LEIBNIZ-
WISSENSCHAFTSCAMPUS
PHOSPHORFORSCHUNG
ROSTOCK

Leibniz-
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Abschlussbericht

Anschubprojekt:
„Phosphorus as a cue regulating microbial N_2O production“

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Vorhabensbezeichnung:	PQ4N
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1. Zusammenfassung und Schlussfolgerung

Phosphorus (P) has been shown to interact with nitrogen (N) transformations in soils, altering microbial sources of nitrous oxide (N_2O) emissions. However, this P-regulated N response remains largely unclear. Interactions with water content as well as P-fertilisation history have rarely been investigated. Within this project, we carried out an incubation and a mesocosm experiment to increase understanding of the interactions.

Using a ^{15}N-labelled NO_3^- tracer in a soil incubation experiment, we studied the influence of P addition on N conversions and N_2O emission under soil water-holding capacities (WHC) of 45 and 60 %. We conclude from the results that P availability could increase N conversion via mineralization, decrease nitrification and increase denitrification, coupled with an increase in N_2O production from denitrification, which was the main N_2O source here despite moderate water contents.

In the mesocosm experiment, we studied effects of P-fertilisation history on reactions of N_2O production processes to P addition. Therefore, soils from two treatments of a long-term P fertilisation experiment were incubated with or without P addition. Fluxes of NO, N_2O and CO_2 were measured continuously and event-based samples taken for isotopomeric measurement of N_2O and molecular analysis of the microbial community. While long-term P fertilisation decreased N_2O production, short-term P addition increased it, leading to largest cumulative N_2O production from the low-P soil with P fertilisation. Preliminary isotopic signatures did not suggest differences in N_2O sources among treatments, but over time of incubation. Molecular results are pending due to constraints caused by pandemic measures.

Overall, the seed project has shown that P has an effect on N conversions and N_2O production. This effect depends on P fertilisation history and probably on the microbial community. These findings will be published in two papers. A grant proposal on this topic seems promising after submitting the manuscripts.

2. Einleitung und Ziele des Projektes

Agricultural soils are an important source of the greenhouse gas nitrous oxide (N_2O). N_2O is mainly produced by microbial transformations of N in soils and is often enhanced where available N exceeds crop demand (Oenema et al., 2005). Different pathways, e.g. denitrification, nitrification, or nitrifier denitrification, are involved in the production of N_2O under a range of soil conditions (Wrage-Mönnig et al., 2018). Among environmental factors, N availability and soil water content are major drivers of N_2O emissions (Butterbach-Bahl et al. 2013; Chen et al., 2014). However, there are also interactions with other elements, e.g. carbon (C) and phosphorus (P) (He and Djikstra, 2015; O'Neill et al., 2020).

Like N, phosphorus (P) is a critical nutrient for plant and microbial growth. P is a primary constituent of biomolecules such as nucleic acids (DNA and RNA), phospholipids and

adenosine triphosphate (ATP), which regulate many biological processes including energy transfer reactions and activation of enzymes (Nannipieri and Paul, 2009; Rouached et al., 2010). Availability of P is essential for the metabolisms of carbon and amino acids, e.g. during photosynthesis and respiration in plants and microbes (Elanchezhian et al., 2015), which play an important role in regulating N cycling and availability. Besides, N transformations in the soil are multi-enzyme processes carried out by diverse microbial species. The effects of P on various enzyme activities and composition of the microbial community could differ, leading to different P sensitivity of e.g. N mineralization, nitrification and denitrification (Olander and Vitousek, 2010).

The N-regulated P response in plant and microbial activity has been well described by recent studies (Medici et al., 2019; Hu et al., 2019), whereas the P-regulated N response remains largely unclear (Hu and Chu, 2019). So far, no robust information is available for P effects and P fertilisation history on sources of N_2O emission. Therefore, in this seed project, we used isotopic approaches to improve knowledge on N_2O sources and N transformations after addition of P fertiliser. In an incubation experiment, interactions between water-holding capacity and P addition were studied. In a mesocosm experiment, we used soils with different histories of P-fertilisation. We hypothesized that 1) P addition would decrease inorganic N in soil and N_2O emissions due to stimulated biological uptake, 2) there would be an interaction between water content and P addition on N_2O emissions, 3) P addition would have no effect on the relative importance of nitrification or denitrification for N conversion or as sources of N_2O and 4) P addition effects would depend on the history of P fertilisation.

3. Material und Methoden

Set-up of the incubation experiment

The soil was obtained from a low-input agricultural system from the experimental station of the University of Rostock, managed in order to decrease soil nutrient levels for experimental purposes. For P, the soil had been classified in the German fertilizer content class A (very low) for the last years. 200 g air-dried soil sieved over 2 mm was placed into 750 mL glass jars with the following four treatment combinations (n = 3): with or without phosphorus (P) at 45 % or 60 % WHC. Jars were divided into two groups, one for gas flux and isotopic gas measurements, and the other for measuring mineral N concentrations and ^{15}N signatures of NO_3^- and NH_4^+. Soils were pre-incubated for two days at 30 % WHC, after which ^{15}N-NO_3^--labelled NH_4NO_3 tracer (10 atom%) at a rate of 50 mg N kg^{-1} were stirred into the soil in all the glass jars. Half of the jars received P (triple super phosphate; 225 mg P kg^{-1} soil) after adjusting to the target soil water content, while the other half received no P addition. The targeted soil water content was maintained daily on a weight basis. The jars were closed for 1 h with air-tight rubber lids with septa before taking the gas samples.

Gas samples were taken at 24 h intervals from the headspace, transferred into helium-flushed and evacuated 12 mL exetainer vials and analyzed for N_2O and its isotopic signatures using a trace gas preparation unit (Trace Gas, Elementar, UK) coupled to an isotope ratio mass spectrometer (IRMS, Isoprime 100, Elementar, GermanyUK). Fluxes were calculated by assuming a linear relationship between concentration and incubation time. For calibration, two working standards were used that had been calibrated against the standards of the laboratory of the Department of Environmental System Science, ETH Zürich (Verhoeven et al. 2019). Sample peak ratios are initially calibrated against an N_2O reference gas peak (100 % N_2O, Air Liquide, Germany) and then corrected for drift and span using the working standards.

The second group of jars was used to extract NH_4^+ and NO_3^- on days 1, 3 and 5 of the incubation by adding 150 mL of 1 M KCl solution to 40 g subsamples of the treated soils, shaking for 1 hour and filtering. The concentrations and ^{15}N enrichment of NH_4^+ and NO_3^- were determined by micro-diffusion (Brooks et al., 1989) and analysis on an elemental analyzer (vario PYRO cube, Elementar, Germany) interfaced to the above IRMS. Our internal standards (wheat flour and sulfanilamide) were calibrated against IAEA-600 and IAEA-NO-3 for ^{15}N. All isotopic values for this experiment are given in at% excess.

Set-up of the mesocosm experiment

The mesocosm experiment was carried out at the facilities of the University of Goettingen, Plant Nutrition and Yield Physiology. Soil was taken from two treatments of the Rostock long-term field experiment (Zicker et al., 2018): a control treatment without added P since 1998 (low P soil) and a treatment with cattle manure + TSP (high P soil; manure applied every three years at about 30 t ha^{-1} and TSP annually at 21.8 kg P ha^{-1} until 2013 and 30 kg P ha^{-1} thereafter). The soils were packed into the mesocosms at a bulk density of 1.46 g cm^{-3}. There were four treatments (five replicates each): low P soil without added P (LP-), low P soil with added P (LP+), high P soil without added P (HP-) and high P soil with added P (HP+). P was added as TSP at 34.4 mg P kg^{-1}, which was supposed to bring the low P soil to the P content of the high P soil. All treatments received 50 mg N kg^{-1} as NH_4NO_3 at the start of incubation and moisture was kept at 75% WHC. To check for potential carbon (C) limitation, on the 6th day of incubation, both glucose (300 mg C kg^{-1}) and NH_4NO_3 (50 mg N kg^{-1}) were added to all treatments.

Fluxes of N_2O, nitric oxide (NO) and carbon dioxide (CO_2) were measured continuously over 13 days. Furthermore, air samples were taken from the pots event-based on days 1, 2, 3, 7, 10 and 13 and measured for stable isotope composition and site preference on the IRMS as above. Isotopic signatures are here reported as δ-values. Samples for soil pH, NH_4^+ and NO_3^- (KCl extraction for concentrations and isotopic composition) and inorganic P content (CAL

extraction) were taken on days 0, 3, 6, 9 and 13. Part of these samples was frozen immediately for later molecular analyses. Due to the outbreak of Covid-19 and following lock-down, sample transfer had to be delayed and molecular analyses will be conducted in the coming weeks.

Statistical analyses

Normality and homogeneity of variances for all variables was verified using the Kolmogorov-Smirnov test before further statistical analysis. In the incubation experiment, a repeated measures analysis of variance (ANOVA) was used to test for main effects of P, WHC, day of measurement, and their interactions. Two-way ANOVA was used to test for P, WHC, and their interaction on cumulative N_2O in soil. Where treatment effects were significant at $P < 0.05$, least significant difference (LSD) tests were used to compare the means of each treatment combination. P values between 0.05 and 0.10 were considered as marginally significant. All analyses were performed with the R software (version 3.6.1) for Windows.

4. Ergebnisse

Incubation experiment

Analysis of the experimental soil before and after incubation showed that the concentrations of NO_3^- were larger than those of NH_4^+. Overall, the NH_4^+ concentrations increased significantly over time ($P = 0.030$), whereas NO_3^- decreased (significant day effect, $P < 0.001$) (Fig. 1a). Both the NH_4^+ and NO_3^- pools were not significantly affected by P treatment. Soil water content significantly ($P < 0.001$) affected the NO_3^- concentration, which was higher at 45 % WHC than at 60%. The NH_4^+ concentration did not change with WHC. There was a significant soil water content × day interactive effect ($P = 0.020$) in NO_3^-, which was larger on day 3 compared to day 5 (Fig. 1a). Overall, the NO_3^- concentration was marginally significantly ($P = 0.070$) increased without P × 45 % WHC compared to the other treatments.

The ^{15}N-enrichment of the NH_4^+ pool slightly increased over time (significant day effect, $P = 0.001$), while that of the NO_3^- pool significantly decreased ($P < 0.001$) (Fig. 1b). P addition did not affect the ^{15}N signature of the NH_4^+ pool, which remained at background levels (0.011 – 0.013 at% excess; $P = 0.003$). Overall, the NO_3^- pool was significantly ($P = 0.001$) more enriched with P addition (2.56 at% excess) than without (2.44 at% excess). With a soil water content of 60 % WHC, the ^{15}N-enrichment of the NH_4^+ pool was slightly increased compared to 45 % (significant WHC effect, $P = 0.003$), while that of NO_3^- remained the same. There was, however, a trend to a significant P × soil water content interactive effect for the ^{15}N-NO_3^- enrichment ($P = 0.050$), which was larger with P × 45 % WHC compared to the other treatments.

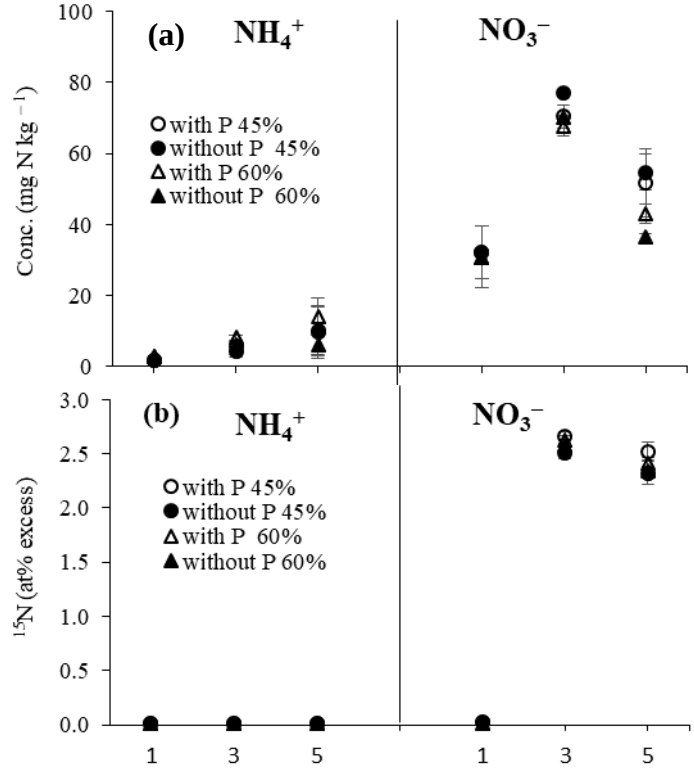

Fig. 1. Mean NH_4^+ and NO_3^- concentrations (a) and the ^{15}N-enrichments (b) as affected by P and soil water content during the 5-day incubation. Error bars represent standard deviation. Data for day 1 were background values taken before the addition of P or ^{15}N-NO_3^-.

N_2O emission and its ^{15}N-enrichment were largest on day 2 (Fig. 2a, b) (significant day effect, $P < 0.0001$, for both N_2O and ^{15}N-N_2O). The addition of P increased the emission of N_2O, which was marginally significant ($P = 0.080$). WHC did not show any significant effect on N_2O emission. The ^{15}N-enrichment of N_2O (Fig. 2b) and cumulative N_2O emission were not significantly different among the treatments. Also, there were no significant interactions between P and WHC for N_2O emission or its enrichment.

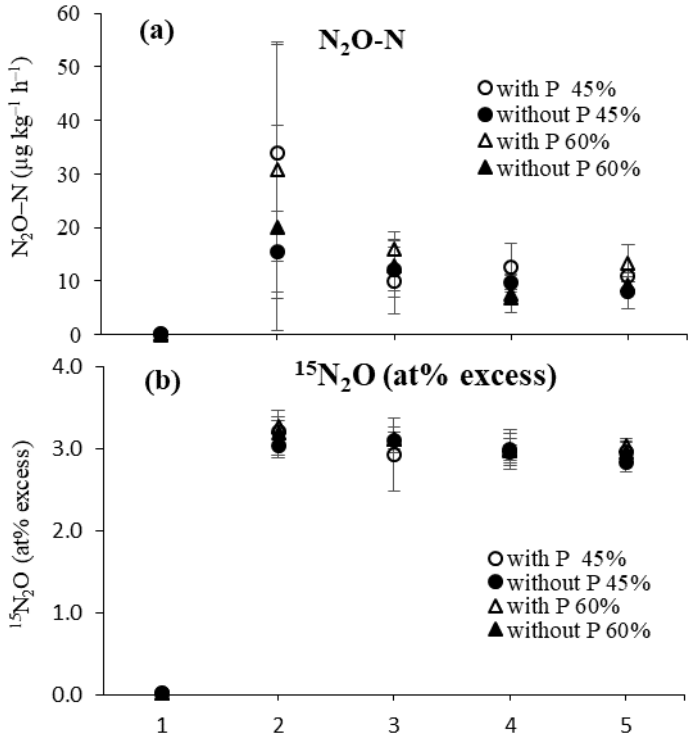

Fig. 2. Mean N_2O–N fluxes (a) and the ^{15}N-enrichment (b) as affected by P and soil water content during the 5-day incubation. Error bars represent standard deviation. Data for day 1 were background values taken before the addition of P or ^{15}N-NO_3^-

Mesocosm experiment (preliminary results)

The soil P content was clearly different in the different treatments: P fertilisation had an influence on soil P, but did not raise the inorganic P content in the LP+ soil to that of the unfertilised HP- soil (data not shown).

Fluxes of N_2O, NO and CO_2 were dynamic over time (Fig. 3). For N_2O, there was a peak emission event at the beginning of the incubation. This was larger and the maximum a little later in the LP than in the HP soils and was slightly increased by P fertilisation in both soils. The addition of glucose and N on the sixth day of incubation led to small peaks of N_2O, especially in the LP+ soil. Furthermore, large NO fluxes were measured afterwards. A first NO peak was similar for all treatments, but it was followed by a second peak in N_2O and NO that was much smaller and a little quicker in both HP incubations. There was also an increased CO_2 flux at this time, which had a similar magnitude for all treatments. Accumulated N_2O emissions (Fig. 4) were slightly increased with P fertilisation, but were larger in LP than HP soils.

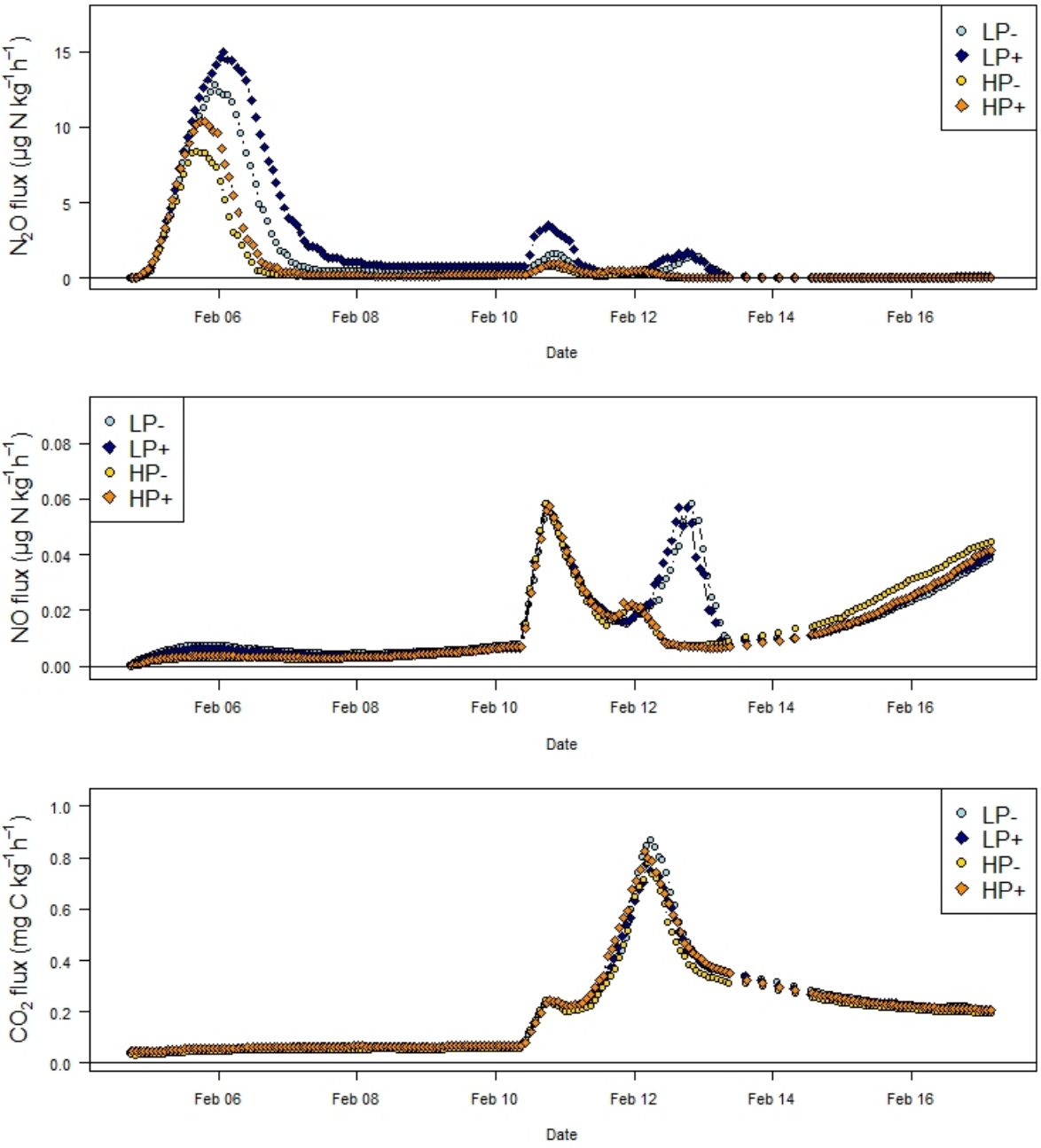

Fig. 3. Fluxes of N_2O, NO and CO_2 over time in treatments differing in P fertilisation. LP: soil low in P, HP: soil high in P, -: no P added, + P added at beginning of incubation. Shown are means (n = 5).

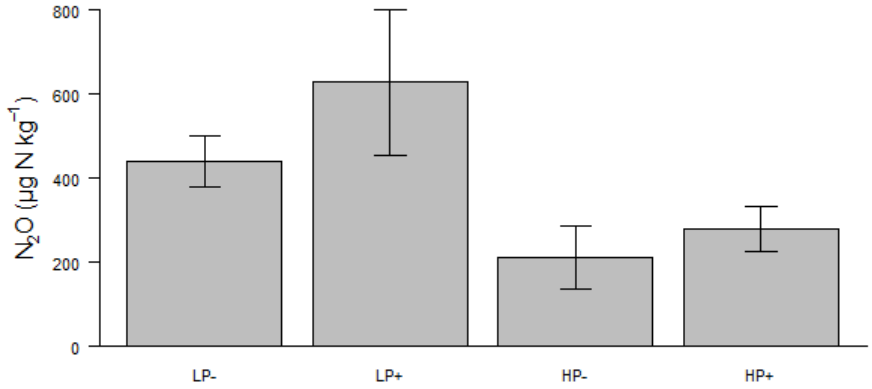

Fig. 4. Accumulated N_2O emissions over the time of the mesocosm experiment. LP: soil low in P, HP: soil high in P, -: no P added, + P added at beginning of incubation. Shown are means and standard deviations (n = 5).

The isotopic signatures of N_2O (Fig. 5) showed large variability over time. The ^{15}N signatures were depleted at the beginning of the incubation and became gradually more enriched towards the end. In contrast, $\delta^{18}O$ signatures were rather depleted on the first day, at around 40 ‰ on the next three sampling days and at about 25 ‰ towards the end of the incubation. The site preference was negative throughout, but especially on days 3, 7 and 10, indicating that the N at the α-position was more depleted than that at the β-position. There were no consistent differences in isotopic signatures among treatments.

5. Diskussion

Incubation experiment

In the following, we will discuss the results according to our hypotheses. We will start with effects of P addition on inorganic N in soil and N_2O emissions, go on with the influence of the interaction between water content and P addition on N_2O emissions, and lastly discuss effects on the relative importance of nitrification or denitrification for N conversion or as sources of N_2O.

Our results did not support the hypothesis that P addition would decrease inorganic N in soil and N_2O emissions by stimulated biological uptake. The addition of P did not affect NO_3^- availability, though P addition appeared to increase both the NH_4^+ concentration and N_2O emission. A similar P addition effect increasing N_2O production has been found in a P-limited grassland soil; however, extractable NH_4^+ and NO_3^- were not significantly affected by the P addition (He and Dijkstra, 2015; Mehnaz and Dijkstra, 2016). Previous studies have demonstrated that alleviation of P limitation may increase or decrease N_2O emission by stimulating biological N uptake (Baral et al., 2014; He and Dijkstra, 2015; Mehnaz and Dijkstra, 2016). Our study did not include plant activity and responses, which might explain a

missing effect of P on inorganic N and N_2O emissions by biological N uptake. Baral et al. (2014) demonstrated that P addition decreased N_2O emissions by increasing plant N uptake in a P-limited arable soil with maize under greenhouse conditions. Conversely, He and Dijkstra (2015) found that P addition did not increase plant N uptake but instead increased N loss in P-limited soil in a mesocosm experiment with grass species. Thus, it seems that if P addition leads to increased plant N uptake, N_2O emissions are decreased, and vice versa.

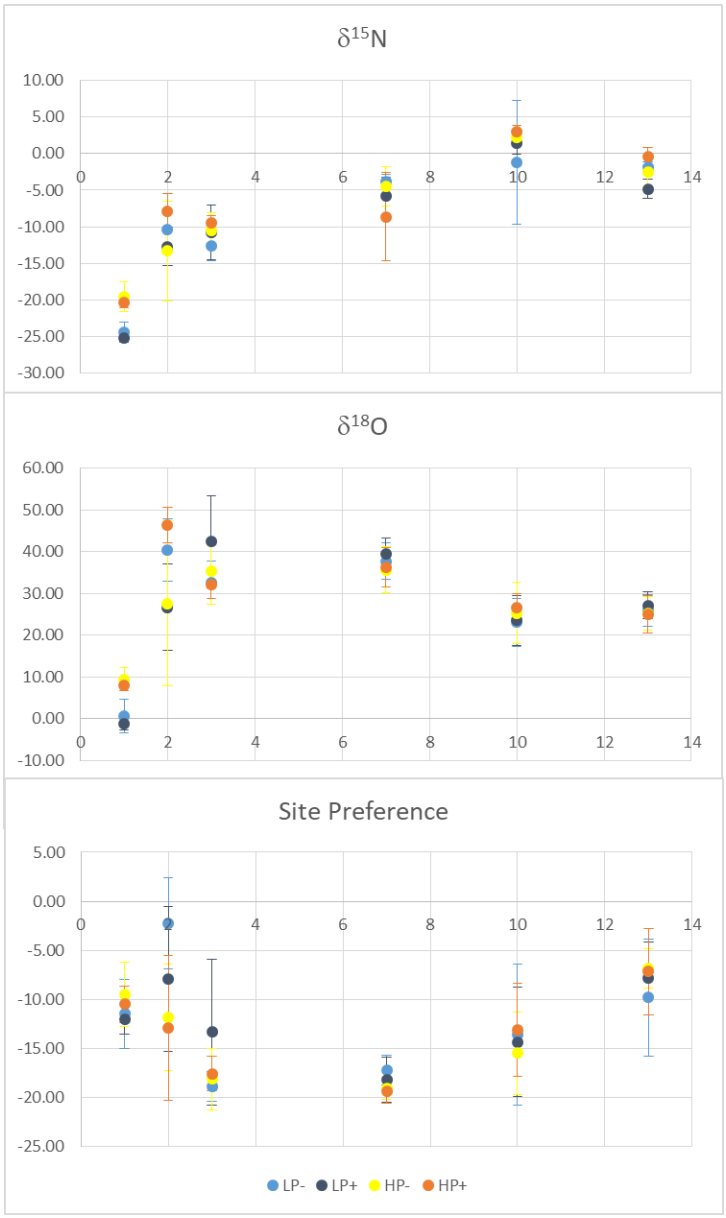

Fig. 5. Isotopic signatures of N_2O over the course of the mesocosm experiment. Shown are means and standard deviations (n = 5). All values are δ-values in ‰.

Our results did not support the second hypothesis of an interaction between soil water content and P addition on N_2O emissions. However, the soil water content influenced NO_3^- availability. The slight increase in NO_3^- concentrations without P at 45 % WHC compared to

the other treatments suggests that NO_3^- reduction to N_2O was affected by the interaction. N_2O emissions were smaller at peak emission and decreased more until day 5 without P under 45 % WHC than 60 %, indicating increased reduction of NO_3^- to N_2O under the higher soil water content. Decreased NO_3^- concentrations under the higher soil water content are also indicative of increased NO_3^- use relative to production and thus in our plantless system potentially larger denitrification relative to nitrification. An increasing water content is known to increase denitrification relative to nitrification and at very large water contents increase N_2O reduction (Butterbach-Bahl et al. 2013).

Our hypothesis that P addition would have no effect on the relative importance of nitrification or denitrification for N conversion or as sources of N_2O was not supported. N transformations and the associated N_2O emissions were stimulated after P addition. Our result is more consistent with a larger increase of mineralization and denitrification compared to nitrification with P addition, which suggests that P availability could regulate inorganic N conversion and associated N_2O production. An increased NH_4^+ concentration with P addition indicated increasing mineralization relative to nitrification. Moreover, the isotopic signatures of the NH_4^+ pool after addition of the $^{15}NO_3^-$ label indicated no occurrence of dissimilatory nitrate reduction to ammonia (DNRA) in this soil. A tendency to more depleted ^{15}N-NH_4^+ signatures with added P hints towards a decrease of nitrification relative to mineralization with P.

Although the overall NO_3^- concentrations were not affected by P treatment alone in our soil incubation experiment, decreased NO_3^- concentrations over time and significantly increased ^{15}N-NO_3^- with added P showed increasing denitrification relative to nitrification. The decline of the ^{15}N-NO_3^- enrichment over time indicates dilution by unlabelled NO_3^- produced from nitrification. As the enrichment remains larger with P addition than without, the influence of nitrification seems decreased with P. Interestingly, the ^{15}N-enrichment of N_2O almost matched that of NO_3^-, indicating that mostly denitrification was responsible for N_2O production, despite the rather dry soil conditions. Similar to our findings, P addition also increased N_2O production by denitrification compared to nitrification where NO_3^- was abundant in a highly P-limited grassland soil (Mehnaz and Dijkstra, 2016). To summarise, our soil incubation experiment indicates that P addition enhanced N conversion via mineralization and N_2O emissions via denitrification, whereas it decreased nitrification relative to the other processes.

Mesocosm experiment

The preliminary results of the mesocosm experiment showed that the effect of P addition on N_2O production indeed depended on the soil's history of P fertilisation. Interestingly, short-term P addition had a stimulating effect on N_2O emission, while long-term P-fertilisation had

a reducing effect. Isotopic results did not indicate different sources of N_2O among treatments. Here, signatures of ^{15}N in soil NH_4^+ and NO_3^- need to be considered for further evaluation. Besides, molecular analyses will indicate whether differences existed among the soils initially and/or after incubation.

6. Weitere Leistungen und Ziele aus dem Projekt

At least two publications will be prepared from this project. One concerning the incubation experiment is almost ready to be submitted – this report was partly based on the text of the manuscript. The other manuscript will be written after submission of the first and analysis of the remaining data for ^{15}N (mineral N in soil) and samples for microbial composition. There are several ideas for research proposals.

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