Nitrous Oxide from Beef Cattle Manure: Effects of Temperature, Water Addition and Manure Properties on Denitrification and Nitrification

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Abstract: Beef feedyards produce nitrous oxide (N₂O), a potent greenhouse gas. Limited research has evaluated the processes that produce feedyard N₂O, and how rainfall and temperature impact N₂O losses. Manure in feedyard pens develops into a complex ecosystem of microbes, extracellular enzymes, feces, and urine, with varying H₂O content. This study aimed to improve understanding of feedyard N cycling under differing environmental conditions by incubation of manure in simulated feedyard pens using large chambers under laboratory conditions. We hypothesized that nitrification was the primary source of feedyard N₂O, with interactions among temperature, H₂O content, and manure properties. Emissions of N₂O were monitored with a real-time N₂O analyzer. Manure samples were taken at intervals for analyses of physicochemical properties, denitrification enzyme activity (DEA), and nitrification activity (NA). Due to equipment limitations, there was only one chamber per temperature tested. Correlation was poor among N₂O emissions and rates of DEA and NA. However, significant relationships were found among key manure characteristics, such as ammonia/ammonium and nitrate/nitrite concentrations, manure dry matter, redox status, and temperature. These data suggest that most N₂O was derived from denitrification in the top 5 cm of the manure pack. Further study is warranted to identify the processes involved in flushes of N₂O emitted immediately after rainfall, possibly due to abiotic chemical reactions that release N₂O sequestered in manure pores.

Keywords: ammonia; denitrifier enzyme activity; manure; nitrifier activity; nitrate; nitrite; rainfall; temperature; wet-dry cycling

1. Introduction

Beef cattle feedyards produce nitrous oxide (N₂O) from manure [1–6]. Nitrous oxide is a potent greenhouse gas (GHG) implicated in climate change due to a global warming potential (GWP) of 265–298 carbon dioxide equivalent (CO₂e). Approximately 25% of total GHG (N₂O and methane (CH₄)) from beef production originates from manure in animal pens and housing [7]. However, few studies
have evaluated the processes via which feedyard N$_2$O is produced and the effects of environmental factors on these processes. Two primary modes of N$_2$O production in soil, manure, sediments, and other substrates are nitrification and denitrification [5,8]. Nitrification and denitrification generally occur under differing conditions (i.e., redox status, available substrates, and microbial community structure and activity) but can occur simultaneously or in tandem in complex ecosystems. Despite significant research on feedyard N$_2$O, a few key questions remain: (1) What processes are involved in feedyard N$_2$O production and how are they affected by temperature and rainfall? (2) What is the primary source of N$_2$O within a feedyard pen, i.e., the surface manure or the deeper, more anaerobic manure pack? A typical Texas feedyard, under typical dry conditions, is presented in Figure 1.

![Commercial feedyard in the Texas Panhandle.](image)

**Figure 1.** Commercial feedyard in the Texas Panhandle. Cattle are kept in soil–surfaced pens at a density of ~15 m$^2$-animal$^{-1}$ for 150 to 180 days. Manure accumulates until cattle are removed for slaughter. The pens develop wet/muddy areas, dry areas, and areas with high urine/feces where cattle congregate. The underlying manure forms a dense “pack” that generally contains more H$_2$O than surface manure.

Feedyard N$_2$O emissions are highly variable over both space and time [4,5]. This variability has been linked to temperature [2,4,9], H$_2$O content [2–4,10], and manure characteristics [1,4,11–13]. Unfortunately, the relationships observed between these variables and N$_2$O losses have been inconsistent, likely due to differences in manure physicochemical properties and treatment effects among studies. Redding et al. [1] used nonflow–through, nonsteady–state (NFT–NSS) chambers and saw high emissions (1.18 mg N$_2$O–N-m$^{-2}$-h$^{-1}$) in northern Australia, but emissions were dramatically lower (0.01 mg N$_2$O–N-m$^{-2}$-h$^{-1}$) at a feedyard in southern Australia. In another study, Parker et al. [2] showed that N$_2$O from dry manure was negligible (0.054 mg N$_2$O–N-m$^{-2}$-h$^{-1}$) but increased to as much as 200 mg N$_2$O–N-m$^{-2}$-h$^{-1}$ for a few hours after a simulated rainfall event. These data were somewhat consistent with the variability found by Redding et al. [1] and Aguilar et al. [10] at regional Australian feedyards and seasonally at a Kansas feedyard, respectively. Using an empirical model, Parker et al. [14] predicted that open–lot feedyards in the Texas panhandle emitted an annual average of 0.12 kg N$_2$O–N-animal$^{-1}$·year$^{-1}$. This unnecessary loss of valuable N could potentially be sustainably...
mitigated by reducing N\textsubscript{2}O emissions and retaining N in manure and composts for later use as crop fertilizer.

In soils, N\textsubscript{2}O is produced via multiple processes, including nitrification, denitrification, coupled nitrification–denitrification, dissimilatory nitrate reduction to ammonium (DNRA), anaerobic ammonia oxidation (Anammox), and various forms of chemodenitrification \[8,15\]. This should also be true for feedyard N transformations because of the complexity and heterogeneity of pen manure and changing weather in a system exposed to ambient conditions; it is unlikely that one single process is responsible for all feedyard N\textsubscript{2}O. The urine and feces excreted within feedyard pens forms an ecosystem of organic matter (OM) and nutrients essential for microbial metabolism (i.e., nitrogen (N), phosphorus (P), and sulfur (S)), bacteria, fungi and actinomycetes, and extracellular enzymes involved in nutrient cycling. Manure accumulates during a typical 150 day cattle finishing period to form a layered “pack” where properties (e.g., density, porosity, OM complexity, ammonia/ammonium (NH\textsubscript{x}) and nitrate/nitrite (NO\textsubscript{x}) availability, and microbial community structure) vary with depth \[16,17\]. To date, the relationship among feedyard N\textsubscript{2}O production, manure depth, and changing temperature and H\textsubscript{2}O content are unclear.

Despite the numerous possible avenues of N\textsubscript{2}O production, denitrification and nitrification are the predominant N\textsubscript{2}O production pathways in soil and manure composts. Nitrification occurs under aerobic conditions and is the stepwise oxidation of NH\textsubscript{4}+ or organic N to NO\textsubscript{3}-. Gross potential nitrification rates, measured as nitrification activity (NA), are used to assess in vitro N transformations \[18\]. Denitrification is a facultative respiratory pathway that occurs under anaerobic/anoxic conditions and in anaerobic microsites \[8,19,20\]. In the absence of O\textsubscript{2}, oxidized N species (i.e., NO\textsubscript{x}, NO, and N\textsubscript{2}O) are reduced and coupled to electron transport phosphorylation. Nitrous oxide is a frequent end–product if conditions are not optimal for the final step of N\textsubscript{2}O reduction to N\textsubscript{2} \[21\]. The rate of potential denitrifier enzyme activity (DEA) in soil is commonly assessed in vitro \[22\], and DEA rates were determined for beef manure under varying conditions \[11,12\].

The N cycle in feedyards differs from agricultural soils because there is no route within animal pens for plant NH\textsubscript{x} or NO\textsubscript{x} uptake. Thus, some excess N remains in the manure, a large portion (~50%) is lost to the atmosphere as ammonia (NH\textsubscript{3}), and smaller amounts of N\textsubscript{2}O, dinitrogen (N\textsubscript{2}), and nitric oxide (NO) are also emitted. Feedyard N can also be lost via runoff and leaching of liquid or particulate forms. The fate of manure N depends on the consortia of microbial species present, activities of enzymes involved in OM degradation and N mineralization, manure chemistry, aeration (i.e., O\textsubscript{2} content), H\textsubscript{2}O content, and substrate availability for microbial energy and respiration. Specific N\textsubscript{2}O production pathways vary with conditions and may occur consecutively or simultaneously in sequestered microsites within the manure pack \[23–25\].

To date, no study has assessed the interaction among N\textsubscript{2}O emissions, key variables, and DEA and NA rates in feedyard manure. The objective of this study was to simulate feedyard conditions in a controlled laboratory setting to improve understanding of feedyard N cycling so that effective N\textsubscript{2}O mitigation methods can be developed and employed. We hypothesized that both nitrification and denitrification contribute to feedyard N\textsubscript{2}O, but their predominance differs with environmental conditions and depth within the manure pack.

2. Experiments

2.1. Large–Chamber Incubation Study

Manure that accumulated during a typical 150 day finishing period was scraped from a pen at a commercial feedyard in the Texas Panhandle and transported to the United States Department of Agriculture–Agricultural Research Service (USDA–ARS) Conservation and Production Research Laboratory in Bushland, TX. The manure was a mixture of unconsolidated, loose surface manure and deeper packed manure from cattle fed a steam–flaked corn–based diet. Selected physicochemical parameters of the manure were as follows: 91% dry matter (DM); 55% OM; 341 mg NH\textsubscript{x}-kg\textsuperscript{-1};
5.7 mg NO₃·kg⁻¹; 2.6% total nitrogen (TN); 27.7% total carbon (TC); C:N ratio of 10.8. The manure was roughly ground (<0.64 mm) with a 15-HP, 420 cc chipper/shredder (Stanley Black & Decker, New Britain, CT, USA). Manure (109 mm depth) was added to each of five 1 m² (surface area) chambers (Figure 2a). Compacted native caliche (calcium carbonate soil; 89 mm) underlaid the manure in each chamber to simulate the relatively impermeable soil–manure interface under regional soil–surfaced feedyard pens [26,27]. The manure was compacted to an approximate dry bulk density of 0.61 g·cm⁻³ with a handheld tamper. The experimental period was 59 days in length and occurred in winter and spring of 2017. Chamber temperatures were controlled with 1.3 × 1.3 × 1.3 m “hotbox” material warmers (Model HB64–1440, Powerblanket, Salt Lake City, UT, USA) with digital temperature controllers and 12 V exhaust fans for venting (Figure 2b). In addition, silicone heating pads with digital temperature controllers (ProTherm Industries, Inc., Hermitage, TN, USA) were bonded to the bottom of each chamber. A single chamber (Chamber 1) did not have thermal regulation and was exposed to ambient temperatures, which varied diurnally and seasonally in an indoor facility without temperature control.

**Figure 2. **Equipment for determining nitrous oxide emissions from feedyard manure: (a) emissions were measured from five 1.0 m² (surface area) chambers sealed with a portable lid. Polyethylene tubing recirculated sampled air between chambers and a Los Gatos N₂O analyzer; (b) chamber temperatures were controlled with vented thermal blankets. Source: Parker et al. [1].
Two simulated 25 mm rainfall episodes were conducted by evenly applying distilled H$_2$O with a handheld watering can to the surface of each manure–filled chamber on 13 February (Day 1) and 17 March 2017 (Day 22). This decreased the manure DM content at 0–5 cm depth to ~77% and 68% on these days, respectively, and ~75% DM at the 5–10 cm depth on both days (Table 1). During the 8.4 week study, the manure in the chambers dried naturally between H$_2$O applications for investigation of dry–wet cycling effects. From Day 1 to 21, the five chambers were maintained at (1) 5.0, (2) 11.2, (3) 21.5, (4) 26.8, and (5) 17.2 °C. These temperatures simulated a range of winter, fall, and spring temperatures in the Texas Panhandle. On Day 22, the second simulated rainfall was applied and the temperatures of three chambers (1, 2, and 5) were increased and maintained at (1) 15, (2) 38.1, and (5) 46.2 °C. Headspace N$_2$O concentrations were measured from each chamber at 30 min intervals on the days of H$_2$O addition, and then daily (~6:00 a.m. Central Standard Time) for the remainder of the study. Details on the chamber system are available in Parker et al. [1,2]. In short, the chambers were fitted with a vented, portable lid that was moved among the chambers. Headspace air was recirculated from the sealed chamber with polyethylene tubing to a real–time N$_2$O analyzer (Model N2O/CO–30–EP Enhanced Performance, Los Gatos Research, Inc., San Jose, CA, USA). Concentrations of N$_2$O were recorded at 1 s intervals during 60 s measurement periods, with flux rates calculated from the slopes of N$_2$O concentrations vs. time using linear regression for 30 s periods. Emissions data were reported by Parker et al. [3] and are presented in Figure 3. Chamber temperatures (±0.1 °C) were monitored with thermistors (model #ACC–SEN–SDIP, Acclima, Inc., Meridian, ID, USA) placed mid–depth in the manure of each chamber.

![Figure 3](image_url)

**Figure 3.** N$_2$O–N fluxes from feedyard manure following two simulated rainfall applications. Emissions were baseline until H$_2$O was added, and then a large peak was observed in all chambers: (a) Days 1–20, and (b) Days 20–59, where temperatures were increased in three chambers and a second, longer–term N$_2$O peak was observed that was related to temperature. Source: Parker et al. [3].
Table 1. Nitrous oxide (N$_2$O) emissions, temperature and manure characteristics in five chambers (1, 2, 3, 4, and 5) that received two simulated rainfall episodes. Chambers were held at temperatures ranging from 5.0 to 46.2 °C, with increases on Day 22 (changes in italics). Samples were taken at (a) 0–5 cm and (b) 5–10 cm depths.

| Day and Chamber | Temp (°C) | N$_2$O–N (mg·m$^{-2}$·h$^{-1}$) | DM (%) | OM (% DM) | Eh (mV) | pH | NH$_x$ (mg·kg$^{-1}$) | NO$_x$ (mg·kg$^{-1}$) | Total N (% DM) | Total C (% DM) |
|----------------|----------|---------------------------------|--------|-----------|---------|----|----------------|----------------|---------------|---------------|
| 1 (1st H$_2$O application) |          |                                 |        |           |         |     |                 |                 |               |               |
| 1              | 5.0      | 1.43                            | 77.6 * | 54.7      | -27.0   | 9.00| 403             | 5.68 b          | 2.49 b        | 26.3 bc        |
| 2              | 11.2     | 4.26                            | 76.3 * | 55.3      | -29.7   | 9.05| 368             | 5.78 b          | 2.62 ab       | 28.4 a         |
| 3              | 21.5     | 66.1                            | 80.6 a | 55.7      | -19.7   | 8.92| 341             | 4.65 b          | 2.58 ab       | 26.5 bc        |
| 4              | 26.8     | 152                             | 68.7 b | 55.1      | -22.3   | 8.86| 402             | 24.2 a          | 2.68 a        | 28.1 ab        |
| 5              | 17.2     | 25.5                            | 81.9 a | 56.0      | -14.0   | 8.94| 352             | 4.27 b          | 2.51 b        | 26.1 c         |
| 9              |          |                                 |        |           |         |     |                 |                 |               |               |
| 1              | 5.0      | 0.21                            | 80.7 b | 56.2      | -15.3   | 8.95 a| 371 a          | 3.85 ab         | 2.67 a        | 28.6 a         |
| 2              | 11.2     | 0.46                            | 87.4 a | 54.3      | -32.7   | 8.82 b| 343 a          | 2.93 b          | 2.53 b        | 26.6 b         |
| 3              | 21.5     | 0.50                            | 86.1 ab| 54.9      | -12.3   | 8.85 ab| 297 bc         | 4.38 a          | 2.62 ab       | 27.6 ab        |
| 4              | 26.8     | 1.58                            | 87.9 a | 56.0      | -21.3   | 8.95 a| 273 c          | 4.99 a          | 2.68 a        | 28.3 a         |
| 5              | 17.2     | 0.45                            | 87.6 a | 55.1      | -12.3   | 8.86 ab| 334 ab         | 4.68 a          | 2.66 ab       | 27.6 ab        |
| 18             |          |                                 |        |           |         |     |                 |                 |               |               |
| 1              | 5.0      | 0.01                            | 88.8 b | 56.0      | -65.7   | 9.07 | 337 a          | 2.65 b          | 2.55          | 26.4 b         |
| 2              | 11.2     | 2.13                            | 91.6 a | 56.4      | -61.3   | 8.94 | 315 a          | 3.10 ab         | 2.63          | 28.3 a         |
| 3              | 21.5     | 0.83                            | 91.8 a | 55.6      | -67.7   | 9.03 | 290 a          | 3.67 ab         | 2.56          | 27.2 ab        |
| 4              | 26.8     | 0.93                            | 92.0 a | 55.3      | -66.3   | 9.04 | 215 b          | 4.98 a          | 2.59          | 26.6 ab        |
| 5              | 17.2     | 4.34                            | 90.1 ab| 57.0      | -68.3   | 9.00 | 312 a          | 4.42 ab         | 2.66          | 28.1 ab        |
Table 1. Cont.

| Day and Chamber | Temp (°C) | N$_2$O–N (mg·m$^{-2}$·h$^{-1}$) | DM (%) | OM (% DM) | Eh (mV) | pH | NH$_4$ (mg·kg$^{-1}$) | NO$_3$ (mg·kg$^{-1}$) | Total N (% DM) | Total C (% DM) |
|-----------------|-----------|-------------------------------|--------|-----------|--------|----|----------------|----------------|----------------|----------------|
| **22 (2nd H$_2$O application)** |           |                               |        |           |        |    |                  |                |                |                |
| 1               | 15.0      | 21.3                          | 70.9   | 57.4      | −16.3  | 9.06| 427              | 13.6            | 2.59            | 28.1           |
| 2               | 38.1      | 94.1                          | 63.1   | 53.4      | −98.3  | 8.85| 458              | 17.6            | 2.59            | 27.2           |
| 3               | 21.5      | 58.7                          | 70.0   | 56.3      | −37.3  | 8.98| 338              | 12.5            | 2.67            | 28.8           |
| 4               | 26.8      | 85.2                          | 63.4   | 55.5      | −69.7  | 8.94| 348              | 14.5            | 2.47            | 26.0           |
| 5               | 46.2      | 106                           | 71.5   | 54.7      | −62.7  | 8.94| 408              | 14.9            | 2.40            | 25.6           |
| **30**          |           |                               |        |           |        |    |                  |                |                |                |
| 1               | 15.0      | 0.22                          | 73.1   | 56.1      | −26.7  | 9.23| 286              | 3.99            | 2.90            | 30.5           |
| 2               | 38.1      | 13.1                          | 89.0   | 52.7      | 2.33   | 9.03| 217              | 3.69            | 2.70            | 28.0           |
| 3               | 21.5      | 3.33                          | 82.3   | 55.4      | −16.3  | 9.22| 150              | 5.99            | 2.75            | 28.0           |
| 4               | 26.8      | 4.50                          | 86.9   | 53.7      | −16.3  | 9.16| 153              | 3.89            | 2.65            | 26.8           |
| 5               | 46.2      | 6.71                          | 92.1   | 55.5      | −23.0  | 8.89| 275              | 3.25            | 2.43            | 25.1           |
| **59**          |           |                               |        |           |        |    |                  |                |                |                |
| 1               | 15.0      | 2.08                          | 87.9   | 56.5      | 52.3   | 8.95| 294              | 3.45            | 2.66            | 28.1           |
| 2               | 38.1      | 0.89                          | 91.7   | 54.9      | 16.0   | 8.96| 183              | 3.83            | 2.66            | 27.7           |
| 3               | 21.5      | 0.41                          | 90.4   | 55.1      | −3.3   | 9.09| 201              | 4.79            | 2.56            | 26.9           |
| 4               | 26.8      | 0.62                          | 91.1   | 55.0      | −11.3  | 9.13| 179              | 5.32            | 2.61            | 27.4           |
| 5               | 46.2      | 0.55                          | 93.4   | 53.8      | 0.00   | 8.94| 209              | 2.66            | 2.50            | 27.0           |
Table 1. Cont.

| Day and Chamber | Temp (°C) | N₂O-N (mg·m⁻²·h⁻¹) | DM (%) | OM (% DM) | Eh (mV) | pH | NH₄⁺ (mg·kg⁻¹) | NO³⁻ (mg·kg⁻¹) | Total N (% DM) | Total C (% DM) |
|-----------------|-----------|---------------------|--------|-----------|---------|----|----------------|----------------|----------------|----------------|
| 1 (1st H₂O application) |           |                     |        |           |         |    |                |                |                |                |
| 1               | 5.0       | 1.43                | 75.1   | 55.6      | −32.7  b| 9.03 a| 404 ab         | 4.19 b         | 2.57           | 28.4           |
| 2               | 11.2      | 4.26                | 71.6   | 55.1      | −30.7 b| 8.97 ab| 405 ab        | 10.7 b         | 2.64           | 28.0           |
| 3               | 21.5      | 66.1                | 83.0   | 54.3      | −32.0 b| 8.94 ab| 357 b         | 4.12 b         | 2.55           | 26.0           |
| 4               | 26.8      | 152                 | 65.6   | 54.7      | −20.7 ab| 8.83 b| 469 a         | 36.2 a         | 2.69           | 27.2           |
| 5               | 17.2      | 25.5                | 77.7   | 55.7      | −13.0 a| 8.96 ab| 370 b         | 6.74 b         | 2.57           | 26.0           |
| 9               |           |                     |        |           |         |    |                |                |                |                |
| 1               | 5.0       | 0.21                | 74.1 b | 54.9      | −21.0  | 8.87 | 497 a         | 8.65 a         | 2.61           | 27.5           |
| 2               | 11.2      | 0.46                | 81.4 ab| 52.9      | −30.3  | 8.84 | 394 ab        | 2.99 b         | 2.57           | 26.6           |
| 3               | 21.5      | 0.50                | 82.1 ab| 53.1      | −18.3  | 8.83 | 294 b         | 3.78 ab        | 2.45           | 25.5           |
| 4               | 26.8      | 1.58                | 84.9 a | 54.2      | −21.7  | 8.85 | 301 b         | 2.76 b         | 2.60           | 27.2           |
| 5               | 17.2      | 0.45                | 84.3 a | 52.7      | −19.3  | 8.81 | 343 b         | 2.86 b         | 2.59           | 26.9           |
| 18              |           |                     |        |           |         |    |                |                |                |                |
| 1               | 5.0       | 0.008               | 84.3   | 55.6      | −59.0  | 9.00 | 369 ab        | 2.96 bc        | 2.58           | 27.1           |
| 2               | 11.2      | 2.13                | 86.7   | 55.0      | −60.0  | 8.99 | 404 ab        | 2.72 c         | 2.58           | 26.9           |
| 3               | 21.5      | 0.830               | 88.0   | 55.2      | −62.7  | 9.02 | 255 d         | 4.40 ab        | 2.60           | 27.1           |
| 4               | 26.8      | 0.930               | 87.1   | 55.2      | −62.0  | 9.07 | 313 c         | 3.67 bc        | 2.61           | 26.7           |
| 5               | 17.2      | 4.34                | 82.7   | 56.2      | −67.3  | 9.00 | 316 bc        | 5.68 a         | 2.65           | 28.1           |
| Day and Chamber | Temp (°C) | N₂O-N (mg·m⁻²·h⁻¹) | DM (%) | OM (%) DM | Eh (mV) | pH | NH₄⁺ (mg·kg⁻¹) | NOₓ (mg·kg⁻¹) | Total N (% DM) | Total C (% DM) |
|----------------|-----------|----------------------|--------|------------|---------|----|----------------|----------------|----------------|----------------|
| 22 (2nd H₂O application) |           |                      |        |            |         |     |                |                |                |                |
| 22 (2nd H₂O application) |           |                      |        |            |         |     |                |                |                |                |
| 1              | 15.0      | 21.3                 | 85.4   | 55.5       | −25.3   | 9.02| 347            | 2.48           | 2.55           | 25.7           |
| 2              | 38.1      | 94.1                 | 68.1   | 53.6       | −146    | 8.79| 450            | 22.5           | 2.58           | 26.5           |
| 3              | 21.5      | 58.7                 | 81.3   | 56.5       | −67.0   | 8.99| 323            | 5.40           | 2.65           | 27.3           |
| 4              | 26.8      | 85.2                 | 66.7   | 52.3       | −80.7   | 8.94| 339            | 7.20           | 2.87           | 28.9           |
| 5              | 46.2      | 106                  | 70.6   | 54.9       | −67.0   | 8.92| 492            | 15.8           | 2.66           | 27.2           |
| 30             |           |                      |        |            |         |     |                |                |                |                |
| 30             |           |                      |        |            |         |     |                |                |                |                |
| 1              | 15.0      | 0.22                 | 63.8   | 55.3       | −22.7   | 9.05| 307            | 8.03           | 2.71           | 26.9           |
| 2              | 38.1      | 13.1                 | 79.3   | 52.5       | −6.6    | 8.97| 220            | 17.4           | 2.69           | 26.1           |
| 3              | 21.5      | 3.33                 | 66.2   | 52.4       | −14.3   | 9.04| 176            | 14.8           | 2.77           | 26.2           |
| 4              | 26.8      | 4.50                 | 78.8   | 52.1       | −22.3   | 9.00| 203            | 5.24           | 2.63           | 26.2           |
| 5              | 46.2      | 6.71                 | 84.3   | 53.6       | −26.3   | 8.82| 337            | 6.10           | 2.73           | 27.7           |
| 59             |           |                      |        |            |         |     |                |                |                |                |
| 59             |           |                      |        |            |         |     |                |                |                |                |
| 1              | 15.0      | 2.08                 | 83.6   | 53.2       | 35.0    | 8.97| 279            | 4.41           | 2.60           | 27.2           |
| 2              | 38.1      | 0.89                 | 87.1   | 50.6       | 15.7    | 8.96| 170            | 2.66           | 2.59           | 25.8           |
| 3              | 21.5      | 0.41                 | 86.0   | 52.9       | 0.33    | 8.92| 247            | 4.58           | 2.65           | 26.4           |
| 4              | 26.8      | 0.62                 | 89.3   | 55.0       | −9.67   | 8.93| 253            | 3.51           | 2.48           | 25.4           |
| 5              | 46.2      | 0.55                 | 91.1   | 53.8       | −12.3   | 8.88| 285            | 2.06           | 2.53           | 26.1           |

N₂O, nitrous oxide; Eh, oxidation–reduction potential; DM, dry matter; OM, organic matter; NH₄⁺, ammonium/ammonia; NOₓ, nitrate/nitrite; total N, total nitrogen; total C, total carbon.

* For each day, values within a column followed by different letters were significantly different (p ≤ 0.05) from other chambers on the same sampling day.
2.2. Manure Collection and Analyses

Manure samples were collected in triplicate from each chamber 2 h after H₂O application on Days 1 and 22, and then at 10:00 a.m. CST on Days 9, 18, 30, and 59. Manures were collected with a small trowel at depths of 0–5 cm and 5–10 cm inside a 30 cm (height) × 7.6 cm (diameter) tin cylinder inserted vertically into the manure. Care was taken to avoid disturbance of the manure both inside (i.e., depth mixing) and near the cylinder (i.e., integrity of emitting surface). For each sample, manure from the top 5 cm was removed manually, placed in polyethylene bags, and then stored on dry ice. The 5–10 cm depth fraction was collected and stored in the same manner.

Within an hour after sampling, oxidation–reduction potential (Eh) was measured according to using a Pocket Pro ORP Tester (Hach Company, Loveland, CO, USA), where manure was slightly wetted with degassed deionized H₂O. Manure pH was measured (1:10 (w/w) with deionized H₂O (pH 8.01)) with an Accumet XL250 pH meter and AccuCap combination pH electrode (Thermo Fisher Scientific, Waltham, MA, USA). Manure DM was determined gravimetrically after drying overnight at 105 °C. Concentrations of OM were determined by loss on ignition at 500 °C. Total N and TC contents were measured with a varioMAX CN analyzer (Elementar Analysensysteme GmbH, Hanau, DEU, Germany). NH₃ and NOₓ were extracted from manure samples with 2.0 M potassium chloride (KCl) for 30 min and quantified colorimetrically with a SEAL Analytical AQ2 Discrete Analyzer (SEAL Analytical Inc., Mequon, WI, USA) and the United State’s Environmental Protection Act Method 350.1 (NH₃) and Method 353.1 (NOₓ) [26].

2.3. Potential Denitrifier and Nitrification Enzyme Activities

Denitrifier enzyme activity was determined according to Woodbury et al. [11] and Ayadi et al. [12]. In brief, 10 g (DM) of each triplicate sample was placed in 250 mL Ankom bottles with sealable sidearm ports (Ankom Technology, Macedon, NY, USA). A solution (90 mL) of 0.5 g·L⁻¹ chloramphenicol, 5 mM glucose, and 10 mM potassium nitrate (KNO₃) was added to each bottle, swirled to mix, and flushed with argon to create anaerobic conditions. The bottles were quickly capped with Ankom RF1 Gas Production Modules programmed to an Ankom Base Coordinator to release headspace gas (Figure 4a). The bottles were shaken at 21 °C for 54 h at 180 rpm. Liquid samples (1.0 mL) were collected with a thin pipette through the bottle side ports at 0, 2, 4, 6, 24, 48, and 54 h (Figure 4b), placed in 1.5 mL microcentrifuge tubes, flash–frozen in dry ice/ethanol, and stored at −80 °C. Samples were then analyzed for NOₓ as previously described. Rates of DEA were calculated with linear regression of NOₓ loss over time according to Ayadi et al. [12]. However, graphs of the data showed that a linear fit for the entire 54 h period was not ideal: R² values ranged from 0.682–0.991. A bimodal data trend indicated there was either (1) more than one mechanism of NOₓ transformation occurring, or (2) a lag in enzyme activation (Figure 5). To accommodate for the different DEA rates over time, linear regression was used to calculate rates for DEA–short (DEAₛ) from 0–24 h incubations and DEA–long (DEAₗ) from 24–54 h incubations. Although linear regression did not always produce the best model fit for DEAₗ data, this method was selected to ease comparison with other DEA rates because of high variability in the patterns of DEAₗ rates over time.

The protocol to assess nitrification as NA was adapted from Ayadi et al. [12] and Woodbury et al. [11]. Manure (5 g DM) was placed in 250 mL Erlenmeyer flasks with 50 mL of 1.0 mM phosphate buffer containing 0.25 mM ammonium sulfate ((NH₄)₂SO₄) and 0.1 M potassium chloride (KClO₃). Flasks were loosely capped with aluminum foil and incubated/shaken at 180 rpm in the dark at 21 °C. As previously described for DEA analyses, liquid samples were removed at intervals of 0, 2, 4, 6, and 24 h, frozen, stored, and later colorimetrically analyzed for NOₓ concentrations. Changes in NOₓ over time were used to calculate NA rates using linear regression of data from 0–6 h (mean R² = 0.902). Quadratic and other regression fits were attempted; however, linear regression produced the highest R² values.

It is important to note that these DEA and NA rates are “potential” gross rates rather than in situ net denitrification and nitrification rates because these assays were designed with optimized reaction conditions (e.g., appropriate redox status, high substrate concentrations, standardized temperatures).
To minimize laboratory hazards, acetylene was not used in the DEA assay to block conversion of N\textsubscript{2}O to N\textsubscript{2}. Furthermore, no instrumentation was available to measure headspace N\textsubscript{2}O; it was assumed that all NO\textsubscript{x} was converted to N\textsubscript{2}O and overestimation of true N\textsubscript{2}O production could have occurred. Due to spatial and temporal variability in feedyard pen manure, in situ feedyard N transformations may be lower and more variable than measured in laboratory DEA and NA assays. However, DEA and NA studies provided background information on potential N transformations in manure over time and with temperature and H\textsubscript{2}O content changes.

**Figure 4.** (a) Denitrifier enzyme activity (DEA) analyses on manure using an Ankom system. (b) Liquid samples were collected through an anaerobic sidearm port on Ankom bottles and analyzed for nitrate/nitrite disappearance over time. The Ankom Gas Production module (lid) released excess headspace gas from bottle at predetermined pressure.

**Figure 5.** Example curve of nitrate/nitrite (NO\textsubscript{x}) loss for denitrification enzyme activity (DEA) studies. There were clear bimodal trends for all plots, where DEA rates changed after 24 h. Thus, DEA rates were also calculated as DEA–short (DEA\textsubscript{s}; 0–24 h) and DEA–long (DEA\textsubscript{l}; 24–54 h).

### 2.4. Statistical Analyses

Data were analyzed with the SAS PROC GLM procedure (SAS Institute Inc., Cary, NC, USA). The experiment included two depths (0–5 cm, 5–10 cm), eight temperatures (5.9, 11.2, 15.0, 17.2, 21.5, 26.8, 38.1, and 46.2 °C), two H\textsubscript{2}O applications (Day 1 and 22), and six manure sampling days. A total of 180 samples were collected. Rates of DEA and NA were calculated as the slope of regression analyses (Microsoft Excel, Microsoft Corp., Redmond, WA, USA). Significant differences among treatments were determined using ANOVA, where \( p \leq 0.05 \). Duncan’s least significant difference test was used.
for mean separations. Pearson’s correlation coefficients were employed to determine positive and negative relationships among variables with Proc CORR.

3. Results and Discussion

3.1. Manure Properties

Selected physicochemical properties of the manures from the two depths, five chambers, and six sampling dates are presented in Table 1, along with N₂O emissions data from Parker et al. [3]. Manure DM content after both artificial rainfall events ranged from 63% to 82% at 0–5 cm and 66% to 85% at 5–10 cm. Overall, manure DM was negatively related to N₂O emissions (p ≤ 0.001) at both depths (Figure 6), indicating that a higher H₂O content led to increased emissions and suggesting denitrification as a N₂O production mechanism. Regression R² values were 0.50 (0–5 cm) and 0.18 (5–10 cm) for N₂O emissions as a function of manure DM, which indicated that the majority of N₂O was derived from the manure surface rather than deeper in the pack. Previous studies used ^1⁵N isotope analyses to determine N₂O production mechanisms in composts and manure-amended soil [28,29]. Water–filled pore space (WFPS) was found to be a key factor in determining N₂O emission process in manure–amended soils, with up to 77% of N₂O originating from heterotrophic denitrification at high WFPS (70% and 80% WFPS) and nitrification at 60% WFPS [30]. The manure used in the current study was considerably drier than the soils of Baral et al. [30], even after simulated rainfall application; however, denitrifiers could be active in sequestered anaerobic microsites within the manure. Maeda et al. [29] reported that the production method for N₂O from dairy manure compost differed with sampling depth, where the isotope site preference, which can indicate origin as nitrification or denitrification, showed that surface samples produced N₂O via bacterial denitrification. In contrast, samples from the core of the compost pile displayed unrecognized isotopic signatures that could not be interpreted. These authors concluded that N₂O from compost largely originated from bacterial denitrification of recently turned material on the surface of the compost pile.

![Figure 6](image-url)

**Figure 6.** Pearson correlation coefficients (r values) of measured variables against nitrous oxide (N₂O) emissions at (a) 0–5 cm depth and (b) 5–10 cm depth. Bars to the right of the y-axis indicate a positive (+) relationship and bars to the left are negatively (−) correlated. Asterisks indicate significant relationships at * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

On days of simulated rainfall, added H₂O did not visibly infiltrate to the 5–10 cm depth and tended to form a crust on the manure surface; however, there were some differences in Eh over time (Table 1). There was a tendency for reduced Eh with simulated rainfall at both depths that increased as...
the manures dried between rainfall events. The relationship between N\textsubscript{2}O emissions and Eh differed with depth. Eh had a strong relationship with emissions at 0–5 cm, where a more negative Eh (reducing environment) was associated with increased N\textsubscript{2}O ($p \leq 0.01$; Figure 6a); however, the relationship was positive at 5–10 cm ($p \leq 0.001$; Figure 6b). The rationale behind this difference was unclear but simulated rainfall generally produced conditions favorable for denitrification (lower Eh and DM content).

Manure is highly buffered due to organic components that control pH via base cation exchange, decarboxylation of organic anions, and other chemical mechanisms [27–30]. In the current study, manure pH was within the narrow ranges of 8.8–9.2 (0–5 cm) and 8.8–9.0 (5–10 cm) (Table 1). Nevertheless, there were some significant pH differences among the chambers; chambers held at higher temperatures tended to have lower manure pH values, but there was no clear relationship between pH and temperature ($R^2 = 0.031$). Negative relationships between N\textsubscript{2}O emissions and manure pH were identified at both depths ($p \leq 0.05$), suggesting greater N\textsubscript{2}O losses at lower pH (Figure 6). However, caution must be used when interpreting these data due to pH effects on other gaseous N species, such as NH\textsubscript{3}; higher manure pH promotes NH\textsubscript{3} volatilization [31–36]. The slightly higher pH observed in some chambers of this study could have favored NH\textsubscript{3} over N\textsubscript{2}O emission, which would reduce the amount of NH\textsubscript{4}\textsuperscript{+} available for nitrification to N\textsubscript{2}O.

Only minor changes in manure TN and TC contents were noted with time, temperature, or depth, where TN and TC ranged from 2.4–2.9% and 25–31% DM, respectively (Table 1). The $R^2$ values for N\textsubscript{2}O against TN and TC were low: 0.02 for both parameters at 0–5 cm, and 0.04 (TN) and 0.01 (TC) at 5–10 cm. This indicated that manure TC and TN contents did not change appreciatively and were not major factors involved in controlling N\textsubscript{2}O losses in this study. The major substrates for denitrification and nitrification, NH\textsubscript{3} and NO\textsubscript{x}, showed some interesting changes during the study period. Concentrations of NH\textsubscript{3} were highest after simulated rainfall applications on days 1 and 22, with mean values of 385 and 396 mg NH\textsubscript{3}·kg\textsuperscript{-1} at 0–5 and 5–10 cm, respectively. Concentrations of NH\textsubscript{3} then decreased over time, particularly at temperatures between 21.5 and 40 °C (Figure 7a,b). This decrease was likely due to NH\textsubscript{3} volatilization or nitrification of NH\textsubscript{3} to NO\textsubscript{x}; however, no corresponding increase in NO\textsubscript{x} was observed (Figure 7c,d; Table 1). Concentrations of NH\textsubscript{x} were positively related to N\textsubscript{2}O emissions at both depths ($p \leq 0.001$) (Figure 6), with $R^2$ values of 0.23 at 0–5 cm and 0.18 at 5–10 cm. At 0–5 cm, NH\textsubscript{x} content tended to be lower at higher temperatures, indicating NH\textsubscript{3} losses or nitrification (Figure 7a). After the second simulated rainfall (Day 22), there were notable increases in NH\textsubscript{x} at 5–10 cm when temperatures were >40 °C (Figure 7b); however, regression analyses of temperature against NH\textsubscript{x} produced poor $R^2$ values ($p < 0.06$) that did not clearly indicate a relationship between NH\textsubscript{x} and temperature within the manure pack. By the end of the experiment, NH\textsubscript{x} concentrations ranged from 179–294 mg·kg\textsuperscript{-1} and 170–285 mg·kg\textsuperscript{-1} at 0–5 and 5–10 cm, respectively (Table 1).

![Figure 7](image-url)
Concentrations of NO\textsubscript{x} at 0–5 cm ranged from 4.3 to 24 mg·kg\textsuperscript{-1} after the first simulated rainfall, then tended to decrease over time until the second simulated rainfall (Table 1, Figure 7c). Interestingly, NO\textsubscript{x} concentrations of Chamber 4 (26.8 °C) at both depths were higher than in the other chambers after the first rainfall application (Figure 7c,d). The rationale behind this NO\textsubscript{x} spike may be related to the interaction of high chamber temperature with H\textsubscript{2}O application to very dry manure. A large flush of N\textsubscript{2}O was emitted at this time (Table 1), which was highest (152 mg N\textsubscript{2}O–N·m\textsuperscript{-2}·h\textsuperscript{-1}) from Chamber 4. This suggests that the high NO\textsubscript{x} content could be a result of conversion of NH\textsubscript{x} to NO\textsubscript{x} via nitrification.
or release of NOx sequestered in manure pores that would then be available for denitrification to N2O. Similar observations of a rapid flush of N2O were observed in wetted soils by Rudaz et al. who found that denitrification was a major source of N2O when soils had a high water content and that denitrifiers adapt quickly to changing conditions to take advantage of available resources. Alternately, this could be due to chemodenitrification following wet–dry cycling, where NO2− reacts with lignins and other forms of manure OM to form nitroso and oximino compounds prior to abiotic N2 and N2O formation [36–41]. Passive diffusive N2O emissions were documented from soil when water displaces the soil air during infiltration [37]. While it was unclear why NOx concentrations in Chamber 4 were higher than in other chambers at the first simulated rainfall, these values quickly decreased and were similar to observations in other chambers for the rest of the study. Correlation analyses revealed a strong positive relationship between NOx and N2O losses ($p < 0.001$) (Figure 6), and regression $R^2$ values were 0.55 (0–5 cm) and 0.33 (5–10 cm). These values support the premise that most N2O was derived from the manure surface. From these limited data, it is difficult to discern if nitrification, denitrification, or some other (bio)physicochemical process were responsible for N2O fluxes. However, data for NOx and NHx suggest that both nitrification and denitrification processes could occur simultaneously after rainfall, as measured concentrations of both substrates tended to increase after H2O application. Anaerobic, reducing conditions created by H2O addition to the manure surface provided an environment for denitrification on the manure surface, while higher NHx concentrations induced by wetting created conditions suitable for nitrification at both depths.

3.2. Nitrous Oxide Emissions

This work builds on that of Parker et al. [3], who reported N2O emissions from this study. In summary, a rapid, large flush of N2O was detected 2 to 11 h after both simulated rainfall events, with magnitudes ranging from <20 to 190 mg N2O–N·m$^{-2}$·h$^{-1}$ (Figure 3). This occurred at all temperatures tested, but the rate was greatest in higher–temperature chambers; emissions at 5.0, 11.2, and 15.0 °C were very low (Figure 5). A similar early N2O flush from agricultural soils was reported by Manalil et al. [42], who proposed that emissions immediately after wetting occurred because H2O displaced O2 during infiltration into manure and created anaerobic conditions that promoted denitrification in a high–N and high–C environment. Xu et al. [37] also saw passive N2O diffusion as water infiltrated soil airspaces and displaced sequestered N2O. Alternate explanations could be (1) displacement and release of N2O sequestered in manure pore spaces, (2) aggregate disruption with release of previously protected substrate, and/or (3) increased soluble substrate availability. Other means of N2O release could be an abiotic physicochemical event such as pH/Eh effects, wet–dry cycling, chemical dissociation of hydroxylamine or other N species to N2O, or complex interactions among biotic and abiotic processes [8,41–43].

At higher temperatures (≥38 °C), a second N2O episode occurred ~3 days after H2O addition (Figure 3b). This emission peak was shorter in height (0.06 to 35 mg N2O–N·m$^{-2}$·h$^{-1}$) than the first episode, but had a longer duration (~2 weeks) and was stimulated by higher temperatures to produce a N2O flush that was greater in both magnitude and duration than the initial N2O peak after wetting. A second emission peak was also observed at 31 °C in an earlier work by Parker et al. [1], who used the same methodologies as the current study. Emissions of N2O during the second episode began ~24 h after H2O application and peaked after 3 days. The absence of the second peak from most chambers at lower temperatures (Figure 3a) was likely due to suboptimal conditions for OM mineralization and microbial/enzymatic processes related to N2O production. A thermostat malfunction occurred in Chamber 3, where the temperature increased to 45 °C for 6 h at 9 days after H2O application, causing a brief spike in emissions and providing further evidence for temperature effects on N2O from feedyards. At higher temperatures, the majority of N2O was derived from the second peak, accounting for 80–89% of cumulative emissions (Figure 3b). These results suggest that maximum cumulative feedyard N2O emissions should occur in warm/hot seasons around a week after a rainfall event; brief, but intense, flushes of N2O occur immediately after rainfall and emit more N2O at higher temperatures.
In soils, monitoring changes in TN, TC, C:N ratios, NH₃, and NOₓ may help infer N₂O production mechanisms. However, feedyard manure is complex and heterogeneous, contains nonlimiting amounts of C and N, and has variable aerobic and anaerobic microsites (e.g., near water troughs and lounging areas, deep in the manure pack) [4,5,9,43]. As manure changes with wetting and drying, there is potential for simultaneous or coupled N₂O production from both denitrification and nitrification [8,11,43]. In addition, N₂O could be produced by a suite of biotic and abiotic reactions related to wet–dry cycling [41]. In a study by Redding et al. [1], N₂O emissions were positively related to manure density, pH, and temperature, whereas negative relationships were identified between N₂O and manure H₂O and organic C contents. Waldrip et al. [4] conducted 15 NFT–NSS measurement campaigns on two commercial Texas feedyards and developed predictive empirical models, where temperature and manure NOₓ and H₂O contents were positively related to measured N₂O losses. Negative relationships were identified between N₂O and manure OM, NH₃, dissolved organic C (DOC), and dissolved N contents, as well as ultraviolet–visible (UV–vis) parameters related to OM complexity/availability.

There is uncertainty about the source of N₂O on feedyards. Is it emitted from the drier, aerobic, loose manure on the pen surface, or is it derived from deeper in the manure pack, where H₂O content is higher, and OM is more mature and stable? Mielke et al. [26] reported feedyard manure bulk densities that ranged from 0.75 to 0.93 g·cm⁻³, which are typical of the deeper manure in the Texas Panhandle. In contrast, bulk densities of <0.50 g·cm⁻³ are common for the loose surface manure. Furthermore, self–sealing of the pores in the deeper layers occurs due to physical compaction and biological gleying, where microbial mucilage clogs pores and creates anaerobic conditions [26,27]. It is unclear to what extent the deeper manure is permeable to gas diffusion leading to N₂O loss to surface air. The regression data of the current study suggested that N₂O losses were more closely related to surface manure characteristics than the pack manure. Further study is required to determine manure pack diffusion of N₂O under feedyard conditions.

3.3. Denitrification Enzyme Activity

Assays of DEA measure the concentration of denitrifying enzymes in a sample on the basis of the principle that denitrification rates are proportional to enzyme concentrations under nonlimiting conditions. The DEA assay conducted for the current study was based on Tiedje [22] and optimized for beef manure [11,12]. The optimized assay considered the high amounts of glucose and KNO₃ needed to provide nonlimiting conditions for feedyard microorganisms [11]. In typical DEA assays, acetylene gas is added to the headspace to block conversion of N₂O to N₂ in the last step of the denitrification pathway. Acetylene was not used in our assays due to safety concerns; thus, measured DEA in this study was a proxy of NOₓ loss in solution rather than N₂O production into the headspace. In preliminary studies, the chloramphenicol concentration (0.5 mg·L⁻¹) was optimized to effectively inhibit de novo enzyme synthesis without influencing DEA rates. The raw data plots of changes in NOₓ concentration over the entire 54 h incubation time were not linear at either depth; average $R^2$ values were 0.889 ± 0.60 (range 0.682 to 0.991). There was a bimodal response, where DEA rates increased after 24 h. Example raw DEA data are presented in Figure 8, which shows NOₓ losses 8 days after the first simulated rainfall event. This bimodal response could be due to the presence of two or more types of denitrification enzymes (one slow and one faster), time required to adapt to conditions, or an unknown phenomenon. Researchers have proposed different sampling times for DEA assays. Smith and Tiedje [43] and Tiedje [22] recommended a 1 h incubation period that should not exceed 2 h. Woodbury et al. [11] adopted this approach and conducted 2 h DEA incubations with beef manure, with samples taken every 15 min. Ayadi et al. [12] adopted a longer–term approach with an incubation time of 56 h. Our approach included both shorter– (DEAₛ, 0–24 h) and longer–term (DEAₐ, 24–54 h) measures to determine DEA rates, with a total incubation time of 54 h.
There was clear variability in DEA rates both among samples and at different depths (Figure 8). This was particularly noticeable at 5–10 cm, where NOx concentrations ranged from −0.20 to 0.48 mmol·g⁻¹ at 30 h, but then rapidly decreased to very low levels in all samples (Figure 8b). The surface samples (0–5 cm) showed highly variable, but generally slower, NOx utilization after 24 h than at 5–10 cm. The data were split into 0–24 h and 24–54 h periods and further evaluated as DEA-short (DEAs) and DEA-long (DEAl), respectively. Rates of DEA, DEAs, and DEAl are presented in Table 2.

Table 2. Denitrifier enzyme activity (DEA) and nitrification enzyme activity (NA) in five chambers (1, 2, 3, 4, and 5) that received two simulated rainfall episodes. Chambers were held at temperatures ranging from 5.0 to 46.2 °C, with increases on Day 22 (changes in italics). Samples were taken at 0–5 cm and 5–10 cm depths. Temperatures denoted in bold italics increased on Day 22. DEA rates were calculated as follows: (1) DEA, 0–54 h incubation; (2) DEAs, 0–24 h incubation; (3) DEAl, 24–54 h incubation.

| Day and Chamber | Temp (°C) | DEA (µmol·g⁻¹·h⁻¹) | DEAs (µmol·g⁻¹·h⁻¹) | DEAl (µmol·g⁻¹·h⁻¹) | NA (µmol·g⁻¹·h⁻¹) |
|-----------------|----------|---------------------|---------------------|---------------------|---------------------|
|                 |          | 0–5 cm | 5–10 cm | 0–5 cm | 5–10 cm | 0–5 cm | 5–10 cm | 0–5 cm | 5–10 cm | 0–5 cm | 5–10 cm |
| 1               | 5.0      | 0.34 b | 0.46 b | 0.24 | 0.23 | 1.14 ab | 0.79 b | 0.18 a | 0.17 |
|                 | 11.2     | 0.69 ab | 0.74 ab | 0.25 | 0.17 | 1.24 ab | 1.45 a | 0.10 b | 0.12 |
|                 | 21.5     | 0.67 b | 0.80 ab | 0.34 | 0.24 | 0.94 b | 1.40 ab | 0.14 ab | 0.16 |
|                 | 26.8     | 0.90 ab | 1.05 a | 0.44 | 0.29 | 1.69 a | 1.96 a | 0.11 ab | 0.11 |
|                 | 17.2     | 0.95 a | 0.94 a | 0.80 | 0.15 | 1.58 a | 1.79 a | 0.11 ab | 0.13 |
| 9               | 5.0      | 0.94 | 3.76 | 0.32 | 0.52 s* | 1.56 | 1.46 | 0.14 b | 0.11 b |
|                 | 11.2     | 0.93 | 0.97 | 0.64 | 0.33 ab | 1.07 | 1.52 | 0.17 ab | 0.15 ab |
|                 | 21.5     | 0.96 | 0.97 | 0.61 | 0.22 ab | 1.16 | 1.66 | 0.20 ab | 0.20 a |
|                 | 26.8     | 0.96 | 0.92 | 0.81 | 0.26 ab | 0.95 | 1.53 | 0.24 a | 0.15 ab |
|                 | 17.2     | 0.89 | 0.88 | 0.27 | 0.14 b | 1.44 | 1.70 | 0.18 ab | 0.12 b |
| 18              | 5.0      | 0.81 | 0.85 a | 0.17 b | 0.23 | 1.50 ab | 1.33 | 0.14 | 0.11 c |
|                 | 11.2     | 0.85 | 0.88 a | 0.17 b | 0.30 | 1.32 ab | 1.33 | 0.14 | 0.13 bc |
|                 | 21.5     | 0.87 | 0.88 a | 0.56 ab | 0.33 | 1.07 ab | 1.28 | 0.18 | 0.16 b |
|                 | 26.8     | 0.84 | 0.43 b | 0.84 a | 0.43 | 0.79 b | 1.30 | 0.16 | 0.23 a |
|                 | 17.2     | 0.78 | 0.61 ab | 0.27 b | 0.43 | 1.36 ab | 1.09 | 0.18 | 0.13 bc |
| 30              | 5.0      | 0.63 | 0.74 | 0.25 | 0.16 b | 1.08 | 1.06 | 0.09 ab c | 0.13 ab |
|                 | 38.1     | 0.68 | 0.94 | 0.52 | 0.65 a | 1.01 | 1.54 | 0.10 ab c | 0.16 ab |
|                 | 21.5     | 0.57 | 0.64 | 0.31 | 0.32 b | 0.90 | 1.03 | 0.12 a | 0.16 a |
|                 | 26.8     | 0.84 | 0.66 | 0.54 | 0.47 ab | 1.22 | 0.95 | 0.05 c | 0.09 b |
|                 | 46.2     | 0.64 | 0.57 | 0.21 | 0.18 b | 1.16 | 1.07 | 0.06 bc | 0.08 b |
| 59              | 15.0     | 0.74 | 1.03 a | 0.33 b | 0.74 | 1.20 | 0.96 ab | 0.10 b | 0.05 b |
|                 | 38.1     | 0.85 | 0.66 b | 0.72 ab | 0.65 | 0.89 | 0.75 b | 0.08 b | 0.14 a |
|                 | 21.5     | 0.82 | 1.02 a | 0.66 ab | 0.82 | 0.83 | 1.04 ab | 0.14 ab | 0.08 b |
|                 | 26.8     | 0.96 | 1.12 a | 0.82 a | 0.90 | 0.87 | 0.90 ab | 0.07 b | 0.16 a |
|                 | 46.2     | 0.85 | 0.94 a | 0.40 ab | 0.35 | 1.02 | 1.24 a | 0.24 a | 0.16 a |

* For each day, values within a column followed by different letters were significantly different among chambers on that particular sampling day (p ≤ 0.05).
The concentration of denitrifying enzymes in a sample on the order of 0.16 µmol g⁻¹ h⁻¹ from 0.20 to 0.48 cm headspace to block convection.

This was over twofold greater than ambient control (15.60 ± 0.05) with mean DEA values of Woodbury et al. [11] and Ayadi et al. [12], which recorded data on DEA (µmol g⁻¹ h⁻¹) concentrations and N₂O emissions. In their study, DEA rates were decreased over the entire 54 h incubation period. The optimized assay considered the high amounts of glucose and KNO₃ in the last step of the denitrification enzyme activity (DEA) study in manure from beef manure, with samples taken every 15 min. Ayadi et al. [12] reported longer-term (24–56 h) in fresh bedded beef manure pack that ranged from 0.8 to 1.4 mmol g⁻¹ h⁻¹. In their study, the DEA rates were decreased at a higher temperature (40 °C) but increased to ~3.5 mmol g⁻¹ h⁻¹ with manure age. This suggests that there is an adaptation period prior to H₂O application, DEA rates were 0.65 ± 0.16 µmol N₂O g⁻¹ h⁻¹ (R² = 0.90). These rates were very low compared to those of Woodbury et al. [11] and Ayadi et al. [12], which recorded data from beef manure in mmol, rather than the µmol g⁻¹ h⁻¹ values of the current study. Two hours after the first H₂O application, manure in Chamber 5 (17.2 °C) at 0–5 cm had higher DEA rates than the ambient control (Chamber 1) (Table 2a). This was followed by a period where all chamber DEA rates were statistically equivalent until Day 59, when DEA in Chamber 5 was 50% lower (0.45 µmol g⁻¹ h⁻¹) than in other chambers (~0.90 µmol g⁻¹ h⁻¹). This was likely due to enzyme inhibition or suboptimal conditions for microbial activity after the temperature of Chamber 5 was increased from 17.2 °C to 46.2 °C on Day 22. This effect was also seen at 5–10 cm, with higher rates in Chambers 4 and 5 (1.05 and 0.94 µmol g⁻¹ h⁻¹, respectively) than ambient control (0.46 µmol g⁻¹ h⁻¹) on Day 1 and reduced DEA in Chamber 5 on Day 59, compared to other chambers. At the lower depth, the chambers with the highest temperatures (Chambers 2 and 5) tended to have lower DEA, suggesting that the high temperatures slowed denitrification under these conditions. Overall, manure DEA rates were not significantly related to N₂O emissions (p = 0.137; Figure 6). However, there were negative relationships between 0–5 cm DEA rates and NO₃⁻ values (p = 0.005) and TC:TN ratio (p = 0.010) (Table 3a). At 0–5 cm, no relationships were found among DEA and temperature, manure DM, or Eh values. DEA was not a good predictor of N₂O emissions at 0–5 cm (p = 0.137, R² = 0.024). Furthermore, no significant relationship was found between DEA and NA rates in surface manure (p = 0.728, R² = 0.001), suggesting that coupled nitrification–denitrification was not the primary source of emissions at 0–5 cm depth.

Using correlation coefficients, only manure pH was found to be significantly related to DEA at 5–10 cm (Table 2b). While DEA at 0–5 cm was negatively (p < 0.05) related to NO₃⁻ concentrations and C:N ratios (Table 2a); these same relationships were not found at 5–10 cm (Table 2b). Ayadi et al. [12] reported longer-term DEA rates (24–56 h) in fresh bedded beef manure pack that ranged from 0.8 to 1.4 mmol g⁻¹ h⁻¹. In their study, the DEA rates were decreased at a higher temperature (40 °C) but increased to ~3.5 mmol g⁻¹ h⁻¹ with manure age. This suggests that there is an adaptation period required for denitrifiers to adjust to higher temperatures.

Calculations for rate of DEA in the current study were broken into 0–24 h for short-term DEA (DEA_s) and longer-term (24–54 h) rates (DEA_l) on the basis of the bimodal nature of the raw data for NO₃⁻ disappearance (Figure 6). Rates for DEA_s were slower than DEA overall 54 h incubation, with mean DEA_s values of 0.41 and 0.22 µmol g⁻¹ h⁻¹ for 0–5 and 5–10 cm, respectively (Table 1). There were very few significant differences in DEA_s among chambers at either depth or between depths; however, at the second H₂O application on Day 22, DEA_s at 0–5 cm in Chamber 2 (40 °C) was over twofold greater than ambient control (15 °C). In addition, there was a trend for increased DEA_s in Chamber 4 (26.8 °C). At this depth, DEA_s was negatively related to manure NH₃ content (p < 0.0001) and C:N ratios (p = 0.003) (Table 3a). At 5–10 cm, H₂O addition did little to affect DEA_s,

Figure 8. Example plots of nitrate/nitrite (NOₓ) disappearance over 54 h of anaerobic incubation for denitrification enzyme activity (DEA) studies in manure from (a) 0–5 cm depth and (b) 5–10 cm depth. These data were used to calculate DEA rates.
which is understandable due to limited visible infiltration of simulated rainfall into the manure. DEA at 5–10 cm was negatively related to manure DM, NH₃, and NOₓ contents, and C:N ratio (p = 0.003 to 0.0001). There was a positive relationship with DEAₙ and TN (p = 0.024) (Table 3b).

Table 3. Pearson’s correlation coefficients (R values) among measured parameters and denitrifier enzyme activity (DEA) and nitrification activity (NA). DEA–short (DEAₛ, 0–24 h incubation) and DEA–long (DEAₗ, 24–54 h incubation) were also analyzed at depths of (a) 0–5 cm and (b) 5–10 cm; p–values for each parameter are in parentheses. Asterisks indicate significance at * p ≤ 0.05, ** p ≤ 0.01, and *** p ≤ 0.001.

| 0–5 cm depth | DEA | DEAₛ | DEAₗ | NA |
|--------------|-----|------|------|----|
| N₂O emissions | -0.156 (0.137) | -0.083 (0.428) | 0.155 (0.139) | -0.369 (0.0003) *** |
| Temperature | -0.139 (0.191) | 0.169 (0.112) | -0.359 (0.0005) *** | -0.038 (0.722) |
| Dry matter | 0.171 (0.103) | 0.114 (0.275) | -0.183 (0.079) | 0.471 (&lt;0.0001) *** |
| Eh | 0.065 (0.540) | -0.018 (0.862) | -0.056 (0.591) | 0.129 (0.218) |
| pH | 0.047 (0.659) | 0.143 (0.180) | -0.152 (0.152) | -0.278 (0.008) ** |
| Total nitrogen | 0.098 (0.352) | 0.082 (0.436) | 0.022 (0.835) | -0.060 (0.570) |
| Total carbon | -0.041 (0.700) | -0.079 (0.452) | 0.088 (0.400) | -0.025 (0.825) |
| Ammonium/ammonia | -0.176 (0.094) | -0.442 &lt;0.0001) *** | 0.464 &lt;0.0001) *** | -0.026 (0.802) |
| Nitrate/nitrite | -0.289 (0.005) ** | -0.098 (0.350) | 0.042 (0.689) | -0.320 (0.002) ** |
| TC:TN | -0.266 (0.010) ** | -0.308 (0.003) ** | 0.134 (0.202) | 0.053 (0.614) |
| DEA | 0.405 &lt;0.0001) *** | 0.426 &lt;0.0001) *** | 0.001 (0.991) | —— |
| DEAS | 0.405 &lt;0.0001) *** | -0.408 &lt;0.0001) *** | -0.118 (0.268) | —— |
| DEAL | 0.426 &lt;0.0001) *** | -0.408 &lt;0.0001) *** | -0.039 (0.712) | —— |
| NA | 0.001 (0.991) | -0.118 (0.268) | -0.039 (0.712) | —— |

| 5–10 cm depth | DEA | DEAₛ | DEAₗ | NA |
|--------------|-----|------|------|----|
| N₂O emissions | -0.085 (0.545) | -0.035 (0.608) | 0.156 (0.142) | -0.244 (0.020) * |
| Temperature | -0.196 (0.064) | 0.069 (0.522) | -0.181 (0.187) | -0.022 (0.834) |
| Dry matter | -0.025 (0.817) | -0.378 (0.0003) *** | -0.108 (0.310) | 0.516 &lt;0.0001) *** |
| Eh | 0.134 (0.208) | -0.028 (0.792) | -0.098 (0.939) | 0.146 (0.170) |
| pH | -0.228 (0.031) * | 0.183 (0.086) | -0.372 (0.0003) ** | 0.125 (0.242) |
| Total nitrogen | 0.003 (0.978) | 0.239 (0.024) * | -0.102 (0.340) | -0.180 (0.090) |
| Total carbon | -0.044 (0.683) | -0.166 (0.121) | -0.025 (0.815) | -0.055 (0.669) |
| Ammonium/ammonia | 0.021 (0.946) | -0.387 (0.0002) *** | 0.339 (0.0013) ** | -0.241 (0.022) * |
| Nitrate/nitrite | -0.001 (0.992) | -0.255 (0.016) * | 0.193 (0.068) | -0.265 (0.012) * |
| TC:TN | -0.060 (0.573) | -0.482 &lt;0.0001) *** | 0.084 (0.429) | 0.136 (0.202) |
| DEA | —— | 0.060 (0.574) | 0.219 (0.038) * | -0.075 (0.483) |
| DEAS | 0.060 (0.574) | —— | -0.291 (0.006) ** | -0.189 (0.076) |
| DEAL | 0.219 (0.038) * | -0.291 (0.006) ** | 0.0004 (1.00) | —— |
| NA | -0.073 (0.483) | -0.189 (0.076) | 0.0004 (1.00) | —— |

1 N₂O: nitrous oxide; Eh, redox potential; TC, total carbon; TN, total nitrogen.

In general, DEA and DEAₛ rates were much lower than Woodbury et al. [11], who reported that 2 h DEA rates in feedyard manure were 29 to 132 mmol·g⁻¹·h⁻¹. The difference between results obtained in the current study and Woodbury et al. [11] could be due to manure characteristics; Woodbury et al. [11] measured seasonal DEA rates at different depths and locations within a feedyard pen with a downdgradient, where DEA of the surface manure was much higher than that of the deeper manure pack. They also noted that rates in the surface layer of unconsolidated manure were 29 to 132 mmol·g⁻¹·h⁻¹ and tended to be higher at the lower side of the pen, where runoff occurred. In contrast, DEA in the manure pack was much lower at 0.0 to 6.8 mmol·g⁻¹·h⁻¹. The differences between the current study and those of Woodbury et al. [11] were likely because our manure was a composite of surface and pack manure after cattle had been transported to slaughter. It was scraped near the soil level, ground, and added to chambers. In contrast, Woodbury et al. [11] measured DEA from fresher manure collected from locations in an occupied cattle pen. It is likely that the lower concentrations of NH₃ and NOₓ (due to NH₃ volatilization over time), combined with the dry nature of the manure in the current study, had some impact on measured DEA rates. From these data, however, it can be discerned that short-term DEA in dry manure was slow but increased over time and with
H2O addition. Temperature did not have a clear-cut effect on DEA but was negatively related to NH3 (p < 0.0001), where higher temperatures would promote NH3 volatilization and manure NH3 losses.

Rates for DEA1 (24–54 h) were up to fourfold higher than DEA and DEA2 rates, ranging from 0.94 to 1.69 µmol·g⁻¹·h⁻¹ at 0–5 cm after the first H2O addition (Table 2). Rates of DEA1 tended to be slightly lower at 5–10 cm that at 0–5 cm and decreased over time. The rate of DEA1 at 0–5 cm was negatively related to temperature (p = 0.0005) and positively related to NH3 contents (p < 0.0001). In addition, NH3 was positively related to DEA1 at 5–10 cm. This could suggest coupled nitrification–denitrification (NH4⁺ to NO3⁻ via nitrification and then NO3⁻ to N2O/N2 via denitrification). At 5–10 cm, the temperature response was negligible (p = 0.087), but pH was negatively related to DEA1 (p = 0.001). However, there were no significant differences among DEA1 in chambers for most of the study, except for reduced rates at 46.2 °C (Chamber 5) on Day 59 at 5–10 cm.

In general, measures of potential DEA did not provide sufficient data to make a hard case about the effects of temperature, rainfall, or depth on denitrification–related N2O emissions from feedyard manure. However, from these data, we can ascertain that short–term DEA rates were very low at both depths, and DEA was unlikely to contribute to the initial peak observed immediately after simulated rainfall addition (Figure 3). The time period for the DEA data would also exclude longer–term DEA from suspicion as the cause of the first observed N2O peak but does not exclude denitrification as the rationale behind the second, temperature–related N2O peak observed (Figure 3b). Relationships identified among DEA, DEA2, and DEA1 data and manure chemical characteristics suggest denitrifier involvement in the N2O production process in feedyard manure (Table 3) and should be further explored.

3.4. Nitrification Enzyme Activity

At the beginning of the experiment, NA rates at 0–5 cm and 5–10 cm ranged from 0.10–0.18 and 0.11–0.17 µmol·g⁻¹·h⁻¹, respectively (Table 2). Although there were some differences in NA among chambers, they were inconsistent between the two sampled depths. At 0–5 cm, there was a tendency for increased NA with temperature, but this same trend was not obvious at 5–10 cm. Nitrification is a key process in the manure N cycle, with NH4 oxidation as the first and limiting step of the nitrification process. Pearson’s correlation coefficients revealed a negative relationship among NA in the surface manure and N2O emissions (p = 0.0003), pH (p = 0.008), and NOx concentrations (p = 0.0018), and a positive relationship with manure DM content (p < 0.0001) (Table 3). Similarly, at 5–10 cm, NA was negatively related to N2O emissions (p = 0.02), NH3 (p = 0.022), and NOx (p = 0.012), and positively related to DM content (p < 0.0001). NA data are presented in Table 2, where H2O addition at 0–5 cm on Day 22 reduced NA, but then NA increased with the highest activity on Day 30 in Chamber 5 (46.2 °C). The same trend was not observed at 5–10 cm. In general, NA was considerably lower than DEA at both depths and was not related to DEA, which should exclude coupled nitrification–denitrification as the predominant pathway(s) of N2O production in feedyard manure.

Although the pH range for this study was narrow (8.81–9.23), there was a significant relationship between pH at 0–5 cm and NA. Nitrification in soils is favored by a DM content of 15–20%, and this trend for higher NA in dryer substrate was observed as a positive correlation with between NA and manure DM (Table 3). Soil and microbial properties have been found to regulate NA, particularly pH and abundance of ammonia–oxidizing bacteria (AOB). Lin et al. [44] found that AOB, particularly Nitrospira Cluster 8a, explained 73% of the variation in soil NA. In the current study, NA values were significantly higher than those of Ayadi et al. [12], who reported NA in the nmol range rather than the µmol range of the current study. Ayadi et al. [12] found that temperature had a major effect, where incubation of bedded beef manure at 40 °C had more than twofold higher NA than that at 10 °C. In contrast, temperature had no significant effect on NA in the current study at either depth (p ≥ 0.72). Given the temperature response observed in N2O emissions (Figure 3b) and the lack of correlation with temperature and NA rates, it could be assumed that the majority of the N2O emitted in the wide peak ~2 days after wetting was derived from denitrification rather than nitrification.
4. Conclusions

Correlation analyses of all variables against N$_2$O emissions proved useful in identifying key parameters involved in N$_2$O production and emission from dry feedyard manure. Fluxes of N$_2$O increased immediately after simulated rainfall and a second, temperature–related N$_2$O flush was observed after the second simulated rainfall application. The variables most highly positively related to N$_2$O flux were NO$_x$ and NH$_x$ concentrations and temperature at both depths ($p \leq 0.01$ to 0.001). Negatively related variables at 0–5 cm were DM ($p \leq 0.001$), NA, Eh, and pH (all $p \leq 0.01$). These data suggest that N$_2$O from feedyards was largely emitted from surface manure (0–5 cm) via denitrification when DM content was lower (i.e., higher H$_2$O content) and Eh, pH, and potential nitrification rates were low. Higher temperatures promoted N$_2$O emissions and influenced rates of DEA. However, analyses of DEA did not show any significant relationship with N$_2$O fluxes. It is possible that DEA rates were already high (or low), and the “optimal” conditions provided in the DEA assay did not stimulate nor inhibit DEA. On the basis of these data, we propose that most N$_2$O was emitted from the surface of the manure–packed chambers via denitrification after simulated rainfall; however, we cannot exclude the contribution of nitrification under drier conditions. Further study, perhaps with isotopic analysis, is warranted to ascertain if the first flush of N$_2$O after simulated rainfall to dry manure was produced biotically or an abiotic N$_2$O–releasing mechanism.

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