Title: Fertilizer ammonium to nitrate ratios determine phosphorus uptake in young maize plants

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Abstract

We investigated the interacting effects of inorganic nitrogen and the main inorganic phosphorus form in dairy manure (dicalcium phosphate, $CaHPO_4$) on growth, nutrient uptake and rhizosphere pH of young maize plants.

In a pot experiment three levels of $CaHPO_4$ (0, 167 and 500 mg P pot^{-1}) were combined with nitrogen (637 mg N pot^{-1}) applied at five NH_4-N:NO_3-N ratios (0:100, 25:75, 50:50, 75:25 and 100:0) and a nitrification inhibitor in a concentrated layer of a typical acid sandy soil from Denmark. ^{15}N-labelled NH_4-N was applied to differentiate the role of nitrification and to partition nitrogen uptake derived from NH_4-N.

Among treatments including nitrogen, shoot biomass, rooting and phosphorus uptake were significantly higher at the five-leaf stage, when $CaHPO_4$ was applied with NH_4-N:NO_3-N ratios of 50:50 and 75:25. In these treatments, rhizosphere pH dropped significantly in direct proportion with NH_4-N uptake. The fertilizers in the concentrated layer had a root inhibiting effect in treatments without phosphorus supply and in treatments with pure NO_3-N or NH_4-N supply.

Increased nitrogen uptake as NH_4-N instead of NO_3-N reduced rhizosphere pH and enhanced acquisition of applied $CaHPO_4$ in young maize plants, which could have positive implications for the enhanced utilization of manure phosphorus.

1 Introduction

Strategies to increase the efficiency of recyclable phosphorus (P) use within regional and global agriculture is a key step towards the sustainable intensification of food production. One option towards greater P sustainability is to minimize the use of mineral fertilizer derived from phosphate rock. This to some extent may be achieved by increasing the utilization of P in livestock manure, which is the largest source of recyclable P in Europe (*Ott and Rechberger*, 2012). Indeed more effective recycling of P in livestock manure could potentially substitute a substantial part of mineral P fertilizer consumption, and aid the transition towards a circular economy for P (*Withers* et al., 2015). However, to achieve a greater integration of livestock manure nutrients on the farm, a better understanding is needed of how the availability of P in livestock manure is regulated in plant-soil systems, especially for the inorganic P forms that prevail in manures.

In northwest Europe, maize (*Zea mays* L.) for silage is an important crop on intensive dairy farms. The P applied with dairy manure often fully matches the P exported from the field with the crop, but in Danish maize production 10-15 kg ha^{-1} of mineral P fertilizer is routinely placed near the seed at sowing (starter P fertilizer) in addition to non-positioned injection of dairy slurry (*Knudsen*, 2010). Starter fertilizer is widely used for maize in many other regions including other northwest European countries (e.g. *Schröder* et al., 1997). This starter fertilizer is considered necessary because a lack of P in the early growing stages can compromise the final crop yield (*Barry and Miller* 1989; *Grant* et al., 2001). However, long-term application of P above crop P demand can lead to P accumulation in soil, which enhances the eutrophication risk in downstream waterbodies (*Kronvang* et al., 2009). A reliance on placed soluble inorganic fertilizer for starter nutrients reflects its immediate availability to plants, but these starter nutrients could potentially be supplied by the dairy manure if the inorganic nutrients contained in dairy manure can be equally relied upon to satisfy crop nutrient demands during the early growth stages. This crop demand could be satisfied, for instance, by placement of injected cattle slurry (e.g. *Schröder* et al., 2015), but the interacting effects of placed inorganic nitrogen (N) and P present in cattle slurry that could affect the availability of injected slurry P must be clarified. Since P is taken up by plants as inorganic orthophosphate from the soil solution, the inorganic P forms in animal manures are more readily available to plants than organic P forms and inorganic P forms constitute up to 92 percent of total P in dairy manure (*Sharpley and Moyer*, 2000). Dicalcium phosphate ($CaHPO_4$, DCP) constitutes more than half of the inorganic P in dairy manure, and the solubility of DCP is strongly dependent on solution pH among other factors (*Güngör* et al., 2007; *Pagliari,* 2014). As manure also contains nutrients other than P, most notably N, nutrient interactions after addition to the soil may affect P availability in the silage maize cropping system. It is unclear though how the supply of ammonium N (NH_4-N), which is the dominant form of inorganic N in dairy slurry (*Webb* et al., 2013) affects the short-term availability of DCP.

Previous studies have shown that rhizosphere pH decreases when plants are supplied with NH_4-N, whereas rhizosphere pH increases when the plants are supplied with nitrate N (NO_3-N) (*Riley and Barber,* 1971). Such pH changes in the rhizosphere may influence the availability of inorganic P present in dairy manure, through pH controls on P speciation, precipitation and sorption processes. For highly soluble mineral P, *Jing* et al. (2010) showed that the combination of localized supply of P with NH_4-N improved maize growth and root proliferation on a calcareous soil. A recent meta-analysis by *Nkebiwe* et al. (2016) also concluded that placement of NH_4-N in combination with highly soluble P was more effective in increasing yield than placement of either NH_4-N or soluble P alone across various crop types. However, it has not previously been studied if less soluble inorganic P forms present in dairy manure such as DCP also become more available to young maize plants, when the plants are supplied with a higher amount of NH_4-N relative to the NO_3-N supply, or if the high application rate of NH_4-N normally applied in slurry could form an unfavorable environment for root growth.

To provide a better mechanistic understanding of the interaction between inorganic N form and DCP via pH changes in the rhizosphere, we mimicked the addition of inorganic N and DCP in dairy manure in a pot trial with maize. We hypothesized that growth and P-uptake in young maize plants would be improved when a higher proportion of NH_4-N was applied relative to NO_3-N due to increased plant availability of DCP induced by a pH decline in the rhizosphere, when N was taken up as NH_4-N. The aim was to determine the effect of increasing NH_4-N:NO_3-N application ratios on pH in the rhizosphere compared to the bulk soil and to study how such pH changes in the rhizosphere affect the availability of DCP, and whether high NH_4N concentrations in the soil restrict root growth.

2 Materials and methods

2.1 Experimental details

Maize was grown in cylindrical 1.9 L pots in a full factorial experiment with four replicates that included three levels of P in DCP (0, 167 and 500 mg P pot^{-1}) and five NH_4-N:NO_3-N ratios (0:100, 25:75, 50:50, 75:25 and 100:0 applied at a total rate of 637 mg N pot^{-1},Table 1) in all combinations with a nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) to reduce the conversion of NH_4-N to NO_3-N. The NH_4-N additions were labelled with ^{15}N to quantify the amount of NO_3-N in soil at harvest derived from the NH_4-N fertilizer due to nitrification, and to quantify the amount of N in plants derived from NH_4-N fertilizer. Additionally three reference treatments (0N treatments) for each P level were tested to study the plant growth response and soil pH development without N application. One reference treatment with 100% NH_4-N and no maize plants, and one with 100% NH_4-N and no DMPP were also included to test the influence of plant growth, nitrification and DMPP, respectively on soil pH and N dynamics.

The 1.9 L pots (inner diameter 103 mm) contained a coarse sandy topsoil (5-15 cm) collected from Jyndevad Experimental Station, Southern Denmark. The soil was sieved (5 mm), mixed and filled in pots to a height of 23.5 cm. The coarse sandy soil, which is a common soil type of Danish agricultural land with maize cropping, had 3% clay (<2 µm), 4% silt (2-20 µm), 91% sand (20 µm to 2 mm), 1.69% carbon and 0.13% N. The soil classifies as Orthic Haplohumod (USDA Soil Taxonomy System). The gravimetric water content at field capacity under pot conditions defined by *Kirkham* (2004) was 28%. At the start of the experiment, the coarse sandy soil had a pH ($CaCl_2$) of 5.4, and Olsen-P content of 21 mg P kg^{-1} (defined as a soil with medium P fertility in *Jordan-Meille* et al., (2012)). The Olsen-P test is the official soil-P test used on all soil types in Denmark and is widely used across Europe on a range of soils, including acid sandy soils (*Jordan-Meille* et al., 2012). Initially, the soil contained 2 mg NH_4-N and 8 mg NO_3-N kg^{-1} dry soil. The soil was carefully packed into the pots in three separate layers: 1568 g of soil was packed into a lower soil layer equivalent to 14.5 cm height, 432 g of soil enriched with the N and P fertilizer treatments constituted the middle soil layer equivalent to 4 cm height, and 502 g of soil equivalent to 5 cm height constituted the upper soil layer (Fig. 1a). A nylon mesh (mesh size=8 mm) separated the middle enriched soil layer from the lower and upper soil layer to be able to identify the middle layer at harvest. In total 2.5 kg soil was packed into each pot at a bulk density of 1.3 g cm^{-3}.

(Figure 1)

The N and P (and DMPP) treatments were mixed into the middle soil layer only in order to simulate a concentrated slurry injection band in forms and concentrations that mimicked the form of N (NH_4-N) and P (DCP) most abundant in dairy slurry. Application of the fertilizers in a concentrated layer simulated placed and injected fertilizer. DCP was applied as dry powder at a rate of 0, 167 or 500 mg P pot^{-1} corresponding to 0, 15 and 45 kg P ha^{-1} based on a plant density of 90,000 plants ha^{-1} (75 cm distance between rows and 15 cm distance within rows). The rate of 15 kg P ha^{-1} represents recommended agronomic practice in Denmark, and the rate of 45 kg P ha^{-1} was chosen to avoid potential P limitation to plant growth. The N fertilizer was applied in increasing proportions of NH_4-N relative to the amount of NO_3-N. The total N application rate was 637 mg N pot^{-1}corresponding to 57 kg N ha^{-1} based in a plant density of 90,000 plants ha^{-1}. The N application rate was based on a typical NH_4-N:P ratio in cattle slurry for the P level of 15 kg P ha^{-1}. NO_3-N was applied as potassium nitrate (KNO_3), and ^{15}N-labelled NH_4-N as ammonium sulphate ($(NH_4)_2SO_4$; 5.7% atom% ^{15}N). To prevent microbial oxidation of ammonium, Vizura ® (BASF, Ludwigshafen, Germany) was added at a rate of 1% of total N applied in all treatments except the reference treatment with 100% NH_4-N and 167 mg P pot^{-1}. The stock solution consisted of 10% (w/w) DMPP ($C_5H_{11}N_2O_4P$) in 40% phosphoric acid (w/w). This stock solution was mixed with the $(NH_4)_2SO_4$ fertilizer solutions and with demineralized water in the 0% NH_4-N treatments adding 9 mg P pot^{-1} from DMPP and phosphoric acid (equivalent to 5.1 and 1.7% of P application in the 167 and 500 mg P pot^{-1} treatments, respectively). Additional nutrients K, S and Mg were applied as solutions to the lower soil layer ten days before sowing at rates per pot of: 804 mg K (as K_2SO_4), 39 mg Mg (as $MgSO_4$) and 381 mg S (as K_2SO_4 and $MgSO_4$), based on the P:K ratio in cattle slurry. Other nutrients were applied to all pots by later surface irrigation 15 days after

sowing at a rate per pot of 2.9 mg Mn, 2.1 mg Zn, 0.4 mg B, 1.2 mg Cu, 0.03 mg Co and 0.5 mg Mo. These additional nutrients were added to eliminate other nutrient effects than P and N.
Maize seeds of an early developing maize hybrid (cv. Emblem, FAO 180; Limagrain) with an average weight of 345 mg were pre-germinated and transplanted at 3 cm depth into the pots containing soil prewetted to 50% field capacity. The pots were then placed in a climate-controlled chamber at a daily average temperature of 15 ˚C with a daily amplitude from 11 to 19 ˚C for the first 10 days, a mean temperature increase of 0.1 ˚C day^{-1} after 10 days, and a relative mean air humidity of 75%. The plants were grown in 16 h photoperiods with light intensities ranging from 170 to 1060 μmol photons m^{-2} s^{-1} to mimic Danish growing conditions in spring. The pots were irrigated with demineralized water to a water content of 60% of field capacity during the first 21 days of growth, and then to 65% from 21 days of growth until harvest (34 days). The position of the pots in the climate chamber was randomly changed every fourth day to minimize any positional effects.

2.2 Plant and soil measurements

The maize plants were harvested destructively by cutting stems 1 cm above soil surface 55 days after sowing. The harvest date was based on the establishment of a clear difference between the P-levels in chlorophyll content indices, plant height and leaf area at the five-leaf stage. The soil column was removed intact using a hydraulic pusher and cut into three layers (upper, middle, lower) using a knife. For each layer, the bulk soil and rhizosphere soil were sampled separately immediately after separation of the column. The rhizosphere soil was defined as the soil adhering to the roots. Root and rhizosphere soil were separated by placing the roots on a 2 mm sieve and gently tapping on the side of the sieve and collecting the soil passing the sieve. Bulk soil was defined as the remaining soil after sampling of roots and adhering soil, and was sieved to 4 mm. All soils were kept at 2 ˚C until analyses were performed two to three days after harvest. Sub-samples of the bulk soil were oven-dried for 24 h at 105 °C. The roots from each layer were washed with deionized water right after separation from the rhizosphere soil. The maize seed was included in the upper root layer. The shoots and roots were oven-dried at 60 ˚C to constant weight (min 48 h) for determination of dry matter (DM) and ground to a fine powder in a ball-mill prior to analysis.

2.3 Analytical methods

Soil pH was measured by glass electrode in 0.01 M $CaCl_2$ suspensions (1:2.5, *w*/w). NH_4-N and NO_3-N in soil were determined by flow colorimetry (Autoanalyzer III, Bran + Luebbe GmbH, Norderstedt, Germany) after shaking fresh soil immediately after sampling with 2 M KCl for 30 minutes (1:4, w/w). To study the rate of nitrification, the amount of ^{15}N-NH_4 and ^{15}N-NO_3 was determined in the soil extract by sequential diffusion analyses (*Sørensen and Jensen*, 1991). Electrical conductivity was measured in the supernatant after shaking 1 g of soil in 50 ml of deionized water for 1 h at 20 °C followed by centrifugation for 10 min at 1831 x g (20 °C).

Total N in shoots and roots and ^{15}N enrichment of shoots, roots and soil extracts were determined at the UC Davis Stable Isotope Facility (UC Davis, CA, USA) using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europe 20-20 isotope ratio mass spectrometer (Sercon Ltd. Cheshire, UK). The P concentration in shoot and root tissue was determined by digesting 300 mg dried plant material in 3 ml H_2O_2 (9.7 M) and 6 ml HNO_3 (14.3 M) under pressure in a microwave. In case of less material than 300 mg, a minimum 100 mg was digested. The P concentration in the diluted digest was determined by ICP-OES (Thermo Fisher Scientific, Waltham, MA). All soil and plant results are expressed on an oven-dry basis.

2.4 Calculations and statistical analysis

Total P uptake (PU) and N uptake (NU) was calculated from DM weights and the P and N concentration in the shoot and root tissue, respectively. The concentration of protons in 0.01 M $CaCl_2$ soil suspension was calculated as $[H^+] = 10^{-soil\ pH(0.01M\ CaCl_2)}$.

Percentage of N in plant derived from NH_4-N fertilizer ($N_{plant}dfNH_4$) was calculated as:

$$N_{plant}dfNH_4 = \frac{^{15}N_{excess}\ plant}{^{15}N_{excess}\ fertilizer} x100$$

where $^{15}N_{excess}$ in plant was calculated as the atom% ^{15}N in the labelled plant minus the atom% ^{15}N of treatments with 100% NO_3-N supply, and $^{15}N_{excess}$ fertilizer is the atom% excess of the added NH_4-N fertilizer (5.3 atom% excess). The quantity of N in plant derived from NH_4-N fertilizer ($QN_{plant}dfNH_4$) was calculated from NU and $N_{plant}dfNH_4$.

The amount of NO_3-N in bulk soil in the lower and middle soil layer derived from NH_4-N fertilizer (NO_3dfNH_4) at harvest was calculated as:

$$NO_3dfNH_4 = {}^{15}N_{excess}NO_3 \text{ x } NO_3\text{-}N \text{ in soil}$$

where the $^{15}N_{excess}$ of NO_3-N is the ^{15}N atom% in the soil extract minus the ^{15}N atom% of treatments with 100% NO_3-N supply, and NO_3-N in soil is the total amount of NO_3-N in the middle and lower soil layer (mg N).

Statistical analysis was conducted using R version 3.2.3 (*R Development Core Team,* 2015). Data normality was verified using the Shapiro-Wilk statistics. Data was logtransformed in cases where homoscedasticity was not obtained from the raw data. One-way analysis of variance (ANOVA) was used to study the effect of NH_4-N:NO_3-N ratio on N and P concentrations in shoots, root and shoot DM yields, NU and PU for each P level. To perform multiple comparisons between the NH_4-N:NO_3-N ratios within each P level the Tukey's honestly significant difference (HSD) test was used. A paired *t*-test was used to test the difference in pH between the rhizosphere and the bulk soil. An unpaired *t*-test was used to test if soil pH differed in treatments with a nitrification inhibitor and without plant, respectively compared to the corresponding treatment with a nitrification inhibitor. Simple linear regression analysis was used to study the relationship between the concentration of protons in the rhizosphere and the amount of N in shoot derived from the NH_4-N fertilizer, and between the concentration of protons in the bulk soil and the amount of NO_3-N in soil deriving from NH_4-N fertilizer in each layer. Significance was declared at the $P \leq 0.05$ level of probability.

3 Results

3.1 Root and shoot biomass

The root and shoot DM yield in the 0N treatments was significantly higher in treatments receiving 167 and 500 mg P pot^{-1} compared to 0 mg P pot^{-1} (Fig. 2), indicating that the plants benefitted from P supply despite the medium soil P status (Olsen-P content of 21 mg P kg^{-1}).

(Figure 2)

The plants receiving N but not P benefitted slightly from increasing NH_4-N:NO_3-N ratio, but the DM yield was much lower than in the reference treatments without N (Fig. 2a).

When 167 and 500 mg P $pot^{-1}$was added, shoot DM yield and root DM yield in the middle and lower soil layer were highest in treatments with a NH_4-N:NO_3-N ratio of 50:50 and 75:25 compared to the other NH_4-N: NO_3-N ratios (Fig. 2). The similarity in DM yields between treatments with an application rate of 167 mg P pot^{-1} and 500 mg P pot^{-1} showed that a rate of 167 mg P pot^{-1} was sufficient to meet the crop P demands.

Treatments applied with only NO_3-N irrespective of the P level had a significantly lower shoot DM yield than the other NH_4-N:NO_3-N ratios, and the root growth was limited in the lower layer. The root and shoot DM yield also decreased, when only NH_4-N was applied compared to a NH_4-N:NO_3-N ratio of 75:25, although this was only significant in treatments with a P supply of 167 mg P pot^{-1} (Fig. 2a). Treatments with only NH_4-N supply had also clearly visible toxicity symptoms as foliar burn and chlorosis of the leaf tips (Fig. 1b, *right*).

3.2 P and N uptake

Total PU was significantly higher in treatments with NH_4-N:NO_3-N ratios of 50:50 and 75:25 receiving 167 mg P pot^{-1} than the other NH_4-N:NO_3-N ratios, and the same tendency was seen in treatments with a P supply of 500 mg pot^{-1} (Table 1). In the 0N treatments, the P concentration and total PU were higher when P was applied compared to the 0N treatment without P supply (Table 1). The NU in 0N treatments did not differ among the P levels (Table 1), and was higher (+10 mg pot^{-1}) than the initial amount of inorganic N in the soil at sowing, indicating that endosperm N and mineralized soil organic N contributed to NU during growth. The N shoot concentration in 0N treatments ranged from 1.2% to 1.7% of DM, whereas it ranged from 5.2% to 6.0% across treatments applied with N and was not significantly different between the NH_4-N:NO_3-N ratios (Table 1).

(Table 1)

3.3 pH and inorganic N in soil

Soil pH generally decreased with increasing proportion of NH_4-N to NO_3-N added with the rhizosphere soil generally having lower pH than the bulk soil (Fig. 3).

(Figure 3)

The amount of P applied was only a significant factor affecting soil pH in the middle layer, with higher soil pH in treatments receiving 167 and 500 mg P pot^{-1} (Fig. 3b), which could be due to the buffering effect of the DCP applied.

In bulk soil, pH in the 0N treatments was 5.4, 5.4 and 5.5 in the upper, middle and lower bulk soil layer, respectively across the three P application levels. pH in the 0N treatments did not change from the initial value (pH 5.4), whereas pH in the bulk soil declined in the upper and lower soil layer by as much as 0.5 pH units as the NH_4-N:NO_3-N ratio increased (Fig. 3a). pH in bulk soil with no maize plant and 100% NH_4-N was not significantly different from the corresponding treatment with a maize plant (Table 2).

(Table 2)

Bulk soil pH in the middle layer was lower in the 100% NH_4-N treatment without DMPP than the corresponding treatment with DMPP (Table 2), and similarly the amount of NO_3-N in soil derived from the NH_4-N fertilizer was higher in the treatment without DMPP than with DMPP (Table 1), indicating a higher nitrification rate in the treatment without DMPP. The relatively stable bulk soil pH in the middle layer with increasing proportion of NH_4-N added (Fig. 3b) does also reflect a local inhibition of nitrification in the middle layer. In agreement with these findings, there was only a weak relationship between NO_3-N derived from NH_4-N fertilizer and the concentration of protons (R^2=0.34, P>0.05) in the middle layer, where DMPP was applied.

A substantial amount of NH_4-N found in the lower soil layer at harvest was derived from NH_4-N fertilizer (from ^{15}N assay, Table 1), which indicates movement of NH_4-N from the middle layer to the lower layer. The pH decline in the lower layer bulk soil, in response to the increasing proportion of NH_4-N applied in the middle layer (Fig. 3a), could therefore be due to nitrification of NH_4-N after transport from the middle layer. This was also reflected in the significant relationship between the NO_3-N derived from the NH_4-N fertilizer and the concentration of protons in the lower bulk soil layer without a local inhibition of the nitrification (R^2=0.95, P<0.001).

The pH decline in the upper layer bulk soil, in response to the increasing proportion of NH_4-N applied (Fig. 3a) could also be due to nitrification of the NH_4-N applied. We did not measure the amount of NH_4-N in the upper soil layer at harvest, but we surmise that water evaporation from the soil surface and movement of water to the upper soil layer due to root water uptake could induce flow transport of NH_4-N in the soil solution from the middle to the upper layer between irrigations.

Treatments with a NH_4-N:NO_3-N ratio of 25:75 or higher had a significantly lower soil pH in the rhizosphere compared to the bulk soil in the upper and lower soil layers (Fig. 3a). In the middle soil layer, the lower pH in the rhizosphere compared to the bulk soil was in general only observed in treatments with P supply (Fig. 3b). In contrast, treatments with a NH_4-N:NO_3-N ratio of 0:100 had significantly higher pH in the rhizosphere in the upper and lower soil layer compared to the bulk soil. The pH in the rhizosphere was 5.5 in all three layers in the 0N treatments, and did not differ from the pH in the bulk soil.

4 Discussion

4.1 Root distribution

The low root biomass in the middle and lower soil layer when N fertilizer was applied as 100% NO_3-N or 100% NH_4-N or when no P was applied in combination with N (Fig. 2) indicated a general root inhibition caused by the N fertilizer applied to the middle soil layer.

The relatively high root biomass in treatments where 50% or 75% of the N supply was applied as NH_4-N combined with 167 or 500 mg P pot^{-1} may reflect a root growth promoting effect of plant available P in the middle and lower soil layer. This is in line with early studies by *Drew and Saker* (1978) who reported an increase in the number of lateral roots in barley in a P enriched zone. The lack of rooting in treatments applied with 100% NO_3-N irrespective of the P level could be due to the inhibitory effect of high nitrate concentrations in the soil solution on root elongation of primary roots, which is also reported in other studies (e.g. *Tian* et al., 2008).

Toxicity effects of pure NH_4-N supply have been reported in previous studies (e.g. *Gerendás* et al., 1997). A toxic effect of 100% NH_4-N supply has also been observed under conditions where pH was controlled (*Li* et al., 2014), and could be due to several processes, such as energy requirements including energy costs for NH_4-N efflux due to limited storage capacity of NH_4-N in the plant (*Britto* et al., 2001) and/or suppression of the photosynthetic rate due to reduced stomatal conductance (*Miller and Cramer,* 2005). It is recognized however, that the relatively better growth response observed in the treatments applied with only NH_4-N than treatments applied with only NO_3-N (Fig. 2) is not in accordance with previous studies (e.g. *Cramer and Lewis*, 1993), but could be due to differences in the experimental conditions such as nutrient supply level and soil buffer capacity.

The lower DM yield and poor root growth in deeper layers in the treatments receiving N but not P compared to the reference treatments without N supply suggest that N application (no matter the NH_4-N:NO_3-N ratio) formed an unfavorable environment in the middle soil layer when no P was applied. An unfavorable environment in the middle layer could be due to the high electrical conductivity (Table 1) caused by the high salt concentrations, which can reduce cell osmotic potential (*Bernstein,* 1975) and hence result in poor plant growth. The lack of rooting into the lower layer could also indicate that there was no need to acquire N from the lower layer, because of a sufficient amount of available N in the middle layer.

The extensive rooting into the lower soil layer for the 0N treatments could reflect the plant´s need to explore a larger soil volume for N due to limited N supply in combination with absence of a root-inhibiting layer, which was present in the treatments with N application. Limited N supply in the 0N treatments was also confirmed by low shoot N concentrations and shoot N:P ratios of <10 (Table 1). According to *Güsewell* (2004) a N:P ratio <10 can indicate N limited biomass production across various terrestrial plant species. Plant growth in the 0N treatments would therefore probably be compromised in the subsequent growing stages due to limited N supply.

4.2 Availability of P and N

The increased P uptake (PU) in treatments with 50% and 75% NH_4-N supply and P supply could be due to an increased solubility of DCP close to the root induced by the larger pH decrease in the rhizosphere. A balanced N and P supply was also reflected in shoot N:P ratios between 12 and 16 in these treatments (Table 1), suggesting that neither N nor P was limiting growth according to *Güsewell* (2004). The lower PU in treatments with 100% NH_4-N supply despite decreasing soil pH again reflects the toxic effect of pure NH_4-N on crop growth, which compromises the higher solubility of DCP induced by the pH decrease in the rhizosphere. The low PU and low P concentrations in shoots in treatments with 100% NO_3-N and P supply could be because of the pH increase in the rhizosphere in the middle layer (Fig. 3b), which makes the DCP less soluble (*Lindsay* et al., 1989) and hence less plant available combined with the poor root growth in the middle layer in these treatments. P shortage in treatments with 100% NO_3-N supply irrespective of P supply, and in treatments with N but no P supply, was also reflected in their high shoot N:P ratios (Table 1).

The N concentrations in the plant tissues were high compared to other pot studies with maize and high N application rates (e.g. *Wu* et al., 2005), which suggest that there was sufficient N supply to the maize plants. The results also show that the plants were able to take up N from N fertilizer applied to the middle soil layer, despite the poor root growth in this layer.

The significant response to P supply in treatments without N supply can be related to the simple dissolution of DCP in an acid soil (*Lindsay* et al., 1989) rather than dissolution caused by treatment related pH decline. Moreover, the 0N treatment without P supply had a higher PU compared to treatments with N but no P (Table 1), because the inhibited root growth in the middle and lower layer in these latter treatments greatly restricted P uptake from the lower soil layer.

4.3 Linking NH_4-N supply, rhizosphere acidification and maize growth

The pH decrease in bulk soil of the lower layer was related to the nitrification of the NH_4-N fertilizer, whereas the stable pH in bulk soil of the middle layer was due to a local inhibition of nitrification. The inhibitory effect on nitrification in the middle layer due to DMPP application is in line with previous work (e.g. *Kong* et al., 2016). The lack of pH difference in the bulk soil between the treatments with and without plants and pure NH_4-N supply also supports that the pH change in the bulk soil was not plant-induced, but rather due to the nitrification of the NH_4-N applied and possibly the mineralization of organic matter.

The lower soil pH recorded in the rhizosphere than in the bulk soil in treatments with a NH_4-N:NO_3-N ratio of 25:75 or higher suggests release of protons from the roots as a consequence of NH_4-N plant uptake. The proton efflux may also be due to other pH regulating processes in the plant such as greater cation than anion uptake or production of organic acids in the plant containing a dissociating proton (*Raven*, 1986) in addition to any nitrification effect in the soil. This was confirmed by the significant linear relationship between the difference in proton concentration between the bulk and rhizosphere soils and the amount of N in the shoot derived from the NH_4-N fertilizer (^{15}N labelled) in treatments supplied with NH_4-N:NO_3-N ratios from 0:100 to 75:25 (Fig. 4).

(Figure 4)

Treatments applied with only NH_4-N did not follow the same pattern because of a restricted N uptake induced by a toxicity effect (Table 1). Hence, the additional soil acidification in the rhizosphere compared to the bulk soil can be attributed to the extrusion of H^+ to counter-balance the NH_4-N uptake, and likewise the pH increase in the rhizosphere in treatments supplied with 100% NO_3-N was due to the release of OH^-/HCO_3^- by the roots as suggested by *Riley and Barber* (1971). The steeper pH decline with a NH_4-N:NO_3-N ratio of 25:75 (Fig. 3) could be due to preferential uptake of NH_4-N (*Lee and Drew*, 1989). This was further supported by the percentage of N in the plant tissue derived from the NH_4-N fertilizer being >25% at this specific NH_4-N level (Table 1).

The small differences in pH between the rhizosphere soil and the bulk soil in treatments without N application indicate a minor importance of plant or microbial mediated acidifying processes in the rhizosphere, which are not coupled to N application, such as excretion of organic anions and associated protons (*Hinsinger* et al., 2003). The lack of any pH drop in the rhizosphere in the middle layer in treatments receiving N but no P was most probably due to the adverse effect of these particular treatments on root growth and function.

The root induced pH change in the rhizosphere was also significant in the upper layer implying that proton release following NH_4-N uptake may not only take place close to where N is taken up, but rather that the whole root system behaves evenly assuming that the highest N uptake took place in the middle soil layer. A study by *Taylor and Bloom* (1998) shows that the pH drop occurs along the entire root, when NH_4-N is applied alone, whereas pH increases in the basal regions of primary root of the maize seedling and decreases in the elongation zone, when NO_3-N is applied alone. However, further root studies of proton fluxes along the root in a system with placed fertilizers with high concentrations of N are needed to confirm this.

4.4 Implications for nutrient management in maize cropping systems

The clear response to added P (whether N was added or not) reaffirms the benefits of starter P fertilizer to young maize plants even on a soil with a medium P status, where P limitation is not expected. Although this growth benefit may not always translate into extra yield at harvest, and the crop recovery of this added P is very low, it is clearly in the farmer's interest to optimize early plant development. Our study suggests that dairy slurry, which has a high proportion of NH_4-N and DCP, could be a good source of both starter N and P to young maize plants due to the beneficial effect of NH_4-N supply and uptake on the availability of DCP due to acidification of the rhizosphere. Preventing nitrification of slurry NH_4-N through the use of an inhibitor is likely to enhance this interaction between NH_4-N and DCP in the rhizosphere, whilst at the same time maximizing the long-term availability of N by reducing the risk of NO_3-N leaching. For example, *Westerschulte* et al. (2016) found in a field trial that addition of a nitrification inhibitor increased the NH_4-N concentration in the slurry injection zone, which may ensure a higher uptake of NH_4-N and hence an improved availability of DCP. It is recognized however, that it is unclear how the positive interacting effects between NH_4-N uptake and DCP availability identified in the present study are affected by other components present in manure such as buffering compounds (*Sommer and Husted,* 1995), which could reduce the rhizosphere acidification if the slurry is placed below the maize row. Moreover, there was limited root growth and nutrient uptake due to N application in our study, but it is unclear whether this would occur when slurry is band applied at operational rates. The current application rate of slurry N to maize in Denmark is around 120 kg NH_4-N ha^{-1} (*Landbrugsstyrelsen,* 2018), which will correspond to a local application rate in the slurry injection zone of 600 kg NH_4-N ha^{-1} near the maize plant, assuming a 15 cm broad slurry band for each maize row with 75 cm distance. Few studies (e.g. *Sawyer and Hoeft*, 1990) report that slurry injection can cause an unfavorable environment for root growth, whereas other field studies (e.g. *Schröder* et al., 1997) do not report any root injuries in the concentrated slurry band. However, further work is needed to investigate if potentially toxicity effects from banded slurry applications and/or interactions with other components in the slurry such as buffering compounds could compromise the positive interacting effects between NH_4-N supply and DCP availability on maize growth during early growth.

5 Conclusions

The major proportion of inorganic P in dairy manure is present as DCP ($CaHPO_4$). Application of DCP increased the growth of young maize plants on a coarse sandy soil with a medium P status under typical Danish environmental conditions. Shoot DM yield and P uptake were significantly higher when DCP was applied in combination with N at NH_4-N:NO_3-N ratios of 50:50 and 75:25. This increased P uptake was explained by the release of protons into the rhizosphere as the proportion of NH_4-N taken up by the plants increased, allowing enhanced dissolution of the DCP. Less root growth were apparent when NO_3-N or NH_4-N was the sole N source, or when N (all NH_4-N:NO_3-N ratios) was applied without P. The absence of the root-inhibiting layer in the treatments without N application explains the relatively high DM yields in these particular treatments. Fertilizer N form therefore had a major effect on P uptake and our results suggest that early growth of maize will benefit from the combined application of both NH_4-N and DCP, if a substantial amount of the NH_4-N is taken up before nitrification.

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Figure captions

Figure 1. a) Schematic view of the cylindrical pot separated in three layers; upper soil layer with maize seed (*red circle*), middle soil layer applied with N and P fertilizers and lower soil layer, b) photos of leaves in treatments applied with 167 or 500 mg P pot^{-1} combined with a NH_4-N:NO_3-N ratio of (*from the left*) 0:0, 0:100, 50:50 and 100:0.

Figure 2. a) Shoot dry matter yield and b) root dry matter yield and the distribution of roots in the three soil layers at 5-leaf stage. Different letters denote significant differences between the three P application rates in combination with 0N application, and significant differences between the NH_4-N:NO_3-N ratios within each P-level (Tukey´s HSD, P<0.05). There was no significant difference between the root dry matter yields for treatments receiving 0 mg P pot^{-1}.

Figure 3. a) pH in bulk soil and rhizosphere at harvest for each soil layer across the three P application levels and b) pH in bulk soil and rhizosphere at harvest in the middle soil layer for each P application level (0, 167 and 500 mg P pot^{-1}). P application level was only a significant variable in the middle soil layer. Asterisks (*) indicate a significant difference between the pH in the rhizosphere and bulk soil within the same NH_4-N:NO_3-N ratio (paired *t*-test, P<0.05). Error bars represent the standard deviations.

Figure 4. Relation between the amounts of N derived from the NH_4-N fertilizer ($QN_{plant}dfNH_4$) in whole plant and the difference in concentration of protons [H^+] in 0.01 M $CaCl_2$ soil suspension between the bulk soil and the rhizosphere for each soil layer. Treatments with 100% NH_4-N supply (open symbols) were not included in the statistical analysis. The solid lines represent the simple linear regression for each layer. Upper layer: R^2=0.76, P<0.05, Middle layer: R^2=0.81, P<0.05, Lower layer: R^2=0.65, P<0.05.

Table 1. Treatment effects on plant and soil at harvest. Plant measurements at harvest: N and P concentration (conc.) in shoot, N:P ratio in shoot, N uptake (NU) in whole plant, percentage of N in plant derived from NH_4N fertilizer ($NdfNH_4N$) and P uptake (PU) in whole plant. Soil measurements at harvest: amount of NO_3N derived from NH_4N fertilizer (NO_3NdfNH_4N) in middle and lower soil layer, amount of NH_4N in lower layer derived from NH_4N fertilizer (NH_4NdfNH_4N) and the electrical conductivity (EC) in middle soil layer with nutrient application. Different letters denote significant differences between the three P application rates in combination with 0N application, and significant differences between $NH_4N:NO_3N$ ratios within each P-level (Tukey´s HSD, *P*<0.05).

Treatment		Plant at harvest						Soil at harvest		
P-level	$NH_4N:NO_3N$ ratio	N conc.	P conc.	N:P ratio	NU	$NdfNH_4N$	PU	NO_3NdfNH_4N	NH_4NdfNH_4N lower layer	EC middle layer
mg pot^{-1}		% of shoot DM			mg pot^{-1}	%	mg pot^{-1}	----------------mg pot^{-1}----------------		µs cm^{-1}
0	0:0	1.69^{a}	0.16^{b}	10	35.9^{a}	-	3.8^{b}	-	-	8
167	0:0	1.23^{b}	0.25^{a}	5	36.3^{a}	-	7.4^{a}	-	-	16
500	0:0	1.22^{b}	0.26^{a}	5	36.5^{a}	-	8.4^{a}	-	-	22
0	0:100	5.39^{a}	0.12^{b}	45	33.4^{b}	0	0.7^{c}	0.0	0.0	62
0	25:75	6.04^{a}	0.13^{b}	46	46.4^{ab}	26	1.1^{b}	28.3	15.2	77
0	50:50	5.99^{a}	0.13^{b}	45	55.3^{a}	41	1.3^{b}	46.7	51.8	83
0	75:25	6.01^{a}	0.14^{b}	42	61.7^{a}	56	1.5^{ab}	55.7	75.0	97
0	100:0	5.56^{a}	0.22^{a}	25	49.5^{a}	83	2.0^{a}	66.0	117.3	96
167	0:100	5.57^{a}	0.12^{c}	46	33.7^{c}	0	0.8^{c}	0.0	0.0	83
167	25:75	5.47^{a}	0.19^{b}	30	65.0^{b}	28	2.4^{b}	29.0	23.7	91
167	50:50	5.39^{a}	0.34^{a}	16	157.0^{a}	46	10.1^{a}	42.3	31.5	106
167	75:25	5.44^{a}	0.34^{a}	16	148.3^{a}	60	9.5^{a}	51.4	74.4	118
167	100:0	5.62^{a}	0.37^{a}	15	73.0^{b}	86	4.5^{b}	60.1	81.5	117
167	100:0, no DMPP	5.87^{a}	0.36^{a}	20	66.0^{b}	87	3.8^{b}	103.2	56.4	140
167	100:0, no plant	-	-	-	-	-	-	65.7	81.6	118
500	0:100	5.19^{a}	0.14^{c}	36	39.9^{b}	0	1.1^{c}	0.0	0.0	90
500	25:75	5.25^{a}	0.30^{b}	19	79.2^{ab}	32	4.8^{b}	26.8	22.9	101
500	50:50	5.36^{a}	0.42^{ab}	13	148.6^{a}	45	12.0^{ab}	43.4	39.7	108
500	75:25	5.53^{a}	0.47^{a}	12	151.8^{a}	59	12.7^{a}	48.2	50.8	127
500	100:0	5.62^{a}	0.56^{a}	10	95.1^{a}	86	9.1^{ab}	63.0	108.5	124

Table 2. pH in bulk soil and rhizosphere for treatments with a nitrification inhibitor (With DMPP), without a nitrification inhibitor (No DMPP) and without a plant (No plant), respectively. The treatments had a NH_4N:NO_3N ratio of 100:0 and a P application rate of 167 mg P pot^{-1}. Asterisks (*) indicate a significant difference compared to the treatment with a nitrification inhibitor (with DMPP) within each column (unpaired t-test, P<0.05).

	pH in bulk soil			pH in rhizosphere		
	Lower	Middle	Upper	Lower	Middle	Upper
With DMPP	5.03	5.60	4.88	4.69	5.21	4.54
No DMPP	4.90	5.45*	4.54*	4.85	5.14	4.26*
No plant	5.08	5.67	5.00	-	-	-