Published April 25, 2016

Journal of Environmental Quality SPECIAL SECTION

MOVING DENITRIFYING BIOREACTORS BEYOND PROOF OF CONCEPT

Performance of Agricultural Residue Media in Laboratory Denitrifying Bioreactors at Low Temperatures

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Abstract

Denitrifying bioreactors can be effective for removing nitrate from agricultural tile drainage; however, questions about cold springtime performance persist. The objective of this study was to improve the nitrate removal rate (NRR) of denitrifying bioreactors at warm and cold temperatures using agriculturally derived media rather than wood chips (WC). Corn (*Zea mays* L.) cobs (CC), corn stover (CS), barley (*Hordeum vulgare* L.) straw (BS), WC, and CC followed by a compartment of WC (CC+WC) were tested in laboratory columns for 5 mo at a 12-h hydraulic residence time in separate experiments at 15.5 and 1.5°C. Nitrate-N removal rates ranged from 35 to 1.4 at 15.5°C and from 7.4 to 1.6 g N m^{-3} d^{-1} at 1.5°C, respectively; NRRs were ranked CC > CC+WC > BS = CS > WC and CC ≥ CC+WC = CS ≥ BS > WC for 15.5 and 1.5°C, respectively. Although NRRs for CC were increased relative to WC, CC released greater amounts of carbon. Greater abundance of nitrous oxide (N_2O) reductase gene (*nosZ*) was supported by crop residues than WC at 15.5°C, and CS and BS supported greater abundance than WC at 1.5°C. Production of N_2O relative to nitrate removal (rN_2O) was consistently greater at 1.5°C (7.5% of nitrate removed) than at 15.5°C (1.9%). The rN_2O was lowest in CC (1.1%) and CC-WC (0.9%) and greatest in WC (9.7%). Using a compartment of agricultural residue media in series before wood chips has the potential to improve denitrifying bioreactor nitrate removal rates, but field-scale verification is needed.

Core Ideas

- A compartment of corn cobs before wood chips increases N removal and reduces C loss.
- Microbial denitrifier populations for crop residues were higher than for wood chips.
- Denitrification was limited by C availability at 1.5°C.
- Nitrate-N removal does not necessarily correlate to N_2O production.
- N_2O production per nitrate-N removed was nearly 4× at 1.5 compared with 15.5°C.

J. Environ. Qual. 45:779–787 (2016)
doi:10.2134/jeq2015.07.0407
Supplemental material is available online for this article.
Received 31 July 2015.
Accepted 25 Nov. 2015.
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Denitrifying bioreactors to remove nitrate from agricultural drainage are a well-demonstrated technology with successful application in many locations (Schipper et al., 2010; USDA–NRCS, 2015). Over the past 20 yr, wood media have been the bioreactor fill of choice due to practicality, longevity, and cost-effectiveness (Robertson, 2010; Schipper et al., 2010). As this technology matures, there are new questions about maximizing nitrate removal and minimizing the release of unintended pollutants (Healy et al., 2015). Despite the general acceptance of wood-based fill, more labile carbon (C) media derived from agricultural residues (e.g., corn [*Zea mays* L.] cobs [CC], barley [*Hordeum vulgare* L.] straw [BS], and wheat [*Triticum aestivum* L.] straw) have repeatedly shown greater nitrogen (N) removal rates than wood (Greenan et al., 2006; Cameron and Schipper, 2010; Warneke et al., 2011a; Krause Camilo et al., 2013). Although agricultural residue–based media have been associated with high levels of nutrient leaching (Gibert et al., 2008; Healy et al., 2012; Krause Camilo et al., 2013), these issues may be mitigated via start-up under cool temperatures, shorter hydraulic retention times, and/or preflushing (Cameron and Schipper, 2010). Labile C media may degrade relatively quickly and require replenishment (Soares and Abeliovich, 1998; Aslan and Türkman, 2005; Greenan et al., 2006; Saliling et al., 2007), but more sophisticated bioreactor designs could facilitate media replacement. There is obvious rationale for using agricultural residues in agriculturally purposed bioreactors, particularly if on-farm equipment could be used for media replacement.

Warneke et al. (2011a) found that the N removal potential of several organic media was more dependent on the quantity of microbially available C than the specific substrate type. Clearly,

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Abbreviations: BS, barley straw; CC, corn cobs; CS, corn stover; HRT, hydraulic residence time; LCI, lignocellulose index; NRR, nitrate removal rate; qPCR, quantitative polymerase chain reaction; TC, total carbon; TOC, total organic carbon; WC, wood chips.

the microbiology of denitrification is important, but understanding of microbial communities within denitrifying bioreactors is only now emerging. The *nosZ* gene encodes nitrous oxide (N_2O) reductase and is widely distributed in bacteria (Sanford et al., 2012; Jones et al., 2013; Graf et al., 2014). The *nosZ* and other structural genes provide quantitative PCR (qPCR) targets, allowing quantification of denitrifier communities that may provide insight into bioreactor performance (Henry et al., 2006; Warneke et al., 2011a; Hathaway et al., 2015; Healy et al., 2015; Porter et al., 2015). Similarly, few studies have looked at the impact of C quality of media types on N removal (Krause Camilo et al., 2013; Schmidt and Clark, 2013).

One important consideration is the release of the potent greenhouse gas N_2O. Denitrifying bioreactor N_2O emissions comprise a small percentage of the system's total N budget, providing generally <5% of influent nitrate (Elgood et al., 2010; Moorman et al., 2010; Woli et al., 2010; Warneke et al., 2011a; Herbstritt, 2014; Ghane et al., 2015; Healy et al., 2015). However, despite low emissions relative to system N budgets, N_2O's high global warming potential can translate to substantial CO_2 equivalents. Thus, continued study of N_2O emissions from varying bioreactor designs and applications is warranted.

Selection of media to improve nitrate removal during early spring is needed. The combination of wet or snowmelt conditions and minimal crop uptake of water and nutrients means early-season drainage is a prime target for nitrate reduction efforts. Jin and Sands (2003) estimated that in Minnesota 50% of the average annual runoff occurs before 11 May when average daily air temperatures are 5°C. Although studies have confirmed that temperature is a critical bioreactor performance predictor (Christianson et al., 2012; Bell et al., 2015), few studies have been conducted at ≤5°C (Welander and Mattiasson, 2003).

The objectives of this laboratory denitrifying bioreactor column study were (i) to test three agricultural residues, and a combination of a residue in series before wood chips, for nitrate removal performance; (ii) to quantify denitrifier populations; and (iii) to measure N_2O production of these treatments, all under both "warm" (15.5°C) and "cold" (1.5°C) temperature regimes.

Materials and Methods

Media Treatments

A randomized complete block design (n = 3) was used to evaluate five materials: wood chips (WC), CC, shredded corn stover (CS), BS, and a combination of corn cobs in series with wood chips (CC-WC) (CC at inlet two-thirds and WC at outlet one-third). Water dosed with nitrate-N was continuously pumped through packed PVC columns for 5 mo at 15.5°C (i.e., "warm run"). The columns were disassembled, sampled for C quality analysis, and freshly repacked for an additional 5-mo run at 1.5°C (i.e., "cold run"). The temperatures were based on summer and early-spring data from bioreactor research in south-central Minnesota (Ranaivoson et al., 2012), which were similar to values from northeastern Iowa (Christianson et al., 2012) and eastern Illinois (David et al., 2016).

Carbon quality was determined as follows. Air-dried media were ground to 40 mesh, and moisture content was determined (105°C overnight) (NREL/TP-510–42621) (Sluiter et al., 2008a). Klason lignin, cellulose, and hemicellulose content after acid hydrolysis were determined using a high-performance liquid chromatograph (Model 1525 Binary Pump, Waters Corp.) and a prepacked carbohydrate analytical column (Model Aminex HPX-87P, Bio-Rad) with a de-ashing cartridge (#125–0118, Bio-Rad) in line and a refractive index detector (Model 2414, Waters Corp.) (NREL/TP-510–42618; flow rate 0.3 mL min^{-1} and 80°C column temperature, all in duplicate) (Sluiter et al., 2011). Ash content was determined via combustion (550°C) (Sluiter et al., 2008b). The lignocellulose index (LCI) was calculated as the ratio of lignin to lignin+cellulose for the initial and spent media (BS, CC, CS, and WC) after the warm and cold runs (Schmidt and Clark, 2013). Additional initial material properties are provided in Supplemental Tables S1 and S2.

Bioreactor Design and Operation

Column bioreactors (15.2-cm-diameter by 49.5-cm-long PVC) were each packed with approximately eight 400-g batches of water-saturated media, leaving a headspace of 13 to 52 mm. Waukegan silt loam (fine-silty over sandy, mixed, superactive, mesic Typic Hapludolls) with a manure history (140 ± 0.5 dry g $column^{-1}$) was mixed uniformly with the media to inoculate the columns, which were then strapped to a steel framework in a walk-in cooler. Drainable pore volumes were established by filling columns with reverse osmosis water, soaking the media overnight, topping off columns in the morning, and draining columns for 24 h. Drainable porosity was calculated as drainable pore volume divided by gross volume occupied by the media. Bottom and top caps were sealed with silicone, and calibrated oxidation–reduction potential sensors were installed in the top caps. To seal the head space, outlet tubing extended 5 to 6 cm above the top cap before looping down to covered effluent containers on the floor. Batches of synthetic water with chemistry resembling Minnesota tile drainage (Ranaivoson et al., 2012; Supplemental Tables S3 and S4) were mixed, tempered in plastic 208-L drums, and transferred to the 640-L supply tank from which a peristaltic pump fed the bioreactors at a design hydraulic residence time (HRT) of 12 h. The influent nitrate-N concentration was 50 mg L^{-1}, ensuring that NNRs were not nitrate limited. For 22 d, reverse-osmosis water was circulated through the columns to identify/fix leaks, calibrate the pump, flush suspended soil particles, and flush rapidly soluble C from the media. At the completion of the warm run, the microbial biomass was sampled 7 to 8 cm from the outlets and inlets of the columns (see below). The columns were cleaned, and the inoculation/packing procedure was repeated with fresh media. Again, reverse-osmosis water was circulated through the columns (11 d), and then synthetic water was introduced (7 d), after which the chamber temperature was lowered from 15.5 to 1.5°C over a period of 21 d. The cold run experimental time began when the temperature stabilized at 1.5°C.

Sample Collection and Analyses

Water

Thrice-weekly water samples (125 mL) were collected in 250-mL polyethylene bottles, acidified (Clesceri et al., 1998), and refrigerated (4°C) for analysis by colorimetry (Lachat QuikChem 8500, Hach Co.; method number 10–107–04–1-A)

for NO_3–N + NO_2–N (referred to as nitrate-N). Total organic C (TOC) and total C (TC) were analyzed by EPA Methods 415.1 and 9060A (Tekmar Dohrmann Phoenix 8000, Teledyne Tekmar). Unacidified samples were measured for pH. Sampling methodology of influent TOC/TC concentrations early in the first experiment was later determined to have resulted in high variation in the inlet TOC levels that likely did not represent the actual inlet TOC/TC concentrations.

Nitrate-N removal rate (NRR) (units g N m^{-3} d^{-1}) was calculated for each water sampling date as the difference between the nitrate-N mass into and out of each column between that sampling date and the previous sampling date using average nitrate-N concentration, divided by gross volume occupied by the column's medium, divided by time between samplings. Cumulative nitrate-N mass (load) reduction was calculated as cumulative mass removed divided by cumulative mass into each column. Periods of 16 and 12 d were excluded due to operator error and pump failure for the warm and cold runs, respectively.

Total C loads produced were calculated as follows:

$$\text{TC load production} = \sum_{i=1}^{n} \text{TC}_{\text{out}} - \sum_{i=1}^{n} \text{TC}_{\text{in}} \quad [1]$$

where TC_{out} is TC mass for the ith of n sampling dates, and TC_{in} is TC mass entering the column over the same sampling periods. Load production for TOC was calculated similarly.

Molecular Analysis of Microbial Populations

Samples of column media were obtained at the end of the experiments and were frozen until processing. DNA was extracted from thawed and chopped subsamples using the Ultra Clean Mega Soil kit (MoBio) according to the manufacturer's instructions. Barley straw and CS were chopped into approximately 1-cm sections. Corn cobs were cut into quarter sections, and small WC were cut into smaller pieces also approximately 1 cm in length. Substrate moisture contents were determined gravimetrically.

Primers for qPCR targeted the *nosZ* gene clade 1 (Henry et al., 2006) and clade 2 (Jones et al., 2013) and the 16S rRNA gene (Fierer et al., 2005). Standards for the *nosZ*1 (clade 1) assay were constructed by extracting genomic DNA from *Pseudomonas stutzeri* (ATCC 14405), PCR amplification with the *nosZ*1 primers, and cloning the PCR product into pCR-4TOPO hosted in *Escherichia coli* (TOPO TA kit, Invitrogen). Plasmid DNA extracted from *E. coli* cultures was quantified, and dilutions of plasmid DNA were used as standards for qPCR. Similar procedures were used to construct standards for the *nosZ* clade 2 qPCR assay. The *nosZ*-II primer set was used with DNA extracted from *Geobacillus thermodenitrificans* DSM 465 (ATCC 29492) to prepare PCR product for cloning. Finally, the Eub 338 (F) and Eub 518 (R) primer set (Fierer et al., 2005) was used for measurement of 16S-rRNA genes in bacteria.

Each qPCR run included positive standards (dilutions of plasmid DNA) and negative standards: water as template (no DNA) or *E. coli* (ATCC 43651) genomic DNA prepared in triplicate wells. Each reaction contained 12.5 μL of 2X QuantiTect Sybr Green Master Mix (Qiagen) including Taq polymerase and 5.0 μL of 6.25 μmol L^{-1} of each *nosZ*1 or *nosZ*-II primer, resulting in 1.25 μmol L^{-1} primer concentration in the well. Each well received 2.5 μL of standard or sample DNA. Thermal cycling conditions for *nosZ* clade 1 included Taq polymerase activation at 95°C for 15 min, 40 cycles of 95°C for 15 s, 53°C for 15 s (annealing), 72°C for 30 s, followed by melting curve analysis from 50 to 90°C. For *nosZ* clade 2, thermal cycling included Taq polymerase activation at 95°C for 15 min and 40 cycles of 95°C for 30 s, 47°C for 1 min (annealing), 72°C for 30 s, followed by melting curve analysis from 50 to 98°C.

N_2O

Effluent water was sampled for dissolved N_2O concentration (dN_2O) using the headspace equilibration technique. Water samples (12 mL) were collected by gas-tight syringe and injected into 40-mL glass vials with septum caps preserved with 0.1 mL H_2SO_4. Vials were equilibrated at 23°C for 1 h; headspace gases were transferred to 9-mL glass vials and analyzed for gas-phase N_2O concentration by gas chromatography (Agilent/Hewlett-Packard) with electron capture detection (Venterea et al., 2010). The dN_2O in the sample was determined using Henry's Law coefficient (Sander, 2015) and accounting for gas-phase N_2O originally present in the vial. Samples were collected on Days 2, 30, 49, 77, 105, and 133 at 15.5°C and on Days 14, 42, 70, 98, and 114 at 1.5°C. The N_2O production rate (pN_2O) was calculated analogously to NRR using Eq. [1] except that a positive value indicates production. Cumulative N_2O production (cpN_2O) was determined by trapezoidal integration of pN_2O versus time. To have a valid comparison of results at 15.5 and 1.5°C, cpN_2O was determined over a common time period (Days 14–114) in both experiments. The cumulative production of N_2O relative to the cumulative removal of nitrate-N was determined over this same 100-d period and expressed as a percentage (rN_2O).

Statistical Analysis

Data were analyzed at $P \leq 0.05$ using the MIXED procedure of SAS (SAS Institute, 2011). Temperature and substrate were considered fixed effects, and block and interactions with block were considered random effects. Sampling time and position within a bioreactor were considered fixed effects and repeated measurements. Due to differences in sampling times, time-series data were analyzed separately by experiment. Data for dN_2O, cpN_2O, and abundance of *nosZ* and the 16S-rRNA genes, expressed as gene copies g^{-1} substrate on a dry weight basis, were logarithm base 10 transformed before statistical analysis to meet the requirements of normality and common variance. Gene abundance data (excluding CC-WC columns) were subjected to a three-factor analysis of temperature, substrate ($n = 4$), and position within bioreactor as main effects. An additional two-factor analysis of temperature and substrate nearest the inlet of the bioreactor ($n = 2$) was conducted for gene abundance data from samples collected from wood chips nearest the outlet of the bioreactor for CC-WC and WC. When significant differences occurred for main effects or interactions, means were compared with independent pairwise t tests using the PDIFF option of the MIXED procedure of SAS. Linear regression equations were developed to describe the response of nitrate-N concentration to sampling time by substrate for both experiments using the REG procedure of SAS. All parameter estimates for these regression models were significant at $P < 0.001$.

Results

Hydrology/Hydraulic Residence Time

Averaged across temperatures, porosity and HRT for WC were significantly greater than all other treatments (Supplemental Tables S5 and S6). Porosity for CC was greater than for CC-WC, and BC and CS were similar to both CC and CC-WC. The HRT for both experiments averaged 12.0 and 14.2 h across temperatures for agricultural residue and WC treatments, respectively (Supplemental Table S6).

Nitrate-N Concentration, Nitrate-N Removal Rate, and Nitrate-N Load Reduction

Effluent nitrate-N concentrations at 15.5°C were highest for WC on all 48 sampling dates, followed by CS and BS (Fig. 1a; Supplemental Table S7). Corn cobs exhibited the lowest effluent nitrate-N concentrations for 20 dates and were not different from CC-WC for the remaining dates. For the first 31 d, BS concentrations were not different from CC or CC-WC, after which time they always exceeded CC. Concentrations for CC-WC were generally lower than CS and BS after Day 80. At 1.5°C, effluent nitrate-N concentrations across all treatments were closer to inlet concentrations than at 15.5°C, and WC effluent nitrate-N concentrations were again highest (Fig. 1b; Supplemental Table S8). Although CC and CC-WC treatments were statistically not different for 50 of the 53 sampling dates and had the lowest concentrations, CC was lower than BS or CS more frequently than was CC-WC.

Average NRRs at 15.5°C ranged from 34.9 to 2.2 g N m^{-3} d^{-1}, with CC > CC-WC > BS = CS > WC (Table 1). At 1.5°C, the range of values was tighter (7.4–1.6 g N m^{-3} d^{-1}), with all agricultural residue treatments again having significantly higher values than WC. The Q_{10} values, which quantify changes in NRR for every 10°C change in water temperature, for the agricultural residue treatments ranged from 2.7 to 3.4, and WC was 1.0 (Table 1).

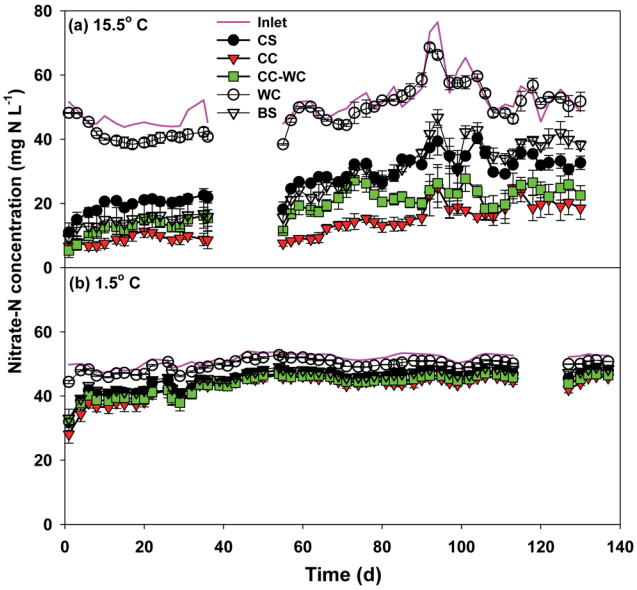

Fig. 1. Inlet and treatment mean nitrate concentrations for the (a) warm (15.5°C) and (b) cold (1.5°C) runs. Data points represent average concentrations from beginning and end of each sampling period. BS, barley straw; CC, corn cobs; CS, corn stover; WC, wood chips. CC-WC refers to CC at inlet two-thirds and WC at outlet one-third.

The average nitrate-N load reduction as a percentage of inlet load for the various treatments ranged from 73 to 5% and from 16 to 4% for the warm and cold runs, respectively (Table 1). The significance of differences among treatments followed the same pattern as for NRR. The load reductions were significantly lower for the cold compared with the warm run for the agricultural residues but not for WC.

C Concentrations and Loads

Concentrations of TOC eluted among treatments differed significantly at 15.5°C but not at 1.5°C (P = 0.004 and 0.582, respectively) (Fig. 2). The substrate × time interaction for TOC was not significant for either run. When averaged across all days, TOC concentrations ranged from 12.2 to 29.4 mg L^{-1} at

Table 1. Nitrate-N removal rates, nitrate-N load reductions as a percentage of the cumulative inlet nitrate-N load throughout the duration of the warm and cold runs, and Q_{10} for the treatments.

Temperature	Substrate†				
	CS	CC	CC-WC	BS	WC
	Nitrate-N removal rate				
°C	g N m^{-3} d^{-1}				
15.5	21.6 (0.5)aC‡	34.9 (0.9)aA	29.3 (1.2)aB	22.4 (0.9)aC	2.2 (0.5)aD
1.5	5.7 (0.2)bAB	7.4 (0.8)bA	6.8 (0.9)bAB	5.1 (0.3)bB	1.6 (0.1)aC
	Cumulative nitrate-N removed				
	%				
15.5	46 (1.2)aC	73 (2.5)aA	62 (2.2)aB	47 (1.6)aC	4.9 (1.0)aD
1.5	12 (0.3)bAB	16 (1.9)bA	15 (0.9)bAB	11 (0.6)bB	3.6 (0.1)aC
	Temperature sensitivity				
	Q_{10}§				
	2.7	3.4	3.1	3.1	1.0

† BS, barley straw; CC, corn cobs; CC-WC, corn cobs in series with wood chips; CS, corn stover; WC, wood chips.

‡ Values are mean (SE). Within a row, means followed by the same uppercase letter are not significantly different at $P \le 0.05$. For each variable, means within a column followed by the same lowercase letter are not significantly different at $P \le 0.05$.

§ Q_{10} is the multiplicative change in nitrate-N removal rates for every 10°C change in water temperature. The Q_{10} values were calculated with means for the warm and cold runs, not by individual columns. Thus, statistical comparison of Q_{10} values were not made.

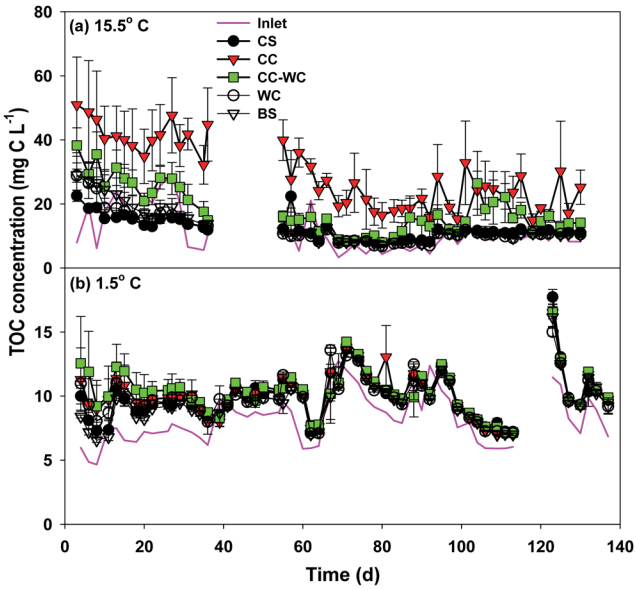

Fig. 2. Total organic C concentration (TOC) of the treatments and inlet for the (a) warm (15.5°C) and (b) cold (1.5°C) runs. BS, barley straw; CC, corn cobs; CS, corn stover; WC, wood chips. CC-WC refers to CC at inlet two-thirds and WC at outlet one-third.

15.5°C and were significantly higher for CC than for the other treatments. Averaged across the cold run, TOC concentrations ranged from 9.3 to 10.2 mg L^{-1}.

As was the case for TOC concentrations, TC concentrations among treatments differed significantly at 15.5°C but not at 1.5°C ($P < 0.001$ and 0.150, respectively) (data not shown). The substrate × time interaction was significant for TC for both warm and cold runs ($P = 0.003$ and 0.049, respectively) (Supplemental Tables S9 and S10). For the warm run, the TC concentrations for CC were highest for all 48 sampling dates. Through Day 76, CC was generally greater than the other treatments. From Days 78 to 130, CC and CC-WC were the same for 18 of 23 dates. The WC and CS treatments tended to have the lowest TC concentrations and differed from one another on only two sampling dates.

Loads of TOC at 15.5°C ranged from 1.0 to 15.7 g column^{-1}, with WC and CS lowest and CC highest. Loads of TC at 15.5°C ranged from 1.2 to 23.7 g column^{-1}, with WC lowest and CC highest (Table 2). At 1.5°C, TOC and TC loads were similar for all treatments, ranging from 1.6 to 2.0 and from 2.5 to 4.2 g column^{-1} for TOC and TC loads, respectively.

Media C Quality

The initial cellulose content of the agricultural residue materials ranged from 41 to 43% and was 36% for WC, and lignin content was 18 to 22% and 29%, respectively (Supplemental Table S11). After the warm run, cellulose contents of the agricultural media decreased to range from 28 to 32%, whereas WC was marginally lower at 32%. The initial LCI ranged from 0.30 to 0.34 for the agricultural residue materials and was 0.45 for WC (Supplemental Fig. S1). The LCI for all treatments increased after the warm run, ranging from 0.37 to 0.43 for the agricultural residues to 0.47 for WC. At 1.5°C, the LCI for BS increased, but for the other media the LCI remained similar to initial values.

Gene Abundance on Substrates

The *nosZ*-F/*nosZ*1-R primer set (Henry et al., 2006) does not amplify all denitrifying bacteria (Verbaendert et al., 2011; Sanford et al., 2012; Jones et al., 2013). Jones et al. (2013) divided denitrifying bacteria on the basis of their *nosZ* sequences into clade 1 and clade 2, with clade 2 including members of the Gram-positive Firmicutes and Gram-negative Bacteriodetes, and developed the *nosZ*-II primers for their amplification. In this study, the *nosZ*-II primer set produced PCR product that matched the expected 720-bp size as determined by gel electrophoresis.

The mean abundances of *nosZ* (clade 1 and clade 2) and the 16S-rRNA gene were significantly different for the substrates ($P \leq 0.007$) but were not affected by temperature or sampling position within the bioreactor when considered as main effects ($P \geq 0.147$). However, there were significant interactions between substrate and position for *nosZ* clade 1 ($P = 0.038$) and between substrate and temperature for clade 2 ($P = 0.004$).

The CC, CS, and BS substrates generally supported greater *nosZ* clade 1 abundances than WC (Table 3). Abundance of *nosZ* clade 1 was greater on CC nearer the outlet position compared with the inlet, but there was no difference in abundance due to position for the other substrates. The abundance of the 16S-rRNA gene also was significantly greater at the outlet (2.28×10^{10} copies g^{-1} substrate) of the CC bioreactor than the inlet (1.26×10^{10} g^{-1}) but only at 15.5°C. The abundance of 16S-rRNA gene is similar to that of the *nosZ* clade 1 gene, indicating that denitrifying bacteria are dominating the microbial community (Supplemental Table S12). The abundances of clade 1 and clade 2 *nosZ* on a volumetric basis are in Supplemental Table S12.

Abundance of *nosZ* clade 2 also was less on WC than on the other substrates (Table 3). At 15.5°C CC supported the greatest clade 2 abundance, but at 1.5°C CS supported

Table 2. Treatment net total C and total organic C loads produced at the reactor outlets throughout the duration of the warm and cold runs. Note that the durations of the warm and cold runs were different, and therefore the loads between the runs should not be compared.

Analyte†	Temperature	Substrate‡				
		CS	CC	CC-WC	BS	WC
	°C	g column^{-1}				
TC	15.5	5.5 (0.1)C§	23.7 (4.9)A	12.2 (1.5)B	6.4 (1.4)C	1.2 (0.2)D
TOC	15.5	1.0 (0.2)C	15.7 (4.8)A	6.0 (1.2)B	1.8 (0.9)B	1.0 (0.3)C
TC	1.5	3.5 (0.1)A	3.9 (0.6)A	4.2 (0.7)A	3.1 (0.2)A	2.5 (0.03)A
TOC	1.5	1.6 (0.1)A	1.8 (0.4)A	2.0 (0.6)A	1.3 (0.1)A	1.6 (0.04)A

† TC, total C; TOC, total organic C.

‡ BS, barley straw; CC, corn cobs; CC-WC, corn cobs in series with wood chips; CS, corn stover; WC, wood chips.

§ Values are mean (SE).Within a row, means followed by the same uppercase letter are not significantly different at $P \leq 0.05$.

Table 3. Effect of substrate and position within the denitrification bioreactor on mean abundance of the N_2O reductase *nosZ* gene (clade 1) and effect of substrate and temperature on mean abundance of the N_2O reductase *nosZ* gene (clade 2).

	Substrate†			
	CC	CS	BS	WC
Position‡	**Abundance (log_{10} gene copy g^{-1} substrate) (clade 1)**			
Inlet	9.59bAB§	10.44aA	9.74aA	8.70aB
Outlet	10.95aA	10.14aAB	9.80aB	8.53aC
Temperature¶	**Abundance (log_{10} gene copy g^{-1} substrate) (clade 2)**			
15.5°C	10.32aA	7.79aB	6.05aC	5.61aC
1.5°C	7.03bA	7.66aA	7.39aA	6.12aA

† BS, barley straw; CC, corn cobs; CS, corn stover; WC, wood chips.

‡ Means are averaged over the 1.5 and 15.5°C treatments.

§ For each variable, means within the same row followed by the same uppercase letter are not significantly different ($P < 0.05$). Means within the same column followed by the same lowercase letter are not significantly different ($P < 0.05$).

¶ Means are averaged over the inlet and outlet sampling positions.

the greatest abundance. The abundance of clade 2 *nosZ* was strongly affected by temperature on CC but not on the other substrates. The abundance of clade 2 *nosZ* on the different substrates is generally substantially less than 1% of the clade 1 *nosZ* abundance (Supplemental Table S12), except for clade 2 on CC at 15.5°C.

The CC-WC bioreactor had samples collected nearer the inlet (CC) and nearer the outlet (WC). The inlet CC supported abundance of clade 1 and clade 2 *nosZ* genes similar to that measured in the CC bioreactor (data not shown). However, the WC at the outlet of CC-WC supported approximately 20-fold greater clade 1 *nosZ* gene abundance than at the outlet of the WC bioreactor (Supplemental Table S13). This stimulatory effect of CC on denitrifiers on WC was only observed at the 15.5°C temperature and was not seen for the clade 2 *nosZ* gene or the 16S-rRNA gene.

Dissolved N_2O Production

At 15.5°C, there was a highly significant ($P = 0.003$) sampling time × media interaction effect on dN_2O. Significant differences in dN_2O by media were observed on all sampling days except Day 77 (Table 4). On Day 2, effluent water from WC contained greater dN_2O than all other treatments. The BS treatment had greater dN_2O than CC-WC (Days 30, 49, 105), WC (Days 49, 105), and CC (Day 30). Corn stover had greater dN_2O than CC-WC (Days 105, 133) and CC (Day 105). At 1.5°C, there was a significant ($P = 0.035$) main effect of media, but the effects of sampling time ($P = 0.085$) and sampling time × media ($P = 0.104$) were not significant. Across all sampling

Table 4. Dissolved N_2O concentration (dN_2O), cumulative N_2O production (cpN_2O), and relative N_2O (rN_2O) in bioreactors operated at different temperatures and containing different agricultural residues as packing media.

Time†	Temperature	Substrate‡					
		All	CS	CC	CC-WC	BS	WC
d	°C						
				dN_2O, μg N L^{-1}			
2	15.5		4.8 (2.1)bB§	13.0 (6.5)aB	8.3 (5.5)aB	8.3 (2.4)cB	505 (117)aA
30			17 (16)bAB	5 (1)aB	2 (2)aB	71 (62)bA	29 (21)bAB
49			157 (92)bAB	204 (137)aAB	18 (10)aB	739 (385)aA	16 (13)bB
77			805 (142)aA	286 (55)aA	39 (27)aA	757 (412)aA	43 (30)bA
105			1025 (501)aA	241 (106)aBC	50 (9)aC	611 (332)aAB	61 (10)bC
133			1135 (649)aA	321 (86)aAB	164 (71)aB	633 (372)aAB	72 (3)bB
14, 42, 70, 98, 114	1.5¶		423 (85)AB	131 (19)B	150 (35)B	627 (64)A	290 (25)AB
				cpN_2O production, g N m^{-3}			
14–114	1.5, 15.5#		44.4 (11.4)A	29.5 (10.4)B	7.7 (3.5)B	56.0 (13.3) A	15.3 (5.1)B
				rN_2O, %			
14–114	1.5, 15.5††		4.84 (1.96)B	1.11 (0.39)C	0.92 (0.48)C	6.42 (2.37)AB	9.71 (3.00)A
	15.5	1.93 (0.65)b					
	1.5	7.47 (1.67)a					

† Time excludes downtime.

‡ BS, barley straw; CC, corn cobs; CC-WC, corn cobs in series with wood chips; CS, corn stover; WC, wood chips.

§ Values are mean (SE). Within a row, means followed by the same uppercase letter are not significantly different at $P \leq 0.05$; within a column for each variable, means followed by the same lowercase letter are not significantly different at $P \leq 0.05$.

¶ For dN_2O at 1.5°C, means and SE represent averages across sampling times.

For cpN_2O produced, means and SE represent averages across temperatures.

†† For rN_2O, means and SE by media represent averages across temperatures, and means and standard errors by temperature represent averages across media.

times, dN$_2$O was greater in BS compared with CC or CC-WC but with no differences between other treatments (Table 4). For cpN$_2$O, the main effect of medium was highly significant (P = 0.002), but the effects of temperature (P = 0.237) and media × temperature (P = 0.175) were not significant. Across both temperatures, cpN$_2$O was greater in both BS and CS compared with WC, CC, and CC-WC. For rN$_2$O, the main effects of temperature (P = 0.047) and media (P < 0.001) were each significant, but the temperature × media interaction effect was not significant (P = 0.054). Across both temperatures, rN$_2$O in CC and CC-WC were significantly less than all other treatments, whereas rN$_2$O in WC was significantly greater than all other treatments except BS (Table 4). The rN$_2$O at 1.5°C was greater than at 15.5°C consistently for all media; across all media, rN$_2$O at 1.5°C was nearly four times greater than at 15.5°C (Table 4).

Discussion

The WC and CC consistently removed the least and most nitrate, respectively, at both temperatures. The mixture of CC and WC, not surprisingly, fell between the two. The poor performance of WC relative to the other materials was within the range previously reported for wood-based media (2–22 g N m^{-3} d^{-1}) (Schipper et al., 2010; Christianson et al., 2012). The effect on WC performance that using fresh material from one living tree (further details are provided in the Supplemental Information) had is unknown. The WC N load reductions of <5% may be due to the relatively large nitrate-N concentration entering the bioreactor. As reported for other types of straw (Soares and Abeliovich, 1998; Aslan and Türkman, 2005; Greenan et al., 2006), BS's relatively high nitrate removal during the start of the warm run faded to one of the poorest performances by approximately Day 80. Corn stover was similar in NRR to BS, becoming less effective over time. The CC supported the greatest NRRs, and our rate of 34.9 g N m^{-3} d^{-1} (15.5°C) is similar to Cameron and Schipper's (2010) NRR of 34.6 g N m^{-3} d^{-1} for CC at 14°C.

The high abundance of the *nosZ* gene on the media/substrates is indicative of denitrification being responsible for nitrate-N removal in these non–N-limited bioreactors. The abundance of clade 1 *nosZ* was similar for CC, CS, and BS but was least for WC. Corn cobs removed more N than CS and BS, but there was little difference in the *nosZ* gene abundance on these three materials. This suggests that the differences in N removal among these materials are due to bioavailable C rather than denitrifier populations. This is consistent with the results from Warneke et al. (2011a), who observed similar differences in N removal and *nosZ* abundance between CC and wood materials with respect to N removal and denitrifier gene abundance. Warneke et al. (2011a) also observed a relationship between N removal and respirable C. We did not measure respirable C expressly, but the TOC loads (Table 2) are a possible indicator of bioavailable C and microbial activity. The TOC and TC loads follow the expected pattern at 15°C: greatest for CC, least for WC, with BS and CS intermediate. The initial LCI of the materials was lowest for CC, with CS and BS intermediate and WC highest. The LCI increases because cellulose degrades more quickly than lignin.

We detected the clade 2 *nosZ* gene on all bioreactor materials. Clade 2 abundances were less than those of clade 1 but greater than that reported by Hathaway et al. (2015) for woodchip bioreactors in Illinois. All materials supported greater clade 2 *nosZ* abundance than soil (Jones et al., 2013), which is consistent with previous comparisons of denitrifiers in soil and bioreactors (Moorman et al., 2010).

Temperature affected both nitrate-N removal and microbial communities. The NRRs were generally 4- or 5-fold lower under cool conditions compared with warm for all the media except WC. The difference in N removal between the two temperatures is much less for WC than for the other materials, which is reflected in its lower Q_{10} value. Clade 1 *nosZ* abundance was similar at the two temperatures, which suggests that the lower N removals seen at the 1.5°C are likely due to reduced microbial activity rather than insufficient denitrifier biomass. Both TOC and TC in reactor effluent were reduced at the 1.5°C compared with 15.5°C, also suggesting reduced microbial activity. The different N removal response to temperature of the bioreactors may be due to differences in the denitrifier communities among the materials.

The CC-WC bioreactors were intermediate in NRRs to CC or WC bioreactors. However, the mean CC-WC NNR (29.3 g N m^{-3} d^{-1}) exceeded the volume-weighted mean NRR of CC and WC (13.0 g N m^{-3} d^{-1}), suggesting that CC had a greater than expected stimulatory effect. The export of TOC from the CC portion of the bioreactor to the WC portion caused an increase in the abundance of *nosZ* on the wood, although only under the higher temperature. This result suggests that more efficient bioreactors could be designed by mixing materials and by their selective placement within the bioreactor.

Nitrate removal rates for WC were consistently the lowest across all media types; thus, there is a strong possibility that denitrifying bioreactor performance could be improved with the use of agricultural residues as fill media. Releases of TOC and TC in bioreactor effluent indicate that CC, CS, and BS are more biodegradable than wood. At the higher temperature, the LCI increased for the agricultural residues, indicating these media were becoming more recalcitrant to a greater extent than under the cool conditions. Schmidt and Clark (2013) reported a similar final LCI of 0.44 for oak and pine denitrification media in small-scale tests, although their initial value of 0.25 was much lower than the treatments here. The WC used here had an initial LCI higher than the other media, indicating that it was the most recalcitrant; this observation may be correlated with this treatment's low NNR. Longer-term studies are needed to determine the longevity of these alternatives to wood.

Dissolved N$_2$O concentrations were generally the lowest for WC, CC, and CC-WC treatments, although CC had a much higher NRR than WC. The low N$_2$O emissions from WC may be due to less denitrification occurring in WC. Conversely, CC might have low N$_2$O emissions because denitrification was efficient. This work does not allow for speculation about the potential N$_2$O emissions from WC at longer HRTs, when nitrate removal would likely have been higher. The WC released greater mean N$_2$O concentrations than CC under cool conditions but generally lower mean N$_2$O concentrations under warm conditions. Warneke et al. (2011b)

also observed this trend with hardwood (eucalyptus) chips and maize cobs; at an outlet temperature of 16.8°C, $d$$N_2O$ concentrations for eucalyptus were higher than for maize cobs, but at 27.1°C maize cob N_2O emissions were higher (all under ~400 μg N_2O–N L^{-1}).

Conclusions

In this controlled laboratory study, denitrification was observed at 1.5°C, a temperature representative of early spring drainage in the northern United States. Although NRRs were lower than at 15.5°C, some nitrate removal can be expected under field conditions when drainage first begins in early spring, with agricultural residues performing better than wood chips. High *nosZ* gene copy numbers were found on all substrates, indicating substantial populations of denitrifiers. The lowest *nosZ* abundance was on the wood chips, which was also the medium with the smallest nitrate-N removal capacity and the greatest $r$$N_2O$ produced. Variations in nitrate removal (denitrification) are likely due to the fraction of bioavailable C that drives microbial denitrification. These results also show that the relative NRR performance of different bioreactor designs does not necessarily predict relative performance with respect to minimizing absolute amounts of N_2O production. Overall, the performance of a compartment of corn cobs in series before wood chips shows promise at the laboratory scale but needs to be evaluated at field scale.

Acknowledgments

The authors thank Dr. Debra Palmquist, Statistician General, USDA–ARS Midwest Area, for her contribution to the experimental design; Todd Schumacher, Biological Science Technician, USDA–ARS St. Paul, MN, for overseeing experimental operations; Taylor Hoffman, Liz Petesch, and Noah Slocum for tending the experiment; University of Minnesota for providing facilities; Ranae Jorgenson, Analytical Chemist, Minnesota Agricultural Utilization Research Institute (AURI), for analyzing material properties; and Alan Doering and Becky Philipp, AURI, for providing project organization. This work was supported by AURI and the Minnesota Corn Research and Promotion Council. Coauthor L.E. Christianson's time was supported through USDA–ARS Agreement No. 59-1930-0-046.

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