Isolation and Characterization of Denitrifiers from Woodchip Bioreactors for Bioaugmentation Application

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Denitrifiers from Woodchip Bioreactors

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ABSTRACT

Aims: This study was done to obtain denitrifiers that could be used for bioaugmentation in woodchip bioreactors to remove nitrate from agricultural subsurface drainage water.

Methods and Results: We isolated denitrifiers from four different bioreactors in Minnesota, and characterized the strains by measuring their denitrification rates and analyzing their whole genomes. A total of 206 bacteria were isolated from woodchips and thick biofilms (bioslimes) that formed in the bioreactors, 76 of which were able to reduce nitrate at 15°C. Among those, nine potential denitrifying strains were identified, all of which were isolated from the woodchip samples. Although many nitrate-reducing strains were isolated from the bioslime samples, none were categorized as denitrifiers but instead as carrying out dissimilatory nitrate reduction to ammonium (DNRA).

Conclusions: Among the denitrifiers confirmed by $^{15}$N stable isotope analysis and genome analysis, Cellulomonas cellacea strain WB94 and Microvirgula aerodenitrificans strain BE2.4 appear to be promising for bioreactor bioaugmentation due to their potential for both aerobic and anaerobic denitrification, and the ability of strain WB94 to degrade cellulose.

Significance and Impact of Study: Denitrifiers isolated in this study could be useful for bioaugmentation application to enhance nitrate removal in woodchip bioreactors.
INTRODUCTION

Increasing nitrate pollution from agricultural runoff has had detrimental impacts on water quality, causing eutrophication in rivers, lakes, and oceans (USEPA 2008). Many strategies have been developed and applied to reduce nitrogen pollution from agricultural environments (MPCA 2014; USEPA 2008). 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 (Addy et al. 2016; Lopez-Ponnada et al. 2017; Schipper et al. 2010). Woodchip bioreactors are designed to enhance microbial denitrification, in which nitrate is reduced to gaseous end products such as $\text{N}_2$ and $\text{N}_2\text{O}$ (Rivett et al. 2008; Seitzinger et al. 2006). The woodchips that are used as a matrix in the 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 (Christianson et al. 2012; Gibert et al. 2008). However, under cold temperatures (<10°C), bioreactor performance decreases due to inhibited microbial activity (David et al. 2016; Ghane et al. 2015; Hartz et al. 2017; Hassanpour et al. 2017; Husk et al. 2017; Schipper et al. 2010; Warneke et al. 2011). This is especially a concern in cooler regions such as northern Europe and the upper Midwest USA where large quantities of nitrate can leach from agricultural lands during periods when water temperatures are low (Jin and Sands 2003).

A potential solution to this problem is biostimulation, in which readily available carbon is supplemented to the woodchip bioreactor to enhance microbial denitrification rates under low temperatures (Roser et al. 2018). However, addition of excess carbon could cause overgrowth of bacteria and formation of thick biofilms in the bioreactor itself, or in the piping, resulting in clogging (Christianson et al. 2016; Gibert et al. 2008; 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.

Another solution to enhance the performance of woodchip bioreactors under low temperature conditions 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. This strategy requires identifying and selecting low temperature-adapted...
denitrifiers. Microorganisms selected for bioaugmentation purposes are often isolated from the contaminated site and are, therefore, native to the specific location (Gentry et al. 2004). It is believed that environment-borne microorganisms can better colonize and survive in the environment than non-native microorganisms (Bouchez et al. 2000; El Fantroussi and Agathos 2005). However, non-native microorganisms can also be successfully used when there are no local species that are able to remove contaminants rapidly or efficiently enough (Tyagi et al. 2011). In addition, it is more practical to inoculate denitrifiers that are commonly present in woodchip bioreactors at multiple locations than isolating bacteria for each location.

A limited number of microbiological studies has been done to identify denitrifiers in woodchip bioreactors. 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. By analyzing the relative abundance of bacterial and archaeal operational taxonomic units (OTUs), they 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. More recently, Jang et al. (2019a) used both culture-independent and –dependent approaches and identified *Pseudomonas* spp. (*Pseudomonadales*), *Polaromonas* spp. (*Burkholderiales*), and *Cellulomonas* spp. (*Actinomycetales*) as representing relevant denitrifiers in a woodchip bioreactor at relatively low temperature conditions (15°C). Some of these potential denitrifiers have also been identified in woodchip bioreactors treating aquaculture effluent (Ahnen et al. 2019; Kiani et al. 2020). However, no study has compared the denitrifying microorganisms among different field woodchip bioreactors. Since performance of woodchip bioreactors may in part depend on the presence of particular denitrifiers, it is important to compare denitrifier populations among different field woodchip bioreactors.

Therefore, the purpose of this study was to: 1) isolate denitrifying bacteria from four different bioreactors in continental climate regions in Minnesota, USA; 2) compare common denitrifiers among sites; 3) determine whether any denitrifiers perform better than others; and 4) and identify the strains that could be useful in enhancing field bioreactor performance under low temperature conditions through bioaugmentation.
MATERIALS AND METHODS

Sample collection

We collected samples from woodchip bioreactors located in Willmar (WB), Blue Earth (BE), Olmsted County (OC) and Lamberton (LB) in Minnesota, USA (Table S1). Bioreactor WB was established in Fall 2010 and consisted of eight distinct 1.7 m x 11.6 m x 0.9 m beds and contained a mix of softwood and hardwood woodchips. A biostimulation experiment was conducted in two of the eight beds in 2017 throughout the spring and summer months, during which acetate was injected to stimulate denitrification (Feyereisen et al. 2018). Addition of acetate, however, resulted in the formation of thick biofilms near the bioreactor inlet pipes, some of which was released as (bioslime and resulted in clogging (Fig. S1). Woodchip samples were collected from this bioreactor in October 2014 as previously described in detail (Ghane et al. 2018). Bioslime samples were collected from the water of the bioreactor inlet pipes. Bioreactor BE was sampled shortly after its establishment in 2016 and consisted of three beds measuring 7.62 m x 41 m x 1.5 m. Bioreactor OC was established in July 2016 and consisted of one bed measuring 6 m x 30.5 m x 1.3 m, which contained fine wood pieces and green cuttings. Clogging occurred near the bioreactor outlet pipe as a result of bioslime formation. The bioslime sample was collected in September 2016. Bioreactor LB was established in 2016 as a cube design composed of a series of layers of crushed limestone and concrete, corn cobs, woodchips and lava rock measuring 1 m x 0.96 m x 0.78 m. The woodchips from this bioreactor were sampled in 2017. Bioreactor LB was subjected to biostimulation in 2016 and 2017 during which acetate was injected to stimulate denitrification. Similar to the biostimulation experiments in the WB bioreactors, clogging occurred at the inlet pipe where the acetate and drainage water converged. Descriptions of each of the bioreactors are presented in Table S1. The ages of the woodchip bioreactors at the time of sampling varied from two months (OC) to four years (WB).

Woodchips were collected from submerged areas of the bioreactors and were immediately placed in a cooler. The bioslime samples from bioreactors WB and LB were collected from inlet tubing where the acetate and drainage water converged, and the bioslime samples from bioreactor OC were collected at the clogged outlet pipe. Samples were immediately placed in a cooler, brought back to the laboratory and stored at 4°C and -20°C for bacteria isolation and DNA extraction, respectively.
Isolation of denitrifiers

Denitrifying microorganisms were isolated from woodchip and biofilm samples according to a previously described method (Jang et al. 2019a). Briefly, 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 repeatedly streaked until individual colonies appeared. Denitrification potential was confirmed for all isolates using the acetylene inhibition method, which prevents the final step in denitrification from \( \text{N}_2\text{O} \) to \( \text{N}_2 \) gas (Yoshinari and Knowles 1976). In brief, fresh cell cultures (300 \( \mu \)l) were inoculated into R2A-NA broth (10 ml) in 27-ml test tubes and incubated under an Ar:C\(_2\)H\(_2\) (90:10) atmosphere (Jang et al. 2019). The amount of \( \text{N}_2\text{O} \) produced was measured using gas chromatography (GC; Model 5890, Hewlett-Packard/Agilent Technologies) equipped with an electron capture detector and PoraPak Q column (Sigma-Aldrich) (Maharjan 2013). In order to differentiate between denitrification and dissimilatory nitrate reduction to ammonium (DNRA), we also measured concentrations of nitrate, nitrite and ammonium using a SEAL AA3 HR AutoAnalyzer. Potential denitrifiers were determined based on the following criteria: ≥40% nitrate-N was reduced; <10% N was converted to ammonium; no nitrite-N was detected and >100 ppm \( \text{N}_2\text{O} \) was produced (Jang et al. 2019a). Because many of the strains produced \( \text{N}_2\text{O} \) greater than the upper quantification limit of the GC, the percentage of nitrate reduced to \( \text{N}_2\text{O} \) could not be calculated for all strains, as previously used to identify denitrifiers (Mahne and Tiedje 1995; Tago et al. 2011; Tiedje 1994).

DNA extraction, PCR, and sequencing

All nitrate-reducing microorganisms were identified by sequencing near full-length of the 16S rRNA gene as previously described (Ishii et al. 2011). First, DNA was extracted from colonies on R2A-NA agar plates by heating cells in 0.05 M NaOH at 95°C for 15 min. After centrifugation at 2,000 g for 5 min, crude DNA extracts were diluted 10-fold with water and used for PCR. The reaction mixture (50 \( \mu \)l) contained 1 \( \times \) Ex Taq buffer (Takara Bio), 0.2 \( \mu \)M of each primer (27F and 1492R), 0.2 mM dNTP, 1 U of Ex Taq DNA polymerase (Takara Bio), and 2 \( \mu \)l 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. 2007).

Denitrification rate measurement

Denitrification rates were measured using $^{15}$N-labeled nitrate and a gas chromatograph-mass spectrometer (GC-MS). In brief, denitrifying bacteria grown in R2A-NA broth under anaerobic conditions were washed in 10 mM piperazine-N, N'-bis (PIPES) buffer (pH 7.4) (Ishii et al. 2016). Washed cells were inoculated to 50 ml PIPES buffer (pH 7.4) supplemented with 10 mM acetate and 5 mM $^{15}$N-labeled nitrate (Cambridge Isotope Laboratories) in 160 ml airtight vials. After exchanging the gas phase with He gas (>99.999%), the vials were incubated at 15°C. Gas samples (10 µl) were taken from the vials at 0, 24, 48, 72, 168, and 336 h after incubation, 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$_2$. A standard curve was created by injecting different volumes of $^{30}$N$_2$ gas and comparing absorbance values to known concentrations. Results were normalized by cell density, measured as optical density at 600 nm wavelength (OD$_{600}$), and reported as pmol $^{30}$N$_2$ produced per $10^6$ cells. The denitrification rate was calculated as pmol/10$^6$ cells/hour for $^{30}$N$_2$ gas based on the slope of the trendline where $^{30}$N$_2$ gas was produced linearly. Triplicate vials were prepared for each strain for statistical analysis.

To examine the denitrification ability of the strains under aerobic conditions (Ji et al. 2015), denitrification rates were also measured in the presence of O$_2$ gas. Vials (n = 3 for each strain) were prepared as described above except the gas phase was not exchanged. Gas samples were taken and analyzed at the same time intervals as described above.
Whole genome sequencing

Whole genomes were sequenced for selected denitrifying strains. Cells grown in R2A-NA at 30°C for 1 to 2 weeks were pelleted by centrifugation, and subjected to DNA extraction using the PowerSoil DNA Isolation kit (Mobio) according to the manufacturer’s instructions. Sequencing libraries were prepared with insert size of 20 kbp by using the PacBio SMRT kit (Pacific Biosciences), and sequenced using the PacBio RS II platform (Pacific Biosciences) at the Mayo Clinic’s Molecular Biology Core. After quality filtering, reads were assembled de novo with the hierarchical genome assembly process (HGAP3) in the SMRT Link portal (v 2.3.0). Genome annotation was carried out 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).

Statistical analysis

R software version 3.3.2 (https://www.R-project.org/) 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.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of the isolated strains have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers MH681433-MH681487 and MH196452-MH196472. The whole genome sequences of strains BE2.4, WB53, and WB94 are also available in the DDBJ/EMBL/GenBank databases under accession numbers PRJNA448131, PRJNA470639, and PRJNA453624, respectively.

RESULTS

Isolation of potential denitrifying bacteria

A total of 206 isolates were obtained from woodchip and bioslime samples collected from four woodchip bioreactor sites in Minnesota (Table S2). Of these, 76 strains reduced nitrate at 15°C. Based on the 16S rRNA gene sequence analysis, they belong to the genera Clostridium (17 strains), Bacillus (15 strains), Microvirgula (12 strains), Enterobacter (8 strains) RaoulUlta (6 strains), Lelliottia (5 strains), and others.
strains), *Cellulomonas* (3 strains), and others (Table 1). *Clostridium* spp. and *Microvirgula* spp. were commonly obtained from the woodchip samples, while *Bacillus* spp. and *Enterobacteriacea* species were commonly and abundantly obtained from the bioslime samples. Bacteria belonging to *Enterobacteriacea* were also frequently isolated from the BE woodchip samples, but not from the WB woodchip samples.

Degrees of nitrate reduction and production of ammonium and/or N₂O under acetylene-inhibition varied by taxa (Table S2). Strains belonging to the family *Enterobacteriacea* (the genera *Buttiauxella*, *Enterobacter*, *Escherichia*, *Kosakonia*, *Lelliottia*, and *Raoultella*) reduced large amounts of nitrate (20-100%, mean 65%) but also produced relatively large amounts of ammonium (7-63%, mean 37%). To distinguish denitrifiers (i.e., those reducing nitrate to N₂ or N₂O gas) from DNRA (i.e., those reducing nitrate to ammonium), we used criteria proposed by Jang et al. (2019a): denitrifiers should (i) reduce ≥40% nitrate-N; (ii) convert <10% N to ammonium; (iii) not accumulate nitrite-N; and (iv) produce a significant amount of N₂O (>100 ppm). As a result, nine strains were identified as low temperature-adapted denitrifiers, belonging to the genera *Clostridium* (4 strains), *Raoultella* (2 strains), *Cellulomonas* (1 strain), *Delftia* (1 strain), and *Microvirgula* (1 strain).

Interestingly, potential denitrifiers were only isolated from woodchip samples. Strains isolated from bioslime samples either did not reduce nitrate sufficiently or produced large amounts of ammonium (i.e., DNRA bacteria). The three bioslime sampling sites contained bacteria known to reduce nitrate to ammonium, such as *Raoultella*, *Enterobacter*, *Aeromonas* and *Lelliottia*, all of which belong to *Enterobacteriaceae*. *Bacillus* spp. were the most common nitrate-reducing bacteria isolated from both the WB bioreactor bioslime and OC bioreactor bioslime samples. Although some strains produced relatively large amounts of N₂O, the majority of the isolates reduced a negligible amount (<10%) of the nitrate-N or accumulated nitrite (0.2-17.5% of the nitrate-N converted to nitrite-N), and therefore were not considered denitrifiers in this study. No potential denitrifiers were identified from any of the biofilm samples across the three sites, indicating that biofilms responsible for clogging in woodchip bioreactors were likely composed of non-denitrifying microorganisms primarily performing DNRA.

**Denitrification rates of the strains**
Five potential denitrifiers were selected, based on their identity and the degree of nitrate reduction, for the $^{15}$N isotope analysis to measure denitrification rates under aerobic and anaerobic conditions. When strains were incubated with $^{15}$N-labeled nitrate under anaerobic conditions, $^{30}$N$_2$ was significantly ($p<0.05$) produced over time in three strains ($Cellulomonas$ sp. WB94, $Delftia$ sp. BE1.2, and $Microvirgula$ sp. BE2.4) (Fig. 1). Interestingly, two of these strains, $Microvirgula$ sp. BE2.4 and $Cellulomonas$ sp. WB94, also significantly ($p<0.05$) produced $^{30}$N$_2$ when they were incubated with $^{15}$N-labeled nitrate under aerobic conditions, suggesting that they are aerobic denitrifiers. However, two strains ($Raoultella$ sp. BE2.1 and $Clostridium$ sp. WB53) did not show significant production of $^{30}$N$_2$ (data not shown), suggesting that they are DNRA bacteria.

Denitrification rates (mean ± SD) of $Cellulomonas$ sp. WB94 under anaerobic and aerobic conditions were 0.088 ± 0.023 pmol/h/10$^6$ cells and 0.102 ± 0.013 pmol/h/10$^6$ cells, respectively, and these values were not significantly different from each other ($p=0.496$ by ANOVA) (Table 2). Similarly, denitrification rates of $Microvirgula$ sp. BE2.4 were not significantly different ($p=0.607$) between anaerobic (0.017 ± 0.007 pmol/h/10$^6$ cells) and aerobic conditions (0.020 ± 0.002 pmol/h/10$^6$ cells). The denitrification rate of $Delftia$ sp. BE1.2 under anaerobic conditions was 1.598 ± 0.752 pmol/h/10$^6$ cells, which was much greater than those of $Cellulomonas$ sp. WB94 and $Microvirgula$ sp. BE2.4 ($p<0.05$ by ANOVA). $Delftia$ sp. BE1.2 also performed denitrification under aerobic conditions but at a significantly lower rate than under anaerobic conditions ($p<0.05$ by ANOVA).

**Detection of denitrification functional genes**

We tried PCR to amplify nitrite reductase genes ($nirK$ and $nirS$), which are considered key functional genes for denitrification, with commonly used primers (Hallin and Lindgren 1999; Henry et al. 2004; Kandeler et al. 2006) to verify that the strains isolated are indeed denitrifiers. However, no PCR-positives were obtained, probably due to the presence of base mismatches on the target gene sequences with the primer sequences. We therefore used a whole genome sequencing approach to detect denitrification functional genes.

The following three strains were selected for whole genome sequencing: $Cellulomonas$ sp. WB94, $Clostridium$ sp. WB53, and $Microvirgula$ sp. BE2.4. A summary of these genome sequences is shown in Table S3. The genome of $Cellulomonas$ sp. WB94 was previously reported (Jang et al.
and contained the nitrate reductase genes \textit{narGHJI} and \textit{napA}, the copper containing nitrite reductase gene \textit{nirK}, and genes related to the biodegradation of complex polysaccharides (Table S4). However, nitric oxide reductase gene \textit{nor} and nitrous oxide reductase gene \textit{nos} were not identified. Based on the ANI analysis, strain WB94 most likely belonged to \textit{Cellulomonas cellasea} (Jang et al. 2019a).

\textit{Clostridium} sp. WB53 was selected due to its ability to remove nitrate, while producing little ammonium. This strain is also obligately anaerobic, as opposed to other known denitrifiers. Based on the genome sequencing, however, we were unable to identify denitrification genes, although the genome contained genes capable of cellulose degradation (Table S5). Based on the ANI analysis, strain WB53 most likely belongs to \textit{Clostridium beijerinckii}.

\textit{Microvirgula} sp. BE2.4 was selected because it produced N\textsubscript{2} from nitrate both under aerobic and anaerobic conditions. Based on the ANI analysis, strain BE2.4 most likely belongs to \textit{Microvirgula aerodenitrificans}. The genome of \textit{Microvirgula} sp. BE2.4 contained a complete set of denitrification genes including nitrate reductase genes \textit{narGHIJ}, cytochrome \textit{cd}\textsubscript{1}-type nitrite reductase gene \textit{nirS}, nitric oxide reductases genes \textit{norBC}, and nitrous oxide reductase gene \textit{nosZ} (Table S6). Interestingly, two \textit{nos} operons were found on the genome of \textit{Microvirgula} sp. BE2.4. Although the gene sequences were highly similar to each other (91.0–99.6%), gene arrangement were different (\textit{nosDFYLCZ} vs. \textit{nosDFYLZC}). The inverse orientation \textit{nosZC} (locus tags DAI18_07220 and DAI18_07225) is likely due to the activity of composite transposon structure with two \textit{IS3} family transposases found adjacent to these genes (locus tags DAI18_07210 and DAI18_07230).

**DISCUSSION**

The main purpose of this study was to obtain denitrifiers that show promise for future bioaugmentation to enhance nitrate removal in woodchip bioreactors. We used a conventional spread-plating technique to isolate potential denitrifiers, which has proved useful in isolating/obtaining denitrifiers (Jang et al. 2019a; Nishizawa et al. 2012). Jang et al. (2019a) used the spread-plating technique to isolate \textit{Cellulomonas} spp. that was also identified as an active denitrifier using a culture-independent analysis.
Out of >200 isolates, only three strains were confirmed as denitrifiers based on the GC-MS analysis. The efficiency of isolation (0.14% overall; 6.5% and 0% from woodchip and bioslime samples, respectively) is much lower than that reported previously (9.7%) (Nishizawa et al. 2012). This difference is likely related to the type of samples used: Nishizawa et al. (2012) isolated denitrifiers from rice paddy soils where denitrification actively occurred; while woodchip and bioslime samples were used in this study.

Many nitrate-reducing strains were isolated from the biofilm samples, although they were not confirmed as denitrifiers. Many of them belonged to the genus *Bacillus* and the family *Enterobacteriacea*. These strains most likely perform DNRA. Similar to this study, *Bacillus* and *Enterobacteriacea* species are known for their ability to carry out the DNRA reaction (Tiedje 1988), although some *Bacillus* sp. are also known to denitrify (Verbaendert et al. 2011).

Isolation of many DNRA bacteria, but no denitrifiers, from the bioslime samples might be due to the enrichment of DNRA bacteria in the bioslime environments. We collected the bioslime samples from the WB and LB woodchip bioreactors which had undergone biostimulation treatment (i.e., addition of acetate, with target C/N ratios of 1.95 and 0.35–1.13, respectively; Table S1) and from the OC woodchip bioreactor, which contained relatively easily degradable carbon such as twigs, bark, green cuttings, and fines. These readily available carbon sources might have increased the C/N ratio in the woodchip bioreactors or inlet pipes, which would select for DNRA bacteria over denitrifying bacteria. A higher ratio of available carbon (i.e. acetate) to electron acceptor (i.e. NO$_3^-$) is more favorable for DNRA because it allows for an additional three electrons per nitrogen atom to be accepted compared to denitrification (Kraft et al. 2014; Tiedje 1988) although other factors such as nitrate and carbon concentrations can be also important (Jia et al. 2019).

Biofilms/bioslimes often form in woodchip bioreactors when too much readily available carbon is added. Formation of biofilms/bioslimes is a problem because it can clog the bioreactor pipes and reduce the flow (Christianson et al. 2016; Gibert et al. 2008; Husk et al. 2017). Our results also suggest that DNRA bacteria can be also enriched in bioslimes, which is not desirable for nitrate removal since the DNRA process preserves N in the environment rather than releasing it as N$_2$ gas.

Based on Jang’s criteria (2019a), five potential denitrifiers were identified; however, only three out of the five strains were confirmed as denitrifiers based on the $^{15}$N analysis. Based on the
Jang’s criteria, denitrifiers should (i) reduce ≥40% nitrate-N; (ii) convert <10% N to ammonium; (iii) not accumulate nitrite-N; and (iv) produce a significant amount of N$_2$O (>100 ppm). This is different from more commonly used criteria: >80% of nitrate-N should be converted to N$_2$O (Mahne and Tiedje 1995). However, Mahne and Tiedje’s criteria are designed for denitrifiers capable of growth at nutrient-rich medium at 30°C, and does not apply to oligotrophic denitrifiers (Ishii et al. 2011; Tago et al. 2011). Since we wanted to obtain as many potential denitrifiers as possible, we used the less stringent Jang’s criteria for denitrifier identification as the initial screening.

For denitrifier confirmation and characterization, we used $^{15}$N isotope analysis and genome sequencing, respectively. While PCR is frequently used to detect denitrification functional genes such as nitrite reductase genes nirK and nirS in isolated strains (Ashida et al. 2010; Ishii et al. 2011), there are no universal primers that can amplify target denitrification genes from all denitrifiers (Bonilla-Rosso et al. 2016; Ma et al. 2019; Penton et al. 2013; Verbaendert et al. 2014). Therefore, denitrification capability should not be ruled out by negative PCR results due to the PCR bias. In our study, nirK and nirS were not detected by PCR, although the strains showed production of $^{30}$N$_2$ by the $^{15}$N stable isotope analysis. Genome sequencing was necessary to detect denitrification functional genes. Whole genome sequencing can also provide a complete picture of all relevant functional genes, including those related to the N cycle and the degradation of woodchips, which would allow for appropriately choosing a denitrifying microorganism for bioaugmentation.

Based on the $^{15}$N isotope analysis, three strains were confirmed as denitrifiers in this study. These strains belonged to the genera Cellulomonas, Delftia, and Microvirgula. Delftia sp. strain BE1.2 was isolated from the BE woodchip bioreactor, and showed the highest denitrification rates under anaerobic conditions. Delftia sp. strains have previously demonstrated efficient denitrification (Zhang and Zhou 2016). However, it did not demonstrate aerobic denitrification, and therefore, was not selected for further genome analysis.

Microvirgula spp. was most frequently isolated from the BE bioreactor (n = 8). While all eight Microvirgula strains showed promising nitrate reduction ranging from 62-100%, seven strains converted >10% of N to ammonium, and therefore, were not considered as denitrifiers based on Jang’s criteria. Only one strain, Microvirgula aerodenitrificans strain BE2.4 was confirmed as a denitrifier. This strain reduced nitrate to N$_2$ gas both under aerobic and anaerobic conditions, similar
to some of the previously reported *Microvirgula* spp. strains (Patureau et al. 1998; Patureau et al. 2001). While denitrification has been largely thought to be an anaerobic process, some microorganisms have been identified that are capable of aerobic denitrification (Ji et al. 2015; Patureau et al. 1998; Patureau et al. 2001; Takaya et al. 2003). Aerobic denitrification would serve an important role in wastewater treatment, particularly in woodchip bioreactors where dissolved oxygen is present at the inlet end of the bed and fluctuating water depth affects oxygen levels within the bed.

The genome of *Microvirgula aerodenitrificans* strain BE2.4 contained a complete set of denitrification genes, further supporting its ability to denitrify. Interestingly, two *nos* operons were found on the genome of *Microvirgula aerodenitrificans* strain BE2.4, one of which were located on a composite transposon structure. This suggests that *nos* genes could be relocated on the genome, and further transferred to other cells if it is moved to a prophage region or other conjugative elements.

Similar to this study, association of denitrification genes (*nir-nor* cluster) on a composite transposon was recently reported in an *Azospirillum* sp. denitrifier (Jang et al., 2019b). These results suggest that denitrification genes might be horizontally transferred among cells.

*Cellulomonas* spp. was also a relatively frequently isolated nitrate reducer (n = 3) from the WB woodchips; however, only one strain WB94 was confirmed as a denitrifier. *Cellulomonas* spp. was previously identified as one of the active denitrifiers in woodchip bioreactor microcosms at low temperature conditions using an RNA-based culture-independent approach (Jang et al. 2019a). Similar to *Microvirgula aerodenitrificans* strain BE2.4, *Cellulomonas cellacea* strain WB94 also showed denitrification both under aerobic and anaerobic conditions. The presence of nitrite reductase gene *nirK* on the genome of strain WB94 supports its denitrification ability.

Strain WB94 also possesses genes related to the biodegradation of complex polysaccharides and can degrade cellulose (Jang et al. 2019a), similar to other *Cellulomonas* sp. strains (Han and Srinivasan 1968; Poulsen et al. 2016; Thayer et al. 1984). The ability to degrade cellulose is deemed useful for bioaugmentation in woodchip bioreactors because: (i) wood is mainly composed of cellulose (Pérez et al. 2002), and (ii) the product of cellulose degradation, glucose, can be used as a source of carbon and electrons for nitrate reduction. In addition to *Cellulomonas cellacea* strain WB94, *Clostridium beijerinckii* strain WB53 possessed genes for cellulose degradation, although this strain did not reduce nitrate to N₂ gas. Strain WB53 also lacked denitrification functional genes,
confirming that this strain is not a denitrifier. Nevertheless, *Clostridium* may play an active role in
woodchip bioreactors as they are known to be able to degrade wood compounds including cellulose,
hemicellulose, xylan and pectin under anaerobic conditions, providing a carbon source for the
denitrifying microbial community (Desvaux 2006; Kosugi et al. 2001).

*Cellulomonas* spp. and *Clostridium* spp. were more frequently isolated from the WB woodchip
bioreactor than the BE woodchip bioreactor. This difference may be related to the age of the
bioreactor. Woodchip samples were collected from the BE woodchip bioreactor shortly after its
establishment, whereas samples were collected from the WB woodchip bioreactor after four years of
operation. 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 (about 9 mg N L\(^{-1}\) \(\text{d}^{-1}\)) (Robertson 2010). Fresh woodchips could provide more readily
available C than aged woodchips, but this C has been shown to be degraded quickly in new
bioreactors (David et al. 2016; Robertson 2010). After this labile C is depleted, cellulose-degrading
microorganisms such as *Cellulomonas* spp. and *Clostridium* spp. may become enriched and play
important roles in providing C in woodchip bioreactors. Additional studies are needed to determine
how a denitrifying community changes over time within a bioreactor.

In conclusion, *Microvirgula aerodenitrificans* strain BE2.4 and *Cellulomonascellacea* strain
WB94 are promising for future bioaugmentation in the field due to their potential for aerobic and
anaerobic denitrification and the possible ability of WB94 to degrade cellulose. Ability to denitrify in
aerobic conditions would be of benefit in treating agricultural drainage water in woodchip bioreactors
where water and oxygen levels greatly fluctuate. It is also important to consider the ability to degrade
woodchip materials to enhance denitrification. Inoculating a strain capable of cellulose degradation
would provide C which would, together with other indigenous woodchip-degrading microorganisms
(e.g., fungi), promote microbial denitrification activity. Our preliminary bioaugmentation trials
showed promising results (Feyereisen et al. 2018); however, further research is necessary to confirm
the efficacy of inoculation of these denitrifiers for enhancing nitrate removal in woodchip bioreactors
under field conditions. Questions to be addressed in future research include whether inoculated strains
actually enhance N removal in woodchip bioreactors under field conditions; how long inoculated
strains can survive in bioreactors; and, if C addition can enhance the abundance of the inoculated and indigenous denitrifiers. We are planning to address these questions in the near future.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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**FIGURE LEGENDS**

Figure 1. Production of $^{30}\text{N}_2$ over time at 15°C under anaerobic (open circle) and aerobic (closed circle) conditions. A, *Cellulomonas* sp. strain WB94; B, *Delftia* sp. strain BE1.2; C, *Microvirgula* sp. strain BE2.4. Mean values of triplicate test tubes are shown. Error bars indicate standard deviation.
Table 1. Identity of the nitrate-reducing strains isolated in this study. Numbers shown in bold are the number of denitrifying strains confirmed based on the Jang’s criteria (Jang et al. 2019). Woodchip bioreactors BE, WB, LB, and OC are located in Blue Earth, Willmar, Lamberton, and Olmsted County, respectively. See Table S1 for detail.

<table>
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<tr>
<th>Identity</th>
<th>Woodchips</th>
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<tr>
<td></td>
<td></td>
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<td></td>
</tr>
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<td>1</td>
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<tr>
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<td>Buttiauxella</td>
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<td></td>
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<tr>
<td></td>
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<tr>
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<td>Microvirgula</td>
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<tr>
<td>unclassified <em>Bacillales</em></td>
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<tr>
<td><strong>Total</strong></td>
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<td>21</td>
</tr>
</tbody>
</table>

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Table 2. Denitrification rates of the strains isolated in this study. Mean ± SD (n = 3) is shown.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Denitrification rate (pmol/h/10⁶ cells)</th>
<th>Statistical significance*</th>
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</thead>
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<tr>
<td></td>
<td>Anaerobic</td>
<td>Aerobic</td>
</tr>
<tr>
<td><em>Cellulomonas</em> sp. WB94</td>
<td>0.088 ± 0.023</td>
<td>0.102 ± 0.013</td>
</tr>
<tr>
<td><em>Microvirgula</em> sp. BE2.4</td>
<td>0.017 ± 0.007</td>
<td>0.020 ± 0.002</td>
</tr>
<tr>
<td><em>Delftia</em> sp. BE1.2</td>
<td>1.598 ± 0.752</td>
<td>0.050 ± 0.013</td>
</tr>
</tbody>
</table>

*NS, not significant
SUPPORTING INFORMATION

Figure S1. Photos of biofilm clogging the WB woodchip bioreactors: a) biofilm inside the woodchip sampling port. b) biofilm accumulation at the inlet pipe and acetate injection site.

Table S1. Descriptions of the four bioreactors from which denitrifying bacteria were isolated.

Table S2. Nitrate-reducing bacteria isolated in this study. Denitrifiers identified based on Jang’s criteria (2019) are shown in red.

Table S3. Summary of the sequenced genomes of Cellulomonas cellasea strain WB94, Clostridium beijerinckii strain WB53, and Microvirgula aerodenitrificans strain BE2.4.

Table S4. Genes related to denitrification and polysaccharide degradation identified on the genome of Cellulomonas sp. strain WB94.

Table S5. Genes related to nitrate/nitrite reductions and polysaccharide degradation identified on the genome of Clostridium sp. strain WB53.

Table S6. Genes related to denitrification identified on the genome of Microvirgula sp. strain BE2.