Received: 15 October 2020 | Accepted: 27 January 2021

DOI: 10.1002/agg2.20149

Agrosystems, Geosciences & Environment OPEN ACCESS

ORIGINAL RESEARCH ARTICLE

Environment

Impact of flow on woodchip properties and subsidence in denitrifying bioreactors

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Assigned to Associate Editor Joshua M. McGrath.

Abstract

Woodchip bioreactors are edge-of-field practices that remove nutrients from agricultural drainage water, with an effective lifespan estimated between 10 and 30 yr. Subsidence, or bioreactor settling and subsequent depression formation, is a concern of producers and stakeholders and little is known regarding its effect on bioreactor performance. Six woodchip bioreactors set at three different hydraulic residence times (HRTs 2, 8, and 16 h) were excavated after 2 yr of operation, with wood samples collected from multiple depths and distances from the bioreactor inlet. Subsidence was observed in all six bioreactors and was greater near the inlet. Particle-size distribution did not change over the study period, indicating that smaller woodchips were not degrading preferentially or washing out of the bioreactor while the macropore space was simultaneously decreasing. Flow path analysis showed an increase in Morrill Dispersion Indices and short-circuiting as well as decreases in drainable porosity and hydraulic efficiency; these changes were uniform across all three HRTs, suggesting that the decline in hydraulic properties was independent of flow. Further, despite increased woodchip decomposition as measured by C/N ratio in the 2-h HRT bioreactors (mean ± SD = 64.9 ± 13.7) compared with the 8- and 16-h HRT systems (90.3 ± 19.0, 95.6 ± 27.2, respectively), denitrification was still supported at all HRTs based on the results from a batch denitrification test. To offset wood aging, bioreactor fill material nearest the inlet could be replenished without excavation of the entire bioreactor.

1 | INTRODUCTION

Excessive nutrient loading resulting in rapid production of biomass and subsequent oxygen depletion has caused the hypoxic zone that has plagued the northern Gulf of Mexico

Abbreviations: DO, dissolved oxygen; DOC, dissolved organic carbon; HRT, hydraulic residence time; LCI, ligno-cellulose index; MDI, Morrill Dispersion Index; S, short circuiting

since the 1970s. The dead zone has been linked to human population growth in coastal areas, increasing agricultural activity in upland watersheds, and growing demand for food and energy (Rabalais et al., 2002). Subsurface agricultural drainage networks in the Upper Mississippi River Basin contribute to eutrophication by serving as efficient conduits from row-crop fields (Ikenberry et al., 2014; Mitsch et al., 2001; Schilling & Helmers, 2008). Further, bioavailable forms of nitrogen such as nitrate have been specifically linked to the Gulf of Mexico hypoxic zone due to increased mobility in the dissolved form. In 2008, the Gulf of Mexico Hypoxia Action Plan called on Midwest states to reduce their contributions to nutrient loading within the Mississippi River Watershed and many conservation practices have been developed to achieve these water quality goals (Iowa Department of Agriculture & Land Stewardship, 2017; Mississippi River/Gulf of Mexico Nutrient Task Force, 2008).

Conservation practices that have demonstrated nitrate reduction practices can be classified as land conversion, in-field, or edge-of-field; examples of land conversion and in-field practices include retiring land, converting to perennial land cover, or changing the timing and rate of nitrogen application in corn (*Zea mays* L.) production (Christianson et al., 2013). However, most in-field management practices only have the capacity to decrease nitrate loads by 4–10% (Randall et al., 2003). Cover crops are an in-field practice that have higher nitrate removal potential (24–60% reduction) but require extensive management on the part of the producer (Kaspar et al., 2007, 2012). Additionally, the economic feasibility of broad land conversion is questionable (Qi et al., 2011; Tomer et al., 2010). Edge-of-field practices have the advantage that they minimally impact crop yield and are low-maintenance, which can otherwise be a barrier to practice implementation (Liu et al., 2018). Therefore, edge-of-field practices with high nitrate load reduction potential are attractive options for meeting nutrient reduction goals.

Since the early 1990s, edge-of-field denitrifying bioreactors have been widely studied due to their potential to significantly reduce nitrate loading, with observed average seasonal load reductions ranging between 40–60%, but with the potential to be as high as 80–95% (Addy et al., 2016; Blowes et al., 1994; Christianson et al., 2012; Greenan et al., 2009; Schipper et al., 2010a). Bioreactors are a habitat for a complex consortium of microbial species (Jang et al., 2019; Porter et al., 2015; Yao et al., 2020), which enable denitrification processes (Dandie et al., 2008; Kraft et al., 2011; Kuypers et al., 2018; Melillo et al., 1984). Denitrifying bioreactors utilize a lignocellulosic substrate (typically woodchips) that a population of microorganisms, including denitrifying species, metabolizes via reduction of nitrate to nitrogen gas (Christianson et al., 2011; Schipper et al., 2010b).

Two main processes strike a balance within denitrifying bioreactors. On one hand, anaerobic conditions have been shown to promote the denitrification process because oxygen is preferred as an electron acceptor over nitrate (Averill & Tiedje, 1982; Tesoriero et al., 2000; Warneke et al., 2011). However, the breakdown of cellulose is a process that is thought to be mainly mediated by various obligate-aerobic fungal species due to their ability to secrete hydrolytic enzymes and physically compromise the woodchip cell walls with their hyphae (Eriksson et al., 1990); anaerobic conditions have been shown to inhibit this catabolic process (Mattila et al., 2020; Tavzes et al., 2001). As the organic fill material in an aging bioreactor is consumed through denitrification, redox potential increases and nitrate removal efficiency decreases in the long term (Easton et al., 2015; Elgood et al., 2010). Further, differences in media redox potential and oxygen levels within denitrifying bioreactors can stratify microorganism populations spatially (Jansen et al., 2019; Porter et al., 2015). The organisms and mechanism of denitrificationand the species performing lignin and cellulose degradation are targets for further study (Brown & Chang, 2014; Janusz et al., 2017).

Extensive engineering optimization of denitrifying bioreactor systems has occurred regarding media, geometry, managing bypass flow, and hydraulic retention time (HRT) (Cameron & Schipper, 2010; Christianson et al., 2011; Hoover et al., 2016; Martin et al., 2019). Hydraulic retention time has been emphasized to maximize the volume treated while still constraining these systems to a reasonable size. Typically, HRT is selected to between 4–8 h to allow for substantial mass removal of nitrate while minimizing by-pass flow, with HRTs less than 6 h, exhibiting decreased cumulative nitrate removal and longer HRTs required to reach the same nitrate removal efficiency at lower temperatures (Addy et al., 2016; Christianson et al., 2011; Hassanpour et al., 2017; Warneke et al., 2011). Denitrifying bioreactor fill material and longevity has not previously been studied as a function of HRT. Recent studies evaluated the lifespan of the bioreactor fill material as these systems were initially designed to require little intervention by producers (Christianson et al.,

Core Ideas

- Hydraulic residence time (HRT) is optimized to maximize nitrate removal in denitrifying bioreactors.
- Three HRTs were controlled in triplicate pilot-scale bioreactors.
- Hydraulic properties did not differ after 3 yr at different HRTs.
- Woodchip degradation is impacted by HRT due to differences in influent nitrate load.

FIGURE 1 The concrete-lined trenches were filled to the top with woodchips in 2014

2020; Ghane et al., 2018). Initial estimations of the timeframe for replenishing the fill material were 10-yr intervals based on the rate of ligno-cellulosic substrate depletion, although decades-long intervals have since been estimated (Moorman et al., 2010; Robertson et al., 2000, 2008). Since woodchip bioreactors are a relatively new conservation practice, there are limited field studies of their performance over longer time scales to validate this assumption. Additionally, few studies have examined the long-term performance and substrate characteristics of a woodchip bioreactor, but differences in woodchip particle-size, C/N ratio, and carbon quality as measured by the lignocellulosic index (LCI) have been demonstrated with degradation resulting in mass loss, lower C/N ratios, and higher LCIs (Ghane et al., 2018; Moorman et al., 2010). Further, woodchip degradation has not yet been evaluated as a function of operational flowrate or HRT. Flowrate is known to be associated with dissolved oxygen (DO) levels within bioreactors and oxygen is associated with rapid woodchip degradation (Ghane et al., 2018). Specifically, the changes in the properties of the woodchips themselves contribute to the changes in overall hydraulic properties of the bioreactors. There have been few long-term monitoring studies of denitrifying bioreactors due to the newness of the technology. Therefore, there is a need to study the effects of HRT on the decomposition of woodchip fill material and thus the implications for woodchip bioreactor lifespan. Determining the effect of HRT on woodchip decomposition will allow better estimation of the design lifetime of denitrifying bioreactors.

The objective of this study is to determine the impact of varying HRT on indicators of bioreactor aging.

2 | MATERIALS AND METHODS

2.1 | Site description

The replicate woodchip bioreactors at the Iowa State University (ISU) Agronomy and Agricultural Engineering Research Farm (42.019861, −93.776872) provide a unique opportunity to evaluate the effect of HRT on the aged characteristics of this conservation practice (Hoover et al., 2017). The experimental set-up at ISU is part of a long-term monitoring study of denitrifying bioreactors, and in 2018 excavation was done to exchange portions of the woodchip media for corn cobs. The site is described extensively in Hoover et al. (2017). Briefly, nine woodchip bioreactors were installed in parallel in September 2014. Each bioreactor consists of a concrete trench filled with a mixture of hardwood chips obtained from Golden Valley Hardscapes, Story City, IA. The trenches were filled to cover the trench sides with woodchips (Figure 1).

A 11,356-L (3,000-gallon) storage tank intercepts a nearby 3.5-cm (12-inch) diameter county tile line and an underground storage cistern supplies water to the bioreactors. Each bioreactor is fitted with its own influent control structure and dosing port. The outlet structures were fitted with stoplog drainage control structures, which can be adjusted to change the active volume of the bioreactors. Three HRTs (2, 8, and 16 h) are controlled in triplicate at this site. The bioreactors were saturated throughout the year, with the flow through the bioreactors occurring during the sampling season. The bioreactors remained saturated during winter months to inhibit rodents from bedding into the woodchips.

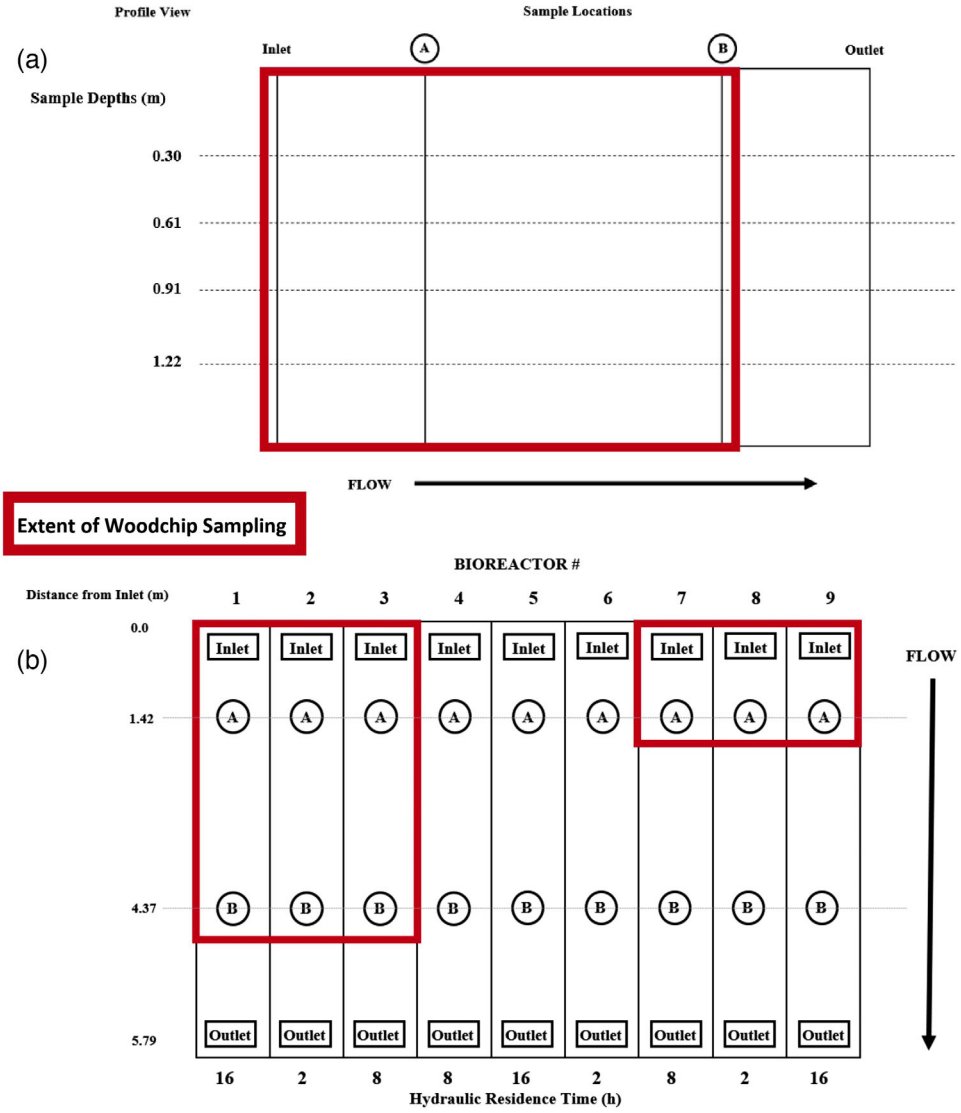

FIGURE 2 (a) Profile view of woodchip sampling plan in 2018. Woodchips were collected from four depths: 0–0.30 m, 0.30–0.61 m, 0.61–0.91 m, and 0.91–1.22 m. (b) Top view of woodchip sampling plan in 2018

2.2 | Woodchip preservation, transport, and analysis

Six woodchip bioreactors were installed in fall 2014 and operated continuously during summer months beginning in 2016 through 2018. Excavation was initiated to change the experimental conditions by replacing the one-fourth or three-fourths proportion of the woodchips closet to the inlet with corncobs. Woodchips were sampled from three locations within replicate denitrifying bioreactors for analysis and a tracer study was conducted to evaluate hydraulic properties.

Three bioreactors were left undisturbed and served as the source of woodchips for a denitrification assay in 2019. The soil caps of the excavated bioreactors were removed to partially expose the tops of the concrete trenches. For all excavated bioreactors, woodchips were sampled from 0.30-, 0.61-, 0.91-, and 1.22-m vertical depths at the inlet and at a location one-third the length of the bioreactor (Figure 2a). To examine impact of distance from bioreactor inlet length on woodchip properties, additional samples from a distance of two-thirds the bioreactor length were taken from an additional three bioreactors, for a total of 8 or 12 sample locations from each bioreactor, depending on the excavation (Figure 2b). The overburden was removed using an excavator and remaining soil material was removed with a shovel. Representative samples from 0–0.3 m were collected using a shovel. Woodchips from the 0.30-to-0.61-m, 0.61-to-0.91-m, and 0.91-to-1.22-m depths were collected by shovel or post hole digger. All samples were mixed in a bucket or plastic tote before storage in a labeled 4-L (1-gallon) resealable plastic bag. All

FIGURE 3 Subsidence was measured during the 2018 excavation from the top of the concrete lining to the top of the woodchips with a tape measure

samples filled at least 75% of the gallon bag. Woodchip samples were stored in a cooler and transported to ISU, where they were stored at 4 ˚C. Subsidence (or settling) of the woodchips below the depth of the original fill on the ISU bioreactors was observed and measured with a tape measure at the Inlet location and Location B, 4.3 m from the inlet, during the 2018 excavation (Figure 3). Observing subsidence as early as 3 yr into bioreactor operation is of note and could be concerning to producers and stakeholders.

2.3 | Tracer study to determine hydraulic characteristics

Potassium bromide (KBr) tracer tests were performed in May 2015 prior to system operation and in May 2018 to study changes in flow characteristics during 2 yr of operation. The bioreactor tracer study water samples were analyzed for Br^- using a Dionex ICS-2100 Ion Chromatography System (Thermo Fisher Scientific) using method EPA 300.1 (Pfaff et al., 1997). The results from the flow path analysis provide insight into the Morrill Dispersion Index (MDI), short-circuiting (S), and hydraulic efficiency (λ) for each bioreactor. Briefly, MDI is a dimensionless measure of the amount of fluid recirculation or deviation from plug flow. An MDI of 1.0 indicates ideal plug flow, and values of 22 or greater indicate complete-mix reactor conditions. Hydraulic efficiency is a different metric for evaluating plug flow conditions, with values between .5 and .75 being considered satisfactory (Hoover et al., 2017). Short-circuiting is indicated with a value near zero, and an S value of 1.0 indicates ideal conditions (no stagnation) (Ta & Birgnal, 1998).

Flow-path analysis was performed as outlined in Hoover et al. (2017). Flow rates were adjusted at the beginning of the study to achieve retention times of approximately 4 h. A 1-L dose of 36.5 g KBr L^{-1} was introduced into each dosing port and outflow samples were collected for 1,180 min in 20-min increments. The Br^- concentrations at 20-min sample intervals were used for MDI calculations to maintain even sampling intervals. Short circuiting, S, was calculated using the Ta and Birgnal (1998) method and hydraulic efficiency was calculated using the Persson et al. (1999) method, as described in Hoover et al. (2017). Drainable porosity was determined by the following: the saturated depth in each bioreactor well was measured and the average saturated depth for each bioreactor was calculated. The bioreactors were gradually drained by gravity so that all the flow volume was measured by the individual flow meters at the outlet of each bioreactor. The final flow volume was recorded after 2 d. Drainable porosity was calculated by dividing the flow volume by the saturated volume of the bioreactor.

2.4 | Woodchip particle size, particle density, and chemical composition analysis

Approximately 750 g of woodchips were weighed and placed in a 23 by 28 cm aluminum pan and oven-dried at 60 ˚C for 48 h or until no change in mass was observed. The mass at which further drying produced no change was used to determine gravimetric moisture content. To determine the C and N content, the woodchips were ground in a coarse Wiley mill (2.0 mm) followed by a fine Wiley mill (1.0 mm) and placed in labeled bags before shipment to the USDA-ARS Laboratory in Saint Paul, MN. Prior (>24 h) to samples being weighed for C/N analysis, paper bags containing the samples were again oven-dried at 60 ˚C to ensure all moisture had been removed. Each sample was analyzed in triplicate for percentages of ash, C, and N. The C/N ratio was calculated. Ash content was determined by combustion at 550 ˚C for 4 h in a muffle furnace. Carbon and N analyses were performed with the Dumas combustion method using an element analyzer (vario MAX cube, Elementar). Dried samples were analyzed for cellulose, lignin, and hemicellulose composition, also called "carbon quality" as in Ghane et al. (2018). Briefly, Klason lignin, glucose, mannose, xylose, galactose, and arabinose

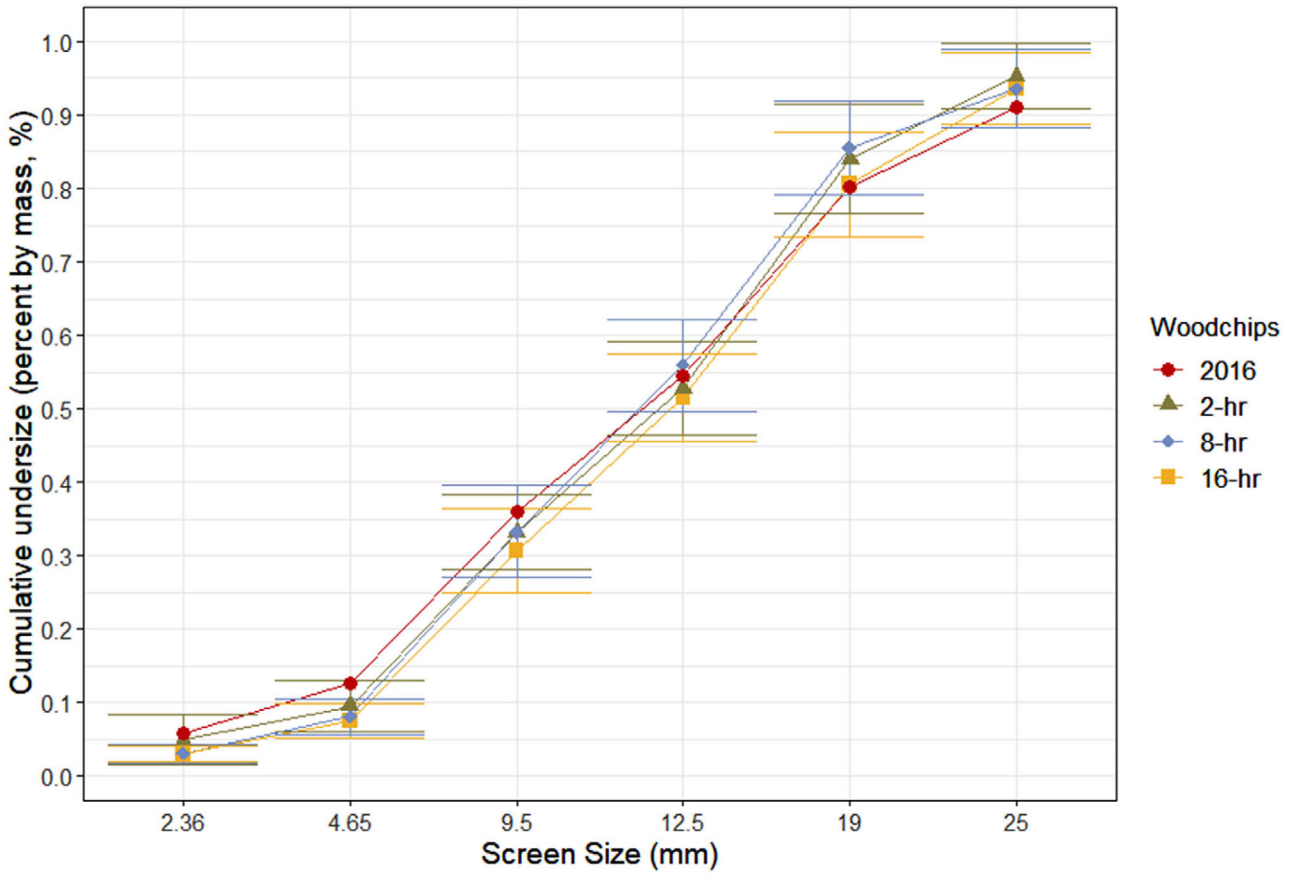

FIGURE 4 Mean particle-size distributions of the aged woodchips compared with the distribution reported in 2016 (Hoover et al., 2017). Error bars represent 1 SD

concentrations after acid hydrolysis were determined twice using a high-performance liquid chromatograph (model 1525 binary pump, Waters Corp.) and pre-packed carbohydrate analytical column (Aminex HPX-87P, Bio-Rad). An in-line de-ashing cartridge (no. 125-0118, Bio-Rad) was used with the carbohydrate analytical column and a refractive index detector (model 2414, Waters Corp.) at a flow rate of 0.3 ml min^{-1} and 80°C column temperature (Sluiter et al., 2012).

Approximately 100 g oven-dried batches of each sample were sieved with a vibratory shaker for 10–15 min through a nested sieve set with the sieve sizes indicated in the axis range of Figure 4 and a catch pan. The mass of woodchip material in each sieve was recorded to determine the particle-size distribution curve and median particle size (D50). To determine woodchip particle density, woodchip samples in the previously described resealable gallon bags were saturated with deionized water for 24 h. Additionally, a sample of fresh woodchips collected from Golden Valley Hardscapes was also saturated in the same way. A corner was cut from each bag and the bags were drained by gravity for 24 h. A 100-ml aliquot of deionized water was added to a 250-ml graduated cylinder and the volume was recorded. The volume change was recorded when 50 g of woodchips were added to the graduated cylinder and fully submerged to determine the particle volume and this volume was used to calculate particle density.

2.5 | Batch kinetic study

To assess the NO_3^-–N removal capability of the aged woodchips, a batch kinetic study was conducted. Four types of woodchips were used in the study: those collected from each of the undisturbed bioreactors at the ISU Agronomy and Agricultural Engineering Farm (2, 8, and 16-h HRT samples) and fresh hardwood chips that had not been used as a denitrifying substrate. Woodchips were excavated from the top 10 cm of bioreactors 4, 5, and 6 (8, 16, and 2 h HRTs, respectively) at a location 1.1 m from the inlet at the ISU Agronomy and Agricultural Engineering Farm in October 2019. Four-liter (1-gallon) resealable bags were filled approximately 75% full of woodchips and transported in a cooler to ISU, where they were stored at 4 °C until the batch study was performed. Nutrient solution (25 L of 30.0 mg L^{-1}) was prepared according to Hoover et al. (2016). Triplicate 10.0-g samples of woodchips

from each bioreactor were aliquoted into clean, quart-sized, glass Mason jars for each time point for a total of 30 jars per bioreactor. The fresh woodchips were saturated with 190 ml of DI water for 48 h at 4 ˚C prior to the test to prevent the woodchips from floating during the batch test. To bring the final concentration of NO_3^-–N to 30.0 mg L^{-1} in each jar, 200 ml of nutrient solution was added to each jar of aged woodchips, and a spike of 10.0 ml of concentrated nutrient solution was added to the jars of fresh woodchips. The jars were gently swirled, sealed, and kept at 21 ˚C during the test. At timepoints of 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 12, 24, 36, and 48 h, three jars from each were destructively sampled. The jars were unsealed, and liquid was decanted into 125-ml Nalgene bottles. The samples were stored on ice before vacuum filtering with a 45-µm filter for NO_3^-–N analysis on the AQ2 (Seal Analytical) with method EPA-114-A, Rev. 7 (equivalent to USEPA method 353.2 ver.2, 1993) (O'Dell, 1993).

2.6 | Data analysis

Data manipulation, statistical analyses, and figure generation were performed using the RStudio software package with R version 4.0.0 (R Core Team, 2020). R packages tidyverse, Hmisc, olsrr, and rstatix were used for data analysis (Harrell et al., 2020; Hebbali, 2020; Kassambara, 2020; Wickham et al., 2019). The distribution of observations for woodchip C/N ratio, woodchip chemical composition, D50 value, and particle density were tested for normality with the Shapiro-Wilk test ($p < .05$). A factorial ANOVA was conducted to compare the main effects of sampling location, depth, and HRT and the interaction effect of location, depth, and HRT on woodchip C/N ratio, woodchip chemical composition, D50 value, and particle density. Location included three levels (Inlet, A, B), depth included four levels (0.30, 0.61, 0.91, and 1.22 m) and HRT included three levels (2, 8, and 16 h). This ANOVA technique was also used to assess statistical differences in the mass retained in each sieve to compare particle-size distributions. A separate factorial ANOVA was performed to compare the main effects of location and HRT and the interaction effect of location and HRT on bioreactor subsidence. Location included two levels (Inlet, B) and HRT included three levels (2, 8, and 16 h). We used a Tukey post hoc analysis between factors in RStudio for both ANOVA analyses to determine differences between main effect means and interactions.

The following analyses were also carried out in R. Two-sided student's *t* tests were used to compare means of the flow characteristics (MDI, S, drainable porosity, and hydraulic conductivity) between years 2016 and 2018. Carbon and N concentrations were compared to literature values with one-sided student's *t* tests. Linear regression was used to fit a zero-order model to the nitrate nitrogen (NO_3^-–N) concentration over time in the batch removal test. Time and three indicator variables representing woodchip type were used as explanatory variables in a multiple linear regression to test for significant differences in the regression slopes.

3 | RESULTS

3.1 | Flow-path analysis

The MDIs determined in 2018 (2.9–3.9) (Supplementary Table S1) were higher than the 2016 MDIs (2.8 ± 0.3) for all bioreactors except for Bioreactor 9 (MDI = 2.7, HRT = 16 h) (Table 1). The highest MDI observed was 3.9 in Bioreactor 5 (HRT = 16 h). This suggests a deterioration of ideal plug flow toward flow with greater dispersion (Metcalf & Eddy, 2003). However, the calculated MDIs in this study are similar to other reported MDI values for field woodchip Bioreactors of 3.5 and 4.2 (Christianson et al., 2011). Similarly, the calculated short-circuiting values (S) are all lower than the mean 2016 value and range from 0.61 in Bioreactor 5 to 0.69 in Bioreactor 7. An S value of 1.0 indicates ideal flow, while an S value of 0.0 indicates short-circuiting (Ta & Brignal, 1998). Short-circuit-like conditions increased in all reactors in 2018 when compared to 2016. All drainable porosity values determined in 2018 are lower than the 2016 average, indicating a decrease in pore volume. The lowest drainable porosity was observed in Bioreactor 2 (0.41 m^3 m^{-3}, HRT = 2-h), and the highest drainable porosity was observed in bioreactors 1 and 6 (0.47, HRT = 16 and 2 h, respectively). All the calculated hydraulic efficiency values in 2018 were greater than 0.50, which is considered satisfactory (Hoover et al., 2016). The lowest hydraulic efficiency was observed in Bioreactor 5 (0.60) and the highest hydraulic efficiency was observed in Bioreactor 9 (0.80). The mean of each flow characteristic determined in 2018 was statistically different than the mean determined in 2016 ($p < .01$, Student's t test, unequal variance).

3.2 | Bioreactor subsidence

The subsidence for six woodchip bioreactors ranged between 10.8 and 25.4 cm, with a mean of 17.3 ± 4.6 cm (Figure 5). Subsidence generally increased at the Inlet location when compared to Location B, 4.3 m from the inlet. The ANOVA main effect for location yielded an F ratio of $F(1, 30) = 17.067$, $p < .05$, indicating a significant difference between the Inlet location and Location B. Additionally, we observed that subsidence increased for the 2-h HRT compared with the 8-h and 16-h HRTs (Figure 5).

TABLE 1 Hydraulic properties of nine denitrifying bioreactors before and after 2 yr of operation

	Unit	2018 Avg. ± SD	2016 Avg. ± SD	Percentage change
Tracer residence time	h	5.3 ± 0.9	2.3 ± 0.3	N/A (test parameter)
MDI		3.3 ± 0.4	2.8 ± 0.3	+17.9%
Short circuiting (S)		0.66 ± 0.02	0.73 ± 0.03	−9.6%
Drainable porosity	$m^3\ m^{-3}$	0.45 ± 0.021	0.51 ± 0.02	−11.7%
Hydraulic efficiency (λ)		0.70 ± 0.06	0.78 ± 0.03	−10.3%

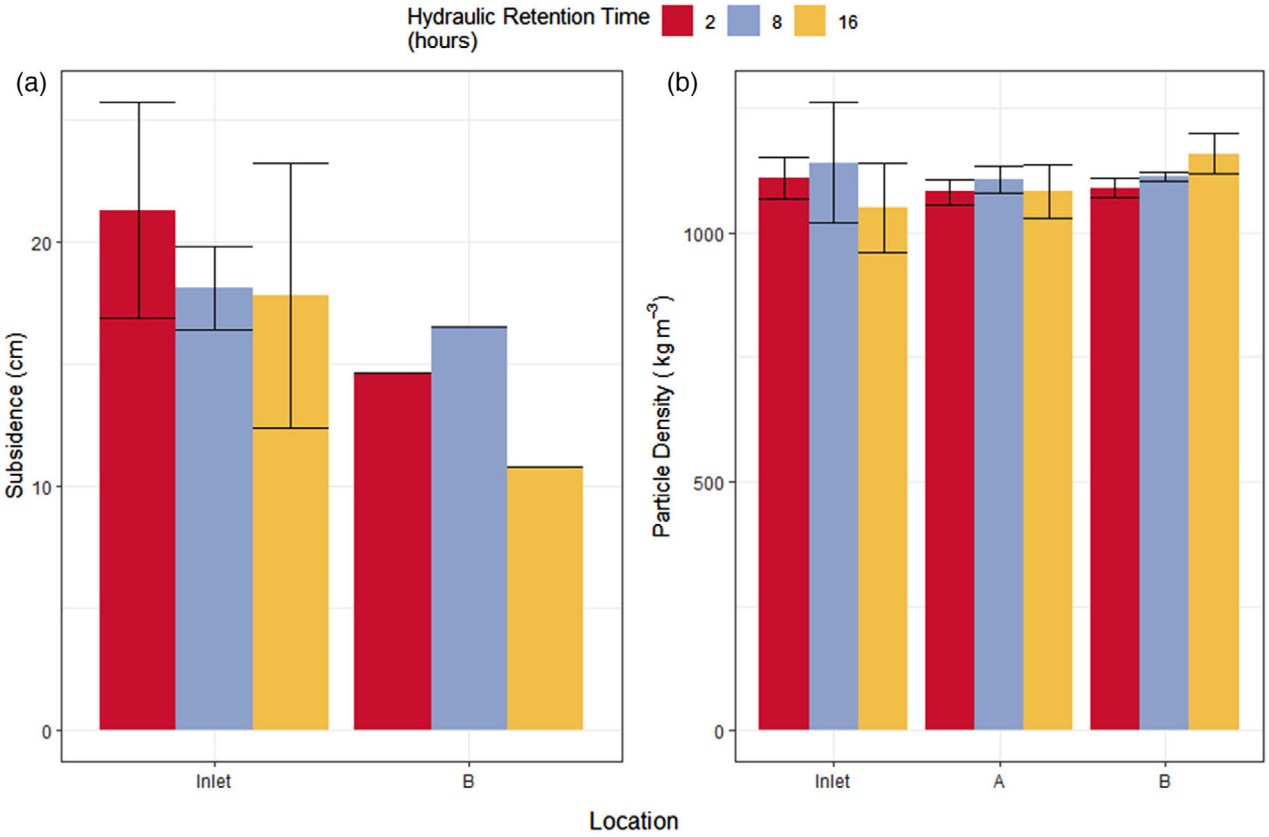

FIGURE 5 (a) Mean subsidence in centimeters at the Inlet and B locations for denitrifying bioreactors operating at three hydraulic retention times. Error bars represent 1 SD. Only one measurement was taken at Location B due to the sampling design. (b) Mean particle density of woodchips sampled from three locations for denitrifying bioreactors operating at three hydraulic retention times. Error bars represent 1 SD

3.3 | Woodchip properties

Particle density was not a parameter that was determined in 2016; therefore, comparisons between years are not discussed here. The particle densities were similar at all three locations for all bioreactors. Particle density was not a factor that was affected by hydraulic retention time, location, or depth (Figure 5). The woodchip particle-size distribution for all HRTs was similar to the distribution reported in 2016 (Figure 4). There was only one analysis conducted for the particle-size distribution in 2016 ($n = 1$), and as a result there is little statistical power to compare to the 2018 woodchips. There was a statistically significant difference in the mass retained in the 19-mm sieve from the 8-h HRT bioreactor compared with both the 2-h and 16-h woodchips ($p < .05$) but no other statistical differences in percentage mass retained within sieves among woodchip type were observed. Results of the 3-way ANOVA showed D50 was not a parameter that appeared to be influenced by HRT, location, depth, or the interaction between these effects $F(12, 24) = 0.548$, $p > .05$).

The reported C/N ratio for fresh mixed hardwood chips from the same supplier as those used in this study is 247 (Christianson et al., 2010). Others have reported C/N ratios ranging between 224 and 496 for hardwood, softwood, or

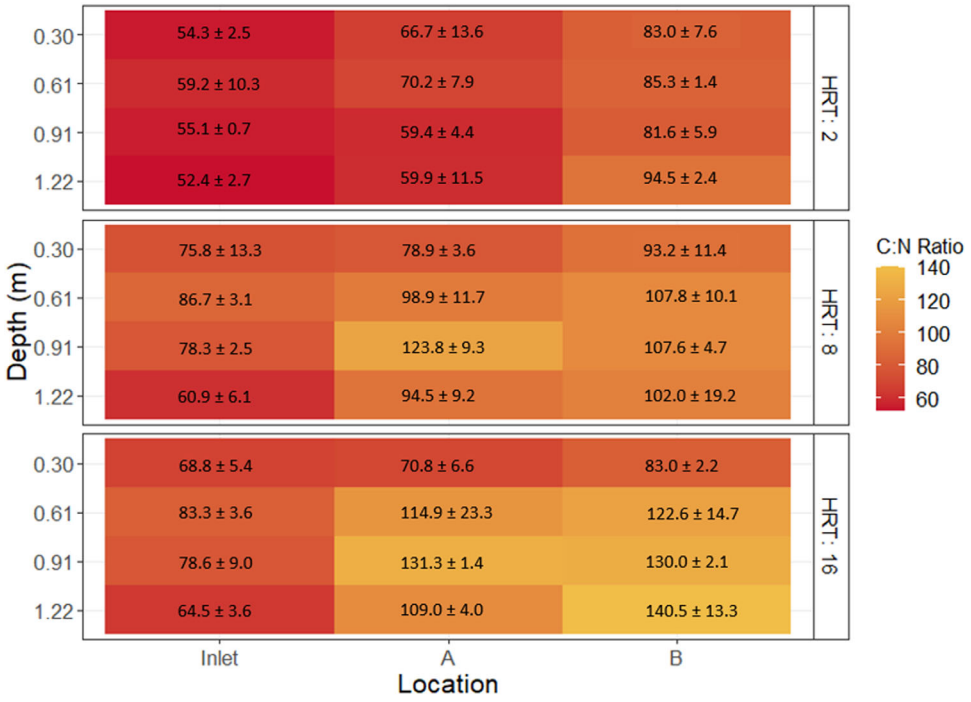

FIGURE 6 Heatmap comparing C/N ratios by HRT, location, and depth. Figure text is C/N ratio ± SD

TABLE 2 Mean C/N ratios for combinations of HRT and Depth and HRT and Location. Within a row, means followed by the same lowercase letter are not significantly different ($p < .05$). Within a column, means followed by the same uppercase letters are not significantly different ($p < .05$)

Depth and location	Hydraulic retention time		
	2 h	8 h	16 h
	C/N ratio (mean ± SD)		
Depth, m			
0.30	65.0 ± 13.2 aA	80.5 ± 10.5 bA	72.4 ± 8.2 abA
0.61	68.8 ± 12.6 aA	95.8 ± 11.8 bB	103.4 ± 22.1 bB
0.91	62.1 ± 11.0 aA	102.4 ± 22.0 bB	109.9 ± 27.1 bB
1.22	63.8 ± 17.6 aA	82.6 ± 20.6 bC	97.5 ± 31.0 cB
Location			
Inlet	55.3 ± 5.9aA	75.4 ± 11.4bA	73.8 ± 9.4bA
A	64.0 ± 9.5aB	99.0 ± 18.2bB	106.5 ± 25.0bB
B	86.1 ± 6.8aC	102.7 ± 12.3bC	119.0 ± 24.3cC

mixed hardwood woodchip varieties (Ghane et al., 2018). The C/N ratios of the aged woodchips were below this range for all hydraulic retention times, locations, and depths (Figure 6). Additionally, the highest C/N ratios were observed in the 16-h HRT bioreactors at Location B, whereas the lowest C/N ratios were observed in the 2-h HRT bioreactors at the Inlet location. There were statistically significant interaction effects between HRT and depth $F(6, 24) = 5.569$, $p < .05$), HRT and location $F(4, 24) = 4.955$, $p < .05$), and depth and location $F(6, 24) = 4.739$, $p < .05$) on C/N ratio. These differences are further displayed in Table 2. The Tukey post-hoc comparisons showed statistically significant differences ($p < .05$) in C/N ratios between all three locations (Inlet, A, B). There were statistically significant differences in C/N ratios in the 2-h HRT and both the 8-h and 16-h HRTs. There were statistically significant C/N ratios between the 0.30-m and 0.61-m depth, the 0.31-m and the 0.91-m depth, and the 0.91-m and the 1.22-m depth. When comparing the combined effect of HRT and depth, there were statistically significant differences between the 2-h HRT bioreactor and the 16-h HRT bioreactor at all depths except 0.30 m. The 0.30-m depth of the 16 h HRT was statistically different than all other depths in that bioreactor. None of the depths at any of the locations within the 2-h HRT bioreactor were significantly different from one another.

TABLE 3 Constituents of woodchips sampled from bioreactors operating at 3 HRTs compared with fresh woodchips. Means within columns followed by the same letters are not significantly different ($p > .05$)

Woodchips	Lignin	Glucose equivalents	Ash	LCI
	%			
2-h HRT	39.8 ± 3.6 a	29.8 ± 2.4 a	22.4 ± 13.3 a	0.61 ± 0.09 a
8-h HRT	36.6 ± 1.4 b	32.4 ± 2.1 b	12.2 ± 6.9 b	0.57 ± 0.06 b
16-h HRT	35.8 ± 3.6 b	33.5 ± 3.6 b	9.9 ± 6.3 b	0.56 ± 0.04 bc
Fresh	30.9 ± 0.03 c	34.0 ± 0.8 b	4.25 ± 0.13 b	0.51 ± 0.01 c

There were statistically significant differences in C/N ratio between the 2-h HRT Inlet location and all other locations in the 8-h and 16-h HRT bioreactors (Table 2). The 2-h Inlet location was statistically different than the 2-h B location. The 2-h A and B locations were significantly different than both the 16-h A and B locations. The 8-h Inlet location was statistically different than the 8-h A and B locations and the 16-h Inlet location was significantly different than the 16-h A and B locations.

Typical carbon concentrations (C%) for woodchips used in denitrifying bioreactors range between 47.0 and 51.0% and typical nitrogen concentrations (N%) range between 0.1 and 0.21% (Ghane et al., 2018). The C% was lower in all bioreactor HRTs in 2018 compared with the mean literature value (one-sided t test, $p < .05$) (data not shown). Additionally, the main effects of location and HRT on C% were significant ($F(2, 24) = 5.018$, $p < .05$ and $F(2, 24) = 7.692$, $p < .05$). Similarly, N% was higher in wood from all bioreactor HRTs compared with the mean literature value ($p < .05$; data not shown). The main effects of location and HRT were significant on N% ($F(2, 42) = 7.421$, $p < .05$; $F(2,24) = 11.741$, $p < .05$).

The effect of HRT on lignin content, glucose content, and ash content were all significant ($F(3, 24) = 18.769$, $p < .05$; $F(3,24) = 5.665$, $p < .05$; $F(3, 24) = 7.750$, $p < .05$) (Table 3). The mean LCI for all aged woodchips was statistically different than that observed in the fresh woodchips ($p < .05$). The glucose content in the 2-h HRT woodchips was statistically different than the 16-h HRT and fresh woodchips ($p < .05$). The mean glucose content of the fresh woodchips was higher by 2.1% than that measured in the aged woodchips ($p < .05$). Additionally, mean ash and mean lignin contents of the fresh woodchips were lower than those measured in the aged woodchips by an average of 10.6 and 6.5%, respectively ($p < .05$). The 2-h HRT woodchips showed the largest percentage decrease compared with the fresh woodchips in glucose (4.2%) and largest percentage increases in lignin (8.9%) and ash (18.2%) content. Statistical differences in ash content between the 2-h HRT woodchips and the 8-h HRT, 16-h HRT, and fresh woodchips were observed ($p < .05$). These results are consistent with the increased LCIs observed in all aged woodchips.

3.4 | Batch kinetic study

The 2-h HRT woodchip NO_3^-–N concentrations observed after 48 h were significantly different (p-adjusted $< .05$) than the 8-h and 16-h HRT woodchips (Figure 7). All aged woodchips showed greater concentration reductions over 48 h compared with the fresh woodchips. The woodchips sampled from the bioreactor operating at the 2-h HRT showed the greatest concentration reduction (6.9 ± 0.57 mg L^{-1} in 48 h) whereas the fresh woodchips produced the smallest concentration reduction (1.1 ± 0.58 mg L^{-1}). The 8-h and 16-h woodchips showed similar concentration reductions over 48 h (4.4 ± 0.25 and 4.3 ± 0.73 mg L^{-1} in 48 h). A yellow color was observed in the liquid of the jars containing the fresh woodchips, which is indicative of dissolved carbon leached from the woodchips; however, dissolved organic carbon (DOC) concentrations were not measured.

Multiple linear regression was used to predict NO_3^-–N concentration based on time and woodchip type. A significant regression equation was found ($F(7, 112) = 102.1$, $p < .05$), with an R^2 of .865. Significant regression coefficients for the interaction of time and indicator variables for 2-h, 8-h, and 16-h HRT woodchips ($p < .05$) indicated that the zero-order kinetic constants for all aged woodchips differed from the kinetic constant calculated for the fresh woodchips. A second multiple linear regression excluding the concentration results was performed to determine whether any of the kinetic constants for the aged woodchips were significantly different. There was a significant regression equation ($F(5, 84) = 155.7$, $p < .05$) with an R^2 of .903. A significant regression coefficient for the interaction of time and the indicator variable for 2-h HRT woodchips ($p < .05$) indicated that the zero-order kinetic constant for the 2-h HRT woodchips was statistically different from both the 8-h and 16-h HRT woodchip constants. A non-significant regression coefficient for the interaction

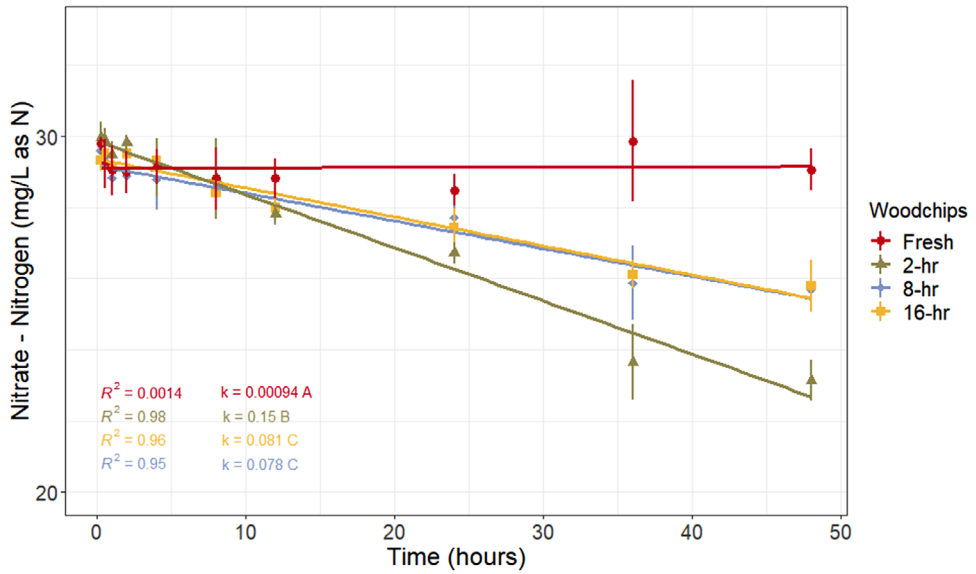

FIGURE 7 Linear regression was used to fit zero-order models to the NO_3^-–N concentration data. Figure label displays zero-order kinetic constants (mg L^{-1} h^{-1}) for linear models fit to the average concentration at each time. The constant for the fresh woodchips is not significantly different from zero ($p > .05$). Capital letters indicate significant differences between kinetic constants ($p < .05$)

of time and the indicator variable for 8-h HRT woodchips ($p > .05$) indicated that the zero-order kinetic constants for the 8-h and 16-h HRT woodchips were not statistically different.

4 | DISCUSSION

Our experiment allowed us to observe changes in woodchip properties and hydraulic characteristics in denitrifying bioreactors operating for 2 yr to better understand the implication of hydraulic retention time as a design criterion. We observed decreases in C/N ratio and increased subsidence over time. There are several potential reasons for observed of subsidence, including damage due to field equipment, woodchips being washed out of the bioreactors, or mass loss from woodchips due to degradation. Subsidence in denitrifying bioreactors is a concern of producers due to aesthetics, but our results indicate that subsidence varied with HRT whereas changes in hydraulic properties did not, due to the differences observed in subsidence by HRT (Figure 5) and lack of statistical differences in hydraulic properties between HRTs (Table 1). Our experiments do suggest that the cause of the subsidence is likely settling due to gravity and independent of the changes in hydraulic properties (MDI, S, λ, drainable porosity) that we observed.

Our results suggest that across all six bioreactors pore space has decreased and flow characteristics have shifted toward unideal conditions during 2 yr of flow. We hypothesize that the decrease in drainable porosity can be attributed to media compaction, or subsidence, due to gravity effects. Due to the controlled conditions at the Iowa State Research Farm, the observed subsidence is not likely attributable to damage by field equipment. Sedimentation is also unlikely in this system because of the use of a reservoir tank prior to the inlet into the bioreactors, although others have hypothesized sedimentation was occurring based on an increase in the proportion of media particles falling under 1.18 mm and an increase in observed ash content (Christianson et al., 2020; Feyereisen & Christianson, 2015; Ghane et al., 2018; Robertson et al., 2008).

Woodchip subsidence has been mentioned in the literature previously within the context that an unsaturated woodchip layer might be advantageous to replenish the bioreactor media after settling or consumption (Christianson & Schipper, 2016). Installation photos from 2014 (Figure 1) show that the woodchips were filled to at least the level of the concrete trench. Overfilling the bioreactor with media may be able to compensate for subsidence over time but with a cost trade-off.

The particle-size distributions for all three HRTs from woodchips sampled in 2018 were identical to the distribution reported for the woodchips in 2016 (Hoover et al., 2017). This means that the proportion of woodchips of each size has remained relatively stable, as opposed to preferential degradation of particles of a given size. Ghane et al. (2018) reported a decrease in the proportion of larger-sized woodchips closest to the Inlet location after 4 yr but did not report a D50 value or particle size distribution for the original woodchips (Ghane et al., 2018). Additionally, Christianson et al. (2020) reported a decrease in D50 over 9 yr. Both these studies also indicated a decrease in porosity, likely from sedimentation as subsidence was not reported. In our study it is more likely that the decrease in porosity was from gravity effects due

to the low likelihood of sedimentation and given that we did not observe a change in particle-size distribution. These results combined show that the initial 2-yr period of bioreactor operation is not when changes in particle size distribution or D50 occur.

Low C/N ratios are indicative of a depletion of carbon relative to nitrogen or accumulation of nitrogen relative to carbon (Moorman et al., 2010). Though aerobic respiration and other anaerobic processes such as dissimilatory nitrate reduction to ammonia (DNRA) may occur within denitrifying bioreactors, we expect that substrate depletion within these systems is proportional to influent nitrate load because denitrification has been shown to be the dominant fate of nitrogen (Gibert et al., 2008; Greenan et al., 2006). During the period of operation between 2016 and 2017, the nitrate load to the 2-h HRT bioreactors was significantly higher than the 8-h and 16-h HRT bioreactors (Martin et al., 2019), explaining why the lowest C/N ratios were observed at all locations and depths in the bioreactors set at 2 h HRT.

An additional potential mechanism of substrate degradation in denitrifying bioreactors is aerobic respiration. Results from an earlier study of these bioreactors between 2016 and 2018 indicated anaerobic conditions for the A (one-third bioreactor length) and B (two-thirds bioreactor length) locations as well as the outlet in all bioreactors. This suggests that aerobic respiration had reduced the oxygen concentration sufficiently between the Inlet and Location A for all HRTs. The presence of anaerobic conditions in the bioreactor at Location A suggests that woodchip degradation due to aerobic respiration primarily occurs between the inlet and Location A. No statistical differences in DO levels were reported between HRTs for any of the locations, providing evidence that differences in nitrate load better explain the observed changes in C/N ratios between bioreactor HRTs (Martin et al., 2019). These results are supported by the conclusions of a different study where greater woodchip decomposition occurred nearest the inlet of denitrification beds (Ghane et al., 2018).

After 2 yr, the observed LCIs had not yet decreased sufficiently to be indicative of decomposition stabilization (~0.70–0.80, DeBusk & Reddy, 1998; Melillo et al., 1984). The LCI for the fresh woodchips presented here (0.51 ± 0.01) is the highest reported for fresh fill material in the literature, with values typically between 0.2–0.25; the highest previously reported value reported for fresh woodchip media is 0.45 (Christianson & Schipper, 2016; Feyereisen et al., 2016).

The spatial sampling design in our study provides potential insight into the chemical and biological stratification within denitrifying bioreactors. If the C/N ratios are taken as spatial indicators of denitrification activity, the results from our study indicate that substrate degradation primarily occurs within the first 75% lengthwise of the denitrifying bioreactor. These results, taken with the subsidence measurements, suggest that recharging the woodchips near the Inlet location while leaving the outlet location undisturbed might be an effective management strategy to prolong the usable lifetime of these systems.

The components of the woodchips described as ash are not capable of being consumed by microbial metabolism and therefore the absolute amount of ash in a sample of woodchips should remain constant. With this in mind, the relative amount of ash should increase as other components are metabolized. This can mean that components that fail to increase in relative abundance at the same rate as ash are being degraded or consumed. Therefore, the smaller increase in percentage lignin compared with the percentage increase in ash can be taken to mean that the absolute amount of lignin decreased overall. These results are consistent with cellulose being the preferred energy source for denitrification.

Another study has reported zero-order reaction constants ranging between 0.38 ± 0.06 mg N L^{-1} h^{-1} for 7-yr-old woodchips and 0.50 ± 0.01 mg N L^{-1} h^{-1} for 2-yr old woodchips (Robertson, 2010). The C/N ratio of these woodchips was not reported, so we cannot compare whether the higher rates observed here result from differences in substrate composition. Despite lower C/N ratios, the aged woodchips still contain sufficient labile C to support denitrification and the results from the chemical analysis of the aged woodchips showed that roughly one-third glucose content remained after 2 yr. Based on the observed denitrification potential of the aged woodchips, we hypothesize that despite the elevated oxygen levels observed near the inlet of the 2-h HRT bioreactors, facultative anaerobic microorganisms are present that can perform denitrification. Further work could characterize the effect of oxygen load on microbial community composition in contrast to the selective pressure of nitrate load.

Our study suggests that the change in flow characteristics (MDI, S, λ) and decrease in drainable porosity is independent of flow rate. This is promising because it implies that designing for a target flow rate will not negatively impact the hydraulic properties of the bioreactor. Additionally, our findings suggest that woodchips are not selectively degraded based on particle-size during the first 2 yr of operation. The spatial changes in C/N ratio could be indicators of denitrification; the woodchip degradation observed nearest the inlet suggests that partial excavation and replenishment of woodchips might serve to prolong the usable lifetime of denitrifying bioreactors. Further research on the microbial characteristics of denitrifying bioreactors is warranted to explore the differences in decomposition and denitrification performance in denitrifying bioreactors operating at different HRTs.

ACKNOWLEDGMENTS

The authors would like to thank Leigh Ann Long for assistance during bioreactor excavation, woodchip sample preservation, and batch study nutrient analysis. We would also like to thank Alexis Slade, Andrew Craig, Ana Barana, Elizabeth

Reike, Laura Alt, and Andrew Hillman for assistance during bioreactor excavation.

AUTHOR CONTRIBUTIONS

Abby Schaefer: Data curation; Formal analysis; Investigation; Methodology; Software; Validation; Visualization; Writing-original draft. **Kyle Werning**: Data curation; Formal analysis; Investigation. **Natasha Hoover**: Data curation; Formal analysis; Investigation; Writing-review & editing. **Ulrike W Tschirner**: Investigation; Methodology; Resources; Writing-review & editing. **Gary W. Feyereisen**: Conceptualization; Methodology; Resources; Writing-review & editing. **Thomas B. Moorman**: Conceptualization; Methodology; Resources; Writing-review & editing. **Adina C. Howe**: Funding acquisition, Supervision; Writing-review & editing. **Michelle L. Soupir**: Conceptualization; Funding acquisition; Methodology; Project administration; Resources; Supervision; Writing-review & editing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Schaefer A, Werning K, Hoover N, et al. Impact of flow on woodchip properties and subsidence in denitrifying bioreactors. *Agrosyst Geosci Environ*. 2021;4:e20149. https://doi.org/10.1002/agg2.20149