Patterns of Denitrification and Methanogenesis Rates from Vernal Pools in a Temperate Forest Driven by Seasonal, Microbial Functional Gene Abundances, and Soil Chemistry

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Abstract

Due to their relatively small sizes, temperate forest vernal pools are less studied than other wetlands, despite being potential biogeochemical hotspots in landscapes. We investigated spatial and temporal factors driving N₂O and CH₄ emission rates from vernal pools in a temperate forest. We determined higher N₂O ($3.66 \pm 0.53 \times 10^{-6}$, $\mu\text{g N}_2\text{O}/\text{m}^2/\text{h}$) and CH₄ ($2.10 \pm 0.7 \times 10^{-3}$, $\mu\text{g N}_2\text{O}/\text{m}^2/\text{h}$) rates in spring relative to fall (~50% and 77% lower for N₂O and CH₄ rates, respectively) and winter (~70% and 94% lower for N₂O and CH₄ rates, respectively). Soil organic matter, nitrate content and bacterial 16S rDNA, *nirS*, and *norB* gene abundances emerged as significant drivers of N₂O rates, whereas, soil pH, organic matter content and *mcrA* abundance were significant drivers of CH₄ rates. Denitrification gene abundances were negatively correlated with N₂O rates, whereas *mcrA* abundance correlated positively with CH₄ rates. Results suggest that CH₄ rates may be directly coupled to methanogen abundance, whereas N₂O rates may be directly impacted by a variety of abiotic variables and indirectly coupled to the abundance of potential denitrifier assemblages. Overall, additional studies examining these dynamics over extended periods are needed to provide more insights into their control.

Keywords Vernal pools · Denitrification · Methanogenesis · *norB* · *mcrA* · Biogeochemistry

Introduction

Vernal pools are ephemeral wetlands formed in relatively small permanent basins/depressions (less than 2 m deep) that experience periodic inundation and drying (Zedler 2003). Vernal pools are variable in size, depth, and volume (Brooks and Hayashi 2002). The relatively shallow depths, small to medium sizes, and ephemeral nature of vernal pools results in considerable mixing between upper (water column) and lower (soil and organic matter) layers during periods of

inundation that differ from larger wetland systems. This mixing changes biotic and abiotic variables at the oxic and anoxic interface (Zedler 2003; Carrino-Kyker and Swanson 2007; Holgerson and Raymond 2016) and impacts biogeochemical processes.

Several abiotic factors impact biogeochemical processes in terrestrial wetland systems. Elevated soil moisture (and reduced soil aeration) (Liu et al. 2011; Signor and Cerri 2013; Butterbach-Bahl et al. 2013; Holgerson and Raymond 2016), and increasing soil temperature (Le Mer and Roger 2001; Butterbach-Bahl et al. 2013) create suitable conditions for enhancing denitrification and methanogenesis in anaerobic microsites within soils. Other soil abiotic factors, such as soil type, pH, nitrate, and organic matter content, also impact these processes (Knowles 1982; Brentrup et al. 2000; Le Mer and Roger 2001; Chapuis-Lardy et al. 2006; Cameron et al. 2013; Signor and Cerri 2013; Butterbach-Bahl et al. 2013; Holgerson 2015). Additionally, biogeochemical processes in wetlands also depend on the presence, composition, and activity of microbial populations that are capable of responding to seasonal changes in abiotic factors (Butterbach-Bahl et al. 2013; Capps et al. 2014). These microbial assemblages are

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crucial for the maintenance of ecosystem processes under fluctuating conditions (Allison and Martiny 2008). However, the impacts of varying environmental conditions on functional groups within assemblages are not straightforward. The uncertainty around this is because microbial diversity and abundances of functional populations vary spatially and seasonally, and the effects of changing environmental variables on functional groups within these assemblages are complex and not well-understood (Braker et al. 2010; Dandie et al. 2011; Hallin et al. 2012; Chen et al. 2015; Saarenheimo et al. 2015).

Few studies have studied the spatial and temporal variations of methanogenesis and denitrification rates in temperate forest vernal pools within landscapes (but see Carrino-Kyker and Swanson 2008; Capps et al. 2014). Temporally, inundated vernal pools are characterized by low dissolved oxygen, high soil moisture, high organic matter content, low pH, low redox, and high enzyme activities (Carrino-Kyker and Swanson 2007; Capps et al. 2014). These conditions result in rates of denitrification (Capps et al. 2014) and methanogenesis rates (Kutzbach et al. 2004) that considerably higher in inundated vernal pool soils than in adjacent *terra firma* sites. Spatially, vernal pools may also be considered biogeochemical “hot spots” where denitrification and methane emission occur at disproportionately higher rates during periods of inundation, compared to the surrounding landscape (Butterbach-Bahl et al. 2013; Capps et al. 2014). This is because vernal pools are linked spatially along hydrologic flow paths within landscapes, sharing precursors that are potentially converted into gases (N_2 , N_2O , CH_4) when microbial assemblages with the potential for transformation are present and responsive (Mayorga et al. 2003). However, the specific location of vernal pools within the surrounding landscape may also be a contributing factor. For example, in general, upland sites across various landscapes are characterized by lower soil moisture content and water holding capacity (Bayabil et al. 2016; Arias-Navarro et al. 2017), which can negatively impact biogeochemical processes in vernal pools located in these regions. In contrast, bottomland regions in floodplains tended to have higher soil moisture content and reduced soil aeration, which can positively enhance anaerobic biogeochemical processes in these vernal pools (Bayabil et al. 2016; Arias-Navarro et al. 2017).

Given the nature of vernal pools, the biogeochemical processes, and abiotic and microbial factors that regulate biogeochemical processes may differ in vernal pools from other wetland types. In this study, we measured denitrification and methanogenesis rates from vernal pools along a topographic gradient (bottomland and upland) across seasons in a temperate forest ecosystem to investigate the nature of the relationships among abiotic and microbial variables and measured rates. We anticipated higher denitrification and methanogenesis rates 1) during periods of inundation, and 2) in bottomland pools relative to upland pools. Abiotic variables

(soil moisture, % soil organic matter, soil nitrate concentration, and soil pH) and microbial community characteristics (16S rRNA, *nirS*, *norB*, *nosZ*, and *mcrA* gene abundances) were quantified and utilized in determining the best predictors of these processes across seasons.

Methods

Study Site

Ten vernal pools at Kent State University’s research forest, Jennings Woods, were sampled. Jennings is a 30 ha hardwood temperate forest with two distinct zones along a topographical gradient (Blackwood et al. 2013). The upland zone is 15–20 m above a riparian border on a plateau above an incline and is characterized by well-drained Chili loam and Geeburg-Glenford silt loam complex soils (fine-loamy Alfisols), with poorly drained vernal pools. The bottomland zone is located at the bottom of the incline within 80 m of the West Branch of the Mahoning River and is characterized by the Geeburg-Glenford silt loam complex and Holly silt loam soil series (a fine-loamy Inceptisol) (Blackwood et al. 2013; Valverde-Barrantes et al. 2015). These distinct spatial zones with different edaphic factors (bulk density, water holding capacity, total nitrogen, and carbon content) were selected to examine the impact of existing vernal pool soil conditions on biogeochemical process rates. Five soils cores from five vernal pools were sampled in the upland (UL) and bottomland (BL) zones. Samples were taken in spring (mid-June, entirely inundated with water) and fall (late September to early October, dry with little surface water) of 2016, and winter (late February–early March, covered with ice) of 2017. We were unable to sample in summer due to extenuating circumstances.

Sample Collection and Processing

Five soil core samples (~7.62 cm long, with litter layer) were randomly collected from each of the ten vernal pools with a soil corer and placed into polyvinyl chloride (PVC) tubes (diameter = 2.5 cm, length = 15.24 cm, volume = 74.80 cm³) and sealed at both ends with rubber septa ($N = 25$ tubes per zone). Soil cores were transferred to PVC tubes in spring, without any concerted effort to drain water. In winter, the frozen surface layer of water from the upper portion of soil cores was removed, before the transfer of soil cores to PVC tubes without any effort to drain water. N_2O emission rates was measured immediately in the field using the acetylene inhibition method (Groffman and Turner 1995). Because soil temperatures in wetlands vary and are dependent on factors, such as the presence of surface water (Kurylyk et al. 2019) and depth, we incubated samples at prevailing atmospheric temperatures during sampling periods. Briefly, 5 ml of acetylene

gas was injected into each tube and point 0 (t_0) (5 ml) headspace samples collected and injected into evacuated 25 ml glass vials for storage. Three additional 5 ml headspace samples were collected at 20-min intervals for 1 h in the field. At each sampling point, 5 ml of headspace removal was followed by re-injection of 5 ml of acetylene into each tube to make up headspace volume and pressure. Samples were incubated in the field at atmospheric temperatures in between sampling periods. To minimize additional disturbances following the initial removal of soil core samples, they were immediately transferred into the PVC tubes. PVC tubes were incubated on adjacent dry sites to avoid variations associated with differences in depth of surface water and sizes of vernal pools within and across seasons. N_2O and CH_4 concentrations were quantified via gas chromatography using a Shimadzu GC-2014 Gas Chromatograph equipped with an electron capture detector (ECD) for N_2O (detector temperature: 350 °C) and a flame ionization detector (FID) for CH_4 (detector temperature: 250 °C). Denitrification rates and methanogenesis rates were calculated from the linear increases (accumulation) in both N_2O and CH_4 gas concentrations (in parts per million, ppm or $\mu\text{g/g}$) over time (slope), standardized per gram of dry soil (dm), and multiplied by headspace volume within PVC tubes, then divided by area of PVC tubes used as $\mu\text{g } N_2O \text{ m}^{-2} \text{ h}^{-1}$ (Collier et al. 2014).

After gas sample collection, soil core samples were transported to the laboratory on ice. In the laboratory, total fresh weights of soil core samples were determined. Soil samples were then mixed thoroughly without sieving and divided into four subsamples. One subsample was used right away in the laboratory for determination of soil moisture content by drying for 48 h at 60 °C, and percent soil organic matter by combusting at 500 °C for 6 h. The second subsample (stored at –20 °C) was used to determine soil nitrate concentrations after incubation in 2 M KCl with shaking for 90 min to extract soil nitrate followed by spectrophotometric quantification of nitrate concentrations via Vanadium (III) reduction (Miranda et al. 2001). The third subsample (~3–5 g) (stored at –20 °C) used to determine soil pH after incubation in 1 M KCL with shaking for 60 min, using a Delta 320 pH meter (Mettler, Toledo, OH, USA). The final subsample (stored –80 °C) was used for DNA extraction.

DNA Extraction and Molecular Analyses

The fourth subsample (~0.2–0.3 g) was used for DNA extraction. Samples were homogenized via vortexing for 15 min before DNA extraction with Qiagen DNeasy PowerSoil DNA extraction kit (Qiagen Inc., Germantown, MD, USA). Bacterial 16S rDNA gene was confirmed in samples using the universal forward (357F) and reverse (1391R) bacterial primer pair (10 mM each) (Turner et al. 2007). The abundances of five genes were quantified: bacterial 16S rRNA gene, nitrite

reductase gene *nirS*, [responsible for reducing nitrite (NO_2^-) to nitric oxide (NO)], nitric oxide reductase gene, *norB* [responsible for reducing nitric oxide (NO) to nitrous oxide (N_2O)], nitrous oxide reductase gene *nosZ* (responsible for the final reduction of N_2O to N_2 gas) (Moreno-Vivián et al. 1999), and methyl co-enzyme M reductase, subunit A gene (*mcrA*), which catalyzes the synthesis of methane from methyl-coenzyme M (Friedrich 2005). Gene abundances were quantified via quantitative polymerase chain reaction (qPCR) in a subset of vernal pool soil core DNA samples (five vernal pools per location and three replicates per vernal pool across three sampling periods). Primer pairs obtained from the literature were used to quantify the abundances of a 180 bp 16S rDNA fragment (Fierer et al. 2005), a 256 bp *nirS* fragment (Graham et al. 2010), a 389 bp *cnorB* fragment (Braker and Tiedje 2003), a 259 bp *nosZ* fragment from the *nosZ* clade I (Henry et al. 2006; Jones et al. 2013), and a 438 bp *mcrA* fragment (Luton et al. 2002). Each 20 μl qPCR reaction mixture contained template DNA, Perfecta SYBR Green FastMix (Quanta bio, Beverly, MA, USA), water, and primers (0.2 μM each). qPCR reactions were carried out with a Stratagene MX3005P Real-time PCR System (Agilent Technologies, Santa Clara, CA, USA). Primer information and qPCR conditions for these genes are shown in Table 1. All runs were followed by a melt curve step comprised of 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s, in order to assess PCR efficiency (i.e., presence of a single distinct peak for each gene). Standard curves for runs were generated using serial dilutions of plasmids acquired following cloning using the TOPO TA Cloning kit (Thermo Fisher Scientific, Waltham, MA, USA). Gene abundances were calculated as copy number/g dry weight. Standards were plasmids containing 16S rRNA, *nirS*, *norB*, and *nosZ* genes from *Pseudomonas aeruginosa* (ATCC number BAA-47; GenBank accession number AE004091), and a plasmid containing the *mcrA* gene from *Methanosarcinia acetivorans*.

Statistical Analyses

All variables were log-transformed for normality before statistical analysis. Analyses were carried out in JMP Pro 13 (SAS Inc. NC, USA). A mixed model analysis (equivalent to a repeated-measures ANOVA) with a residual covariance structure was used to examine the impact of season, vernal pool location, and their interaction on measured variables and rates. Season, location (BL and UL), and season $\times$ location were fixed effects, and individual vernal pools were treated as random effects to account for lack of independence among samples from individual sites within and across seasons, followed by post hoc student's *t* test for pairwise comparisons. Data used in the mixed model analyses are provided in Table S1. Subsequently, stepwise multiple regression analyses were carried out to determine the abiotic (nitrate

Table 1 Genes, primer information, and qPCR cycling conditions for amplifying denitrification and methanogenesis-related genes

Genes	Primers	Conditions	Size	Reference
16S rDNA	Forward (5-ACTCCTACGGGAGGCAGCAG-3) Reverse (5-ATTACCGCGGCTGCTGG-3)	96 °C for 3 min 40 cycles of 95 °C for 30 s, 58 °C for 60 s, and 72 °C for 60 s	180 bp	Fierer et al. 2005
<i>nirS</i>	<i>nirS-1F</i> (CCT AYT GGC CGG CRC ART) <i>nirS-3R</i> (GCCGCCGTCRTGVAGGAA)	96 °C for 3 min 40 cycles of 95 °C for 30 s, 65 °C for 60 s, and 72 °C for 60 s	256 bp	Graham et al. 2010
<i>norB</i>	<i>cnorB-2F</i> (GACAAGNNNTACTGGTGGT) <i>cnorB-6R</i> (GAANCCCCANACNCCNGC)	96 °C for 3 min 40 cycles of 95 °C for 30 s, 56 °C for 90s, and 72 °C for 60 s	389 bp	Braker and Tiedje 2003
<i>nosZ</i>	<i>nosZ1F</i> (WCSYTGTTCMTGACAGCCAG) <i>nosZ1R</i> (ATGTCGATCARCTGVKCRTTYTC)	96 °C for 3 min 40 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s	259 bp	Henry et al. 2006
<i>mcrA</i>	<i>mcrA-F</i> (GGTGGTGTGGMGATTACAC ACARTAYGCWACAGC) <i>mcrA-R</i> (TTCATTGCRTAGTTWGGRTAGTT-3)	96 °C for 3 min 40 cycles of 95 °C for 60 s, 58 °C for 60 s, and 72 °C for 60 s	438 bp	Luton et al. 2002

(W = A/T, S = C/G, Y = C/T, M = A/C, R = A/G, K = G/T, and V = A/C/G)

concentration, % organic matter, soil moisture, and pH) and microbial (16S rDNA, *nirS*, *norB*, *nosZ*, and *mcrA*) variables that were the best predictors of N₂O and CH₄ emission rates. Analyses were carried out using an all possible combination fit model option with selection of the most parsimonious model according to the lowest Akaike information criterion (AIC). The all possible combination fit model option performs variable selection, parameter estimation, as well as minimizes/eliminates collinearity among variables. Best models in each instance were selected based on a combination of lowest AIC, highest R², and the Mallows statistic value, Cp, which best approximates the number of predictors used in generating the regression model (Mallows 1973). Finally, least-squares linear regression analyses were used to examine individual relationships among identified predictors and N₂O and CH₄ emission rates. Data used in the stepwise regression analyses are provided in Table S2.

Results

Measured N₂O emission rates ranged from 0 to 17.4 µg N₂O/m²/h across all seasons and sites. There was a significant season by site interaction ($P = 0.01$), indicating that the impact of location on rates was different across seasons. Within seasons, N₂O emission rates in bottomland (BL) vernal pools was ~35% higher relative to upland (UL) vernal pools in fall and winter; rates were higher in BL vernal pools (~46%) than in UL vernal pools in winter ($P = 0.01$), but not in fall (Fig. 1a). In contrast, N₂O emission rates were higher in UL vernal pools (~12%) relative to BL vernal pools in spring, but not statistically significant (Fig. 1a). Furthermore, by site, rates in UL vernal pool sites differed significantly among seasons,

whereas denitrification rates in BL vernal pool sites did not (Fig. 1a). Across seasons, average N₂O emission rates in bottomland (BL) vernal pools ($23.3 \pm 3.14 \times 10^{-7}$, mean $\pm$ S.E.M, standard error of the mean) was slightly higher than in upland (UL) pools ($21.4 \pm 3.20 \times 10^{-7}$), although this was not statistically significant. Finally, there was an overall seasonal effect on N₂O emission rates ($P < 0.0001$). Rates were highest in spring ($3.66 \pm 0.53 \times 10^{-6}$, mean $\pm$ S.E, standard error), intermediate in fall ($1.83 \pm 0.21 \times 10^{-6}$) and lowest in winter ($1.10 \pm 0.13 \times 10^{-6}$) (Fig. 1a).

Measured CH₄ emission rates ranged from 1.56×10^{-6} to 30.06×10^{-4} µg CH₄/m²/h across all sites and dates. Season had a significant impact ($P < 0.0001$), with rates highest in spring ($21.03 \pm 7.05 \times 10^{-5}$, mean $\pm$ S.E), followed by fall ($4.80 \pm 1.22 \times 10^{-5}$), and lowest in winter ($1.22 \pm 0.27 \times 10^{-5}$) (Fig. 1b). There was no season by site interaction ($P = 0.50$) nor significant site effect ($P = 0.22$), CH₄ emission rates were on average higher in BL vernal pools ($12.0 \pm 4.4 \times 10^{-5}$) relative to UL pools ($6.3 \pm 2.42 \times 10^{-5}$) throughout the study.

Vernal pool soil abiotic variables are summarized in Table 2. Season also significantly affected soil moisture ($P < 0.0001$), soil % organic matter content ($P < 0.0001$), and pH ($P < 0.001$). On average, soil moisture (g H₂O/g dry soil) was highest in spring (1.87 ± 0.20) and winter (1.90 ± 0.14), and lowest in fall (0.86 ± 0.07). Soil % organic matter was highest in fall ($10.61 \pm 0.62\%$) and significantly lower in winter (5.98 ± 0.47) and spring (6.28 ± 0.28). Finally, soil pH was high in fall (4.90 ± 0.07), intermediate in spring (4.45 ± 0.04), and least in winter (4.20 ± 0.21). There were no significant effects of site or season by site interactions for these variables. There was, however, was a significant season ($P = 0.003$) and site ($P = 0.0005$) effect on soil nitrate concentrations. On average, vernal pool soil nitrate concentrations

Fig. 1 N_2O and CH_4 emission rates across seasons. Season, location, and season $\times$ location for **a** N_2O emission rates ($\mu\text{g N}_2\text{O m}^{-2} \text{h}^{-1} \times 10^{-6}$) and **b** CH_4 emission rates ($\mu\text{g CH}_4 \text{m}^{-2} \text{h}^{-1} \times 10^{-6}$) from bottomland (BL) and upland (UL) vernal pools soil. In the figure, *, **, and *** represents

significant differences among seasons, and different letters represent significant season by location differences at $P=0.05$. N_2O and CH_4 emission rates are non-transformed in the figure. Bars represent standard error of means

were highest in fall (0.78 ± 0.22 , $\mu\text{g NO}_3/\text{g dm}$), intermediate in winter (0.29 ± 0.04), and lowest in spring (0.16 ± 0.04). Average nitrate concentration was significantly higher in bottomland vernal pools (0.70 ± 0.09) relative to upland vernal pools (0.13 ± 0.12) across seasons. There was no significant season by site interaction on vernal pool soil nitrate concentrations ($P=0.07$).

Season significantly impacted abundances of microbial 16S rDNA ($P < 0.0001$) and *norB* ($P < 0.0001$) gene abundances, with no significant site effect or season by site interactions (Fig. 2). On average, 16S rRNA gene abundance was highest in winter ($125.3 \pm 9.0 \times 10^5$, copy number/g dry mass), followed by fall ($71.7 \pm 8.7 \times 10^5$), and spring ($23.4 \pm 2.5 \times 10^5$). *norB* gene abundance was highest in fall ($14.9 \pm 1.5 \times 10^5$), followed by winter ($5.6 \pm 0.50 \times 10^5$) and spring ($1.6 \pm 0.18 \times 10^5$). In contrast, season ($P=0.07$), site ($P=$

0.20), and their interaction ($P=0.62$) did not significantly impact *nosZ* gene abundances, whereas the interaction between site and season significantly impacted *nirS* gene abundances ($P=0.04$). On average, *nirS* gene abundance was highest in fall ($1.2 \pm 0.20 \times 10^5$), and not statistically different (i.e. comparable) in spring ($0.53 \pm 0.11 \times 10^5$) and winter ($0.78 \pm 0.13 \times 10^5$). The abundances of the *nirS* gene were higher in BL pools than UL pools across seasons (Fig. 2). Finally, the abundance of the methanogenesis-related *mcrA* gene was only significantly impacted by season ($P < 0.0001$) (Fig. 2). *mcrA* gene abundances were highest in the spring ($1.1 \pm 0.35 \times 10^5$, copy number/g dry mass), reduced in fall ($0.16 \pm 0.04 \times 10^5$), and lowest in winter ($0.025 \pm 0.0053 \times 10^5$) following the methane emission rate pattern.

From the stepwise multiple regression analyses, a combination of the abiotic (% organic matter and nitrate

Table 2 Seasonal differences in vernal pool soil abiotic parameters across bottomland (BL) and upland (UL) vernal pools

Abiotic soil variables	Spring	Fall	Winter
Soil moisture ^a	BL 1.94 ± 0.32^A	BL 0.83 ± 0.13^B	BL 1.71 ± 0.12^A
	UL 1.80 ± 0.21^A	UL 0.88 ± 0.07^B	UL 2.05 ± 0.25^A
% Organic matter	BL 6.13 ± 0.36^B	BL 9.40 ± 0.56^A	BL 5.50 ± 0.85^B
	UL 6.44 ± 0.44^B	UL 11.86 ± 1.07^A	UL 6.46 ± 0.42^B
pH	BL 4.62 ± 0.06^B	BL 5.30 ± 0.09^A	BL 4.40 ± 0.40^B
	UL 4.28 ± 0.03^B	UL 4.53 ± 0.04^A	UL 4.03 ± 0.2^B
Nitrate ^b	BL $0.21 \pm 0.06^{B,C}$	BL 1.42 ± 0.20^A	BL 0.46 ± 0.06^B
	UL 0.11 ± 0.05^C	UL $0.15 \pm 0.36^{B,C}$	UL 0.13 ± 0.03^C

Season significantly impacted soil moisture, % organic matter, soil nitrate content, and pH. Values in the table are non-transformed abiotic soil variables. Different letters represent significant season or site effects differences at $P=0.05$

^a Soil moisture content expressed as g $\text{H}_2\text{O}/\text{g}$ dry weight of the entire soil sample

^b Nitrate expressed as $\mu\text{g NO}_3$ per/g dry weight of the entire soil sample. Significant site effect was uncovered only for nitrate ($P=0.0005$)

Fig. 2 Changes in gene abundances across seasons. Raw gene abundances (copy number g^{-1} dry mass $\times 10^6$) of bacterial, denitrification-related, and methanogenesis-related genes within and across seasons for bottomland and upland vernal pool soil core samples across seasons. *, **, and *** represents significant differences among seasons, and different letters represent significant season by location differences at $P=0.05$. Bacterial abundances are non-transformed in the figure. Bars represent standard error of means

concentration) and microbial (16S rDNA, *nirS*, and *norB* gene abundances) variables emerged as the best set of predictors of N_2O emission rates ($\text{AICc} = 38.70$, $R^2 = 0.46$, $\text{Cp} = 5.87$; number of predictors = 5) (Table 3). The coefficients of the abiotic variables in the regression model were positively related to N_2O emission rates, as expected. However, the

Table 3 Best fit models for N_2O and CH_4 emission rates (selected by AIC)

Best fit model components	Coefficients	<i>P</i> value
Denitrification rates		
Log % Organic matter	0.48	0.04
Log Soil nitrate	0.26	0.005
Log 16S rRNA	-0.27	0.02
Log <i>nirS</i>	-0.17	0.03
Log <i>norB</i>	-0.28	0.006
Methanogenesis rates		
Log % Organic matter	0.30	0.50
Log Soil pH	3.43	0.04
Log <i>mcrA</i>	0.50	< 0.0001

The abiotic and microbial variables contributing to the model as predictors of denitrification and methanogenesis rates, their coefficients, and associated P values across seasons. Significant variables are italicized at $P=0.05$

coefficients of the microbial variables were negatively related (Table 3). Soil organic matter, soil pH, and *mcrA* gene abundances were selected as the best set of predictors of CH_4 emission rates ($\text{AICc} = 133.6$, $R^2 = 0.36$, $\text{Cp} = 2.13$; number of predictors = 4). All three coefficients were positively related (Table 3). Data used in the stepwise regression analyses are provided in Table S2.

Discussion

Soil moisture and atmospheric temperature are recognized major drivers of landscape-level biogeochemical process rates due to their respective impacts on soil oxygen availability (increasing soil anaerobiosis) and increased microbial enzymatic activities in well-studied wetland systems (Butterbach-Bahl et al. 2013; Holgersson 2015). However, the impacts of other drivers and more importantly, their interactions, on these landscape-level biogeochemical process rates and the ability to predict landscape-level responses in less-studied systems remains a challenge (Butterbach-Bahl et al. 2013). Capps et al. (2014) in a seminal study, demonstrated that vernal pools within a temperate forest landscape were a significant nitrogen removal “hotspots” relative to broader adjacent *terra firma* sites, and suggested the need for further spatial and temporal examinations of biogeochemical process rates in these systems. We uncovered significant season- and site-specific differences in N_2O emission rates and significant seasonal differences in CH_4 emission rates, as well as season- and site-specific differences among measured soil abiotic and microbial variables in vernal pool soil samples in this study. The seasonal patterns of N_2O and CH_4 emission rates from vernal pools in this study are consistent with reported seasonal patterns from other wetlands, such as vernal pools (Kutzbach et al. 2004; Capps et al. 2014), ditches (Hansen et al. 2016), and estuaries and mangrove systems (Allen et al. 2007). Overall, our examination of drivers of both emission rates in vernal pools suggests that the impacts of biotic and abiotic variables on these rates are context-dependent and complex, underscoring the influence of spatial and temporal heterogeneity within landscapes on biogeochemical processes.

Drivers of Denitrification Rates

Organic matter and nitrate concentration were significant and positive predictors of denitrification rates in this study, as expected because this process utilizes organic matter as an electron donor and nitrate as an electron acceptor in the first step of denitrification (i.e., nitrate reduction) (Hansen et al. 2016; Tomasek et al. 2017; Sabha et al. 2017). The positive impacts of soil nitrate and organic matter content are consistent with other studies that demonstrate positive influences of nitrate concentrations on denitrification rates in sediments (Kjellin

et al. 2007; Groffman et al. 2009; Song et al. 2012) from comparatively larger wetland types, agricultural soils (Signor and Cerri 2013), and one singular study of vernal pools (Carrino-Kyker et al. 2012), as well as positive impacts of organic matter on denitrification rates across a variety of systems (Brentrup et al. 2000; Cameron et al. 2013; Capps et al. 2014). The importance of soil nitrate and organic matter content in predicting denitrification rates in wetlands, in general, might be particularly crucial in vernal pools in temperate forests. This is because these vernal pools receive significant seasonal organic matter input from external sources (leaf fall from surrounding tree stands, and insect remains), as well as experience considerable changes in temperature due to their shallow depths (Williams 2006; Kurylyk et al. 2019). These seasonal inputs vary among vernal pools in different locations in an ecosystem, and can potentially impact physicochemical properties of vernal pools under rapidly changing season-associated environmental conditions (pH, nitrate, and temperature) leading to different seasonal and site rates (Brentrup et al. 2000; Cameron et al. 2013). Differences organic matter content and overall N₂O emission rates between BL and UL sites in this study may be related to differences in dominant forest tree species (and consequently, leaf input), the decomposition rates of these leaf inputs, and underlying soil properties (bulk density, water holding capacity, etc.), and at the specific study sites as previously reported (Blackwood et al. 2013). The UL site is dominated by red maple (*Acer rubrum*) and sugar maple (*Acer saccharum*) followed by American beech (*Fagus grandifolia*), and black cherry (*Prunus serotina*), whereas the BL site is dominated by *Acer rubrum*, Spanish oak (*Quercus palustris*), black ash (*Fraxinus nigra*), American sycamore (*Palanus occidentalis*), and bitternut hickory (*Carya cordiformis*) (Blackwood et al. 2013). The presence of beech in the UL site and its complete absence in the BL site may impact decomposition rates (and consequently higher organic matter and lower nitrate content in UL sites), as beech litter tends to have slower decomposition rates compared to other temperate forest tree species (Gosz et al. 1973; Berger et al. 2015). This results in overall slower decomposition rates in mixed tree litter input studies (Jacob et al. 2010). The high soil nitrate content in fall may be due to high microbial and macroinvertebrate decomposing activities following leaf litter input), whereas the intermediate nitrate content by winter may be due to slowed down microbial and macroinvertebrate decomposing activities, and lowest nitrate content in spring may be attributed to thawing and increased microbial utilization and possibly leaching into deeper layers of vernal pool soils.

Interestingly, soil moisture was not a significant predictor of N₂O emission rates in this study. High soil moisture, and consequently low dissolved oxygen, are essential variables that often drive soil denitrification rates in terrestrial systems, but are not as crucial in wetland ecosystems (Vargas et al.

2018). Major wetland systems experience consistently low dissolved oxygen and high moisture content, leaving other variables, such as organic matter and nitrate content as the limiting variables (Taylor and Townsend 2010; Hansen et al. 2016). Unlike larger and deeper wetlands, the shallow and ephemeral nature of vernal pools dictates that, at some point, there is water limitation. In this study, there was a 54% decrease in soil moisture content in vernal pools in fall relative to spring. Although considerable, this 54% decrease in soil moisture did not appear to be a significant driver of N₂O emission rates. A possible explanation of this might be that there was reduced evaporative water loss (desiccation) from vernal pools as a result of increased forest vegetation cover during the fall. Thus, perhaps, forested vernal pools that are shaded by dense vegetation during periods of high temperature and low rainfall, may not be completely desiccated even if there is no actual surface water. It remains to be determined if this is solely a climatic effect of the study period, or if this relationship between moisture content and denitrification rates in vernal pools changes with varying annual temperature and precipitation regimes.

There were three significant microbial predictors of N₂O emission rates from vernal pools (16S rRNA, *nirS*, and *norB*); however, abundances of these genes were all unexpectedly negatively related to N₂O emission rates. This trend did not change following normalizing functional gene copy numbers to 16S rRNA gene copy numbers (data not shown). These denitrification-related microbial gene abundances have been used as indicators or predictors of denitrification in several studies, with mixed results. For example, studies have reported positive correlations between denitrification rates and *nirS* gene abundances from a variety of systems (Morales et al. 2010; Braker et al. 2010; Graham et al. 2010; Saarenheimo et al. 2015; Yang et al. 2017; Tomasek et al. 2017), suggestive of linkages between gene abundances and process rates since all denitrifiers have the *nir* genes (Zumft 1997). The detection of *nirS* is crucial because it is a definitive marker for denitrifying bacteria and is not found in nitrifying bacteria, unlike *nirK* (Casciotti and Ward 2006, 2001). Additionally, most *nirS*-containing (nitric oxide, NO-producing) denitrifiers also contain the *norB* (nitrous oxide, N₂O-producing) gene (Philippot 2002; Zumft 1997), and have similar gene expression patterns (Yoshida et al. 2012). This co-occurrence is significant because NO is toxic to bacteria and requires a rapid and efficient conversion of NO to N₂O (Sabba et al. 2017). However, similar to the data for vernal pools reported here, Chen et al. (2015) found a negative correlation between denitrification rates and denitrification-related gene abundances in grassland soils, while other studies have reported no significant correlations (Ducey et al. 2010; Dandie et al. 2011).

The observed negative relationships between gene abundances and N₂O emission rates in vernal pools were unexpected. A common explanation for lack of expected positive

relationships between gene abundances and microbe-mediated rates is that these gene abundances do not necessarily translate directly to actual gene expression. While this would be adequate to explain a casual relationship, it does not entirely explain the negative relationships observed here. The impacts of environmental variables on specific genotypes or functional groups in driving biogeochemical processes are not straightforward, particularly for denitrification. Denitrification is biochemically similar to aerobic respiration, with denitrification-related genes widespread among phylogenetically diverse facultatively anaerobic bacteria (Martiny et al. 2015). This makes it particularly challenging to assign nitrate reducing or denitrifying functions to the organisms carrying denitrification related genes. For example, fluctuating environmental variables (temperature, salinity) were shown to change the abundances and community compositions of denitrifying bacterial taxa in soils, resulting in incongruent patterns between selected gene abundances and denitrification rates (Braker et al. 2010; Hallin et al. 2012). Thus, in this study, the lack of congruence between gene abundances and denitrification activity may be attributed to specific small populations of denitrifying bacteria selected for by environmental conditions within vernal pools that are poised to take advantage of prevailing conditions but were undetected in this study. The shallow, and organic matter-rich nature of forested vernal pools relative to larger and deeper wetland types and non-forested vernal pools suggest perhaps that factors regulating microbial diversity, abundance, and activity are still largely unknown. Overall, the presence and ubiquity of denitrification genes across several bacterial phyla may have masked the detection of any positive associations between measured N_2O emission rates and denitrification gene abundances in vernal pools in this study.

Drivers of Methanogenesis Rates

Vernal pools and small temporary ponds tend to be rich in organic matter and relatively acidic to neutral in pH, emitting disproportionately more methane than surrounding *terra firma* sites (Kutzbach et al. 2004; Holgerson 2015; Holgerson and Raymond 2016). Much like denitrification, the seasonal relationship between CH_4 emission rates and inundation observed in this study is consistent with reported methane emission patterns in vernal pools (Capps et al. 2014) and other types of wetlands (Le Mer and Roger 2001; Allen et al. 2007; Hansen et al. 2016). Positive predictors of methanogenesis in this study were soil pH and organic matter content. The dominant sources of biogenic methane in wetlands are hydrogenotrophic methanogenesis, which is more pronounced in comparatively acidic soils and is limited by hydrogen (H_2) availability, and acetotrophic/acetoclastic methanogenesis, which becomes more dominant and relevant when hydrogen has been consumed, as in comparatively

alkaline soils (Conrad 1999). Both sources of methane are connected to the availability of organic matter since both substrates are by-products of organic matter fermentation. The relatively high soil organic matter content in the studied vernal pools in this study may explain the positively correlated, but not statistically significant relationship between CH_4 emission rates and soil organic matter. Whereas, the positive correlation between CH_4 emission rates and the slightly acidic soil pH in this study is perhaps suggestive of hydrogenotrophic methanogenesis underscored by the relatively low pH of the studied vernal pools following fermentation of organic matter (Conrad 1999; Le Mer and Roger 2001; Holgerson 2015; Holgerson and Raymond 2016).

Unlike denitrification, the abundance of the methanogenesis-related *mcrA* gene was positively and significantly correlated with measured CH_4 emission rates. This positive correlation between *mcrA* gene abundance is consistent with reports from studies in anaerobic reactors (Morris et al. 2014), rice paddies (Ma et al. 2012; Bao et al. 2014), streams (Crawford et al. 2017), and bogs (Milferstedt et al. 2010). This suggests a more direct linkage between *mcrA* gene abundance and methanogenesis. This linkage may be attributed to the fact that methanogenesis (both hydrogenotrophic and acetotrophic) is an extremely conserved microbial functional trait involving several gene subsystems present in a diverse but phylogenetically unique microbial group (Friedrich 2005; Martiny et al. 2015). Furthermore, unlike denitrifiers, methanogens are obligate anaerobes incapable of surviving using alternate respiratory mechanisms. This complex methanogenesis gene system is not readily amenable to horizontal gene transfer, and thus, the presence of the most conserved methanogenesis gene, *mcrA*, tends to be a good indicator of methanogenesis rates (Martiny et al. 2015). Thus, in this study, the observed pattern of CH_4 emission rates from vernal pools can be primarily attributed to the abundance of methanogens which vary between dry and wet periods in soils (He et al. 2015) and wetlands (Fu et al. 2015), the tightly-regulated methanogenesis gene system, the obligately anaerobic nature of methanogens in contrast to the derived nature of denitrification-related genes, and the heterotrophic nature of denitrifiers and nitrate reducers. This study is the first to our knowledge to report this positive relationship between CH_4 emission rates and methanogen abundance in forested vernal pools.

Conclusion

Although soil moisture and atmospheric temperature are conventionally recognized predictors of biogeochemical process rates in wetland systems, we demonstrate in this study that other soil abiotic variables, such as nitrate, pH, and organic matter content may be relevant drivers of biogeochemical processes, such as denitrification and methanogenesis in ephemeral,

shallow forested vernal pool wetland systems across seasons. Denitrification and methanogenesis rates in vernal pools in temperate northeastern forests were context-dependent and differentially impacted by microbial and abiotic variables. The spatial and temporal variations of these proximal soil and microbial variables within the studied vernal pools in the forested landscape, and how these impacted measured biogeochemical process rates (i.e., C and N cycling), underscore the “hot spot” nature of these wetland systems within landscapes. Vernal pools may be potential sites of significant N and C removal processes during “hot moments” when factors besides soil moisture and elevated temperature become relevant for vernal pool microbial assemblages. Long-term studies (≤ 2 years of seasonal data) examining the relationships among environmental, and microbial (composition and functional gene abundance) parameters, and biogeochemical process rates in these systems will provide more insights into the controls of these dynamics. Overall, studies of this nature provide insights into other factors that drive biogeochemical process rates in wetlands. They also improve the accuracy of emission factor assignments to wetland ecosystems (varying in size and nature) in greenhouse gas emission models within landscapes for improved predictive capabilities in response to changing climate (Sherlock et al. 2002; Li et al. 2002; Davidson and Kanter 2016; Gaillard et al. 2017).

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Compliance with Ethical Standards

Conflicts of Interest The authors declare no conflict of interest.

Ethical Statement No animal rights were violated in the execution of this study. Guidelines of the Kent State University’s Office of Research Compliance and Kent State Institutional Animal Care and Use Committee (IACUC) did not apply to the use of insects.

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