

Plant and Soil

Nitrogen but not phosphorus addition affects symbiotic N₂ fixation in grasslands located on four continents --Manuscript Draft--

Manuscript Number:	PLSO-D-22-00086
Full Title:	Nitrogen but not phosphorus addition affects symbiotic N ₂ fixation in grasslands located on four continents
Article Type:	Research Article
Keywords:	Grasslands; Legumes; Nitrogen addition; Nutrient Network (NutNet); Phosphorus addition; Symbiotic N ₂ fixation; ¹⁵ N natural abundance method
Corresponding Author:	Eduardo Vázquez, Ph.D. Swedish University of Agricultural Sciences: Sveriges lantbruksuniversitet Uppsala, SWEDEN
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Swedish University of Agricultural Sciences: Sveriges lantbruksuniversitet
Corresponding Author's Secondary Institution:	
First Author:	Eduardo Vázquez, Ph.D.
First Author Secondary Information:	
Order of Authors:	Eduardo Vázquez, Ph.D. Per-Marten Schleuss Elizabeth T. Borer Miguel N. Bugalho Maria C. Caldeira Nico Eisenhauer Anu Eskelinen Philip A. Fay Sylvia Haider Anke Jentsch Kevin P. Kirkman Rebecca L. McCulley Pablo L. Peri Jodi Price Anna E. Richards Anita C. Risch Christiane Roscher Martin Schütz Eric W. Seabloom Rachel J. Standish Carly J. Stevens Michelle J. Tedder

	Risto Virtanen																						
	Marie Spohn																						
Order of Authors Secondary Information:																							
Funding Information:	<table border="1"> <tr> <td>Deutsche Forschungsgemeinschaft (SP1389/6-1)</td> <td>Dr. Marie Spohn</td> </tr> <tr> <td>National Science Foundation Research Coordination Network (NSF-DEB-1042132)</td> <td>Dr. Elizabeth T. Borer</td> </tr> <tr> <td>National Science Foundation Research Coordination Network (NSF-DEB-1234162)</td> <td>Dr. Elizabeth T. Borer</td> </tr> <tr> <td>National Science Foundation Research Coordination Network (NSF-DEB-1831944)</td> <td>Dr. Elizabeth T. Borer</td> </tr> <tr> <td>Institute on the Environment (DG-0001-13)</td> <td>Dr. Elizabeth T. Borer</td> </tr> <tr> <td>Bundesministerium für Forschung und Technologie (FKZ 031B0516C)</td> <td>Dr. Anke Jentsch</td> </tr> <tr> <td>Deutsche Forschungsgemeinschaft (FZT 118)</td> <td>Dr. Nico Eisenhauer</td> </tr> <tr> <td>Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa (DL 57/2016/CP1382/CT0030)</td> <td>Dr. Maria C. Caldeira</td> </tr> <tr> <td>Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa (UID/BIA/50027/2013)</td> <td>Dr. Maria C. Caldeira</td> </tr> <tr> <td>European Regional Development Fund (POCI-01-0145-FEDER-006821)</td> <td>Dr. Maria C. Caldeira</td> </tr> <tr> <td>Connecting Europe Facility (UID/AGR/00239/2019)</td> <td>Dr. Maria C. Caldeira</td> </tr> </table>	Deutsche Forschungsgemeinschaft (SP1389/6-1)	Dr. Marie Spohn	National Science Foundation Research Coordination Network (NSF-DEB-1042132)	Dr. Elizabeth T. Borer	National Science Foundation Research Coordination Network (NSF-DEB-1234162)	Dr. Elizabeth T. Borer	National Science Foundation Research Coordination Network (NSF-DEB-1831944)	Dr. Elizabeth T. Borer	Institute on the Environment (DG-0001-13)	Dr. Elizabeth T. Borer	Bundesministerium für Forschung und Technologie (FKZ 031B0516C)	Dr. Anke Jentsch	Deutsche Forschungsgemeinschaft (FZT 118)	Dr. Nico Eisenhauer	Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa (DL 57/2016/CP1382/CT0030)	Dr. Maria C. Caldeira	Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa (UID/BIA/50027/2013)	Dr. Maria C. Caldeira	European Regional Development Fund (POCI-01-0145-FEDER-006821)	Dr. Maria C. Caldeira	Connecting Europe Facility (UID/AGR/00239/2019)	Dr. Maria C. Caldeira
Deutsche Forschungsgemeinschaft (SP1389/6-1)	Dr. Marie Spohn																						
National Science Foundation Research Coordination Network (NSF-DEB-1042132)	Dr. Elizabeth T. Borer																						
National Science Foundation Research Coordination Network (NSF-DEB-1234162)	Dr. Elizabeth T. Borer																						
National Science Foundation Research Coordination Network (NSF-DEB-1831944)	Dr. Elizabeth T. Borer																						
Institute on the Environment (DG-0001-13)	Dr. Elizabeth T. Borer																						
Bundesministerium für Forschung und Technologie (FKZ 031B0516C)	Dr. Anke Jentsch																						
Deutsche Forschungsgemeinschaft (FZT 118)	Dr. Nico Eisenhauer																						
Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa (DL 57/2016/CP1382/CT0030)	Dr. Maria C. Caldeira																						
Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa (UID/BIA/50027/2013)	Dr. Maria C. Caldeira																						
European Regional Development Fund (POCI-01-0145-FEDER-006821)	Dr. Maria C. Caldeira																						
Connecting Europe Facility (UID/AGR/00239/2019)	Dr. Maria C. Caldeira																						
Abstract:	<p>Background and aims: The amount of nitrogen (N) derived from symbiotic N₂ fixation by legumes in grasslands might be affected by anthropogenic N and phosphorus (P) inputs, but the underlying mechanisms are not known.</p> <p>Methods: We evaluated symbiotic N₂ fixation in 17 grasslands on four continents that are subjected to the same full-factorial N and P addition experiment, using the ¹⁵N natural abundance method.</p> <p>Results: N as well as combined N and P (NP) addition reduced legume biomass by 65% and 45%, respectively, compared to the control, whereas P addition had no significant impact. Element addition had no significant effect on the symbiotic N₂ fixation per unit legume biomass. In consequence, the amount of N fixed annually per grassland area was less than half in the N addition treatments compared to control and P addition, irrespective of whether the dominant legumes were annuals or perennials.</p> <p>Conclusion: Our results reveal that N addition mainly impacts symbiotic N₂ fixation via reduced biomass of legumes rather than changes in N₂ fixation per unit legume biomass. The results show that soil N enrichment by anthropogenic activities significantly reduces N₂ fixation in the world's grasslands, and these effects cannot be reversed by additional P amendment.</p>																						
Suggested Reviewers:	<p>Wolfgang Wanek University of Vienna: Universitat Wien Wolfgang.wanek@univie.ac.at Use of stable isotopes in plants and soils</p> <p>Georg Cadisch University of Hohenheim: Universitat Hohenheim georg.cadisch@uni-hohenheim.de Expert in N₂ fixation by legumes</p> <p>Ken Giller</p>																						

Wageningen UR: Wageningen University & Research
ken.giller@wur.nl
Expert in N₂ fixation by legumes

William Burchill
Teagasc Food Research Centre Moorepark
william.burchill@teagasc.ie
Expert in N management in grassland and N₂ fixation by legumes

Andreas Lüscher
Agroscope
andreas.luescher@usys.ethz.ch
Expert in symbiotic N₂ fixation in grasslands



Dr. Eduardo Vázquez

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Department of Soil and Environment; Biogeochemistry of
Forest Soils
Lennart Hjelms väg 9,
750 07 Uppsala, Sweden.
eduardo.vazquez.garcia@slu.se,

To
Plant and Soil
Editorial Board

Dear Editor,

On behalf of all authors, I send you a manuscript titled:

**Nitrogen but not phosphorus addition affects symbiotic N₂ fixation in
grasslands located on four continents**

, for possible publication in Plant and Soil.

The main scientific question addressed by the study is how soil nitrogen (N) and phosphorus (P) enrichment affect the symbiotic N₂ fixation rate of legumes in grasslands on four continents. Up to now, no study has systematically explored how this affects symbiotic N₂ fixation in grasslands on different continents using a global experiment, as we do in this study.

This study shows that N addition mainly impacts symbiotic N₂ fixation via reduced biomass of legumes rather than changes in N₂ fixation per unit legume biomass. Further, the effect of N inputs cannot be alleviated by P inputs, irrespective of whether the grasslands are dominated by annual or perennial legumes.

We are looking forward to hearing from you on this manuscript.

Yours sincerely,
Eduardo Vázquez

[Click here to view linked References](#)

1 Nitrogen but not phosphorus addition affects symbiotic N₂ fixation in 2 grasslands located on four continents

3 Eduardo Vázquez^{1,2*}, Per-Marten Schleuss¹, Elizabeth T. Borer³, Miguel N. Bugalho⁴, Maria C. Caldeira⁵,
4 Nico Eisenhauer^{6, 7}, Anu Eskelinen^{6, 8, 9}, Philip A. Fay¹⁰, Sylvia Haider^{6, 11}, Anke Jentsch¹², Kevin P.
5 Kirkman¹³, Rebecca L. McCulley¹⁴, Pablo L. Peri¹⁵, Jodi Price¹⁶, Anna E. Richards¹⁷, Anita C. Risch¹⁸,
6 Christiane Roscher⁶, Martin Schütz¹⁸, Eric W. Seabloom², Rachel J. Standish¹⁹, Carly J. Stevens²⁰,
7 Michelle J. Tedder¹³, Risto Virtanen⁹, Marie Spohn^{1,2}

8 ¹Department of Soil Ecology, Bayreuth Center of Ecology and Environmental Research (BayCEER),
9 University of Bayreuth, Dr.-Hans-Frisch-Straße 1-3, 95448, Bayreuth, Germany

10 ²Department of Soil and Environment, Swedish University of Agricultural Sciences (SLU), Lennart
11 Hjelms väg 9, 75007 Uppsala, Sweden

12 ³Dept. of Ecology, Evolution, and Behavior, University of Minnesota, St. Paul, MN 55108, USA

13 ⁴Centre for Applied Ecology “Prof. Baeta Neves” (CEABN-InBIO), School of Agriculture, University of
14 Lisbon, 1349-017, Lisbon, Portugal

15 ⁵Forest Research Centre, School of Agriculture, University of Lisbon, 1349-017, Lisbon, Portugal

16 ⁶German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig; Puschstrasse 4, 04103
17 Leipzig, Germany

18 ⁷Institute of Biology, Leipzig University, Puschstrasse 4, 04103 Leipzig, Germany

19 ⁸Helmholtz Centre for Environmental Research (UFZ), Physiological Diversity, Permoserstrasse 15,
20 04318 Leipzig, Germany

21 ⁹Ecology & Genetics, University of Oulu, PO Box 3000, FI-90014, Oulu, Finland

22 ¹⁰USDA-ARS Grassland Soil and Water Research Laboratory, Temple, TX 76502, USA

23 ¹¹Martin Luther University Halle-Wittenberg, Institute of Biology / Geobotany and Botanical Garden,
24 Am Kirchtor 1, 06108 Halle, Germany

25 ¹²Disturbance Ecology, Bayreuth Center of Ecology and Environmental Research (BayCEER), University
26 of Bayreuth, Universitaetsstr. 30, 95447 Bayreuth, Germany

27 ¹³School of Life Sciences, University of KwaZulu-Natal, Carbis Road, 3209, Pietermaritzburg, South
28 Africa

29 ¹⁴Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY 40546, USA

30 ¹⁵Instituto Nacional de Tecnología Agropecuaria (INTA) - Universidad Nacional de la Patagonia Austral
31 (UNPA) - CONICET CC 332, (CP 9400), Río Gallegos, Santa Cruz, Argentina

32 ¹⁶Institute for Land, Water and Society, Charles Sturt University, Albury, New South Wales, 2640,
33 Australia

34 ¹⁷CSIRO Land and Water, 564 Vanderlin Drive, Berrimah, Northern Territory, 0828 Australia

35 ¹⁸Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Zuercherstrasse 111, 8903,
36 Birmensdorf, Switzerland

37 ¹⁹Harry Butler Institute, Murdoch University, 90 South Street, Murdoch, Western Australia 6150,
38 Australia

39 ²⁰Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, United Kingdom

40 **Corresponding author:**

41 Eduardo Vázquez.

42 Department of Soil and Environment, Swedish University of Agricultural Sciences (SLU), Lennart Hjelm
43 väg 9, 75007 Uppsala, Sweden

44 Email: eduardo.vazquez.garcia@slu.se

45 ORCID: 0000-0003-4662-1921

46 **Acknowledgements**

47 We thank Renate Krauss and Fabrizio Protti for laboratory assistance and the Analytical Chemistry
48 laboratory (CAN) of the Bayreuth Center of Ecological and Environmental Research (BayCEER) for
49 performing parts of the chemical analyses. In addition, we thank the stable isotope laboratory of
50 BayCEER for performing the isotope analyses. E.V. would like to thank Nikola Teutscherová for her
51 comments on a previous version of this manuscript. MCC and MNB thank to Companhia das Lezírias
52 for granting access to the study site.

53

54

55

56 **Abstract**

57 Background and aims:

58 The amount of nitrogen (N) derived from symbiotic N₂ fixation by legumes in grasslands might be
59 affected by anthropogenic N and phosphorus (P) inputs, but the underlying mechanisms are not
60 known.

61 Methods:

62 We evaluated symbiotic N₂ fixation in 17 grasslands on four continents that are subjected to the same
63 full-factorial N and P addition experiment, using the ¹⁵N natural abundance method.

64 Results:

65 N as well as combined N and P (NP) addition reduced legume biomass by 65% and 45%, respectively,
66 compared to the control, whereas P addition had no significant impact. Element addition had no
67 significant effect on the symbiotic N₂ fixation per unit legume biomass. In consequence, the amount of
68 N fixed annually per grassland area was less than half in the N addition treatments compared to control
69 and P addition, irrespective of whether the dominant legumes were annuals or perennials.

70 Conclusion:

71 Our results reveal that N addition mainly impacts symbiotic N₂ fixation via reduced biomass of legumes
72 rather than changes in N₂ fixation per unit legume biomass. The results show that soil N enrichment
73 by anthropogenic activities significantly reduces N₂ fixation in the world's grasslands, and these effects
74 cannot be reversed by additional P amendment.

75 **Keywords**

76 Grasslands; Legumes; Nitrogen addition; Nutrient Network (NutNet); Phosphorus addition; ¹⁵N
77 natural abundance method.

78 **1. Introduction**

79 Grasslands cover approximately 40% of the terrestrial ice-free surface of the Earth and provide diverse
80 ecosystem services including climate regulation, plant diversity maintenance and support for
81 pollinators while contributing to human nutrition (Lamarque et al. 2011). In particular, legumes are
82 one of the key plant functional groups in grasslands for their capacity to increase the nitrogen (N)
83 availability by symbiotic N₂-fixation, which, in turn, enhances the grassland net primary productivity,
84 mitigates environmental pollution, and increases forage quality and productivity, critical for livestock
85 production (Lüscher et al. 2014; Suter et al. 2015). However, anthropogenic N inputs (in the form of
86 fertilizers, manure, and atmospheric deposition) are changing the supply of N relative to phosphorus
87 (P) in grasslands (Peñuelas et al. 2013) which can affect the symbiotic N₂ fixation by legumes (Høgh-
88 Jensen et al. 2002; Carlsson and Huss-Danell 2003; Stevens et al. 2004; Craine and Jackson 2010). N
89 and P availability can affect symbiotic N₂ fixation by changing the legume biomass production in
90 grasslands and the contribution of N derived from symbiotic N₂ fixation to the total N content of
91 legumes (proportion of legume N derived from atmosphere, %Ndfa) (Høgh-Jensen et al. 2002; Carlsson
92 and Huss-Danell 2003; Nyfeler et al. 2011; Peoples et al. 2012). Therefore, understanding the effects
93 of N and P inputs on symbiotic N₂ fixation by legumes is crucial to maintain grassland biodiversity and
94 functionality.

95 Nitrogen inputs can affect the legume biomass production and the %Ndfa and thus, symbiotic N₂
96 fixation by legumes in grasslands (West et al. 2005; Nyfeler et al. 2011; Peoples et al. 2012; Oberson
97 et al. 2013). The %Ndfa often declines with availability of both ammonium and nitrate in soil (Leidi and
98 Rodríguez-Navarro 2000; Peoples et al. 2012), because symbiotic N₂ fixation is energetically expensive,
99 and legumes take up reactive N, if available. In addition, grasses and non-leguminous forbs often
100 displace legumes at high N availability due to their higher competitiveness for light (Soussana and
101 Tallec 2010; Tognetti et al. 2021). Thus, anthropogenic N inputs to grasslands, can lead to decreased
102 N₂ fixation by legumes affecting the grassland functioning.

103 Symbiotic N₂ fixation by legumes in grasslands can also be influenced by P inputs (Høgh-Jensen et al.
104 2002; Edwards et al. 2006). Low soil P levels reduce the activity of N₂-fixing legume-associated bacteria
105 (Edwards et al. 2006) due to the high ATP requirements of N₂ fixation (Valentine et al. 2017). At the
106 plant level, long-term P deprivation decreases nodular P concentration and reduces the energy status
107 of the nodules and their capacity to assimilate N₂, shifting the source of legume N nutrition from
108 atmospheric N₂ fixation towards uptake of reactive soil N (Valentine et al. 2017). Low P availability
109 could reduce the rate of N₂ fixation per unit legume biomass, removing the advantage that legumes
110 might have over non-N₂-fixing plants in N-poor conditions and reducing legume biomass production
111 (Edwards et al. 2006). Thus, it can be expected that P addition increases legume biomass production
112 and symbiotic N₂ fixation. Furthermore, it has been observed that simultaneous additions of N and P
113 (NP) can offset the negative effect of N addition on N₂ fixation resulting in higher symbiotic N₂ fixation
114 compared to only N addition in tropical leguminous trees (Zheng et al. 2016). However, a recent study
115 revealed that P addition enhances legume abundance in grasslands but does not mitigate the negative
116 N effect when both elements are added simultaneously (Tognetti et al. 2021). Nevertheless, the extent
117 to which the NP addition affects the N₂ fixation rates in grasslands remains to be studied.

118 Most studies on the effect of N and P addition on N₂ fixation in grasslands differ in experimental design,
119 climate, soil type, element addition rate, type of fertilizer used, and sampling procedure which leads
120 to biases and uncertainties hampering our understanding of the main drivers of symbiotic N₂ fixation
121 at the global scale (Zheng et al. 2019). Therefore, a standardized and globally replicated experiment is
122 needed to gain insight into N₂ fixation. Recent work using a standardized and globally replicated
123 experiment has shown that legume biomass production declines with N addition (Tognetti et al. 2021),
124 but the extent to which element addition affects symbiotic N₂ fixation rates was not evaluated.

125 Here, we use a standardized evaluation of symbiotic N₂ fixation in a globally coordinated grassland
126 experiment, replicated at sites spanning a wide range of climatic and edaphic characteristics, to shed
127 light on the response of symbiotic N₂ fixation in grasslands to N and P inputs. To evaluate the influence

128 of single and combined N and P additions under various environmental conditions, we studied
129 symbiotic N₂ fixation in 17 grassland sites on four continents that are part of the Nutrient Network
130 project (NutNet, <https://nutnet.org>) (Borer et al. 2014, 2017). At each of the 17 study sites, the
131 following four treatments were replicated three times: N addition, P addition, N and P addition, and a
132 control without element addition. We determined symbiotic N₂ fixation based on the natural
133 abundance of ¹⁵N in plant biomass. This approach relies on the distinct isotopic N signature of
134 atmospheric N₂ and reactive soil N, which affects the plant N isotopic signature, depending on the
135 source from which plants take up N (Amarger et al. 1979; Hoegberg 1997). We hypothesized that (i)
136 N addition leads to a reduction in symbiotic N₂ fixation since it decreases legume biomass and N₂
137 fixation per unit legume biomass, (ii) P addition enhances symbiotic N₂ fixation by increasing legume
138 biomass and N₂ fixation per unit legume biomass, and (iii) the combined application of N and P
139 increases N₂ fixation because it offsets the N-induced P deficiency caused by N application. Our study
140 goes far beyond the study by Tognetti *et al.* (2021) who only investigated legume biomass because it
141 explores the N₂ fixation rate.

142 **2. Material and methods**

143 **2.1. Study sites**

144 The 17 study sites (Table 1, Figure S1) are part of the Nutrient Network Global Research Cooperative
145 (NutNet, <https://nutnet.org>) (Borer et al. 2014, 2017) and were selected according to the criterion that
146 legumes were recorded in a minimum of six out of the 12 experimental plots (Table S1). The selected
147 sites are distributed across four continents (Table 1, Figure S1) covering a wide range of climatic
148 conditions: Mean annual temperature (MAT) ranged between -3.1 and 27.3 °C and the mean annual
149 precipitation (MAP) from 243 to 1222 mm. Sites were located between 51 and 2320 m above sea level
150 (Table 1).

151 An identical experiment is replicated at each site with four treatments: control (Ctrl; no element
152 addition), N addition (N; 100 kg N ha⁻¹yr⁻¹ as slow-release urea with δ¹⁵N close to 0‰ (Choi et al. 2017)),

153 P addition (P; 100 kg P ha⁻¹ yr⁻¹ as triple superphosphate (Ca(H₂PO₄)₂·H₂O)), and combined N and P
154 addition (NP). All treatments are replicated three times (n=3) at each site, and the experiments are
155 organized in a randomized block design with 25 m² plots (5 x 5 m). All sites follow the same protocol
156 and design (Borer et al. 2014).

157 The climatic data were derived from (Hijmans et al. 2005) based on the location of each site (Table 1).
158 Nitrogen deposition (as kg N ha⁻¹ yr⁻¹) was estimated based on the location of each site (longitude and
159 latitude) using the model output of Ackerman *et al.* (2019) for the year 2016 (Table 1). The soil
160 properties of the control treatment at the time of establishment of the experiment are summarized in
161 the Table S2. The methods used for soil analysis have been described in Seabloom *et al.* (2021).

162 **2.2. Plant sampling**

163 Aboveground biomass was sampled at the time of peak biomass using a standardized protocol (Borer
164 et al. 2014) between the years 2015 and 2020 (Table 1). Two 10 x 100 cm strips (covering area of 0.2
165 m²) of vegetation were clipped directly above the soil surface in a subplot of 1 x 1 m within each plot.
166 The clipped plant biomass was sorted into the three functional groups: grasses, non-leguminous forbs
167 and legumes, and oven-dried at 60°C to a constant mass prior to weighing. Hereafter, we will refer to
168 aboveground plant biomass as plant biomass. Representative subsamples of the biomass of the three
169 plant functional groups from all plots were sent to the University of Bayreuth (Germany) for further
170 analyses. The two most abundant grass, forb and legume species based on the cover estimates in the
171 control plots for each year of sampling at each site are shown in Table S3. In addition, the sites were
172 classified as grassland with perennial or annual legumes according to the life cycle (perennial or annual)
173 of the two most abundant legume species (Table S3).

174 **2.3. Plant C, N and P concentration and stable isotopes determination ($\delta^{15}N$)**

175 In total, 490 dried plant samples were processed. Plant biomass of each functional group was cut with
176 scissors, homogenized and ground in a ball mill. The total C and N concentration and the isotopic
177 composition were analyzed using continuous-flow isotope ratio mass spectrometry (NA 1108

178 elemental Analyzer, CE Instruments, Milano, Italy) coupled via ConFlo III open-split interface (Finnigan
179 MAT, Bremen, Germany) to a delta S isotope ratio mass spectrometer (Finnigan MAT, Bremen,
180 Germany) at the University of Bayreuth. The isotopic composition of N was expressed in δ notation,
181 which represents the ‰ of variation compared to the international standard for natural ^{15}N abundance
182 measurements (atmospheric N isotope ratio). In addition, the total P concentration of plant biomass
183 was determined by ICP-OES (Vista-Pro radial, Varian, Aschaffenburg, Germany) after nitric acid
184 digestion.

185 **2.4. Calculations**

186 The proportion of legume N in aboveground biomass derived from the atmosphere (%Ndfa), via N_2
187 fixation by legumes, was calculated following the approach described by Amarger et al. (1979) and
188 Hoegberg (1997) using equation 1:

$$189 \quad Ndfa (\%) = (\delta^{15}\text{N}_{reference} - \delta^{15}\text{N}_{legume}) / (\delta^{15}\text{N}_{reference} - B) \times 100 \quad (\text{Eq. 1}),$$

190 where $\delta^{15}\text{N}_{reference}$ is the $\delta^{15}\text{N}$ of a non-fixing reference plant, $\delta^{15}\text{N}_{legume}$ is the $\delta^{15}\text{N}$ of the legume
191 aboveground biomass (called legume $\delta^{15}\text{N}$ hereafter) in the evaluated plot, and B is the legume $\delta^{15}\text{N}$
192 fully relying on atmospheric N_2 fixation which accounts for any internal isotopic fractionation of the
193 legume (Hoegberg 1997). We considered the mean of the $\delta^{15}\text{N}$ of the two non-fixing functional groups
194 (grasses and forbs) as the reference, which was calculated separately for each plot at each site (and is
195 called reference $\delta^{15}\text{N}$ hereafter).

196 We used the lowest legume $\delta^{15}\text{N}$ value of all plots at each site as the B value (Eq. 1), similar to previous
197 studies (Hansen and Vinther 2001; West et al. 2005; Roscher et al. 2011; Oberson et al. 2013). This
198 approach relies on the assumption that the legumes in the plot with the lowest legume $\delta^{15}\text{N}$ receive
199 100% of the N from symbiotic N_2 fixation. We used the lowest legume $\delta^{15}\text{N}$ from all four treatments
200 because the legume $\delta^{15}\text{N}$ was not significantly affected by element addition (see below in the Results

201 section), similar to previous studies that considered different element addition treatments (Oberson
202 et al. 2013; Tzanakakis et al. 2017).

203 We consider the isotopic signature of the added N does not bias the estimation of %Ndfa since %Ndfa
204 is calculated based on the difference in the isotope signature of N in legume and reference of the same
205 plot growing in the same soil under the same conditions. Further, Eq. 1 calculates the difference of the
206 isotope signature of legume and reference not in absolute terms but relative to the difference between
207 the isotopic signature of the reference and the legume with the lowest $\delta^{15}\text{N}$ value at each site (B).

208 We detected a relative ^{15}N -depletion in reference $\delta^{15}\text{N}$ compared to legume $\delta^{15}\text{N}$ (i.e., lower reference
209 $\delta^{15}\text{N}$ than legume $\delta^{15}\text{N}$) at seven sites [Koffler (Canada), Hopland, Spindletop and Bunchgrass (USA),
210 Bad Lauchstädt and Bayreuth (Germany) and Val Mustair (Switzerland)] which challenged the
211 estimation of %Ndfa using Eq. 1 because it resulted in negative %Ndfa estimates. We observed a
212 decrease in reference $\delta^{15}\text{N}$ (mean of grasses and forbs) with increasing elevation of the study site
213 ($r^2=0.301$, $p=0.024$, Figure S2), and no significant relationship between elevation and legume $\delta^{15}\text{N}$
214 ($p=0.337$). This observation is consistent with previous studies, showing that $\delta^{15}\text{N}$ of non-fixing plants
215 decreases with increasing elevation (Jacot et al. 2000; Craine and Lee 2003; Huber et al. 2007; Zhou et
216 al. 2016). We adjusted the reference $\delta^{15}\text{N}$ for the effect of elevation (elevation adj. $\delta^{15}\text{N}_{\text{reference}}$),
217 assuming that all sites would be located at an elevation of 0 m a.s.l., using the slope of the regression
218 line describing the relationship between the $\delta^{15}\text{N}$ of the reference and elevation (see Figure S2), as:

$$219 \text{ Elevation adj. } \delta^{15}\text{N}_{\text{reference}} (\text{‰}) = \delta^{15}\text{N}_{\text{reference}} (\text{‰}) - [-0.002 * \text{Elevation (m)}] \quad (\text{Eq. 2}).$$

220 Next, Ndfa (%) was calculated based on the Elevation adj. $\delta^{15}\text{N}_{\text{reference}}$ as follows:

$$221 \text{ Ndfa (\%)} = (\text{Elevation adj. } \delta^{15}\text{N}_{\text{reference}} - \delta^{15}\text{N}_{\text{legume}}) / (\text{Elevation adj. } \delta^{15}\text{N}_{\text{reference}} - B) \times 100 \quad (\text{Eq. 3}).$$

222 Further details about the elevation adjustment and %Ndfa calculation are presented as Supporting
223 Information.

224 Legume N uptake was calculated based on the legume N concentration at peak biomass as:

225 $Legume\ N\ uptake\ (mg\ N\ g^{-1}yr^{-1}) = legume\ N\ concentration\ (mg\ N\ g^{-1}) \times yr^{-1}$ (Eq. 4).

226 N_2 fixation per legume biomass was calculated for the plots with legumes as:

227 $N_2\ fixation\ per\ legume\ biomass\ (mg\ N\ g^{-1}yr^{-1}) = Legume\ N\ uptake\ (mg\ N\ g^{-1}yr^{-1}) \times Ndfa\ (\%) \times 0.01$
228 (Eq. 5).

229 Symbiotic N_2 fixation per area grassland was calculated as follows:

230 $N_2\ fixation\ per\ area\ (g\ N\ m^{-2}\ yr^{-1}) = legume\ biomass\ (g\ m^{-2}) \times N_2\ fixation\ per\ legume\ biomass\ (mg\ N\ g^{-1}yr^{-1})$
231 (Eq. 6).

232 Symbiotic N_2 fixation per total grassland biomass (which grassland biomass being the sum of the
233 biomasses of all three functional groups) was calculated for all plots as:

234 $N_2\ fixation\ per\ total\ biomass\ (mg\ N\ g^{-1}\ yr^{-1}) = N_2\ fixation\ per\ area\ (g\ N\ m^{-2}\ yr^{-1}) \times 1000 / total\ biomass$
235 $(g\ m^{-2})$ (Eq. 7).

236 **2.5. Calculation and statistics**

237 We calculated means and standard errors of all four treatments across all 17 sites. If legumes were
238 absent in a plot, the legume N stock (in $g\ ha^{-1}$) or legume N uptake of this plot was assumed to be zero.

239 If the legume biomass was zero, we assumed that symbiotic N_2 fixation per unit biomass or area was
240 also zero. When calculating the mean of the N or P concentration of the biomass of all three functional
241 groups, we considered only plots with biomass of the respective functional group. Similarly, when
242 calculating the mean of symbiotic N_2 fixation per legume biomass, only plots with legume biomass > 0
243 and valid %Ndfa were considered. In contrast, when calculating symbiotic N_2 fixation per unit area of
244 grassland or total grassland biomass, all plots with legumes and valid %Ndfa as well as plots without
245 legumes were considered.

246 Data were analyzed using linear mixed models with the software SPSS 27 (IBM SPSS, Inc., Chicago,
247 USA). Before analysis, all variables except the $\delta^{15}\text{N}$ values (including both positive and negative values)
248 were log-transformed. The different element addition treatments (Ctrl, N, P, NP) and site- and plot-
249 level covariates were used as fixed factors, and block as a random factor where block was nested within
250 site. The site-level covariates included in the linear mixed model were MAP, MAT, water availability
251 index (MAP/potential evapotranspiration), the estimates of N deposition and the legume proportion
252 of biomass in the control treatments at each site. Because sites were set up in different years, the
253 number of years of element addition was considered as a site-level covariate, because the element
254 addition started in different years (redundant in most of the evaluated parameters with no significant
255 effect). Soil properties summarized in Table S2 (except total organic carbon (TOC), which was highly
256 correlated with total nitrogen (TN)) were included in the linear mixed model as plot-level covariates.
257 The interactions between treatment and the plot-level covariates were initially considered in the
258 model, although after a selection based on Akaike Information Criterion only the interactions
259 'treatment x TN' and 'treatment x soil pH' remained in the final model as covariates. When a significant
260 treatment effect ($p < 0.05$) was found, LSD post hoc test ($p < 0.05$) was used for comparison of means
261 of the element addition treatments.

262 Additionally, we evaluated how the life cycle of dominating legumes (perennial or annual) at each site
263 affected legume biomass, legume N concentration, legume $\delta^{15}\text{N}$, %Ndfa and symbiotic N_2 fixation per
264 area of grassland. A linear mixed model was used with treatment (Ctrl, N, P and NP), life cycle of
265 dominating legumes (annual or perennial), their interaction and the site- and plot-level covariates as
266 fixed factors, and block as a random factor nested within site. The model was performed as previously
267 described.

268 We calculated the response to nutrient addition of legume biomass, %Ndfa and N_2 fixation per unit
269 area as:

$$270 \text{ Response} = \text{Ln} ((Y_{\text{treatment}} + 1) / (Y_{\text{control}} + 1)) \quad (\text{Eq. 8}),$$

271 where $Y_{\text{treatment}}$ is the value of legume biomass, %Ndfa or N_2 fixation per unit area in the N, P or NP
272 addition treatment and Y_{control} is the mean value of legume biomass, %Ndfa or N_2 fixation per unit area
273 in the control. The response was calculated separately for each site. We added 1 to the numerator and
274 denominator to remove zeros before the logarithmic transformation.

275 We performed stepwise multiple regression analyses to evaluate the impact of site- and plot-level
276 covariates on the response of legume biomass, %Ndfa and N_2 fixation per unit area to nutrient addition.
277 Stepwise multiple regressions analyses were performed using the site-scale factors (MAP, MAT, water
278 availability, N deposition and the legume proportion of biomass in the control treatment at each site)
279 and plot-scale soil properties (TN, carbon-to-nitrogen ratio (C:N ratio), available P, and soil pH).
280 Collinearity was evaluated based on the variance inflation factor. The multiple regression analyses
281 were performed separately for the three different element addition treatments (i.e., N, P and NP).

282 **3. Results**

283 ***3.1. Plant aboveground biomass***

284 On average across sites, N and P addition increased total plant biomass by 32 and 28%, respectively,
285 compared to the control (Figure 1). The combined addition of N and P increased the total plant biomass
286 by 72%, from a mean of 3040 kg ha⁻¹ in the control to 5222 kg ha⁻¹ in the NP treatment. The biomass
287 of both the grasses and the forbs increased significantly with NP addition (Figure 1) but did not respond
288 significantly to N or P addition alone.

289 Legume biomass was highly variable among sites and ranged from 0.1 kg ha⁻¹ (Potrock, Argentina) to
290 1082 kg ha⁻¹ (Bad Lauchstädt, Germany) in the control treatment (Table S4). Compared to the control,
291 biomass of legumes was reduced by 65% and 45% in the N and NP treatments, respectively, while in
292 the P treatment was increased by 77% (although this difference was not statistically significant) (Figure
293 1). Compared to the P treatment, biomass of legumes was significantly reduced by 81% and 69% by N
294 and NP addition. The percent of legumes in the total biomass was 9.9% in the control, 4.7% in the N,

295 12.0% in the P, and 3.3% in the NP treatment (Figure 1). No significant interaction between the
296 treatments and the life cycle of dominant legumes (annual or perennial) was observed on legume
297 biomass, although legume biomass was significantly higher in the grasslands dominated by annual than
298 by perennial legumes (Figure S3A). Similar differences between P and N and NP treatments were
299 observed in the response ratio to nutrient addition of legume biomass (Figure S4A).

300 ***3.2. Nitrogen and phosphorus concentrations in aboveground biomass***

301 The addition of N significantly increased plant N concentrations (Fig. 2A), while the addition of P
302 significantly increased plant P concentrations (Fig. 2B) in both grasses and forbs compared to the
303 control. In contrast, legume N concentration was not affected by N addition, whereas legume P
304 concentration was enhanced by both the P and NP treatments (Figure 2A, B). No significant interaction
305 between the treatments and the life cycle of dominating legumes (annual or perennial) was observed
306 in legume N concentration (Figure S3B). The biomass N:P ratio in the control treatment was 8.5 for
307 grasses, 7.3 for forbs, and 13.9 for legumes (Figure 2C). The N:P ratio of legumes was significantly
308 reduced by the addition of P (N:P = 8.7) and NP (N:P = 7.9) compared to the control and the N treatment
309 (Figure 2C). Similarly, P addition decreased the N:P ratio of grasses and forbs (Figure 2C).

310 The legume N stock was significantly higher in the control (0.71 g N m⁻²) and P treatment (1.34 g N m⁻²)
311 than in the N (0.24 g N m⁻²) and NP (0.36 g N m⁻²) treatment (Figure S5A). The P stock of grasses and
312 forbs was increased by P and NP addition in comparison to the control, while the P stock of legumes
313 was only increased in the P treatment compared to the control (Figure S5B).

314 ***3.3. Plant isotopic composition***

315 We observed a decrease in $\delta^{15}\text{N}$ of the reference functional groups (grasses and forbs) in the control
316 treatment with increasing elevation of the study site ($r^2=0.301$, $p=0.024$, Figure S2). Therefore, we
317 adjusted the reference $\delta^{15}\text{N}$ of all plots and treatments for elevation (see section 2.4). After the
318 recalculation of the reference $\delta^{15}\text{N}$, the mean of the elevation-adjusted $\delta^{15}\text{N}_{\text{reference}}$ was +0.81‰ in the

319 control treatment (Table S5). The $\delta^{15}\text{N}_{\text{reference}}$ before and after the elevation-adjustment was
320 significantly higher in the N and NP treatments than in the control and P treatment (Table S5). The
321 legume $\delta^{15}\text{N}$ across all sites was unaffected by treatments (Table S5).

322 **3.1. Symbiotic N_2 fixation per unit legume biomass**

323 The mean %Ndfa in the control treatment was 65.8% across all 17 sites (Figure 3A). No significant
324 difference in %Ndfa among treatments was found. However, %Ndfa was slightly higher in the P and NP
325 treatments (69.8 and 70.9%, respectively) than in the control and N treatments (65.8 and 64.2%,
326 respectively). Similarly, no significant difference was observed in the %Ndfa response to nutrient
327 addition (Figure S4B). The response of %Ndfa to NP addition was positively related to the proportion
328 of legumes in the total biomass in the control treatment, initial soil N and water availability index, while
329 it was negatively related to N deposition (Table 2, Table S6). There were no significant linear regression
330 models ($p > 0.05$) for the %Ndfa response to N or P addition. We observed a significantly higher %Ndfa
331 in the ten sites dominated by perennial legumes (74.0%) compared to the seven sites dominated by
332 annual legumes (58.0%) (Figure 4). In addition, the interaction between treatment and life cycle of
333 dominating legumes revealed that the single N addition limited the differences in %Ndfa between
334 annual and perennial legumes (Figure 4).

335 Mean N_2 fixation per unit legume biomass in the control treatment across all 17 study sites was 18.1
336 $\text{mg N g}^{-1} \text{yr}^{-1}$ legume biomass (Figure 3B). No significant difference among treatments in N_2 fixation per
337 legume biomass was observed due to the lack of element addition effect on legume N concentration
338 and %Ndfa. However, N_2 fixation per unit legume biomass was slightly higher in the NP treatment (20.2
339 mg N g^{-1} legume biomass yr^{-1}) than in the other three treatments (Figure 3B).

340 **3.5. Symbiotic N_2 fixation per unit area grassland**

341 Symbiotic N_2 fixation per unit area in the control treatment across all 17 study sites was 3.5 kg N ha^{-1}
342 yr^{-1} (Figure 3C). It ranged from 0.002 $\text{kg N ha}^{-1} \text{yr}^{-1}$ (Potrock, Argentina) to 11.9 $\text{kg N ha}^{-1} \text{yr}^{-1}$ (Bad

343 Lauchstädt, Germany). Across all sites, N₂ fixation per area was 1.39 kg N ha⁻¹yr⁻¹ in the N treatment
344 and 2.13 kg N ha⁻¹yr⁻¹ in the NP treatment. Thus, N₂ fixation was significantly reduced in the N and NP
345 treatment by 60 and 39%, respectively, compared to the control. Similarly, N₂ fixation per unit area
346 was also 63% lower in the N and 43% lower in the NP treatment than in the P treatment (3.71 kg N ha⁻¹
347 yr⁻¹) (Figure 3C). In contrast, P addition had no significant effect on N₂ fixation per area compared to
348 the control. The number of years of element addition was a redundant site-level covariate in the linear
349 mixed model, indicating that the different ages of the sites did not influence the estimation of N₂
350 fixation in the present study. In addition, no significant effect of the life cycle of the dominant legumes
351 (annual or perennial) on N₂ fixation per area was observed (Figure S3D). Similarly, the response to N
352 and NP addition of the N₂ fixation per unit area was significantly lower than the response to P addition
353 (Figure S4C). Soil pH, N deposition, MAT and MAP were negatively related with the response of N₂
354 fixation per unit area to N addition ($r^2 = 0.550$, $p = 0.001$, Table 2, Table S6). In addition, the response
355 of N₂ fixation per unit area to P addition was negatively related with soil N ($r^2 = 0.162$, $p = 0.009$, Table
356 2, Table S6), while the response of N₂ fixation per unit area to NP addition was positively related with
357 soil N, MAT and N deposition ($r^2 = 0.406$, $p = 0.001$, Table 2, Table S6).

358 The mean N₂ fixation per unit total biomass in the control treatment was 1.23 mg N g⁻¹yr⁻¹ across all
359 17 study sites (Figure 3D). The N₂ fixation per total biomass was reduced by 60% under N and 73%
360 under NP addition compared to the control (Figure 3D). Similarly, N₂ fixation per total biomass was
361 lower in the N and NP treatments compared to the P treatment.

362 **4. Discussion**

363 Our results reveal that the addition of N decreased the rate of N₂ fixation per grassland area compared
364 to the control, and this effect was not reversed by additional P amendment (Figure 3C). The reduced
365 N₂ fixation per area grassland was caused by the reduction in legume biomass, and not by an altered
366 N₂ fixation rate per unit legume biomass. Tognetti et al. (2021) recently showed that legume biomass
367 was negatively affected by N addition in grasslands on several continents. Our study goes further,

368 demonstrating that this reduction in legume biomass causes the N₂ fixation rate per unit area to
369 decrease from 3.50 kg N ha⁻¹ yr⁻¹ to 1.39 kg N ha⁻¹ yr⁻¹ due to N addition across all sites. Further, we
370 found that N₂ fixation per legume biomass was not significantly affected by N or P addition, which is
371 an important finding since it suggests that the expression of the enzyme that fixes N₂ is not
372 downregulated in legumes in response to high availability of reactive N.

373 **4.1. Symbiotic N₂ fixation per area decreased by N addition**

374 We found that N addition significantly reduced the rate of symbiotic N₂ fixation per area of grassland
375 due to a reduction in legume biomass without effect on the N₂ fixation per unit legume biomass. The
376 negative effect of N addition on symbiotic N₂ fixation is consistent with previous single-site studies
377 observing a reduction in symbiotic N₂ fixation by N addition (West et al. 2005; Nyfeler et al. 2011;
378 Peoples et al. 2012; Burchill et al. 2014; Tzanakakis et al. 2017) and a recent study showing that also
379 non-symbiotic N₂ fixation in the soil is decreased by N addition at sites of the Nutrient Network
380 experiment (Schleuss et al. 2021). Our results suggest that continuous anthropogenic N enrichment of
381 grasslands can lead to a decrease in legume biomass production, which in the long-term can limit
382 symbiotic N₂ fixation. The most plausible explanation for reduced rates of N₂ fixation per unit area with
383 N addition is that higher soil N availability allows grasses and forbs to outcompete legumes (via
384 competition for light) and reduce legume biomass (Suding et al. 2005; Soussana and Tallec 2010;
385 Tognetti et al. 2021). The size of the response of symbiotic N₂ fixation per area to N addition was
386 affected by several abiotic factors. Addition of N reduced N₂ fixation per area more strongly at sites
387 with higher soil pH, atmospheric N deposition, MAT, and MAP (Table 2, Table S6). The reasons for this
388 could be that i) legumes and rhizobium strains from neutral and alkaline sites are less tolerant to soil
389 acidification caused by urea addition (Hungria and Vargas 2000), and that ii) N addition has a larger
390 effect at sites where the anthropogenic N input through atmospheric N deposition is already large, and
391 iii) N₂ fixation is more sensitive to N addition at sites where the N₂ fixation is not constrained by
392 temperature or water availability (Houlton et al. 2008; Tognetti et al. 2021).

393 In contrast to our second hypothesis, we observed similar rates of symbiotic N₂ fixation per area of
394 grassland in the control and P treatment, which can be attributed to a lack of P limitation of N₂ fixation
395 in the control treatment as further indicated by the low N:P ratio of legume biomass (13.9) (Güsewell
396 2004). The reason for the lack of effect to P addition is likely that legumes have evolved very effective
397 mechanisms to increase their P uptake from different soil P pools such as the release of phosphatases
398 or organic acids into the rhizosphere (Nuruzzaman et al. 2006). The negative relationship between the
399 response to P addition of N₂ fixation per area and soil N (Table 2, Table S6) might indicate that at N-
400 limited sites, legumes could invest the added P in symbiotic N₂ fixation to overcome the N limitation
401 (McKey 1994; Houlton et al. 2008; Soussana and Tallec 2010).

402 The application of P in combination with N did not counterbalance the negative impact of N addition
403 on symbiotic N₂ fixation per area, in contrast to our third hypothesis. Our results indicate that the
404 negative impact of N addition on symbiotic N₂ fixation per area is not a result of N-driven P deficiency
405 because in this case, combined addition of NP would have offset the negative impact of single N
406 addition. Thus, the addition of P does not seem to be a suitable strategy to enhance symbiotic N₂
407 fixation in a scenario of anthropogenic N enrichment of grasslands.

408 We observed no significant differences in the symbiotic N₂ fixation per area between the sites with
409 annual or perennial legumes, because the higher biomass at sites dominated by annual legumes was
410 counterbalanced by the higher %Ndfa of the sites dominated by perennials, irrespective of treatment
411 (Figure 4, Figure S3). This shows that there are no substantial differences in N₂ fixation on an area basis
412 between grasslands dominated by annual and perennial grasslands.

413 Across treatments, we found relatively low symbiotic N₂ fixation per area grassland (3.4 kg N ha⁻¹ yr⁻¹
414 in the control) compared to other studies (Carlsson and Huss-Danell 2003; Nyfeler et al. 2011; Peoples
415 et al. 2012; Oberson et al. 2013). The reason for this seems to be our focus on natural and semi-natural
416 grasslands with natural abundance of legumes (i.e. legumes were not deliberately introduced for the
417 study), and the inclusion of some sites with low overall biomass production. The rate of N addition

418 used in the present study ($100 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) exceeds any present and even projected atmospheric N
419 deposition levels (Ackerman et al. 2019). However, considering the common fertilization rates used in
420 managed grasslands ranging from 20-30 up to $400 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ (Oenema et al. 2012; Klaus et al. 2018),
421 the experimental rate used in our study mimics a realistic situation for many grasslands. The reduction
422 of symbiotic N_2 fixation in grasslands by N addition increases the dependence of grassland biomass
423 productivity on fertilization, which has several economic and environmental drawbacks (Lüscher et al.
424 2014).

425 ***4.2. Symbiotic N_2 fixation per unit legume biomass not affected by element addition***

426 Although relatively high rates of N and P were added, we found no significant response of N_2 fixation
427 per unit legume biomass to N, P or NP addition (Figure 3B). We found no significant effect of element
428 addition on N_2 fixation per legume biomass due to the lack of element addition effect on legume N
429 concentration and %Ndfa. Our finding is in disagreement with previous field studies in grasslands
430 reporting a positive effect of P addition and a negative effect of N addition on %Ndfa (Høgh-Jensen et
431 al. 2002; Carlsson and Huss-Danell 2003; West et al. 2005; Burchill et al. 2014; Tzanakakis et al. 2017).
432 The lack of response of N_2 fixation per unit legume biomass to P addition could indicate that symbiotic
433 N_2 fixation was not limited by P in the majority of grasslands included in the present study, as P addition
434 should increase N_2 fixation per unit biomass under strong P limitation due to the high ATP requirements
435 of N_2 fixation (Almeida et al. 2000; Høgh-Jensen et al. 2002; Edwards et al. 2006). Similarly, the lack of
436 differences between N and NP addition on N_2 fixation per unit biomass indicates that N addition did
437 not induce a P limitation of N_2 fixation as previously described in pot experiments or tree plantations
438 (Leidi and Rodríguez-Navarro 2000; Zheng et al. 2016). Otherwise, combined NP would have increased
439 the N_2 fixation per unit biomass compared to single N addition. The reason why N addition did not
440 cause a P limitation of N_2 fixation is likely that soil P availability is relatively high since the sites are
441 located in the temperate zone which is dominated by relatively young soils (Figure S1).

442 The lack of response of N₂ fixation per unit biomass to N addition contrasts with previous results
443 (Carlsson and Huss-Danell 2003) including experiments using urea as N source (Burchill et al. 2014), as
444 in our study. We speculate that N addition had no significant effect on N₂ fixation per unit biomass
445 because grasses and forbs were N limited (as indicated by the low N:P ratio of grasses and forbs in the
446 control treatment), and their efficient uptake of additional N reduced the availability of added N to
447 legumes, as described in previous studies (Nyfeler et al. 2011; Peoples et al. 2012; Oberson et al. 2013).
448 Another explanation might be that most legume species are permanent, rather than facultative N₂
449 fixers and cannot shift their N source in spite of increased soil N availability (Menge et al. 2009).

450 We observed a higher N₂ fixation per unit biomass in the grasslands dominated by perennial legumes
451 compared to the sites dominated by annual legumes. The reason for this could be that perennials can
452 build up a symbiosis with N₂ fixation that last for several years, whereas annuals have to establish a
453 new symbiosis with N₂ fixing microorganisms every year which makes this symbiosis likely less effective
454 (Primieri et al. 2022). The differences in N₂ fixation per unit biomass between annual and perennial
455 legume sites disappeared in the N treatment (Fig. 4D). This finding suggests that N addition has a very
456 similar effect on N₂ fixation per unit biomass irrespective of whether the grassland is dominated by
457 annual or perennial legumes.

458 **4.3. $\delta^{15}\text{N}$ patterns in plant functional groups**

459 We observed a significant negative correlation between site elevation and the $\delta^{15}\text{N}$ of the reference
460 plants (grass and forbs; Figure S2), which suggests low soil $\delta^{15}\text{N}$ at high elevations. Similar observations
461 were described in previous global reviews (Amundson et al. 2003) and in studies about altitudinal
462 gradients (Vitousek et al. 1989; Jacot et al. 2000; Craine and Lee 2003; Huber et al. 2007; Zhou et al.
463 2016). The underlying reason is the relationship between elevation and MAT and MAP, two of the main
464 drivers of plant $\delta^{15}\text{N}$ as described by Craine *et al.* (2009) and Zhou et al. (2016). The elevation-
465 dependent $\delta^{15}\text{N}$ of non-fixer plants (reference plants) likely caused difficulties when applying the ^{15}N
466 natural abundance method to determine symbiotic N₂ fixation about altitudinal gradients in previous

467 studies (Vitousek et al. 1989; Jacot et al. 2000). The unique global design of this study allowed us to
468 correct this elevation effect on reference plants and to determine symbiotic N₂ fixation across a large
469 number of sites on different continents. The relationship between elevation and δ¹⁵N of grasses and
470 forbs identified here will likely be of use also in future studies. However, external inputs of ¹⁵N-depleted
471 N, such as cattle urine, large inputs of legume-derived N or atmospheric N deposition cannot be
472 dismissed as another factor affecting δ¹⁵N of grasses and forbs (Jacot et al. 2000; Hansen and Vinther
473 2001; Gehring and Vlek 2004). Further details about the elevation adjustment and ¹⁵N natural
474 abundance method are presented as Supporting Information.

475 **5. Conclusions**

476 We found that N addition significantly decreased symbiotic N₂ fixation by legumes per area grassland
477 across 17 grasslands distributed in four continents, as hypothesised. This was caused exclusively by the
478 negative effect of N addition on legume biomass, and not by an effect on the N₂ fixation per unit
479 biomass. In contrast to our second hypothesis, P addition did not increase the symbiotic N₂ fixation per
480 area grassland due to the lack of effect on legume biomass and on N₂ fixation per unit legume biomass.
481 In addition, the application of P in combination with N did not counterbalance the negative impact of
482 N addition on symbiotic N₂ fixation per area. Further, the unique global design of this study allowed us
483 to derive an equation to correct for the effect of elevation on the isotope signature of N in grasses and
484 non-fixing forbs which will be useful in future studies. Taken together, our results show that soil N
485 enrichment by anthropogenic activities significantly reduces N₂ fixation in the world's grasslands, and
486 these effects cannot be reversed by additional P amendment. This reduction in symbiotic N₂ fixation
487 can ultimately change the ecological functioning of grasslands, affecting their net primary productivity
488 as well as their above and belowground biodiversity, forage quality and provision of ecosystem
489 services.

490 **References**

491

- 492 Ackerman D, Millet DB, Chen X (2019) Global Estimates of Inorganic Nitrogen Deposition Across Four
493 Decades. *Global Biogeochemical Cycles* 33:100–107. <https://doi.org/10.1029/2018GB005990>
- 494 Almeida JPF, Hartwig UA, Frehner M, et al (2000) Evidence that P deficiency induces N feedback
495 regulation of symbiotic N₂ fixation in white clover (*Trifolium repens* L.). *Journal of Experimental*
496 *Botany* 51:1289–1297. <https://doi.org/10.1093/jxb/51.348.1289>
- 497 Amarger N, Mariotti A, Mariotti F, et al (1979) Estimate of symbiotically fixed nitrogen in field grown
498 soybeans using variations in ¹⁵N Natural abundance. *Plant and Soil* 52:269–280.
499 <https://doi.org/10.1007/BF02184565>
- 500 Amundson R, Austin AT, Schuur EAG, et al (2003) Global patterns of the isotopic composition of soil
501 and plant nitrogen. *Global Biogeochemical Cycles* 17:. <https://doi.org/10.1029/2002GB001903>
- 502 Borer ET, Grace JB, Harpole WS, et al (2017) A decade of insights into grassland ecosystem responses
503 to global environmental change. *Nature Ecology and Evolution* 1:1–7.
504 <https://doi.org/10.1038/s41559-017-0118>
- 505 Borer ET, Harpole WS, Adler PB, et al (2014) Finding generality in ecology: A model for globally
506 distributed experiments. *Methods in Ecology and Evolution* 5:65–73.
507 <https://doi.org/10.1111/2041-210X.12125>
- 508 Burchill W, James EK, Li D, et al (2014) Comparisons of biological nitrogen fixation in association with
509 white clover (*Trifolium repens* L.) under four fertiliser nitrogen inputs as measured using two
510 ¹⁵N techniques. *Plant and Soil* 385:287–302. <https://doi.org/10.1007/s11104-014-2199-1>
- 511 Carlsson G, Huss-Danell K (2003) Nitrogen fixation in perennial forage legumes in the field. *Plant and*
512 *Soil* 253:353–372. <https://doi.org/10.1023/A:1024847017371>
- 513 Choi W, Kwak J, Lim S, et al (2017) Synthetic fertilizer and livestock manure differently affect $\delta^{15}\text{N}$ in
514 the agricultural landscape: A review. *Agriculture, Ecosystems & Environment* 237:1–15.
515 <https://doi.org/https://doi.org/10.1016/j.agee.2016.12.020>
- 516 Craine JM, Jackson RD (2010) Plant nitrogen and phosphorus limitation in 98 North American
517 grassland soils. *Plant and Soil* 334:73–84. <https://doi.org/10.1007/s11104-009-0237-1>
- 518 Craine JM, Lee WG (2003) Covariation in leaf and root traits for native and non-native grasses along
519 an altitudinal gradient in New Zealand. *Oecologia* 134:471–478.
520 <https://doi.org/10.1007/s00442-002-1155-6>
- 521 Edwards EJ, McCaffery S, Evans JR (2006) Phosphorus availability and elevated CO₂ affect biological
522 nitrogen fixation and nutrient fluxes in a clover-dominated sward. *New Phytologist* 169:157–
523 167. <https://doi.org/10.1111/j.1469-8137.2005.01568.x>
- 524 Gehring C, Vlek PLG (2004) Limitations of the ¹⁵N natural abundance method for estimating
525 biological nitrogen fixation in Amazonian forest legumes. *Basic and Applied Ecology* 5:567–580.
526 <https://doi.org/10.1016/j.baae.2004.09.005>
- 527 Güsewell S (2004) N:P ratios in terrestrial plants: Variation and functional significance. *New*
528 *Phytologist* 164:243–266. <https://doi.org/10.1111/j.1469-8137.2004.01192.x>

- 529 Hansen JP, Vinther FP (2001) Spatial variability of symbiotic N₂ fixation in grass-white clover pastures
530 estimated by the 15N isotope dilution method and the natural 15N abundance method. *Plant*
531 *and Soil* 230:257–266. <https://doi.org/10.1023/A:1010390901845>
- 532 Hijmans RJ, Cameron SE, Parra JL, et al (2005) Very high resolution interpolated climate surfaces for
533 global land areas. *International Journal of Climatology* 25:1965–1978.
534 <https://doi.org/10.1002/joc.1276>
- 535 Hoegberg P (1997) Tansley Review No. 95 15N natural abundance in soil-plant systems. *New*
536 *Phytologist* 137:179:203. <https://doi.org/https://doi.org/10.1046/j.1469-8137.1997.00808.x>
- 537 Høgh-Jensen H, Schjoerring JK, Soussana JF (2002) The influence of phosphorus deficiency on growth
538 and nitrogen fixation of white clover plants. *Annals of Botany* 90:745–753.
539 <https://doi.org/10.1093/aob/mcf260>
- 540 Houlton BZ, Wang YP, Vitousek PM, Field CB (2008) A unifying framework for dinitrogen fixation in
541 the terrestrial biosphere. *Nature* 454:327–330. <https://doi.org/10.1038/nature07028>
- 542 Huber E, Wanek W, Gottfried M, et al (2007) Shift in soil-plant nitrogen dynamics of an alpine-nival
543 ecotone. *Plant and Soil* 301:65–76. <https://doi.org/10.1007/s11104-007-9422-2>
- 544 Hungria M, Vargas MAT (2000) Environmental factors affecting N₂ fixation in grain legumes in the
545 tropics with an emphasis on Brazil. *Field crops research* 65:151–164.
546 [https://doi.org/10.1016/S0378-4290\(99\)00084-2](https://doi.org/10.1016/S0378-4290(99)00084-2)
- 547 Jacot KA, Lüscher A, Nösberger J, Hartwig UA (2000) Symbiotic N₂ fixation of various legume species
548 along an altitudinal gradient in the Swiss Alps. *Soil Biology and Biochemistry* 32:1043–1052.
549 [https://doi.org/10.1016/S0038-0717\(00\)00012-2](https://doi.org/10.1016/S0038-0717(00)00012-2)
- 550 Klaus VH, Kleinebecker T, Busch V, et al (2018) Land use intensity, rather than plant species richness,
551 affects the leaching risk of multiple nutrients from permanent grasslands. *Global Change*
552 *Biology* 24:2828–2840. <https://doi.org/10.1111/gcb.14123>
- 553 Lamarque P, Tappeiner U, Turner C, et al (2011) Stakeholder perceptions of grassland ecosystem
554 services in relation to knowledge on soil fertility and biodiversity. *Regional Environmental*
555 *Change* 11:791–804. <https://doi.org/10.1007/s10113-011-0214-0>
- 556 Leidi EO, Rodríguez-Navarro DN (2000) Nitrogen and phosphorus availability limit N₂ fixation in bean.
557 *New Phytologist* 147:337–346. <https://doi.org/10.1046/J.1469-8137.2000.00703.X>
- 558 Lüscher A, Mueller-Harvey I, Soussana JF, et al (2014) Potential of legume-based grassland-livestock
559 systems in Europe: A review. *Grass and Forage Science* 69:206–228.
560 <https://doi.org/10.1111/gfs.12124>
- 561 McKey D (1994) Legumes and nitrogen: The evolutionary ecology of a nitrogen-demanding lifestyle.
562 In: Sprent JI, McKey D (eds) *Advances in Legume Systematics 5: the Nitrogen Factor*. Royal
563 Botanic Gardens, Kew, pp 211–228
- 564 Menge DNL, Levin SA, Hedin LO (2009) Facultative versus obligate nitrogen fixation strategies and
565 their ecosystem consequences. *American Naturalist* 174:465–477.
566 <https://doi.org/10.1086/605377>

567 Nuruzzaman M, Lambers H, Bolland MDA, Veneklaas EJ (2006) Distribution of carboxylates and acid
568 phosphatase and depletion of different phosphorus fractions in the rhizosphere of a cereal and
569 three grain legumes. *Plant and Soil* 281:109–120. <https://doi.org/10.1007/s11104-005-3936-2>

570 Nyfeler D, Huguenin-Elie O, Suter M, et al (2011) Grass-legume mixtures can yield more nitrogen than
571 legume pure stands due to mutual stimulation of nitrogen uptake from symbiotic and non-
572 symbiotic sources. *Agriculture, Ecosystems and Environment* 140:155–163.
573 <https://doi.org/10.1016/j.agee.2010.11.022>

574 Oberson A, Frossard E, Bühlmann C, et al (2013) Nitrogen fixation and transfer in grass-clover leys
575 under organic and conventional cropping systems. *Plant and Soil* 371:237–255.
576 <https://doi.org/10.1007/s11104-013-1666-4>

577 Oenema J, van Ittersum M, van Keulen H (2012) Improving nitrogen management on grassland on
578 commercial pilot dairy farms in the Netherlands. *Agriculture, Ecosystems and Environment*
579 162:116–126. <https://doi.org/10.1016/j.agee.2012.08.012>

580 Peñuelas J, Poulter B, Sardans J, et al (2013) Human-induced nitrogen-phosphorus imbalances alter
581 natural and managed ecosystems across the globe. *Nature Communications* 4:1–10.
582 <https://doi.org/10.1038/ncomms3934>

583 Peoples MB, Brockwell J, Hunt JR, et al (2012) Factors affecting the potential contributions of N₂
584 fixation by legumes in Australian pasture systems. *Crop and Pasture Science* 63:759–786.
585 <https://doi.org/10.1071/CP12123>

586 Primieri S, Magnoli SM, Koffel T, et al (2022) Perennial, but not annual legumes synergistically benefit
587 from infection with arbuscular mycorrhizal fungi and rhizobia: a meta-analysis. *New Phytologist*
588 233:505–514. <https://doi.org/10.1111/NPH.17787>

589 Roscher C, Thein S, Weigelt A, et al (2011) N₂ fixation and performance of 12 legume species in a 6-
590 year grassland biodiversity experiment. *Plant and Soil* 341:333–348.
591 <https://doi.org/10.1007/s11104-010-0647-0>

592 Schleuss PM, Widdig M, Biederman LA, et al (2021) Microbial substrate stoichiometry governs
593 nutrient effects on nitrogen cycling in grassland soils. *Soil Biology and Biochemistry* 155:.
594 <https://doi.org/10.1016/j.soilbio.2021.108168>

595 Soussana JF, Tallec T (2010) Can we understand and predict the regulation of biological N₂ fixation in
596 grassland ecosystems? *Nutrient Cycling in Agroecosystems* 88:197–213.
597 <https://doi.org/10.1007/s10705-009-9335-y>

598 Stevens CJ, Dise NB, Mountford JO, Gowing DJ (2004) Impact of Nitrogen Deposition on the Species
599 Richness of Grasslands. *Science* 303:1876–1879. <https://doi.org/10.1126/science.1094678>

600 Suding KN, Collins SL, Gough L, et al (2005) Functional- and abundance-based mechanisms explain
601 diversity loss due to N fertilization. *Proceedings of the National Academy of Sciences of the*
602 *United States of America* 102:4387–4392. <https://doi.org/10.1073/pnas.0408648102>

603 Suter M, Connolly J, Finn JA, et al (2015) Nitrogen yield advantage from grass-legume mixtures is
604 robust over a wide range of legume proportions and environmental conditions. *Global Change*
605 *Biology* 21:2424–2438. <https://doi.org/10.1111/gcb.12880>

606 Tognetti PM, Prober SM, Báez S, et al (2021) Negative effects of nitrogen override positive effects of
607 phosphorus on grassland legumes worldwide. *Proceedings of National Academy of Sciences of*
608 *the United States of America* 118:. <https://doi.org/10.1073/pnas.2023718118>

609 Tzanakakis V, Sturite I, Dörsch P (2017) Biological nitrogen fixation and transfer in a high latitude
610 grass-clover grassland under different management practices. *Plant and Soil* 421:107–122.
611 <https://doi.org/10.1007/s11104-017-3435-2>

612 Valentine AJ, Kleinert A, Benedito VA (2017) Adaptive strategies for nitrogen metabolism in
613 phosphate deficient legume nodules. *Plant Science* 256:46–52.
614 <https://doi.org/10.1016/j.plantsci.2016.12.010>

615 Vitousek PM, Shearer G, Kohl DH (1989) Foliar ^{15}N natural abundance in Hawaiian rainforest:
616 patterns and possible mechanisms. *Oecologia* 78:383–388.
617 <https://doi.org/10.1007/BF00379113>

618 West JB, HilleRisLambers J, Lee TD, et al (2005) Legume species identity and soil nitrogen supply
619 determine symbiotic nitrogen-fixation responses to elevated atmospheric $[\text{CO}_2]$. *New*
620 *Phytologist* 167:523–530. <https://doi.org/10.1111/j.1469-8137.2005.01444.x>

621 Zheng M, Li D, Lu X, et al (2016) Effects of phosphorus addition with and without nitrogen addition on
622 biological nitrogen fixation in tropical legume and non-legume tree plantations.
623 *Biogeochemistry* 131:65–76. <https://doi.org/10.1007/s10533-016-0265-x>

624 Zheng M, Zhou Z, Luo Y, et al (2019) Global pattern and controls of biological nitrogen fixation under
625 nutrient enrichment: A meta-analysis. *Global Change Biology* 25:3018–3030.
626 <https://doi.org/10.1111/gcb.14705>

627 Zhou Y, Cheng X, Fan J, Harris W (2016) Patterns and controls of foliar nitrogen isotope composition
628 on the Qinghai-Tibet Plateau, China. *Plant and Soil* 406:265–276.
629 <https://doi.org/10.1007/s11104-016-2882-5>

630

631 **Statements and Declarations**

632 **Funding**

633 EV, PMS and MS have been funded by the German Research Foundation through the Emmy Noether-
634 program (grant SP1389/6-1). This work was generated using data from the Nutrient Network (NutNet,
635 <https://nutnet.org>) experiment, funded at the site-scale by individual researchers. Coordination and
636 data management have been supported by funding to E. Borer and E. Seabloom from the National
637 Science Foundation Research Coordination Network (NSF-DEB-1042132) and Long-Term Ecological
638 Research (NSF-DEB-1234162 and NSF-DEB-1831944 to Cedar Creek LTER) programs, and the Institute
639 on the Environment (DG-0001-13). We also thank the Minnesota Supercomputer Institute for hosting
640 project data and the Institute on the Environment for hosting Network meetings. AJ acknowledge the
641 German Ministry for Education and Research (BMBF) for funding this research within the Biodiversa
642 project SUSALPS; grant number: FKZ 031B0516C. NE and SH acknowledge support from the German
643 Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, funded by the German Research
644 Foundation (FZT 118). MNB also acknowledges funding through FCT contract DL
645 57/2016/CP1382/CT0030 and the COMPETE program through FEDER and FCT funding
646 UID/BIA/50027/2013 and POCI-01-0145-FEDER-006821 and CEF by UID/AGR/00239/2019.

647 **Competing interest**

648 The authors have no relevant financial or non-financial interests to disclose.

649 **Authors' contributions**

650 *EV, PMS and MSp conceived the ideas and designed methodology; EV, PMS and MSp collected the data;*
651 *EV, PMS and MSp analyzed the data; EV, PMS and MSp led the writing of the manuscript. All authors*
652 *contributed critically to the drafts and gave final approval for publication.*

653 **Data availability**

654 Data will be publicly available on the Environmental Data Initiative platform.

Table 1: Continent, country, region/state, site name, latitude and longitude, elevation, mean annual precipitation (MAP), mean annual temperature (MAT), potential evapotranspiration (PET), estimated atmospheric nitrogen deposition (Ndep), year of study establishment and seasons of element addition (i.e., seasons from the establishment to the sample collection) of the 17 NutNet sites included in the study.

Continent	Country	Region/State	Site	Latitude (°)	Longitude (°)	Elevation (m)	MAP (mm)	MAT (°C)	PET (mm)	Ndep Kg N ha ⁻¹	Year of establish.	Seasons of element addition
Africa	South Africa	KwaZulu-Natal	Mt. Gilboa	29.3 S	30.3 E	1748	867	13.1	1194	5.6	2010	10
Africa	South Africa	KwaZulu-Natal	Ukulunga	29.7 S	30.4 E	843	838	18.1	1393	5.6	2009	7
America	Argentina	Santa Cruz	Potrok Aike	51.0 S	70.4 W	150	243	6.3	2923	0.5	2015	2
America	Canada	Ontario	Koffler Reserve	44.0 N	79.5 W	301	834	6.4	835	10.0	2010	5
America	USA	California	Hopland REC	39.0 N	123.1 W	598	939	12.3	1194	2.5	2007	11
America	USA	Kentucky	Spindletop	38.1 N	84.5 W	271	1166	12.5	1139	10.7	2007	10
America	USA	Oregon	Bunchgrass	44.3 N	122.0 W	1318	2160	5.5	860	3.2	2007	11
America	USA	Texas	Temple	31.0 N	97.3 W	184	870	19.1	1463	7.5	2007	8
Europe	Finland	Lapland	Saana	69.0 N	20.8 E	600	400	-3.1	339	3.5	2014	3
Europe	Germany	Bavaria	Bayreuth	49.9 N	11.6 E	340	724	8.3	756	14.6	2016	3
Europe	Germany	Saxony-Anhalt	Bad Lauchstädt	51.4 N	11.9 E	51	489	8.9	117	14.8	2015	2
Europe	Germany	Thuringia	Jena	50.9 N	11.5 E	320	597	8.0	724	14.6	2013	4
Europe	Portugal	Ribatejo	Companhia das Lezírias	38.0 N	8.0 W	200	642	16.5	1220	3.0	2012	4
Europe	Switzerland	Graubünden	Val Mustair	46.6 N	10.4 E	2320	950	0.3	442	21.7	2008	7
Europe	United Kingdom	North West	Lancaster	54.0 N	2.6 W	180	1222	8.0	599	10.2	2008	9
Oceania	Australia	Northern Territory	Kidmand Springs	16.1 S	131 E	87	749	27.3	2046	1.9	2014	1
Oceania	Australia	Western Australia	Pingelly Paddock	32.5 S	117.0 E	338	446	16.2	1427	1.0	2013	2

Table 2: Regression models of the response of legume biomass, the proportion of N derived from atmosphere (%Ndfa), and symbiotic N₂ fixation per unit area to element addition (nitrogen (N), phosphorus (P) and their combined application (NP)) as a function of site-scale environmental factors (MAP, MAT, water availability (Aw), N deposition (Ndep) and legume proportion of biomass in the control treatment at each site (prop)) and plot-scale soil properties (total nitrogen (TN), soil carbon to nitrogen ratio (C:N), available phosphorus (P) and soil pH). A dash (-) indicates that no significant ($p < 0.05$) model was found)

Dependent variable (y)	Response to	Regression model	r ²	p-value
Response of legume biomass	N addition	$y = 5.792 - 0.752pH - 0.128MAT - 0.110Ndep - 2.150TN + 0.006P$	0.660	0.001
	P addition	$y = 2.360 - 6.091TN - 0.082 MAT$	0.431	0.001
	NP addition	$y = 1.570 - 6.009TN - 0.111MAT$	0.472	0.001
Response of %Ndfa	N addition	–	–	–
	P addition	–	–	–
	NP addition	$y = -0.061 + 0.007Prop - 0.027Ndep + 0.449TN + 0.073Aw$	0.613	0.001
Response of N ₂ fixation	N addition	$y = 3.389 - 0.481pH - 0.048Ndep - 0.036 MAT - 0.001 MAP$	0.550	0.001
	P addition	$y = 0.529 - 2.430TN$	0.186	0.009
	NP addition	$y = 0.817 - 2.403TN - 0.042MAT - 0.046Ndep$	0.406	0.001



Click here to access/download
colour figure
Fig 1.docx



Click here to access/download
colour figure
Fig 2.docx



Click here to access/download
colour figure
Fig 3.docx



Click here to access/download
colour figure
Fig 4.docx

