Mechanisms for Nitrogen Oxide Formation during Ensiling of Dairy Feeds

Paper # 100

Michael Kosusko, U.S. Environmental Protection Agency, Office of Research and Development, 109 T.W. Alexander Drive, E343-02, Research Triangle Park, NC 27711; **Sona Chilingaryan,** U.S. Environmental Protection Agency, Region 9, 75 Hawthorne Street, San Francisco, CA 94105; and **Peter G. Green, Mathew Cohen,** and **Frank M. Mitloehner,** University of California at Davis, One Shields Avenue, Davis, CA 95616

ABSTRACT

Silage (ensiled feed), as a dairy's greatest operational cost, is its most critical feed commodity. Ensiling is the process of converting entire harvested feed plants such as corn, sorghum, or alfalfa into fermented, stable anaerobic animal feed (i.e., silage). The continued use of silage is essential to a highly productive and economically viable industry. Previous work has shown that silages are a major source of volatile organic compounds (VOC) and a potential source of nitrogen oxides (NO_x) from dairies contributing to the emission inventories for the San Joaquin Valley and South Coast Air Basin in California. Both VOC and NO_x are precursors to the formation of ozone and $PM_{2.5}$. The emissions of ozone and fine particulate matter ($PM_{2.5}$) are long-standing air quality challenges in many areas of the country, but particularly in these California locations. As a result, California has been diligently identifying, understanding and reducing all sources of VOC and NO_x emissions.

The emission of NO_x has been observed during the ensiling process[1, 2]. Since substantial NO_x is not inherently present in corn, it is not released by the processing of corn NO_x is generated during the early days of the ensiling process by an unknown mechanism. The underlying question for this investigation was whether the production of NO_x is due to biological activity from the growth of microbes or whether the production of NO_x is enzymatic, using precursor compounds already present in the harvested plant matter. Hence, our goal was to better understand mechanisms that could generate NO_x emissions from silage. To understand the mechanism for NO_x generation, NO_x emissions during ensiling were compared between untreated control samples and treatment by (a) sterilizing the microbes that are inherently present in chopped corn, including all parts of the plant, to discern whether NO_x formation during ensiling is microbial or is due to pre-existing plant enzymes, and (b) testing three chemical inhibitors to limit the activity of the peroxidase enzyme that is the most likely candidate to produce NO_2 from nitrate.

This paper will describe our test procedures, the results from testing, and conclusions and recommendations resulting from this effort.

Commented [KM1]: QA suggests title change to Mechanisms for Nitrogen Oxide Formation… Should we change the title?

Commented [KM2R1]: The recommended change has been made.

Commented [KM3]: Per QA: What is the relationship between NO_2, nitrate, silage and peroxidase enzyme?

Commented [KM4R3]: This is discussed later in the manuscript.

INTRODUCTION

Nitrogen oxide gases (NO_x) are important air pollutants, particularly in regions with summer-time ozone and wintertime fine particulate matter ($PM_{2.5}$) exceedances such as the San Joaquin Valley of California[3, 4]. This experiment investigated the mechanisms that may be causing the formation of nitrogen oxide gases that occur very early in the ensiling process and may be primarily enzymatic (i.e., pre-existing biochemical action on precursors present in the harvested plant) or microbial. This study pertains to mechanisms only, not a field study, nor an effort to measure emissions or plan mitigation or controls.

To store animal feed from the time of harvest over a period of many months (often up to a full year), the feed is ensiled to produce an acidified product that, kept sealed tight from exposure to air, remains stable at ambient temperature. Ensiling is the process of converting the entire harvested feed plant such as corn, sorghum, or alfalfa into fermented, stable anaerobic animal feed (i.e., silage). The common practices of pickling or making sauerkraut are somewhat analogous. The ensiling process is completed in large air-tight structures (silos) or in large piles that are covered with plastic sheeting to minimize exposure to air and the elements. The anaerobic conditions in silos and piles allow anaerobic bacteria to partially break down and acidify the feed plant material both stabilizing it and making it more digestible for dairy cows. In many parts of the country, such as the San Joaquin Valley that can grow their own feed crops, silage (ensiled feed) is a dairy's most critical feed commodity and its greatest operational cost. The continued use of silage is essential to a highly productive and economically viable industry. Previous work has shown that silages are a major source of volatile organic compounds (VOC) from dairies contributing to the San Joaquin Valley's (SJV's) emissions inventory[5, 6]. The emission of NO_x has also been observed during the ensiling process[1, 2] and at dairies[7]. Because NO_x can be directly toxic and contributes to the regional air quality problem of ozone and $PM_{2.5}$, its formation is important to understand.

In addition, there is a California Air Resources Board (CARB)-funded project underway to measure the amount of NO_x and VOC emissions from silage at dairy locations in the field in California. The co-authors of this manuscript at the University of California at Davis, are also involved in the CARB-funded project. The investigation described in this paper was completed to provide information on a side issue of the overall CARB-funded effort, the generation of NOx during the initial, aerobic stage of ensiling. Ozone formation and $PM_{2.5}$ are long-standing air quality challenges in many areas of the country, especially in regions with hot sunny summers and cold winters with valley geography which traps air emissions near the ground. The San Joaquin Valley is such a region. As a result, California air pollution agencies have been diligently identifying, understanding and reducing all sources of VOC and NO_x emissions.

The purpose of this effort was to determine what mechanisms in the ensiling process could produce nitrogen-containing air emissions by identifying which mechanisms could suppress NO_x creation and to answer the following questions:

1. What mechanism(s) in the ensiling process create nitrogen-containing air pollutant emissions, particularly NO_x?

Commented [KM5]: Per QA: The Introduction should define all the terms (i.e., what is a silo? How does a silo work? What is silage?) and explain why it is important to solve this problem.

The present text makes the problem sound like a case of intellectual curiosity, which indeed it may be, but if funding is being supplied by EPA and CARB, there must be an environmental reason to do the work. 'There are X-many silos in the geographical area that produce Y-quantity of NO_x, causing an environmental problem.'

Commented [KM6R5]: Language has been added to address the first part of this comment.

The project team has specifically avoided making a statement such as that recommend in the second part of this comment because a quality study of NO_x emissions from the initial stages of ensiling has not been completed and the industry is very sensitive to the suggestion that significant NO_x is emitted during ensiling.

Commented [KM7]: From Li: Cite 1 or 2 research papers here.

Commented [KM8R7]: Placeholders inserted.

Commented [KM9]: Per Nancy Levin of Region 9: SJV dairies use silage, but some areas used dry feed (e.g., South Coast). I'm not sure about other parts of country. Provide caveat?

Commented [KM10R9]: New language has been added and needs to be reviewed by the authors.

Commented [KM11]: Per Nancy Levin of Region 9: Provide a footnote on this project, and it's relationship and/or relationship of researchers, if any, to this study.

Commented [KM12R11]: I took a shot at this.

2. What are the potential ways of suppressing those mechanisms?

To understand the mechanisms, NO_x emissions were compared to control samples by completing treatments either (a) sterilizing the microbes inherently present in chopped corn, or (b) using chemical inhibitors to limit the activity of the peroxidase enzyme, which plausibly produces NO_2 from nitrate[8]. Radiation sterilization (by electron beam) was tested to discern whether NO_x formation during corn ensiling is microbial or due to pre-existing plant enzymes. Three possible chemical inhibitors (azide, cystine and vanadate) of the peroxidase enzyme thought to be responsible for NO_x formation were tested.

These experiments were designed to elucidate the possible mechanism(s) of NO_x formation, not to attempt quantification or control of emissions.

EXPERIMENTAL METHODS/MATERIALS/PROJECT APPROACH

Test Description

NO_x is generated by an unknown mechanism during the early days of the ensiling process. It is not known whether NO_x arises directly from nitrate via the action of peroxidase, or through other pathways. A wide variety of nitrogen-containing compounds are found in all plants, including amino acids such as in proteins. The deepest underlying question is whether the production of NO_x is due to biological activity from the growth of microbes during the ensiling process, or whether the production of NO_x is enzymatic, using precursor compounds already present in the harvested plant matter. To distinguish between these two options, we ensiled material that had been sterilized by radiation[9] and compared emissions of NO_x with emissions of NO_x from untreated control samples as described below.

Commented [KM13]: Per QA: What constituted 'control samples'?

Commented [KM14R13]: See added language referring to language later in the paper.

Published results suggested that the key enzyme involved in production of NO_x is peroxidase (8). To test this hypothesis, we added each of three established chemical peroxidase inhibitors and compared NO_x production to the control. The three plausible inhibitors represent different classes of chemicals: one metal, one nitrogen compound and one amino acid. The first is a naturally occurring trace micronutrient and the last is a natural component in protein. If neither vanadate[10], azide[11] nor cystine[12] were to inhibit NO_x production, then the responsible enzyme (whether pre-existing or microbially generated) would be unlikely to be peroxidase.

Methods and Materials

Harvesting

Whole-plant corn was harvested with approximately 30 % dry matter using a commercial flail chopper, providing a chopped forage material with a cut length between 1 and 2 cm. Fresh material was collected (during the process shown in Figures 1 and 2) and immediately

Figure 1. Chopped corn being delivered by truck into the ensiling machine.

Figure 2. The open tray area for chopped corn collection, and the silage bags (white) being filled at the dairy.

transported in a covered truck to the laboratory. For the chopped corn, nitrate content was measured in samples shipped to a commercial laboratory[13].

Commented [KM15]: From Li: Call Figures and Please follow the figure requirements: http://ace2015.awma.org/technical-program/author-resources/

Commented [KM16R15]: Done.

Sample Preparation

The goal of ensiling is to produce air tight storage and mini-silos are expected to accurately simulate actual silage piles. Mini-silos are five-gallon buckets, previously fitted with a gas-tight sampling port to collect emitted gases into Tedlar bags for gas composition (and volume) measurements. The mini-silos have been used successfully in previous corn silage experiments[14, 15]. For this effort, the mini-silos were thoroughly cleaned and fitted with new bags and valves. Figure 3 shows mini-silos in use.

Chopped feed corn was harvested and chopped by a commercial operator and then transported to the laboratory to fill five sets of five replicate buckets. All bucket contents for each set of replicates were vigorously mixed using an electrical 'cement' mixer (to homogenize the feed) prior to loading, packing and sealing. Packing was at the typical density of silage piles, which corresponds to 3.5 to 3.9 kg per five-gallon container. Packing in the mini-silos was at the typical dry matter content of 300 to 320 g/kg. Dry matter is determined by net loss of mass upon drying in an oven to constant weight. After compaction of the chopped corn forage, mini-silos were sealed with the addition of white silicon caulking around the lid threads to provide an inert gas-tight seal.

Sample Treatment

In addition to a control sample, four independent treatments were used in this experiment. Electron-beam irradiation at 45 kilogray (kGy) was used to sterilize the microbes inherently present in the chopped forage. Sodium vanadate addition, sodium azide addition, and L-cystine addition were used to test peroxidase inhibition. The control sample passed through all of the handling steps including the addition of sprayed water, mixing, packing and sealing. The potential inhibitors were diluted shortly before use into 1 L of double-deionized water to be sprayed (0.2 L per mini-silo) onto the chopped corn during mixing.

Commented [KM17]: Per Li, Need to check this part.

Commented [KM18R17]: Will check after submittal to STICS review.

The first set of five buckets had no treatment and were marked N1-N5. The second set was treated with sterilization (S) and marked S1-S5. One set was used for each of the three plausible inhibitors of peroxidase [Sodium Vanadate (V1-V5), L-cystine (C1-C5) and Sodium Azide (A1-A5)].

Sterilization of the second sample set was accomplished commercially with electron-beam exposure at a level known to kill microbes to >99.99 %. The dosage was 45 kGy. This dosage can penetrate up to 15 cm through water and is projected at the sample from opposing sides. This dosage is used to sterilize medical instruments and other small apparatus, which may be made of steel, and successfully sterilizes both their exterior and interior surfaces.

Commented [KM19]: Per Li: Check this statement.

Commented [KM20R19]: Will check after submittal to STICS review.

For the third sample set, vanadium (in the form of sodium vanadate), a trace micro-nutrient, typically found at a level of approximately 2 ppb (2 ng/g) in animal feed, was used. Sodium

Figure 3. Gas collection bags directly attached to the mini-silos, with no gas in the nearest bag, but visible volumes in the two bags beyond.

vanadate has been reported to be an inhibitor of peroxidase (10). We supplemented to 20 ppb in the experimental samples.

For the fourth sample set, L-cystine (the dimer of the amino acid cysteine), common in animal feed, at levels of approximately 0.2 % (2 mg/g) was used. L-cystine has been reported to be an inhibitor of peroxidase (12). We supplemented to double this level in the experimental samples.

For the fifth sample set, sodium azide, reported as an inhibitor of peroxidase (11), is not detectable in normal feed samples but has been used at 1 mg/kg to inhibit microbial activity in laboratory buffer solutions (such as in liquid chromatography), so we used that level in our experiment.

Evaluation of Silage Quality

The chemical composition of the silage samples was analyzed using the standard tests for nutritional content: dry matter (DM) and nitrate ion (NO_3) before ensiling, and after ensiling dry matter (DM), nitrate ion (NO_3), pH, total volatile fatty acids (VFAs), lactic acid, acetic acid, and total titratable acidity. These tests establish the proper completion of the ensiling process and verify the suitability of the feed.

Dry matter (DM) is determined by net loss of mass in an oven, weighing to constant weight. The following procedures were conducted for the commercial laboratory analysis. The silage sample was first extracted in preparation for testing. (The fermented feed sample was mixed and a 25 g wet sample was taken and diluted with 200 mL deionized water. The sample mixture sat overnight, then was blended for two minutes and filtered through coarse filter paper.) For pH and Titratable Acidity, 30 mL of extract was introduced to a Mettler DL12 Titrator. The pH was read and the sample was titrated with 0.1N NaOH to a pH of 6.5. For Ammonia, 25 mL of extract was mixed with 75 mL deionized water and introduced into a Labconco Rapidstill II model 65200 analyzer. The sample was titrated with 0.1 N HCl to determine Ammonia. To determine L-lactic acid, a 1:1 ratio of extract to deionized water was introduced to an YSI 2700 Select Biochemistry Analyzer and the result was multiplied by four to obtain total lactic acid. For Acetic Acid and Total VFA, 3 mL of extract was filtered through a 0.2 µm filter membrane, and a 1.0 µL sub-sample was injected into a Perkin Elmer AutoSystem gas chromatograph using a Restek column packed with Stabilwax-DA.

NO_x Emissions during Ensiling

Nitric oxide (NO), nitrogen dioxide (NO_2) and total oxides of nitrogen (NO_x) were measured during the ensiling process using a chemiluminescence $NO/NO_2/NO_x$ analyzer. The detection limit of the analyzer for NO, NO_2 and total NO_x was 5 ppb. Precision was 10 % (or better) and accuracy was 10 % (except higher near the detection limit). The measurement range was from 5 ppb to 5 ppm, using appropriate dilution with flow meters at the inlet of the instrument.

Gas sampling commenced the next day, as soon as gas was generated (see Figure 3) in sufficient volumes. We calibrated with our certified gas cylinder (NO_2 in air, 10 ppm by volume) diluted to

1 ppm. Gas measurements continued until insufficient volume (less than 1 liter) was being generated (after approximately 2 weeks).

Volumes sampled ranged from 1 L to 5 L. Gas sample collection was manually controlled using a 5-liter Tedlar bag directly connected to a Teflon tube (6.35 mm ID, 0.20 m long) to the $NO/NO_2/NO_x$ analyzer. The gas samples were frequently measured for gas concentrations at six- to eight-hour intervals until the depletion of sample inside the gas bags. Gas emissions were reported as nanoliters, nL – that is, parts-per-billion by volume (nL/L) multiplied by L. The amount of gas produced was determined by multiplying the volume fraction measured (nL/L or ppb) by the volume sampled (L), where the latter was determined by the time of gas signal (minutes) and the fixed sampling rate of 0.5 L/minute. Since the sample volume changes for each sampling period, the total emitted volume is more representative of the NO_x generation rate than concentration.

The vendor reported that nitrogen dioxide has 54.5 % recovery after 24 hours in 1-liter Tedlar bags. Measurements occurred in six- to eight-hour time periods – much faster than 24 hours. In addition, we used 5-liter bags with a much lower surface area to volume ratio, both implying better recovery of NO_x than the vendor test. The vendor's (SKC) report can be found at http://www.skcinc.com/instructions/1805.pdf (accessed March 25, 2015).

Commented [MK21]: From QA: This publication indicates that the bag material is Tedlar, but the UCD project report indicates that the bag material is Teflon. Did we have this discussion previously and resolved this issue? Photo 3 shows that the bags appear to be clear, which is consistent with their being made from FEP Teflon rather than Tedlar, which is more translucent.

Commented [KM22R21]: FEP Teflon has 0% recovery. I would hope it wasn't used. I will work with Peter Green to resolve this issue after submittal to STICS review.

Commented [KM23]: From QA: Please supply date of last access.

Commented [KM24R23]: Done.

RESULTS AND DISCUSSION

Raw Corn Quality

The nitrate level in the material for ensiling was 0.04 % (in all three sub-samples), relatively low in the range seen for animal feeds. Up to 0.44 % is always considered safe to feed, while levels up to 1 % are fed with appropriate limits as a portion of the total diet. This low level of nitrate might have resulted in lowered emissions, if nitrate indeed is the precursor. However, the relationship between initial nitrate and emissions has not been studied.

Ensiled Corn Quality

Table 1 describes the quality of the ensiled corn for each of the five treatments. According to the commercial laboratory used for this effort, corn silage should have the following properties:

- Initial and final % Dry Matter (DM) should be consistent and appropriate (32-35 %).
- Final pH should be in the range 3.7 to 4.5.
- Total volatile fatty acids (VFAs) should be in the range 3.6 to 9.3 % of DM.
- Lactic Acid should be in the range 2.4 to 6.5 % of DM.
- Acetic Acid should be in the range 0.8 to 3.2 %.

Table 1. Mean, standard deviation and statistical significance relative to no treatment (N).

Treat-ment		Total NO_x nL	%DM	NH_3 % of DM	pH	VFA (tot.) % of DM	Lactic Acid % of DM	Lac/ VFA %	Acetic Acid % of DM	Acidity meq /100g
A (Azide) vs. N	mean	29683	32.66	0.41	3.84	5.75	4.68	84.10	1.05	7.09
	std. dev.	26700	0.55	0.03	0.03	0.10	0.08	0.55	0.04	0.28
	ANOVA	p = 0.18	p = 0.15	p = 0.22	p < 0.001	p = 0.005	p < 0.001	p < 0.001	p < 0.001	p = 0.21
C (Cystine) vs. N	mean	6574	32.64	0.45	3.94	5.88	4.42	75.00	1.45	6.49
	std. dev.	9915	0.76	0.03	0.02	0.20	0.15	1.41	0.12	0.65
	ANOVA	p = 0.46	p = 0.34	p = 0.85	p = 0.45	p = 0.005	p = 0.01	p = 0.92	p = 0.25	p = 0.29
N (No Treat-ment)	mean	11175	32.22	0.45	3.95	5.46	4.10	75.00	1.36	6.85
	std. dev.	8660	0.43	0.06	0.02	0.14	0.16	2.00	0.11	0.28
	ANOVA	NA	NA	NA	NA	NA	NA	NA	NA	NA
S (Steril-ized) vs. N	mean	1126	33.46	0.40	4.23	3.71	2.82	76.20	0.89	3.60
	std. dev.	345	0.65	0.05	0.10	0.34	0.33	3.70	0.12	0.42
	ANOVA	p = 0.03	p = 0.006	p = 0.19	p < 0.001	p < 0.001	p < 0.001	p = 0.58	p < 0.001	p < 0.001
V (Vana-date) vs. N	mean	25610	32.60	0.33	3.90	6.03	4.74	78.60	1.30	6.68
	std. dev.	45112	0.39	0.03	0.01	0.33	0.09	3.65	0.30	0.48
	ANOVA	p = 0.50	p = 0.16	p = 0.004	p = 0.001	p = 0.007	p < 0.001	p = 0.10	p = 0.68	p = 0.51

ANOVA: Analysis of Variance
NO_x: Nitrogen Oxides (nL)
%DM: Percent Dry Matter
NH_3: Ammonia (NPN)
VFA: Volatile Fatty Acids
Lac: Lactic Acid
Acidity: Titratable Acidity
meq: milli equivalents

All replicates (five each) of all five treatments ensiled well, based on final production of acidity (low pH), and specific tests for lactic acid and VFAs. Final dry matter content was also within a narrow range and indicated successful ensiling for all 25 samples. Final dry matter was slightly higher for the sterilized (S) treatment, possibly due to lowered microbial respiration (hydrolysis) and lower gas volume emission.

Other measurements of the final silage showed statistically significant differences ($p < 0.01$) between the treatments and no treatment. Table 1 lists the silage data with mean, standard deviation, and statistical significance ('p'-value) relative to no treatment (N). The magnitude of change for some parameters is modest, but the change does indicate that the chemical inhibitors were applied and mixed successfully, and did affect the ensiling process. For example, the sterilized (S) samples had a higher pH and percent dry matter and lower acidity, VFAs, lactic acid and acetic acid. The samples treated with sodium azide (A) had higher VFAs, lactic acid and

Commented [KM25]: Per Li: Please follow the table requirements: http://ace2015.awma.org/technical-program/author-resources/.

Commented [KM26R25]: Table has been recast into A&WMA format.

Commented [KM27]: From QA: Columns in the Table should be centered. Column headings should be in BOLD font.

Commented [KM28R27]: Table has been recast into A&WMA format.

lower pH and acetic acid. The samples treated with sodium vanadate (V) had higher VFAs and lactic acid and lower pH and ammonia. The samples treated with l-cystine (C) had higher VFAs and lactic acid.

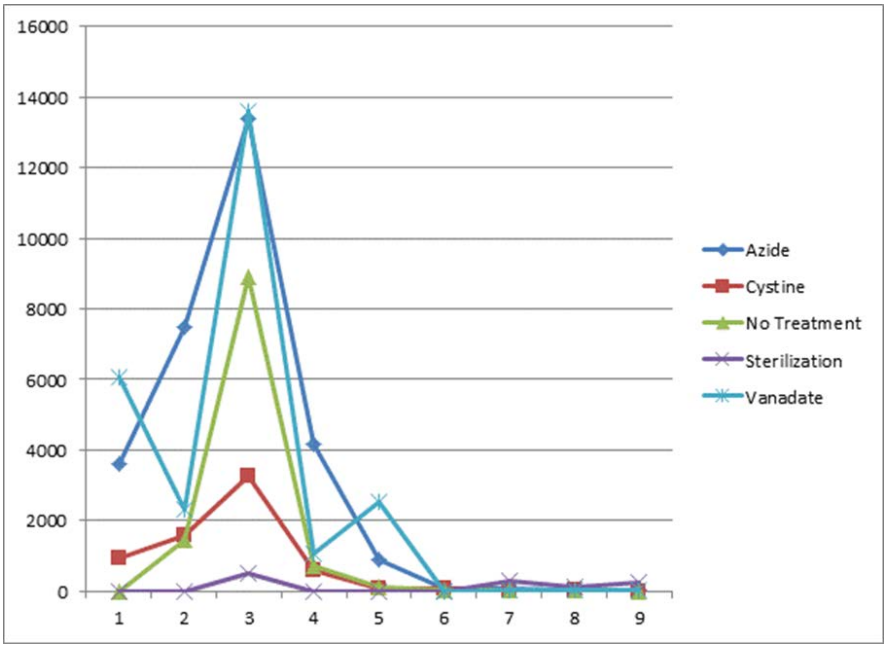
Commented [KM29]: From QA: Were these values consistent for all of the replicate samples with a given treatment? Please supply a table of the reported values so the variation between replicates can be evaluated.

Commented [KM30R29]: The combined data and statistics are provided in Table 1.

NO_x Emissions

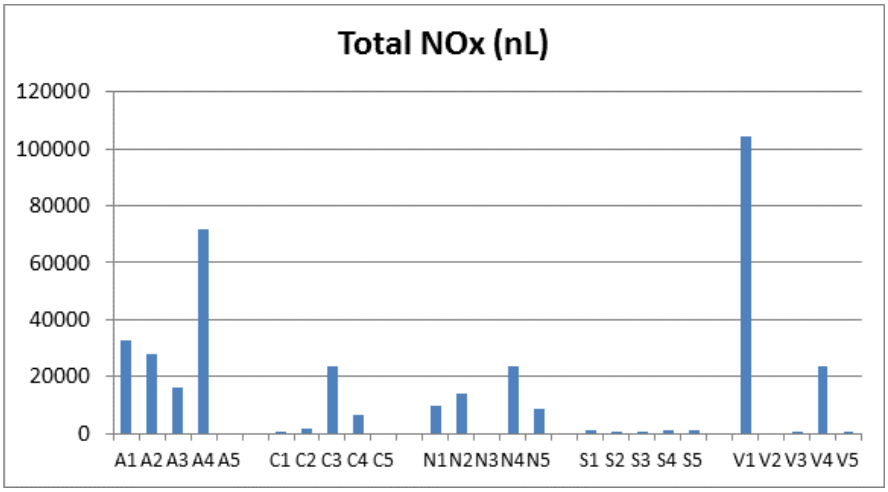
Figure 4 presents the time course of overall NO_x production (volume of gas multiplied by concentration, as sampled every six to eight hours). NO_x production started within one to two days, peaking at three days, and tailed off at six to eight days. NO_x production varied greatly, however, in each of the treatment sets – including four (out of 25 total) that produced periods of observable volumes of gas but with NO_x below the detection limit of 5 ppb, by volume. The four mini-silos that had periods of no detectable NO_x (<5 ppb) were N4, V4, A1 and A3. Overall, despite having two non-detects, the azide (A) treatment had the highest mean production of NO_x. It is possible that this highest mean production of NO_x happened due to the presence of nitrogen in the azide treatment.

Figure 4. Total NOx (nL) vs. day after ensiling.



As shown in Table 1 and Figure 5, NO_x measurement replicates were highly varied. Total NO_x is calculated as the sum of emissions measured periodically. No treatments showed statistically significant differences from the control sample. This observation is not surprising. Due to natural

Figure 5. Total NO_x (nL) for each sample, grouped in the five treatments.



variation, replicates of chopped field corn will have different constituents (physical, chemical and biological), so it is expected that trace gas emissions will also vary among the replicates, perhaps with standard deviations of the same order of magnitude as the mean. The sterilized sample (S) has the closest to significant results with $p = 0.3$ and mean NO_x generation ten times lower than the control.

The most striking pattern was with the samples treated with electron beam sterilization (S) to see if that would be sufficient to limit ensiling and restrict gas production to existing enzymes only – not those produced by microbial reproduction over time. Promptly after treatment, the samples treated with electron beam sterilization all showed orange staining in the attached gas sampling bag. This staining is shown in Figure 6. Unfortunately, the staining reaction prevented the bag from inflating so that sampling was not possible until the bags were replaced after day 1. Although NO_x emissions from the first day after sterilization were not sampled because of the staining reaction, NO_x production during day 1 is anticipated to be quite low. As shown in Figure 4, the production of NO_x in unsterilized mini-silos peaked on day 3 with very few emissions during day 1. We believe that the same would be true for the sterilized samples.

Given the lower NO_x production by the sterilized silage (S) samples and the lower production of acidity and the lower production of VFAs and lactic acid, it appears that sterilization reduced the ensiling of these samples, indicating a bacterial mechanism for ensiling and NO_x generation.

Commented [MK31]: QA comment: It may be worth noting that the orange staining in the bags may be caused by the decomposition of the orange plastic bucket by the electron beam. See Azume et al. (1986) for a discussion of volatiles emitted by low-density polyethylene film that was irradiated by an electron beam.

Commented [KM32R31]: I will work to resolve this issue after the paper has been submitted to STICS review.

Figure 6. The 25 mini-silos, with the orange-stained bags on the sterilized samples at right.

Commented [KM33]: From QA: Did the mini-silos all start out with white paper inside the bags? If the staining reaction has discolored the paper in the bag, this observation should be pointed out.

Commented [KM34R33]: I will work to resolve this issue after the paper has been submitted to STICS review.

SUMMARY

The emission of NO_x has been observed during the ensiling process even though substantial NO_x is not inherently present in corn. The NO_x is generated by an unknown mechanism during the early days of the ensiling process. The underlying question for this effort was whether the production of NO_x is due to biological activity from the growth of microbes or the production of NO_x is due to enzymatic action, using precursor compounds already present in the harvested plant matter. NO_x emissions were compared between control and treatment by (a) sterilizing the microbes that are inherently present in chopped corn to discern whether NO_x formation during ensiling is microbial, and (b) testing three chemical inhibitors to limit the activity of the peroxidase enzyme that is the most likely candidate to produce NO_2 from nitrate.

Five replicates were prepared for each of the four treatments and the control. The resulting feed corn was properly ensiled in all 25 mini-silos. That is, the pH was lowered into the range of 3.7 to 4.5 considered normal by the commercial testing laboratory, and production of acids, such as lactic acid, was raised to its desirable range of 3.4 to 6.5 % of dry matter.

Based on this work, it seems plausible that the mechanism that creates NO_x emissions in the early phase of the ensiling process is caused by microbes. Electron-beam sterilization (S) lowered NO_x emissions by a statistically significant amount ($p = 0.03$) and partially prevented microbes from achieving the full acidity of the ensiled product.

Chemical inhibition of the pre-existing enzyme peroxidase did not have a conclusive effect on NO_x emissions, even though three different types of inhibitors were tested, and all affected the ensiling process. Enzymes other than peroxidase in the plant material remain a possible source of NO_x generation.

It is unknown whether the sterilization process might have affected the peroxidase enzymes, and whether the chemical inhibitors might have affected the microbes. Both would be interesting questions to pursue in a future study.

The variability of the NO_x results prevents us from drawing any specific conclusions about the influence of those treatments on NO_x emissions. Variation between replicates limited the statistical significance of chemical inhibition on NO_x emissions. More consistency between replicates, or more replicates, could enable distinguishing an effect. More frequent sampling, such as with an automated system, would limit sample bag recovery losses and could aid in characterizing peak emissions.

ACKNOWLEDGMENTS

The authors would like to thank a cooperating dairy and our stakeholder group for its participation and support. Bob Wright of EPA's National Risk Management Research Laboratory (NRMRL) has provided QA support above and beyond the call of duty during this effort. We thank him.

REFERENCES

Commented [KM35]: Per QA: Reference #7 is incomplete; volume and pages of the article are omitted.

Commented [KM36R35]: Correction will be made for final paper.

1. Peterson, W.H.; Burris, R.H.; Sant, R.; Little, H.N. *J. Agric. Food Chem.* **1958**, *6*, 2, 121-126.

2. Maw, S.J.; Johnson, C.L.; Lewis, A.C.; McQuaid, J. B. *Environ. Mon. Assess*. **2002**, *74*, 2009-2015.

5. Howard, C.; Kumar, A.; Malkina, I.; Mitloehner, F.M.; Green, P.G.; Flocchini, R.; Kleeman, M. *Environ. Sci. Tech.* **2010**, *44*, 2309-2314.

6. Malkina, I.L.; Kumar, A.; Green, P.G.; Mitloehner, F.M. *J. of Environ. Qual*. **2010**, *40*, 1-9.

7. Hasson, A.S.; Ogunjemiyo, S.O.; Trabue, S.; Ashkan, S.; Scoggin, K.; Steele, J.; Olea, C.; Middala, S.; Vu, K.; Scruggs, A.; Addala, L.R.; Nana L. *Atmos. Environ.* **2013**, *XX*, YYYY-YYYY.

8. Augusto, O.; Bonini, M.G.; Amanso, A.M.; Linares, E.; Santos, C.C.X.; DeMenezes, S.L. *Free Radical Biol. Med.* **2002**, *32*, 841-849.

9. Winters, A.L.; Cockburn, J.E.; Dhonaoa, M.S.; Merry, R.J. *Appl. Microbiol.* **2000**, *89*, 442-451.

10. Serra, M.A.; Sabbioni, E.; Marchesini, A.; Pintar, A.; Valoti, M. *Biol. Trace Elem. Res.* **1990**, *23*, 151-164.

11. Li, C.; Ziesmer, S.C.; Lazcano-Villareal, O. *J. Histochem. Cytochem.* **1987**, *35*, 1457-1460.

12. Carvalho, D.P.; Ferreira, A.C.; Coelho, S.M.; Moraes, J.M.; Camacho, M.A.S.; Rosenthal, D. *Brazilian J. Med. Biol. Res.* **2000**, *33*, 355-361.

13. *Nitrate in Forages, Potentiometric Method (986.31)*; Association of Official Analytical Chemists; Official Methods of Analysis, 15th Edition. 1990.

KEYWORDS

A&WMA, NO_x emissions, silage, ensilage, dairy, corn.