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Can the presence of plantain (*Plantago lanceolata* L.) improve nitrogen cycling of dairy grassland systems on peat soils?

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ABSTRACT

Reactive nitrogen (N) losses, and in particular nitrous oxide losses, from dairy grasslands on peat soils are generally high as a result of relative high soil organic matter contents, potential N mineralisation rates and shallow groundwater levels. Effects of the inclusion of the temperate forage species plantain (*Plantago lanceolata* L.) (PL), which produces secondary compounds with biological nitrification inhibition capacity, on the fate of soil mineral N were studied in a combined mesocosm and field experiment. The experiments comprised four treatments differing in intentional herbage share of plantain versus perennial ryegrass (*Lolium perenne* L.) (100%PL, 66%PL, 33%PL and 0%PL). Potential nitrification in the mesocosm experiment was significantly lower at 100%PL versus 0%PL ($p = 0.018$), but soil nitrate concentrations were not. Nitrous oxide fluxes reduced by 39% ($p = 0.021$) in the presence of plantain in the field experiment, without an obvious link to the quantity of plantain. N use efficiency of plantain tended to increase with the quantity of plantain in the sward in the mesocosm experiment ($p = 0.098$), but not in the field experiment. Our results suggest that the presence of plantain can affect the fate of soil mineral N of dairy grasslands on peat soils.

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Plantain; nitrogen; peat; grassland; nitrification; nitrous oxide

Introduction

Agriculture is the largest contributor to global reactive nitrogen (N) production (Galloway et al. 2008). This causes environmental pollution, disrupts N cycles of natural ecosystems and results in a significant biodiversity loss (Galloway et al. 2008; Erisman et al. 2013). It is clear that reactive N exceeds the limits of interference with the global N cycle (Rockström et al. 2009). In commercial dairy farms N losses during conversion of N into milk and meat are often high, and include gaseous forms (N_2 , N_2O , NO_x , NH_3) as well as losses to groundwater through erosion, runoff or leaching of nitrate (NO_3^-) (Jarvis 1993;

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Kohn et al. 1997; Nevens et al. 2006; Powell et al. 2010). Dairy grassland systems on peat soils are particularly prone to high N₂O losses as a result of relative high soil organic matter contents, high potential N mineralisation rates and shallow groundwater levels (Koops et al. 1996; Van den Pol-van Dasselaar et al. 1998).

Due to the increase of mineral N use in agriculture since the introduction of mineral fertilisers, NO₃⁻ nowadays accounts for >95% of N plant uptake in modern agriculture (Subbarao et al. 2015), despite the fact that NO₃⁻ is more easily lost from the soil through leaching or denitrification than NH₄⁺. Inhibition of soil nitrification has therefore been identified as a potential measure to increase herbage mineral N uptake efficiency and to reduce NO₃⁻ leaching and gaseous N emissions (mainly N₂O and N₂) (Di et al. 2009; Butterbach-Bahl et al. 2013; Ruser and Schulz 2015; Subbarao et al. 2015; Bowatte et al. 2016).

Synthetic nitrification inhibitors such as dicyandiamide (DCD) and 3,4-dimethylpyrazole phosphate (DMPP) have shown to reduce the release of compounds such as N₂O from soil to the environment under different conditions (Ruser and Schulz 2015). But, further study on their efficacy under different conditions and long term applications is still needed, as well as on the impact of their toxicity on plant growth and human health (Ruser and Schulz 2015; Yang et al. 2016). Biological nitrification inhibition (BNI) might be an interesting alternative to reduce N losses from agriculture to the environment (Subbarao et al. 2015). BNI is defined as the release of compounds from plant roots that directly influence nitrifying organisms, i.e. via the reduction of ammonia monooxygenase (AMO) enzymatic activity by inhibiting or inactivating AMO enzymatic pathways (Arp and Stein 2003; Subbarao et al. 2015). For example, the tropical pasture species *Brachiaria* spp., which is adapted to low-N environments, can release significant amounts of compounds with BNI capacity depending on the presence of NH₄⁺ in the soil (Subbarao et al. 2007). However, more recently the presence of compounds with BNI capacity has been identified in plantain (*Plantago lanceolata* L.) (Dietz et al. 2013). Plantain is a pasture species which is known to grow on a wide variety of soils in temperate climates, is highly responsive to soil N, has good forage quality, has been cultivated to produce large amounts of forage (Stewart 1996; Lee et al. 2015) and could therefore be included in grassland mixtures in the search for improving farming system N use efficiency. The major secondary metabolites of plantain that have been identified as potentially causing BNI due to their chemical structure, are aucubin, catalpol, verbascoside and their aglyca (Dietz et al. 2013).

Several studies performed on sand and loamy sand soils have reported effects of plantain on soil nitrifying activity and the fate of soil mineral N. Verhagen et al. (1995) observed more than 200-fold lower numbers of nitrifying bacteria as well as lowered potential NH₄⁺-oxidising activities in the presence of plantain, after application of NH₄⁺ fertiliser. Dietz et al. (2013) found that aucubin as well as soil incubated with plantain leaf material negatively affected soil mineralisation and nitrification. Carlton et al. (2019) observed a lower abundance of NH₄⁺-oxidising bacteria and a significant decrease of NO₃⁻ eutrophication if plantain was present in the forage in a ryegrass-clover sward on which urine-N was applied in patches at a rate of 7.0 g m⁻². Luo et al. (2018) and Simon et al. (2019) observed lower N₂O emissions in the presence of plantain, after cattle urine applications on silt loam soils.

There are no known reports on the effects of plantain or other plants secreting compounds with potential BNI capacity on N cycling under nutrient rich conditions such as dairy grasslands on peat soils. Therefore, we studied the effects of the presence of

plantain in a mixture with perennial ryegrass (*Lolium perenne* L.) on the fate of soil mineral N in a combined mesocosm experiment with peat soil and in a field experiment on a dairy grassland on peat soil. We hypothesised that the presence of plantain in a mixture with perennial ryegrass, compared to a monoculture of perennial ryegrass, (1) reduces the process of soil nitrification, (2) lowers soil NO_3^- concentrations, (3) reduces soil N_2O emissions and (4) leads to an increased efficiency of herbage N uptake.

Material and methods

Mesocosm experiment

The mesocosm experiment comprised four treatments differing in proportions of plantain (PL) and perennial ryegrass (RG) (100%PL, 66%PL, 33%PL and 0%PL). Thirty-two mesocosms were allocated to the treatments according to a completely randomised design, resulting in eight replicates per treatment. Mesocosms were made up of glass containers (25 cm × 25 cm × 30 cm height) which were filled with 5 cm of gravel, which in turn was topped with root fabric and 25 cm of soil. Within the gravel, a porous cup (Rhizon SMS, Eijkelkamp, Giesbeek, the Netherlands) was placed in order to collect water leaching from the system. Peat soil (terric histosol, IUSS Working Group WRB 2015) from a depth of ±10–20 cm below surface was obtained from a perennial dairy grassland with a mean groundwater table of 60 cm below surface (Knowledge Transfer Centre (KTC), Zegveld, the Netherlands, 52°08'25.7" N, 4°50'22.0" E) (see Table 1 for soil parameters). Before filling, the soil was thoroughly mixed by hand. Mesocosms were placed in a temperature-regulated water bath and maintained at ± 12°C soil temperature using a cryostat (ThermoFlex 1400, ThermoFisher Scientific, Waltham, MA, USA). The experiment was performed in the greenhouse facilities of Radboud University (Nijmegen, the Netherlands), and ran from 28 July till 23 December 2017 (148 days). Within the facilities, the temperature was maintained between 16.6 and 17.5°C, relative humidity at 50% and irradiance at 111 W m⁻² or higher for 16 h per day, using grow lights if sunlight was not sufficient. Mesocosms received on average twice a week equal amounts of demineralised water.

Table 1. Properties of soil used ($n = 2$ per experiment) for the mesocosm and field experiment and monthly mean air temperatures and precipitation sums for the field experiment.

			Mesocosm experiment		Field experiment		
			Mean	Range	Mean	Range	SD
Soil	Organic matter	%	47.8	1.0	44.2	3.7	
	Lutum	%	36	1	27	2	
	Total C	g 100 g ⁻¹	26.2	1.2	20.3	4.7	
	Total N	mg 100 g ⁻¹	2244	89	2028	64	
	Total P	mg 100 g ⁻¹	187	22	195	–	
	P _{AL}	mg 100 g ⁻¹	7.2	0.4	12.2	3.5	
	Total K	mg 100 g ⁻¹	14	–	36	1	
	pH-KCl	–	4.6	0.0	4.9	0.2	
	Temperature	Mar. – Oct. 2017	°C			14.0	
Mar. – Oct. 2018		°C			14.6		5.0
Mar. – Oct. 1999–2018		°C			13.6		4.1
Precipitation	Mar. – Oct. 2017	mm month ⁻¹			72		38
	Mar. – Oct. 2018	mm month ⁻¹			43		26
	Mar. – Oct. 1999–2018	mm month ⁻¹			69		17

Note: SD = standard deviation.

After an acclimatisation period of 17 days, plantain (*Plantago lanceolata* L. var. 'Hercules', Pelgrum Vink Materials, Lobith, the Netherlands) was planted at rates of 24, 16, 8 or 0 seedlings per mesocosm and ryegrass (*Lolium perenne* L. var. 'Barimero' (50%) and var. 'Toronto' (50%), BG3 Superplus, Barenbrug Holland, Nijmegen the Netherlands) was sown at rates of 0.0, 1.5, 3.0 and 4.5 g m⁻² in the treatments 100%PL, 66%PL, 33%PL and 0%PL, respectively. The day of seeding and planting was considered as day 0 of the experiment. Plantain seeds were placed on a humid paper cloth at room temperature and seedlings were planted three days after germination.

Above ground herbage was harvested at ±5 cm stubble height on days 41, 61, 95 and 116. At each harvest, the total herbage of each mesocosm was manually separated into plantain and ryegrass, and the dry matter (DM) content of both species was determined by oven drying at 70°C for 48 h. Ryegrass and plantain harvested at day 116 were also analysed for total N content using an elemental analyser (EA NA1500 Carlo Erba, Thermo-Fisher Scientific, Waltham, MA, USA) after thoroughly grinding the dried plant material using a mixer mill (MM400, Retsch, Haan, Germany).

On days 5, 74, 95 and 116 soil cores and soil leachate samples were collected. Leachate was obtained via the porous cups by connecting a vacuumed syringe to the cups until 20 mL fluid was collected. Soil cores (0–20 cm depth) were obtained by using a 17 mm diameter gouge, and the resulting hole was filled with a 20 mm diameter PVC pipe which was closed off on top to prevent interactions other than soil surface interactions with the atmosphere. Soil samples were weighed and split into two equal parts. Samples were incubated in 100 mL HDPE-bottles either with 50 mL type I purified water in order to remove water soluble ions or with 50 mL of 0.2M NaCl solution in order to remove cations. Incubated samples were shaken at 105 rpm for 2 h at room temperature and a porous cup was placed in each HDPE-bottle connected to a 100 mL vacuumed bottle to obtain fluid samples. NO₃⁻ and NH₄⁺ concentrations in the soil fluid and leachate samples were measured colorimetrically with two continuous flow AutoAnalyzer III systems (Bran + Luebbe, Norderstedt, Germany), using hydrazine sulphate and salicylate, respectively (for details see Geurts et al. 2008). Soil core sample DM contents were determined by oven drying at 70°C for 48 h in order to express results per kg of soil DM.

At day 148, 0–10 cm depth soil cores of treatment 100%PL and 0%PL were taken for analyses of DM content and NO₃⁻ and NH₄⁺ concentrations as described above, and for analyses of potential nitrification (ISO 15685:2012). For potential nitrification, soil was sieved over a 5 mm screen in order to remove roots. Autotrophic NH₄⁺ oxidising bacteria were exposed to ammonium sulphate in soil slurry buffered at pH 7.2. Oxidation of the nitrite, performed by nitrite-oxidising bacteria in the slurry, was inhibited by the addition of sodium chlorate. The subsequent accumulation of nitrite was measured over a 6 h incubation period, and taken as an estimate of the potential activity of NH₄⁺ oxidising bacteria at the time of sampling. For details see Belser and Mays (1980).

Field experiment

The field experiment was established by sowing plantain and ryegrass at 18 May 2017 on a dairy grassland on peat with a mean groundwater table of 60 cm below surface (KTC

Zegveld, 52°08'23.3"N, 4°50'12.9"E). Ten days before sowing, the field was treated with 2.5 kg ha⁻¹ glyphosate (Roundup[®]+, Monsanto, Saint Louis, MO, USA), and on the day before sowing, the present grass sod was rotavated at a depth of ±10 cm. The treatments 100%PL, 66%PL, 33%PL and 0%PL were allocated to twenty-four plots of 2.5 × 10.0 m according to a complete randomised block design, resulting in six replicates per treatment. Plantain seed was sown at 1.00, 0.66, 0.33 and 0.00 g m⁻² for the treatments 100%PL, 66% PL, 33%PL and 0%PL, respectively, and ryegrass seed was sown at 3 g m⁻² in all treatments (Rommelink et al. 2018), following advised sowing rates for monocultures and mixtures and reported sowing rates for plantain monocultures by Minneé et al. (2013) and Lee et al. (2015). The same varieties and seedlines were used in the mesocosm and field experiment.

In June 2017, above ground herbage was mulched and left in place, and in September 2017, herbage was harvested and removed from the fields (mean stubble height ± 5 cm). In March 2018, all plots were fertilised with 5.0 g N m⁻², 8.3 g K m⁻² and 1.8 g P m⁻² and in May 2018 with 6.6 g K m⁻². N, K and P were applied in the form of calcium ammonium nitrate (CAN), potassium chloride and diphosphorus pentoxide, respectively. On 12 September 2018 all fields were again fertilised with 5.0 g CAN-N m⁻² prior to the measurements of soil N₂O fluxes.

Soil samples (0–10 cm depth) were taken before the start of the experiment and in November 2018 and were pooled per sampling moment for further analyses (Table 1). In the year of establishment (2017) and measurements (2018), average temperatures during the growing season (Mar. – Oct.) were 0.4°C and 1.0°C higher, respectively, compared to 20 year averages (KNMI, de Bilt, the Netherlands, Table 1). Monthly precipitations were on average 3 mm higher (2017) and 26 mm lower (2018) compared to 20 year averages. The growing season of 2018 had the 5th highest mean potential precipitation deficit since 1901 (Sluijter et al. 2018). Between 1 May and 31 July 2018, cumulative precipitation was 55 mm while 20 year averages for that period were 216 mm.

Herbage yields were determined at a stubble height of ±5 cm on 16 May, 13 June, 24 July, 4 September and 29 October 2018 using a 'Haldrup' small plot harvester (J. Haldrup, Løgstør, Denmark). Two subsamples were taken per harvest date and plot. The first was used for analyses of total herbage DM content (oven drying at 70°C for 48 h). The second subsample of at least 0.2 kg was manually separated into plantain, grasses and other herbs and forbs to obtain the sward species composition, as described in Hoekstra et al. (2018). The separated plantain, total grasses and other herbs and forbs were analysed for DM and plantain and total grasses were analysed for total N contents (NEN-ISO 5983-2, Kjeldahl method, Eurofins Agro, Wageningen, the Netherlands).

Soil N₂O fluxes were measured five times with six to eight day intervals from 20 September 2018 onwards. Within each of the 24 plots three fixed locations were randomly chosen, and each measurement day sealed off airtight for at least 30 min (exact time was recorded) using 3.1 L polyethylene caps equipped with tube fittings. The build-up of N₂O concentrations in the caps was measured using a photo-acoustic multi-gas monitor (Innova 1312, Innova AirTech Instruments, Ballerup, Denmark) by connecting Teflon tubes to the fittings. Fluxes were corrected for background concentrations, determined each fifth measurement. After correction, flux data were averaged per field. Cumulative soil N₂O fluxes were calculated assuming a linear flux between the measurement dates. For more details see Velthof et al. (2002).

Statistical analyses

All statistical analyses were done in R (R Core Team 2017). Soil and leachate NO_3^- and NH_4^+ concentrations measured in the mesocosm experiment were log-transformed before analyses since data did not fit a normal distribution. Analyses for statistical differences between treatments of soil, leachate, herbage and cumulative N_2O data were done by one or two-way ANOVA in which mesocosms and plots were considered as an experimental unit in the mesocosm and field experiment, respectively. Block effects of the replications were included in the analyses of the field experiment data. Differences between treatments were analysed by Least Significant Differences (LSD). In all analyses a p -value ≤ 0.05 was considered as significant.

Models for the approximation of herbage N uptake of both experiments were fitted by stepwise regression. Model selection was done based on the lowest Akaike information criterion. Following the hypotheses that plantain can increase plant N use efficiency, candidate predictors for total herbage, grass and plantain herbage N uptake were plantain and grass DM yield. Linear and quadratic effects of candidate predictors were allowed.

Results

Mesocosm experiment

Potential soil nitrification at day 148 of the experiment was significantly lower for the 100%PL treatment compared to 0%PL (39.7 versus 65.0 $\mu\text{mol NO}_2 \text{g}^{-1} \text{h}^{-1}$, $p = 0.018$). Soil NO_3^- concentrations were negatively correlated to potential nitrification ($r = -0.83$, $p = 0.011$ for 100%PL and $r = -0.75$, $p = 0.033$ for 0%PL) (Figure 1). The presence and amount of plantain did not affect leachate and soil NO_3^- and NH_4^+ concentrations, since there were no significant differences between the treatments at any of the sampling moments (Table 2). Soil NH_4^+ concentrations decreased during the experiment, while NO_3^- concentrations were highest at day 95 and lower at the subsequent sampling days.

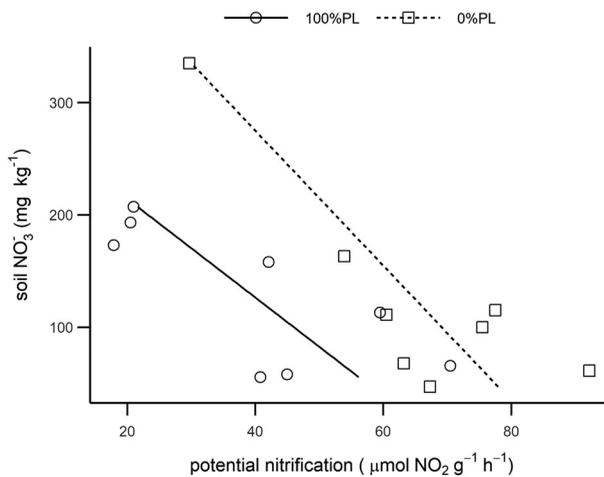


Figure 1. Mesocosm experiment. Correlation between soil potential nitrification and soil NO_3^- concentrations in treatments 100%PL (open dots, $r = -0.83$, $p = 0.011$) and 0%PL (squares, $r = -0.75$, $p = 0.033$) at day 148.

Table 2. Mesocosm experiment: Back log-transformed mean values of leachate and soil NO₃⁻ and NH₄⁺ concentrations, and soil moisture concentrations at different sampling days.

Parameter	Depth (cm)	Unit	Day	Treatment				<i>p</i> -value	SEM
				100% PL	66%PL	33%PL	0%PL		
Leachate NO ₃ ⁻		mg L ⁻¹	5	0.37	0.43	0.34	0.46	0.912	0.198
			74	0.16	0.23	0.15	0.24	0.425	0.083
			95	0.27	0.20	0.21	0.19	0.585	0.058
			116	0.45	0.44	0.55	0.53	0.977	0.587
Leachate NH ₄ ⁺		mg L ⁻¹	5	3.73	2.44	3.23	3.15	0.247	0.225
			74	4.28	2.84	4.07	3.51	0.206	0.255
			95	5.28	3.76	4.57	2.39	0.216	0.343
			116	4.95	3.78	3.79	3.17	0.559	0.369
Soil NO ₃ ⁻	0–20	mg kg ⁻¹ DM soil	5	6.6*					
			74	103.9	90.6	86.7	77.8	0.521	5.42
	0–20		95	564.1	544.4	441.2	436.5	0.798	54.40
			116	45.4	40.8	36.0	51.8	0.540	4.52
	0–10		148	111.1	ND	ND	108.5	0.942	16.60
Soil NH ₄ ⁺	0–20	mg kg ⁻¹ DM soil	5	62.0*					
			74	111.8	142.3	73.2	117.8	0.113	14.02
	0–20		95	22.3	15.5	18.9	16.0	0.618	2.09
			116	2.5	2.1	1.5	2.8	0.635	0.67
	0–10		148	1.2	ND	ND	8.9	0.354	3.99
Soil moisture	0–20	g kg ⁻¹ soil	5	660*					
			74	727	724	690	710	0.704	7.1
	0–20		95	647 ^a	618 ^b	607 ^b	600 ^b	0.010	3.7
			116	560	560	547	574	0.925	10.1
	0–20		148	593	ND	ND	583	0.525	7.3
	0–10								
Total biomass DM yield		g m ⁻²	cum	294 ^b	430 ^a	483 ^a	465 ^a	<0.001	32.5
Plantain DM yield		g m ⁻²	cum	294 ^a	173 ^b	94 ^c		<0.001	14.9
Ryegrass DM yield		g m ⁻²	cum		258 ^b	390 ^a	465 ^a	<0.001	26.6
Total biomass N yield		g m ⁻²	cum	3.85 ^b	5.03 ^a	5.04 ^a	4.13 ^{ab}	0.045	0.19
Plantain N yield		g m ⁻²	cum	3.85 ^a	1.85 ^b	0.93 ^c		<0.001	0.26
Ryegrass N yield		g m ⁻²	cum		3.19	4.10	4.13	0.118	0.18

Note: Cumulative dry matter (DM) yields of herbage harvested at days 41, 61, 95 and 116, and N-contents and N yields of herbage harvested at day 116 of the experiment. Values not sharing the same superscript letter differ significantly ($p < 0.05$). DM = dry matter, cum = cumulative data, ND = not determined and SEM = standard error of the mean. Note the difference in soil sampling depth for the soil NO₃⁻, NH₄⁺ and moisture concentrations.

*Pooled sample analysis

Soil moisture concentration was significantly higher for 100%PL versus the other treatments at day 95 ($p = 0.004$), but not significantly different between treatments at any other sampling time.

Plants did not flower during the experiment. Cumulative herbage DM yield after 116 days of growth was significantly lower for 100%PL compared to the other treatments ($p < 0.001$, Table 2). At day 116, treatment 100%PL showed a lower N yield compared to the treatments 66%PL and 33%PL ($p = 0.045$). Ryegrass made up the greatest part of the cumulative DM yield and the N yield at day 116 in the 66%PL and 33%PL treatments. Stepwise regression revealed that plantain N yields tended to increase quadratically ($p = 0.098$) in addition of a linear effect ($p = 0.014$) to plantain DM yields, while ryegrass N yields only increased linearly per unit of ryegrass DM yield ($p < 0.001$) (Figure 2, Table S1). Total herbage N yields (plantain + ryegrass) were significantly ($p < 0.001$) positively correlated to PL and ryegrass DM yields; however, the total herbage N yield increase

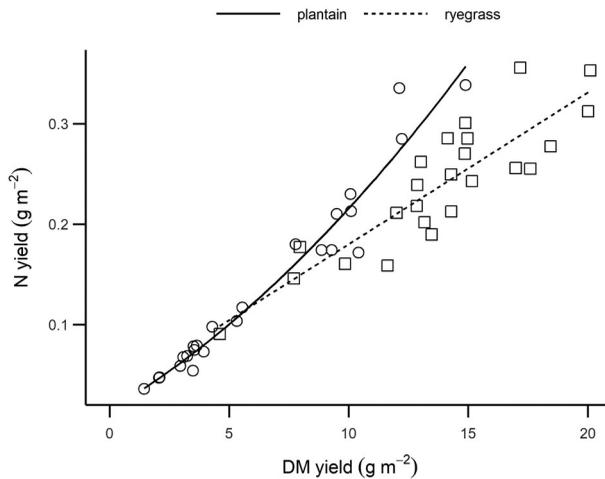


Figure 2. Mesocosm experiment. Nitrogen (N) yields of plantain (*open dots*) and ryegrass (*squares*) versus plantain and ryegrass dry matter (DM) yields at day 116 of the experiment, grown in a monoculture or in a plantain ryegrass mixture. For model details on the fitted lines see Table S1.

was greater per gram of plantain DM yield ($1.94 \cdot 10^{-2} \text{ g g}^{-1}$) than per gram of ryegrass DM yield ($1.60 \cdot 10^{-2} \text{ g g}^{-1}$), respectively.

No significant correlations were observed between soil NO_3^- or NH_4^+ concentrations and plantain or ryegrass N uptake at day 116, between soil NH_4^+ concentrations and potential nitrification at day 148 or between plantain or ryegrass N uptake at day 116 and potential nitrification at day 148.

Field experiment

N_2O -N fluxes were significantly higher for 0%PL compared to the other treatments at 21 days after the CAN-N application ($p = 0.009$, Figure 3). Cumulative N_2O -N fluxes differed significantly for all combined plantain treatments compared to the control without plantain ($p = 0.021$) (Figure 4). Overall, the highest mean N_2O -N fluxes ($0.53 \pm 0.53 \text{ mg m}^{-2} \text{ h}^{-1}$) were observed 15 days after the N application and after the first significant precipitation during the measurement period (32 mm, Figure 4). After day 15, observed mean fluxes were lower at each subsequent measurement day. The lowest mean N_2O -N fluxes were observed at 35 days after the CAN-N application ($0.12 \pm 0.06 \text{ mg m}^{-2} \text{ h}^{-1}$).

At each harvest, plantain had produced inflorescences while ryegrass had not. Except for the fifth harvest, treatments significantly affected the total herbage DM yield ($p < 0.001$ for all four harvests) (Figure 5). At the first harvest, herbage DM yields were highest for 0%PL and lowest for 100%PL, while at the second, third and fourth harvest yields were the lowest for 0%PL and at the third harvest highest for 100%PL. Total grasses N yield at the second harvest was negatively associated with the DM yield of plantain ($p = 0.049$), while total grasses N yield at the fourth harvest tended to be positively associated with the DM yield of plantain ($p = 0.066$) (Figure 7, Table S2). Total grasses N yields in the first, third and fifth harvest and plantain N yields in all harvests were only related linearly to total grasses and plantain DM yields respectively (Figures 6 and 7, Table S2).

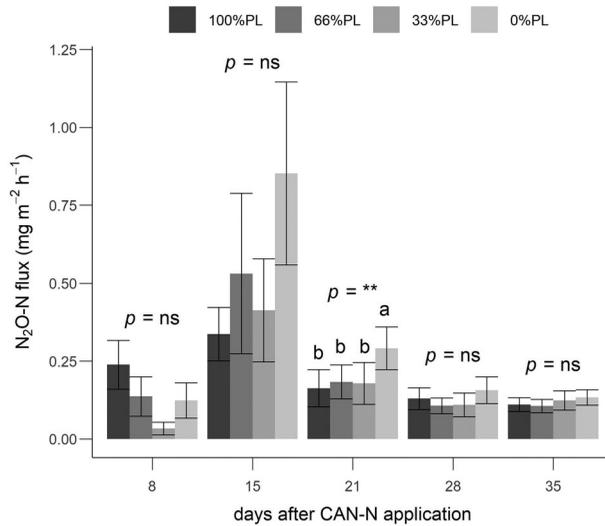


Figure 3. Field experiment. $\text{N}_2\text{O-N}$ fluxes at days 8 till 35 after CAN-N application (5 g m^{-2}). Bars represent standard errors. Bars with the same letters are not significantly different ($p > 0.05$).

Discussion

Mesocosm experiment

The nearly 40% lower potential nitrification found for a monoculture of plantain versus a monoculture of ryegrass at day 148 of the experiment is in line with the suggested BNI capacity of plantain (Dietz et al. 2013; Subbarao et al. 2015). However, in contrast to our hypothesis that the presence of plantain would lead to lower soil NO_3^- concentrations,

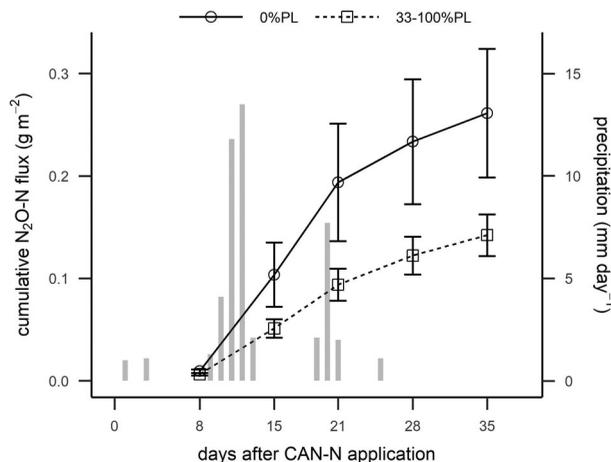


Figure 4. Field experiment. Precipitation (grey bars, KNMI, Zegveld, the Netherlands) since CAN-N application (5 g m^{-2}) and cumulative $\text{N}_2\text{O-N}$ fluxes between 8 till 35 days after CAN-N application of treatment 0%PL (open dots, solid line) versus all treatments with plantain (33%PL, 66%PL and 100%PL) (squares, dashed line). Error bars represent $\text{N}_2\text{O-N}$ fluxes standard errors. Cumulative $\text{N}_2\text{O-N}$ fluxes were significantly lower in the presence of plantain ($p = 0.021$).

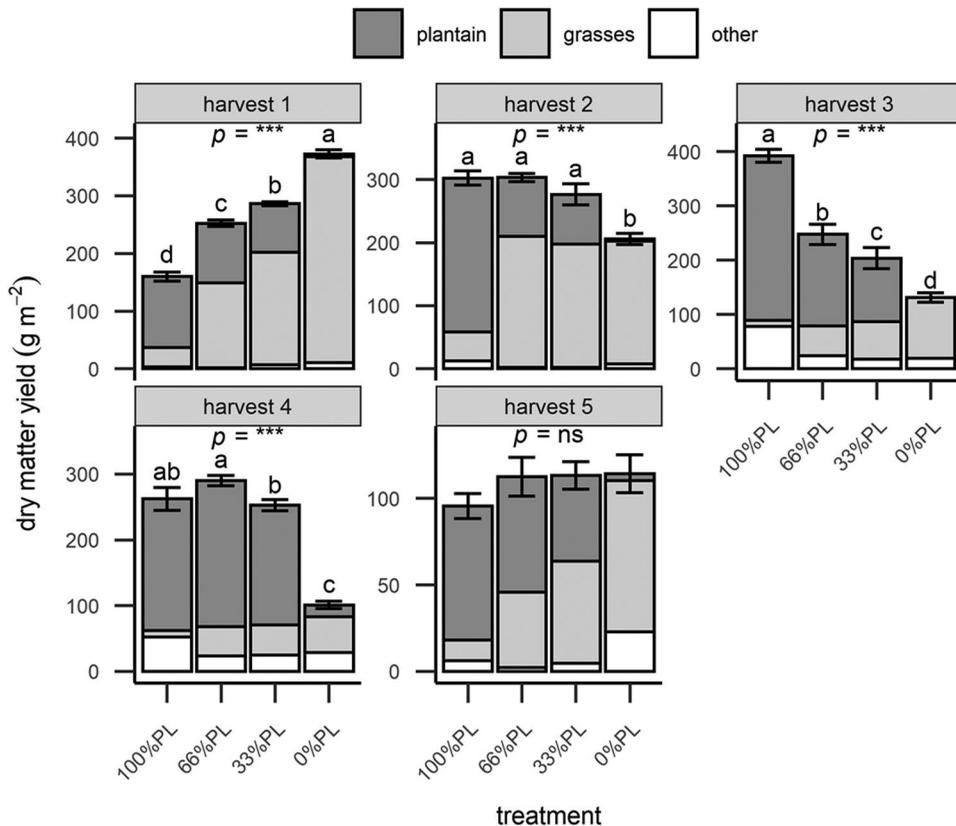


Figure 5. Field experiment. Total herbage dry matter yields per harvest and treatment, and contribution of plantain (dark grey fill), grasses (light grey fill) and other herbs and forbs (white fill) to the total herbage dry matter yield. *p*-values and errors bars represent probabilities for statistical differences and standard errors of the total herbage dry matter yield. Total herbage dry matter yields of bars with the same letters are not significantly different ($p > 0.05$).

no differences between treatments were observed. Soil NO₃⁻ concentrations were rather variable, possibly as the result of spatial and temporal heterogeneity of denitrification patterns caused by the nature of the process (e.g. Van den Pol-van Dasselaar et al. 1998). Furthermore, soil NO₃⁻ concentrations were lower when potential nitrification was higher (Figure 1). Low NO₃⁻ concentrations could be the result of a greater microbial and/or bacterivore activity coinciding with higher soil respiration and mineralisation rates (Bloem et al. 1994). Higher respiration rates can result locally in micro patches with lower soil oxygen concentrations favouring denitrification and decreasing soil NO₃⁻ concentrations (Firestone and Davidson 1989).

Plants may also influence nitrification in soils by altering the identity or activity of the microbial community as the result of effects on the physical and/or chemical soil environment (e.g. pH, moisture). Additionally, plants influence nitrification by competing with the microbial community for N and thus reducing the amount of NH₄⁺ available for nitrifiers (Bowatte et al. 2016). Since in our experiment effects of exudated secondary metabolites with BNI capacity on nitrification were not directly assessed, we cannot exclude effects on potential nitrification caused by the soil environment or by competition for

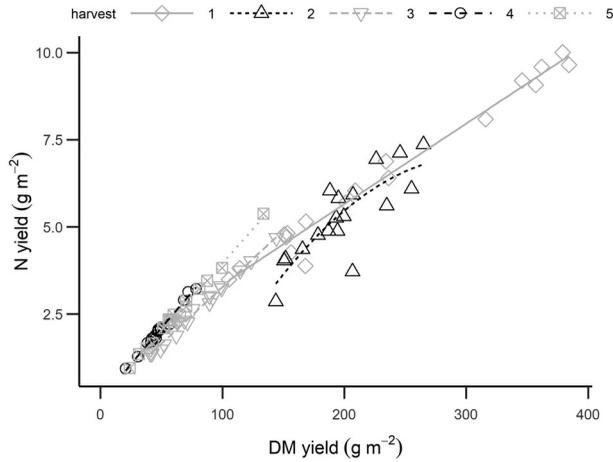


Figure 7. Field experiment. Grasses nitrogen (N) yields versus grasses dry matter (DM) yields per harvest, grown in a monoculture or in a plantain ryegrass mixture, respectively. For model details on the fitted lines see Table S2.

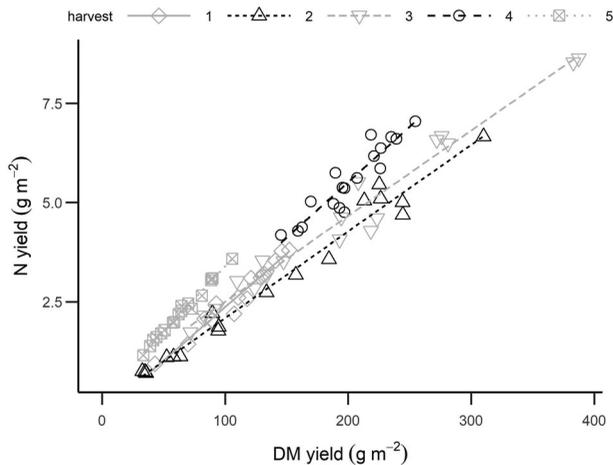


Figure 6. Field experiment. Plantain nitrogen (N) yields versus plantain dry matter (DM) yields per harvest, grown in a monoculture or in a plantain ryegrass mixture, respectively. For model details on the fitted lines see Table S2.

N. However, several studies showed that secondary metabolites with BNI capacity are produced by plantain and are present in above ground biomass and roots (Marak et al. 2000; De Deyn et al. 2009; Dietz et al. 2013; Miehe-Steier et al. 2015; Box and Judson 2018). Box and Judson (2018) reported for the plantain var. ‘Hercules’, used in our experiments, mean leaf concentrations of $39.1 \text{ mg g}^{-1} \text{ DM}$ verbascoside, $39.1 \text{ mg g}^{-1} \text{ DM}$ aucubin and $<5 \text{ mg g}^{-1} \text{ DM}$ catalpol over a year on a silt loam soil in New Zealand. Miehe-Steier et al. (2015) showed that leaf aucubin, catalpol and verbascoside concentrations are similar to root concentrations under high nutrient and high light conditions. Moreover, Fuchs and Bowers (2004) showed that aucubin and catalpol concentrations in plantain leaves increased from

0.5% dry weight at the age of 7 weeks to 6% dry weight at the age of 11 weeks. Therefore, it seems likely that in our experiment, in treatments with plantain, compounds with BNI capacity were present in the roots and rhizosphere. However, to confirm the hypotheses that the reduced potential nitrification resulted of the presence of compounds with BNI capacity, more detailed research is needed on the bacterial activity and the release and mode of action of these compounds in the rhizosphere.

According to Verhagen et al. (1995), to determine the exclusive effects of secondary metabolites on inhibition of nitrification, soil mineral N conditions should not be limiting for this process. Effects of competition for N on nitrification seemed unlikely in our experiment due to the high soil organic matter, NO_3^- and NH_4^+ concentrations. In comparison, in our experiment soil NO_3^- and NH_4^+ concentrations were on average about 1.000 times higher than in experiments on sandy soils reported by Dietz et al. (2013), who observed lower soil NO_3^- and NH_4^+ concentrations after incubation with aucubin, catalpol or a plantain liquid extract compared to a water incubation. Possibly, in our experiment the level and heterogeneity of soil NO_3^- and NH_4^+ concentrations were such that the potential effects of compounds with BNI capacity on these concentrations were too limited to be determined. Moreover, genotype may have played a role, since we used a different plantain variety than Dietz et al. (2013) and concentrations of BNI compounds have been shown to be genotype specific (Box and Judson 2018).

The observed quadratic increase of plantain N yields at higher plantain DM yields partly confirmed our hypothesis that the presence of plantain can increase the N use efficiency of grassland on peat soil since on the other hand no effects of the presence and yield of plantain on ryegrass N yields were observed (Figure 2). In our experiment plant N contents were low ($1.94 \pm 0.32 \text{ g kg}^{-1} \text{ DM}$) compared to field conditions (typically summer and autumn N contents without fertilisation are $\geq 2.40 \text{ g kg}^{-1} \text{ DM}$) (Vellinga and Andre 1999; Sonneveld and Lantinga 2011; Deru et al. 2019). Soil mineral N, P_{AL} , K and pH conditions were not likely to have limited plant N uptake (CBGV 2017). Therefore, besides a potential effect of compounds with BNI capacity in the treatments with plantain, likely other chemical, biological and/or physical soil properties affected herbage N uptake in general in the experiment. However, since soil NO_3^- and NH_4^+ concentrations between treatments did not differ significantly (Table 2), apparently these conditions did not differently affect soil mineral N concentrations between treatments.

Field experiment

In the presence of plantain cumulative N_2O fluxes were 39% lower compared to the monoculture of perennial ryegrass (Figures 3 and 4). These substantially lower N_2O fluxes in the presence of plantain confirmed our hypothesis that plantain can reduce N_2O formation in grassland on peat soil compared to ryegrass. Measured N_2O fluxes were in range with others studies on drained peat grasslands (Velthof et al. 1996; Koops et al. 1997; Van den Pol-van Dasselaar et al. 1998). During the N_2O flux measurement period, herbage N uptakes between treatments were similar, indicating no differences were present in available mineral N for herbage uptake between treatments.

Similar to our results, Luo et al. (2018) found lower N_2O fluxes when urine-N was applied at a rate of 62 g m^{-2} on a monoculture of plantain compared to ryegrass and

lucerne on a well-drained silt loam soil, and suggested the effect of the BNI potential of plantain as the dominant mechanism reducing N_2O fluxes. Simon et al. (2019) observed a linear decreasing N_2O emission factor as the proportion of plantain increased from 0% to 100% in a ryegrass/white clover sward, after a urine-N application of 61 g m^{-2} on a silt loam soil. In our experiment, above ground biomass of plantain (plantain made up 43%–86% of the total harvested herbage DM at 29 October in treatments 33%PL, 66%PL and 100%PL) was not correlated to cumulative N_2O fluxes. Thus, if the BNI potential of plantain was the dominant mechanism reducing N_2O fluxes, apparently the presence rather than the amount of plantain was an important factor for the reduction of the N_2O flux, at least within the studied conditions. For example differences in applied N sources, soil N concentrations and soil types resulted in different conditions between studies. Luo et al. (2018) and Simon et al. (2019) applied before N_2O measurements urine-N on silt loam soils while in the current study CAN-N was applied on peat soil. Urine-N consists mainly of urea which first needs to be transformed to NO_3^- to be available for denitrification, while CAN-N consists of 50% NH_4^+ and 50% NO_3^- . Furthermore, urine applications temporarily increase water filled pore space (WFPS) and possibly pH which both can (in)directly stimulate denitrification (Luo et al. 2018). Peat soils differ in many aspects from mineral soils which can (in)directly affect soil N cycling (Pihlatie et al. 2004); for example peat soils generally contain relative higher mineral N and organic carbon pools, have a relative higher moisture retention capacity, often have a lower pH and a different soil texture compared to mineral soils. In comparison, the grassland on silt loam soil used by Luo et al. (2018) had nearly four times lower total N and soil organic matter contents compared to the peat soil in the current study (Table 1). Moreover, in the current study measurements were only in the first year after glyphosate treatment and soil tillage, which likely both (in)directly affected the soil food web compared to an undisturbed grassland system (Wardle 1995), and likely thus also affected soil N cycling.

The sharp increase in the N_2O flux observed after the first significant precipitations nine to thirteen days after fertiliser application (total 32 mm, Figure 4) is probably the result of an increase in soil moisture content since denitrification activity and WFPS in the top soil are positively correlated (Koops et al. 1996). Significant differences in soil moisture contents between the treatments seemed unlikely as we measured nitrous oxide fluxes under relative (very) dry field conditions, resulting from the high potential precipitation deficit preceding the measurement period (Sluijter et al. 2018). Luo et al. (2018) found WFPS to be lower in a monoculture of plantain and lucerne compared to ryegrass, and lower N_2O fluxes after urine applications on plantain versus lucerne or ryegrass pastures. However, Luo et al. (2018) concluded that effects of plantain on N_2O fluxes could not have only been induced by different soil moisture contents since they observed lower N_2O fluxes at plantain at similar WFPS contents between lucerne and plantain in their study. Therefore, it seems likely that also in our experiment root-released compounds with BNI capacity at least partially contributed to the lower N_2O flux observed in the presence of plantain.

The negative effect of plantain DM yield on grasses N yields at the second harvest and linear relations between herbage N uptake and herbage DM growth at other harvests (Figures 6 and 7, Table S2) suggested that our hypothesis of plantain increasing soil mineral N use efficiency in a grassland on peat soil had to be rejected, at least within

the conditions of the field experiment. Lower total grasses N yields associated with higher plantain DM yields indicate increased herbage N uptake competition rather than an increase of available N for plant uptake.

Herbage N contents in the field experiment were well in range with other field measurements (Vellinga and Andre 1999; Sonneveld and Lantinga 2011; Deru et al. 2019). Possibly, the tendency towards the positive effect of plantain presence on plantain N uptake efficiency as observed in the mesocosm experiment was overruled by the relative higher herbage N uptake rate in the field experiment. Soil temperatures in the field experiment (increasing from about 11°C around 1 May to more than 20°C around 1 August) exceeded soil temperatures in the mesocosm experiment (maintained around 12°C), which positively affected soil mineralisation (Bloem et al. 1994). Furthermore, long periods without precipitation in the second, third and fourth harvest of the field experiment may have limited denitrification and leaching of NO_3^- to below the main rooting depth, leaving relative more NO_3^- available for herbage uptake.

Conclusions

Plantain grown in mesocosms significantly reduced soil potential nitrification but did not affect soil NO_3^- concentrations. Plantain N uptake efficiency increased at a higher herbage plantain share in the mesocosm experiment, but the plantain share did not affect herbage N uptake efficiency under field conditions. Under field conditions, N_2O fluxes were nearly 40% lower in the presence of plantain, without an obvious link to the amount of plantain present. Therefore, our results strongly suggest that the presence of plantain influences the fate of soil mineral N in a dairy grassland on peat soil, but more integrated research is needed to fully confirm this.

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Disclosure statement

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