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Denitrifying bioreactor microbiome: Understanding pollution swapping and potential for improved performance

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Abstract

Denitrifying woodchip bioreactors are a best management practice to reduce nitrate–nitrogen (NO_3–N) loading to surface waters from agricultural subsurface drainage. Their effectiveness has been proven in many studies, although variable results with respect to performance indicators have been observed. This paper serves the purpose of synthesizing the current state of the science in terms of the microbial community, its impact on the consistency of bioreactor performance, and its role in the production of potential harmful by-products including greenhouse gases, sulfate reduction, and methylmercury. Microbial processes other than denitrification have been observed in these bioreactor systems, including dissimilatory nitrate reduction to ammonia (DNRA) and anaerobic ammonium oxidation (anammox). Specific gene targets for denitrification, DNRA, anammox, and the production of harmful by-products are identified from bioreactor studies and other environmentally relevant systems for application in bioreactor studies. Lastly, cellulose depletion has been observed over time via increasing ligno-cellulose indices, therefore, the microbial metabolism of cellulose is an important function for bioreactor performance and management. Future work should draw from the knowledge of soil and wetland ecology to inform the study of bioreactor microbiomes.

1 | INTRODUCTION

Human activities, including fertilizer production, preferential planting of legumes, and burning fuels, have doubled fixed nitrogen levels since pre-industrial times, and this has implications for climate change, acid rain, and water quality (National Academy of Engineering, 2019). New engineering strategies are needed to "manage the nitrogen cycle," one of the National Academy of Engineering Grand Challenges for Engineering. Fertilization of cropland paired with high-yielding crop genetics has provided a consistent food supply for our expanding human population. If fixed nitrogen inputs in our agricultural systems are not fully converted to food crops, it can cycle from fixed to mobile forms, ultimately draining to surface and ground waters or fluxing from agricultural soils into the atmosphere. To prevent this nitrogen leaching, excess pools of fixed nitrogen can be decreased in engineered denitrification systems, which convert this nitrogen to N_2 gas.

Abbreviations: anammox, anaerobic ammonium oxidation; ARISA, automated ribosomal intergenic spacer analysis; DNRA, dissimilatory nitrate reduction to ammonia; FARISA, fungal automated ribosomal intergenic spacer analysis; GHG, greenhouse gas; HRT, hydraulic residence time; qPCR, quantitative polymerase chain reaction; rRNA, ribosomal ribonucleic acid; TAN, total ammonia nitrogen; TRFLPs, terminal restriction fragment length polymorphisms.

A major contributing factor to the productivity of the Upper Midwest and other semihumid-to-humid agricultural regions is subsurface tile drainage lines that have been installed to lower the water table and increase the viability of crops (Gramlich et al., 2018; Helmers et al., 2012; Mehan et al., 2019). These tile drainage lines have increased annual streamflow and serve as a vector to export nitrogen from fields (Schilling et al., 2009). Although classified in the United States as a nonpoint source of pollution because the nutrients originate from diffuse sources across the agricultural landscape, tile lines can discharge nitrate concentrations as high as 77 mg N/L into downstream water bodies (Ikenberry et al., 2014). Typical annual flow-weighted nitrate-nitrogen (NO_3–N) concentrations in the Midwest range between 6.9–31.8 mg/L; however, tile line nitrate export peaks during periods of heavier flows (Ikenberry et al., 2014; Jaynes, 2012).

When considering mitigation strategies, woodchip bioreactors (Figure 1) have been identified as a promising practice for removing NO_3–N from agricultural drainage (INRS, 2017; Addy et al., 2016). Among several edge-of-field practices analyzed (wetlands, buffers, bioreactors, and controlled drainage), woodchip bioreactors were estimated to be the most cost-effective practice for nitrogen reduction on a dollar per mass-removed basis (INRS, 2017). Briefly, denitrifying bioreactors are a best management practice that promotes nitrate removal by providing a carbon substrate for denitrifying microorganisms (Figure 1). These systems have been widely studied, and previous literature reviews have described both the design (geometry, media type, hydraulic residence time, site selection, etc.) and general performance (nitrate removal and influencing environmental factors) (Addy et al., 2016; Christianson et al., 2021; Christianson, Bhandari, & Helmers, 2012).

Core ideas

- Denitrifying bioreactor researchers must address pollution swapping to advance implementation.
- GHG, methylmercury, and sulfate reduction have been observed in denitrifying bioreactors.
- Gene targets for detection of pollution swapping processes are identified.
- Bioreactor substrate is a potential target for microbial community management.

Under ideal conditions, microbial denitrification would be the primary microbial process occurring within the bioreactor (Figure 1). Realistically, the conditions within the bioreactor are not homogenous in terms of flow, temperature, or dissolved oxygen (Christianson, Helmers et al., 2013; Martin et al., 2019). When considering flow through the bioreactor, it is likely that there are pockets of low-flow or no-flow near the corners or edges of the reactor. In addition, research has shown that microbial communities can be different and diverse in the water surrounding the carbon source when compared with the water within the carbon source or within its biofilm layer (Aalto et al., 2020; Grießmeier et al., 2017; Yamashita et al., 2011). Thus these variations in environmental conditions will also affect the microbial community (Andrus, 2011; Herbert et al., 2014; Porter et al., 2015),

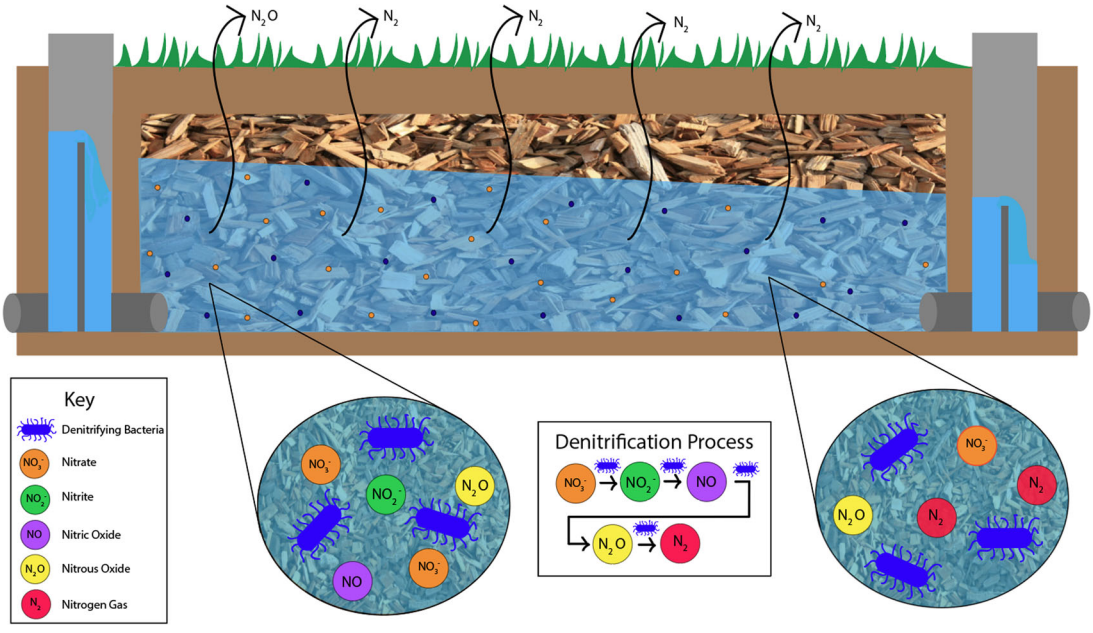

FIGURE 1 Denitrifying bioreactor and resulting denitrification process

making it likely that a variety of microbial processes including denitrification, sulfate reduction, and methanogenesis are occurring at any given time.

With the extensive study of and future planned installations of carbon-based denitrification bioreactors, the limited attention to the study of microbial processes is quite surprising. Central to the performance of bioreactors is its associated microbiome, which contains a complex microbial community interacting with available carbon, nitrogen, and other nutrients. Here, we conduct a review of the role of the microbial community on potential biochemical outcomes of denitrifying bioreactors, including both desirable and harmful effects. This paper serves the purpose of identifying potential strategies to improve bioreactor performance while also reducing potential harmful by-products, specifically through raising awareness of the need for a greater understanding of the microbial processes within denitrifying bioreactors.

2 | DENITRIFICATION MECHANISMS IN NATURAL AND ENGINEERED SYSTEMS

Denitrification is a four-step process, and each step is enzyme-catalyzed. Genes associated with denitrification have been used as targets for identifying community members that are important in the denitrification process and include membrane-bound nitrate reductase (*narG*), nitrite reductase (*nirS, nirK)*, nitric oxide reductase (*nor*), and nitrous oxide (N_2O) reductase (*nosZ*); however, *nirS*, *nirK*, *and nosZ* are the most commonly used targets (Kraft et al., 2011, Table 1). Although *nirS* and *nirK* are structurally dissimilar and coordinate different metal ion cofactors in their catalytic sites, they are functionally equivalent (Kraft et al., 2011). Copy numbers of the *nosZ* gene have been used as a proxy for denitrification potential, and *nirS* gene copy numbers have been positively correlated with denitrification rate in woodchip bioreactors (Fatehi-Pouladi et al., 2019; Ilhan et al., 2011; Warneke, Schipper, Matiasek et al., 2011). Further, it is hypothesized that communities of microorganisms work together to carry out the process of denitrification, especially because most microorganisms do not possess all the enzymes required to complete the entire process (Kuypers et al., 2018). Denitrifying communities can be thought of as possessing highly ordered divisions of labor that allow each member to have their metabolic needs met, a type of interaction known as syntrophy (De Roy et al., 2014). These communities form complex and dynamic relationships that respond to changes in their environment (Gonze et al., 2018).

Although the engineering design aspects of denitrification bioreactors have been well-explored in the literature, less is understood regarding the microbial community structure and function and role on bioreactor performance. However, the role of microbial communities in other natural and engineered systems can inform the role of microbes in bioreactors. In soils, the structure of denitrifying microbial communities is influenced by nitrate, dissolved nitrogen and carbon, soil structure, pH, soil nutrients, and cropping system (Enwall et al., 2010; Regan et al., 2017). Similarly, in wastewater treatment, nitrification and denitrification are coupled, and internal (carbon-containing wastewater) or external (methanol additive) electron donors are used to reduce nitrate to N_2 (Xiao et al., 2021). The performance and stability of these treatment systems have been closely linked to the microbial community structure and population dynamics, which are impacted by factors such as dissolved oxygen, pH, HRT, and temperature (Chen et al., 2017).

As in soils and wastewater treatment systems, microorganisms within woodchip bioreactors drive the transformation of key forms of bioavailable nitrogen, ammonium, and nitrate, the former of which is an oxidizable cation while the latter is a reduceable anion. The subsequent nitrogen cycling within the bioreactor depends on the specific microbes and substrates present in bioreactors. Specifically, this membership and the available metabolites influence the oxidative state of nitrogen, specifically, the number of electrons associated with the nitrogen atom (Jeannotte, 2014; Kraft et al., 2011; Petersen et al., 2012; Reisinger et al., 2016). Levels of bioavailable nitrogen are modulated by microbial activity that changes the oxidation state of molecular nitrogen. Some nitrogen-cycling microbes fix nitrogen using an assimilatory or a dissimilatory nitrogen reduction pathway. In assimilatory nitrate reduction, the key enzymes are in the cytoplasm and are used to build biomass. In dissimilatory nitrate reduction, the key enzymes are membrane-bound and used for respiration.

Within woodchip bioreactors, there can exist microorganisms that can carry out metabolic activity under the presence and absence of oxygen (e.g., both aerobic and anaerobic conditions), and these microbes are called facultative anaerobes. To be capable of such metabolic flexibility, these microorganisms must have a system in place to determine when oxygen is no longer available. For example, in *Escherichia coli* , this feedback system has been determined to be regulated by the gene associated with fumarate nitrate reductase (Unden and Schirawski, 1997). The gene encoding fumarate nitrate reductase is activated in the absence of oxygen, initiating the transport of nitrate into the cell. Several transporters are involved in this process across bacteria and archaea, and the detection of their encoding genes can also be used as evidence that denitrifying microorganisms might be present (Kaft et al., 2011; Kuypers et al., 2018).

TABLE 1 Summary of studies using molecular techniques to study denitrifying bioreactors

Source	Media type	Influent NO_3–N concentration	Experiment scale (reactor volume)	HRT	Gene target	Method	Study outcome
		mg L^{-1}					
Yamashita et al., 2011	Cedar woodchips	0–45.2	1 L	6–24 h	*dsrb*	PCR	Higher sulfate reduction was observed in the deep-layer biofilm than in the total biofilm inside the woodchips.
Warneke, Schipper, Matiasek et al., 2011	Maize cobs, wheat straw, green waste, sawdust, pine woodchips, eucalyptus woodchips	14.4–17.2	200 L	33.1–54.3 h	*nirS, nirK*	qPCR	Positive correlation between total *nir* copy numbers and mass removal rate of nitrate.
Ilhan et al., 2011	Woodchips	~5.48	20 ml	0, 2, 5, 20, 45 d	*nosZ*	qPCR	Denitrifier abundance was temporarily inhibited by enroflaxacin and sulfamethazine, but uninhibited by atrazine
Andrus, 2011	Woodchips	14	55.8–84.6 m^3	1.4–4.4 h	*nosZ*	T-RFLP, ARISA, and FARISA	Distal controls on microbial community structure were inlet nitrate concentration, pH, woodchip moisture content, depth, and sampling port temperature. Community composition is linked to nitrate removal; bacteria mediate denitrification while fungi may form commensal relationships with denitrifiers or mediate woodchip decomposition.
Herbert et al., 2014	Crushed rock, water-saturated sawdust, and sewage sludge	30.4	27 m^3	24 h	*nirS, nirK, nos*ZI, *nos*ZII, anammox-specific 16S rRNA genes	qPCR	Spatial variability in the bioreactor community (nirS, nirK, nosZI) were observed which was likely due to varying hydraulic conditions within the bioreactor.
Porter et al., 2015	Woodchips	3.92–12.16 (average)	44.0–84.6 m^3	0.2–29 d	*nosZ* clade I	FARISA and ARISA	Microbial community composition is related to depth and seasonal variations in temperature, moisture content, and bioreactor inundation
Hathaway et al., 2017	Woodchips	15	18.2 L	7.5–8.8 h	*nosZ*I and *nosZ*II	ARISA	Denitrifying bacterial community was resistant to changes based on fluctuating water levels.
Zhao et al., 2018	Poplar woodchips	50.6	7.26 L	9.4–52.4 h	16S *rRNA*	Amplicon sequencing	Denitrifiers, carbonaceous-compound-degrading bacteria, and fermentative bacteria co-existed in the woodchip-based solid-phase denitrification bioreactor
Fatehi-Pouladi et al., 2019	Maple hard woodchips	202–307	220 L	7.9 d	*NirS, nirK*	qPCR	Positive correlation between nitrate reduction and denitrifying genes.

(Continues)

TABLE 1 (Continued)

Source	Media type	Influent NO_3–N concentration	Experiment scale (reactor volume)	HRT	Gene target	Method	Study outcome
Jang et al., 2019	Woodchips	~50	5 ml microcosm incubation & 295.6 m^3 woodchip bioreactor	48 h (microcosm)	*nirK*, 16S *rRNA*	qPCR, amplicon sequencing	*Cellulomonas sp*. denitrifiers may degrade woodchips to provide electron donors to themselves and other denitrifiers in woodchip bioreactors at low temperatures.
Von Ahnen et al., 2019	Poplar woodchips	~35	5.34 L	8.4 h	*nirS, nirK*	qPCR, amplicon sequencing	Salinity resulted in an altered microbiome with a promotion of autotrophic denitrifiers. A lower overall denitrification potential was also observed.
Kiani et al., 2020	Woodchips, industrial potato waste, biochar, and/or dried moss	34.7	2.51 L	48 h	16S *rRNA*	PCR, amplicon sequencing	The bioreactors containing the woodchip and potato residue mixture developed distinct microbial communities from the bioreactors containing the other media combinations. The bioreactors containing mixed media developed vertically-stratified communities, with distinct communities forming in the woodchip layers compared to the layer of the second media-type.
Aalto et al., 2020	Woodchips	5.02–12.7	300, 660, and 1440 m^3	10–18 h	*nirK, nirS, fungal nirK, nosZI, nosZII, nrfA*	qPCR	NO3-removal rates were linked to the denitrifying community diversity. A core proteobacterial group drives denitrification, while Bacteroidetes dominated the DNRA-carrying microbes across the three bioreactors included in the study.
Gorski et al., 2020	Redwood woodchips or woodchip and topsoil mixture	3–12	7.28 L	9.6 ± 3.9 h	*16S rRNA*	Amplicon sequencing	The carbon-rich permeable reactive barrier treatment was associated with lower overall diversity and a greater relative abundance of groups known to degrade carbon and metabolize nitrogen in the underlying soil.
Hellman et al., 2020	Pine woodchips, barley straw, and bottle sedge	22.3–32.9	0.54 L	1.5–7.2 d	16S *rRNA*, *nirS, nirK, nosZI, nosZII, nrfA*	Amplicon sequencing, qPCR	Different denitrifying bioreactor substrates formed distinct microbial communities. All substrate types showed an increase in abundance of nitrous oxide; reducing capacity was observed over the study period.

Note: ARISA, automated ribosomal intergenic spacer analysis; FARISA, fungal automated ribosomal intergenic spacer analysis; HRT, hydraulic residence time; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; T-RFLP, terminal restriction fragment length polymorphisms.

3 | FACTORS AFFECTING THE MICROBIAL COMMUNITY WITHIN WOODCHIP BIOREACTORS

Woodchip bioreactor microbial communities have been studied indirectly through the measurements of denitrification rates and nitrate removal efficiency at the lab, pilot, and field scales. Denitrifying bioreactor performance is evaluated in two ways: on a nitrogen–mass removal (g NO_3–N d^{-1}) basis (Warneke, Schipper, Bruesewitz et al., 2011) or on a nitrogen–concentration reduction (mg L^{-1} NO_3–N) basis (Addy et al., 2016). Reporting denitrification performance on a mass basis per volume of bioreactor and unit time (g NO_3–N m^{-3} d^{-1}) facilitates comparison between various bioreactor designs. Current design standards require either a 20% annual reduction in NO_3–N load from the effluent flow of the bioreactor or treatment of at least 15% of peak flow events with a minimum hydraulic residence time (HRT) of 3 h (USDA, 2020). Other reviews have addressed the engineering design of these systems and their general performance (Addy et al., 2016; Christianson et al., 2021; Christianson, Bhandari, & Helmers, 2012).

Studies have assessed the presence and spatial variability of important microorganisms in promoting denitrification in woodchip bioreactors. Transcript levels of *nirK* were elevated in denitrifying microcosms compared to nondenitrifying microcosms, and *Pseudomonas* spp., *Polaromonas* spp., and *Cellumonas* spp. were identified as important bacteria for denitrification at low temperatures (Jang et al., 2019). Denitrification mechanisms present along the height of an upflow bench reactor have also been investigated, showing that carbon-degrading, denitrifying, and fermentative microorganisms were important for bioreactor performance (Zhao et al., 2018). Additionally, the abundance of each type of microorganism was determined to be correlated to resource availability (nitrate, dissolved carbon), which varied along the height of the reactor.

Other efforts to describe the microbial communities in bioreactors have included the characterization of total microbial community membership and structure through sequencing phylogenetic markers of bacteria, archaea, and fungi. These approaches target amplifying conserved DNA regions and using sequencing to distinguish variable regions that can be used to identify specific taxa within the community. Gene targets that are often used include the 16S small subunit ribosomal ribonucleic acid (rRNA) gene for bacteria and archaea and the internal transcribed spacer regions between ribosomal deoxyribonucleic acid units for fungi (Johnston-Monje & Lopez Mejia, 2020). Gene fingerprinting approaches, including automated ribosomal intergenic spacer analysis (ARISA), fungal automated ribosomal intergenic spacer analysis (FARISA), and terminal restriction fragment length polymorphisms (TRFLPs), are also techniques that have been previously used (Hathaway et al., 2017; Porter et al., 2015).

3.1 | Carbon substrate availability impacts microbial community and denitrification

Several studies have demonstrated that carbon substrates can have a significant role in microbial community and bioreactor performance (Healy et al., 2012; Healy et al., 2015). In addition, carbon-degrading and fermentative microorganisms have been shown to provide carbon to the denitrifiers, evidence of the links between carbon and nitrogen metabolism in these communities (Zhao et al., 2018).

Most studies of denitrifying bioreactor substrate have focused on carbon/nitrogen ratio and carbon quality, or the lignin concentration in the woodchips (Ghane et al., 2018). Carbon/nitrogen ratio has been shown to decrease in proportion to nitrate load (Ghane et al., 2018; Moorman et al., 2010; Schaefer et al., 2021). In addition, carbon quality decreases over time as sugars are preferentially consumed over lignin, which is more recalcitrant to microbial degradation based on its chemical structure (Schaefer et al., 2021; van der Lelie et al., 2012). Other work has shown that cellulose and hemicellulose can be converted to carbon dioxide (CO_2) and methane (CH_4) under anaerobic conditions, but the anaerobic breakdown of lignin has not been demonstrated (Ko et al., 2009). NO_3–N removal rates greater than 100 g m^{-3} d^{-1} have been observed in bench-scale bioreactors where denitrification was stimulated with acetate, which is a form of soluble, bioavailable carbon (Roser et al., 2018), approximately 10 times greater than typical removal rates observed (Addy et al., 2016). This work suggests that carbon availability impacts or stimulates the denitrifying microbial community.

Although it is widely thought that anaerobic conditions are necessary for denitrification to occur within denitrifying bioreactors, it has also been shown that some organisms primarily responsible for the breakdown of cellulose and lignin require oxygen (Brown and Chang, 2014; Tavzes et al., 2001). Recent studies of denitrifying bioreactors have investigated the effects of cyclical aerobic and anaerobic periods (Maxwell et al., 2019). It is thought that the aerobic periods stimulate the release of labile carbon from the woodchips. This could be because it is hypothesized that lignin degradation is performed most efficiently by aerobic, heterotrophic basidiomycete (white-rot) fungi, though there is increasing evidence of bacterial species that are capable of lignin degradation (Toljander et al., 2006; Rashid et al., 2015; Janusz et al., 2017). Microbial lignin degradation has been primarily studied in white-rot fungi, a group well-adapted to perform lignin degradation due to the extra-cellular enzymes

they produce, which are necessary because lignin cannot be endocytosed (Dashtban et al., 2010). There is also evidence that bacterial and fungal species work together to breakdown ligno-cellulose materials, where bacteria consume the products of fungal wood degradation such that the lignin-degrading enzymes are not hindered by feedback inhibition (van der Lelie et al., 2012). Bacterial classes that have been shown to contain the crucial prokaryotic ligninolytic enzyme laccase include Actinomycetes, α-Proteobacteria, and γ-Proteobacteria (Bugg et al. 2011; Huang et al. 2013). Further, *Sphingobacterium* (from the order Bacteroidetes) produces manganese superoxide dismutase and therefore is capable of oxidizing lignin through the hydroxyl radical mechanism (Rashid et al., 2015).

Cellulose is the woodchip compound that is most quickly consumed as the energy source for microbial transformations within woodchip bioreactors, and therefore microbial cellulose metabolism is an important function with respect to denitrifying bioreactor performance (Schaefer et al., 2021). Cellulolytic activity is thought to be distributed across the entire fungal kingdom and bacterial species that are fermentative anaerobes, aerobic gram-positive bacteria, and aerobic gliding bacteria (Lynd et al., 2002). Because of the presence of both aerobic and anaerobic conditions within denitrifying bioreactors, it would be beneficial for metabolic activities responsible for electron donor availability to be possible under a range of dissolved oxygen levels. *Cellulomonas* are key cellulose degraders that are facultative anaerobes, and their detection in denitrifying bioreactors may be associated with increased nitrogen removal efficiency if they in fact are responsible in part for woodchip degradation (Bagnara et al., 1987). Clostridiales and Bacteroidetes have also been identified as organisms involved in the hydrolysis of cellulose within denitrifying bioreactors (Grießmeier et al., 2017). Given that work integrating carbon and nitrogen dynamics and microbial community metabolism is extremely limited, there is a need for systematically obtaining these measurements simultaneously to provide insights into broader bioreactor performance.

4 | ROLE OF THE MICROBIAL COMMUNITY ON WOODCHIP BIOREACTOR PERFORMANCE

Despite the implementation of standards for denitrifying bioreactor design, variable nitrate removal rates have been observed in bioreactors ranging from 7 to 100% removal or from 0.38 to 121 g NO_3–N m^{-3} d^{-1} mass removal in lab-, pilot-, and field scale bioreactors (Bell, 2013; Christianson et al., 2011; Chun et al., 2010; Hoover et al., 2016; Hua et al., 2016; Jaynes et al., 2016; Martin et al., 2019; Roser et al., 2018; Woli et al., 2010). Some of the variability comes from different operating conditions such as temperature, dissolved oxygen, and HRT (Addy et al., 2016; Christianson, Bhandari, Helmers, Kult et al., 2012). Similar factors have been identified as impacting denitrifying microbial communities, including carbon availability, the presence of oxygen, and pH (Wallenstein et al., 2006). Thus, designs that consider both the engineered system and the biological system are necessary to stimulate the denitrifying activity of the microorganisms to further enhance bioreactor performance. However, the application of these approaches to provide insights into the microbial communities of bioreactors is relatively limited. A 2-yr study in Illinois sampled woodchip and water samples to track the microbial community composition of pilot-scale bioreactors and their response to environmental change (Porter et al., 2015). Researchers found that the community varied with respect to both season and bioreactor depth. Saturation levels could vary with bioreactor depth, therefore the community variation observed could be correlated to woodchip moisture content. Hathaway et al. (2017) also reported relationships between bioreactor water level and microbial community structure in a laboratory-based study. In addition, Porter et al. (2015) reported that samples collected 125 d apart were less similar than samples collected 300 d apart, showing that this bioreactor community cycles on a roughly annual basis. Others have reported 125-d cycles for denitrifying bioreactors, with community structures correlated with temperature, inlet nitrate concentration, pH, moisture content, and depth (Andrus, 2011). Another pilot-scale study conducted by the same group investigated whether denitrifying bioreactor microbial communities were similar to those found in soil or wetland environments (Hathaway et al., 2015), reporting that the bioreactor, soil, and both constructed and natural wetlands, contained distinct microbial communities. These findings suggested that other factors dictate community structure beyond the desired function of denitrification. Given the lack of consistency between denitrification and targeted genes and variation in multiple studies, results indicate the community structure of bioreactors is variable, and researchers have not yet identified the links between microbial community membership and denitrification.

4.1 | Dissimilatory nitrate reduction to ammonia and anaerobic ammonium oxidation

Other nitrogen-transforming pathways besides denitrification have been observed in woodchip bioreactors. For example, significant total ammonia nitrogen (TAN) production in woodchip bioreactors has been observed at multiple operating HRTs (Martin et al., 2019). It is hypothesized that the primary mechanism of TAN production in woodchip bioreactors is dissimilatory nitrate reduction to ammonia (DNRA) as opposed to nitrogen mineralization because the carbon/nitrogen ratio

of the substrate is typically higher than 16:1, above which nitrogen mineralization does not occur (Enwezor, 1975). Shorter HRTs have produced more TAN at the lab-scale (Healy et al., 2012); however, TAN production has been estimated to account for <4% of nitrogen removal (Greenan et al., 2006). Generally, outlet concentrations of TAN are approximately 0.1 mg L^{-1} or less (Martin et al., 2019; Herbstritt, 2014). Total ammonia nitrogen production, and specifically DNRA, has been shown to be influenced by pH, substrate availability, nitrate scarcity, and anaerobic conditions (Mohan and Cole, 2007). In bioreactors, DNRA is most likely to occur when there are low levels of NO_3–N and high levels of available carbon (Kiani et al., 2020; Aalto et al., 2020; Grießmeier et al., 2017; Manca et al., 2020).

Recent research has shifted to identifying the members of the microbial community responsible for DNRA. The gene that is used to target community member involvement in DNRA is *nrf*A (Aalto et al., 2020). The rate of DNRA has been positively correlated to the ratio of *nrfA/nir* indicating the relative abundance of these genes may influence the pathway of N removal (Aalto et al., 2020). Members of Ignavibacteriales may be involved in the switch from denitrification to DNRA (Grießmeier et al., 2017). Although study of archaeal contributions to the microbial transformations within denitrifying bioreactors has been limited, the subgroup Bathyarchaeota has been identified in estuarine sediments and may contribute to DNRA (Lazar et al., 2016).

Another nitrogen-transforming process that has recently been observed in bioreactor systems is anaerobic ammonium oxidation (anammox). A study with similar influent NO_3–N and ammonium (NH_4–N) levels (15.3 and 15.7 mg L^{-1}, respectively) evaluated the potential for both denitrification and anammox in several mediums (Rambags et al., 2019). In that study, the denitrification removal rate ranged from 0.7–2.6 g N m^{-3} d^{-1}, while the anammox removal rate ranged similarly from 0.6–3.8 g N m^{-3} d^{-1}. To the best of the authors' knowledge, this was the first study to document substantial anammox and denitrification in bioreactors. Worthy of investigation in future studies, media type had a significant impact on NH_4–N removal rate with mature and fresh coconut husk media having significantly higher removal than fresh and mature woodchips and gravel media (Rambags et al. 2019).

While the occurrence of anammox has not been well-studied or observed in bioreactor systems, research to identify the community members involved has been conducted, albeit limited. The abundance of the target gene, *hzoA*, for anammox has been observed to be extremely low in these systems with the abundance of sequences in one study being less than 5 sequences for *hzoA* compared to ~141,000–255,000 sequences related to N_2 fixation (Aalto et al., 2020). Another study observed that the abundance of denitrification genes was a magnitude of approximately two times greater than the abundance of anammox 16S rRNA genes (Herbert et al., 2014). At the lab scale, the detection of the order Planctomycetales nearly ubiquitously in a study that examined nitrogen-removal pathways besides denitrification suggests that group is involved in the anammox process (Grießmeier et al., 2017). Much of the research thus far has demonstrated a low level of potential for anammox to occur which has been supported with analysis of the microbial community in these studies. However, when high levels of both NO_3–N and NH_4–N are present, both denitrification and anammox can occur (Rambags et al., 2019). Future research should expand to further evaluate the conditions and mediums under which anammox occurs. Additional research into the microbial community involvement in anammox in bioreactor studies is also warranted due to its limited nature.

4.2 | Harmful gas production

When the denitrification process is not completed, there are concerns that intermediate by-products will be produced. There are also concerns for additional unintended processes to occur when the denitrification process nears nitrate depletion. Briefly, these include production of N_2O, CH_4, CO_2, and methylmercury, and reduction of sulfate (which is linked to methylmercury production). Under low-flow conditions, concerns for production of CH_4, CO_2, and methylmercury and reduction of sulfate become elevated (Figure 2). Under higher-flow conditions, incomplete denitrification could result in elevated N_2O production.

4.2.1 | Nitrous oxide

One of the intermediate products during the denitrification process is N_2O, a highly water soluble gas (Weiss and Price, 1980; Chen et al., 2014). For a bioreactor to be considered sustainable, the percent of NO_3–N removed as N_2O should be at least less than the percent of NO_3–N removed as N_2O in the natural environment if the bioreactor were not existing (Davis et al., 2019). The amount of NO_3–N that would be removed in the environment as N_2O can be considered using the default emission factor (EF5) of 0.0075 kg N_2O–N per kg NO_3–N leached (De Klein et al., 2006). Studies generally show that N_2O emissions associated with bioreactors are relatively low with emissions of N_2O from the surface of bioreactors being observed in the range of 0.002–0.89% of the NO_3–N removed from woodchip bioreactors (Christianson, Hanley et al., 2013; David et al., 2016; Davis et al., 2019; Ghane et al., 2015; Woli et al., 2010), with the majority of N_2O emissions being observed in the dissolved form. In a pilot-scale study, the N_2O surface emissions from the bioreactors were only 0.1, 2.6, and 0.8% of the total N_2O produced, while the total N_2O observed corresponded to 5.19, 0.38, and 0.50% of the NO_3–

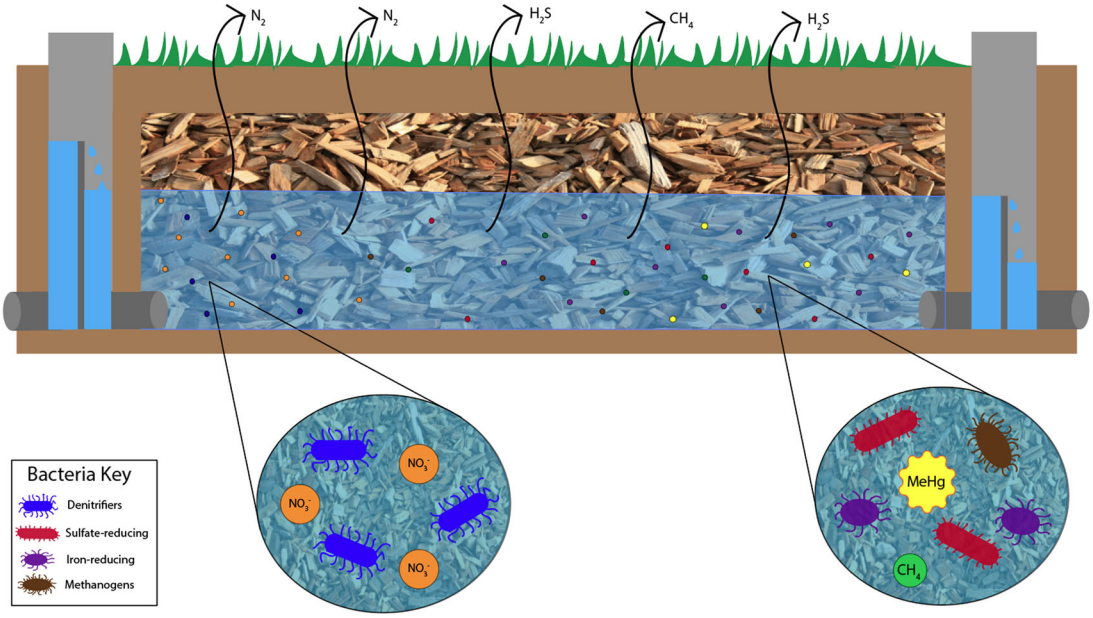

FIGURE 2 Nonideal bioreactor conditions and examples of other microbial processes and by-products (MeHg, methylmercury; CH_4, methane) that can result

N removed at 2, 8, and 16 h HRTs, respectively (Davis et al., 2019). These results indicate the shorter HRT of 2 h is not ideal in terms of N_2O production, likely due to insufficient time for the denitrification process to occur.

The phenomenon of N_2O production has been studied for additional bioreactor media types as well. Nitrous oxide emissions in the range of 1.45–2.15 mg N_2O-N m^{-2} d^{-1} were observed for woodchips and lower than 0.6 mg N_2O-N m^{-2} d^{-1} for the other media types (cardboard, lodgepole pine needles, barley straw, and a soil control) (Healy et al., 2012). In a similar study using the same media types, low N_2O emissions for all media in the range of 0.04–8.80 mg N_2O-N m^{-2} d^{-1} were observed, with the highest concentrations generally occurring at the greatest hydraulic loading rate (Healy et al., 2015). In all cases, this is a relatively small portion of the NO_3–N that is being removed but should still be acknowledged because its global warming potential is 298 times greater than that of CO_2 (Forster et al., 2007). All of the surface N_2O emissions are lower than those reported for a nearby 20-ha row crop field with average emissions of 24.1 mg N m^{-2} d^{-1} (David et al., 2016). Insignificant differences in N_2O emissions from tile drainage and denitrification walls have been observed; treatment of tile drainage through a woodchip bioreactor also corresponds to reduced NO_3–N loading downstream and subsequent N_2O emissions from downstream denitrification (Moorman et al., 2010). In addition, research has shown that the N_2O emissions can peak toward the beginning of bioreactors and be reduced to similar levels as in the incoming tile drainage at the outlet of the bioreactor as the denitrification process continues across the bioreactor length (Fenton et al., 2016; Manca et al., 2020). Therefore, N_2O emissions from denitrifying bioreactors have been identified to be minimal in the overall nitrogen budget and when compared with row crop production (Table 2). The denitrification process in an engineered system, such as a woodchip bioreactor, can be better controlled and designed to maximize NO_3–N removal and minimize N_2O production than if the tile drainage is left untreated.

Understanding the role of the microbial community and its role in N_2O production can help to maximize NO_3–N removal while minimizing the production of N_2O. Research has shown that the *nosZ* gene is responsible for N_2O reduction while the Nir genes are responsible for nitrite reduction. Graf et al. (2014) investigated 652 organisms, finding that 80% of the *nirS* organisms investigated also contained the *nosZ* gene, while in contrast only 30% of the *nirK* organisms had the *nosZ* gene. These findings indicate that the *nirS* gene may be an influential gene in terms of N_2O reduction and complete denitrification. Additional factors contributing to greater formation of N_2O include the ratio of NO_3–N to labile carbon (with an abundance of NO_3–N contributing to greater N_2O), low pH, high levels of oxygen, and lower temperature (Chapin et al., 2012; Grießmeier et al., 2019). Greater understanding of the microbial community and influences on the abundance of *nirS* and *nosZ* genes is warranted to ensure a low risk of N_2O production from these systems.

4.2.2 | Methane and carbon dioxide

Following depletion of nitrate, additional gases, including CH_4 and CO_2, may be produced by methanogens and sev-

TABLE 2 Summary of nitrous oxide (N_2O) emissions reported in denitrification bioreactor studies

Source	Scale	Reported N_2O emissions		Normalized N_2O emissions		Study outcome
		Quantity	Unit	Quantity	Unit	
Woli et al., 2010	Field	0.01–0.13	mg N m^{-2} h^{-1}	0.24–3.12	mg N m^{-2} d^{-1}	Dissolved N_2O was not measured, but surface emissions were found to be negligible.
Warneke, Schipper, Bruesewitz et al., 2011	Field	Surface: 42.8–110.3; dissolved: 0.09–0.51;(4.30% of N removed)	µg N m^{-2} min^{-1} kg d^{-1}.(%)	Surface: 61.6–159; Dissolved: 102–580;(4.30% of N removed)	mg N m^{-2} d^{-1};(%)	Low surface emissions of N_2O were observed (1% of N removed) with greater levels of N_2O in the dissolved phase.
Healy et al., 2012	Lab	<0.60 to 2.15	mg N m^{-2} d^{-1}	<0.60 to 2.15	mg N m^{-2} d^{-1}	Highest N_2O emissions occurred in the control soil. Studied multiple media types.
Christianson, Hanley et al., 2013	Pilot	0.02–1.74; (< 0.32% of N removed)	mg N m^{-2} h^{-1};(%)	0.48–41.8; (< 0.32% of N removed)	mg N m^{-2} d^{-1};(%)	Low levels of N_2O–N were observed in both the dissolved and surface emissions. Soil covers show promise for reduced surface emissions.
Fenton et al., 2016	Pilot	≤70	mg N m^{-2} d^{-1}	≤70	mg N m^{-2} d^{-1}	N_2O emissions were greatest in the beginning of the bioreactor and decreased further in the bioreactor
Healy et al., 2015	Lab	0.04–8.80	mg N m^{-2} d^{-1}	0.04–8.80	mg N m^{-2} d^{-1}	Highest N_2O emissions occurred at the higher hydraulic loading rate (shortest HRT). Studied multiple media types.
Ghane et al., 2015	Field	0.01–0.29;(0.002% of N removed)	µg N m^{-2} min^{-1};(%)	0.014–0.42; (0.002% of N removed)	mg N m^{-2} d^{-1};(%)	Surface emissions of N_2O were determined to be very low, and dissolved emissions were recommended for future studies.
David et al., 2016	Field	0.32 and 0.41;(0.44% and 0.89% of N removed)	kg N yr^{-1};(%)	9.74 and 12.5; (0.44% and 0.89% of N removed)	mg N m^{-2} d^{-1};(%)	Surface N_2O emissions were found to be low but higher than other soil capped bioreactors but were much less than a nearby row crop field. Dissolved N_2O was not measured.
Davis et al., 2019	Pilot	0.002–4.17; (5.19%, 0.38%, and 0.50% of N removed)	g N_2O-N d^{-1};(%)	0.34–719; (5.19%, 0.38%, and 0.50% of N removed)	mg N m^{-2} d^{-1};(%)	The greatest N_2O emissions occurred at the 2-hr HRT, with dissolved N_2O representing the majority of the emissions.
Manca et al., 2020	Field (wall)	-188.5–46.0	mg N m^{-2} d^{-1}	-188.5–46.0	mg N m^{-2} d^{-1}	Negative fluxes of N_2O indicated the walls acted as a sink for N_2O, supporting complete denitrification

Note: HRT, hydraulic residence time.

eral other microbes present in the bioreactor. The oxidation of organic carbon for denitrification results in the production of carbon dioxide (Korom, 1992), which because of its role in greenhouse gas (GHG emissions), is of interest to bioreactor sustainability. The release of CO_2 in bioreactors is primarily due to the decomposition of the media, which has been linked to several phyla including Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, and Spirochaetes (Greißmeier et al., 2017). The carbon source used in bioreactors would degrade over time regardless of its use in the bioreactor, meaning there is not a net increase in CO_2 emissions from decomposition (Warneke, Schipper, Bruesewitz et al., 2011). The microbial community linked to degradation of the carbon source, and therefore CO_2 production, was discussed in Section 3.1 and is not further discussed here. Concern for CH_4 production in these systems is generally greater than the concern for CO_2 due to its 25 times greater global warming potential (Forster et al., 2007). In contrast to N_2O, CH_4 is less soluble in water (Yamamoto et al., 1976; Chen et al., 2014). Because of its lower solubility, there is a greater need to monitor CH_4 at the surface of the bioreactor, although monitoring of CH_4 in the dissolved form is still important. CH_4 emissions that have been observed in bioreactors (Table 3) are comparable to that of a riverside floodplain with median emissions of 0.0079–2.06 g CH_4–C m^{-2} d^{-1} (Sha et al., 2011). Most studies of CH_4 emissions have focused on surface emissions from woodchip bioreactors, which have been observed to range from 0.00031 to 0.0077 g CH_4–C m^{-2} d^{-1} (Table 3) (Ghane et al., 2015; Warneke, Schipper, Bruesewitz et al., 2011). One study measured both surface and dissolved CH_4, finding 84–99% of the emissions being in the dissolved form (Davis et al., 2019). Surface emissions of CO_2 have ranged from 4.80 to 180 g CO_2–C m^{-2} d^{-1}, again with most of these emissions being associated with the inevitable decay of the woodchips (Ghane et al., 2015; Warneke, Schipper, Bruesewitz et al., 2011; Woli et al., 2010). Additional media have been tested and compared with woodchips, finding that GHG emissions were mainly influenced by CH_4 emissions, which accounted for 91, 86, and 54% of the emissions for barley straw, cardboard, and lodgepole pine woodchips, respectively (Healy et al., 2012). One parameter that has been identified as influencing the CH_4 emissions is the hydraulic residence time, with greater emissions at longer HRTs (Davis et al., 2019; Healy et al., 2012; Healy et al., 2015). The same trend has also been observed for CO_2 emissions (Healy et al., 2012; Healy et al., 2015).

The release of these gases directly or in the dissolved form is a concern moving forward and potentially a barrier in increasing field installation of denitrifying bioreactors. In general, the N_2O emissions observed have been minimal (5.19% or less of the N removed in the system) (Table 2, Figure 3), with the majority (54%) of the GHG emissions being from CH_4 for woodchip media (Table 3) (Healy et al., 2012). The concern for N_2O emissions is lowered at longer HRTs while concerns for CH_4 and CO_2 increase (Davis et al., 2019). The risk of complete nitrate reduction has been emphasized, indicating this may create conditions where CH_4 is produced. Methanogens, the group of bacteria responsible for producing the CH_4 gas, are believed to be outcompeted by the denitrifying bacteria when nitrate concentrations remain high (Liu et al., 2017; Schipper et al., 2010). However, in one study, an abundance of methanogens was observed at high NO_3–N concentrations, indicating simultaneous methanogenesis and denitrification were occurring (Grießmeier et al., 2017). All methanogens extend from Archaea and can be further classified into three subgroups depending on their use of substrate: hydrogenotropic, aceticlastic, and methylotrophic (Lyu et al., 2018). Regardless of subgroup, all methanogenic pathways require the methyl-coenzyme M reductase enzyme (*mcrA* genes) (Lloyd, 2015; Lyu et al., 2018). Recent research is extending the current knowledge of methanogens. Presently, four phyla have been identified (Euryarchaeota, Crenarchaeota, Halobacterota, and Thermoplasmatota) as having methanogens (Lyu et al., 2018). It is believed that much is still to be discovered about the breadth and diversity of methanogens (Pandey et al., 2015). Because of the variations in conditions where methanogens or CH_4 production have been observed, a greater understanding of the conditions that methanogens exist and CH_4 is released under is warranted to enhance future bioreactor designs (Christianson et al., 2011). Specifically, the design of bioreactors may be able to be improved in the future to prevent these conditions from occurring as a result of future research. A first step to improve bioreactor design and operation describes the HRT range where the negative emissions are minimized in pilot-scale bioreactors (Davis et al. 2019). Additional research into this area is warranted to represent

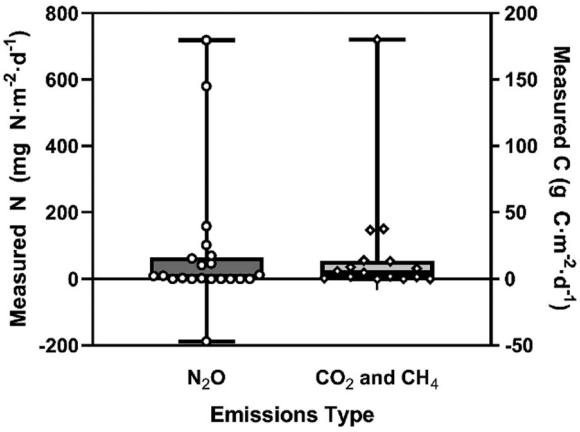

FIGURE 3 Box and whisker plot visually summarizing the data presented in Tables 2 and 3 for nitrous oxide (N_2O), methane (CH_4), and carbon dioxide (CO_2). The box represents the quartiles while the whiskers represent the minimum and maximum values of the data

TABLE 3 Summary of methane (CH_4) and carbon dioxide (CO_2) emissions reported for denitrification bioreactors

Source	Scale	Reported CH_4 and/or CO_2 emissions		Normalized CH_4 and/or CO_2 emissions		Study outcome
		Quantity	Unit	Quantity	Unit	
Woli et al., 2010	Field	CO_2: 0.20–7.5	g C m^{-2} h^{-1}	CO_2: 4.80–180	g C m^{-2} d^{-1}	CO_2 released likely due to the decay of the woodchips.
Warneke, Schipper, Bruesewitz et al., 2011	Field	CH_4: 0.27;CO_2: 5.48–25.8	g C d^{-1}; mg m^{-2} min^{-1}	CH_4: 0.00031;CO_2: 7.89–36.8	g C m^{-2} d^{-1}	CH_4 emissions were low likely due to higher NO_3–N levels; CO_2 measured did not indicate a net increase to the atmosphere after considering the natural CO_2 released to the atmosphere due to wood decay.
Healy et al., 2012	Lab	1.8–13.9	g C m^{-2} d^{-1}	1.8–13.9	g C m^{-2} d^{-1}	GHG emissions (CO_2 equivalents) were dominated by CH_4 emissions. Studied multiple media types.
Healy et al., 2015	Lab	CH_4: ≤8.9;CO_2: ≤5.7	g C m^{-2} d^{-1}	CH_4: ≤8.9;CO_2: ≤5.7	g C m^{-2} d^{-1}	CH_4 emissions were generally greatest at shortest hydraulic loading rates (longest HRTs), as were CO_2 emissions. Studied multiple media types.
Ghane et al., 2015	Field	CH_4: 0.59–5.15;CO_2: 9.14-26.2	µg m^{-2} min^{-1}; mg m^{-2} min^{-1}	CH_4: 0.00085–0.0077;CO_2: 13.2-37.8	g C m^{-2} d^{-1}	CH_4 was measured during high summer temperatures and is expected to be lower during other periods of the year; CO_2 emissions were comparable to agricultural soils but generally slightly higher.
Davis et al., 2019	Pilot	CH_4: 0.51, 1.5, and 1.69	g C m^{-3} d^{-1}	CH_4: 0.56, 1.65, and 1.86	g C m^{-2} d^{-1}	CH_4 production was greatest at the 8- and 16-h HRTs, with between 84–99% of emissions being in the dissolved phase.

Note: GHG, greenhouse gas emissions; HRT, hydraulic residence time.

field-scale bioreactors that operate under a more comprehensive range of uncontrolled field conditions as well as further investigation into the methanogenic microbial community present in these systems.

4.3 | Sulfate reduction and methylmercury formation

Sulfate reduction occurs in denitrifying bioreactors when nitrate is nearly or completely removed and is a concern for various reasons. Specifically, sulfate reduction corresponds to a loss of the carbon source intended for denitrification, produces an odorous hydrogen sulfide gas, and is linked to methylmercury production (Christianson Bhandari, Helmers, Kult et al., 2012, Shih et al., 2011; Hudson and Cooke, 2015). An often-overlooked concern with sulfate reduction in bioreactors is the highly toxic hydrogen sulfide gas produced. At high enough concentrations, loss of consciousness, smell, and even death can occur (Guidotti, 2010), representing an area of caution for those working with these systems. It has been determined that smaller bioreactors may lower the risk of these possible by-products while being more efficient at nitrate removal on a volumetric basis (Christianson, Christianson et al., 2013). There has been concern about denitrifying bioreactors producing methylmercury under sulfate-reducing conditions for some time, but this has been minimally investigated. Mercury is abundant in the environment, coming from natural sources (volcanoes, forest fires, etc.) and anthropogenic sources (burning of coal, oil, wood, etc.) that can travel great distances in the atmosphere before being deposited back to the surface of the earth (US EPA, 2020). Research has shown that mercury methylation occurs in surface water bodies under conditions similar to those found in denitrifying bioreactors under sulfate-reducing conditions (Gilmour et al., 1992). To confirm this, experiments were conducted under both sulfate-reducing and sulfate-inhibiting conditions, and the

subsequent methylmercury production was monitored. Methylmercury production was directly related to the sulfate concentration initially introduced and was lowest when a sulfate reduction inhibitor, sodium molybdate, was present (Gilmour et al., 1992). Iron-reducing bacteria have also been identified as causes of methylmercury production in freshwater sediments (Fleming et al., 2006). The gene cluster *hgcAB* was proven as a prediction mechanism for methylmercury production in sulfate-reducing bacteria, iron-reducing bacteria, methanogens, and some *Firmicutes;* previously, mercury methylation had been only confirmed in iron- and sulfate-reducing bacteria in the Deltaproteobacteria family (Gilmour et al., 2013). The prevalence of mercury methylation genes has been identified globally, primarily in anaerobic environments (Podar et al., 2015). Similar studies to investigate the source of mercury methylation have not been conducted in woodchip bioreactors yet, likely due to the cost of sample analysis. In addition, although there has been documentation of sulfate-reducing conditions within bioreactor systems, investigation into the microbial community associated with sulfate reduction has been minimal. The dissimilatory sulfite reductase gene, *dsrAB*, has been used in wetlands to identify the abundance of sulfate-reducing bacteria (Faulwetter et al., 2013; Pester et al., 2012). Only one study to the best of the authors knowledge has identified specific sulfate-reducing bacteria in bioreactors. That study identified *Desulfomicrobium baculatum* and *Desulfobulbus rhabdoformis* as dominant sulfate-reducing bacteria in the surface-layer and deep-layer biofilms of woodchips (Yamashita et al., 2011).

At long HRTs, nitrate can be almost completely removed, allowing sulfate reduction to occur (Woli et al., 2010; Christianson, Bhandari, & Helmers, 2012; 2016), which validates the concern for methylmercury production in denitrifying bioreactors. Two studies have been conducted that confirmed increases in methylmercury in bioreactors. Methylmercury production has been correlated with warmer conditions under which nitrate was completely removed and when nitrate levels were below 0.5 mg L^{-1}, which allowed for sulfate-reducing conditions to occur (Shih et al., 2011; Hudson and Cooke, 2015). These studies show there is a legitimate concern regarding the production of methylmercury in denitrifying bioreactors; however, additional research is needed to further our understanding of its risk. In particular, studies to confirm the mechanisms that cause methylmercury to be produced in denitrifying bioreactors need to be conducted, similar to the studies to confirm the mechanisms in freshwater sediments through the use of qPCR, inhibiting of sulfate or iron-reducing conditions, or use of sulfate-inhibitors (Gilmour et al., 1992; Fleming et al., 2006; Gilmour et al., 2013). Once the cause is confirmed, further research into the conditions under which it occurs under can be conducted. The design and operation of bioreactors could once again be improved with this information. Methylmercury is considered to be one of the most abundant water contaminants with a great potential to bioaccumulate, leading to adverse effects for both birds and mammals consuming aquatic species (Sams, 2004). Concern for methylmercury production in denitrifying bioreactors and the lack of knowledge around its formation in these systems have limited bioreactor installation, especially in areas upstream of drinking water sources (Adam Schneiders, personal communication 12 June 2019). Therefore, this is another area of importance in denitrifying bioreactor design and performance.

5 | CONCLUSIONS

Although studies have explored the presence of denitrification genes and microbial communities in denitrification bioreactors, variation in bioreactor performance and early results of conducted studies imply that improved understanding of the microbial community is needed to improve bioreactor design to enhance denitrification and minimize pollutant swapping concerns. Future areas of research are recommended in the following areas:

1. In denitrification bioreactors, little attention has been given to the microorganisms that degrade cellulose and lignin, which provide the electron donors for denitrification. Consistency in electron donor availability will lead to consistency in denitrification performance. Therefore, further study of factors that influence cellulose and lignin metabolism genes and community members that promote substrate degradation is warranted.
2. Additional microbial processes, including DNRA and anammox have been observed in bioreactor systems. These microbial processes may have been overlooked in the past as studies have primarily focused on denitrification as the mechanism for nitrogen transformation in bioreactors. Gene targets are presented, and further study is warranted due to the limited research into these processes in bioreactors.
3. The role of the microbial community in harmful by-product formation (GHG production, sulfate reduction, and subsequent methylmercury production) warrants study as the pathways are hypothesized to be microbially-mediated. HRT has been implicated as a design factor that contributes to by-product production, but the mechanism by which HRT impacts the microbial community should be examined.
4. Varying substrates to select for differing microbial communities is also a promising avenue to promote consistency in these systems and to potentially reduce by-product formation.

Woodchip denitrifying bioreactors are a promising conservation practice for NO_3–N reduction within the agroecosystem. The research reviewed has indicated low risks for GHG or methylmercury production. As we move to implement more of these bioreactors across the landscape, additional research into these harmful by-products and the microbial community is warranted to ensure that a scaling up of by-products does not occur and that these systems are being managed and designed for long-term sustainability.

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AUTHOR CONTRIBUTIONS

Lindsey Hartfiel: Conceptualization; Formal analysis; Investigation; Methodology; Visualization; Writing-original draft. Abby Schaefer: Conceptualization; Formal analysis; Investigation; Methodology; Validation; Writing-original draft. Adina Howe: Funding acquisition; Supervision; Writing-review & editing. Michelle 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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