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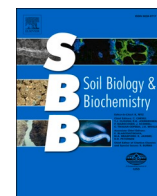
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Plant communities can attenuate flooding induced N₂O fluxes by altering nitrogen cycling microbial communities and plant nitrogen uptake

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ABSTRACT

Plant communities comprising species with different growth strategies and belonging to different functional groups can ensure stable productivity under variable climatic conditions. However, how plant communities can influence the response of nitrogen (N) cycling, in particular, soil microbial N cycling communities, N leaching and N₂O fluxes under flooding, and their capacity to suppress flooding-induced N₂O fluxes, remains unresolved. The aim of this study was to examine the effect of different plant communities composed of grasses and/or legumes on N cycling soil microorganisms and N₂O fluxes, and how these effects are influenced by flooding. Our field experiment consisted of monocultures and two- and four-species mixtures of two grass and two legume species with different growth strategies (slow- and fast-growing species), grown in a fertilised sandy soil in the Netherlands. One year after plant establishment, we imposed paired control and flooding treatments for three weeks. We found that flooding significantly reduced plant N uptake and increased N₂O fluxes. This increase was associated with higher abundances of N cycling microbial communities (except for ammonia-oxidising bacteria). Legume presence increased N₂O fluxes, irrespective of the legume growth strategy or flooding, but this was not driven by changes in N cycling microbial communities; instead, it was related to an increase in soil nitrate availability. Mixing grasses with legumes promoted high plant N uptake and reduced N losses under control and flooded conditions, in particular when combining slow-growing species, and in the four-species mixture. Our results show that flooding exerted a strong influence on N cycling by increasing N leaching, N₂O fluxes, microbial community abundances and decreasing plant N uptake. However, plant communities with slow-growing strategy had lowest relative abundance of *nosZII* bacteria and ameliorated flooding effects by both reducing N losses and enhancing plant N uptake.

1. Introduction

Intensively managed grasslands receive abundant nitrogen (N) fertilisers to produce high quality food for a growing world population (Lassaletta et al., 2016). Higher N availability in soils may, however, result in higher N losses with negative impact to the environment via nitrate (NO₃⁻) leaching and nitrous oxide (N₂O) emissions (Butterbach-Bahl et al., 2013). Nitrate leaching losses from soil to water can cause severe health problems, eutrophication of surface water and groundwater contamination (Di and Cameron, 2002), whereas N₂O is a powerful greenhouse gas and ozone-depleting substance (Ravishankara et al., 2009; Alexander et al., 2013) with 273 times higher global

warming potential than CO₂ on a mass basis (over a 100-year horizon; Canadell et al. (2021)). A reduction in N fertilisation levels, an increase of N uptake by the plants, and the reduction of N₂O to N₂, can reduce N₂O fluxes, and the extent to which these can reduce the emission of N₂O is mediated by the soil microbial community (Grados et al., 2022).

Production of N₂O is mainly caused by two well-studied microbial processes: nitrification and heterotrophic denitrification. The first step of nitrification consists of the oxidation of ammonia (NH₃) to nitrite (NO₂⁻) via hydroxylamine (NH₂OH) during which N₂O is emitted as a by-product. This step can be performed by ammonia-oxidising archaea (AOA) and bacteria (AOB), as well as by comammox (Daims et al., 2015), and the enzyme responsible for the reaction is encoded by the

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amoA gene (Kowalchuk and Stephen, 2001). Denitrification is the stepwise reduction of soluble NO_3^- or NO_2^- to gaseous compounds NO , N_2O or N_2 (Wrage et al., 2001; Butterbach-Bahl et al., 2013). The reduction of NO_2^- to NO is catalysed by nitrite reductases encoded by the *nirK* and *nirS* genes, while the reduction of N_2O to N_2 is catalysed by the N_2O -reductases, encoded by the *nosZI* or *nosZII* genes, representing the only known sink for N_2O in the biosphere (Hallin et al., 2018). Previous studies reported that denitrification is the main N_2O producing process in anaerobic conditions at high soil water content (>70% WHC) while N_2O is mostly emitted by nitrification in aerobic soil at lower soil water content (Bateman and Baggs, 2005). These processes may occur simultaneously in different microsites of the same soil (Stevens et al., 1997), but there is still uncertainty associated with which processes are predominantly contributing to N_2O fluxes (Zhang et al., 2022) and, particularly during and after a flood event.

Climate change has increased the frequency and intensity of extreme rainfall events (IPCC, 2022), and therefore flooding events are becoming more common in many ecosystems across the world (Beniston et al., 2007; Schiermeier, 2011). This is concerning because floods reduce gas diffusivity and O_2 availability in soil, resulting in anaerobic microsites that favour denitrification (Xu et al., 2016; Nguyen et al., 2018), and consequently N_2O production (Butterbach-Bahl et al., 2013; Harrison-Kirk et al., 2013; Congreves et al., 2018). During flooding, NH_4^+ can accumulate, as anaerobic conditions prevail throughout the soil profile inhibiting nitrification of NH_4^+ to NO_3^- (Zhang et al., 2018). In addition, NO_3^- in the soil may be moved down to the shallow groundwater as leachate, and N_2O may become entrapped in soil pores (Clough et al., 2005). After floodwater removal, and the re-oxygenation of the soil, the accumulated NH_4^+ can be progressively nitrified to NO_3^- , increasing N_2O fluxes (Yang et al., 2017; Sánchez-Rodríguez et al., 2019).

Soil microorganisms are key actors in the soil N cycle. It is, however, still unknown what the impact of flooding is on the abundance of the different microbial guilds involved in soil N cycling. Although some studies have examined the influence of flooding on N cycling soil communities (Stres et al., 2008; Chen et al., 2017), how plant communities modulate these microbial responses to flooding has been overlooked.

Growing plants from different functional groups together can reduce the need for mineral N inputs and can promote plant community resilience to floods (Oram et al., 2020, 2021). Legumes can form a symbiotic relationship with bacteria that can fix N from the air and have

correspondingly lower soil N uptake rates than grasses and forbs, potentially resulting in increased levels of soil mineral N (Palmberg et al., 2005; Niklaus et al., 2006) and higher risk of NO_3^- leaching (Scherer-lorenzen et al., 2003). The effect of legumes on N_2O fluxes is however not consistent, with studies finding no effect (Barneze et al., 2020) or an increase in N_2O fluxes from fertilised grassland experiments (Abalos et al., 2021). Under flooding conditions, a recent mesocosm study found that legume monocultures had lower productivity and higher N_2O fluxes than grasses (Oram et al., 2021). However, when legumes are grown in mixtures with grasses, there is a potential to diminish flooding induced N_2O if the companion species is very efficient in taking up the available mineral N (Oram et al., 2020). These findings are promising yet remain to be tested under more realistic field conditions, with inclusion of the functional shifts in the microbial communities that underpin the soil N cycle to help unravel the underlying mechanisms. Although Abalos et al. (2021) evaluated N cycling including N cycling microbial communities in a field experiment with grassland communities, the experiment did not include more than one legume species and flooding was not included in the study design.

Apart from the plant functional groups, the plant growth strategy and plant resource acquisition strategy (acquisitive-conservative, Reich (2014)) is of relevance in relation to plant responses to flood and impact on N losses. Fast-growing plant species (with acquisitive traits) often have higher N uptake, and reduce N_2O fluxes (Abalos et al., 2018) and N leaching (de Vries and Bardgett, 2016). Furthermore, plant communities with fast-growing legumes and grasses can increase productivity and plant N content with lower soil mineral N compared to fertilised grassland without legumes (Barneze et al., *under review*), thereby potentially lowering N losses (N leaching and N_2O fluxes). Whether these findings are also observed under flooded conditions remains uncertain, and the links with N cycling microbial communities have never been studied.

The aim of this study was to assess the potential of contrasting grass and legume species and their mixtures to reduce N losses while maintaining high N uptake under flooding conditions, as well as their effects on the abundances of N cycling microbial communities. We hypothesised that H1) flooding will increase N_2O fluxes and the relative abundance of soil denitrifiers; H2) legume presence will increase N input, N losses and abundances of N cycling microbial communities in fertilised soils under control and flooded conditions, and H3) this legume effect will be offset by mixing legumes with grasses, especially under flooding conditions.

2. Material and methods

2.1. Study site

The experimental site was located at Nergena, Wageningen, the Netherlands (51° 59' 43.3" N, 5° 39' 17.6" E, 9 m a.s.l.). The site is under maritime temperate climate, with mean annual temperature of 9.4 °C and mean annual precipitation of 780 mm. The soil is a typical endoaquoll (Soil Survey Staff, 2014) with 84% sand, 10% silt and 6% clay. Initial analyses of the properties of the upper 15 cm of the soil profile were: total N content 1.5 g kg⁻¹, total organic C content 21 g kg⁻¹, C:N ratio 14, plant available P 7.2 mg kg⁻¹, pHCaCl₂ 5.6, and bulk density 1.25 g cm⁻³.

2.2. Experimental design

The field experiment was established in September 2019 and comprised 11 distinct plant communities, replicated five times in a completely randomised block design. The plant community consisted of: monocultures of two grass species (*Lolium perenne* and *Festuca arundinacea*) and two legume species (*Trifolium pratense* and *Lotus corniculatus*), all two-species combinations (*L. perenne* + *F. arundinacea*, *L. perenne* + *T. pratense*, *L. perenne* + *L. corniculatus*, *F. arundinacea* + *T. pratense*, *F. arundinacea* + *L. corniculatus*, *T. pratense* + *L. corniculatus*),

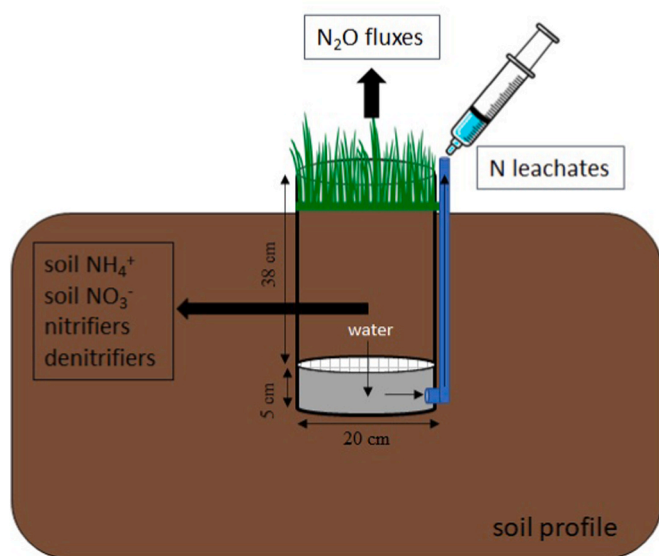


Fig. 1. Schematic of the assembled soil monolith (20 cm diameter × 38 cm height) used in the flooding field experiment at Wageningen, The Netherlands. The blue drainage tube connects the bottom of the monolith to the soil surface, allowing for leachate sampling.

and a four-species mixture (*L. perenne* + *F. arundinacea* + *T. pratense* + *L. corniculatus*), (Fig. S1, Barneze et al. *under review*). The plant communities were weeded by hand as needed (primarily in March–April 2020) to maintain the original plant community composition. The field plots were harvested three times (11th May, 6th July, and 10th August of 2020), followed by N-fertilisation in May and July of 50 kg N ha⁻¹ as calcium ammonium nitrate (totalling 100 kg N ha⁻¹). The plots also received a dose of 63 kg K ha⁻¹ as potassium sulphate in May 2020.

Two intact soil monoliths (20 cm diameter × 38 cm depth) were taken from each field plot (Fig. S1, Barneze et al. *under review*) in September 2020, one for the control (non-flooded) and one for the flood treatment (totalling 110 monoliths). Additionally from 15 selected field plots (all replicates of *L. perenne*, *T. pratense* and *L. perenne* + *T. pratense*), 30 extra monoliths were also taken (15 for the control and 15 for the flood treatment) totalling 140 monoliths (Fig. S2). These monoliths were not fertilised. The monoliths were cored using PVC cylinders (20 cm diameter 40 cm height) that were pushed into the soil and lifted out of the soil, retaining the soil monolith in the PVC cylinder. PVC caps (20 cm diameter × 5 cm height) with a mesh in the middle that prevented root penetration and soil loss but allowed soil water drainage were used to cover the bottom of each monolith. A thin drainage tube of similar length as the PVC cylinder was attached to the outside of the cylinder and connected to a small drainage hole in the bottom side of the closing cap of the monolith to collect the leachate from the soil monolith using a syringe, as shown in the schematic of Fig. 1. The monoliths were then placed back into the soil (still in their PVC cylinder) in custom-made holes in an adjacent area within the field to keep the soil monoliths under realistic soil profile temperature conditions during the whole experiment and to facilitate easy access to measure gas fluxes. The control (non-flooded) and flood monoliths were placed next to each other conferring a split plot design. Each field block (five blocks in total) contained now 22 monoliths (11 monoliths under control and flooding conditions) with 70 cm between each monolith (Fig. S2).

Before the flood was imposed, the monoliths were fertilised with an equivalent rate of 50 kg N ha⁻¹ as calcium ammonium nitrate totalling 150 kg N ha⁻¹, applied during the plant growing season. This level of fertilisation was consistent with typical semi-intensively managed grassland in temperate climate (Sutton et al., 2011; Suter et al., 2015). The flood treatments were then imposed on the October 5, 2020 with an equivalent to 100 mm of rainfall (around 3 L water per monoliths, in accordance with Stocker et al. (2013)). After 3 weeks, the surface water and the leachate at the bottom of the monoliths were collected from the drainage tube (Fig. 1) with a syringe, and the total volume of leachate recorded and kept at 2 °C for further analysis.

Meteorological data (rainfall and air temperature) were recorded at the nearest meteorological station, which was within 4 km of the field site. The air temperature inside and outside the chamber was measured at each monolith and used to correct the concentrations of N₂O inside the chamber. The microclimatic conditions were measured using thermal and soil moisture microclimatic sensors (TMS-4, TOMST Ltd., Prague, Czech Republic). The sensors are stand-alone, fully automatic, and measured the temperature at 15 cm above-ground, near the ground and 8 cm below-ground, and soil moisture at an interval of 15 min (Wild et al., 2019). The sensors were installed randomly in 14 non-fertilised monoliths (seven sensors in the control (non-flooded), and seven in the flooding monoliths).

2.3. Nitrous oxide measurements

Measurements of N₂O were made using polypropylene opaque flux chambers (20 cm diameter × 14 cm height) with two septa connected to Teflon tubes (Abalos et al., 2021; Oram et al., 2021). For each gas flux measurement, the chambers were attached to the monoliths and the headspace samples were taken approx. 30 min after chamber closure with tubes connected to a photoacoustic infrared spectroscopy Gaser-aOne gas analyser (Gaser Ltd, Turku, Finland). Samples of the ambient

air entering were also measured (once every 10 samples) and used to correct the gas fluxes. The precise closing period of the chambers was noted, and linearity of the gas concentration in each monolith was tested regularly (Chadwick et al., 2014). Gases were sampled before the flood was imposed, immediately after (within 12h of flooding), and daily during the first four days of the experiment, then two to three times a week up to day 67, when fluxes subsided. Cumulative N₂O flux was calculated by linear interpolation of the average N₂O fluxes between the measurements and integrating the results over the total period.

2.4. Plant and root analyses

Above-ground biomass was harvested to 2 cm above the soil surface before imposing the flood treatment to confirm that the initial biomass in the cores with the same plant communities was similar. Above-ground biomass was also harvested directly after the flood event (T1) and after 5 weeks of recovery at the end of the experiment (T2). Above-ground biomass was sorted per plant species, dried at 70 °C for 72 h, and weighed. Below-ground biomass was collected by taking one soil core (7.5 cm diameter × 15 cm depth) at the end of the experiment. These soil cores were then washed over a 0.5 mm sieve and root material dried at 70 °C for 72 h.

Leaf subsamples were ground, ball-milled into a fine powder, weighed into tin cups (approx. 4 mg cup⁻¹) and analysed for leaf C and N content at the UC Davis Stable Isotope Facility (California, USA) using an PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20-20 IRMS (Sercon Ltd., Cheshire, UK). Plant N uptake was estimated as the product of percentage N content and dry matter production (concentration × biomass).

2.5. Soil and leachate analysis

Soil cores (1.5 cm diameter × 15 cm depth) were taken from the main field experiment within each plot at the time of the intact monolith collection to assess the soil N levels (T0 – before flood event), and from the monoliths after each plant biomass harvest directly after the flood event (T1) and after 5 weeks of recovery at the end of the experiment (T2). Gravimetric moisture content was determined after soil drying at 105 °C for 24 h. A soil subsample was frozen (–20 °C) after sieving until further microbial analyses. Soil mineral N (NH₄⁺-N and NO₃⁻-N) was measured after extraction with 0.01 M CaCl₂ (Houba et al., 2000) in a 1:10 (soil weight: extractant volume, dry weight basis) and analysed by colorimetry (Brann en LuebbeTraACS 800 Autoanalyzer, Skalar Analytical B.V. Breda). Leachate samples were analysed by colorimetry to determine NH₄⁺-N and NO₃⁻-N in the solution samples.

2.6. Soil microbial communities

To quantify the abundances of microbial communities involved in N cycling, DNA was extracted from 250 mg of each soil sample before imposing flood (T0) (Table S1) and directly after the flood event (T1) from all the monoliths using the DNeasy PowerSoil-htp 96-well DNA isolation kit (Qiagen, France). Total bacterial community was quantified using 16S rRNA primer-based real-time quantitative PCR (qPCR) assays (Muyzer et al., 1993). The *amoA* gene was used as molecular markers to quantify the bacterial and archaeal ammonia-oxidisers (AOB and AOA, respectively) while the *nirK* and *nirS* genes were used to quantify the denitrifiers (Leininger et al., 2006; Bru et al., 2011). The N₂O-reducers were quantified using the *nosZI* and *nosZII* genes (Henry et al., 2006; Jones et al., 2013). qPCR reactions were carried out in a ViiA7 (Life Technologies, United States) in a 15 µl reaction volume containing 7.5 µl of Takyon MasterMix (Eurogentec, France), 1–2 µM of each primer, 250 ng of T4 gene 32 (MPBiomedicals, France), and 1 ng of DNA. Standard curves were obtained using serial dilutions of linearised plasmids containing appropriated cloned targeted genes from bacterial strains or environmental clones. No template controls gave null or

Table 1

Flood effects on soil temperature (5 cm below surface) and soil moisture before and during the flood. Values represent the average for all dates ± 1 standard error ($n = 11$ before flooding, and $n = 19$ during flooding). Different letters indicate significant differences ($P < 0.05$) based on a Tukey posthoc test.

	Flood treatment	Soil temperature ($^{\circ}\text{C}$)	Soil moisture (v:v %)
Before flooding	Control	13.3 ± 0.34	0.36 ± 0.02
	Flood	13.3 ± 0.33	0.35 ± 0.05
During flooding	Control	10.61 ± 0.33	0.30 ± 0.006^a
	Flood	10.63 ± 0.32	0.38 ± 0.003^b

negligible values. The presence of PCR inhibitors in DNA extracted from soil was estimated by mixing a known amount of standard DNA with soil DNA extract prior to qPCR. No inhibition was detected in any case. The relative abundances of the N cycling microbial communities were calculated based on the ratio of the functional gene copy numbers to the total 16S rRNA gene copy numbers, yielding a percentage of the abundance of the studied microbial communities relative to the total bacterial community abundance.

2.7. Statistical analyses

Linear mixed effects (LME) models (*nlme* package, Pinheiro et al. (2017)) were used to test the effect of flooding only (FLOOD), or the interaction between flooding and legume presence (FLOOD \times LEG), or the interaction between flooding and plant communities (11 plant communities) (FLOOD \times PLANTCOM) for the entire experimental period on N cycling (cumulative N_2O fluxes, plant N uptake, above- (sum of the three harvests) and below-ground plant biomass, soil $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ concentrations, leachate $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ concentrations) and soil N cycling microbial community (relative abundance of functional genes, *nirK*, *nirS*, AOA, AOB, *nosZI*, *nosZII* at T1). Fixed effects were flood, or flood and legume presence and their interaction, or flood and plant communities and their interaction. The random effect was block nested with plot to account for the split plot design. Some of the drainage tubes in the monoliths to collect the leachate were blocked at the time of collection, thus, these samples were not included in the analyses of $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ leaching, these monoliths comprised number 7, 14, 21 from block A, 32, 44, 52 from block B, 58, 65, 78 from block C, 86, 92, 96, 109 from block D and 118, 122, 138 from block E.

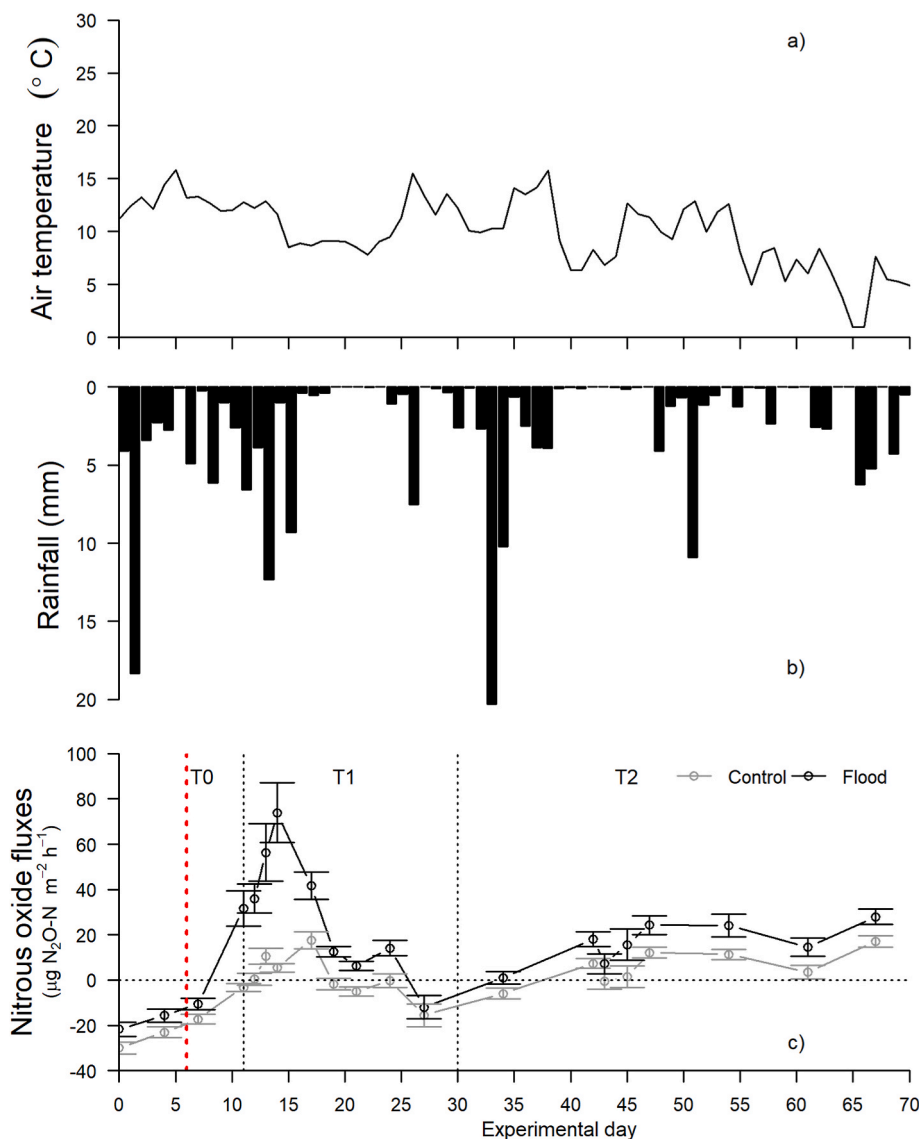


Fig. 2. (a) Air temperature ($^{\circ}\text{C}$), (b) rainfall (mm), and (c) daily N_2O fluxes from the soil under control and flooded conditions during the experimental period. Vertical bars show ± 1 standard error ($n = 55$). Red dashed line indicates the N-fertiliser application, and the black dashed lines represent the start and end of the flooding treatment. T0, before flooding; T1, during flooding and T2, after flooding.

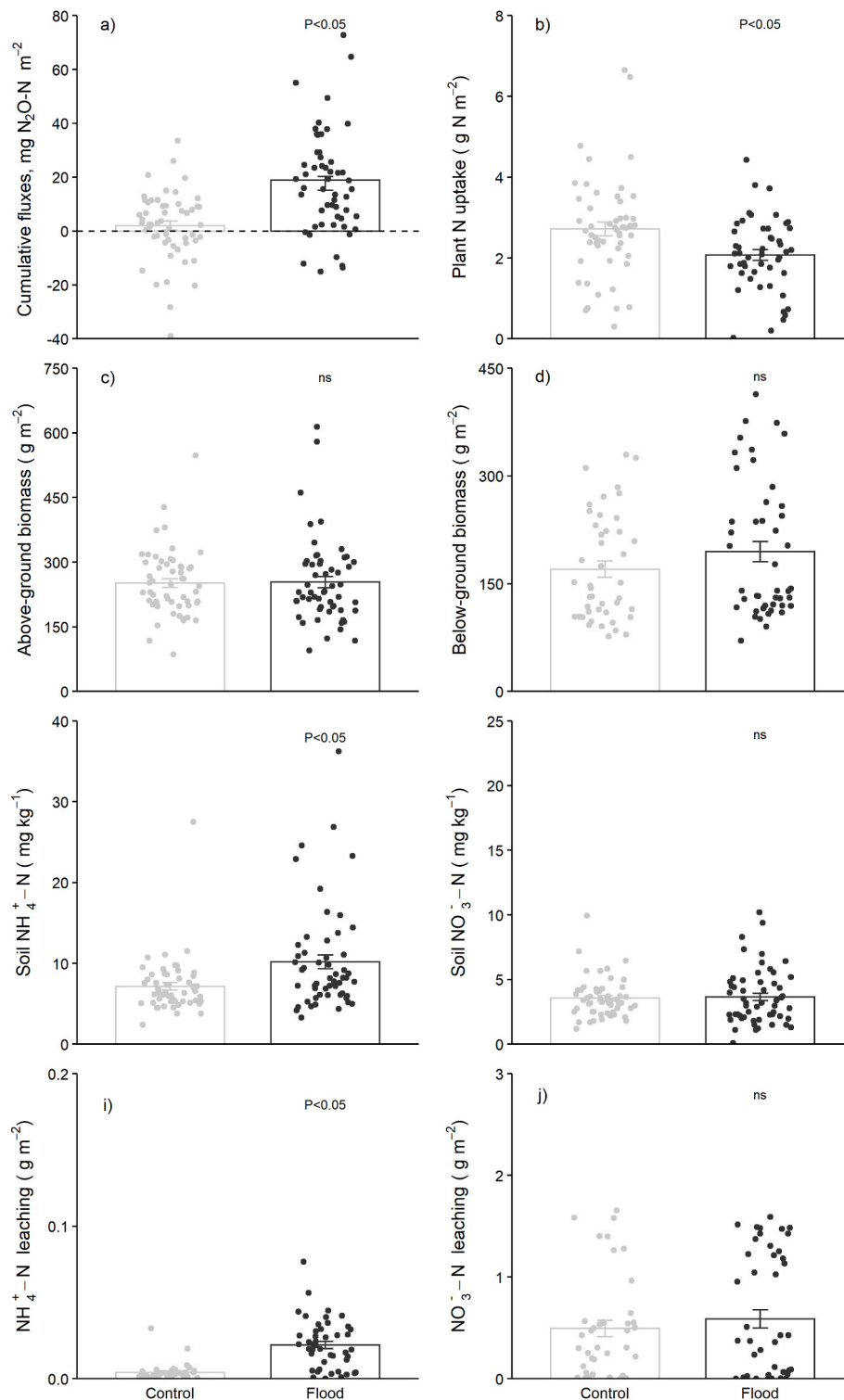


Fig. 3. Flood effects on a) cumulative N_2O fluxes, b) plant N uptake, c) above-ground biomass (cumulative for the three harvests), d) below-ground biomass, e) soil $\text{NH}_4^+\text{-N}$ for T1, f) soil $\text{NO}_3^-\text{-N}$ for T1, g) $\text{NH}_4^+\text{-N}$ leaching and h) $\text{NO}_3^-\text{-N}$ leaching. Bars are mean \pm SE ($n = 55$). Dots indicate values of individual plots. Significant differences between control and flood ($P < 0.05$) are based on a Tukey posthoc test (ns: non-significant).

Spearman rank correlation analysis was conducted to examine relationships between N cycling microbial communities (*nirK*, *nirS*, AOA, AOB, *nosZI*, *nosZII*) and N pools (plant N uptake, cumulative N_2O fluxes, N leaching and soil mineral N) from T1. The relative abundance of N cycling microbial communities was used to perform all statistical analysis. A principal component analysis (PCA) was carried out (FactoMineR, L   et al. (2008)) to group the plant communities on the basis of

their N pool values under control and flood conditions.

All data were checked for normality and equal variances using residual plots and log-transformed where necessary before analysis (i.e., below-ground biomass, soil mineral N and genes abundances). Weight functions (*varIdent*) were used to account for unequal variances following (Zuur et al., 2011), i.e., cumulative N_2O fluxes, plant N uptake, above-ground biomass, and soil $\text{NH}_4^+\text{-N}$. The significance of the

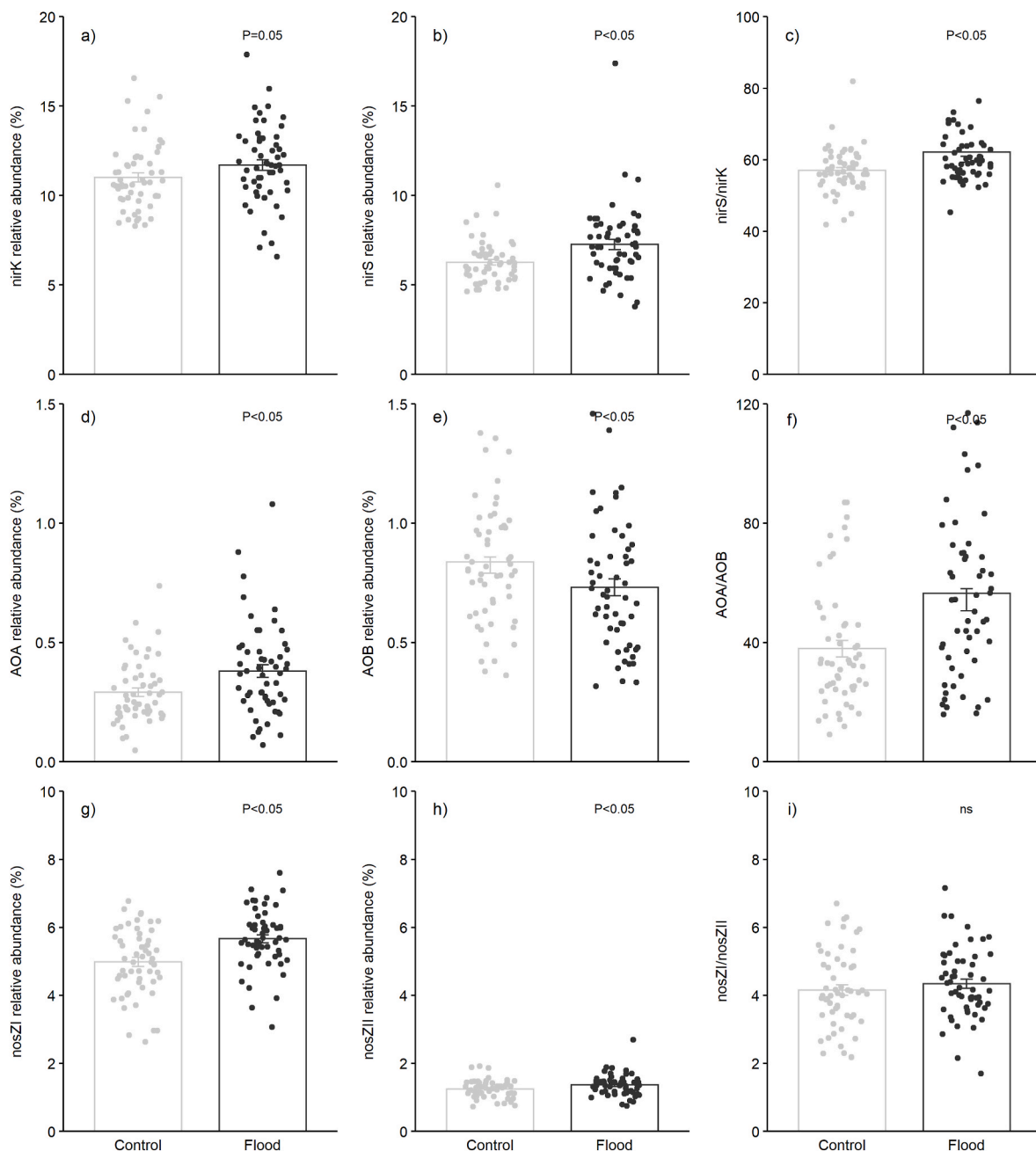


Fig. 4. Flood effects on the relative abundance of a) *nirK*, b) *nirS*, c) *nirS/nirK*, d) AOA, e) AOB, f) AOA/AOB, g) *nosZI*, h) *nosZII* and i) *nosZI/nosZII*. Bars are mean \pm SE (n = 55). Dots indicate values of individual plots. Significant differences between control and flood ($P < 0.05$) are based on a Tukey posthoc test (ns: non-significant).

fixed effects was determined by comparing models with and without the factor of interest using a likelihood-ratio test. Tukey post hoc testing was performed using pairwise comparisons between different plant communities at $P \leq 0.05$ with the *emmeans* package (Lenth, 2020). All statistical analysis was carried out in the R programming language 4.0.2 (R Core Team, 2020).

3. Results

3.1. Flooding affected soil moisture and daily N_2O fluxes

Flooding did not affect soil temperature ($P > 0.05$), while mean soil moisture was as intended significantly higher in flooded compared to the control (non-flooded) treatments during the three-week flooding (P

< 0.05 , Table 1). During the experiment, mean air temperature was around 10°C and total rainfall was 181 mm, with peaks on day 1 and day 33 (Fig. 2ab). There was an immediate steep increase in N_2O fluxes from the soil soon after N-fertilisation and after imposing the flooding compared to the control treatment (Fig. 2c), with an N_2O flux peak reaching $74 \mu\text{g } N_2O \text{ m}^{-2} \text{ h}^{-1}$. Flux rates then declined to background values by day 20. After ceasing the flooding (surface and leachate water removed), there was a slight increase in N_2O fluxes. Fluxes from the flooding treatments remained above those of the control ($P < 0.05$) until the end of the experiment on day 67.

3.2. Flooding increased N cycling and soil microbial communities

Cumulative N_2O fluxes were significantly greater in the flooding

Table 2

The effect of flood (FLOOD), legume presence (LEG) and their interaction (FLOOD x LEG) on cumulative N₂O fluxes, plant N uptake, above- and below-ground biomass, soil NH₄⁺-N and NO₃⁻-N and NH₄⁺-N and NO₃⁻-N leaching. Data are mean ± SE (flood: n = 55, leg: n = 80, non-leg: n = 30). Significance tests using likelihood ratio test (LRT) comparing models with or without parameters of interest, where degree of freedom shows the difference in degrees of freedom between the models. Significant effects ($P < 0.05$) are shown in bold.

	Cumulative N ₂ O fluxes mg m ⁻²	Plant N uptake g N m ⁻²	Above-ground biomass g m ⁻²	Below-ground biomass g m ⁻²	Soil NH ₄ ⁺ -N mg kg ⁻¹	Soil NO ₃ ⁻ -N mg kg ⁻¹	NH ₄ ⁺ -N leaching g m ⁻²	NO ₃ ⁻ -N leaching g m ⁻²
FLOOD								
Control	2.01 ± 1.7	2.7 ± 0.2	251.4 ± 10.4	170.2 ± 11.2	7.1 ± 0.4	3.6 ± 0.2	0.004 ± 0.0009	0.49 ± 0.08
Flood	18.9 ± 2.8	2.1 ± 0.1	253.5 ± 13.3	194.7 ± 14.1	10.2 ± 0.9	3.6 ± 0.3	0.022 ± 0.002	0.59 ± 0.09
LEG								
Non-leg	2.2 ± 3.2	2.6 ± 1.8	266.9 ± 12.1	124.4 ± 4.0	8.2 ± 1.1	2.3 ± 0.1	0.008 ± 0.002	0.09 ± 0.04
Leg	13.5 ± 2.1	2.3 ± 0.1	247.0 ± 10.6	205.1 ± 11.3	8.9 ± 0.6	4.1 ± 0.2	0.02 ± 0.002	0.72 ± 0.07
FLOOD	LRT=25.9, P<0.0001	LRT=11.6, P=0.0007	LRT = 0.4, P = 0.50	LRT = 1.0, P = 0.30	LRT=15.3, P=0.0001	LRT = 1.6, P = 0.21	LRT=35.9, P<0.0001	LRT = 0.01, P = 0.91
LEG	LRT=10.6, P=0.001	LRT = 2.6, P = 0.11	LRT=3.8, P=0.05	LRT=32.3, P<0.0001	LRT = 1.07, P = 0.30	LRT=45.4, P<0.0001	LRT = 2.4, P = 0.12	LRT=46.9, P<0.0001
FLOOD*LEG	LRT = 0.44, P = 0.59	LRT = 0.22, P = 0.64	LRT = 1.5, P = 0.22	LRT = 0.5, P = 0.47	LRT = 0.60, P = 0.44	LRT = 2.9, P = 0.08	LRT = 3.9, P=0.05	LRT = 0.72, P = 0.39

Table 3

The effect of flood (FLOOD), legume presence (LEG) and their interaction (FLOOD x LEG) on the relative abundance of *nirK*, *nirS*, *nirS/nirK*, AOA, AOB, AOA/AOB, *nosZI*, *nosZII* and *nosZI/nosZII*. Data are mean ± SE (flood: n = 55, leg: n = 80, non-leg: n = 30). Significance tests using likelihood ratio test (LRT) comparing models with or without parameters of interest, where degree of freedom shows the difference in degrees of freedom between the models. Significant effects ($P < 0.05$) are shown in bold.

	<i>nirK</i>	<i>nirS</i>	<i>nirS/nirK</i>	AOA	AOB
FLOOD					
Control	11.0 ± 0.2	6.2 ± 0.1	57.0 ± 0.8	0.3 ± 0.02	0.8 ± 0.03
Flood	11.7 ± 0.3	7.3 ± 0.3	62.1 ± 2.2	0.4 ± 0.03	0.7 ± 0.03
LEG					
Non-leg	11.7 ± 0.4	6.7 ± 0.3	57.5 ± 1.3	0.3 ± 0.02	0.7 ± 0.04
Leg	11.2 ± 0.2	6.8 ± 0.2	60.3 ± 1.5	0.3 ± 0.02	0.8 ± 0.03
FLOOD	LRT = 4.7, P=0.03	LRT = 14.1, P=0.0002	LRT = 7.5, P=0.006	LRT = 6.9, P=0.001	LRT = 5.3, P=0.02
LEG	LRT = 1.9, P = 0.16	LRT = 0.02, P = 0.89	LRT = 2.3, P = 0.12	LRT = 2.1, P = 0.14	LRT = 3.6, P = 0.05
FLOOD x LEG	LRT = 0.001, P = 0.93	LRT = 0.01, P = 0.92	LRT = 0.01, P = 0.9	LRT=3.6, P=0.05	LRT = 0.004, P = 0.94
	AOA/AOB	<i>nosZI</i>	<i>nosZII</i>	<i>nosZI/nosZII</i>	
FLOOD					
Control	37.9 ± 2.7	4.9 ± 0.1	1.2 ± 0.03	4.1 ± 0.1	
Flood	56.5 ± 4.2	5.7 ± 0.1	1.4 ± 0.04	4.4 ± 0.1	
LEG					
Non-leg	46.1 ± 4.2	5.2 ± 0.2	1.3 ± 0.04	4.2 ± 0.2	
Leg	47.6 ± 3.3	5.4 ± 0.1	1.3 ± 0.03	4.3 ± 0.1	
FLOOD	LRT = 13.3, P=0.0003	LRT = 17.5, P<0.0001	LRT = 5.3, P=0.02	LRT = 1.6, P = 0.21	
LEG	LRT = 0.00, P = 0.98	LRT = 1.4, P = 0.24	LRT = 0.62, P = 0.43	LRT = 0.12, P = 0.72	
FLOOD x LEG	LRT = 2.5, P = 0.11	LRT = 0.9, P = 0.34	LRT = 0.01, P = 0.91	LRT = 0.35, P = 0.54	

than in the control (non-flooded) treatments, while flooding decreased N uptake by the plants ($P < 0.05$, Fig. 3ab). Above- and below-ground biomass were unaffected by flooding ($P > 0.05$, Fig. 3cd). Soil and leachate NH₄⁺-N levels increased under flooding ($P < 0.05$, Fig. 3eg), while soil and NO₃⁻-N leaching were unaffected by flooding ($P > 0.05$, Fig. 3fh). Flooding also significantly increased the relative abundance of archaeal ammonia oxidisers (AOA) and denitrifiers (*nirK*, *nirS*, *nosZI*, *nosZII*) ($P < 0.05$, Fig. 4), but decreased the abundance of bacterial ammonia oxidisers (AOB) ($P < 0.05$, Fig. 4e). The *nirS/nirK* and AOA/AOB ratios were significantly increased by flooding ($P < 0.05$, Fig. 4cf), while no effect was observed on the *nosZI/nosZII* ratio ($P > 0.05$, Fig. 4i).

3.3. Legumes affected N₂O fluxes and NO₃⁻-N leaching, but not the abundances of N cycling microbial communities, irrespective of flooding

Plant communities with legumes showed significantly higher cumulative N₂O fluxes and NO₃⁻-N leaching compared to plant communities with only grasses, whereas plant N uptake did not significantly differ between the plant communities (Table 2). Above-ground biomass was lower in plant communities with legumes, while below-ground biomass was significantly higher ($P < 0.05$, Table 2). Levels of soil NH₄⁺-N were not different between plant communities with vs without legumes, but levels of soil NO₃⁻-N and NO₃⁻-N leaching were significantly higher with legumes ($P < 0.05$, Table 2). Presence of legumes had no effect on the relative abundances of N cycling microbes (Table 3). However, under flooding conditions legume presence tended to increase the abundance of AOA (FLOOD × LEG: $P = 0.05$, Fig. S3, Table 3), but this increase in AOA was not related to an increase in N₂O fluxes (Fig. S4). The relationship between cumulative N₂O fluxes and AOA abundance was positive in plant communities with only grasses, under both control and flooding conditions. However, in plant communities with legumes subjected to flooding, cumulative N₂O fluxes and AOA abundance were negatively related ($P < 0.05$, Fig. S4).

3.4. Plant communities as a driver of N cycling

There was an interaction between plant communities and flooding affecting cumulative N₂O fluxes (PLANTCOM × FLOOD, $P < 0.05$, Fig. 5a, Table S2). Flooding significantly increased cumulative N₂O fluxes (compared to the control) in the *T. pratense* and *L. corniculatus* monocultures, the legume mixture (*T. pratense* + *L. corniculatus*), and in the grass mixture *F. arundinacea* + *L. perenne*, but not in the grass-legume mixtures (Fig. 5a). Either in the control or flooding treatments, *L. corniculatus* combined with the slow-growing grass (*F. arundinacea*) reduced N₂O fluxes considerably compared to *L. corniculatus* monoculture ($P < 0.05$, Fig. 5a), and showed low N₂O fluxes overall.

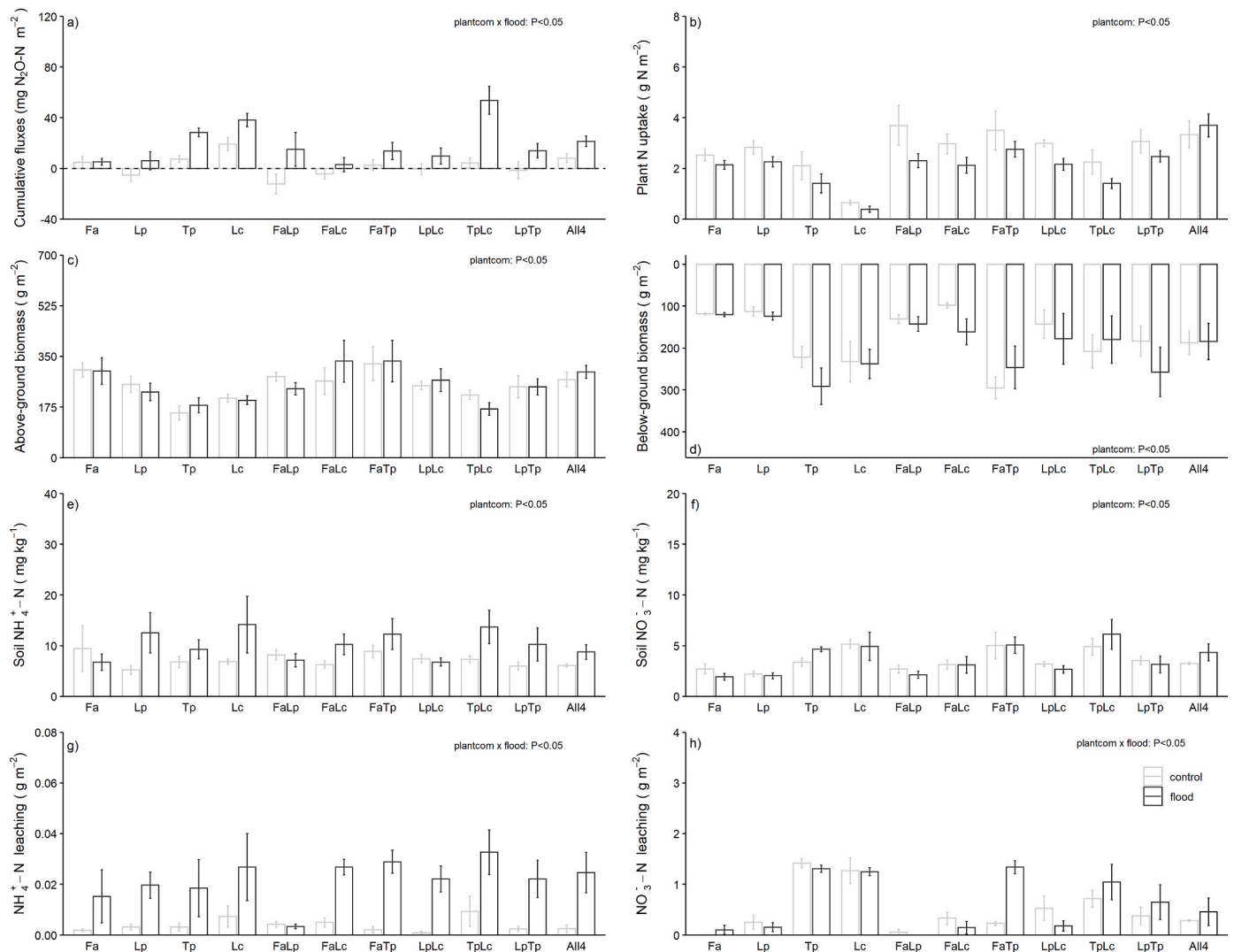


Fig. 5. The interactive effect of flood and plant communities on a) cumulative N_2O fluxes, b) plant N uptake, c) above-ground biomass (cumulative for the three harvests), d) below-ground biomass, e) soil $\text{NH}_4^+\text{-N}$, f) soil $\text{NO}_3^-\text{-N}$ for T1, g) $\text{NH}_4^+\text{-N}$ leaching, and h) $\text{NO}_3^-\text{-N}$ leaching. Bars are mean \pm SE ($n = 5$). Significant differences between plant communities and flooding ($P < 0.05$) based on Tukey posthoc test (ns: non-significant). *Lolium perenne* (Lp), *Festuca arundinacea* (Fa), *Lotus corniculatus* (Lc), *Trifolium pratense* (Tp).

There were some differences between the different plant communities in relation to plant N uptake, and above- and below-ground biomass (PLANTCOM, $P < 0.05$, Fig. 5b,c,d, Table S2). Legume monocultures had the lowest, while the four-species mixture had the highest plant N uptake (Fig. 5b). Above-ground biomass was largest in monocultures of *F. arundinacea*, and in mixtures containing this grass species (*F. arundinacea* + *L. perenne*, *F. arundinacea* + *T. pratense* and the four-species mixture) (Fig. 5c). In contrast, below-ground biomass was largest in monocultures of *T. pratense* and in combinations of this legume species with grass (*F. arundinacea* + *T. pratense*) (Fig. 5d). Overall, the grasses had lower below-ground biomass compared to the legume monocultures (Fig. 5d).

Soil $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ levels were highest in the monoculture of *T. pratense* at T1 ($P < 0.05$, Fig. 5e,f, Table S2). The interaction between plant communities and flooding showed that $\text{NH}_4^+\text{-N}$ leaching under flooding conditions was increased in the plant communities of *F. arundinacea*, *T. pratense*, *L. corniculatus* and *F. arundinacea* + *L. perenne* (PLANTCOM \times FLOOD, $P < 0.05$, Fig. 5g, Table S2). Also for N leaching, there was a significant interactive effect between plant communities and flooding with lower $\text{NO}_3^-\text{-N}$ leaching losses in response to flooding in *L. perenne* + *L. corniculatus* and an increase in $\text{NO}_3^-\text{-N}$ leaching in

response to flooding in *F. arundinacea* + *T. pratense* (PLANTCOM \times FLOOD, $P < 0.05$, Fig. 5h, Table S2).

There were several significant differences in the relative abundance of the N cycling microbial communities in relation to plant communities, irrespective of flooding (Fig. 6, Table S3). AOB relative abundance was highest in the legume mixture (*T. pratense* + *L. corniculatus*) and lowest in the grass monoculture *L. perenne* and the grass legume mixture *L. perenne* + *L. corniculatus* ($P < 0.05$, Fig. 6e, Table S3). There were two significant interactions between plant communities and flooding: AOA/AOB relative abundance was greater in mixtures containing *F. arundinacea* (*F. arundinacea* + *T. pratense* and *F. arundinacea* + *L. perenne*) under flooding conditions as compared to all other plant communities without or with flooding (PLANTCOM \times FLOOD, $P < 0.05$, Fig. 6f, Table S3). The abundances of *nosZII* were highest in the legume monoculture *L. corniculatus* and lowest in the grass monoculture *L. perenne* and grass-legume mixture *F. arundinacea* + *L. corniculatus* ($P < 0.05$, Fig. 6h, Table S3).

3.5. Relationships between N cycling microbial communities and N pools

Cumulative N_2O fluxes were positively correlated to several N

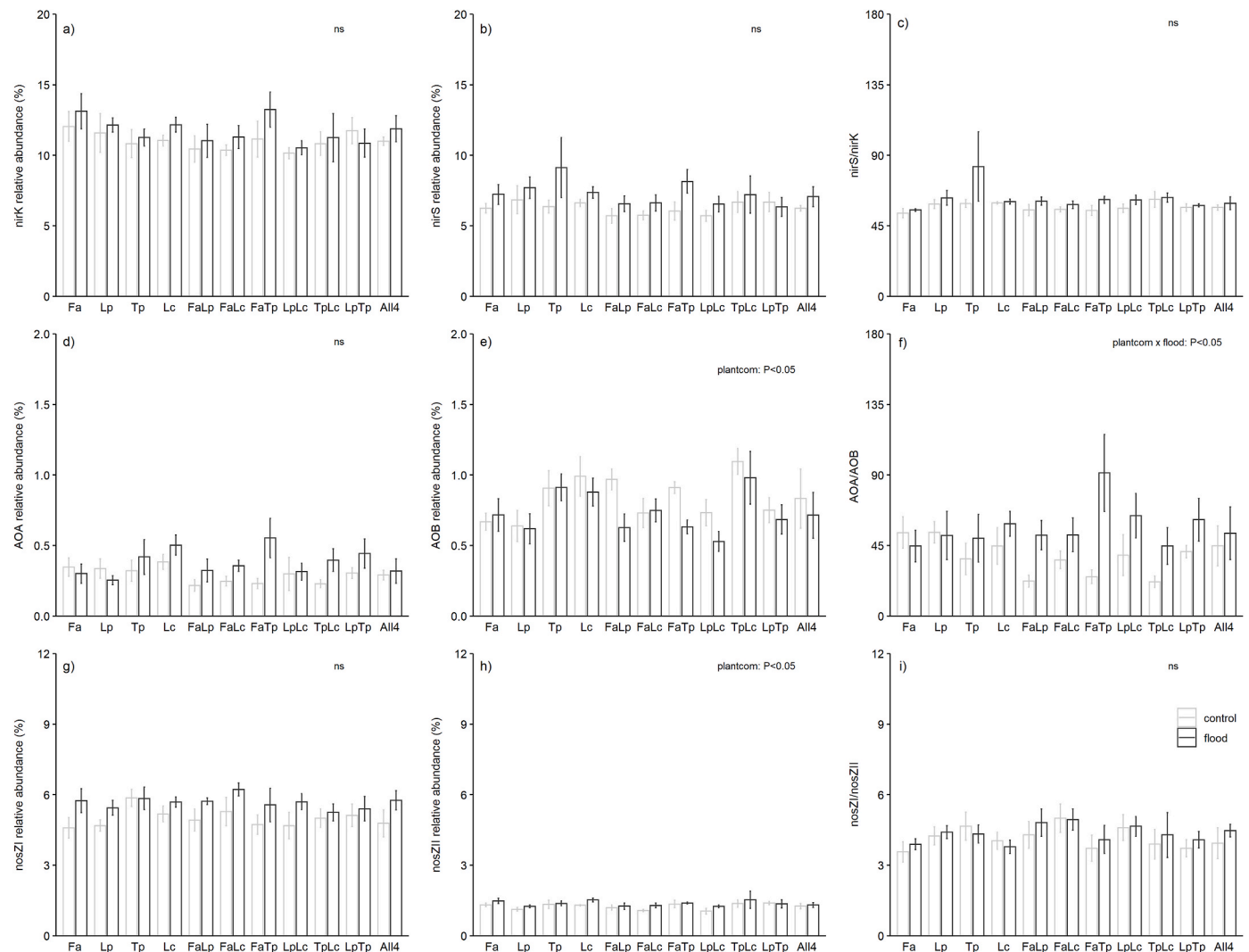


Fig. 6. The interactive effect of flood and plant communities on the relative abundance of a) *nirK*, b) *nirS*, c) *nirS/nirK*, d) AOA, e) AOB, f) AOA/AOB, g) *nosZI*, h) *nosZII* and i) *nosZI/nosZII*. Bars are mean \pm SE ($n = 5$). Significant differences between plant communities and flooding ($P < 0.05$) based on Tukey posthoc test (ns: non-significant). *Lolium perenne* (Lp), *Festuca arundinacea* (Fa), *Lotus corniculatus* (Lc), *Trifolium pratense* (Tp).

cycling microbial communities (*nirS*, *nosZI*, *nosZII*, AOA), and negatively related to plant N uptake, irrespective of flooding ($P < 0.05$, Fig. 7, Table S4). In control conditions, plant N uptake was negatively related to the relative abundances of AOB and *nosZI*, while under flooding there was no relation between plant N uptake and the relative abundances of the N-cycling microbes (Fig. 7, Table S4). Soil and leachate N pools were related to the relative abundances of AOA and AOB; NO_3^- -N leaching was positively related to AOB and AOA, and NH_4^+ -N leaching was positively related to AOA ($P < 0.05$, Fig. 7, Table S4). Soil moisture was positively related to cumulative N_2O fluxes, soil NH_4^+ -N, N in leachate, and relative abundances of denitrifiers (*nirK* and *nirS*) and AOB ($P < 0.05$, Fig. 7, Table S4).

4. Discussion

4.1. Understanding flooding effects on N cycling

Agreeing with our first hypothesis, flooding modified N pools and the abundance of N cycling microbial communities; flooding increased N_2O fluxes and the relative abundance of denitrifiers and AOA, but decreased AOB. We also found the common pattern of higher N_2O fluxes as soil moisture increases (Brown et al., 2012; Liu et al., 2015). This pattern is more commonly observed under relatively high water-filled pore space

(WFPS > 70%), and is often attributed to the formation of anoxic soil microsites enhancing denitrification (Bollmann and Conrad, 1998; Bateman and Baggs, 2005). Therefore, the higher N_2O fluxes observed after flooding could be due to increases in denitrifier abundances as well as in denitrification activity. However, we found that the abundances of both N_2O producers (*nirK* and *nirS*) and N_2O reducers (*nosZI* and *nosZII*) were higher after flooding but, as we did not measure N_2 emissions directly, it is not possible to determine the extent to which the $\text{N}_2\text{O}:\text{N}_2$ denitrification end product ratio was affected by flooding.

The contrasting effects of flooding on bacterial (AOB) and archaeal ammonia-oxidisers (AOA) can be related to their different physiologies (Offre et al., 2014; Hink et al., 2018). Some studies indicate that AOA are more tolerant to low- O_2 conditions than AOB (French et al., 2012; Wang et al., 2015), which may explain the increase of AOA with flooding. We also found a negative correlation between soil moisture and AOB, with no effects on AOA abundances (Table S1). In agreement with our findings, Li et al. (2020) in a meta-analysis also found a decrease in AOB with increases in precipitation, and Horz et al. (2004) found a reduction in AOB abundance when high soil moisture limits O_2 diffusion through the soil. In contrast, the stimulation of AOA abundance could be a result of the increase in substrate availability after the flood (i.e., increased soil NH_4^+ -N, Verhamme et al. (2011)) rather than higher soil moisture. It is often assumed that the higher N_2O fluxes induced by floods are due to

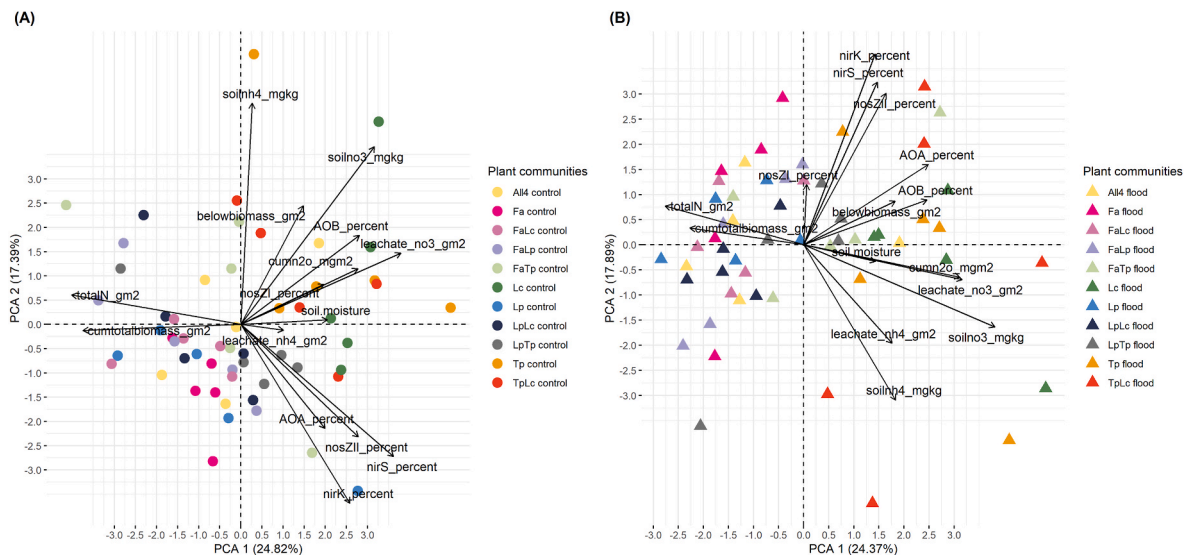


Fig. 7. PCA biplots of the N pools (Cum. N_2O , cumulative N_2O fluxes over the experimental period; Plant N uptake, above-ground T1 plant N uptake; Soil $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$; Leachate $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$, leachate collected at the end of the flooding event), N cycling microbial communities (*nirK*, *nirS*, *nosZI*, *nosZII*, AOA, AOB, *nirS/nirK*, *nosZI/nosZII* and AOA/AOB, microbial gene relative abundances and soil moisture during T1 (A) under control and (B) under flooding conditions. *Lolium perenne* (Lp), *Festuca arundinacea* (Fa), *Lotus corniculatus* (Lc), *Trifolium pratense* (Tp).

transient anaerobic conditions and changes in the availability of C and N (Khalid et al., 2019), but our results show that increased abundance of N_2O -producing communities may be an overlooked mechanism behind flood-induced N_2O fluxes, which could last longer than the flood itself.

4.2. Legumes augment N losses with no effect on the microbial communities

Partly in agreement with our second hypothesis, legumes augmented N losses via N_2O fluxes and N leaching under non-flooded and flooding conditions. The increase in N_2O fluxes due to legumes under control and flooding conditions in fertilised grasslands confirmed previous studies (Oram et al., 2020; Abalos et al., 2021; Cummins et al., 2021) and contradicted another (Barneze et al., 2020). The lack of legume effect on N_2O fluxes in the latter study could be explained by the short duration of that experiment, with not enough time for N fixation and/or for the fixed N to be released after decomposition. The increase in N leaching confirmed findings of studies with low-diversity grass-legume mixtures and high legume biomass (Scherer-lorenzen et al., 2003). The increase in N_2O fluxes and N leaching induced by legumes is normally associated with lower soil mineral N uptake compared to other plant functional groups, and larger inputs of N to the soil system by plant mortality and decomposition of their N-rich tissue (Table 3), rather than from the biological N fixation process itself (Rochette and Janzen, 2005).

Although we found an increase in N_2O fluxes with legumes, this could not be linked to an effect of legumes on denitrifier abundance. This contradicts the study of Abalos et al. (2021), which did find an increase in denitrifier abundances in plant communities with legumes. The reason might be that the samples in the experiment of Abalos et al. (2021) were collected two years instead of one year after the plants were sown, giving more time to the legumes to modify these N cycling microbial communities and processes. In addition, the temperature during our flooding experiment in the field was rather low (as it was autumn), which likely slowed down microbial respiration and growth (Sabey et al., 1959; Sánchez-Rodríguez et al., 2019). In the flooded plant communities, the higher soil $\text{NO}_3^-\text{-N}$ availability with legumes probably stimulated N_2O fluxes. Understanding to what extent the influence of legumes on N losses depends on their capacity to affect soil microbial communities will require dedicated experiments under controlled conditions, which can be later applied in long-term field experiments.

In terms of growth strategies, we found that the conservative legume species (*L. corniculatus*) had higher N_2O fluxes and lower plant N uptake compared to the acquisitive legume species (*T. pratense*), irrespective of flooding. This may be because despite the differences in N uptake, the root biomass was very similar for both legumes, and root biomass is an important driver of N_2O fluxes especially during floods (Oram et al., 2020). When *L. corniculatus* was combined with a slow-growing grass (*F. arundinacea* + *L. corniculatus*), N_2O fluxes, soil mineral N ($\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$) and *nosZII*-bacteria abundance were lower compared to the legume monoculture, probably because the grass was efficient in taking up N (Suter et al., 2015). Therefore, to promote N uptake and counteract N_2O fluxes and N leaching from fertilised grasslands that risk flooding, we recommend to grow legumes in combination with grasses, especially slow-growing legume species.

4.3. Effect of plant communities on N cycling

In general, our results confirm that higher plant N uptake can reduce N_2O fluxes (Fig. 3, Table 2) under current rainfall conditions (Abalos et al., 2014) by reducing soil mineral N available for denitrifiers. This pattern has also been observed in forest soils (Bohlen et al., 2001). Indeed, plant N uptake was negatively related to cumulative N_2O fluxes and also to soil $\text{NO}_3^-\text{-N}$ content and denitrifier abundance (Table S1). Our findings are in line with Moreau et al. (2015) who found that NO_3^- -reducing microorganisms may be adversely affected by plants with a high N uptake rate. This trend is also visible under flooding conditions in our experiment, contradicting results from earlier greenhouse experiments which did not find a relation between above-ground biomass and N_2O fluxes (Oram et al., 2020, 2021). These differences may be because our findings were obtained under more realistic field conditions, including differences in soil temperature between the studies (being lower in our field experiment) and a wider variation in climatic conditions. Additionally, our experiment included two legume species (with different growth strategies) in monocultures and also in grass-legume mixtures.

When the availability of soil mineral N limits plant growth, grass-legume mixtures often have higher plant N uptake compared to grass monocultures (Nyfeler et al., 2011; Suter et al., 2015). This is explained by several potential processes: symbiotic N_2 fixation by legumes which adds atmospheric N to the plant and soil N pool, N transfer from legumes

to grasses, increase soil exploitation by deep and shallow rooting and temporal niche complementarity (Suter et al., 2015). However, in our study plant N uptake was similar for grass-legume mixtures and grass or legume monocultures (Table 2). Yet, legumes in monoculture and in mixture without grasses increased N₂O fluxes and soil NO₃-N concentrations, suggesting that in these plant communities soil microbial communities were better competitors for N than the plants, or the plants were limited by other elements than N.

Overall, under control (non-flooded) conditions, grass and grass-legume mixtures with *F. arundinacea* (*F. arundinacea* + *L. perenne* and *F. arundinacea* + *T. pratense*) were able to achieve the highest plant N uptake with low N losses, whereas under flooding conditions the grass-legume mixtures containing the slow-growing legume species (*L. corniculatus*) reduced N₂O fluxes with higher plant N uptake (Fig. S5). Although the species used in our study are common for managed grasslands, we had only two grasses and two legumes, and therefore our results are difficult to generalise and to transfer to other fast-vs slow-growing grasses and N fixers. Therefore, follow-up studies are needed, in particular examining the responses under more extreme weather conditions and across seasons.

5. Conclusions

This study showed that flooding exerted considerable changes in N cycling by increasing N leaching, N₂O fluxes, shifting the abundances of N-cycling microbial communities and decreasing plant N uptake. However, plant communities with slow-growing strategy could ameliorate these flood effects by reducing N losses and enhancing plant N uptake under flooded conditions. Overall, our findings demonstrate that the composition of plant communities affects ecosystem functions as well as their ability to withstand extreme weather events. Given that extreme weather events are predicted to become more frequent and intense as a result of climate change, this is of relevance for grasslands management in the future.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.soilbio.2023.109142>.

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