

Grassland productivity is limited by multiple nutrients

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Terrestrial ecosystem productivity is widely accepted to be nutrient limited¹. Although nitrogen is deemed a key determinant of aboveground net primary production^{2,3}, the prevalence of co-limitation by nitrogen and phosphorus is increasingly recognized⁴⁻⁸. However, the extent to which terrestrial productivity is co-limited by nutrients other than nitrogen and phosphorus, such as potassium and micronutrients, has remained unclear. Here, we report results from a standardized factorial nutrient addition experiment, in which we added nitrogen (N), phosphorus (P), and potassium combined with a selection of micronutrients ($K_{+\mu}$), alone or in concert, to 42 grasslands spanning five continents, and monitored aboveground net primary production. Nutrient availability limited productivity at 31 of the 42 grassland sites studied. Pairwise combinations of N, P, and $K_{+\mu}$ co-limited aboveground net primary production at 24 of the 42 sites, and nitrogen limitation peaked in cool, high latitude sites. Our findings indicate widespread variation in the combination of nutrients that limit aboveground grassland productivity, and highlight the importance of potassium and micronutrients, and an apparent absence of limitation by the nutrients we considered at some sites. This variation in nutrient limitation must be considered when assessing the ecosystem-scale consequences of nutrient enrichment.

1 Terrestrial ecosystem productivity is widely accepted to be nutrient limited¹, and many
2 studies have focused on limitation by a single nutrient, nitrogen (N)^{2,3}. In grasslands, and in
3 other systems, the role of additional nutrients is increasingly recognized. A recent meta-analysis
4 of 1400 N and phosphorus (P) fertilization studies⁴ showed limitation of terrestrial productivity
5 by both N and P. Furthermore, these nutrients often were synergistically co-limiting, where
6 together they limited productivity more than the sum of their individual limitations. This meta-
7 analysis is the most comprehensive assessment of ecosystem nutrient limitation to date.
8 However, the global extent and magnitude of multiple limitation by nutrients other than N and P
9 remains poorly understood in natural systems^{4,5,9}, including grasslands^{3,10}, a critically endangered
10 biome that accounts for approximately one-third of Earth's terrestrial net primary production¹¹.
11 More importantly, multiple nutrient limitation has not been experimentally tested in grasslands
12 on a global scale using a standardized experimental approach. The potential for synergistic co-
13 limitation of grasslands by multiple nutrients, or conversely, failing to account for the absence of
14 single or multiple nutrient limitation means we may misestimate the magnitude and extent of
15 nutrient limitation of terrestrial net primary productivity.

16 Humans now produce more reactive N than is produced from all natural terrestrial
17 sources, primarily as fertilizers, industrial products, and through fossil fuel combustion^{12,13}.
18 Anthropogenic increases in atmospheric N will result in further terrestrial N deposition, altering
19 ecosystem function^{12,14-17} and potentially increasing limitation by other nutrients, such as P,
20 potassium (K), or trace elements¹⁶. K occurs in high concentrations in plant tissues¹⁸ and its
21 uptake is correlated with that of other nutrients^{19,20}. Our understanding of limitation by nutrients
22 other than N in grasslands lags that of agro-ecosystems, where the importance of P, K, and
23 micronutrients is better understood²⁰.

24 Here we report the frequency, magnitude, and global extent of nutrient limitation of
25 grassland ANPP by N, P, and K combined with micronutrients ($K_{+\mu}$) at 42 grassland sites in the
26 Nutrient Network (NutNet; Supplementary Material and ²¹). All sites conducted the same
27 standardized N-P- $K_{+\mu}$ addition experiment, the definitive test for nutrient limitation. This
28 approach overcomes major constraints of previous meta-analyses⁴, differing nutrient addition
29 rates and methodologies and the rarity of factorial nutrient treatments in the underlying studies⁵.

30 Sites were located on five continents (Supplementary Figure 1) and spanned a 23-fold
31 range in ANPP (Supplementary Figure 2A), over 25° in absolute latitude (actual latitudes 54° N
32 to 37° S), nearly 3,500 m in elevation, and wide ranges in mean annual precipitation (260 – 1900
33 mm), mean annual temperature (0.3 – 22° C; Supplementary Figure 1), soil texture, and pre-
34 treatment soil N, P, and K pools (Supplementary Table 1). The sites included both native and
35 previously cultivated grassland, and some sites were managed with burning, grazing, or other
36 practices. Thus, we were able to evaluate single and multiple-nutrient limitation of ANPP at
37 global, continental, and site spatial extents, and as influenced by management, climate, and soils.

38 N, P, and $K_{+\mu}$ were factorially applied annually to replicated 5 m² plots at the beginning
39 of each site's growing season at rates commonly used in grassland fertilization experiments^{22,23}.
40 The $K_{+\mu}$ treatment included a micronutrient mix in the first treatment year only, to avoid
41 micronutrient toxicity. Nutrient limitation of ANPP was quantified by harvesting current year
42 standing crop biomass for 3 to 5 years. and computing the log response ratio (LRR), the natural
43 logarithm of the ratio of treatment plot to control plot ANPP, a metric commonly used in meta-
44 analyses²⁴

45 Multiple nutrient limitation of ANPP often occurs in the form of co-limitation⁵. Co-
46 limitation is synergistic when the response to multiple nutrients is greater than the sum of the
47 responses to each nutrient added individually, additive if the multiple-nutrient response equals
48 the sum of the individual nutrient responses, and sub-additive if less than the sum of the single-
49 nutrient responses⁵. Across all sites and years, the combined addition of N and P increased
50 ANPP by an average of 40% over controls (LRR= 0.34), compared to increases of only 18%
51 (LRR = 0.16) for N individually and 9% for P individually (LRR = 0.09; $p = 0.03$, Figure 1A,
52 Supplementary Figure 3). This provides clear evidence for globally-averaged synergistic co-
53 limitation of ANPP by N and P in these grasslands. Synergistic co-limitation by N and P across
54 sites and years emerged from frequent occurrence of NP co-limitation at the site level. Some
55 form of NP co-limitation averaging 67% (LRR = 0.51) occurred at 60% of the sites, including
56 sites on all five continents, with synergistic NP co-limitation at 13 sites and sub-additive co-
57 limitation at 12 sites, (Supplementary Table 3). Globally-averaged synergistic co-limitation of
58 grassland ANPP by N and P contradicts the long-held perception that N is the predominant
59 nutrient limiting grassland productivity^{3,10} and highlights a critical role for P.

60 There was no globally-averaged single or multiple-nutrient limitation involving $K_{+\mu}$ ($p >$
61 0.32 , Supplementary Table 2). However, this did not preclude frequent site-level single and
62 multiple-nutrient $K_{+\mu}$ limitation of grassland ANPP. ANPP was limited at 3 sites by $K_{+\mu}$ alone
63 and co-limited by $NK_{+\mu}$ at 18 sites, both averaging 48% (LRR = 0.39; Figure 1A, Supplementary
64 Table 3). Additionally, ANPP was co-limited by $PK_{+\mu}$ at 9 sites by an average of 52% (LRR =
65 0.42), approaching the magnitude of ANPP limitation at sites where N (57%) or P (54%)
66 individually limited ANPP (Figure 1A). Site-level $NK_{+\mu}$ and $PK_{+\mu}$ co-limitation occurred in
67 synergistic, additive, and sub-additive forms, and in total, single or multiple-nutrient limitation

68 involving $K_{+\mu}$ occurred at 24 sites again occurring on all five continents (Supplementary Table
69 3). This finding represents the broadest assessment to date of potential $K_{+\mu}$ limitation of
70 grassland ANPP, and suggests that single- and multiple-nutrient K limitation, potentially
71 augmented by micronutrient addition during year 1, occurs more frequently than previously
72 recognized^{20,25}.

73 Although single and multiple-nutrient limitation of ANPP was widespread, there were
74 nonetheless 15 sites with no evidence for multiple nutrient limitation, and at 12 of the 15 also no
75 evidence for single-nutrient limitation (Supplementary Table 3). The presence and magnitude of
76 nutrient limitation may depend on site climate, soil development, or fertility^{26,27}. For these
77 reasons, greater site-level limitation of ANPP by one nutrient may correlate with greater
78 limitation by one or more additional nutrients. Indeed, site-level individual nutrient limitation of
79 ANPP increased with limitation by other individual nutrients (R^2 0.08 – 0.15; Figure 2A,B,D),
80 and ANPP limitation by nutrient pairs increased with that of a third individual nutrient (R^2 0.07 –
81 0.21; Figure 2C,E,F). These correlations suggest that site-level attributes may predict the
82 magnitude of single and multiple nutrient limitation of ANPP.

83 Sites differed in various potential qualitative and quantitative predictors of the presence
84 and magnitude of nutrient limitation of ANPP (Supplementary Table 1). We found no evidence
85 that nutrient limitation differed among the qualitative predictors continent, history of
86 management with burning, grazing, and previous cultivation, and predominant soil texture
87 (Supplementary Figure 4, Supplementary Table 4). Quantitative predictors were examined at the
88 19 longest running sites, where estimates of site mean nutrient limitation should best correspond
89 to site mean climate and soil attributes. N limitation increased with decreasing site mean annual
90 temperature (MAT; $R^2 = 0.40$, $p = 0.002$, Figure 3A) and peaked at temperate latitude sites

91 (~45°; $R^2 = 0.38$, $p = 0.009$, Figure 3B) with lower MAT ($R^2 = 0.66$, $p < 0.0001$, Figure 3B
92 inset). Limitation of ANPP by other single or multiple nutrient combinations was not correlated
93 with soil texture fractions, pre-treatment soil nutrient contents, soil pH, or climate variables ($p >$
94 0.05 , data not shown). These findings suggest that N limitation predominated at cooler
95 temperate latitudes, as others have predicted²⁶, and validate the power of these experiments to
96 detect spatial trends in ecosystem nutrient limitation.

97 The magnitude of single or multiple-nutrient limitation of grassland ANPP may increase
98 through time (Supplementary Figure 2B). To isolate the temporal signal of nutrient addition
99 effects on ANPP, we calculated new ANPP response ratios using the pre-treatment year (i.e.,
100 year 0) ANPP for each treatment plot rather than the control plot, to control for plot-to-plot
101 spatial variation in ANPP. For 37 sites with no missing ANPP data in years 1-3, the ANPP
102 response to nutrient addition increased through time in all treatments, ANPP increased each year
103 more in treatments containing N ($p < 0.005$, Figure 4, Supplementary Table 2) compared to the
104 P, $K_{+\mu}$, and $PK_{+\mu}$ treatments. Thus, the larger global-averaged increase in ANPP with N than P or
105 $K_{+\mu}$ (Figure 1A) can be explained partly by both smaller and later manifestation of P and $K_{+\mu}$
106 effects. The full extent of nutrient limitation of grassland ANPP, particularly for nutrients other
107 than N, may require additional years to emerge.

108 Multiple nutrient limitation of ANPP in these grasslands was spatially and temporally
109 varied, often included K and micronutrients, but sometimes none of the nutrients we considered.
110 Thus, while N was an important limiting nutrient in many grasslands, P and $K_{+\mu}$ were also
111 limiting in many instances, both alone and in combination with each other and with N. These
112 estimates for globally-averaged nutrient limitation in these grasslands are conservative because

113 nutrient limitation increased in successive treatment years. The magnitude of nutrient limitation
114 may require revision upward if the increases continue in future years.

115 Multiple nutrient limitation in grasslands, especially synergistic co-limitation, makes the
116 consequences of anthropogenic nutrient inputs in these and other systems more difficult to
117 predict. For example, nutrient co-limitation may actually restrict the impacts of single nutrient
118 inputs such as N deposition¹⁷, but ecosystems may respond more rapidly than expected to
119 subsequent inputs of the co-limiting nutrient. Considering only one of the co-limiting nutrients
120 would risk reaching erroneous conclusions about the consequences of future inputs for
121 biodiversity or ecosystem goods/services provision^{14,15,28,29}. It is imperative to consider multiple
122 nutrient constraints on primary productivity in grasslands and other ecosystems.

123 **Full Methods** and any associated references are available in the online version of the paper at
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128 **Methods**

129 *Study system*

130 The rationale, goals, and experimental protocols used at grasslands in the Nutrient
131 Network (NutNet) are described in Borer et al.²¹, and are summarized here. This study used
132 aboveground net primary productivity (ANPP) data from a standardized nutrient addition
133 experiment conducted at 42 NutNet sites in eight countries (Australia, Canada, China,
134 Switzerland, Tanzania, United Kingdom, USA, South Africa) on five continents (Australia [N =

135 4], Africa [N = 4], Europe [N = 5], Asia [N = 1], North America [N = 28]; Supplementary Figure
136 1, Table 1).

137 The grassland types included alpine/montane grasslands, shortgrass, mixed, and tallgrass
138 prairies, desert and semi-arid grasslands, old fields and pastures, savanna and shrub-steppe, and
139 annual grasslands. Sites span wide ranges of mean annual precipitation (MAP; 252 - 1898 mm
140 y^{-1}), mean annual temperature (MAT; 0.3 - 22.1 °C; Supplementary Figure 1), elevation (50 -
141 3500 m), and absolute latitude (over 25°, actual latitudes 54° N to 37° S, Supplementary Table
142 1). Site climate data were derived from the WorldClim database³⁰ (version 1.4.). Local
143 investigators classified sites as to management practice (burned, grazed, other management) and
144 native or anthropogenic (restored grassland and agricultural pasture) origin. Sites collected pre-
145 treatment soil nutrient content and texture data.

146 At each site, N, P, and K were added in full factorial combination (8 treatments including
147 control plots) to 5 x 5 m plots in a randomized complete blocks design with N=3 for most sites
148 (range 2-6). This experimental scale is well-suited for the relatively short-statured herbaceous
149 vegetation in grasslands. Micronutrients (6% Ca, 3% Mg, 12% S, 0.1% B, 1% Cu, 17% Fe,
150 2.5% Mn, 0.05% Mo, and 1% Zn) were added with K only in year 1 to avoid possible
151 micronutrient toxicity. Nutrient additions began at most sites in 2008 (N = 28), and additional
152 sites began in 2009 (N = 9), 2010 (N = 3) and 2011 (N = 2).

153 ***Sampling***

154 Total ANPP ($g\ m^{-2}\ yr^{-1}$) was estimated annually from clip samples of current year peak
155 aboveground biomass from two 0.1 m^2 quadrats per plot. Samples were dried to constant mass at
156 60°C.

157 *Statistical Analyses*

158 We tested for nutrient treatment effects on ANPP using linear mixed models in
159 SAS/STAT v 9.2. First, we tested for variation among sites in nutrient main and interactive
160 effects and their variation with treatment year (Equation 1):

$$\begin{aligned} 161 \quad \ln(\text{ANPP})_{ijkl} = & \mu + \text{nutrients}_i + \text{nutrients}(\text{site})_{ij} + \text{nutrients}(\text{year})_{ik} \\ 162 \quad & + \text{nutrients}(\text{site} \times \text{year})_{ijk} + e_{ijkl}. \quad (1) \end{aligned}$$

163 where *nutrients* refers to the eight factorial combinations of added N, P, and K. Nutrient
164 treatments (i) were nested within site (j), within year (k), and within site x year (jk). Year was
165 not treated as a repeated effect because each site experienced each year differently, and thus both
166 the year effects and the covariance among years were not the same at each site. There were 42
167 sites with three years of data, 33 sites with four years, and 22 sites with five years. Model (1) was
168 tested using all available treatment years, and with only the first three treatment years to
169 ascertain whether any nutrient x year interactions were caused by sites added later with fewer
170 years of nutrient addition.

171 We tested whether ANPP responses to nutrient treatments varied with continent,
172 management, dominant soil texture, and origin by replacing the site term in Eq. 1 with each of
173 the site classification and soils variables, one at a time. Europe and Asia were combined in tests
174 of continent because of the single Asian site.

175 *Calculation of nutrient effect sizes*

176 Effects sizes of the nutrient treatments were calculated and graphed as natural-log
177 response ratios (LRR). LRRs were calculated in two ways; first, by comparing the ANPP of each

178 treatment plot to the ANPP of the control plot in the same block (2), to isolate treatment effects;
179 and second, by compared treatment plot ANPP to that of its pre-treatment (i.e., year 0) ANPP
180 (3). This isolated the temporal signal of nutrient addition responses.

$$181 \quad \mathbf{LRR = \ln[ANPP_{\text{trt}}/ANPP_{\text{control}}]} \quad (2)$$

$$182 \quad \mathbf{LRR = \ln[ANPP_{\text{trt}}(\text{year X})/ANPP_{\text{trt}}(\text{year 0})]} \quad (3)$$

183 We estimated a critical threshold LRR (4) for categorizing the significance of site LRRs. An
184 LRR was considered statistically significant if it exceeded a value corresponding to the critical
185 Z-score (1.65) at $p = 0.05$, computed as:

$$186 \quad \mathbf{\text{Threshold LRR} = 1/7 * (LRR_N + LRR_P + LRR_K + \dots LRR_{NPK})/\text{pooled SD.}} \quad (4)$$

187 *Correlations of site variables with nutrient effects*

188 We used linear regression analyses to test whether LRRs for each single nutrient and
189 nutrient pair were predicted by site latitude (absolute value), elevation, mean annual precipitation
190 (MAP), mean temperature annual (MAT), potential evapotranspiration (PET), soil fractions of
191 sand, silt, and clay, pre-treatment soil N, P, and K contents, and soil pH. We presented
192 regressions only where $p < 0.05$, and fit polynomial curves where indicated by evaluation of
193 residuals.

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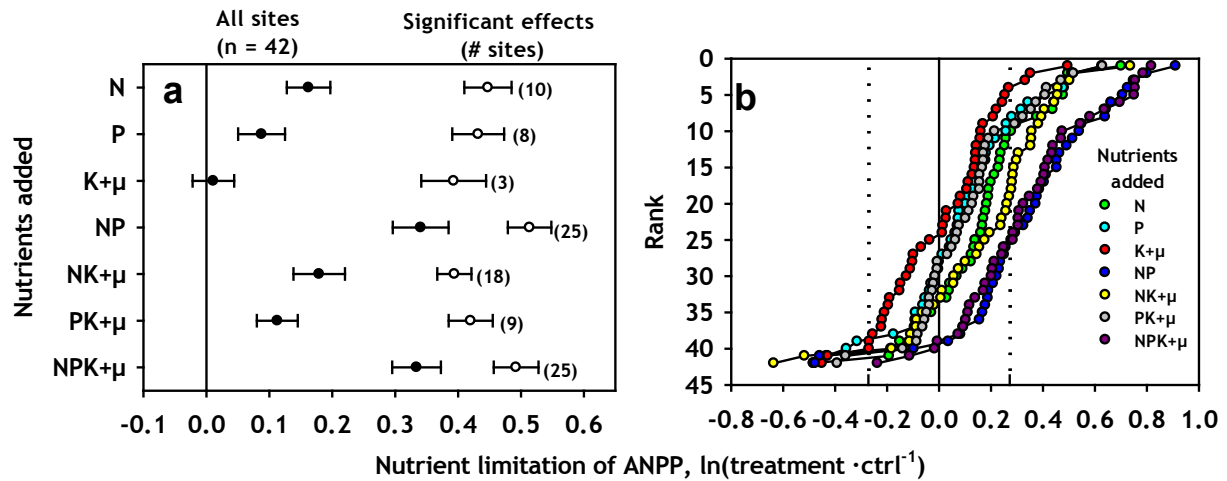
274 This work was generated using data from the Nutrient Network (<http://www.nutnet.org>)
275 experiment.

276 **Author Information** The data reported in this paper are available through
277 <http://www.nutnet.org>. Reprints and permissions information is available at
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281 (philip.fay@ars.usda.gov).

Figure 1

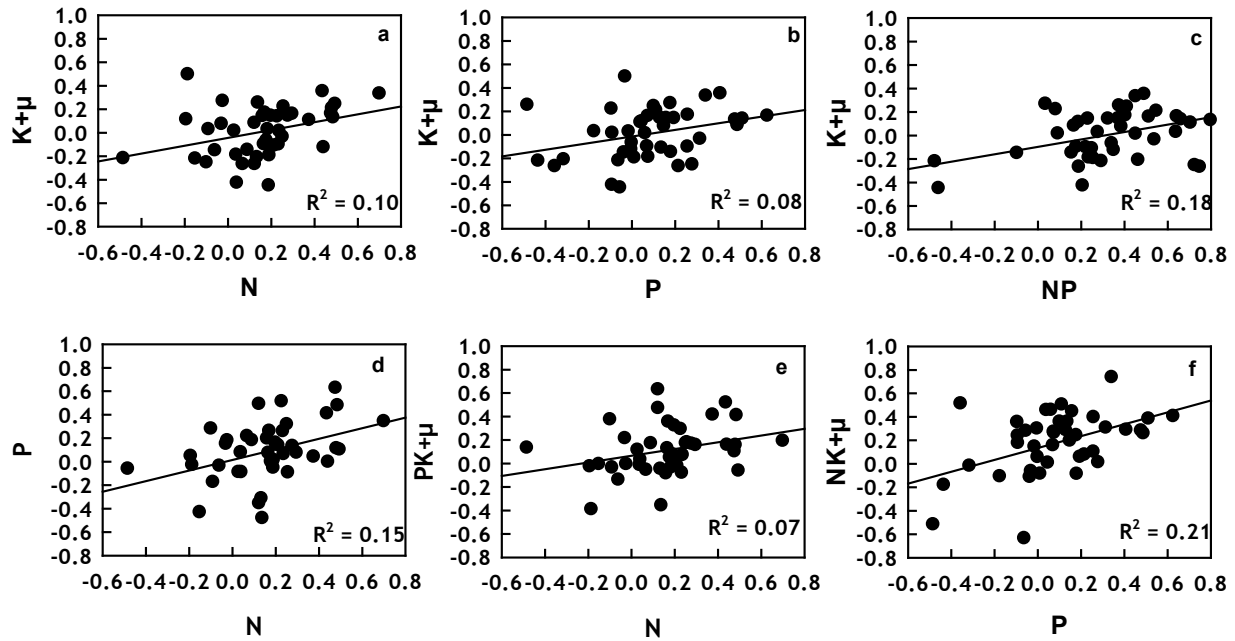
282



283 **Figure 1.** Nutrient limitation of aboveground net primary productivity (ANPP; log response
 284 ratio, $\ln[\text{treatment} \cdot \text{control}^{-1}]$,) by nitrogen (N), phosphorus (P), and potassium plus year 1
 285 micronutrients (K+ μ). a) Mean \pm SE over all years available at each site. Means for all sites are
 286 closed symbols. Means of sites where individual nutrient treatments had significant positive
 287 effects (defined in panel b) are open symbols. b) Site nutrient limitation of ANPP ranked by the
 288 magnitude of limitation, averaged over all available years. Vertical dashed lines mark the effect
 289 sizes approximating a 0.05 significance level for negative and positive nutrient effects on ANPP.
 290 Note that sites rank in a different order in each nutrient treatment.

291

Figure 2



292

293 **Figure 2.** Correlations of nutrient limitation of aboveground net primary productivity (ANPP);
294 log response ratios, $\ln[\text{treatment} \cdot \text{control}^{-1}]$) among single and paired nutrients. a,b,d)

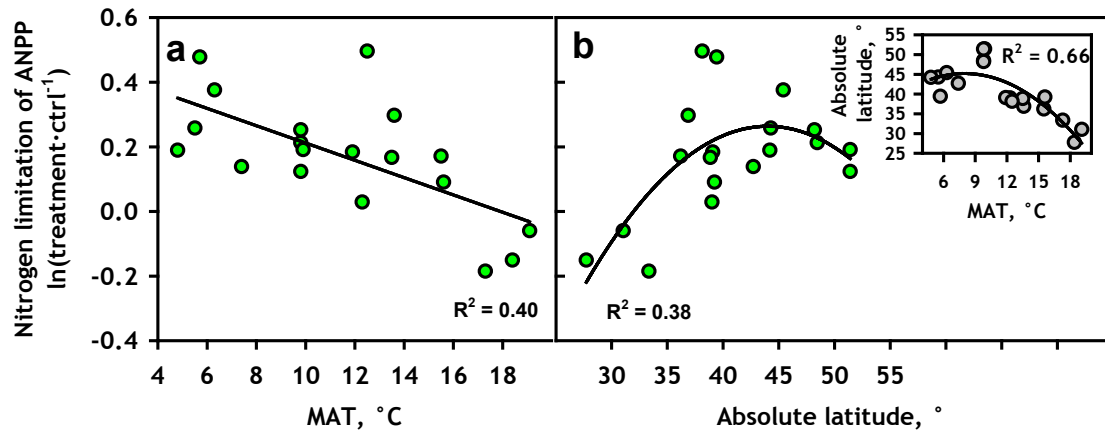
295 Correlations among single nutrients. c,e,f) Correlations of nutrient pairs with the third nutrient.

296 Coefficients of determination (R^2) from linear regression ($0.002 \leq P \leq 0.05$).

297

298

Figure 3



299

300

301 **Figure 3.** Predictors of nitrogen (N) limitation of aboveground net primary productivity (ANPP;

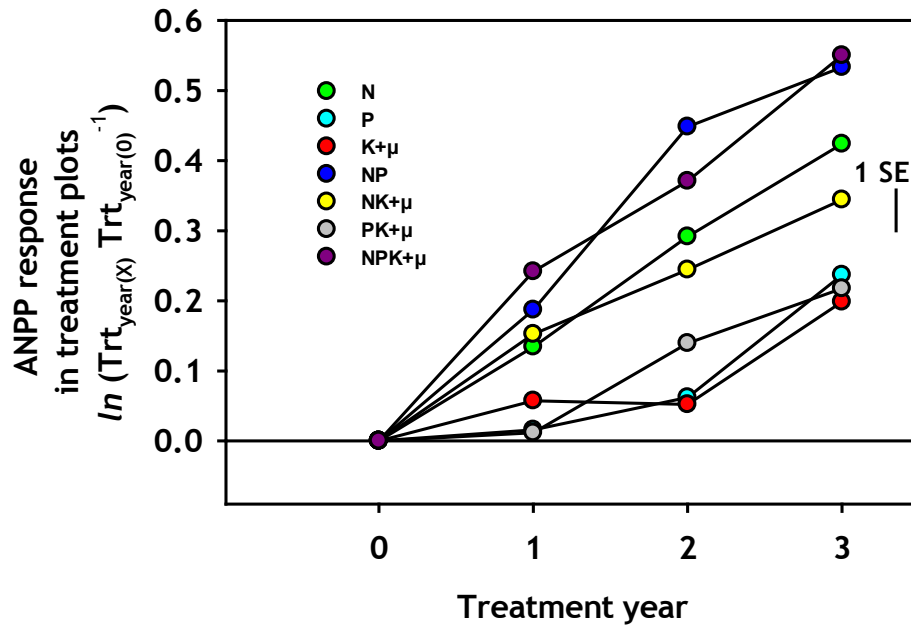
302 log response ratio, $\ln[\text{treatment} \cdot \text{control}^{-1}]$). a) site mean annual temperature (MAT), b) site

303 latitude, in degrees away from the equator, and inset) MAT relationship with site latitude. N

304 limitation values are means of five continuous years of nutrient treatment from 19 sites.

305

Figure 4



306 **Figure 4.** Aboveground net primary productivity (ANPP) responses to factorial nitrogen (N),
307 phosphorus (P) and potassium plus year 1 micronutrient ($K_{+\mu}$) treatments by year in 37
308 grasslands with three continuous years of ANPP data. Data represent the mean natural log
309 response ratio of ANPP in the treatment year to ANPP in the pre-treatment year in the same
310 plot, which controls for plot-to-plot variation.