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Urea Hydrolysis and Calcium Carbonate Precipitation in Gypsum-Amended Broiler Litter

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Abstract

Broiler (*Gallus gallus domesticus*) litter is subject to ammonia (NH_3) volatilization losses. Previous work has shown that the addition of gypsum to broiler litter can increase nitrogen mineralization and decrease NH_3 losses due to a decrease in pH, but the mechanisms responsible for these effects are not well understood. Therefore, three laboratory studies were conducted to evaluate the effect of gypsum addition to broiler litter on (i) urease activity at three water contents, (ii) calcium carbonate precipitation, and (iii) pH. The addition of gypsum to broiler litter increased ammonium concentrations ($p < 0.0033$) and decreased litter pH by 0.43 to 0.49 pH units after 5 d ($p < 0.0001$); however, the rate of urea hydrolysis in treated litter only increased on Day 0 for broiler litter with low (0.29 g H_2O g^{-1}) and high (0.69 g H_2O g^{-1}) water contents, and on Day 3 for litter with medium (0.40 g H_2O g^{-1}) water content ($p < 0.05$). Amending broiler litter with gypsum also caused an immediate decrease in litter pH (0.22 pH units) due to the precipitation of calcium carbonate ($CaCO_3$) from gypsum-derived calcium and litter bicarbonate. Furthermore, as urea was hydrolyzed, more urea-derived carbon precipitated as $CaCO_3$ in gypsum-treated litter than in untreated litter ($p < 0.001$). These results indicate that amending broiler litter with gypsum favors the precipitation of $CaCO_3$, which buffers against increases in litter pH that are known to facilitate NH_3 volatilization.

Core Ideas

- The rate of urea hydrolysis briefly increases when gypsum is added to broiler litter.
- Treating broiler litter with gypsum causes an immediate decrease in litter pH.
- Calcium carbonate precipitation in gypsum-amended litter decreases litter pH.

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Gypsum ($CaSO_4 \cdot 2H_2O$) has previously been investigated as a broiler litter amendment to reduce ammonia (NH_3) volatilization (Sampaio et al., 1999; Oliveira et al., 2003, 2004; Loch et al., 2011; Mishra et al., 2013; Burt et al., 2017), but the mechanism responsible for NH_3 abatement has not been identified. For instance, Sampaio et al. (1999) observed a decrease in NH_3 volatilization from gypsum-amended litter compared with unamended litter and hypothesized that the reduction was due to a decrease in microbial activity associated with NH_3 production. Sampaio et al. (1999) also suggested an alternative hypothesis where NH_3 volatilization is reduced due to a reaction between gypsum and ammonium carbonate [$(NH_4)_2CO_3$], which traps NH_3 in litter as ammonium sulfate [$(NH_4)_2SO_4$] (Eq. [1]) (Teuscher and Adler,1965).

$$(NH_4)_2CO_3 + CaSO_4 \rightarrow (NH_4)_2SO_4 + CaCO_3 \quad [1]$$

Oliveira et al. (2003) observed a reduction in NH_3 volatilization in gypsum-amended poultry litter and hypothesized that gypsum's high capacity to absorb moisture from litter reduced the activity of NH_3–producing bacteria, which in turn would have resulted in a lower litter pH than unamended poultry litter. Ammonia-producing bacteria are bacteria that can hydrolyze litter urea, which consumes hydrogen (H^+) ions and leads to an increase in pH. Thus, a reduction in these bacteria would lead to a lower pH when compared with unamended poultry litter. Recently, Burt et al. (2017) investigated the effects of flue-gas desulfurization gypsum (FGDG) on urea-degrading (ureolytic) bacteria (UDB) and NH_3 volatilization from broiler litter and confirmed that the addition of 20% FGDG decreased litter pH and reduced UDB and NH_3 losses. Based on cumulative carbon dioxide (CO_2) measurements from gypsum-amended litter and unamended litter, the authors speculated that the addition of FGDG decreased litter pH in part due to precipitation of calcium carbonate ($CaCO_3$). Calcium carbonate precipitation and its effect on litter pH have not been investigated and should be considered when trying to identify possible mechanisms of NH_3 abatement in gypsum-amended litter.

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Abbreviations: dH_2O, deionized water; dwe, dry weight equivalent; FGDG, flue-gas desulfurization gypsum; PLUP, poultry litter urease producer; UDB, urea-degrading bacteria.

Microbial-induced carbonate precipitation is a common process found in a variety of terrestrial and aquatic environments (Douglas and Beveridge, 1998; Ehrlich, 1998; Castanier et al., 1999; Hammes et al., 2003). Microbial-induced carbonate precipitation occurs as a byproduct of metabolic processes such as photosynthesis (Douglas and Beveridge, 1998; Yates and Robbins, 1999), sulfate reduction (Warthmann et al., 2000), and urea hydrolysis (Stocks-Fischer et al., 1999; Fujita et al., 2000, 2008; Hammes et al., 2003). Calcium carbonate formation resulting from urea hydrolysis is often associated with precipitation in soils and geological sediments (Fujita et al., 2000), and it has been shown that significant amounts of $CaCO_3$ can be produced by this process in 48 h or less (Hammes et al., 2003). Calcium carbonate precipitation resulting from urea hydrolysis is controlled by four factors: (i) the concentration of dissolved inorganic carbon (C), (ii) the pH, (iii) the calcium (Ca^{2+}) concentration, and (iv) the availability of nucleation sites (Castanier et al., 1999; Hammes and Verstraete, 2002; Gat et al., 2014). The first two factors controlling $CaCO_3$ are directly affected by urease activity, whereas the last factor (nucleation sites) is generally influenced by the amount of negatively charged bacterial cell components (Schultze-Lam et al., 1996; Kawaguchi and Decho, 2002).

Most published literature on $CaCO_3$ produced by urea hydrolysis focuses on using pure cultures of UDB to produce carbonates for construction remediation (Le Métayer-Levrel et al., 1999; Stocks-Fischer et al., 1999; Ramachandran et al., 2001; Sarda et al., 2009; Chahal et al., 2011), heavy metal removal (Fujita et al., 2000; Warren et al., 2001), and wastewater treatment (Hammes and Verstraete, 2002; Hammes et al., 2003). Key findings of this research show that the rate of $CaCO_3$ precipitation is directly linked to the rate of urea hydrolysis (Fujita et al., 2000), and that calcite is the dominant form of $CaCO_3$ produced by urea hydrolysis (Castanier et al., 1999; Stocks-Fischer et al., 1999, Fujita et al., 2000; Anbu et al., 2016). In addition, Hammes et al. (2003) observed that the rate of urea hydrolysis in certain ureolytic isolates increased when Ca^{2+} was added to growth media, and this led to rapid crystallization of $CaCO_3$ aggregates. Calcium carbonate precipitation was also facilitated by using a mixed culture of ureolytic and non-ureolytic bacterial species due to an increase in nucleation sites (Gat et al., 2014).

Given the results discussed above, gypsum-amended broiler litter should be a suitable environment for $CaCO_3$ precipitation due to its rich nitrogen (N) content, abundance of ureolytic and non-ureolytic microorganisms, and source of Ca^{2+}. Uric acid and urea are the most common forms of organic N in litter, and they account for 70 to 80% of the total N content (Groot Koerkamp, 1994; Kelleher et al., 2002). In broiler litter, uricase produced by uric acid-degrading (uricolytic) microbes degrades uric acid to urea, and urea is then hydrolyzed by urease enzymes produced by ureolytic bacteria and fungi (Rothrock et al., 2010). Urea hydrolysis produces ammonium (NH_4^+), bicabonate (HCO_3^-), and an increase in pH (Kissel et al., 1988). Gypsum is a moderately soluble Ca-salt, and its dissolution should create a source of Ca^{2+} that can react with litter carbonates as described by Burt et al. (2017), which should decrease both litter pH and subsequent NH_3 volatilization.

Currently, there are no known studies that evaluate the effect of gypsum on urease activity or $CaCO_3$ precipitation in broiler litter. Therefore, the objectives of this study were to evaluate the effect of gypsum on broiler litter urease activity at three water contents, calcium carbonate precipitation, and pH. Based on the literature surveyed, we hypothesized that the addition of gypsum to broiler litter would increase the rate of urea hydrolysis and decrease litter pH due the precipitation of $CaCO_3$.

Materials and Methods

Broiler litter samples were collected from a broiler house in Georgia. The samples were passed through a 2-mm sieve and analyzed for total C and N, pH, electrical conductivity, water potential, and water content. Total C and N in broiler litter were determined by dry combustion (Nelson and Sommers, 1982); pH was measured using an AB150 pH meter by Fisher Scientific in a 1:5 (litter/deionized water [dH_2O]) ratio, and electrical conductivity was measured in a 1:5 (litter/dH_2O) ratio using a CDM80 conductivity meter (Radiometer America). Broiler litter water potential was measured using a WP4C dewpoint potentiameter (Decagon), and water content was determined gravimetrically by drying at 65°C for 48 h. Physiochemical descriptions of the broiler litters used in this study are shown in Table 1.

Experiment 1: Effect of Gypsum on Urease Activities in Broiler Litter at Different Water Contents

The effect of gypsum on urease activity in broiler litter was examined at three water contents (0.31 [low], 0.44 [medium], and 0.78 [high] g H_2O g^{-1}) over the course of 5 d. These water contents were selected based on studies by Groot Koerkamp (1998), who suggested that maximum NH_3 volatilization and optimum microbial growth occurs between 0.37 and 0.56 g H_2O g^{-1}, and that water contents outside of this range can inhibit growth due to limited water or anaerobic conditions. Broiler litter was air dried for 24 h and then wetted with dH_2O to achieve desired water contents. Broiler litter across all water contents was then incubated on the laboratory bench (23°C) for 72 h to allow microbial populations to adapt to the new water contents. Treatments included unamended litter and litter + 20% reagent-grade gypsum at each of the three water contents described above. For each water content, there were 36 replications of each treatment, all arranged in a completely randomized design. Each experimental unit consisted of 5 g of broiler litter (dry weight equivalent [dwe]) placed in a 0.95-L container. One gram of reagent-grade gypsum was added to treatments with gypsum, and all containers were incubated at 29°C. The addition of gypsum to broiler litter altered the water content of litters due to the addition of dry material (1 g $CaSO_4 \cdot 2H_2O$ = 0.791 g $CaSO_4$ + 0.209 g H_2O). The adjusted water content for gypsum-amended litter was calculated for all water contents to correct for the addition of gypsum: 0.29 (low),

Table 1. Initial physiochemical characteristics of broiler litter used in laboratory experiments.

Parameter	Unit	Value
Water content	g H_2O g^{-1}	0.33
Water potential	MPa	−15.70
pH	−log[H^+]	7.17
Electrical conductivity	μS cm^{-1}	17.76
Total C	g kg^{-1}	252.5
Total N	g kg^{-1}	34.4
C/N ratio	–	7.34

0.40 (medium), and 0.69 (high) g H_2O g^{-1}. It has been shown that 1.5 molecules of gypsum's structural water are loosely held, whereas the remainder is strongly bound by $CaSO_4$ (Mandal and Mandal, 2002). Therefore, 1.5 molecules of gypsum's structural water were included in the adjusted water contents. On Days 0, 1, 3, and 5, eight experimental units from each treatment were retrieved to measure the rate of urea hydrolysis. Day 0 measurements were performed after 1 g of gypsum was applied to designated experimental units. At each time, four experimental units from each treatment received 25 mL of 1000 mg L^{-1} urea solution, whereas the other four experimental units did not receive urea. All experimental units were then incubated on the laboratory bench (23°C) for 12 min to allow for urea hydrolysis. During urea hydrolysis, all experimental units were incubated on the laboratory bench instead of in the incubator due to limited workable space within the incubator. In addition, incubating experimental units on the laboratory bench minimized temperature fluctuations during urea hydrolysis that would have occurred when urea solution was added to the experimental units and when experimental units were retrieved after the 12-min incubation time. After 12 min, urea and NH_4^+–N were extracted by adding 250 mL of 2.5 mol KCl L^{-1} containing 100 μg g^{-1} of Ag_2SO_4 solution. All experimental units were then shaken in a reciprocating shaker at 120 oscillations min^{-1} for 15 min and filtered through a 0.45-μm filter. Extracts were analyzed for urea and NH_4^+–N following the colorimetric procedure described by Keeney and Nelson (1982). The experimental units that did not receive urea solution were used to estimate background concentrations of urea in broiler litter. The average amount of urea extracted from these background experimental units was combined with the amount of urea that was added to each experimental unit of the same treatment to estimate the total amount of urea present before hydrolysis. To calculate the rate of urea hydrolysis (μg urea g^{-1} dry litter min^{-1}), the amount of urea remaining after 12 min was subtracted from the total amount of urea present in each experimental unit (Eq. [2]). On Day 5, the pH of four additional experimental units was measured for gypsum-amended and unamended litter by using a 1:5 (litter/0.01 mol $CaCl_2$ L^{-1} solution) ratio. Measurements of litter pH were only conducted on Day 5 due to limited incubator space and the time-sensitive nature of the experimental units used to measure the rate of urea hydrolysis.

$$\frac{\left[\text{Background urea } (\mu g\, g^{-1})_{(\text{Broiler litter})} + \text{urea } (\mu g\, g^{-1})_{(\text{solution})}\right] - \left[\text{remaining urea } (\mu g\, g^{-1})_{(\text{after hydrolysis})}\right]}{12 \text{ min}} \quad [2]$$

Experiment 2: Effect of Gypsum on Carbonate Precipitation in Broiler Litter

A second experiment was conducted to measure the amount of $CaCO_3$ that precipitates from the hydrolysis of urea in the presence of gypsum. This experiment used procedures similar to those of Bundy and Bremner (1972), which are used to determine inorganic C in soils. Treatments for this experiment included: (i) broiler litter (1 g dwe) + urea (20 mg), (ii) broiler litter (1 g dwe) + urea (20 mg) + gypsum (0.2 g), and (iii) broiler litter (1 g dwe) alone. There were four replicates for each treatment, all arranged in a completely randomized design. Each experimental unit consisted of slurry created by mixing 1.33 g of broiler litter (0.33 g H_2O g^{-1}) with 20 mL of CO_2–free dH_2O in a sealed 240-mL French square bottle. A slurry was used to facilitate dissolution and hydrolysis of the added urea. Reagent-grade gypsum (0.2 g) was added to broiler litter on a dry-weight basis. Each bottle was equipped with a 7-mL 4 mol L^{-1} KOH trap to trap CO_2. All experimental units were placed on a rotary shaker set at 150 oscillations min^{-1} for 48 h. After 48 h, experimental units were removed from the shaker, and the KOH traps were retrieved to measure the amount of CO_2 evolved from respiration and urea hydrolysis. The content of each trap was poured into a 50-mL centrifuge tube, and 9.3 mL of 0.75 mol $BaCl_2$ L^{-1} was added to each tube. All tubes were then centrifuged at 3000 rpm for 5 min. After centrifuging, a 5-mL aliquot was taken from each tube and diluted with 10 mL of dH_2O. This solution was then titrated with 0.1 mol HCl L^{-1} to a pH of 8.3 to measure the amount of unreacted KOH. The amount of CO_2 trapped was calculated from the difference between initial and final amounts of KOH.

After removal of the KOH traps, the broiler litter slurry was transferred to a filtering unit with a 0.45-μm filter. Each bottle was rinsed twice with dH_2O to ensure complete transfer of broiler litter to the filtering unit. The resulting filtrate was titrated to pH 4.5 with 0.1 mol HCl L^{-1} to measure the amount of HCO_3^- and CO_3^{2-} in solution. The amount of $CaCO_3$ that precipitated as a solid was measured using a procedure similar to Bundy and Bremner (1972). The filter paper containing broiler litter from the previous step was retrieved from each filtering unit and placed into a 250-mL French square bottle equipped with a 7-mL 4 mol KOH L^{-1} trap. All bottles were then sealed, and 50 mL of air was removed from each bottle using a syringe. Twenty milliliters of 2 mol HCl L^{-1} was then injected into each bottle through a septum at the top of the bottle, and all experimental units were allowed to stand on the laboratory bench for 24 h. After 24 h, the KOH traps were retrieved, and the amount of CO_2 captured was determined as previously described.

Experiment 3: Effect of Gypsum on Broiler Litter pH

To determine the change in pH that occurs during the first 24 h after the addition of gypsum to broiler litter, a third study was conducted in which the pH of unamended and litter amended with 20% gypsum was measured during a 24-h incubation. There were three replicates of each treatment, all arranged in a completely randomized design. Each experimental unit consisted of 1.33 g of broiler litter (0.33 g H_2O g^{-1}) plus 10 mL of dH_2O placed in a 125-mL Erlenmeyer flask. Gypsum (0.2 g) was added to broiler litter treatments on a dry-weight basis. Forty milliliters of 2.5 mol KCl L^{-1} containing 100 μg g^{-1} of Ag_2SO_4 solution was then added to each experimental unit, and pH was measured immediately. The pH of all experimental units were measured after 0, 1, 2, 3, 4, and 24 h. Day 0 measurements were performed after 0.2 g of gypsum was applied to designated experimental units.

To better understand the immediate decrease in pH observed in gypsum-amended litter compared with unamended litter, the litter pH buffering capacity and the initial amount of HCO_3^- in litter were determined. The initial amount of HCO_3^- in litter was determined using procedures similar to those of Bundy and Bremner (1972). The litter pH buffering capacity was determined using a titrimetric method described by Cassity-Duffey et al. (2015). There were four replicates used to determine the initial amount of HCO_3^- in broiler litter, and each experimental

unit consisted of 10 g of broiler litter (0.33 g H_2O g^{-1}) placed in a 250-mL Erlenmeyer flask with 200 mL of dH_2O. All experimental units were thoroughly mixed and filtered immediately through a 0.45-μm filter. A 20-mL aliquot of each filtrate was placed in a 250-mL square bottle that contained a 2.5-mL 0.87 mol KOH L^{-1} trap for capturing CO_2. All bottles were then sealed, and 50 mL of air was removed from each bottle using a syringe. Next, 5 mL of 0.098 mol HCl L^{-1} was then injected into each bottle through a septum at the top of the bottle, and all experimental units were allowed to stand on the laboratory bench for 24 h. After 24 h, the KOH traps were removed and the contents were poured into a 50-mL centrifuge tube and brought to a final volume of 50 mL using dH_2O. Individual traps were then titrated according to Bundy and Bremner (1972).

Statistical Analysis

Data were statistically analyzed using InfoStat 2012 (Di Rienzo et al., 2012). Data from all the experiments were analyzed using a one-way ANOVA as a completely randomized design. Significant differences among treatment means were determined using Fisher's protected LSD at $p = 0.05$.

Results

Experiment 1: Effect of Gypsum on Urease Activities in Broiler Litter with Different Water Contents

Significant differences between the rates of urea hydrolysis in gypsum-treated and untreated litter were observed on Day 0 for the low ($p = 0.016$) and high ($p = 0.048$) water contents (Fig. 1A and 1C), and on Day 3 ($p = 0.011$) for the medium water content treatment (Fig. 1B). On Day 5, the rate of urea hydrolysis in broiler litter at the different water contents was not affected by the addition of 20% gypsum. At the end of the incubation, the slowest rates of urea hydrolysis were observed in unamended litter with medium water content (90.6 ± 8.9 μg g^{-1} min^{-1}), as well as in unamended and gypsum-amended litter with low water contents (96.7 ± 6.3 and 96.5 ± 1.0 μg g^{-1} min^{-1}, respectively). During this time, an intermediate rate of urea hydrolysis was observed in gypsum-amended litter with medium water content (105.4 ± 26.9 μg g^{-1} min^{-1}), but this rate of urea hydrolysis was not significantly different from unamended litter at the same water content ($p = 0.22$). After 5 d, the greatest rates of urea hydrolysis occurred in gypsum-amended and unamended litter with high water contents (151.3 ± 20.7 and 164.3 ± 4.3 μg g^{-1} min^{-1}).

Treating broiler litter with 20% gypsum significantly increased NH_4^+–N concentrations in litter with low, medium, and high water content after 5 d (Fig. 2A–2C) ($p < 0.0033$). The addition of gypsum to broiler litter with low and high water contents increased NH_4^+–N concentrations by 8 to 23% on Day 0 ($p < 0.001$), and this increase in NH_4^+–N concentrations coincided with increased rates of urea hydrolysis (Fig. 1A and 1C). After Day 0, NH_4^+–N concentrations in unamended and gypsum-amended litter with low and high water contents remained fairly constant for the duration of the experiment, and after 5 d, there was 10 to 14% more NH_4^+–N in gypsum-amended litter than in unamended litter ($p < 0.0033$). The concentration of NH_4^+–N (13%) in broiler litter with medium water content also increased significantly on Day 0 when litter was treated with 20% gypsum

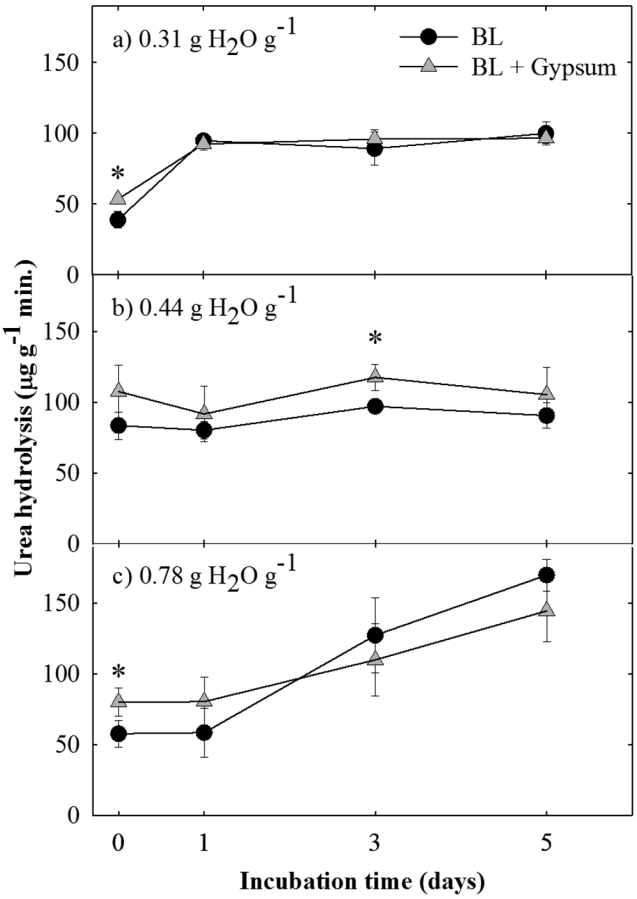

Fig. 1. Urease activity in litter with different water contents, with (0.2 g g^{-1} dry litter) and without gypsum and incubated at 29°C for 5 d (Exp. 1). (a) Rates of urea hydrolysis in litter with 0.31 g H_2O g^{-1} (adjusted water content for gypsum-amended litter: 0.29 g H_2O g^{-1}); (b) rates of urea hydrolysis in litter with 0.44 g H_2O g^{-1} (adjusted water content for gypsum-amended litter: 0.40 g H_2O g^{-1}); (c) rates of urea hydrolysis in litter with 0.78 g H_2O g^{-1} (adjusted water content for gypsum-amended litter: 0.69 g H_2O g^{-1}). All symbols represent the mean of four replicates, error bars represent SD, and asterisks (*) indicate significant differences according to Fisher's LSD at $p < 0.05$. BL, broiler litter.

(Fig. 2B) ($p = 0.0032$), but this increase did not coincide with a significant increase in the rate of urea hydrolysis (Fig. 1B) ($p = 0.12$). Ammonium concentrations in unamended litter and gypsum-amended litter with medium water content remained constant for the duration of the experiment (Fig. 2B), and after 5 d, there was 16% more NH_4^+–N in gypsum-amended litter than in unamended litter ($p = 0.0030$).

At the end of the experiment, the greatest concentration of NH_4^+–N in broiler litter occurred in gypsum-amended litter with high water content (9965 ± 344 μg g^{-1}), followed by unamended litter with high water content (8998 + 220 μg g^{-1}) and gypsum-amended litter with low water content (6681 ± 199 μg g^{-1}) (Fig. 2A–2C). Intermediate concentrations of NH_4^+–N were measured in gypsum-amended litter with medium water content (5830 ± 198 μg g^{-1}) and unamended litter with low water content (5759 ± 336 μg g^{-1}). After 5 d, the lowest concentration of NH_4^+–N occurred in unamended litter with medium water content (4881 ± 251 μg g^{-1}) (Fig. 1B). Additional experimental units were used to measure the pH of both treatments for all water contents after urea hydrolysis on Day 5 (Fig. 2A–2C). The pH values for unamended litter and gypsum-amended litter increased with

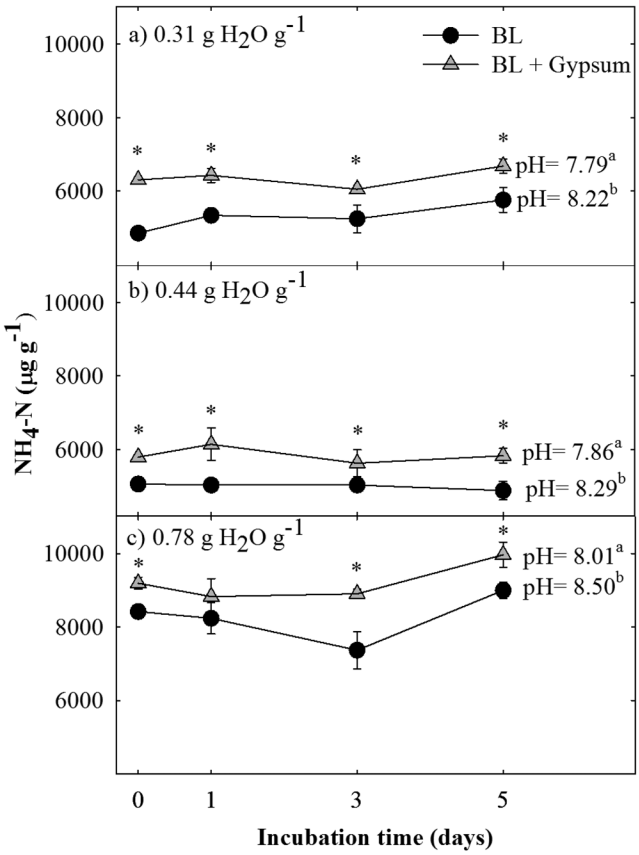

Fig. 2. Concentrations of NH_4^+–N in litter with different water contents, with (0.2 g g^{-1} dry litter) and without gypsum and incubated at 29°C for 5 d (Exp. 1). (a) Concentration of NH_4^+–N in litter with 0.31 g H_2O g^{-1} (adjusted water content for gypsum-amended litter: 0.29 g H_2O g^{-1}); (b) concentration of NH_4^+–N in litter with 0.44 g H_2O g^{-1} (adjusted water content for gypsum-amended litter: 0.40 g H_2O g^{-1}); (c) concentration of NH_4^+–N in litter with 0.78 g H_2O g^{-1} (adjusted water content for gypsum-amended litter: 0.69 g H_2O g^{-1}). All symbols represent the mean of four replicates, error bars represent SD, and asterisks (*) indicate significant differences according to Fisher's LSD at $p < 0.05$. pH was measured on Day 5 after urea hydrolysis for both treatments across all water contents, and means followed by different letters are significantly different according to Fisher's LSD at $p < 0.05$. BL, broiler litter.

increasing water content, and pH values for gypsum-amended litter were significantly lower (0.43–0.49 units) than those of unamended litter ($p = 0.0001$).

Experiment 2: Effect of Gypsum on pH and Carbonate Precipitation in Broiler Litter

In Exp. 2, 20 mg of urea powder was added to broiler litter treatments to calculate the distribution of urea-C (4 mg) in gypsum-amended litter and unamended litter (Table 2). In unamended litter, similar amounts of urea-C were recovered as CO_2 gas and $CaCO_3$ (38 and 36%, respectively), and the remainder of urea-C was recovered as HCO_3^-/CO_3^{2-} in solution (26%). In contrast, the majority of urea-C in gypsum-amended litter was recovered as $CaCO_3$ (59%), whereas lesser amounts of urea-C were recovered as CO_2 (gas) and HCO_3^-/CO_3^{2-} (aqueous) (27 and 12%, respectively). The results of the urea-C balance show that significantly more urea-C precipitated as $CaCO_3$ in gypsum-amended litter than in unamended litter (Table 2) ($p = 0.001$).

Experiment 3: Effect of Gypsum on Broiler Litter pH

Given the above results, a third study was conducted to identify the mechanism responsible for the decrease in litter pH that occurs immediately after gypsum application. The addition of gypsum to broiler litter significantly decreased litter pH by 0.22 units at time zero ($p < 0.0001$) (Fig. 3). After incubating both litters for 1 h, the pH of gypsum-amended litter significantly decreased by 0.18 pH units, whereas the pH of unamended litter only decreased by 0.02 pH units ($p < 0.0001$) (Fig. 3). The pH of both litters continued to decrease during the course of the incubation. After 24 h, amending broiler litter with gypsum significantly decreased litter pH (7.51) compared with unamended litter (7.89) ($p = 0.004$). To better understand the immediate decrease in pH that was observed in amended litter, the pH buffering capacity and the initial amount of HCO_3^- in litter were determined. Broiler litter used for all experiments had a pH buffer capacity of 229 mmol (pH unit) $^{-1}$ kg^{-1} dry litter, and enough HCO_3^- in solution to produce 50.6 ± 5.9 mmol H^+ kg^{-1} when converted to CO_3^- (data not shown).

Discussion

The addition of gypsum to broiler litter has been shown to decrease NH_3 volatilization (Sampaio et al., 1999; Oliveira et al., 2003; Loch et al., 2011; Burt et al., 2017), but the mechanism responsible for NH_3 abatement has not been identified. Recently, Burt et al. (2017) observed decreases in litter pH, NH_3 volatilization, and cumulative CO_2 emission along with increases in N mineralization concentrations when broiler litter was treated with 20% FGDG. These authors hypothesized that the decrease in NH_3 volatilization and the increase in N mineralization resulted from an increase in urease activity and the precipitation of $CaCO_3$, but these two factors were not measured.

In the present study, amending broiler litter with 20% reagent-grade gypsum increased NH_4^+–N concentrations by 10 to 16% after 5 d and decreased litter pH by 0.43 to 0.49 units across three different water contents (Fig. 2A–2C). These results are similar to findings by Burt et al. (2017), who observed an increase in NH_4^+–N concentrations and a decrease in litter pH in gypsum-amended litter compared with unamended litter. More specifically, Burt et al. (2017) found that amending broiler litter with 20% FGDG significantly increased NH_4^+–N concentrations and decreased litter pH during the first 24 h of the incubation.

Table 2. Distribution of urea-C evolved as CO_2, urea-C present as soluble HCO_3^- and CO_3^{2-}, and urea-C present in solid-phase $CaCO_3$ in broiler litter (BL) with (0.2 g g^{-1} dry litter) or without gypsum incubated for 48 h at 25°C (Exp. 2). Values presented were corrected by subtracting values determined for unamended litter without additional urea.

Treatment	CO_2 in traps	HCO_3^- and CO_3^{2-} in solution	$CaCO_3$ (solid)	Total urea-C
	mg urea-C g^{-1} BL			
BL	1.46 (0.23)b†	1.03 (0.29)b	1.39 (0.19)a	3.96 (0.24)
BL + gypsum	1.03 (0.23)a	0.44 (0.083)a	2.11 (0.26)b	3.67 (0.24)
p-value	0.0361	0.0023	0.0010	0.14

† Within a column, means followed by different letters are significantly different according to Fisher's LSD at $p < 0.05$.

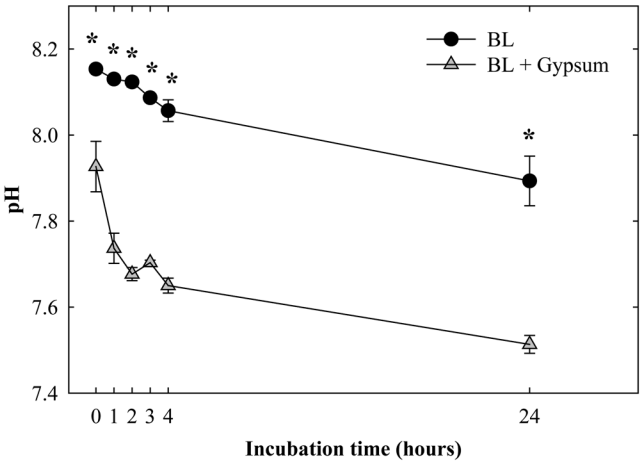

Fig. 3. pH in broiler litter with (0.2 g g^{-1} dry litter) or without gypsum incubated at 25°C for 24 h (Exp. 3). All symbols represent the mean of four replicates, error bars represent SD, and asterisks (*) indicate significant differences according to Fisher's LSD at $p < 0.05$. BL, broiler litter.

After the initial increase of NH_4^+–N in gypsum-amended litter, concentrations remained fairly constant for the duration of the experiment (21 d). Researchers hypothesized that the addition of FGDG to broiler litter created osmotic stress on UDB, which caused these organisms to take in osmolytes (such as urea) to help counter the negative effects of dehydration and solute concentration. This in turn increased intracellular urease activity, which led to a significant increase in NH_4^+–N. The authors also suggested an alternative hypothesis in which cells that died as a result of the stress released intracellular enzymes that increased urea hydrolysis.

In the first experiment of the present study, the addition of gypsum did increase the rate of urea hydrolysis in broiler litter, but this effect was only observed on Day 0 in litter with low and high water content, and on Day 3 in litter with medium water content (Fig. 1A–1C). The increase in urea hydrolysis that was observed in gypsum-amended litter with low and high water content on Day 0 did coincide with a significant increase in NH_4^+–N concentrations (Fig. 2A and 2C). After the initial increase of NH_4^+–N in gypsum-amended litters, NH_4^+–N concentrations remained fairly constant for the duration of the experiment. Given the findings by Burt et al. (2017) and results of the present study, it seems likely that the addition of gypsum to broiler litter does create stress on urease-producing microorganisms, and that this causes an increase in urease activity. However, this increase in urease activity does not appear to be sustained for an extended amount of time, possibly due to the death of certain urease-producing microorganisms and/or the degradation of extracellular urease.

It should be noted that adding gypsum to designated experimental units altered the final water content of litters, and changes in water content have been shown to stress microorganisms and alter microbial activity (Iovieno and Baath, 2008; Chowdhury et al., 2011; Murakami et al., 2011). In addition, before Exp. 1 commenced, litter was air dried for 24 h and then rewetted to achieve the desired water contents. The addition of gypsum to broiler litter decreased water content in litters with low (0.31 g H_2O g^{-1}), medium (0.44 g H_2O g^{-1}), and high (0.78 g H_2O g^{-1}) water contents by 0.02, 0.04, and 0.09 g H_2O g^{-1}, respectively. Although the final water contents of amended litters were slightly lower than those of unamended litters, we do not believe this had a significant impact on microbial activity because the adjusted water contents of these litters were still within the water content levels described by Groot Koerkamp (1998). Furthermore, drying and rewetting events can alter microbial activity, but it has been shown that microbial populations fully recover from these events after 24 to 48 h (Iovieno and Baath, 2008). Therefore, the 72-h incubation period that preceded Exp. 1 should have provided enough time for microbial activity to stabilize before the experiment began.

The limited impact of gypsum on urea hydrolysis in broiler litter is not surprising when results by Hammes et al. (2003) are considered. In their study, sterile solutions containing urea and 30 mM $CaCl_2$ were used to study the effect of Ca^{2+} on urease activity in environmental ureolytic bacteria isolates that were closely related to the *Bacillus sphaericus* group. Results of this research showed that the presence of Ca^{2+} increased urease activity of isolates that were closely related to *Bacillus pasteurii* by 4- to 10-fold but had no effect or slightly decreased urease activity in other isolates. Hammes et al. (2003) hypothesized that increases in urease activity of certain isolates were caused by a cellular detoxification response to excess Ca^{2+} in solution. Therefore, it seems possible that urease activity in certain ureolytic microorganisms increased due to a detoxification response created by the dissolution of gypsum in broiler litter. Our study differs from the current literature in that broiler litter contains numerous strains of urease-producing bacteria and fungi (Rothrock et al., 2008, 2010), and most of the UDB (90%) belong to a group of urease producers that are only found in broiler litter (Rothrock et al., 2008). Phylogenetic analysis of the urease gene (*ureC*) found in broiler litter urease producers (PLUPs) indicated that the closest match in the GenBank database was to the *ureC* gene from *Pseudomonas aeruginosa* (AE004901). There are currently no known studies that investigate the effect of Ca^{2+} on urease activity in *P. aeruginosa* or PLUP isolates. Therefore, future studies that examine gypsum's effect on ureolyitc microorganisms in broiler litter should consider measuring the effect of different Ca^{2+} concentrations on urease activity in PLUP isolates and ureolytic fungi to better understand gypsum's effect on urease activity in broiler litter.

In addition to investigating the effect of gypsum on urease activity, we were also interested in identifying the mechanism responsible for decreases in litter pH that have been documented in other studies (Oliveira et al., 2003; Loch et al., 2011; Burt et al., 2017), because litter pH is one of the most prominent factors affecting NH_3 volatilization (Elliott and Collins, 1982; Carr et al., 1990). Urea hydrolysis in litter generally increases litter pH due to consumption of H^+ (Eq. [3]) (Kissel and Cabrera, 1988), which favors the formation of volatile NH_3 (Reece et al., 1979).

$$CO(NH_2)_2 \text{ (urea)} + 2H_2O + H^+ \rightarrow NH_4^+ + HCO_3^- \quad [3]$$

It was hypothesized by Mishra et al. (2013) and Burt et al. (2017) that as litter pH increases from consumption of H^+, HCO_3^- will deprotonate to form CO_3^{2-} (Eq. [4]), and the dissolution of gypsum in litter would provide Ca^{2+} that reacts with CO_3^{2-}, forming $CaCO_3^-$ (Eq. [5]).

$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+ \quad [4]$$

$$Ca^{+2} + CO_3^{2-} \rightarrow CaCO_3 \quad [5]$$

The formation of $CaCO_3$ would then consume CO_3^{2-}, leading to the release of additional H^+ and CO_3^{2-} from Eq. [4] that can buffer against pH increases caused by urea hydrolysis.

The reactions described by Mishra et al. (2013) and Burt et al. (2017) are supported by the results of our urea-C balance, in which less urea-derived C was recovered as CO_2 (gas) and HCO_3^-/CO_3^{2-} (aqueous) when gypsum was added to broiler litter (Table 2). These differences were due to significantly more $CaCO_3$ precipitating in gypsum-amended litter than in unamended litter ($p = 0.0010$), which led to a decrease in litter pH according to the reactions described above (Eq. [3] and [4]). These findings are in agreement with results by Burt et al. (2017), in which the addition of FGDG to broiler litter decreased litter pH and CO_2 emissions as compared with unamended litter.

In addition to the decrease in litter pH that was observed in gypsum-amended litter after 5 d, results of a third experiment showed that amending broiler litter with 20% gypsum significantly decreased litter pH after 24 h of incubation as compared with unamended litter (Fig. 3). During this 24-h incubation, the largest decrease in pH occurred in gypsum-amended litter at time zero. To better understand the immediate decrease in pH that was observed in the preceding experiment in gypsum-amended litter, the litter pH buffering capacity and the initial amount of HCO_3^- in litter were determined. Measurements of litter pH buffering capacity and HCO_3^- in solution indicated that there was enough HCO_3^- in solution to decrease litter pH by 0.22 pH units. Therefore, it seems likely that the immediate decrease in litter pH (0.23 pH units) that was observed when gypsum was added to litter (Fig. 3) was due to the precipitation of $CaCO_3$ from litter HCO_3^-, as described by Eq. [3] and [4].

It has been hypothesized that the reduction in NH_3 loss and the accumulation of NH_4^+ in litter is due to a reaction described by Teuscher and Adler (1965), in which there is a reaction between gypsum and $(NH_4)_2CO_3$ (Eq. [1]). This reaction would cause $(NH_4)_2SO_4$ to form instead of NH_3, thereby decreasing NH_3 losses. We believe that the mechanism described in Teuscher and Adler (1965) and in Tubail et al. (2008) does not accurately reflect what is occurring in gypsum-amended broiler litter. First, the dominant ionic C species from urea hydrolysis is HCO_3^-, not CO_3^{2-}. Even at pH 8.5, HCO_3^- would be the dominant species, although a small proportion of CO_3^{2-} would also form. Furthermore, the presence of gypsum does not convert $(NH_4)_2CO_3$ to $(NH_4)_2SO_4$ and $CaCO_3$. As shown by Fenn and Kissel (1975), $CaCO_3$ appears to be consumed in the presence of $(NH_4)_2SO_4$ according to pH measurements, with soil pH being negatively correlated to $(NH_4)_2SO_4$ application rate between pH 7.1 and 7.6. A soil would not contain $CaCO_3$ at pH 7.1. This indicates that $CaCO_3$ was consumed in the reaction, not formed. Although both sulfate (SO_4^-) and NH_4^+ may exist in solution, this does not imply that $(NH_4)_2SO_4$ has been formed. Ammonium sulfate is formed only when it precipitates out of solution (to form ammonium sulfate crystals) when enough water is lost.

Our results show that amending broiler litter with 20% gypsum significantly increased the concentration of NH_4^+–N in litter, but there was a limited effect on the rate of urea hydrolysis across three different water contents. Furthermore, urease activity, NH_4^+–N concentrations, and litter pH increased as the water content of litter increased. After 5 d, the greatest rates of urea hydrolysis, pH values, and concentrations of NH_4^+–N in litter were observed in litter with high water content. In Exp. 2, significantly more urea-C was converted to $CaCO_3$ in gypsum-amended litter compared with unamended litter, which resulted from more Ca^{+2} ions in solution and from the deprotonation of HCO_3^-, a process that buffered against increases in pH that accompany urea hydrolysis. Given these results, we believe that the addition of gypsum to broiler litter favors microbially induced carbon precipitation; therefore, Eq. [3–5] accurately describe the proposed mechanism that $CaCO_3$ precipitation in gypsum-amended litter buffers against large increases in pH that are known to facilitate NH_3 loss.

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