.961	0.994
cDNA18	0.966	2012	2937.3	9.094	0.995
cDNA19	0.967	1960	2815.2	9.016	0.995
cDNA20	0.969	1929	2751.6	9.090	0.995
cDNA21	0.966	2009	2931.0	9.117	0.995
cDNA22	0.966	2169	3019.9	9.239	0.994
cDNA23	0.966	2146	2980.5	9.276	0.995
cDNA24	0.962	2258	3265.6	9.407	0.996
cDNA25	0.967	2119	2832.1	9.269	0.995
cDNA26	0.964	2208	3147.2	9.373	0.996
cDNA27	0.965	2072	3134.6	9.218	0.995
cDNA28	0.966	2135	3003.2	9.347	0.996
cDNA29	0.963	2243	3145.0	9.333	0.995
cDNA30	0.966	2093	2915.7	9.051	0.992
cDNA31	0.964	2107	3136.8	9.161	0.994
cDNA32	0.968	1993	2825.1	9.123	0.994
cDNA33	0.967	2031	2942.4	9.154	0.994
cDNA34	0.964	2185	3238.6	9.401	0.996
cDNA35	0.966	2122	2962.4	9.294	0.995
cDNA36	0.967	2108	2953.5	9.278	0.995
cDNA37	0.963	2210	3177.3	9.420	0.996
cDNA38	0.965	2227	3080.0	9.417	0.996
cDNA39	0.966	2170	3074.3	9.335	0.995

Moreover, the addition of nitrate influenced the β diversity as well. Microbial communities in the microcosms incubated with nitrate (i.e., treatments WINA and WIN) clustered differently from those in the microcosms incubated without nitrate (i.e., treatments WIA and WI) based on principal coordinate analysis (PCoA) plots with Bray-Curtis dissimilarity for both DNA (**Figure 2-3A**) and cDNA (**Figure 2-3B**) samples. No clustering of microbial communities was observed by acetate addition (**Figures 2-3A and 2-3B**), suggesting that the addition of an external carbon source such as acetate had minimal impact on α and β diversities of the microbial communities.

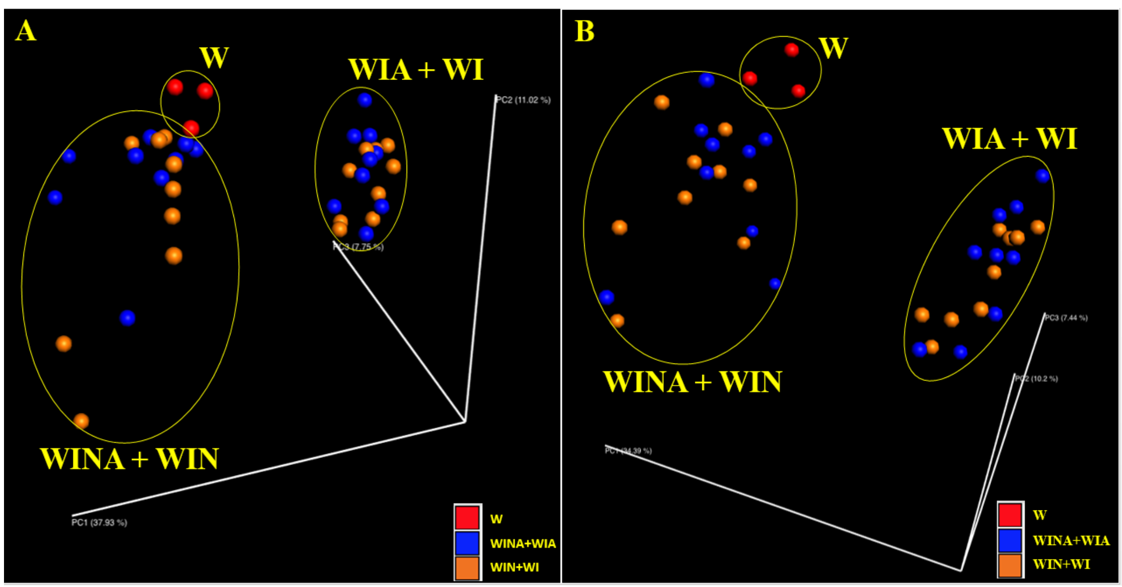


Figure 2-3. Principal coordinate analysis (PCoA) plots showing β diversity between microbial communities for (A) DNA and (B) cDNA samples. The β diversity was calculated using Bray-Curtis dissimilarity. Legends: Red, microcosms without incubation (i.e., treatment W); blue, microcosms incubated with acetate (i.e., treatments WINA and WIN); and orange, microcosms incubated without acetate (i.e., treatments WIN and WI). Microbial communities in the microcosms incubated with nitrate (i.e., treatments WINA and WIN) were clustered together.

Microbial taxa responsive to denitrification

The OTUs responsive to the denitrification-inducing conditions were identified by comparative 16S rRNA (gene) analysis (**Figure 2-4**). OTUs (266 and 232) were identified as having different relative abundance between three sample types (i.e., microcosms incubated with nitrate [treatments WINA and WIN], microcosms incubated without nitrate [treatments WIA and WI], and no incubation control [treatment W]) by ANOVA test (FDR $p < 0.05$), for DNA and cDNA samples, respectively. Among the 266 OTUs identified in the DNA analysis, those classified to the genera *Dechloromona*s, *Flavobacterium*, *Hydrogenphaga*, *Janthinobacterium*, *Mycoplana*, *Polaromonas*, and *Pseudomonas* were significantly more abundant in microcosms incubated with nitrate addition than those incubated without nitrate (**Figure 2-4A**). Among the 232 OTUs identified in the RNA (cDNA) analysis, those classified to the genera *Agrobacterium*, *Cellulomonas, Cryobacterium*, *Devosia*, *Mycoplana*, *Polaromonas*, *Propionicimonas*, *Pseudomonas, and Sphingobium* were significantly more abundant in microcosms incubated with nitrate addition than those incubated without nitrate (**Figure 2-4B**). Since these OTUs increased their abundance in response to denitrifying conditions, they are most likely denitrifiers or nitrate reducers. *Pseudomonas* and *Polaromonas* were significantly more abundant in denitrifying conditions than non-denitrifying conditions for both DNA and RNA samples, indicating that they were active and rapidly growing denitrifiers in the woodchip samples at relatively cold conditions (15°C).

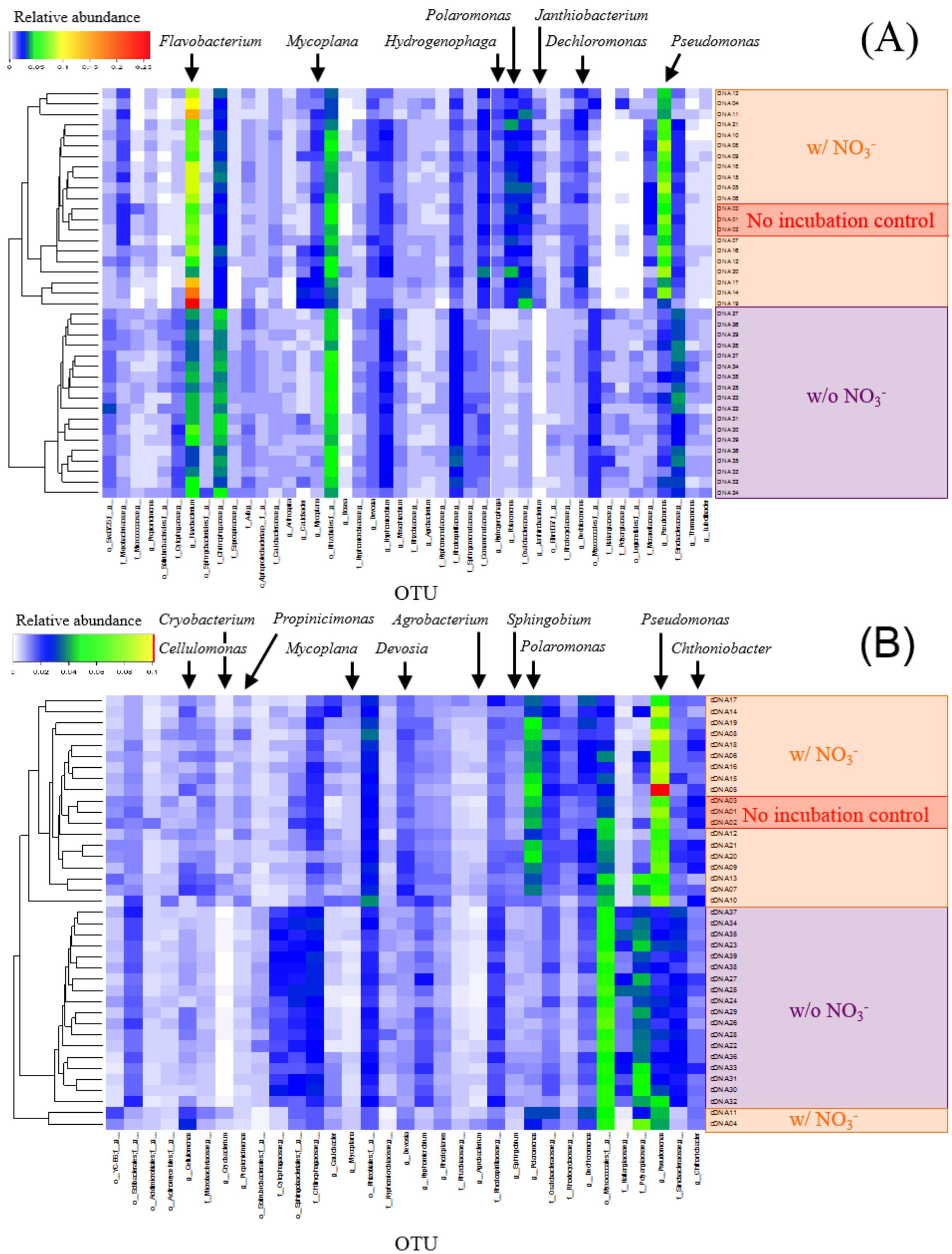


Figure 2-4. Heatmaps showing relative abundance of sequence reads in operational taxonomic units (OTUs) for (A) DNA and (B) cDNA samples. Only OTUs that showed different abundance between incubation conditions are shown. Assigned genus names are shown for the OTUs that showed significant differences between the three sample types (i.e., microcosms incubated with nitrate [treatments WINA and WIN], microcosms incubated without nitrate [treatments WIA and WI], and no incubation control [treatment W]) by analysis of variance (ANOVA) test.

Denitrifiers isolated from the woodchip bioreactors

A total of 21 isolates were identified as nitrate-reducing and N_2O-producing bacteria by the acetylene inhibition assay. Most isolates belonged to three genera: *Cellulomonas* (3 strains), *Clostridium* (14 strains), and *Microvirgula* (3 strains). Since bacteria reducing nitrate to ammonium (i.e., dissimilatory nitrite reduction to ammonium; DNRA) can also produce N_2O in the acetylene inhibition test (Tiedje, 1994), we measured concentrations of nitrate and ammonium to discriminate DNRA bacteria from denitrifying bacteria. Bacteria that reduced >10% of nitrate to ammonium were

Table 2-4: Nitrate reducing and denitrifying strains obtained in this study. Strains shown in bold reduced ≥40% nitrate, converted <10% of nitrate to ammonium, and produced significant amount of N2O (>50 ppm), and therefore, were considered as denitrifiers

Isolate ID	Proportion of N converted to ammonium (%)	Nitrate reduced (%)	N_2O produced (ppm)	Identification (genus)
WB17	40.9	98.3	1401.1	*Microvirgula*
WB18	44.9	98.3	1479.3	*Microvirgula*
WB19	4.1	BDL	63.1	*Clostridium*
WB21	5.8	BDL	224.0	*Clostridium*
WB22	42.1	98.4	1496.5	*Microvirgula*
WB23	7.0	BDL	9.5	*Clostridium*
WB24.2	7.3	BDL	7.1	*Clostridium*
WB26	BDL	33.5	2.5	*Clostridium*
WB29	BDL	32.0	1.3	*Clostridium*
WB39	7.1	39.4	68.8	*Clostridium*
WB40	2.0	3.8	5.1	*Clostridium*
WB49	6.8	39.6	103.2	*Clostridium*
WB53	**0.8**	**58.2**	**843.6**	***Clostridium***
WB66	**5.0**	**45.1**	**112.1**	***Clostridium***
WB76	**BDL**	**44.4**	**147.0**	***Clostridium***
WB80	**5.9**	**49.8**	**603.2**	***Clostridium***
WB81	4.1	47.5	0.3	*Clostridium*
WB91	7.0	38.7	169.7	*Desulfobacterium*
WB94	**6.5**	**49.2**	**116.0**	***Cellulomonas***
WB102	2.5	60.3	BDL	*Cellulomonas*
WB104	73.1	29.1	994.9	*Cellulomonas*

considered as DNRA bacteria. By this analysis, four strains of *Clostridium* spp. and one *Cellulomonas* spp. strains remained as denitrifying bacteria (**Table 2-4**).

The genus *Cellulomonas* was commonly detected by both culture-dependent and –independent approaches. Compared with the control microcosms, the abundance of members of the genus *Cellulomonas* significantly increased in the RNA samples collected from the denitrifying microcosms ($p < 0.05$ by ANOVA), but not in those collected from the non-denitrifying microcosms ($p = 0.33$ by ANOVA) (**Figure 2-5**). Taken together, these results suggested that *Cellulomonas* spp. strains are likely one of the most active denitrifying bacteria in the woodchip bioreactor samples.

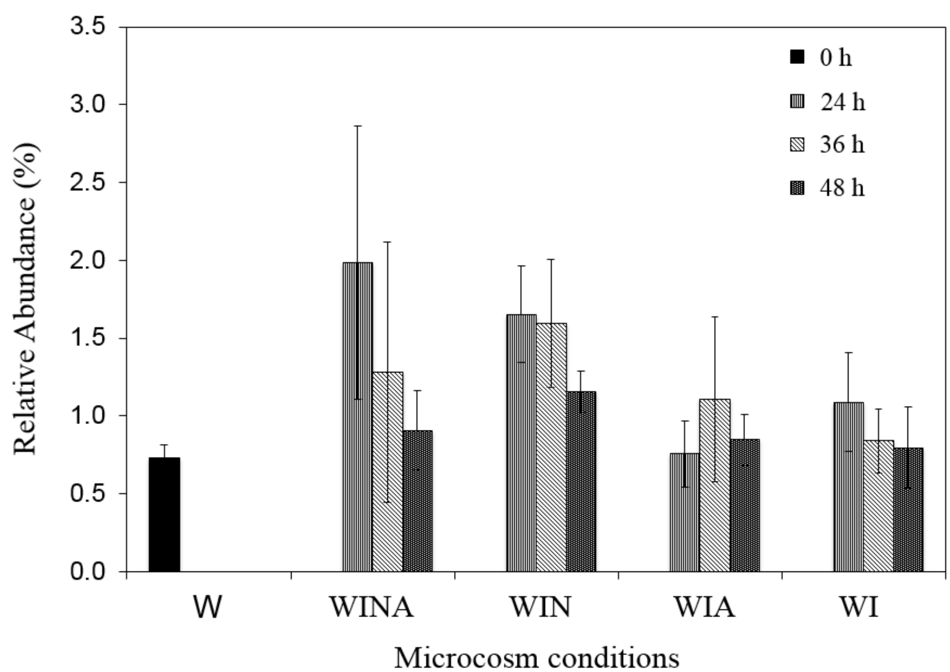


Figure 2-5. Relative abundance (%) of Cellulomonas rRNA in the sequencing libraries. Mean ± SD (n = 3) is shown. Legend: W, woodchip microcosms without incubation; WINA, woodchip microcosms incubation with nitrate and acetate; WIN, woodchip microcosm incubation with nitrate; WIA, woodchip microcosm incubation with acetate; WI, woodchip microcosm incubation.

Whole genome sequencing of Cellulomonas sp. strain WB94

The presence of denitrification functional genes could not be detected by PCR with commonly used primers. To identify genes related to denitrification and cellulose degradation, we sequenced the genome of *Cellulomonas* sp. strain WB94 using the PacBio platform. The genome of strain WB94 (accession number NZ_QEES00000000) was represented by seven contigs, with a total genome size of 3,868,980 bp and mole% G+C content ranging from 0.70 to 0.73% (**Table 2-5**). The genome contained 3,387 protein-coding sequences (CDS), 137 pseudogenes, 46 tRNAs, six rRNAs (two rRNA operons), and three noncoding RNAs. The average nucleotide identity (ANI) between the genomes of strain WB94 and *Cellulomonas cellasea* DSM 20118 were 98%, which is greater than the cutoff value for species discrimination (95% to 96%) (Goris et al., 2007; Richter and Rosselló-Móra, 2009). Therefore, strain WB94 most likely belonged to *Cellulomonas cellasea.*

Table 2-5. Summary of the sequenced genome of Cellulomonas sp. strain WB94.

Contig No.	Accession number	Size (bp)	GC content (%)
0	NZ_QEES01000002.1	2,780,765	71.9
1	NZ_QEES01000005.1	329,035	70.2
2	NZ_QEES01000001.1	235,040	70.3
3	NZ_QEES01000007.1	151,423	71.1
4	NZ_QEES01000004.1	162,011	72.2
5	NZ_QEES01000003.1	157,415	72.7
6	NZ_QEES01000006.1	53,291	71.9

The genome of strain WB94 harbored the nitrate reductase genes *narIJHG* and the dissimilatory NO-forming nitrite reductase gene *nirK* (**Table 2-6**), suggesting that strain WB94 can reduce nitrate to nitrite and to nitric oxide. The deduced NirK amino acid sequence was most closely related to the NirK from *Actinosynnema mirum*

DSM43827 [CP001630], but was also similar to those from other *Cellulomonas* species (>57% identity) (**Figure 2-6**). Other denitrification-related genes were not found on the genome. Additionally, the genome contained the assimilatory NAD(P)H-dependent nitrite reductase genes *nirBD*, suggesting that strain WB94 can use nitrate and nitrite as a N source. The genome also contained genes related to the biodegradation of complex polysaccharides, including cellulose, xylan, starch and glycogen (**Table 2-6**).

Table 2-6. Genes associated with denitrification or polysaccharide catabolism identified on the genome of Cellulomonas sp. strain WB94.

	Function	Gene	Locus_tag	Product
Denitrification	Nitrate Reduction	*narI*	DDP54_03075	respiratory nitrate reductase subunit gamma
		narJ	DDP54_03080	nitrate reductase molybdenum cofactor assembly chaperone
		narH	DDP54_03085	nitrate reductase subunit beta
		narG	DDP54_03090	nitrate reductase subunit alpha
	Nitrite reduction	*nirD*	DDP54_03030	nitrite reductase (NAD(P)H) small subunit
		nirB	DDP54_03035	nitrite reductase (NAD(P)H
			DDP54_03150	Molybdopterin-binding nitrite reductase
		nirK	DDP54_17680	NO-forming nitrite reductase
Polysaccharide catabolism	Cellulose degradation		DDP54_00625	endoglucanase
		malQ	DDP54_01650	4-alpha-glucanotransferase
			DDP54_0629	1,3-beta-glucanase
		malQ	DDP54_17500	4-alpha-glucanotransferase
			DDP54_09215	cellobiose phosphorylase
	Hemicellulose degradation		DDP54_00375	1,4-beta-xylanase
	Starch degradation		DDP54_12980	alpha-amylase
			DDP54_13300	alpha-amylase
			DDP54_12980	alpha-amylase
			DDP54_13300	alpha-amylase
			DDP54_15400	glucoamylase
			DDP54_1540	glucoamylase

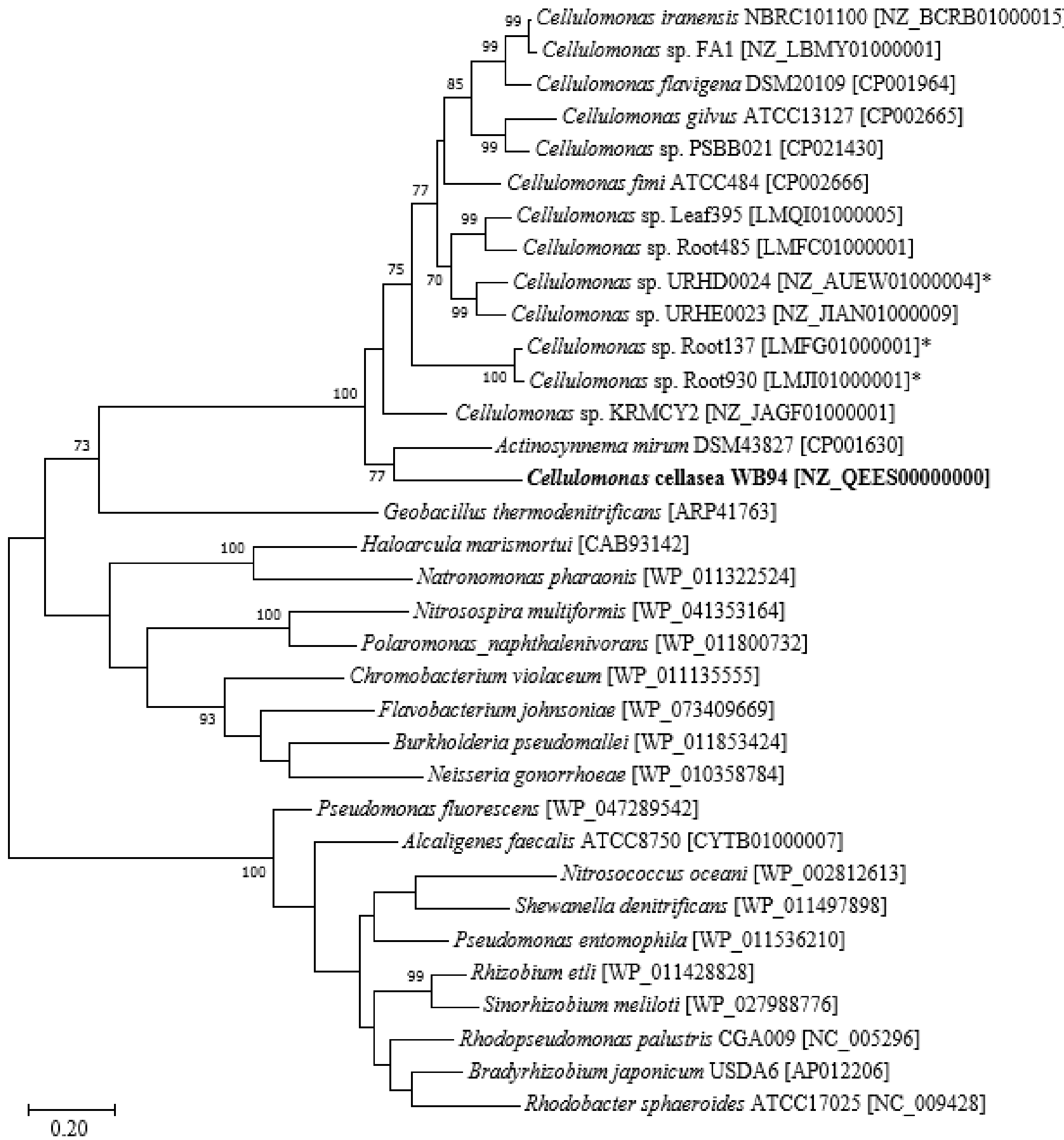


Figure 2-6. Phylogenetic tree generated based on the deduced NirK sequences using the maximum likelihood method. GenBank accession numbers are shown in square brackets. Bootstrap values (%) were generated from 1000 replicates, and the values >70% are shown.

Role of Cellulomonas spp. in the woodchip bioreactors

To verify the role of *Cellulomonas* spp. in the woodchip bioreactor microcosm, we measured the transcription levels of *Cellulomonas nirK* (**Figure 2-7**). Levels of *nirK* transcription were significantly higher in the denitrifying microcosms than in non-denitrifying microcosms (p <0.01 by ANOVA). Interestingly, however, the *nirK* transcription levels in the no incubation controls were also significantly greater than those in the non-denitrifying microcosms (p <0.01 by ANOVA) but not significantly different from those in the denitrifying microcosms (p = 0.87). The biodegradation of cellulose by C*ellulomonas* sp. strain WB94 was also verified by using the cellulase assay (data not shown).

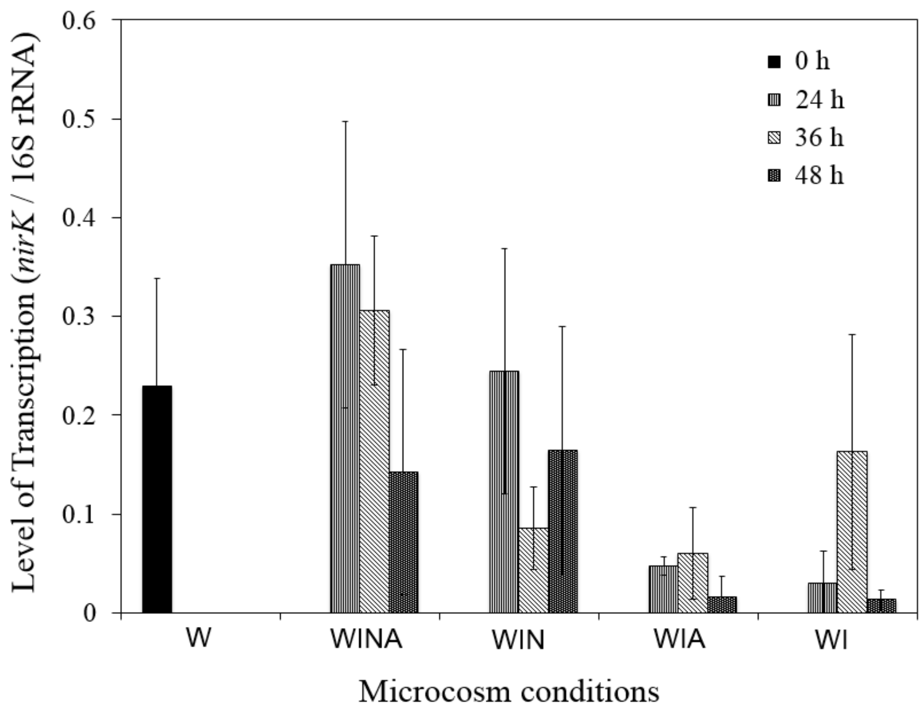


Figure 2-7 Transcription level of Cellulomonas nirK in the woodchip microcosms. Transcription levels were normalized by the amount of the 16S rRNA. Mean ± SD (n = 3) is shown. Legend: W, woodchip microcosms without incubation; WINA, woodchip microcosms incubation with nitrate and acetate; WIN, woodchip microcosm incubation with nitrate; WIA, woodchip microcosm incubation with acetate; WI, woodchip microcosm incubation.

DISCUSSION

While woodchip bioreactor technology is a promising approach to reduce nutrient loading from agricultural fields to surrounding and downstream water bodies (Christianson et al., 2012a), limited research exists identifying low temperature-adapted denitrifiers in these bioreactors. In this study, we used both culture-dependent and –independent approaches to identify nitrate-reducing and denitrifying microorganisms active at low temperature conditions in a woodchip bioreactor.

Similar amounts of N_2O were produced from triplicate woodchip bioreactor microcosms, suggesting that denitrification occurred reproducibly in the microcosms used in this study. The amount of N_2O significantly increased after 12-h incubation at 15°C, suggesting that the microorganisms actively performed denitrification after 12 h. Addition of acetate did not increase the amount of N_2O produced, indicating that carbon was not limited. This lack of improvement in nitrate removal rate with acetate addition to woodchips is in contrast to a recent laboratory column study that showed enhanced performance at 15 and 5°C (Roser et al., 2018). Others have shown that woodchip nitrate removal performance is negatively affected as the woodchips age (Robertson, 2010; David et al., 2016). Thus, in the current study, even though 4-year old woodchips were used, there was still enough C available for denitrification from the woodchips that the addition of readily available C (acetate) did not enhance denitrification rate. This difference could be methodological or attributed to a robust microbial community in this experiment.

A comparative 16S rRNA (gene) sequencing approach was used to identify nitrate-reducing and denitrifying microorganisms. A similar approach was previously successfully used to identify denitrifying bacteria in rice paddy soils (Ishii et al. 2009). While this previous study used conventional clone library analysis with >1,000 clones/library, here we used Illumina MiSeq high-throughput sequencing technology with >20,000 reads/sample. As a result, we sequenced enough DNA to cover the majority of microorganisms in the samples. In addition, Ishii et al. (2009) only used DNA samples, whereas here we sequenced the 16S rRNA (gene) from both DNA and RNA to identify total and metabolically active microorganisms, respectively. Sequencing 16S rRNA was previously shown useful to detect metabolically active microorganisms (Gremion et al., 2003; Yoshida et al., 2012) because more ribosomes are present in metabolically active cells than resting or starved cells (Nomura et al., 1984). Microbial community structures were different between DNA- and RNA-based analyses, similar to previous studies (Gentile et al., 2006; Moeseneder Markus et al., 2006; Lanzén et al., 2011), suggesting that only parts of the microbial populations were active in the environments.

Several genera were identified as potential nitrate-reducing and denitrifying bacteria. *Pseudomonas* spp. and *Polaromonas* spp. were commonly detected both by DNA- and RNA-based analyses. The genus *Pseudomonas* includes well-studied denitrifying strains such as *Pseudomonas stutzeri* strain ZoBell and *Pseudomonas aeruginosa* strain PAO1 and is reported to be one of the most active denitrifiers in natural environments (Knowles, 1982). In addition, some strains such as *P. aeruginosa* strain PKE117 and *Pseudomonas putida* strain mt-2 strains are reported to have strong extracellular lignin peroxidase activities to degrade woodchips (Yang et al., 2007; Ahmad et al., 2010), suggesting that *Pseudomonas* spp. could perform denitrification and use woodchips as a C source. *Polaromonas* species are also known to be psychrophiles with temperature optima 4-12°C (Irgens et al., 1996). Nitrate reduction of the *Polaromonas* strains have been reported (Mattes et al., 2008; Margesin et al., 2012), and a complete set of denitrification functional genes is present in the draft genome of *Polaromonas glacialis* R3-9 strain (GenBank accessions NZ_KL448323 and NZ_KL448327) (Wang et al., 2014), suggesting that *Polaromonas* spp. could perform denitrification at low temperature conditions.

Some genera were detected by the DNA- or the RNA-based analyses, but not by both methods. For example, the genera *Cellulomonas*, *Cryobacterium*, *Propionicimonas*, *Devosia*, *Agrobacterium*, and *Sphingobium* were detected only by the RNA-based analysis. The difference may be due to the growth rates of bacteria. Metabolically active cells may also replicate and increase their rRNA gene copies in the environment; however, there is a time lag between metabolic activity and genome replication (Rolfe et al., 2012). Therefore, active but slow-growing bacteria may not always be detected by the DNA-based analysis.

Cellulomonas spp. were commonly detected by both culture-independent analysis and culture-dependent isolation methods. Other genera identified as nitrate-reducing and denitrifying bacteria by the culture-independent analysis were not obtained by our isolation method, probably due to the bias caused by the medium used (i.e., R2A-NA). Growth media can largely influence results of bacterial isolation (Davis et al., 2005). Although denitrification by *Cellulomonas* strains has not been reported thus far, an incomplete set of denitrification functional genes (e.g., *narG* and *nirK*) is present in several genomes of the *Cellulomonas* sp. strains (GenBank accessions CP001964, CP002665, CP002666, and CP021430). Our *Celluomonas* sp. strain WB94 also possessed denitrification functional genes, including *narG* and *nirK*, and was able to reduce nitrate. The *nirK* of strain WB94 was similar to those from other *Cellulomonas* species. Transcription levels of the *Cellulomonas nirK* were significantly higher in the denitrifying microcosms than the non-denitrifying microcosms, suggesting that *Cellulomonas* strains were actively involved in denitrification process in woodchip bioreactors. Genes responsible for nitric oxide (NO) reductase were not found on the genome. Since WB94 produced N_2O by the acetylene inhibition assay, this strain should have NO reductase on its genome. Further data mining is necessary to identify the NO reductase of this strain.

Cellulomonas spp. are also well known for their ability to use endoglucanases and exoglucanases to degrade cellulose (Thayer et al., 1984). Our strain, *Cellulomonas* sp. strain WB94, also had the ability to degrade cellulose. In addition, various genes related to the biodegradation of complex polysaccharides were found on the genome of strain WB94. These results suggest that *Cellulomonas* spp. could play an important role in nitrate reduction as well as the degradation of woodchips.

CONCLUSION

Based on a series of culture-independent and –dependent analyses, we identified *Pseudomonas* spp., *Polaromonas* spp., and *Cellulomonas* spp. as being important bacteria responsible for nitrate reduction and denitrification in woodchip bioreactor microcosms under relatively cold temperature conditions. Since *Cellulomonas* spp. identified in this study can also degrade cellulose and other complex polysaccharides, they may provide a C source and electron donors to themselves and other denitrifiers in woodchip bioreactors. By inoculating these cold-adapted denitrifiers (i.e., bioaugmentation), it might be possible to increase the nitrate removal rate of woodchip bioreactors under cold temperature conditions.

This microcosm-based study was designed to mimic field conditions of N concentration and temperature, but the study's batch method differed from the continuous flow of field bioreactors. To examine if the low temperature-adapted denitrifiers identified in this study are also active in the field conditions, it is necessary to analyze samples collected from the field, which should be done in the future.

3. Comparison of the denitrifying microbial communities between four woodchip bioreactors in Minnesota

ABSTRACT

Woodchip bioreactors are a feasible strategy to prevent nitrate in agricultural wastewater from reaching bodies of water and causing eutrophication. These rely on denitrification, a microbial respiration in which nitrate is reduced to dinitrogen gas. Under cold temperatures, bioreactor efficiency is low due to inhibited microbial activity. This study employed a culture-dependent approach to isolate and characterize low temperature-adapted denitrifying microorganisms from four different bioreactors in Minnesota. A total of 207 bacteria were isolated from both submerged woodchips and from biofilms causing clogging in bioreactor pipes, 79 of which were able to reduce nitrate. Denitrification potential was determined based on nitrate-N reduction and conversion of nitrate-N to ammonium-N using segmented flow analysis, and N_2O production using gas chromatography. The denitrification rate of seven potential denitrifiers was measured using ^{15}N-labelled nitrate. Two isolates, *Cellulomonas* isolate WB94 and *Microvirgula* isolate BE2.4, demonstrated promising nitrate reduction and a consistent denitrification rate. No potential denitrifiers were isolated from the biofilm samples and it is likely that these biofilms clogging woodchip bioreactors are composed mostly of microbes performing dissimilatory nitrate reduction to ammonium. The composition of the isolated denitrifiers varied among the bioreactors. *Microvirgula*, an aerobic denitrifier, made up the majority of the isolates from a newly established woodchip bioreactor, while *Clostridium*, an obligate anaerobe, made up the majority of the isolates from a woodchip bioreactor established four years prior to sampling, indicating that age may play a role in bioreactor denitrifier community.

INTRODUCTION

Increasing nitrate pollution from agricultural runoff has had detrimental impacts on water quality, as evidenced by the hypoxic zone in the Gulf of Mexico. To mitigate this, the United States Environmental Protection Agency (USEPA) called for a minimum of a 45% reduction in total nitrogen loads to the Mississippi River. As Minnesota is one of the major contributors of nutrients to the Mississippi River, the Minnesota Pollution Control Agency has also set reduction goals of 45% for nitrogen and phosphorus loads to the Mississippi River. Woodchip bioreactors are one approach to achieve this goal, and their use is becoming an increasingly common method for reducing the flow of nitrate from agricultural wastewater to surface waters (Gibert et al. 2008; Schipper et al. 2010b). In a woodchip bioreactor, water diverted from an agricultural field flows through a below-ground trench filled with woodchips. Nitrate in the water is reduced sequentially to dinitrogen (N_2) gas by denitrification, a microbial respiration in which nitrate is used as the terminal electron acceptor (Seitzinger et al. 2006; Rivett et al. 2008). The woodchips in woodchip bioreactors provide a carbon source and an electron donor to denitrifying microorganisms (Gibert et al. 2008). Woodchip bioreactors have been successful at removing nitrate, with almost 100% nitrate load reductions reported in some cases (Gibert et al. 2008; Christianson et al. 2012b). However, under cold temperatures, bioreactor performance decreases due to inhibited microbial activity (Schipper et al. 2010a; Warneke et al. 2011; Ghane et al. 2015; Hartz et al. 2017; Hassanpour et al. 2017; Husk et al. 2017). This is a concern in Minnesota where spring melt contributes large quantities of runoff when the water temperature is still very low. It is believed that supplementing the woodchips with a more readily available carbon source would enhance microbial denitrification under low termperatures (Feyereisen et al. 2016). However, doing so risks clogging the bioreactor inlet and outlet pipes as biofilms accumulate, a common problem among field woodchip bioreactors (Gibert et al. 2008; Christianson et al. 2016; Husk et al. 2017). It is unknown whether these commonly found biofilms are composed of denitrifying microorganisms or how they play a role in denitrifying woodchip bioreactors. Gaining a better understanding of denitrifying microorganisms and biofilms may help enhance bioreactor efficiency, particularly under cold conditions.

Most comparative studies on nitrate-removing practices have focused on design and management of the bioreactor, rather than the microorganisms present. In two meta-analyses of woodchip bioreactors, Christianson et al. (2012b) and Addy et al. (2016) compare different woodchip bioreactors based on factors such as retention time, influent and effluent nitrate-N concentrations, and wood source. These factors are important in establishing a bioreactor and evaluating its success, but do not consider the microbial contribution and differences in community structure between sites. In a lab-based study, Grießmeier et al. (2017) set up bioreactors using nitrate-contaminated drainage water and fresh woodchips to analyze the microbial composition under different nitrate load concentrations. In analyzing the relative abundance of bacterial and archaeal operational taxonomic units (OTUs), Grießmeier et al. (2017) found differences in the microbial community structure with *Pseudomonadales* being a relevant denitrifier at low nitrate concentrations and *Rhodocyclales* and *Rhizobiales* predominating at higher nitrate conditions. However, there have not been any studies to date that have compared the denitrifying microorganisms present between bioreactors in the field. It is possible that the successfulness of different bioreactors is in part a result of the microorganisms present and their denitrification abilities. Therefore, the purpose of this study was to: 1) isolate denitrifying bacteria from four different bioreactors in Minnesota; 2) compare common denitrifiers among sites; and 3) determine whether any denitrifiers perform better than others and identify those that could be useful in enhancing field bioreactor performance through bioaugmentation.

METHODS

Sites and sample collection

Denitrifying microorganisms were isolated from either biofilms or woodchips in four existing woodchip bioreactors located in Willmar (bioreactor WB), Blue Earth (bioreactor BE), Olmsted County (bioreactor OC) and Lamberton (bioreactor LB), Minnesota. Descriptions of each of the bioreactors are presented in **Table 3-1**.

The ages of the woodchip bioreactors varied from three months (bioreactor BE) to four years (bioreactor WB). Woodchips were collected from submerged areas of the bioreactors and were immediately placed in a cooler. Clogging as a result of biofilm formation occurred in three of the woodchip bioreactors (**Figure 3-1**): bioreactor OC, which contained fine wood pieces and green cuttings; bioreactor WB, which contained only soft hardwood woodchips, but was supplemented with acetate in 2017 throughout the spring and summer months; and bioreactor LB which contained a mix of material including corn cobs and woodchips and was also supplemented with acetate. The biofilm samples from bioreactors WB and LB were collected from inlet tubing where the acetate

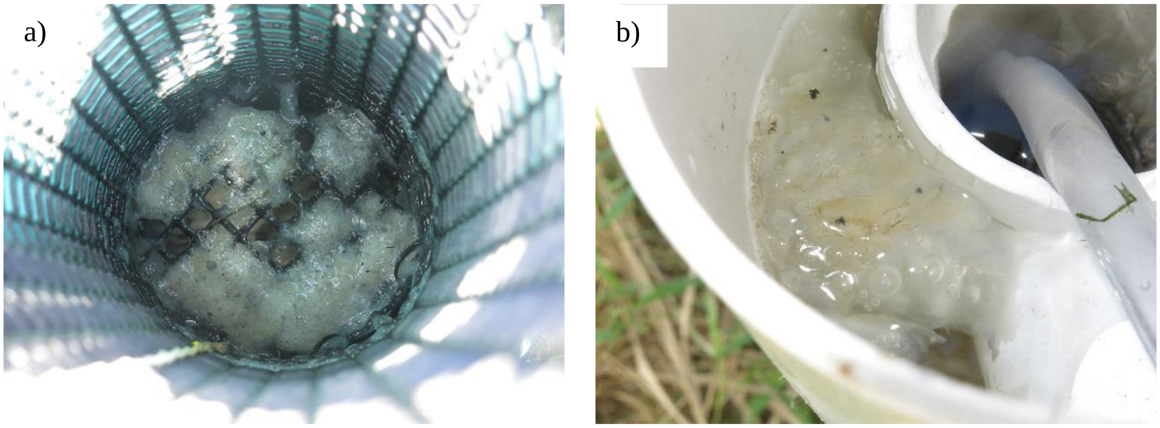


Figure 3-1: Photos of biofilm clogging woodchip bioreactors: a) biofilm inside the woodchip sampling port. b) biofilm accumulation at the inlet pipe and acetate injection site.

and drainage water converged, and the biofilm samples collected from bioreactor OC were collected at the clogged outlet pipe. Samples were frozen for further use.

Table 3-1: Descriptions of the four bioreactors from which denitrifying bacteria were isolated

Bioreactor ID	**OC**	**BE**	**WB**	**LB**
Location of bioreactor	Olmsted County, MN 43°59'46.70"N 92°17'10.67"W	Blue Earth, MN 43°41'42.25"N 94° 7'21.52"W	Willmar, MN 45° 3'0.17"N 95° 0'6.64"W	Lamberton, MN 44°14'35.83"N 95°18'16.01"W
Date	Built: May 2016 Start up: July 2016 Sampled: Sept. 2016	Built: Dec 2015 – Mar 2016 Start up: April 2016 Sampled July 2016	Built: Fall 2010 Re-built: Fall 2014 Sampled: Fall 2014 (woodchips) and June 2017 (biofilm)	Built April 2016 Start up: May 2016 Sampled: June 2017
Size	1 bed: 6m x 30.5m x 1.3m	3 beds: 7.62m x 41m x 1.5m	Original: 1 bed 1.7m x 107m x 1.4m Rebuilt 8 beds: 1.7m x 11.6m x 0.9m	Cube nominal size: 1041L; avg dimension of materials layers in cubes: L 1m x W 0.96m x H 0.78m

Bed Materials	During sampling, appeared to contain twigs, bark, green cuttings, fines.	Bed 3: finely ground cottonwood; Bed 2: similar to bed 3, but mixed with large woodchips Bed 1: large, clean uniform woodchips	Mixed hardwood and softwood chips; not many fines	5-7cm Phosphorous-sorbing material (crushed limestone; steel slag; crushed concrete) 20cm Corn Cobs 20cm Woodchips 7-10cm Lava Rock
Flow rate	22-42 L/min	492 L/min	9.5 L/min	3.8 L/min
HRT	50 – 90 hours	≈6 hours	9 – 10 hours	3 – 4 hours
Influent NO_3-N concentration (mg N L^{-1})	17 - 18	15 - 25	15 - 20	2016: 15 - 23.4 2017: 17.15 - 17.72
Effluent NO_3-N concentration (mg N L^{-1})	0.1	12 – 16	0.2 – 0.4	2016: 3.6 – 17.2 2017: 12.2 - 17.2
Inlet pH	6.31	7.2 – 8.0	7.56	7.38 average (2016-17)
Outlet pH	6.05	7.2 – 7.9	7.51	2016: 5.96 – 7.44 2017: 6.79 – 8.88
Inlet TC/TOC	0.1	5 – 11 (DOC)	3 (DOC)	NA
Outlet TC/TOC	50	6 – 9 (DOC)	4 (DOC)	NA
Acetate Added?	No	No	Yes	Yes
Acetate Rate (mL/min)	n/a	n/a	200	2016: 67.1 2017: 78.2
Acetate C concentration (g C L^{-1})	n/a	n/a	28 g C/L	12.5 g C/L
≈C:N ratio	n/a	n/a	1.95	2016: 0.16 – 0.97 2017: 0.35 – 1.13
Type of sample collected	Biofilm	Woodchips	Woodchips and biofilm	Biofilm Sampled in the inlet pipe prior to reaching the bioreactor cubes.

Isolation

Denitrifying microorganisms were isolated at 15°C according to the methods outlined in Chapter 2. Briefly, woodchip and biofilm samples were suspended in phosphate buffered saline (PBS, pH 7.4) and then plated on R2A agar containing 5 mM nitrate and 10 mM acetate (R2A-NA). Plates were incubated anaerobically at 15°C using an AnaeroPak system (Mitsubishi Gas Chemical) and continually restreaked until individual colonies appeared. Denitrification potential was confirmed for all isolates using the acetylene inhibition method which prevents the final step in denitrification from N_2O to N_2 gas so that N_2O gas can be measured using gas chromatography (Yoshinari and Knowles 1976). While this method does not differentiate between N_2O and N_2, it confirms a gaseous end product. Dissimilatory nitrate reduction to ammonium (DNRA) is a microbial respiration that can be carried out under similar conditions to denitrification. This process preserves N in the environment, rather than releasing it as N_2 gas, and can therefore contribute to N pollution, rather than mitigate it. DNRA has been shown to be the primary nitrate reduction pathway in some systems (Bernard et al. 2015). In order to differentiate between denitrification and DNRA, we also measured concentrations of nitrate, nitrite and ammonium using the SEAL AA3 HR AutoAnalyzer.

Identification

All nitrate-reducing microorganisms were identified based on 16S rRNA gene sequencing. First, DNA was extracted by heating cells at 95°C for 15 min and then diluted 10-fold for PCR. The reaction mixture (50 μl) contained 1x Ex Taq buffer (Takara Bio, Otsu, Japan), 0.2 μM of each primer (27F and 1492R; ref), 0.2 mM of each dNTP, 1

U of Ex Taq DNA polymerase (Takara Bio), and 2 μl of DNA template. PCR was performed using a Veriti Thermal Cyclers (Life Technologies) and the following conditions: initial annealing at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1.5 min, and one cycle of 72°C for 7 min. Amplification was confirmed using agarose gel electrophoresis. PCR products were purified using AccuPrep PCR Purification Kit (Bioneer) and then quantitated using PicoGreen dsDNA quantitation assay (Thermo Scientific). The purified PCR products were bidirectionally sequenced using the Sanger method at the University of Minnesota Genomics Center. The resulting forward (27F) and reverse (1492R) reads were aligned using the phred, phrap, consed software (Ewing et al. 1998) and strain identity was determined using Naïve Bayesian classifier (Wang et al. 2007a).

Denitrification rate measurement

Potential cold-adapted denitrifiers chosen for further testing were selected based on the following criteria: ≥40% nitrate-N was reduced, <10% N was converted to ammonium, no nitrite-N was detected and a significant amount of N_2O (>50 ppm) was produced.

Denitrification rates were measured using ^{15}N-labeled nitrate and a gas chromatograph-mass spectrometer (GC-MS). Having confirmed that these strains are capable of reducing nitrate, the ^{15}N-labeled nitrogenous gases would allow us to track nitrate through the denitrification process, showing whether or not complete denitrification is occurring. In brief, denitrifying bacteria grown in R2A-NA broth under anaerobic conditions were washed in piperazine-N, N′-bis (PIPES) buffer (pH 7.4).

Potential denitrifiers were incubated at 15°C in triplicate 50 ml PIPES buffer (pH 7.4) containing 10 mM acetate and 5 mM ^{15}N-labeled nitrate in 160 ml airtight bottles. The gas phase was exchanged for He. 10 µl gas samples were taken at hours 0, 24, 48, 72 and 1 week and 2 weeks and immediately analyzed using a GCMS-QP2010 SE (Shimadzu) equipped with Rt-Q-BOND column (30 m × 0.32 mm × 10 µm; Restek) to measure the absorbance values of $^{30}N_2$ and $^{46}N_2O$. A standard curve was created by injecting different volumes of $^{30}N_2$ gas and comparing absorbance values to known concentrations. Data was analyzed according to this standard curve and results were calculated as pmol-N $^{46}N_2O$ and $^{30}N_2$ produced per cell, based on OD_{600} values. The denitrification rate was calculated as pmol-N/cell/hour for $^{30}N_2$ gas based on the slope of the trendline where $^{30}N_2$ gas was produced linearly.

Aerobic denitrification confirmation

While denitrification is thought to be an anaerobic process, some microorganisms have been identified that are capable of aerobic denitrification (Takaya et al. 2003) and explanations for compatible aerobic respiration and denitrification have been hypothesized (Chen and Strous 2013). Aerobic denitrification would serve an important role in wastewater treatment, particularly in woodchip bioreactors where fluctuating water depth corresponds to a fluctuation in oxygen levels. To test for aerobic denitrification, potential denitrifying bacteria were incubated under the same conditions as the denitrification rate test except the gas phase was not exchanged. Gas samples were taken at the same time intervals.

Statistical analysis

The PAST software was used to perform one-way ANOVA tests to analyze statistical differences between denitrification rate time points and a p-value of ≤ 0.05 was used to indicate statistically significant differences (Hammer et al. 2001).

RESULTS

Isolated potential denitrifying bacteria

A total of 207 microorganisms were isolated at 15°C from woodchip or biofilm samples from four woodchip bioreactors in Minnesota. Of these, 79 were identified as cold-adapted nitrate-reducers. **Table 3-2** shows the identity and denitrification potential of all nitrate-reducing isolates across all sites. While nitrate reduction and ammonium production varied widely, overall little to no nitrite was produced. As shown in **Figure 3-2**, the composition of the nitrate-reducing isolates varied widely across sites.

From the bioreactor BE woodchip samples, a total of 25 bacteria were isolated, all of which were confirmed nitrate reducers belonging mostly to the genera *Microvirgula* and *Enterobacter*. Six isolated bacteria were considered to be potential denitrifiers ($\geq$40% nitrate reduced, <10% ammonium produced, >50 ppm N_2O produced) and belonged to a more diverse group of genera, including *Microvirgula, Delftia, Raoultella, Clostridium* and *Buttiauxella.*

From bioreactor WB, a total of 104 and 16 bacterial strains were isolated under denitrifying conditions from the woodchip and biofilm samples, respectively. Of these, 21 isolates from the woodchips and five isolates from the biofilm were confirmed nitrate-reducers and both samples contained unique microorganisms. A total of five potential denitrifiers were identified from the WB woodchips, four of which belonged to the genus *Clostridium* and one to *Cellulomonas*. Of the five nitrate-reducers isolated from the WB biofilm, many demonstrated relatively high nitrate reduction (32- 62%), but correspondingly high ammonium conversion, indicating that these strains are likely performing DNRA.

A total of nine nitrate-reducing bacteria were isolated from the LB bioreactor biofilm, none of which were identified as potential denitrifiers. Similarly, no potential denitrifiers were isolated from the OC woodchip bioreactor, which belonged almost exclusively to the genus *Bacillus.* The isolated nitrate-reducers from the OC biofilm produced little ammonium, but nitrite was detected in all but one sample.

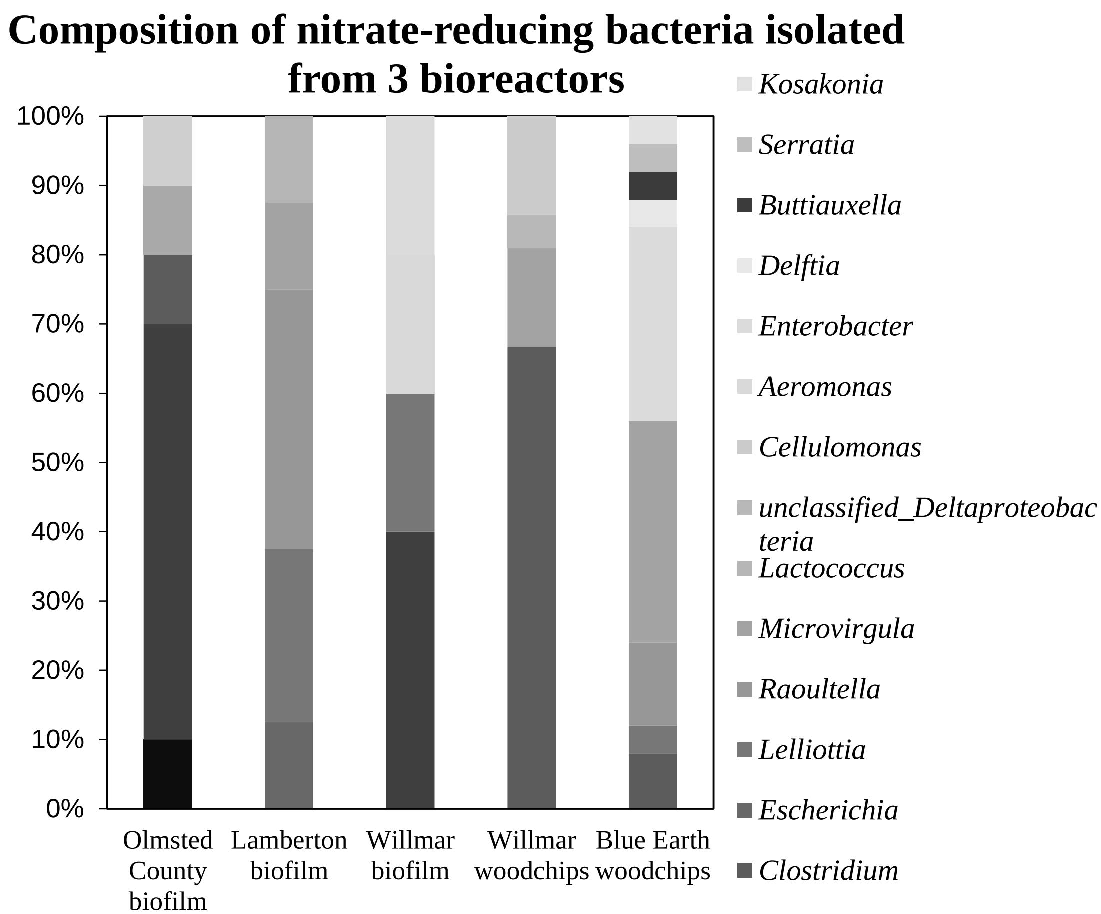


Figure 3-2: The composition of nitrate-reducing bacteria isolated from 4 bioreactors at the genus level.

Table 3-2: Nitrate-reducing bacteria isolated from woodchip bioreactors in Minnesota and their N transformations

Isolate ID	Source	Proportion of N converted to ammonium (%)	Nitrate reduced %	%N to nitrite	N2O produced (ppm)	Identification (genus)
BE1.1	BE woodchips	32.7	100.0	0.0	2020.2	*Enterobacter*
BE 1.2	BE woodchips	7.9	69.8	0.0	1532.5	*Delftia*
BE 1.3	BE woodchips	27.3	99.8	0.0	3348.4	*Enterobacter*
BE 1.4	BE woodchips	34.3	100.0	0.0	2786.8	*Microvirgula*
BE 1.5	BE woodchips	21.6	81.0	0.0	2695.1	*Microvirgula*
BE1.6	BE woodchips	48.0	87.5	0.0	1861.0	*Kosakonia*
BE 1.7	BE woodchips	29.5	74.6	0.0	18.0	*Enterobacter*
BE 2.1	BE woodchips	6.8	70.1	0.0	1815.5	*Raoultella*
BE 2.2	BE woodchips	14.9	73.6	0.0	2072.2	*Clostridium*
BE 2.3	BE woodchips	33.5	99.8	0.0	3693.0	*Microvirgula*
BE 2.4	BE woodchips	BDL	62.1	0.0	2072.2	*Microvirgula*
BE2.5	BE woodchips	7.8	66.8	0.0	316.2	*Raoultella*
BE 2.6	BE woodchips	BDL	62.1	0.0	88.8	*Buttiauxella*
BE2.7	BE woodchips	21.8	78.4	0.0	1898.2	*Enterobacter*
BE 3.1	BE woodchips	18.1	74.8	0.0	2111.1	*Serratia*
BE3.2	BE woodchips	36.8	99.9	0.0	939.6	*Enterobacter*
BE 3.3	BE woodchips	16.5	73.9	0.0	1567.9	*Microvirgula*
BE 3.4	BE woodchips	35.2	100.0	0.0	2919.7	*Raoultella*
BE 3.5	BE woodchips	33.6	50.0	0.0	917.4	*Lelliottia*
BE 3.6	BE woodchips	26.7	60.1	0.0	2073.4	*Enterobacter*
BE 3.7	BE woodchips	73.6	100.0	0.0	3504.3	*Microvirgula*
BE 3.8	BE woodchips	66.2	99.9	0.0	3706.9	*Microvirgula*

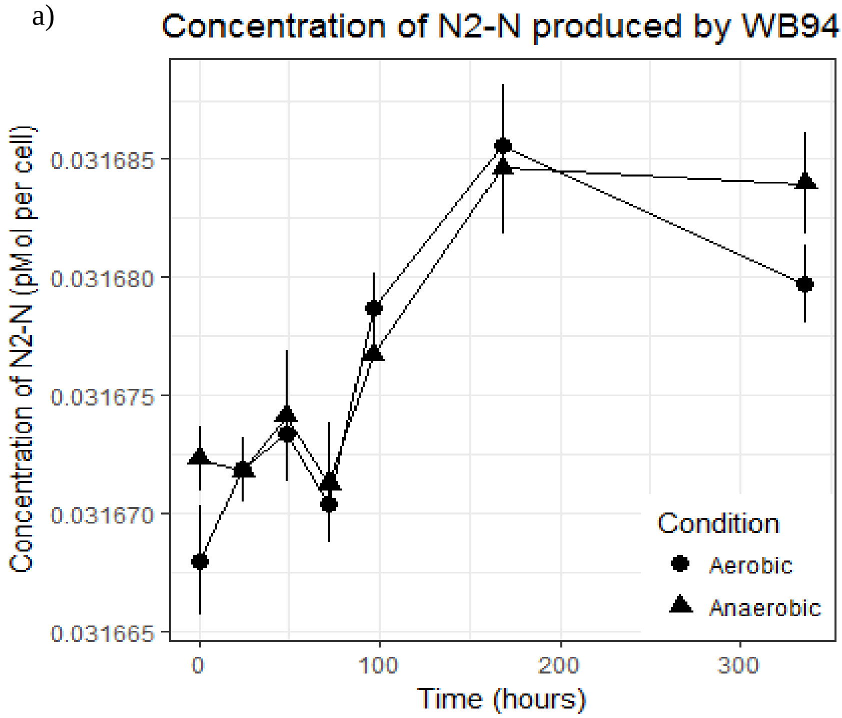
BE 3.9	BE woodchips	39.1	56.9	0.0	1669.0	*Enterobacter*
BE 3.10	BE woodchips	74.7	100.0	0.0	3333.5	*Microvirgula*
BE 3.11	BE woodchips	4.8	62.4	0.0	64.9	*Clostridium*
WB17	WB woodchips	40.9	98.3	0.0	1401.1	*Microvirgula*
WB18	WB woodchips	44.9	98.3	0.0	1479.3	*Microvirgula*
WB19	WB woodchips	4.1	BDL	0.0	63.1	*Clostridium*
WB21	WB woodchips	5.8	BDL	0.0	224.0	*Clostridium*
WB22	WB woodchips	42.1	98.4	0.0	1496.5	*Microvirgula*
WB23	WB woodchips	7.0	BDL	0.0	9.5	*Clostridium*
WB24	WB woodchips	7.3	BDL	0.0	7.1	*Clostridium*
WB26	WB woodchips	BDL	33.5	0.0	2.5	*Clostridium*
WB29	WB woodchips	BDL	32.0	0.0	1.3	*Clostridium*
WB39	WB woodchips	7.1	39.4	0.0	68.8	*Clostridium*
WB40	WB woodchips	2.0	3.8	0.0	5.1	*Clostridium*
WB49	WB woodchips	6.8	39.6	0.0	103.2	*Clostridium*
WB53	WB woodchips	0.8	58.2	0.0	843.6	*Clostridium*
WB66	WB woodchips	5.0	45.1	0.0	112.1	*Clostridium*
WB76	WB woodchips	BDL	44.4	0.0	147.0	*Clostridium*
WB80	WB woodchips	5.9	49.8	0.0	603.2	*Clostridium*
WB81	WB woodchips	4.1	47.5	0.0	0.3	*Clostridium*
WB91	WB woodchips	7.0	38.7	0.0	169.7	*unclassified_Deltaproteobacteria*
WB94	WB woodchips	6.5	49.2	0.0	116.0	*Cellulomonas*
WB102	WB woodchips	2.5	60.3	0.0	BDL	*Cellulomonas*
WB104	WB woodchips	73.1	29.1	0.0	994.9	*Cellulomonas*
2A1	WB biofilm	73.0	62.7	0.0	259.0	*Bacillus*
2A1.1	WB biofilm	65.9	59.2	0.0	168.2	*Aeromonas*
2B3	WB biofilm	38.8	59.3	0.0	837.3	*Lelliottia*
6A1	WB biofilm	58.4	32.2	0.0	1247.7	*Bacillus*

6B1	WB biofilm	60.3	40.4	0.0	1908.7	*Enterobacter*
BA1.12	LB biofilm	25.4	49.4	0.0	620.3	*Escherichia*
BA1.2	LB biofilm	46.1	42.8	0.0	453.3	*Lelliottia*
BA1.3	LB biofilm	54.4	49.5	0.0	299.3	*Raoultella*
BA2.2	LB biofilm	63.4	45.8	0.0	439.8	*Raoultella*
BB1.1	LB biofilm	49.4	64.7	0.0	57.8	*Raoultella*
BB1.2	LB biofilm	47.8	102.3	0.0	1231.3	*Microvirgula*
BB1.3	LB biofilm	6.8	21.1	0.0	73.9	*Lactococcus*
BB2.1	LB biofilm	35.8	43.1	0.0	509.3	*Lelliottia*
BB2.2	LB biofilm	54.4	20.4	0.0	459.7	*Lelliottia*
H6	OC biofilm	9.2	6.2	0.0	739.4	*Mariniluteicoccus*
H13	OC biofilm	13.1	BDL	2.2	832.3	*Bacillus*
H16	OC biofilm	8.4	BDL	2.1	2292.2	*Bacillus*
H20	OC biofilm	9.6	BDL	2.1	941.8	*Bacillus*
H25	OC biofilm	7.0	5.0	1.8	908.9	*Bacillus*
H26	OC biofilm	6.1	3.4	0.8	878.6	*Bacillus*
H29	OC biofilm	8.4	BDL	1.8	1060.6	*unclassified_bacillales*
H30	OC biofilm	8.4	27.3	0.5	884.9	*Bacillus*
H31	OC biofilm	4.4	18.2	0.2	1019.3	*Clostridium*
H32	OC biofilm	5.0	3.7	1.8	685.9	*Bacillus*
H33	OC biofilm	8.8	BDL	1.3	935.3	*Bacillus*
H34	OC biofilm	BDL	50.7	2.0	641.8	*Bacillus*
H37	OC biofilm	BDL	32.6	1.5	1102.6	*Bacillus*
H41	OC biofilm	17.8	29.6	17.5	1181.9	*Bacillus*
H43	OC biofilm	4.4	10.5	3.9	1120.1	*Bacillus*
H45	OC biofilm	BDL	41.9	1.2	635.0	*Bacillus*

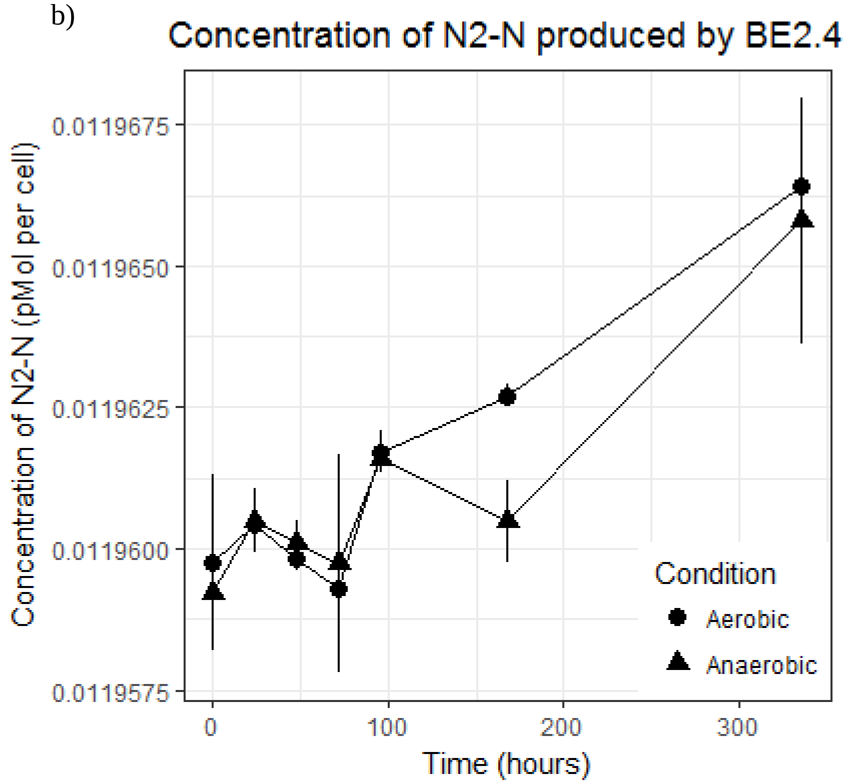
Denitrification rate

Seven potential denitrifiers were selected to be tested for aerobic and anaerobic denitrification rates using ^{15}N-labeled nitrate. Five were isolated from the BE bioreactor (*Buttiauxella*, *Raoultella*, *Delftia*, *Microvirgula* and *Clostridium*) and two from the WB bioreactor woodchips (*Cellulomonas* and *Clostridium*). Four other potential denitrifiers were identified based on our criteria, three additional *Clostridium* from the WB bioreactor and one *Raoultella* from the BE bioreactor, but these were excluded due to repetition. Most of the results showed some increase in $^{30}N_2$, but had too great a variation to conclude whether denitrification was occurring, particularly for the aerobic conditions (**Figure 3-3,a-g**). Isolate BE2.4, belonging to *Microvirgula*, however, showed significant increases in $^{30}N_2$ aerobically at each time point following hour 72 (**Figure 3-3b**). Anaerobic $^{30}N_2$ did not increase significantly until after the first week (hour 168). This suggests that isolate BE2.4 may be a strong aerobic denitrifier. Surprisingly, *Cellulomonas* isolate WB94 also displayed aerobic denitrification, with significant increases in $^{30}N_2$ between hours 72 to 168 under both aerobic and anaerobic conditions (**Figure 3-3a**). The production of $^{46}N_2O$ was also measured and is displayed in **Figure 3-4**. There was a significant increase in $^{46}N_2O$ aerobically produced by BE2.1 during the first 48 hours. $^{46}N_2O$ increased significantly towards the end of the experiment in both the WB94 (**Figure 3-4a**) and BE2.4 (**Figure 3-4b**) aerobic incubations (between 72 and 336 hours for BE2.4 and between 72 and 168 for WB94), and anaerobic conditions (between 168 and 336 hours for BE2.4 and 72 and 168 for WB94). The denitrification rates, based on the slope of the trend line during the time that $^{30}N_2$ gas was produced, varied between samples and between aerobic and anaerobic conditions. The time range during which $^{30}N_2$ gas was produced and the rate are shown in **Table 3-3**.

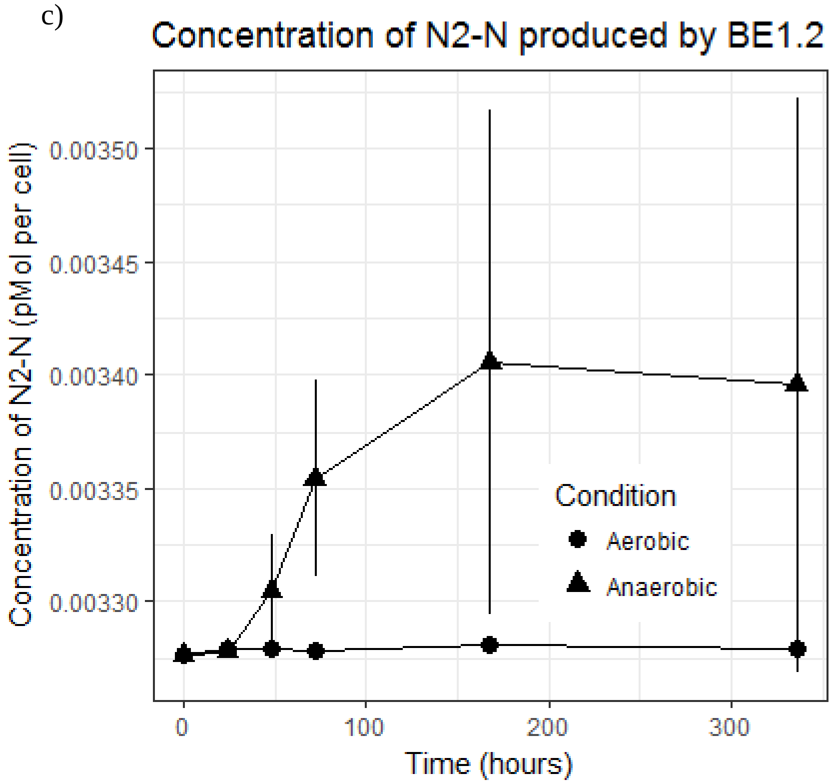
a)


Concentration of N2-N produced by WB94
Concentration of N2-N (pMol per cell)
0.031685
0.031680
0.031675
0.031670
0.031665
0
100
200
300
Time (hours)
Condition
Aerobic
Anaerobic

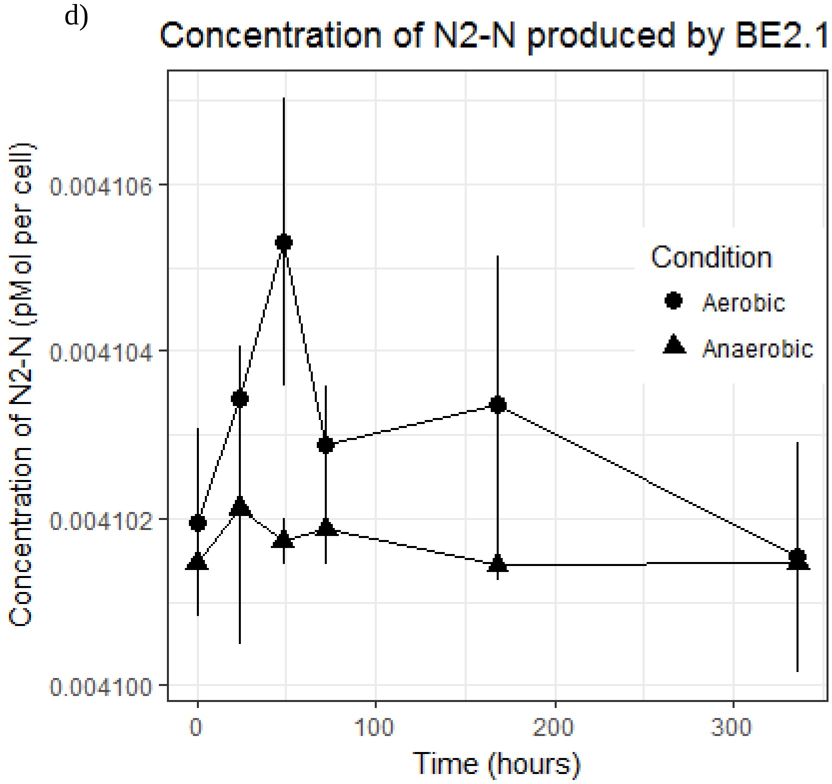
b)


Concentration of N2-N produced by BE2.4
Concentration of N2-N (pMol per cell)
0.0119675
0.0119650
0.0119625
0.0119600
0.0119575
0
100
200
300
Time (hours)
Condition
Aerobic
Anaerobic

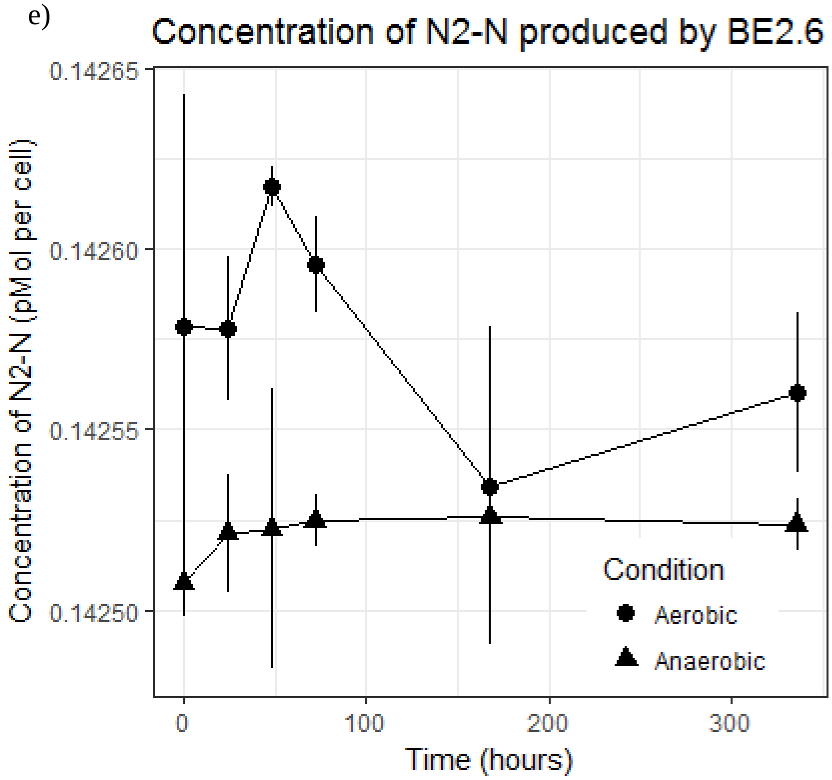
c)


Concentration of N2-N produced by BE1.2
Concentration of N2-N (pMol per cell)
0.00350
0.00345
0.00340
0.00335
0.00330
0
100
200
300
Time (hours)
Condition
Aerobic
Anaerobic

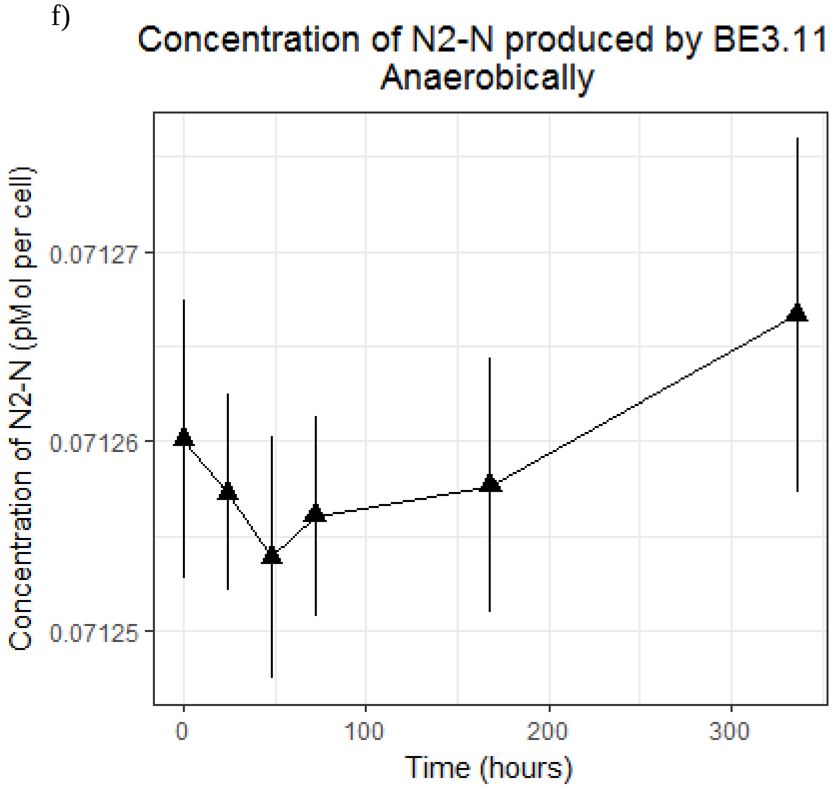
d)


Concentration of N2-N produced by BE2.1
Concentration of N2-N (pMol per cell)
0.004106
0.004104
0.004102
0.004100
0
100
200
300
Time (hours)
Condition
Aerobic
Anaerobic

e)


Concentration of N2-N produced by BE2.6
Concentration of N2-N (pMol per cell)
0.14265
0.14260
0.14255
0.14250
0
100
200
300
Time (hours)
Condition
Aerobic
Anaerobic

f)


Concentration of N2-N produced by BE3.11
Anaerobically
Concentration of N2-N (pMol per cell)
0.07127
0.07126
0.07125
0
100
200
300
Time (hours)

g)

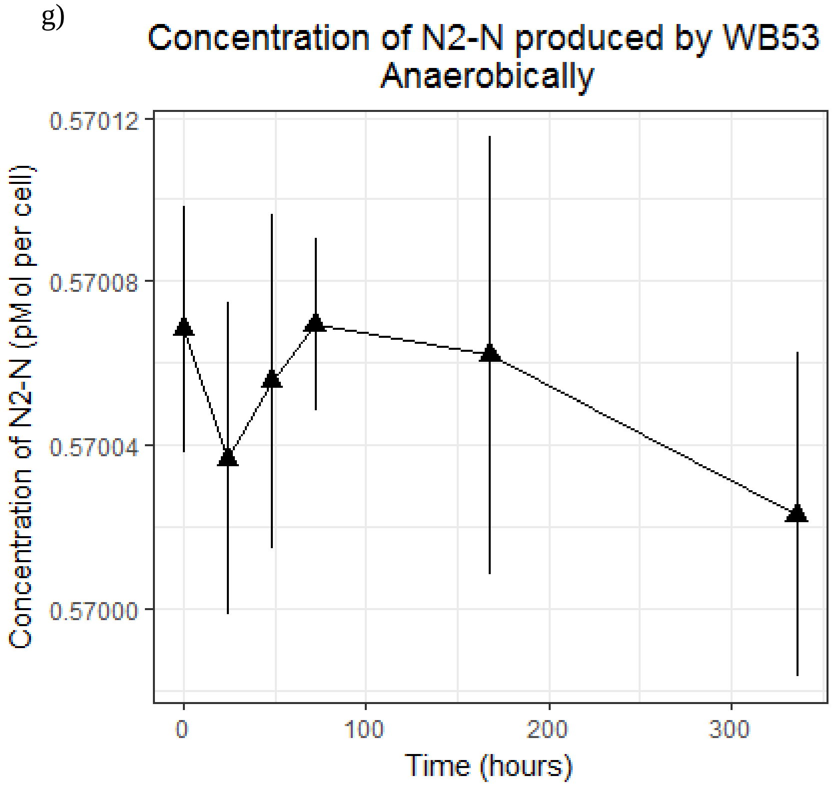
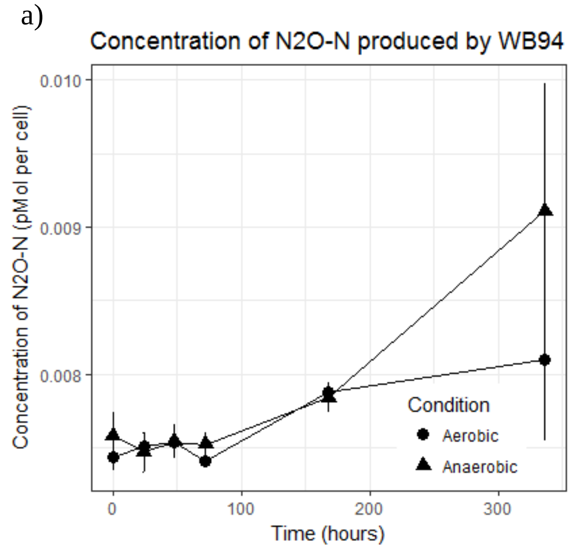
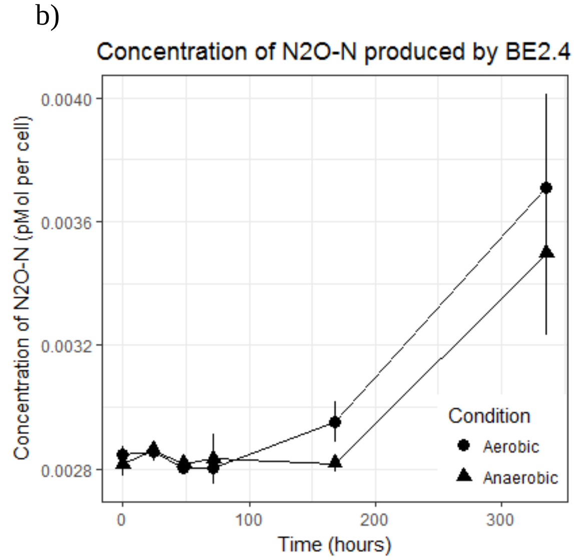


Figure 3-3: Concentration of $^{15}N_2$-N per cell measured over time for each of the seven potential denitrifiers (a-g). Error bars represent standard deviation for triplicate incubations. Note that the concentrations vary between samples.

a)



b)



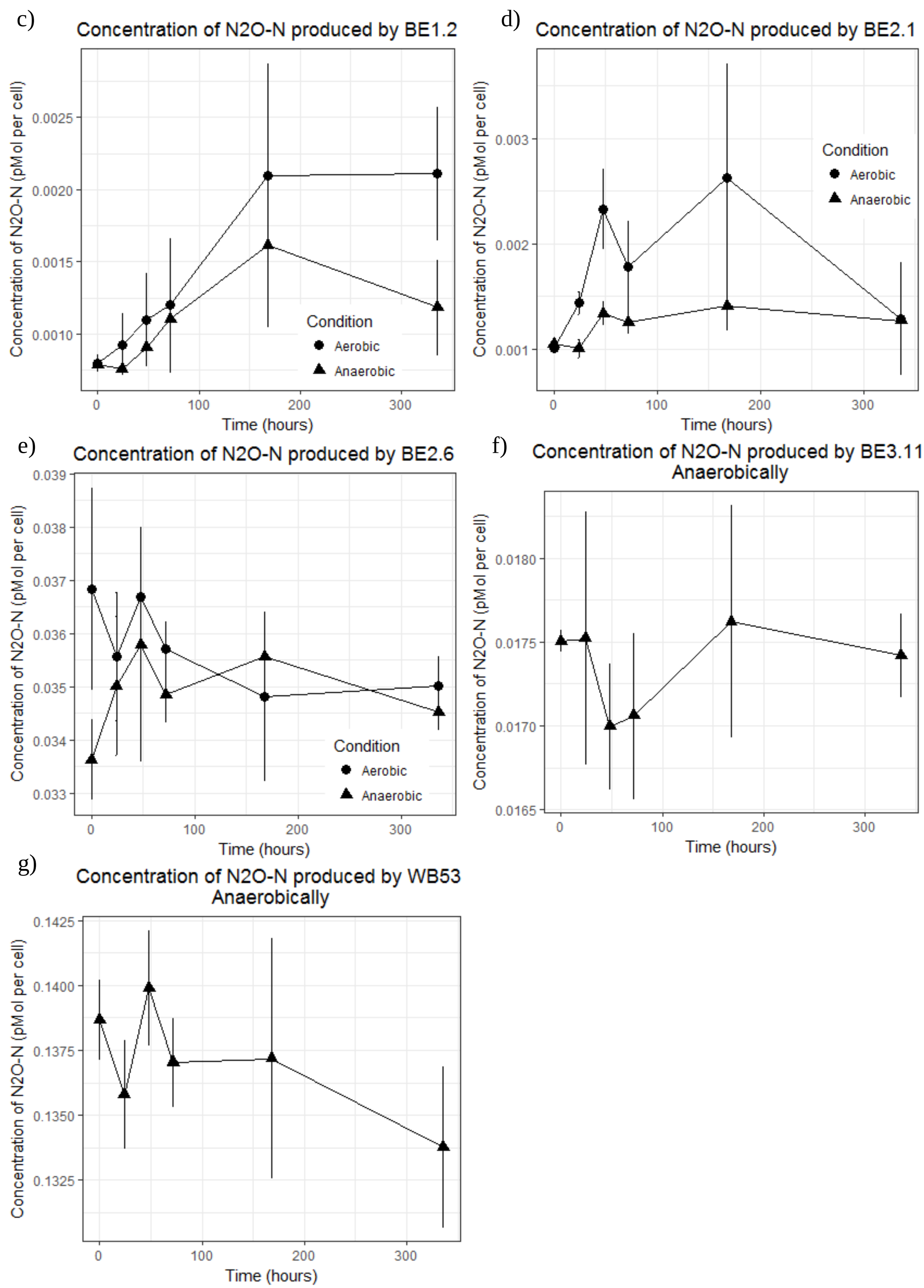


Figure 3-4: Concentration of $^{46}N_2O$-N per cell measured over time for each of the seven potential denitrifiers (a-g). Error bars represent standard deviation for triplicate incubations Note that the concentrations vary between samples.

Table 3-3: The denitrification rate for each sample and the time points that $^{30}N_2$ gas was produced, shown as mean ± SD.

Sample	Hours N_2 produced	pmol-N/cell/hour
WB94 aerobic	0-168	9.67E-08 ± 5.77E-09
WB94 anaerobic	0-168	6.67E-08 ± 3.06E-08
BE2.4 aerobic	0-336	2.00E-08 ± 4.05E-24
BE2.4 anaerobic	0-336	1.60E-08 ± 6.93E-09
BE1.2 aerobic	0-336	4.80E-09 ± 4.91E-09
BE1.2 anaerobic	0-168	9.33E-07 ± 9.29E-07
BE2.1 aerobic	0-48	7.00E-08 ± 3.00E-08
BE2.1 anaerobic	0-48	5.33E-09 ± 1.53E-09
BE2.6 aerobic	24-72	3.67E-07 ± 2.08E-07
BE2.6 anaerobic	0-72	1.93E-07 ± 2.72E-07
BE3.11 anaerobic	48-336	4.33E-08 ± 2.08E-08
WB53 anaerobic	24-72	1.70E-07 ± 7.63E-07

DISCUSSION

The majority of the potential denitrifiers were isolated from the BE bioreactor woodchips followed by the WB bioreactor woodchips. The BE bioreactor, which was the newest bioreactor at the time of sampling, contained fresh and uniform soft hardwood woodchips and we isolated a more diverse community of nitrate-reducing bacteria than at the other bioreactor sites. The genus *Microvirgula*, which was the dominant taxon at the BE bioreactor, is known to harbor strains capable of denitrification in the presence of oxygen, a useful ability in woodchip bioreactors where fluctuating levels of oxygen are present (Patureau et al. 1998; Patureau et al. 2001; Takaya et al. 2003). Our results supported this as BE2.4 demonstrated the ability to reduce nitrate to N_2 gas both aerobically and anaerobically. BE2.4 also produced $^{30}N_2$ gas consistently over the two week sampling period at a rate of 2.00E-08 aerobically and 1.60E-08 pmol-N/cell/hour anaerobically. While all eight *Microvirgula* isolates from bioreactor BE showed promising nitrate reduction ranging from 62-100%, the remaining seven converted >10% of N to ammonium. Similarly, the *Enterobacter* strains in this study reduced 74-100% nitrate-N, but much of the nitrate-N was converted to ammonium (27-39%). This is expected as *Enterobacter* is a known facultative aerobe capable of nitrate reduction to ammonium (Fazzolari et al. 1990; Tiedje et al. 1988). Other nitrate-reducing bacteria isolated in this study including *Serratia*, *Lelliottia*, *Buttiauxella*, and *Kosakonia* fall under Enterobacteriaceae and are also known to be capable of nitrate reduction to ammonium (Tiedje et al. 1988). Isolate BE1.2 belonged to the genus *Delftia*, which has previously demonstrated efficient denitrification (Zhang et al. 2016) and may have the ability to denitrify aerobically (Wang et al. 2007b). BE1.2 was able to denitrify anaerobically at a rate of 9.33E07 pmol-N/cell/hour between hour 0 to one week, but did not demonstrate aerobic denitrification. Three isolates belonged to the genera *Raoultella* and *Buttiauxella*, both of which are known nitrate reducers to ammonium, and seemingly reduced nitrate completely according to segmented flow analysis, however neither BE2.1 nor BE2.6 produced a significant amount of N_2 gas, indicating that they are in fact likely carrying out DNRA. While the denitrification rates were higher for BE2.1 and BE2.6, the time points that $^{30}N_2$ gas was produced were limited (0-48 hours or 0-72 hours).

The three biofilm sampling sites contained similar bacteria known to reduce nitrate to ammonium, such as *Raoultella*, *Enterobacter*, *Aeromonas* and *Lelliottia*, and our results reflected this. One *Microvirgula* strain was isolated from the Lamberton bioreactor biofilm, but it was not classified as a denitrifier according to our definition because it converted >10% N to ammonium-N. *Bacillus spp*. were the most common nitrate-reducing bacteria isolated from both the WB bioreactor biofilm and OC bioreactor biofilm, but despite positive GC results indicating that some of the strains may be potential denitrifiers, the majority of the isolates reduced a negligible amount of nitrate (<10% nitrate-N) or produced nitrite (0.2-17.5% of the nitrate-N converted to nitrite-N). *Bacillus* is also known to harbor many DNRA bacteria (Tiedje 1988). No potential denitrifiers were identified from any of the biofilm samples across the three sites, indicating that biofilms responsible for clogging in woodchip bioreactors are likely composed of non-denitrifying microorganisms primarily performing DNRA. This result is consistent with Tiedje (1983), who proposed that a high C/N ratio would select for DNRA bacteria over denitrifying bacteria. A higher ratio of available carbon (ie. acetate) to electron acceptor (ie. NO_3^-) is more suitable for DNRA because it allows for an additional three electrons to be accepted compared to denitrification (Tiedje 1983).

The WB bioreactor contained both hardwood and softwood woodchips that had been submerged for four years prior to sampling. Three of the 21 isolates from the WB woodchips were identified as *Microvirgula*, although these particular isolates converted 40-44% of the nitrate-N to ammonium, implying that they may be performing DNRA. Interestingly, strains belonging to the genus *Clostridium*, a known obligate anaerobe, made up the majority of the nitrate-reducing woodchip isolates, despite no obligate anaerobe capable of denitrification having been identified previously. While the denitrification rates of BE3.11 and WB53 were comparable to those of the other potential denitrifiers, $^{30}N_2$ gas increased over a short time period and the results varied too much to confirm whether complete denitrification to N_2 gas was occurring. Nevertheless, *Clostridium* may play an active role in woodchip bioreactors as they are known to be able to degrade wood compounds including hemicellulose, xylan and pectin under anaerobic conditions, providing a carbon source for the denitrifying microbial community (Kosugi et al. 2001; Desvaux 2006). Three additional nitrate-reducers from the WB woodchips were identified as *Cellulomonas* which is another genus known for its ability to degrade cellulose (Han et al. 1968; Thayer et al. 1984; Poulsen et al. 2016). *Cellulomonas* isolate WB94 was tested for aerobic and anaerobic denitrification over time and was shown to produce $^{30}N_2$ consistently over a one week period both under anaerobic (6.67E08 pmol-N/cell/hour) and aerobic (9.67E08 pmol-N/cell/hour) conditions. While some *Cellulomonas* strains are known to be capable of nitrate reduction to nitric oxide, a full set of denitrification genes have not been found (GenBank: AEE45473.1). It is possible that WB94 may be capable of complete denitrification, however more research is needed to confirm the presence of all denitrification genes and to further confirm aerobic denitrification.

The nitrate-reducing communities isolated from the two woodchip sample sites, WB and BE, were distinct from one another, indicating that age of the bioreactor may play a major role in denitrifier community composition. The BE bioreactor, which was the most recently established of the four bioreactors was composed mainly of the aerobic denitrifier, *Microvirgula*, implying that an anaerobic denitrifying community may not have been established at the time of sampling. The WB woodchip bioreactor, on the other hand, had been in use for four years prior to sampling and was composed mainly of the obligate anaerobe, *Clostridium*, and the cellulose-degrading *Cellulomonas*. These two genera may have an important role in long-established bioreactors. Previous studies comparing bioreactor efficiency between variably-aged woodchips show that nitrate removal rates decrease by about 50% during the first year and then become stable for years thereafter (Robertson 2010). It is possible that these two cellulose-degrading genera are integral in providing usable carbon for denitrification in aged woodchip bioreactors that contain little labile carbon. Additional studies are needed to determine how a denitrifying community changes over time within a bioreactor.

No denitrifiers were isolated from biofilm samples, demonstrating that clogging due to warming temperatures and the addition of a readily available carbon source is composed of non-denitrifying microorganisms. From this study, *Cellulomonas* isolate WB94 and *Microvirgula* isolate BE2.4 are the recommended candidates for bioaugmentation in the field due to their potential for aerobic and anaerobic denitrification and the possible ability of WB94 to degrade cellulose. More research is needed to confirm the presence of complete denitrification genes in these two isolates and of any genes involved in the degradation of complex polysaccharides.

4. Genomes of four nitrate-reducing bacteria isolated from woodchip bioreactors in Minnesota

ABSTRACT

Nitrate in agricultural wastewater can lead to algal blooms and eutrophication. Edge-of-field bioreactors known as woodchip bioreactors can prevent nitrate in wastewater from reaching surface waters by utilizing microbial denitrification to remove nitrate from the system. However, woodchip bioreactors experience low efficiency under cold temperatures, so one strategy to enhance bioreactors in the early spring involves inoculating the bioreactors with cold-adapted denitrifying microorganisms. Four previously identified, low temperature-adapted nitrate-reducing bacteria were subjected to whole genome sequencing to identify a strain useful for bioreactor inoculation. The selected strains were *Cellulomonas* sp. strain WB94, *Clostridium* sp. strain WB53, *Microvirgula* sp. strain BE2.4 and *Lelliotia* sp. strain BB2.1. Only *Microvirgula* sp. strain BE2.4 contained a complete set of denitrification genes, although all of the isolates were able to reduce nitrate. *Cellulomonas* sp. strain WB94, *Clostridium* sp. strain WB53 and *Lelliotia* sp. strain BB2.1 contained genes involved in cellulose-degradation, an important process in woodchip bioreactors. Based on the results, it was suggested that a combination of *Microvirgula* sp. strain BE2.4 and *Cellulomonas* sp. strain WB94 be used to inoculate a woodchip bioreactor due to their roles in denitrification.

INTRODUCTION

Denitrification, the anaerobic reduction of nitrate or nitrite to nitric oxide, nitrous oxide or nitrogen gas, is an important biochemical pathway carried out by a diverse group of microorganisms (Coyne, 1978; Tiedje 1994). It is one of the main pathways by which nitrogen reenters the atmosphere in gaseous form and is an important tool in treating wastewater. In Minnesota, 70% of nitrogen found in surface water is believed to have originated from agriculture, making it the largest contributor to nitrogen pollution (MPCA 2013). Excess nitrogen in the environment can lead to eutrophication and hypoxia, so many strategies to reduce nitrogen pollution relate to enhancing denitrification in agricultural wastewater. One strategy that is becoming increasingly popular involves diverting agricultural runoff or tile drainage through an underground trench filled with woodchips known as a woodchip bioreactor (Gibert et al. 2008; Schipper et al. 2010b; Lopez-Ponnada et al. 2017). In this system, the agricultural wastewater contains nitrate which is used as a terminal electron acceptor in the absence of oxygen while the woodchips are used as an electron donor and a carbon source for microbial denitrification (Tiedje 1994). Alternative carbon sources such as corn cobs and wheat straw have been used successfully in the past, although require more frequent maintenance to replace the degraded carbon (Greenan et al. 2006).

A major problem facing denitrifying bioreactors is their low efficiency during cold temperatures due to inhibited microbial activity (Christianson et al. 2012b; Bell et al. 2015; Feyereisen et al. 2016). One study found nitrate removal rates in a woodchip bioreactor to fluctuate throughout the year between 0.01 and 4.64 mg N L^{-1} h^{-1}, which corresponded to temperatures ranging from 4.4°C to 24.1°C (Ghane et al. 2015). They also found that nitrate removal decreased greatly below 10°C. This is a concern in Minnesota where it's estimated that 74% of the annual drainage runoff occurs between March and June when water temperatures are still very low (Jin and Sands 2003). A potential solution to this problem is bioaugmentation, in which low temperature-adapted denitrifying microorganisms are inoculated into a denitrifying bioreactor, establishing themselves as part of the community and increasing nitrate removal. For successful bioaugmentation, we need to understand their ecology and physiology.

Nitrate reduction falls under three major categories: dissimilatory nitrate reduction to ammonium (DNRA), in which nitrate is reduced to ammonium for respiration; assimilatory nitrate reduction, in which nitrate is reduced to ammonium and incorporated into the cell for growth; and denitrification, in which nitrate is reduced completely to dinitrogen gas (Tiedje 1988; Jetten 2008). Denitrification is a respiratory process used to derive energy through the sequential reduction of NO_3^- to inert N_2 gas via NO_2^-, NO and N_2O intermediaries. It is the preferred microbial process in treating wastewater because nitrogen is completely released from the system as a gaseous end product.

Often, the presence of nitrite reductase genes, *nirK* and *nirS*, are used as molecular targets for denitrifying microbial communities and are detected using PCR-based approaches (Nogales et al. 2002; Yoshida et al. 2012; Bonilla-Rosso et al. 2016). These approaches require primers targeting universally conserved regions of the targeted genes, making them susceptible to bias (Penton et al. 2013; Verbaendert et al. 2016). Denitrification capability cannot be ruled out by negative PCR results using functional gene-targeting primers due to this bias. Another problem in detecting only the *nirK* and *nirS* genes is that these genes do not imply complete denitrification. The release of the greenhouse gas N_2O as a result of incomplete denitrification due to environmental conditions or lack of a nitrous oxide reductase gene is a concern with woodchip bioreactors that rely on microbial denitrification (Moorman et al. 2010; Baily et al. 2012). Additionally, non-denitrifying microorganisms may contain nitrogen reduction genes for non-respiratory N assimilation or detoxification purposes (Tavares et al. 2006; Malm et al. 2009). Nitrogen oxide reductases are also found in bacteria reducing nitrate to ammonium (Rodionov et al. 2005; Simon and Klotz 2013). Therefore, any microorganisms used for bioaugmentation in the field should have a complete set of denitrifying genes, including nitrate reductase gene *narG*, nitrite reductase gene *nirS* or *nirK*, nitric oxide reductase gene *norB*, and nitrous oxide reductase gene *nosZ*. Whole genome-sequencing provides a complete picture of all relevant functional genes that would allow for appropriately choosing a denitrifying microorganism for field bioaugmentation.

In Chapters 2 and 3, potential cold-adapted denitrifying bacteria were isolated from woodchip bioreactors in Minnesota based on the following criteria: greater than 49% nitrate-N was reduced, less than 10% N was converted to ammonium, no nitrite-N was detected and >50 ppm nitrous oxide was produced. In this study, four of the previously identified, low temperature-adapted nitrate-reducing bacteria were subjected to whole genome sequencing in order to identify genes responsible for denitrification and other important processes in woodchip bioreactors, such as cellulose degradation, and determine whether any strain in particular could be used for bioaugmentation.

METHODS

Strain selection

The denitrifying bacteria subjected to whole genome sequencing were previously isolated and identified in Chapters 2 and 3 and selected based on their denitrification potential which took into account N transformations involving NO_3^-, NO_2^-, NH_4^+, N_2O and N_2. Briefly, microorganisms were isolated at 15°C under anaerobic conditions from four woodchip bioreactors in Minnesota. Out of a total of 207 isolated bacteria, 79 were confirmed to be capable of nitrate reduction based on gas chromatography measuring N_2O production in the presence of acetylene (Yoshinari and Knowles 1976). Segmented flow analysis using the SEAL AA3 HR AutoAnalyzer to measure nitrate, nitrite and ammonium was also employed to confirm nitrate-reduction and distinguish between denitrification and dissimilatory nitrate reduction to ammonium (DNRA). Of the confirmed nitrate-reducers, the following isolates were selected for whole genome sequencing: *Cellulomonas* isolate WB94 and *Clostridium* isolate WB53 from the Willmar bioreactor; *Microvirgula* isolate BE2.4 from the Blue Earth bioreactor; and *Lelliotia* isolate BB2.1 from the Lamberton bioreactor.

The first bioreactor, located in Willmar, MN, was sampled four years after its establishment and consisted of 8 distinct 1.7m x 11.6m x 0.9m beds. The majority of the 21 nitrate-reducing bacteria isolated from the Willmar site belonged to the genus *Clostridium*, which is a known obligate anaerobe. Despite many of the *Clostridium* isolates from this bioreactor demonstrating denitrification abilities, no known denitrifiers are obligate anaerobes, and previous studies show that *Clostridium* most likely reduces nitrate to ammonium (Fujinaga et al. 1999). Isolate WB53, however, demonstrated high nitrate reduction under low temperatures (58% nitrate-N reduced) and was selected for whole genome sequencing. Two *Cellulomonas* strains were isolated from the Willmar woodchip bioreactor, one of which, WB94, demonstrated potential denitrification ability with over 40% nitrate-N reduced and less than 10% total N converted to ammonium. Additionally, *Cellulomonas* is known to harbor strains capable of degrading cellulose, a potentially useful characteristic for application to woodchip bioreactors.

The second bioreactor, in Blue Earth, MN, was sampled shortly after its establishment in 2016 and consisted of 3 beds measuring 7.62m x 41m x 1.5m. Isolate BE2.4, belonging to the genus *Microvirgula*, was isolated from this site and selected for efficient nitrate removal and minimal ammonium production. Additionally, *Microvirgula* are known to be able to denitrify under aerobic conditions.
Aerobic denitrifiers may play an important role in field bioreactors as they are capable of denitrifying under fluctuating water and oxygen levels, making them a potentially useful strain for bioaugmentation.

A third woodchip bioreactor, located in Lamberton, MN, was a cube design composed of a series of layers of crushed limestone and concrete, corn cobs, woodchips and lava rock measuring 1m x 0.96m x 0.78m. This bioreactor was subjected to biostimulation by using acetate injections to stimulate denitrification. Clogging occurred at the inlet pipe where the acetate and wastewater converged and samples of the clogging biofilm were collected to isolate potential denitrifying microorganisms. Isolate BB2.1, a confirmed nitrate-reducer belonging to the genus *Lelliottia*, was isolated from the biofilm samples and was selected for whole genome sequencing as a representative biofilm strain to determine its role in denitrification and woodchip bioreactors.

Whole gene sequencing

Each isolate was incubated in R2A broth containing 10mM acetate and 5mM nitrate (R2A-NA) at 30°C for 1 to 2 weeks. The pelleted cells were then subjected to DNA extraction using the PowerSoil DNA Isolation kit (MOBIO, Carlsbad, CA), according to the manufacturer's instructions. Sequencing libraries were prepared using the PacBio SMRT kit (Pacific Biosciences), and the genome was analyzed using the PacBio RS II platform (Pacific Biosciences). Extracted DNA was used to generate a SMRTbell library (20 kbp insert) and the Mayo Clinic's Molecular Biology Core performed whole genome sequencing using the PacoBioRS II platform (Pacific Biosciences, Menlo Park, CA, USA). After quality filtering, reads were assembled *de novo* with the hierarchical genome assembly process (HGAP3) in the SMRT Link portal (v 2.3.0) and genome annotation was carried out by the NCBI Prokaryotic Genome Annotation Pipeline (Angiuoli et al. 2008). Average Nucleotide Identity (ANI) values were calculated using JSpecies (Richter et al. 2015).

RESULTS

Cellulomonas sp. strain WB94

The genome of strain WB94 was represented by seven contigs with a total size of 3,868,980 bp and GC content ranging from 70 to 73% (**Table 4-1**). The genome contained 3,394 protein-coding sequences (CDS), 129 pseudogenes, 46 tRNAs, six rRNAs (two rRNA operons), and three noncoding RNAs. The ANI values between the genomes of strain WB94 and *Cellulomonas cellasea* DSM 20118 were 98.014%, which is greater than the cutoff value for species discrimination (95% to 96%) (Richter et al. 2009, Goris et al. 2007). Therefore, strain WB94 most likely belongs to *Cellulomonas cellasea*.

The genome of strain WB94 harbored nitrate reductase genes *narIJHG* and dissimilatory NO-forming nitrite reductase gene *nirK* (Table 2), suggesting that strain WB94 can reduce nitrate to nitrite and to nitric oxide. The NirK sequence was closely related to the NirK from *Actinosynnema mirum* DSM43827 (CP001630), but was also similar to those from other *Cellulomonas* species (**Figure 2-5**, phylogenetic tree). Other denitrification related genes were not found on the genome. The genome also contained assimilatory NAD(P)H-dependent nitrite reductase genes *nirBD*, suggesting that strain WB94 can use nitrate and nitrite as a N source. In addition to the genes related to the N

Table 4-1: Features of the genome sequencing results for each of the isolates.

Isolate	Contig number	Accession number	Size (bp)	GC content (%)
Cellulomonas WB94	0	NZ_QEES01000002.1	2,780,765	71.9
	1	NZ_QEES01000005.1	329,035	70.2
	2	NZ_QEES01000001.1	235,040	70.3
	3	NZ_QEES01000007.1	151,423	71.1
	4	NZ_QEES01000004.1	162,011	72.2
	5	NZ_QEES01000003.1	157,415	72.7
	6	NZ_QEES01000006.1	53,291	71.9
Clostridium WB53	1	CP029329.1	4,258,017	29.3
	2	CP029330.1	29,114	28.4
Microvirgula BE2.4	2	CP028518.1	5,968	64.5
	3	CP028519.1	4,121,243	64.3
Lelliottia BB2.1	0	CP028520.1	4,607,442	55
	2	CP028521.1	126,628	50.7

Table 4-2: Genes identified on the genome of Cellulomonas sp. strain WB94 relating to denitrification and relevant complex polysaccharide degradation.

Isolate	Gene family	Function	Gene	locus_tag	Protein
Cellulomonas sp. strain WB94	Nitrogen reduction	Nitrate Reduction	*narI*	DDP54_03075	respiratory nitrate reductase subunit gamma
			narJ	DDP54_03080	nitrate reductase molybdenum cofactor assembly chaperone
			narH	DDP54_03085	nitrate reductase subunit beta
			narG	DDP54_03090	nitrate reductase subunit alpha
		Nitrite reduction	*nirD*	DDP54_03030	nitrite reductase (NAD(P)H) small subunit
			nirB	DDP54_03035	nitrite reductase (NAD(P)H
				DDP54_03150	Molybdopterin-binding nitrite reductase
			nirK	DDP54_17680	NO-forming nitrite reductase
	Polysaccharide degradation	Cellulose degradation		DDP54_00625	endoglucanase
			malQ	DDP54_01650	4-alpha-glucanotransferase
				DDP54_0629	1,3-beta-glucanase
			malQ	DDP54_17500	4-alpha-glucanotransferase
				DDP54_09215	cellobiose phosphorylase
				DDP54_16385 DDP54_06345 DDP54_11510 DDP54_11820 DDP54_12400 DDP54_16380	beta-glucosidase
		Hemi-cellulose degradation		DDP54_00375	1,4-beta-xylanase
		Starch degradation		DDP54_12980 DDP54_13300 DDP54_12980 DDP54_13300	alpha-amylase
				DDP54_1540 DDP54_1540	glucoamylase
				DDP54_14360	amylo-alpha-1,6-glucosidase
				DDP54_06195 DDP54_06215 DDP54_06455	alpha-glucosidase

cycle, the genome contained genes related to the biodegradation of complex polysaccharides, including cellulose, xylan, starch and glycogen (**Table 4-2).**

Clostridium sp. strain WB53

Strain WB53 had a length of 4,287,191 bp represented by two contigs (**Table 4-1**). The GC content was 29.3%. The genome contained 3,597 protein-coding sequences (CDS), 159 pseudogenes, 85 tRNAs, 43 rRNAs and five noncoding RNAs. The ANI values between the genomes of strain WB53 and *Clostridium beijerinckii* NCIMB 14988 were 98.486% which is greater than the cutoff value for species discrimination (95% to 96%) (Richter et al. 2009, Goris et al. 2007). Therefore, strain WB53 most likely belongs to *Clostridium beijerinckii*.

Strain WB53 was selected due to its ability to remove nitrate, while producing little ammonium. This suggests that strain WB53 was able to reduce nitrate to N_2 (i.e., denitrification) as opposed to DNRA, however all other *Clostridium* strains are known to reduce nitrate to ammonium (Fujunaga et al. 1999). Based on the genome sequencing, we

Table 4-3: Genes identified on the genome of Clostridium sp. strain WB53 relating to denitrification and relevant complex polysaccharide degradation.

Isolate	Gene family	Function	Gene	locus_tag	Protein
Clostridium sp. strain WB53	Nitrogen reduction	Nitrate reduction	*narE*	DIC82_01435	molybdopterin molybdenumtransferase MoeA
			nrt	DIC82_00300	nitrate ABC transporter substrate-binding protein
		Nitrite reduction	*nirA*	DIC82_16990	ferredoxin--nitrite reductase
			nirC	DIC82_01080	nitrite transporter
			nirA	DIC82_08130	ferredoxin--nitrite reductase
	Polysaccharide degradation	Cellulose degradation		DIC82_05520	4-alpha-glucanotransferase
				DIC82_10720	alpha-glucosidase
				DIC82_01970 DIC82_02210 DIC82_02235 DIC82_02785 DIC82_18880	6-phospho-beta-glucosidase

were unable to identify a full set of denitrification genes in strain WB53, although this strain does have the ability to reduce both nitrate and nitrite (**Table 4-3**). Additionally, it was found that strain WB53 harbors genes capable of cellulose degradation.

Microvirgula sp. strain BE2.4

The genus *Microvirgula* is known to harbor strains capable of aerobic denitrification and was shown that strain BE2.4 is likely an aerobic denitrifier (Chapter 3). Interestingly, strain BE2.4 was found to contain two nitrous oxide reductase genes, *nosZ*, as well as a complete set of denitrification genes including nitrate reductase genes *narGHIJ*, dissimilatory NO-forming nitrite reductase gene *nirS*, and nitric oxide reductases *norBD* (**Table 4-4**). A phylogenetic analysis of NirS and NosZ can be found in **Figures 4-1** and **4-2**, respectively.

Table 4-4: Genes identified on the genome of Microvirgula sp. strain BE2.4 relating to denitrification and relevant complex polysaccharide degradation.

Isolate	Gene family	Function	Gene	locus_tag	Protein
Microvirgula sp. strain BE2.4	Nitrogen reduction	Nitrate reduction	*narG*	DAI18_04280	nitrate reductase subunit alpha
			narH	DAI18_04275	nitrate reductase subunit beta
			narI	DAI18_04265	respiratory nitrate reductase subunit gamma
			narJ	DAI18_04270	nitrate reductase molybdenum cofactor assembly chaperone
		Nitrite reduction	*nirS*	DAI18_08065	nitrite reductase
		Nitric oxide reduction	*norB*	DAI18_12345	nitric-oxide reductase large subunit
			norD	DAI18_12365	nitric oxide reductase
		Nitrous oxide reduction	*nosZ*	DAI18_13200 DAI18_07220	nitrous-oxide reductase
			nosL	DAI18_13215 DAI18_07200	nitrous oxide reductase accessory protein NosL
			nosD	DAI18_13230 DAI18_07185	nitrous oxide reductase family maturation protein NosD

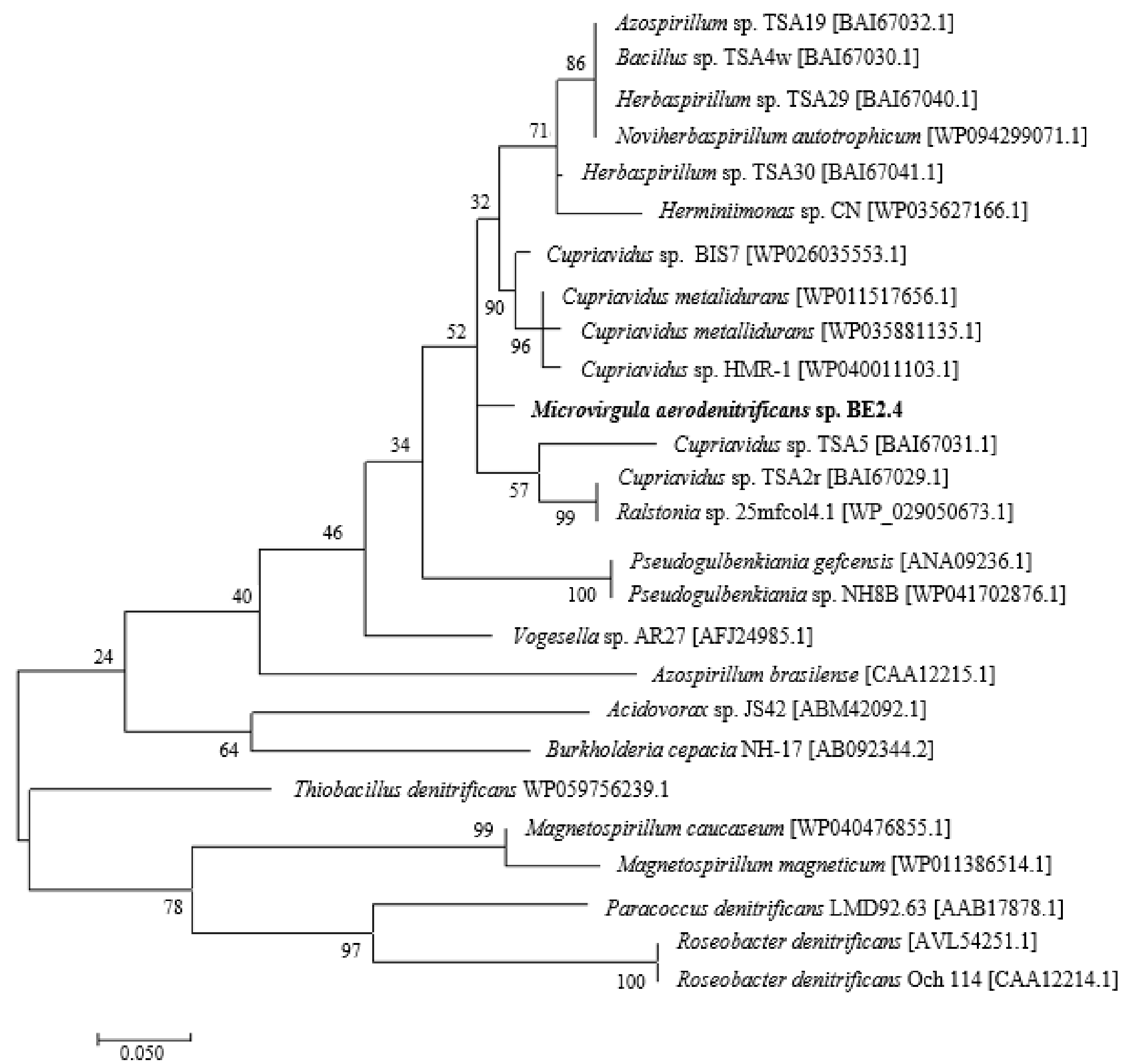


Figure 4-1 Phylogenetic tree based on NirS sequences using MEGA 7.0.26 and neighbor joining method (1,000 replications).

Lelliottia sp. strain BB2.1

The genome of *Lelliottia* sp. strain BB2.1 had a length of 4,734,070 bp and was represented by two contigs (Table 1). Its GC content was 54.9%. The genome contained 4,467 protein-coding sequences (CDS), 177 pseudogenes, 84 tRNAs, 25 rRNAs and eight

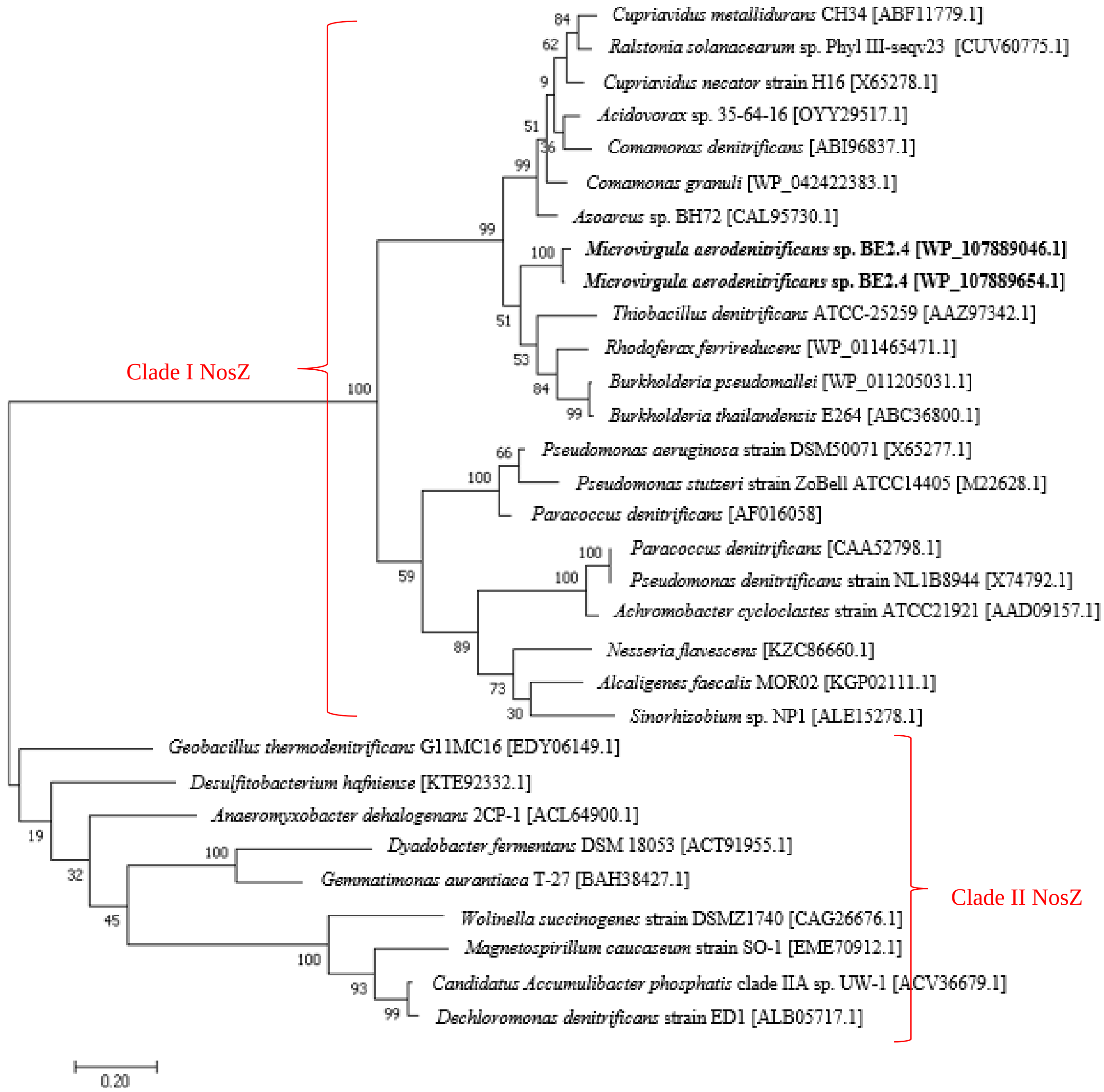


Figure 4-2: Phylogenetic tree based on NosZ sequences using MEGA 7.0.26 and neighbor joining method (1,000 replications).

noncoding RNAs. The ANI values between the genomes of strain BB2.1 and *Lelliottia amnigena* CHS 78 were 98.015%, which is greater than the cutoff value for species discrimination (95% to 96%) (Richter et al. 2009, Goris et al. 2007). Therefore, strain BB2.1 most likely belongs to *Lelliottia amnigena.*

Strain BB2.1 was found to harbor nitrate reductase genes *narIJHG,* nitrite reductase genes *nirBD* and nitric oxide reductase gene *norV* (**Table 4-5**). Additionally, strain BB2.1 contained ammonia monooxygenase *amoA,* glutamine-synthase encoding *glnA* involved in ammonium assimilation, as well as genes involved in degrading polysaccharides including cellulose, cellobiose, glycogen and starch

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Table 4-5: Genes identified on the genome of Lelliottia sp. strain BB2.1 relating to denitrification and relevant complex polysaccharide degradation.

Isolate	**Gene family**	**Function**	**Gene**	**locus_tag**	**Protein**
Lelliottia sp. strain BB2.1	Nitrogen reduction	Nitrate reduction	*narQ*	DAI21_11360	nitrate/nitrite two-component system sensor histidine kinase
			narX	DAI21_14475	nitrate/nitrite two-component system sensor histidine kinase
			narG	DAI21_14485 DAI21_15365	nitrate reductase subunit alpha
			narH	DAI21_14490 DAI21_15360	nitrate reductase subunit beta
			narJ	DAI21_14495 DAI21_15355	nitrate reductase molybdenum cofactor assembly chaperone
			narI	DAI21_14500 DAI21_15350	respiratory nitrate reductase subunit gamma
		Nitrite reduction	*nirD*	DAI21_06345	Involved in reducing nitrite to ammonium to detoxify nitrite accumulation in anaerobic nitrate-respiring cells and regenerate NAD+; bounds to NirB, the cytoplasmic subunit, whose expression is induced at high nitrate concentrations
			nirB	DAI21_06350	nitrite reductase (NAD(P)H)
		Nitric oxide reduction	*norW*	DAI21_09660	NADH:flavorubredoxin reductase NorW
			norR	DAI21_09670	nitric oxide reductase transcriptional regulator norR
			norV	DAI21_09665	anaerobic nitric oxide reductase flavorubredoxin

	Ammonium	Ammonium oxidation	*amoA*	DAI21_22440	ammonia monooxygenase
		Ammonium assimilation	*glnA*	DAI21_04665	Functions in the assimilation of ammonia
	Polysaccharide degradation	Cellulose degradation		DAI21_05430 DAI21_17160	beta-glucosidase
				DAI21_04155 DAI21_04265 DAI21_08900 DAI21_09120 DAI21_09630 DAI21_11020 DAI21_11240 DAI21_12410 DAI21_12450 DAI21_17685 DAI21_19655 DAI21_21715	6-phospho-beta-glucosidase
				DAI21_19625	alpha-glucosidase
		Starch degradation	*malZ*	DAI21_21845	maltodextrin glucosidase
		Hemicellulose degradation		DAI21_01595	1,4-beta-xylanase

DISCUSSION

Four nitrate-reducing bacteria were subjected to whole genome sequencing to identify denitrification genes. They were selected based on N_2O production via the acetylene inhibition method and percent NO_3^- -N reduced measured using segmented flow analysis. All of the strains were able to reduce nitrate, however only *Microvirgula* sp. strain BE2.4 was found to contain a complete set of denitrification genes. *Cellulomonas* sp. strain WB94, *Microvirgula* sp. strain BE2.4, and *Lelliottia* sp. strain BB2.1 contained the complete *narGHJI* operon, which encodes for the three subunits that make up the nitrate reductase as well as the respiratory nitrate reductase chaperone (Gonzalez et al. 2017). *Lelliottia* sp. strain BB2.1 had additional sensor kinase-encoding *narQ* and *narX* while *Clostridium* sp. strain WB53 had only *narE* on its genome.

Clostridium sp. strain WB53 also contained an ABC-type nitrate transporter (NRT) which is a nitrate-nitrite transporter involved in nitrate assimilation (Aichi et al. 2006). The transported nitrate or nitrite is subsequently reduced to ammonium for cell growth.

Each isolate also contained at least one nitrite reductase. *Microvirgula* sp. strain BE2.4 contained the cd_1 nitrite reductase encoded by *nirS* which is a commonly found, haem-containing dissimilatory nitrite reductase (Rinaldo et al. 2017). NirS also reduces oxygen, whereas the other nitrite reductase, NirK, is inactivated by oxygen (Fulop et al. 1995; Chen and Strous 2013). *Cellulomonas* sp. strain WB94 contained the other nitrite reductase, the copper-containing nitrite reductase encoded by *nirK*, Additionally, both *Cellulomonas* sp. strain WB94 and *Lelliottia* sp. strain BB2.1 contained *nirBD*, the cytoplasmic sirohaem nitrite reductase, which is believed to be involved in nitrite assimilation and detoxification by reducing nitrite to ammonium (Lin and Stewart, 1997; Rodionov 2005; Malm 2009; Cole 2017). *Clostridium* sp. strain WB53 contained two *nirA* genes and one *nirC* gene. NirA is distinct from both respiratory NiR and assimilatory NiR as it does not contain sirohaem, haem or copper and has been linked to energy production rather than nitrate assimilation (Fujinaga et al. 1999).

The next step in denitrification is nitric oxide reduction to nitrous oxide via a nitric oxide reductase, of which there are many types (Zumft 2005). One type, cytochrome *c*-dependent nitric oxide reductases NorB and NorC, are only found in denitrifying bacteria (Mesa et al. 2002; Zumft 2005; Tosha and Shiro 2017). The NorB-encoding *norB* gene was detected on the genome of *Microvirgula* sp. strain BE2.4, as was *norD*, which encodes for a presumed cytoplasmic protein that affects both *nirS* and *norB* expression (Mesa et al. 2002; Zumft 2005; Tosha and Shiro 2017). *Lelliottia* sp.

strain BB2.1 contained another type of nitric oxide reductase, the quinol-dependent *norVWR* operon, encoding for a flavorubredoxin, a NADH:flavorubredoxin oxidoreductase, and a regulator (Zumft 2005). This operon is responsible for nitrosative stress response and has been found in bacteria that reduce nitrate to ammonium (Zumft 2005; Cole 2017). We were unable to detect nitric oxide reductases on the genomes of *Cellulomonas* sp. strain WB94 and *Clostridium* sp. strain WB53. As mentioned above, the presence of *nirS* and *nirK* are often used as indicators of denitrification, however these results showed that the presence of *nirS* or *nirK* does not necessarily imply complete denitrification as our results indicated that *Cellulomonas* sp. strain WB94 was unable to further reduce NO. Thus, while *Cellulomonas* sp. strain WB94 is able to reduce NO_3^- to a gaseous product, it is not capable of complete denitrification to N_2 gas. Because N_2 production was detected in Chapter 3, it is possible, however, that additional denitrification genes are present on the genome of *Cellulomonas* sp. strain WB94 and that they are unknown as of yet or require further data mining.

Clostridium sp. strain WB53 did not contain a nitric oxide reductase either, although this is likely because NO_2^- was reduced in the previous step and assimilated into the cell. And, while *Lelliottia* sp. strain BB2.1 contained nitrite and nitric oxide reductases, producing N_2O gas as detected in the acetylene inhibition test in Chapter 3, it does not have a nitrous oxide reductase, implying that the nitrate is not reduced to N_2 gas, but to N_2O or ammonium. Interestingly, ammonia monooxygenase encoding gene *amoA* was detected on the genome of *Lelliottia* sp. strain BB2.1, implying that strain BB2.1 may be capable of reducing NO_3^- to N_2O gas in a non-respiratory process, or to NH_4^+ via NirBD and from NH_4^+ to NO_2^- or N_2 through nitrification (Levy-Booth 2014; Bernard et al. 2015). These processes have been found in other non-denitrifying bacteria performing DNRA (Sun et al. 2017).

The final step in denitrification, the two-electron reduction of N_2O to N_2 and water, is catalyzed by the copper-dependent nitrous oxide reductase, NosZ. There are two *nosZ* clades, with the “typical” clade I consisting of *Alpha*-, *Beta*-, and *Gamma-proteobacteria*, and the “atypical” clade II consisting of a diverse range of bacteria and archaea (Jones et al. 2013; Pauleta et al. 2017). The two clades share <50% amino acid sequence similarity (Yoon et al. 2016). Interestingly, two distinct clusters of NosZ-encoding and accessory genes were found on the genome of *Microvirgula* sp. strain BE2.4. The two *nosZ* genes were 96% identical and belonged to clade I. Redundancy in denitrification functional genes has previously been reported but the purpose is unknown (Jones et al. 2008; Heylen and Keltjens 2012; Sun et al. 2017). That only *Microvirgula* sp. strain BE2.4 contained a *nos* gene is not surprising as it has previously been reported that 70% of genomes containing *nirK* do not have *nosZ*, while only 20% of *nirS* genomes do not contain *nosZ* (Graf et al. 2014). In phylogenetic comparisons between denitrification functional genes and 16S rRNA taxonomic classification, *nosZ* tends to show the greatest level of congruence (Dandie et al. 2007; Jones et al. 2008). *Microvirgula* has comprised only two species since the discovery of *Microvirgula aerodenitrificans* by Patureau et al. in 1998 and based on 16S rRNA sequence comparisons, does not cluster strongly with other nearby genera (Patureau et al. 1998; Ji et al. 2015). The *nosZ* phylogenetic tree (**Figure 4-4**) is consistent in this regard.

Microvirgula sp. strain BE2.4 was shown to be capable of aerobic denitrification in Chapter 3. It is believed that aerobic denitrification requires the Nap operon which encodes for the periplasmic nitrate reductase because the transport of nitrate to the cytoplasmic nitrate reductase Nar is inhibited in the presence of oxygen (Argandoña et al. 2006; Ji et al. 2015). However, a proposed alternative location of the NarG active site in the periplasm is becoming increasingly accepted (Matinez-Espinosa et al. 2007; Chen and Strous 2013; Simon and Klotz 2013). *Microvirgula* sp. strain BE2.4, which was capable of aerobic denitrification, did not have a *nap* gene on its genome. This may indicate that the NarG active site for this strain is located in the periplasm, or that further data mining for a Nap operon is needed.

Another ability deemed useful for bioaugmentation of woodchip bioreactors was the degradation of complex polysaccharides such as cellulose, starch, xylan, maltose, and cellobiose. These genes were detected on the genomes of *Cellulomonas* sp. strain WB94, *Lelliottia* sp. strain BB2.1 and *Clostridium* sp. strain WB53. Wood, the primary carbon source in woodchip bioreactors, is composed primarily of cellulose (45% of the dry weight), hemicellulose (25-30%) and lignin (25-30%) (Pérez et al. 2002). Other components that do not contribute to cell wall structure include starches and glycosides (Pettersen 1984; Smith et al. 2005). Cellulose is a macromolecule made up of glucose subunits that form repeating units of cellobiose (Béguin and Aubert 1994). Enzymes for cellulose degradation include endoglucanases and exoglucanases that hydrolyze cellulose bonds, releasing cellobiose, and β-glycosidases that break down the cellobiose into two glucose molecules (Pérez et al. 2002). Xylan is the primary carbohydrate in hemicellulose and its biodegradation requires xylanses which break down hemicellulose into monomeric sugars and acetic acid (Pérez et al. 2002). Lignin is an aromatic polymer with a highly complex structure and high molecular weight, rendering it more resistant to biodegradation (Pérez et al. 2002). Genes encoding for enzymes involved in cellulose and hemicellulose were detected in the three isolates, however no laccases or lignin-degrading peroxidases were found. The breakdown of cellulose is an important microbial interaction, providing carbon and energy sources for all microorganisms living in the surrounding environment (Leschine 1995; Pérez et al. 2002).

CONCLUSION

Based on whole genome sequencing results, only one nitrate-reducing isolate contained a complete set of denitrification genes. Others that had demonstrated potential denitrification through segmented flow analysis to measure nitrate reduced and ammonium produced and the acetylene inhibition method to confirm N_2O production were not capable of complete denitrification, reducing NO_3^- instead to NO, N_2O or NH_4^+. This again highlights the difficulty in identifying microorganisms capable of complete denitrification through gas measurements and PCR-based approaches. While the representative biofilm isolate, *Lelliottia* sp. strain BB2.1, was not capable of complete denitrification, it contributed to the woodchip bioreactor community through breakdown of cellulose and hemicellulose, and through nitrate, nitrite and nitric oxide reduction. Based on this study, however, only *Microvirgula* sp. strain BE2.4 fits the criteria for a low temperature-adapted, complete denitrifying microorganism. The ability to denitrify aerobically is another advantage as BE2.4 can denitrifying completely under fluctuating oxygen levels in the field. Within a woodchip bioreactor, it is also important to consider the degradability of the carbon source, and so inoculating the bioreactor with a second strain capable of cellulose degradation would provide easily degradable sugars to promote microbial activity. Inoculating *Cellulomonas* sp. strain WB94 along with BE2.4 to the bioreactor is a useful bioaugmentation strategy for future research.

5. Algae Bioreactor to Remove Manganese from Groundwater

ABSTRACT

High levels of manganese in drinking water can cause health problems, but common treatment methods require cost-intensive chemicals, conditions and maintenance. In this study, a novel algae bioreactor was established to remove manganese from water. In this bioreactor, the algae provides fixed carbon for manganese-oxidizing microorganisms that oxidize the dissolved manganese, removing it from solution. Biofilm samples composed of the algae and manganese-oxidizing microorganisms were collected from a manganese-oxidizing waterfall in Hokkaido, Japan. Using a culture-dependent approach, 68 manganese-oxidizing bacteria and fungi were isolated from the biofilm samples, including known oxidizers *Bosea*, *Pseudomonas*, *Plectosphaerella* and *Phoma* and some not previously known to oxidize manganese such as *Aeromonas, Skermanella*, *Ensifer* and *Aspergillus*. A culture-independent approach was also employed using biofilm samples from the manganese-oxidizing waterfall and biofilm samples that had been incubating in the manganese-removing bioreactor to determine how abundant the isolated manganese-oxidizing bacteria are in an actively oxidizing environmental sample. Both sites were dominated by *alpha*- and *beta-Proteobacteria*, but the isolated manganese-oxidizers were not detected at the genus level.

INTRODUCTION

While manganese is an essential element required for life, exposure to elevated manganese can result in severe health problems (Blanc 2017; Tarale et al. 2018). This is a concern in Minnesota where more than 60% of groundwater wells exceed the EPA recommended level of drinking water manganese of 50 µg/L (MPCA, 1998). Manganese in groundwater is stable at its soluble, Mn^{2+} form, so many current methods to oxidize and subsequently remove manganese from drinking water require costly equipment and specific environmental conditions (Roccaro et al. 2007; Tobiason et al. 2016). However, it has long been known that diverse microorganisms are able to catalyze the oxidation of Mn^{2+} to sparingly soluble $Mn^{3/4+}$, precipitating it out of solution (Tebo & Emerson 1986; Tebo & He 1999; Tebo et al. 2004). These microorganisms offer a unique opportunity to affordably treat drinking water, as well as remediate metal-contaminated sites due to the scavenging ability of biogenic manganese oxides (Scott & Morgan 1995; Nelson et al. 2002; Tebo et al. 2004; Grangeon et al. 2010; Droz et al. 2015; Chen et al. 2017). The activity of manganese oxidizing microorganisms in biofiltration to treat drinking water has previously been studied and has demonstrated promising manganese removal (Katsoyiannis and Zouboulis 2004; Burger et al. 2008; Hoyland et al. 2014).

The microorganisms capable of manganese oxidation that have been identified so far belong to diverse phylogenetic lineages, including *Firmicutes*, *Actinobacteria* and the α, β, and γ *Proteobacteria* branches of the bacterial domain (Tebo et al. 2004; Tebo et al. 2005). So far, only heterotrophic bacteria and fungi have been found to oxidize manganese and some of the most commonly studied manganese oxidizers include *Leptothrix discophora* SS-1 (Boogerd and DeVrind 1987; Nelson et al. 1999), *Pseudomonas putida* strain GB-1 (Banh et al. 2013; Geszvain et al. 2013; Wright et al. 2018), *Bacillus* sp. strain SG-1(Francis et al. 2002; Soldatova et al. 2012) and *Plectosphaerella cucumerina* (Santelli et al. 2010ab; Santelli et al. 2011). While the mechanism and reason for manganese oxidation is not entirely known (Ehrlich et al. 2015), it has recently been proposed that manganese oxidation can occur extracellularly through reactive oxygen species, such as superoxide (Learman et al. 2011). The discovery of widespread superoxide production ability in bacteria by Diaz et al. (2013) shows that manganese oxidation may be more common than previously thought.

The purpose of this study was to characterize the manganese-oxidizing microbial community at a site known for high biogenic manganese oxidation using a culture-dependent approach and assess how prevalent manganese-oxidizing bacteria are in the environment using a culture-independent approach. Manganese oxidation and removal will be tested by the establishment of a novel bioreactor that used algae to provide fixed carbon to manganese-oxidizing microorganisms. Going forward, this information can be used to inform future studies of biological manganese oxidation and demonstrate the reliable removal of excess manganese from potential drinking water.

METHODS

Field site

Onneto Yuno-taki is a waterfall in Hokkaido, Japan located in the Akan National Park, one of the earliest established National Parks in Japan. It is an area of volcanic activity and its hot springs were once a popular site for swimming until it was designated a national monument in the 1980s due to the unique microbial communities present at the site and their ability to produce manganese oxides. It is now estimated that 1.1 tons of manganese oxide are produced annually at this site (Mita et al. 1994), as shown by the distinct black coloring of the falls (**Figure 5-1**). The water contains >3 ppm (>55 µM) Mn^{2+} and the temperature and the pH of the water are around 32°C and 7.36, respectively. Detailed water chemistry of Yuno-taki falls can be found in Mita et al. (1994). It is believed that biofilms composed of algae and bacteria are responsible for

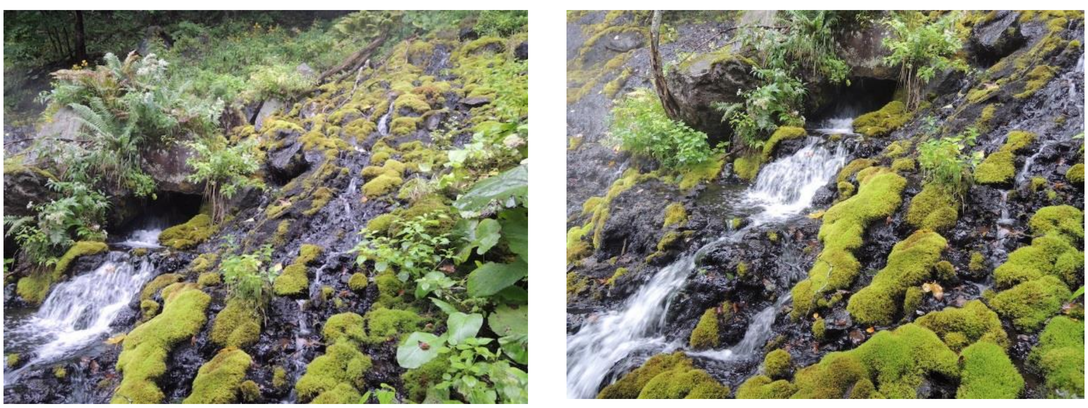


Figure 5-1: Photos taken of Onneto Yuno-taki falls at time of sampling, August 2016.

Mn^{2+} oxidation at this site.

Sample collection from the field

Both biofilm and water samples were collected from Yuno-taki falls on August 22, 2016 to grow algae for the establishment of a bioreactor. Biofilm samples (*n*=6) were collected in sterile 50 mL falcon tubes. Three samples were immediately frozen in dry-ice chilled ethanol for culture-independent analyses, while the other three samples were transported on ice for culture-dependent analyses. Hot spring water (1 L) was collected in a sterile water bottle and transported on ice. Dry ice-frozen biofilm samples were stored in a -80°C freezer immediately after arrival at the laboratory in the University of Minnesota for microbial community analyses.

Batch bioreactor

One of the unfrozen biofilm samples (ca. 50 mL) was placed in a plastic container (9.75” x 6.75” x 1.75”) and incubated with the hot spring water at 32°C under light to promote algal growth for approximately one month. The biofilm samples were then transferred to a small batch bioreactor (9.75” x 6.75” x 1.75”) containing 600 mL medium (**Table 5-1**). The medium was designed to reflect the environmental conditions found in the waterfall (Mita et al. 1994) and was circulated via a peristaltic pump.

Table 5-1: Final concentration (mmol) of artificial medium components used to promote manganese oxidation.

Chemical	Final concentration (mmol)
$MnCl_2$	5.55×10^{-2}
$NaNO_3$	14.7
$ZnSO_4$	1.53×10^{-4}
$CuSO_4$	1.73×10^{-3}
$Co(NO_3)_2$	1.70×10^{-4}
KCl	0.818
$FeSO_4$	1.79×10^{-4}
$MgSO_4$	5.14
$CaCl_2$	0.873
$NaHCO_3$	3.88
NaF	1.68×10^{-2}
Cl_2Ni	3.41×10^{-4}
$CaSO_4$	2.00
H_3BO_3	46.0
$NaMoO_4$	1.78

Continuous-flow bioreactor

Once algae biomass reached approximately 250 mL in the batch bioreactor, it was transferred to a continuous flow bioreactor containing four compartments (**Figure 5-2**) that mimic the passive and cascading flow of the waterfall. Compartment A (2.1 L) was

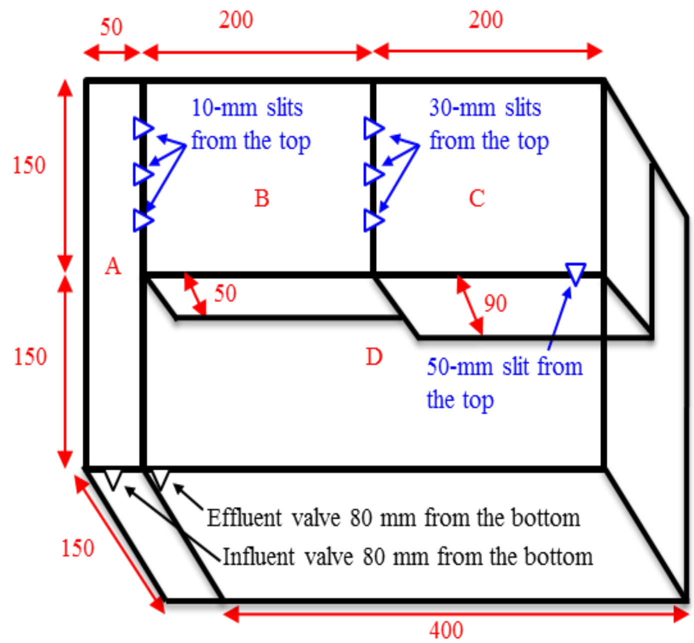


Figure 5-2: Diagram of the continuous flow bioreactor with compartments A, B, C and D. Medium enters via the influent valve in compartment A and flows through compartments B, C and D. The effluent valve is located in compartment D.

used to warm the influent water up to 32°C. Approximately 50 mL of biomass was designated for compartment B (0.6 L) and 100 mL of biomass for compartment C (1.2 L). Bubblers were inserted into compartments B and C to aerate and circulate the water throughout the compartments and promote manganese oxidation. Compartment D (2.4 L) received the rest of the biomass (~100 mL). This compartment is expected to be used in the future for the anoxic remediation of sulfate. The flow rate of the reactor was adjusted to calculate the appropriate hydraulic retention time for complete manganese oxidation in compartments B and C.

Manganese concentration measurements

Concentrations of Mn^{2+} and total Mn were measured using the formaldoxime method (Brewer & Spencer 1971), while $Mn^{3/4+}$ concentrations were measured using the leucoberbelin blue (LBB) method (Krumbein & Altman 1973).

Isolation and identification of manganese-oxidizing microorganisms

One gram fresh biofilm sample was vortexed with phosphate buffered saline (PBS, pH 7.4). The biofilm suspension was then plated onto five types of agar media, each amended post-autoclaving with 200 µM $MnCl_2$ except BG-11: K (Templeton et al. 2005); BG-11 (Rippka et al. 1979); HEPES-buffered AY (Miyata et al 2004); COMBO (Kilham et al. 1998); and WC (Guillard 1975). The K, BG-11, HEPES-buffered AY, COMBO, and WC media were designed for the growth of bacteria, cyanobacteria, fungi and algae, respectively. Plates were incubated at 30ºC for 2 weeks.

Manganese oxidizers were identified by the production of black/brown precipitates. These colonies were continually re-streaked onto fresh media until well-isolated colonies were obtained. Each isolate was tested for manganese oxidation using the LBB colorimetric assay as described above. LBB reacts with $Mn^{3/4+}$ and turns blue, so a positive reaction confirmed manganese oxidation.

Genomic DNA was extracted from each colony by heating cells in 100 µl 0.05 M NaOH at 95°C for 15 min (Ashida et al., 2010). After centrifugation at 13,000 *g* for 5 min, the supernatant was diluted ten-fold in MilliQ water and used for PCR to amplify the bacterial 16S rRNA gene and the fungal internal transcribed spacer (ITS) region between 18S and 28S rRNA genes. The reaction mixture (50 µl) for bacterial identification contained 1× Ex Taq buffer (Takara Bio, Otsu, Japan), 0.2 µM of each primer (m-27F and m-1492R; (Tyson et al., 2004)), 0.2 mM of each dNTP, 1 U of Ex Taq DNA polymerase (Takara Bio), and 2 µl of DNA template. PCR was carried out using a Veriti Thermal Cycler (Life Technologies) and the following conditions: initial annealing at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 90 s, and one cycle of 72°C for 7 min. Amplification was confirmed using gel electrophoresis.

The reaction mixture (50 µl) for fungal ITS amplification differed slightly, and contained 1× Ex Taq buffer (Takara Bio, Otsu, Japan), 1 µM of each primer (ITSF-1, ITS4 (White et al. 1990; Gardes and Bruns 1993), 0.1 mM of each dNTP, 10 U of Ex Taq DNA polymerase (Takara Bio), and 2 µl of DNA template. The Veriti Thermal Cycler was set to the following conditions: initial annealing at 95°C for 3 min, followed by 30 cycles of 92°C for 30 s, 55°C for 30 s and 72°C for 1 min, and one cycle of 72°C for 10 min.

Once amplification was confirmed, PCR products were purified using AccuPrep PCR Purification Kit (Bioneer) and then quantitated using PicoGreen dsDNA quantitation assay. The purified PCR products were bidirectionally sequenced using the Sanger method at the University of Minnesota Genomics Center. The forward and reverse reads were aligned using the phred, phrap, consed software (Ewing et al. 1998) and strain identity was determined by using Naïve Bayesian classifier (Wang et al. 2007).

Culture-independent microbial community analysis

MiSeq 16S rRNA gene sequencing analysis was used to compare microbial communities in the original waterfall samples and those in the bioreactor, and to determine whether the manganese-oxidizing community, based on the culture-dependent results, increased in abundance under our lab conditions. Dry ice-frozen biofilm samples collected from the waterfall (*n*=3) and biofilm samples collected from compartments B, C and D in the continuous flow bioreactor after a six month incubation were used in this analysis. These samples were kept at -80°C until used.

Genomic DNA was extracted from the biofilm samples by using the Fast DNA SPIN Kit for Soil (MP Biomedicals). In addition to DNA, RNA was also extracted from the waterfall samples by using the FastRNA Pro Soil-Direct Kit (MP Biomedicals), to determine whether there was a difference between the total community and active community. The RNA was purified with the TURBO DNA-free Kit (Ambion, Austin, TX) and then subjected to PCR targeting the 16S rRNA gene to ensure no DNA contamination. Complementary DNA (cDNA) was generated using the PrimeScript RT Reagent Kit (Takara Bio, Mountain View, CA) according to the manufacturer's instructions.

The V4 region of the 16S rRNA gene and 16S rRNA was amplified with the 515F-806R primer set for all DNA and cDNA samples, respectively, as described in Caporaso et al. 2012. Paired-end sequencing using Version 3 (300bp read length) of the Illumina MiSeq platform was carried out by the University of Minnesota Genomics Center.

The paired-end raw reads were assembled, quality-filtered and trimmed using NINJA-SH7 (Al-Ghalith et al. 2017) and the resulting multi-fasta files were run through the NINJA-OPS pipeline which clusters assembled sequences into operational taxonomic units (OTUs) at 97% sequence similarity, relying on the Burrows-Wheeler alignment and BowTie2 (Al-Ghalith et al. 2016). Taxonomic assignment was based on the Greengenes 97 reference data set (McDonald et al. 2011). The resulting OTU tables in BIOM 1.0 format were further analyzed using QIIME 1.9.1 and the PAST software was used to perform one-way ANOVA tests to analyze statistical differences between samples, with a p-value of ≤ 0.05 used to indicate statistically significant differences (Hammer et al. 2001).

RESULTS & DISCUSSION

Manganese oxidation in the bioreactor

The 600 ml batch bioreactor was consistently able to remove almost all dissolved manganese within four hours (**Figure 5-3**). For the continuous flow bioreactor with a flow rate of approximately 7.90 mL/minute, it was found that nearly all of the dissolved manganese was oxidized within four hours in compartment B and six hours in compartment C. This corresponded to a hydraulic retention time of 76 minutes in compartment B and 152 minutes in compartment C, totaling 3.80 hours for all manganese oxidation to occur, 57% of which was believed to occur in compartment B. After these parameters were adjusted, manganese oxidation was tested weekly and the results showed that all dissolved manganese was consistently oxidized within compartments B and C (**Figure 5-4**).

Identification of the Mn-oxidizing microorganisms

A total of 90 black/brown precipitate-forming microorganisms were isolated using AY, BG-11, WC and COMBO agar media, 68 of which were confirmed as manganese-oxidizing microorganisms via the LBB colorimetric assay described above (**Table 5-2**).

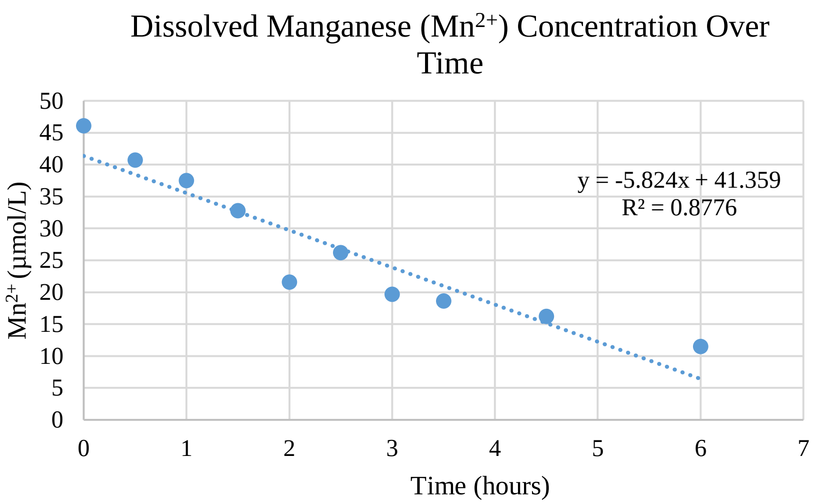


Figure 5-3: Dissolved manganese concentration in the batch bioreactor measured over time.

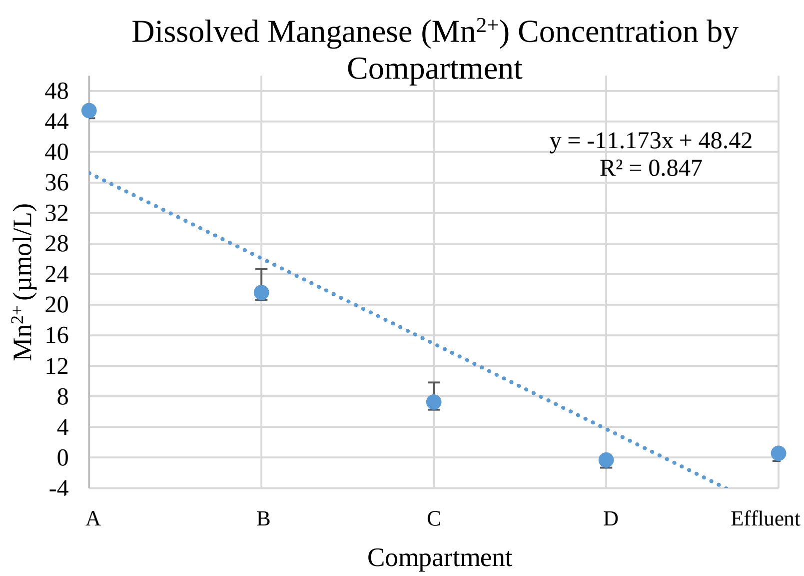


Figure 5-4: Dissolved manganese concentration in the continuous flow bioreactor measured across each compartment.

Of the isolated manganese-oxidizers, 64 were identified as bacteria, belonging mostly to the genera *Bosea* and *Pseudomonas* (**Figure 5-5**), and four were fungi, belonging to the genera *Plectosphaerella*, *Aspergillus* and *Phoma*. Some of the bacterial isolates belong to genera known to harbor common manganese-oxidizers including *Bosea* (Furuta et al. 2015; Biwako et al. 2016; Marcus et al. 2017), *Pseudomonas* (Francis & Tebo 2001; Santelli et al. 2010a; Carmichael et al. 2013; Santelli et al. 2014), and *Flavobacterium* (Santelli et al. 2010a; Carmichael et al. 2013; Santelli et al. 2014). *Aeromonas* (*Gammaproteobacteria*), *Skermanella* (*Alphaproteobacteria*), *Ensifer* (*Alphaproteobacteria*) and *Ochrobactrum* (*Alphaproteobacteria*) have not previously been confirmed as manganese-oxidizing bacteria, although there are a growing number of recognized manganese-oxidizing *Alphaproteobacteria* (Dick et al. 2008; Santelli et al. 2010a). All but four of the isolated manganese-oxidizing bacteria found in this study belong to the taxa *Alphaproteobacteria* and *Gammaproteobacteria*. The remaining

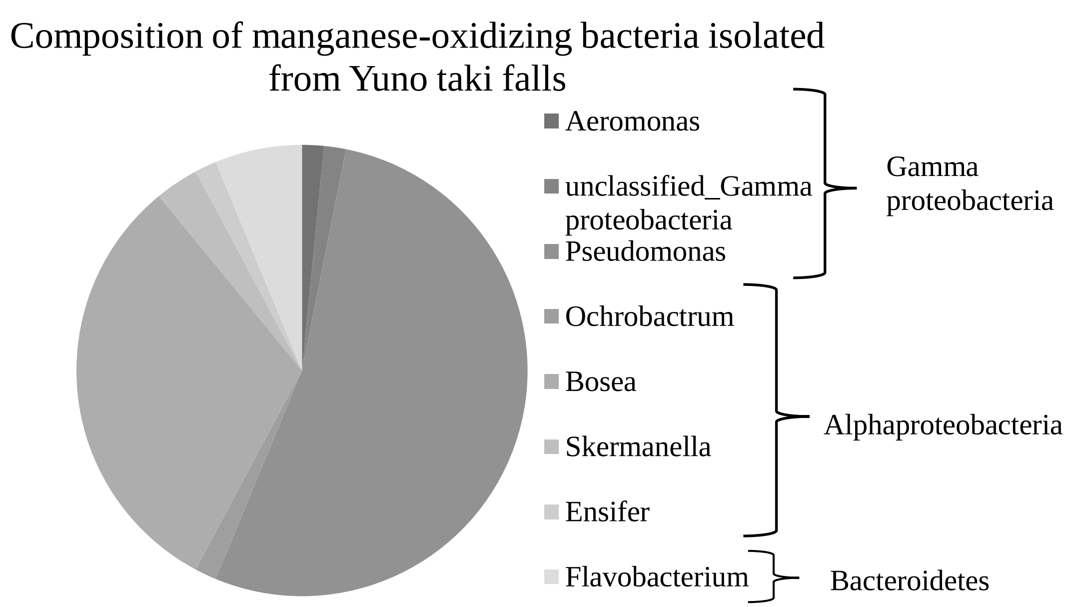


Figure 5-5: Composition of the manganese-oxidizing bacteria isolated from Yuno taki falls at the genus level.

four isolated manganese-oxidizing bacteria are *Flavobacterium*, belonging to the phylum *Bacteroidetes*.

Of the manganese-oxidizing fungi isolated in this study, *Plectosphaerella* and *Phoma* were previously identified manganese-oxidizers (Santelli et al. 2010ab; Santelli et al. 2011). *Aspergillus* has not previously demonstrated manganese-oxidation, however this genus has been known to solubilize manganese oxides (Zhan et al. 2012; Mohanty et al. 2017b). All four of the isolated fungi in this study belonged to the phylum *Ascomycota*, which harbors common manganese oxidizing fungi. In fact, only fungi belonging to *Ascomycota* and *Basidiomycota* have been found to oxidize manganese so far and many isolation studies are dominated by *Ascomycota* (Miyata et al. 2006; Santelli et al. 2010ab; Santelli et al. 2011; Santelli et al. 2014).

Table 5-2: Total isolated manganese-oxidizing microorganisms from Yuno-taki falls.

Isolate ID	Agar used	Bacteria	Fungi	Identity
A1	AY	x		*Pseudomonas*
A5	AY	x		*Pseudomonas*
A7	AY	x		*Pseudomonas*
A9	AY		x	*Plectosphaerella*
A10.2	AY	x		*Pseudomonas*
A10.1	AY	x		*Pseudomonas*
A11	AY	x		*Pseudomonas*
A12	AY	x		*Pseudomonas*
A13.2	AY			*Pseudomonas*
A13.1	AY	x		*Pseudomonas*
A14	AY	x		*Pseudomonas*
A16	AY	x		*Pseudomonas*
A17	AY	x		*Ochrobactrum*
A18	AY	x		*Pseudomonas*
A19	AY	x		*Pseudomonas*
A20	AY	x		*Pseudomonas*
A22	AY		x	*Aspergillus*
A23	AY	x		*Pseudomonas*
A24	AY	x		*Pseudomonas*
A24.2	AY	x		*Pseudomonas*

A25	AY	x		*Pseudomonas*
B1	BG-11	x		*Pseudomonas*
B2	BG-11	x		*Pseudomonas*
B4	BG-11	x		*Pseudomonas*
B5	BG-11	x		*Pseudomonas*
B6	BG-11	x		*Bosea*
B7	BG-11	x		*Skermanella*
B9	BG-11	x		*Pseudomonas*
B10	BG-11	x		*Bosea*
B11	BG-11	x		*Bosea*
B14	BG-11	x		*Pseudomonas*
B17	BG-11	x		*Pseudomonas*
B18	BG-11	x		*Aeromonas*
B19	BG-11	x		*Pseudomonas*
B20	BG-11	x		*Pseudomonas*
B21	BG-11	x		*Bosea*
B23	BG-11	x		*Bosea*
B27	BG-11	x		*Skermanella*
B29	BG-11	x		*Pseudomonas*
B30	BG-11	x		*Pseudomonas*
B33.2	BG-11		x	*Phoma*
B34	BG-11	x		*Flavobacterium*
B34.2	BG-11	x		*Flavobacterium*
B35	BG-11	x		*Pseudomonas*
B36	BG-11	x		*Pseudomonas*
C1.2	COMBO	x		*Pseudomonas*
C8.2.1	COMBO	x		*unclassified_Gamma proteobacteria*
C9	COMBO		x	*Plectosphaerella*
W1	WC	x		*Bosea*
W3	WC	x		*Bosea*
W4	WC	x		*Bosea*
W5	WC	x		*Bosea*
W7	WC	x		*Bosea*
W9	WC	x		*Ensifer*
W10	WC	x		*Flavobacterium*
W11	WC	x		*Bosea*
W12	WC	x		*Bosea*
W13	WC	x		*Bosea*
W15	WC	x		*Pseudomonas*
W16	WC	x		*Bosea*
W17	WC	x		*Bosea*
W18	WC	x		*Flavobacterium*

W19	WC	x		*Bosea*
W20	WC	x		*Bosea*
W21	WC	x		*Bosea*
W22	WC	x		*Bosea*
W23	WC	x		*Bosea*
W24	WC	x		*Flavobacterium*

Microbial communities in the Mn oxidizing biofilms in waterfall and bioreactor samples

Principal coordinates analysis (PCoA) performed using QIIME 1.9.1 showed that the DNA samples from the waterfall clustered separately from the cDNA waterfall samples, indicating that the active waterfall community is a distinct subset of the overall community (**Figure 5-5a**). Between the waterfall and the bioreactor samples, distinct clustering was also found (**Figure 5-5c**) confirming a shift in the microbial community pre- and post-bioreactor incubation, however no clustering occurred between each of the bioreactor compartments (**Figure 5-5b**). It was expected that compartment D, where no manganese-oxidation had been occurring, would have a distinct microbial community from compartments B and C, however this was not the case. As mentioned above, compartments B and C will eventually be coupled with anoxic sulfate reduction in compartment D, however compartment D had not yet been enriched for sulfate-reducing bacteria. This was confirmed by the absence of any sulfate reduction occurring (**SI Figure S5-1**). It is likely that at the time of sampling, compartment D was not anoxic enough for sulfate reduction to occur due to lack of biomass providing anoxic microenvironments and combined light cycles with compartments B and C to promote algal growth and photosynthesis. Therefore, no significant shift in community structure was observed.

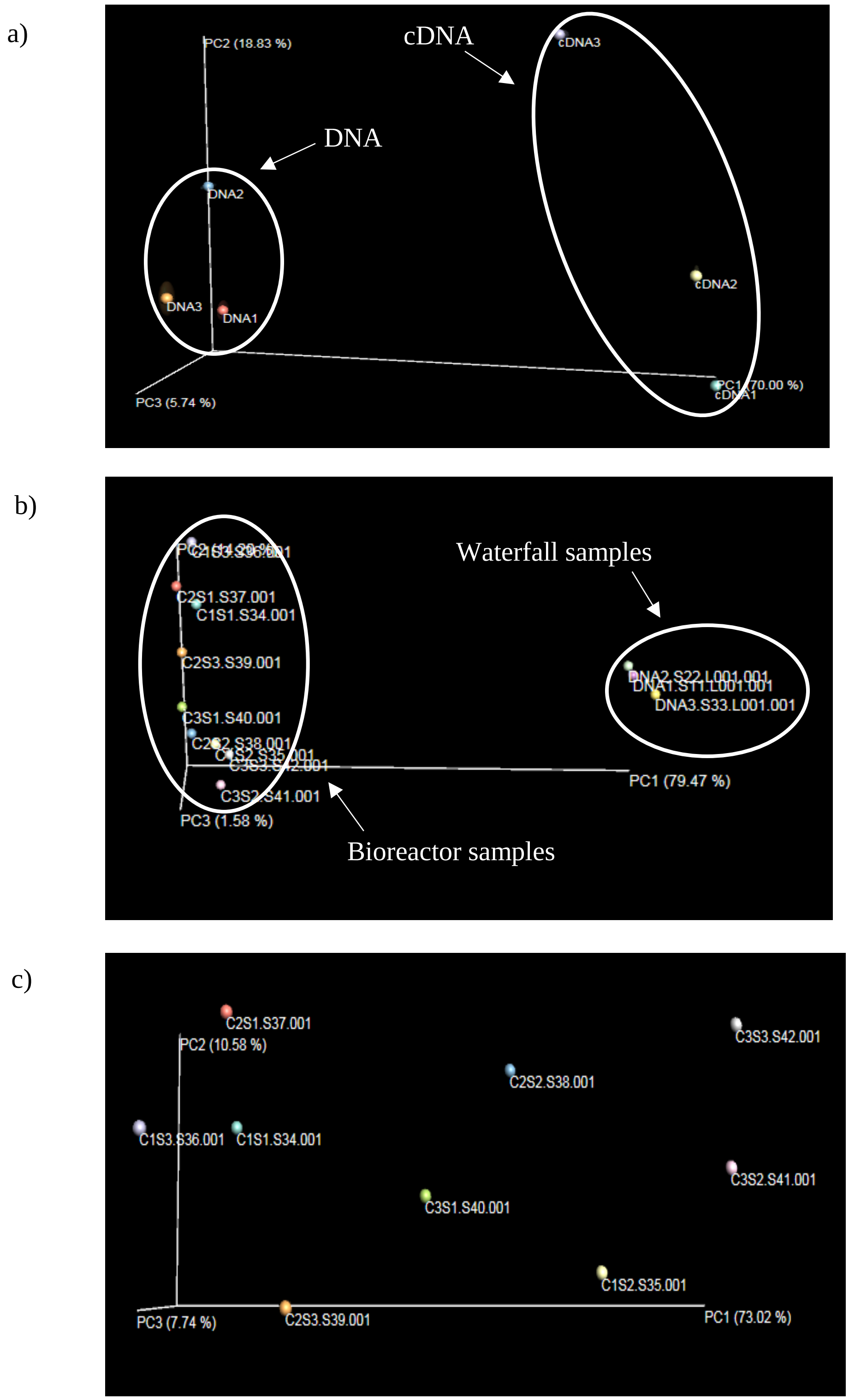


Figure 5-6: Principal coordinates analysis (PCoA) analysis plots based on weighted UniFrac distance measurements comparing: a) DNA and cDNA in the Yuno-taki waterfall samples; b) DNA samples from compartments B, C and D in the continuous flow bioreactor; and c) all bioreactor DNA samples and Yuno-taki waterfall DNA samples.

Table 5-3: Species richness of bacterial 16S rRNA shown as mean ± SD.

Source	Sample	chao1	Observed OTUs	Inverse Simpson	Shannon
Waterfall	3 DNA samples	2090.10 ± 391.93	1565.50 ± 122.33	18.47 ± 3.16	6.16 ± 0.11
Bioreactor	Compartment B DNA	585.56 ± 201.14	436.00 ± 141.42	20.95 ± 2.26	5.53 ± 0.22
	Compartment C DNA	639.13 ± 76.90	429.33 ± 39.55	15.35 ± 3.33	4.99 ± 0.38
	Compartment D DNA	571.71 ± 11.39	380.00 ± 44.24	11.76 ± 1.88	4.67 ± 0.07

Compared to the bioreactor samples, the Yuno-taki waterfall samples had significantly higher Shannon and chao1 indices compared to all three bioreactor compartments (P-values ≤ 0.05) and total OTUs and all other diversity indices generally decreased across the three bioreactor compartments (**Table 5-3**). Species richness is based on the number of phylotypes and all phylotypes are weighted equally, so uncertainty and rare phylotypes present in the waterfall samples could influence the results (Hughes et al. 2001).The Yuno-taki waterfall samples contained a significantly greater number of phylotypes, but this may be a result of the bioreactor being enriched specifically for manganese oxidation. The Shannon index takes into account the relative abundance of phylotypes and measures the uncertainty in the species identity of a sample (Jost 2006; Bent and Forney 2008). This was still significantly higher for the waterfall samples than each of the bioreactor compartments, but less so, with a P-value of 0.01 for Shannon compared to a P-value of 0.003 for chao1. Inverse Simpson indices were significantly different only between compartments B and D and the waterfall and compartment D. The inverse Simpson index also takes into account the relative abundance of phylotypes and is less affected by rare phylotypes (Bent and Forney 2008). That compartment B had a greater inverse Simpson index than the waterfall samples, despite having significantly lower species richness, could be attributed to differences in the frequencies of more abundant phylotypes (Lande et al. 2000).

Within the waterfall samples, *Proteobacteria* and *Cyanobacteria* were the dominant taxa in the cDNA and DNA samples, respectively, followed by *Nitrospirae* and *Acidobacteria* in both cDNA and DNA (**Figure 5-6**). The majority of the sequences classified as "*Cyanobacteria*" were chloroplasts from *Chlorophyta*, *Stramenopiles* and *Streptophyta* (lower taxa unknown), indicating that these likely originated from green algae, which are believed to be the primary producers in this environment. *Cyanobacteria* have been found at other manganese-rich sites and are known to be capable of superoxide production, a proposed mechanism for manganese oxidation (Kustka et al. 2005; Marshall et al. 2005; Learman et al. 2011; Santelli et al. 2014). *Proteobacteria,* including *Bradyrhizobiaceae* (genus unknown), *Burkholderia*, and *Nevskia* were significantly more represented in the cDNA samples, as were *Anaerolineae, Chloracidobacteria*, and *Gaiellaceae*, implying that these taxa are more active at Yuno-taki falls. Both *Anaerolineae*, belonging to the phylum *Chloroflexi*, and *Chloracidobacteria*, an *Acidobacteria*, may be capable of anoxygenic photosynthesis and *Chloracidobacteria* is the only *Acidobacteria* containing chlorosomes (Bryant et al. 2007; Klatt et al. 2011). Additionally, some *Burkholderia* strains are known to harbor *moxA*, a gene proposed to be involved in manganese oxidation (Ridge et al. 2007; Dick et al. 2008) and several members of *Burkholderiales* are known to oxidize manganese (Marcus et al. 2017). Conversely, *Stramenopiles, Streptophyta, Acidimicrobiales* (lower taxa unknown) and *Pedomicrobium* were significantly more abundant in the DNA samples (P-values $\leq$0.05).

The bioreactor samples were also dominated by *Proteobacteria* followed by *Bacteroidetes* (**Figure 5-7**). Within the bioreactor, there were some noticeable differences between compartments. The relative abundance of *Saprospiraceae* (genus unknown) was significantly higher in compartment D than compartment B, where it made up to 27% of the overall community, compared to up to 6.10% of the total community in compartment B. *Saprospiraceae* is known to harbor genera capable of degrading complex polysaccharides and is commonly isolated from activated sludge, but has not been associated with manganese oxidation nor sulfate reduction (Xia et al. 2008; Feng et al. 2015). *Dechloromonas*, a *Proteobacteria*, was significantly more abundant in compartment B compared to compartment D. *Dechloromonas* can degrade chlorobenzoate, aromatic hydrocarbons and perchlorate (Salinero et al. 2009).

Interestingly, the microbial community analyses of both the waterfall samples and bioreactor samples showed very low abundance of the isolated manganese-oxidizing bacteria from this study at the genus or family level (**Table 5-4**). This indicates that either a small proportion of the overall community is carrying out manganese-oxidation, or that culture bias was present and we were unable to isolate many manganese-oxidizing bacteria through the methods used. Previous studies have also highlighted the difficulty in isolating manganese-oxidizing microorganisms through culture-based approaches (Santelli et al. 2014) and it is well-known that culturing methods are limited to only a fraction of the overall community (Curtis et al. 2002).

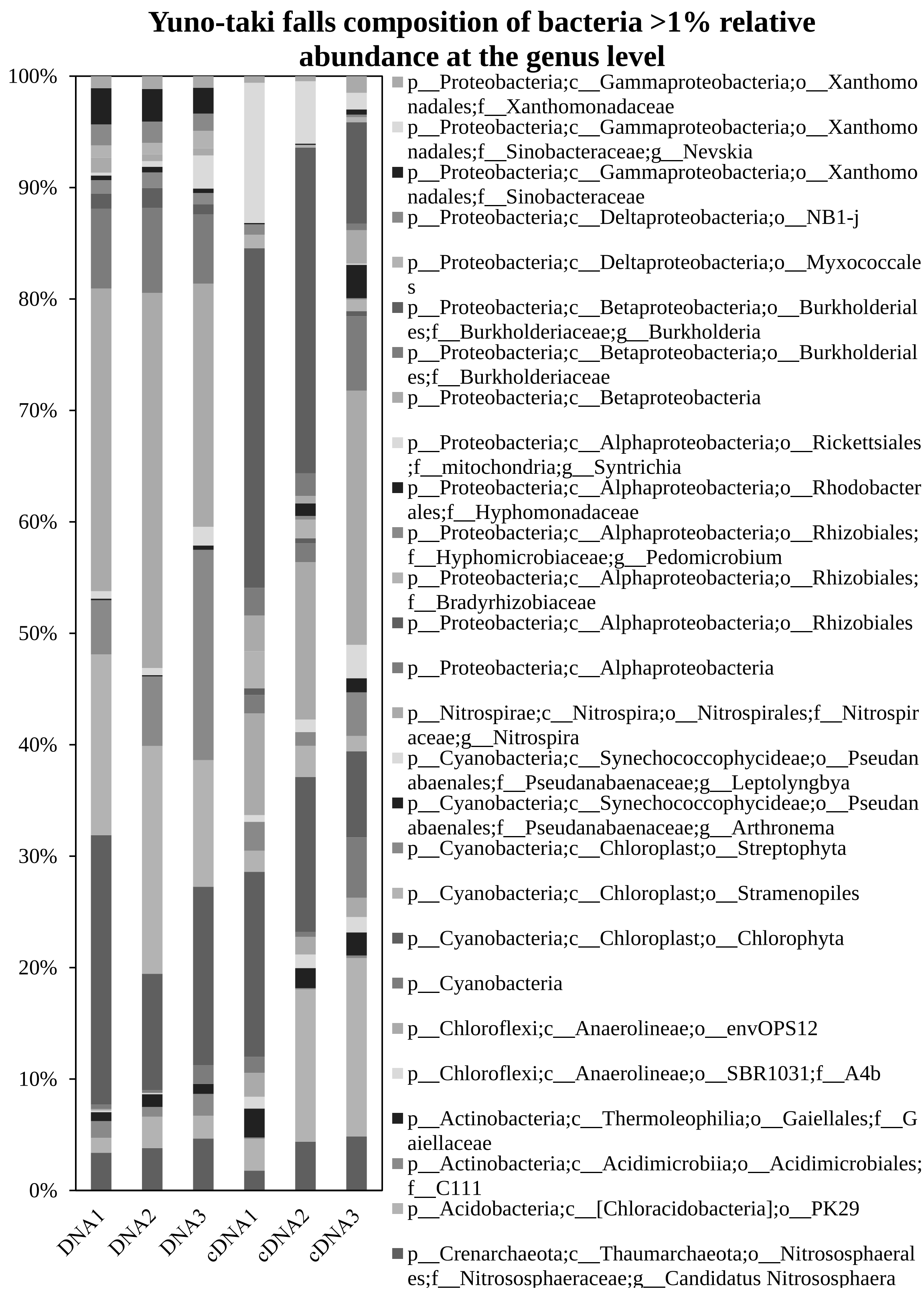


Figure 5-7: Relative abundance of bacteria (>1%) of the waterfall DNA and cDNA samples at the genus level.

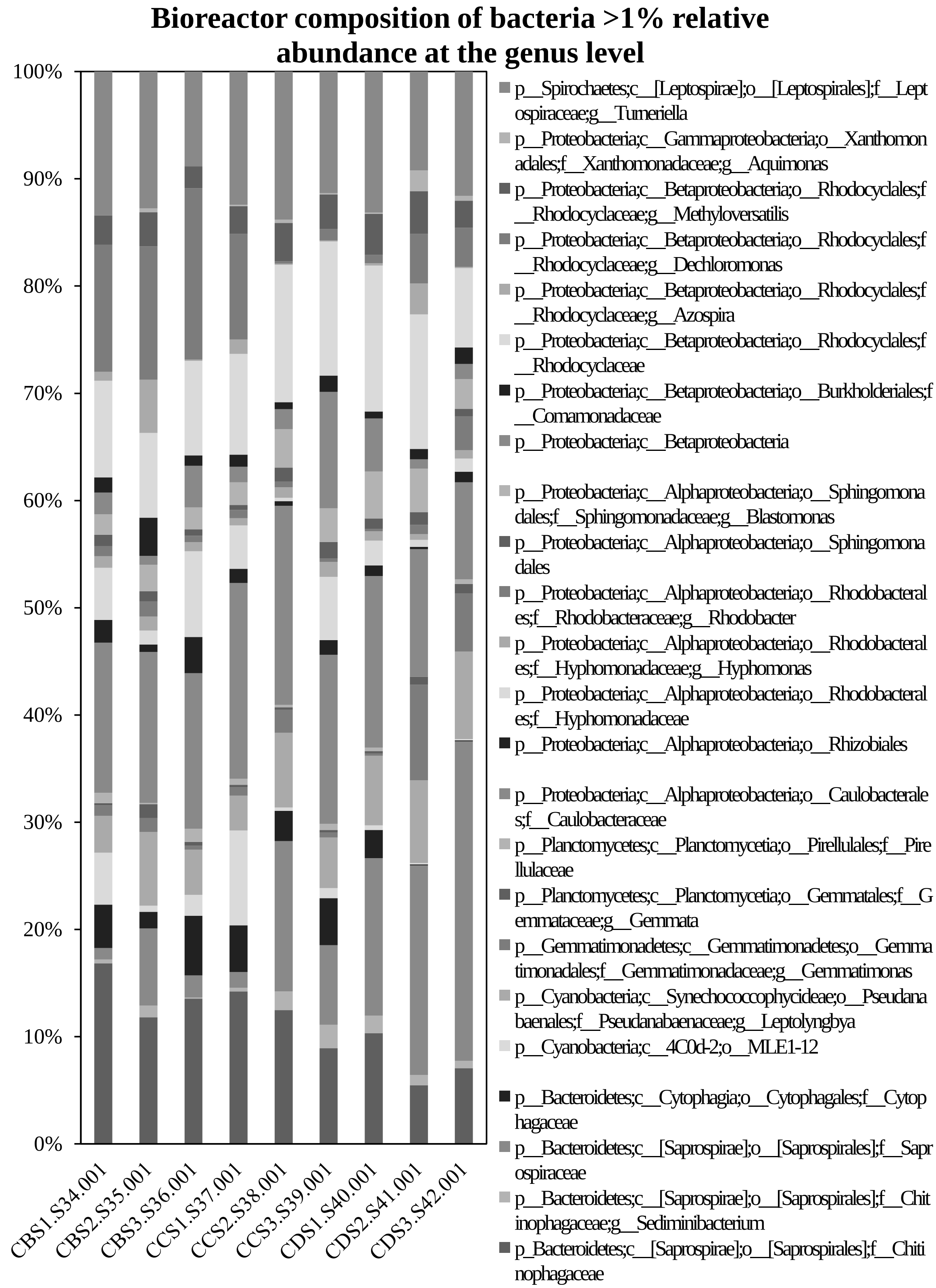


Figure 5-8: Relative abundance of bacteria (>1%) in the bioreactor chambers B, C and D (CC, CB, CD) DNA samples at the genus level.

Table 5-4: Percent composition of bacterial families corresponding to the isolated manganese-oxidizing genera present in the microbial communities, shown in mean ± SD.

Family	Waterfall samples		Bioreactor DNA samples		
	DNA	cDNA	B	C	D
Pseudomonadaceae (Pseudomonas)	0.07 ± 0.06%	0.07 ± 0.06%	0%	0%	0%
Bradyrhizobiaceae (Bosea, Ochrobactrum)	0%	0.2 ± 0.1%	0.03 ± 0.06%	0.03 ± 0.06%	0.10%
Flavobacteriaceae (Flavobacterium)	0%	0%	0%	0%	0%
Rhodospirillaceae (Skermanella)	0.07 ± 0.06%	0.07 ± 0.06%	0%	0%	0.07 ± 0.11%
Aeromonadaceae (Aeromonas)	0%	0%	0%	0%	0%
Rhizobiaceae (Ensifer)	0%	0.07 ± 0.06%	0.03 ± 0.06%	0%	0%

At higher taxa, our results are consistent with other studies focusing on microbial communities in manganese-rich sites. For example, the dominant phyla found in this study (ie. *Acidobacteria*, *Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria* and *Proteobacteria*) have all been commonly found at other manganese-containing sites (Santelli et al. 2010a Pereira et al. 2014; Santelli et al. 2014; Chaput et al. 2015; Bohu et al. 2016). *Bacteroidetes* made up an average of 24% of the bioreactor total communities and this is the phylum to which *Flavobacterium* belongs, a well-known manganese oxidizer. Since we isolated several Mn-oxidizing *Flavobacterium* sp. strains, the high proportion of *Bacteroidetes* may represent a changing or growing manganese-oxidizing community. *Actinobacteria* and *Acidobacteria* were significantly more abundant in the manganese-oxidizing compartment B than in the non-oxidizing compartment D. While we did not isolate manganese-oxidizing *Actinobacteria*, such strains have previously been isolated (Carmichael et al. 2013). In addition, *Acidobacteria* are a common soil bacterium found in acid mine drainage and contaminated sites containing high manganese concentrations, and therefore likely play a role in metal and manganese cycling (Reis et al. 2013; Pereira et al. 2014; Chaput et al. 2015). Additionally, *Gemmatimonadetes*, which has previously been found in mining environments both contaminated with and uncomtaminated with acid mine drainage, was detected in the bioreactor samples but not the waterfall samples (Pereira et al. 2014). This taxon is often found in manganese-rich sites and may also be involved in metal cycling, although no manganese oxidizing members have been isolated (Pereira et al. 2014; Chaput et al. 2015; Bohu et al. 2016).

CONCLUSION

A bioreactor was successfully established in this study based off of a natural manganese-oxidizing waterfall containing elevated levels of manganese and was shown to consistently oxidize all dissolved manganese. Sixty-eight manganese-oxidizing microorganisms were isolated, several of which were not previously known manganese-oxidizers including *Aeromonas*, *Skermanella*, *Ensifer*, *Ochrobactrum* and the fungus *Aspergillus*. Microbial community analyses of both the waterfall and the bioreactor demonstrated a shift in the microbial community, although the isolated manganese-oxidizers made up a small proportion of the overall communities from both samples. Further research is needed to analyze the fungal communities from both sites.

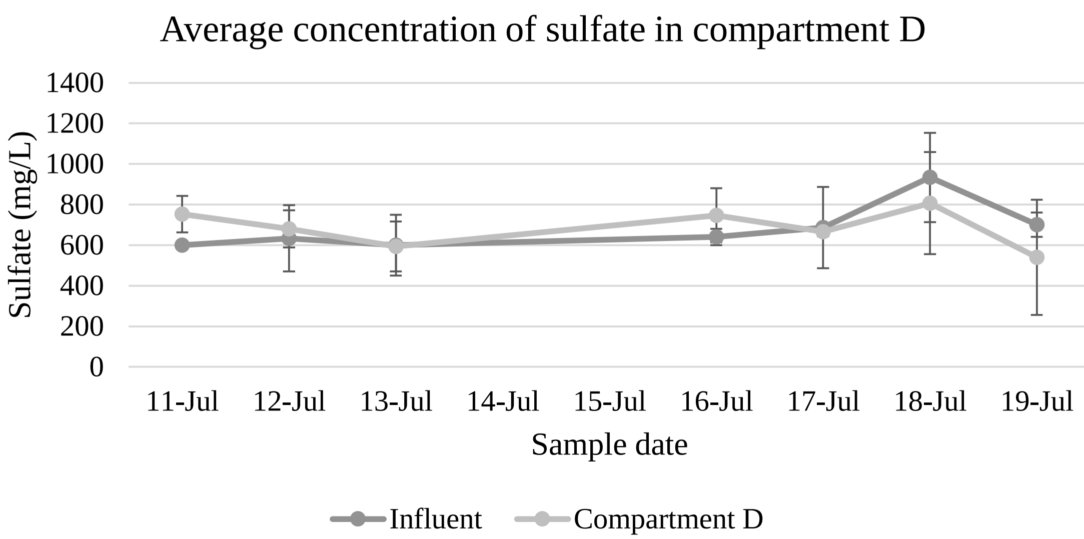
More focus is also needed on coupling manganese oxidation with sulfate reduction in compartment D of this bioreactor to target remediation efforts in Minnesota, particularly southwestern Minnesota where sulfate co-occurs with manganese at concentrations exceeding 1,300 mg/L sulfate and 1,000 µg/L manganese (Kroening and

Ferrey 2013). While this exceeds the secondary drinking water standard of 250 mg/L for sulfate, the primary concern in Minnesota is its toxicity to wild rice and the threat to this economically and culturally important natural resource. The next steps will involve enriching compartment D for anoxic sulfate reduction.

Additionally, this research uses synthetic water. Future research will include operating the bioreactor with actual ground water sampled from southwestern Minnesota where high manganese and sulfate concentrations are present in order to assess the applicability of using this bioreactor for water treatment in Minnesota.

SUPPORTING INFORMATION

Figure S5-1: The concentration of sulfate in the influent and in compartment D of the manganese-oxidizing bioreactor, sampled daily. The results do not show any consistent sulfate reduction.



6. CONCLUSION

6-1 Bioremediation of nitrogen

The preceding four chapters have described unique solutions to environmental and human health issues which utilize microorganisms for safe and effective remediation. A common component of bioremediation involves bioaugmentation, biostimulation or a combination of the two. Chapters 2 through 4 addressed both of these strategies in regards to denitrifying woodchip bioreactors. As described in Chapter 3, biofilm accumulation resulting in bioreactor clogging is a common problem, especially when biostimulation is employed. These biofilms are likely not composed of the targeted denitrifying microorganisms, but non-denitrifying microorganisms performing dissimilatory nitrate reduction to ammonium (DNRA). A high C/N ratio has been shown to select for these non-denitrifiers, and so any biostimulation efforts should take into account this potential outcome by modifying the injection flow rate and the C/N ratio. An alternative to injecting a more easily available carbon source is combining biostimulation with bioaugmentation by inoculating the bioreactor with a cellulose- or complex polysaccharide-degrading microorganism. In this way, carbon is released from the woodchips within the bioreactor, avoiding biofilm accumulation at the convergence of the carbon injection and the bioreactor inlet, where clogging often occurs. *Cellulomonas* sp. strain WB94 contained genes related to cellulose and hemicellulose degradation and laboratory tests indicated that it can degrade cellulose under aerobic and anaerobic conditions. Additionally, strain WB94 played a role in denitrification, as seen by an increased relative abundance in the microcosms in Chapter 2, a relatively high denitrification rate in Chapter 3 and the presence of nitrate and nitrite reductase genes in

Chapter 4. Based on this study, *Cellulomonas* sp. strain WB94 was a good candidate for bioaugmentation and biostimulation.

Bioaugmentation using a low temperature-adapted denitrifying microorganism should also be employed. Chapters 2 through 4 described the many methods used to select a strain for bioaugmentation, including the percent nitrate-N reduced, the percent nitrate-N converted to ammonium and nitrite, and the production of gaseous end products, nitrous oxide via the acetylene inhibition method, and dinitrogen gas using ^{15}N-labelled nitrate, under low temperatures. The selection process highlighted problems with common methods used to measure and confirm denitrification. For example, the acetylene inhibition method did not discriminate between bacteria capable of nitrous oxide reduction and those that weren't. Additionally, detecting denitrification functional genes such as *nirK* and *nirS* through PCR-based approaches does not imply complete denitrification and not detecting these genes similarly does not imply that the microorganism is a non-denitrifier due to the biases inherent in choosing a primer. Whole genome sequencing revealed that only *Microvirgula* sp. strain BE2.4 contained a full set of denitrification genes. Strain BE2.4 was also able to maintain a high denitrification rate both aerobically and anaerobically, as depicted in Chapter 3. Therefore, both *Microvirgula* sp. strain BE2.4 and *Cellulomonas* sp. strain WB94 were selected for field bioaugmentation studies. These two strains were inoculated into the Willmar woodchip bioreactor repeatedly over two week intervals from May to June 2018. Data collection is ongoing but it is expected that the combination of these two strains will enhance nitrate removal and complete denitrification at the Willmar woodchip bioreactor.

6-2 Manganese-oxidation in the algae bioreactor

Chapter 5 explored the ex situ bioremediation of manganese-contaminated groundwater that used a bioreactor containing a microbial consortium collected from a natural manganese oxidizing waterfall. The heterotrophic manganese oxidizers utilized the fixed carbon provided by the algae as a carbon substrate and successfully removed all of the manganese from solution. From the original waterfall samples, 68 manganese-oxidizing bacteria and fungi were isolated and belonged to both genera known to oxidize manganese such as *Bosea*, *Pseudomonas*, *Flavobacterium*, *Plectospharaella*, and *Phoma*, and those not previously known to oxidize manganese including *Aeromonas*, *Skermanella*, *Ensifer*, *Ochrobactrum* and *Aspergillus*. While these bacteria were undetected at the genus level in both the waterfall and the bioreactor microbial community analyses, it may be that many microorganisms capable of manganese oxidation were not isolated. The number of known manganese-oxidizers is continually growing and it is becoming apparent that manganese oxidation is more widespread than previously believed.

The next steps for the bioreactor will involve pairing manganese oxidation with sulfate reduction and then applying this bioreactor to local sites in Minnesota. Although sulfate was only mentioned briefly in Chapter 5, it is a growing concern in Minnesota due to its toxicity to wild rice, an important economic and cultural resource to the state, and there is interest in creating legislation for monitoring sulfate. Sulfate co-occurs with manganese, particularly in southwestern Minnesota, so after enriching compartment D for sulfate reduction, water contaminated with both sulfate and manganese in Minnesota will be treated using this novel algae bioreactor. This will test whether the microorganisms, which have been indigenous to the water used so far in the bioreactor, will be able to adapt and continue removing manganese in contaminated water to which they are not native. Manganese-oxidizing bacteria and fungi that were isolated from the original samples could be used to inoculate the bioreactor in the future if necessary, using the bioaugmentation technique.

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