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1 **Title:** Grassland responses to elevated CO₂ determined by microbial competition for phosphorus.

2 **Authors:** J. Ben Keane^{1,2}, Iain P. Hartley³, Christopher R. Taylor^{1,4}, Jonathan R. Leake¹, Marcel R.
3 Hoosbeek⁵, Franco Miglietta⁶, Gareth K. Phoenix¹.

4 **Affiliations:**

5 **1** Plants Photosynthesis and Soil, School of Biosciences, University of Sheffield, Western Bank,
6 Sheffield, S10 2TN

7 **2** Department of Environment and Geography, Wentworth Way, University of York,
8 Heslington, York, YO10 5NG, UK

9 **3** Geography, Faculty of Environment, Science and Economy, University of Exeter, Amory
10 Building, Rennes Drive, Exeter, EX4 4RJ, UK

11 **4** Soil and Ecosystem Ecology, Earth and Environmental Sciences, University of Manchester,
12 Manchester, UK

13 **5** Soil Chemistry, Wageningen University, PO Box 47, 6700AA, Wageningen, The Netherlands

14 **6** Istituto Di Biometeorologia - Consiglio Nazionale Delle Ricerche, Sede centrale: via Giovanni
15 Caproni 8, Firenze, 50145, Italia

16

17 **Abstract**

18 **Rising atmospheric CO₂ has stimulated plant productivity, with terrestrial ecosystems**
19 **currently absorbing nearly one-third of anthropogenic CO₂ emissions. Increases in**
20 **photosynthesis can subsequently lead to increased carbon (C) storage in plants and soil.**
21 **However, there is growing evidence that nitrogen (N) availability constrains elevated CO₂**
22 **(eCO₂) responses, yet we know much less about the role of phosphorus (P) limitation on**
23 **productivity under eCO₂. This is important because P-limited ecosystems are globally**
24 **widespread, and the biogeochemical cycles of N and P differ fundamentally. We conducted**
25 **a free-air-CO₂-enrichment (FACE) experiment for three years on two contrasting P-**
26 **limited grasslands in the Peak District National Park of northern England under long-**
27 **term nutrient manipulation. Here we show that competition between plants and microbes**
28 **for P can determine plant productivity responses to eCO₂. In a limestone grassland,**
29 **aboveground productivity increased (16%) and microbial biomass P remained unchanged,**
30 **whereas in an acidic grassland, aboveground productivity and P uptake declined (11%**
31 **and 20%, respectively), but P immobilisation into microbial biomass increased (36%).**
32 **Our results demonstrate that strong competition with microbes can cause plant P uptake**
33 **to decline under eCO₂, with implications for the future productivity of P-limited**
34 **ecosystems in response to climate change.**

35

36 **Main Text**

37 The ability of terrestrial ecosystems to sequester more C as CO₂ levels rise represents potentially the
38 single most important biogeochemical feedback limiting climate change¹. This ability can be
39 constrained substantially by nutrient availability¹⁻⁴, with Earth system models indicating that
40 nitrogen (N) limitation reduces this capacity for greater C sequestration by as much as 58%⁵.
41 However, between a third and a half of terrestrial ecosystems are limited by P, not N^{6,7}. Despite the
42 spatial extent of P limitation, we know very little about how P limitation affects ecosystem
43 responses to eCO₂, thus significantly limiting our ability to predict future rates of C uptake by the
44 terrestrial biosphere^{8,9}.

45 Crucially, understanding of eCO₂ responses in N-limited ecosystems does not transfer to P-limited
46 ecosystems because the mechanisms differ so greatly. While C can be utilised by soil microbes to
47 fix atmospheric N, there is no biological equivalent that may increase ecosystem P stock, which is
48 reliant principally on weathering of mineral P. Thus, total ecosystem P stocks will only increase if
49 weathering of P-containing minerals is enhanced. Where the primary minerals have already been
50 weathered, and secondary mineral P is insoluble, competition between plants and microbes for P
51 may control productivity responses. Similarly, in acidic soils inorganic P is likely bound to
52 abundant iron (Fe) and aluminium (Al)¹⁰, to which plant and microbe access may be extremely
53 limited and thus competition for organic P is more intense. Furthermore, unlike the N cycle, soil
54 microbial biomass (MBP) is typically a larger store of P than above ground plant biomass (AGBP),
55 especially during ecosystem retrogression¹¹, where ecosystems become increasingly P-limited
56 during soil development as they age. Thus, changes in competition between plants and microbes
57 could potentially control the response of P limited ecosystems to eCO₂, but this has not been
58 investigated to date.

59 Many of the strategies employed by plants to acquire P require C, either through mycorrhizal
60 symbiosis, or the production of C-rich compounds such as organic acids to mobilise organic- and
61 mineral-bound P, or root surface enzymes such as phytases and phosphatases to mineralise organic

62 P sources¹². Therefore, the expected increase in C entering ecosystems via photosynthesis under
63 eCO₂ may then provide a mechanism by which P limited productivity is alleviated, consequently
64 increasing C sequestration. However, it has been shown that changes in C input can shift investment
65 by soil microbes away from C acquisition to N and P acquisition under eCO₂¹³, and the
66 consequences for competition between plants and microbes is unclear¹⁴.

67 Despite growing recognition of the global importance of P limitation, few studies have quantified
68 changes in P cycling under eCO₂. In an Australian Eucalypt forest¹⁵ of proven P-limitation, net
69 primary productivity did not increase in response to eCO₂, with forest maturity or P-limitation
70 proposed as potential explanations^{16,17}. Meta-analysis of eCO₂ productivity responses indicated that
71 P availability is likely an important constraint¹⁸, but without direct experimental evidence from
72 proven P-limited ecosystems, major uncertainty surrounds eCO₂ responses of P-limited ecosystems.
73 This holds especially true for grasslands, which represent 20% of terrestrial net primary
74 productivity¹⁹ and are the most spatially extensive P-limited ecosystem in temperate regions⁷.

75 To address this, we established a Free Air Carbon dioxide Enrichment (FACE) experiment, where
76 intact soil-turf monoliths were exposed to either ambient CO₂ (ca. 410 ppm) or eCO₂ of 600 ppm.
77 The monoliths used for the experiment were extracted from a long-term N and P manipulation study
78 established in 1995 on two adjacent naturally P-limited grasslands²⁰: a limestone grassland and an
79 acidic grassland, which represent different stages of ecosystem retrogression. The limestone soil sits
80 over a potential source of readily weatherable mineral P in calcium phosphates (Ca; Ca-P). This
81 contrasts with the acidic soil, where Ca is 95% less abundant, and inorganic P is likely bound to
82 abundant Fe and Al, to which plant and microbe access may be extremely limited, and thus
83 competition for organic P is more intense¹⁰. In addition to control (no nutrient addition) plots,
84 nutrient inputs have been manipulated through long-term (ca. 25 years) additions of P (35 kg P ha⁻¹
85 y⁻¹ (P)), and two levels of N addition (35 kg N ha⁻¹ y⁻¹ (LN) and 140 kg N ha⁻¹ y⁻¹ (HN)). Addition
86 of P alleviates the P-limitation of productivity and N additions simulate atmospheric N deposition:
87 in line with the latest understanding of N loading impacts²⁷, these are now also alleviating P-

88 limitation, potentially by more weathering of Ca-P in the limestone soil, and possible stimulation of
89 organic P mineralisation by microbial phosphatases^{13,21}. CO₂ fumigation began in 2018 with the
90 FACE system supplying eCO₂ during the hours of daylight from April until November in each year
91 for three years (2018-2020).

92 *Aboveground biomass responses to eCO₂*

93 There were contrasting responses to eCO₂ of aboveground primary productivity between the two
94 ecosystems (grassland x CO₂ interaction; $F_{1,60} = 8.60$, $p < 0.01$; Fig. 1). Cumulative above ground
95 biomass production in the limestone grassland increasing 16% in response to eCO₂, ($p = 0.03$; Fig.
96 1) whilst in the acidic grassland it declined by 11% ($p = 0.05$, Fig. 1). These contrasting responses
97 were consistent through years 2-3 (2019-2020) and multiple harvests within these years (Fig. 1; $F_{1,}$
98 $_{449} = 14.67$ $p < 0.0001$, and Extended Data Fig. 1). The increased productivity in the limestone
99 grassland is broadly in line with previously reported eCO₂ responses^{22,23}, where swards increased by
100 7- 20% in the first 3-4 years of fumigation. The decline in productivity in the acidic grassland was
101 unexpected but not unprecedented: of 139 studies in a meta-analysis, two grassland systems showed
102 significant declines in productivity under eCO₂¹⁷. The mechanisms for driving these previously
103 reported reductions are not understood, but our outdoor study of grasslands supplied with eCO₂ after
104 several decades of different nitrogen and phosphorus addition treatments, provide this mechanistic
105 understanding of the contrasting plant responses for the first time.

106 *Nutrient treatment response of biomass*

107 Aboveground biomass increased with both nitrogen and phosphorus additions in both ecosystems
108 ($F_{1,60} = 23.15$, $p < 0.0001$, Extended Data Fig. 1). Total productivity was in the order 0N < LN < HN
109 < P across both grasslands, though the acidic grassland was less sensitive to N addition (grassland x
110 nutrient, $F_{3,60} = 1.32$, $p > 0.2$). Productivity only increased under HN (by 29%) in the acidic grassland
111 relative to controls ($p < 0.005$), whereas LN increased productivity in the limestone (by 39%;
112 $p < 0.007$), but did not further increase under HN. In both grasslands, P addition gave the highest

113 shoot productivity, confirming this as the most limiting nutrient in these ecosystems. While N-
114 loading may exacerbate P-demand, the increase in productivity arising from the N treatments is in
115 line with current understanding that N addition can alleviate P-limitation in the long-term, by
116 enhancing weathering of Ca-P, and mineralisation of organic P by microbial phosphatases^{13,21}.
117 Reflecting this, across all the nutrient treatments, positive relationships were observed between
118 available P and above ground biomass production in both grasslands (Fig. 2).

119 *Competition for P drives contrasting productivity responses*

120 Three growing seasons of eCO₂, caused no change in the limestone grassland MBP ($p > 0.5$),
121 contrasting with the 36% increase in MBP in the acidic grassland across all nutrient treatments ($p <$
122 0.03 , Extended Data Fig. 2). These responses were reflected in strong eCO₂ effects on the ratios of
123 above ground plant biomass P (AGBP) to MBP in the two grasslands ($F_{1,185} = 6.78$, $p = 0.01$). Whilst
124 eCO₂ tended to increase AGBP:MBP in the limestone grassland this was not significant, in the
125 acidic grassland it highly significantly decreased it ($p < 0.003$). These contrasts gave a significant
126 interaction between CO₂ treatment and grassland type ($F_{1,185} = 8.94$, $p = 0.004$, Fig. 3). The
127 AGBP:MBP responses to eCO₂ were apparent from the end of the first year of enrichment and
128 persisted throughout the experiment (Fig. 3).

129 Increases in microbial P pools causing immobilisation, can reduce plant productivity²⁴. The decline
130 in the AGBP:MBP under eCO₂ in the acidic grassland implies that competition between soil
131 microbes and plants for P intensified, increased plant growth limitation by P, and reduced shoot
132 productivity. It has long been thought that, in the short term at least, microbes are the better
133 competitors²⁵ for limiting nutrients, and microbial N uptake can be an order of magnitude greater
134 than by plants in grasslands²⁶. None-the-less, while competition for P can be intense²⁷, it is less
135 well-understood²⁸ and our work suggests an important role for this in mediating eCO₂ productivity
136 responses. The contrasting, lack of decline in AGBP:MBP under eCO₂ in the limestone grassland
137 shows no evidence of microbial-driven exacerbation of plant P-limitation and allowed the positive

138 plant productivity and shoot P content response to eCO₂ in this grassland (see NPP and AGBP,
139 Table 1).

140 There was no evidence of CO₂ × nutrient treatment interactions on plant productivity (Supp
141 Fig 3; p < 0.7). Thus, the contrasting responses of the two grasslands appear to be driven by
142 differences in microbial immobilisation even in plots receiving P additions. In the acidic
143 grassland, eCO₂ still reduced plant productivity in plots receiving P, probably because the
144 increase in immobilisation was greatest in this treatment; with microbial biomass P
145 increased by a factor of 1.7 (Table 1). In this context, and in contrast to previous studies^{9,29},
146 the ecosystems had undergone nutrient manipulation for > 25 years, thus the CO₂ effects on
147 each grassland-nutrient combination represented responses of grasslands that have largely
148 stabilised to their new nutrient status rather than responding to a recent step-change in
149 nutrient loading. Competition between plants and microbes is not just controlled by the
150 microbes. Differences in plant species present between the two grasslands could also play a
151 role. For example, low-P specialist groups such as sedges are abundant in the limestone
152 grassland¹⁰, which could explain some of the differences between grasslands. However,
153 plant community composition changed substantially with long-term nutrient manipulations,
154 including an increase in sedge abundance in the N treatments and a decline with P addition.
155 Despite these changes, a consistent positive eCO₂ biomass response was seen across all
156 nutrient treatments. This suggests that it is the key differences in the soil biogeochemistry
157 and potential for P to be immobilised under eCO₂ that explains the overall differences
158 between the grasslands.

159 The key to the differences in the outcome of microbial-plant competition may lie in
160 differences in soil P chemistry between the two grasslands. In both cases, recently-fixed C
161 that enters soil via roots and associated mycorrhizal fungi³⁰ includes enzymes and organic
162 acids, that liberate P needed to sustain a positive plant growth response³¹ to eCO₂. In terms
163 of differences in organic P cycling, the lower soil pH in the acidic grassland may have

164 resulted in reduced total microbial phosphatase activity; it has been shown that reductions in
165 the abundance of alkaline phosphatases in low pH soils cannot necessarily be compensated
166 by increased acid phosphatase activities³². This may in turn promote greater competition
167 between plants and microbes for P³³. There are also important differences in mineral P
168 pools. In the limestone grassland soil, abundant calcium phosphates (Ca-P) offer potential
169 new P inputs, the weathering of which may be enhanced by eCO₂ increasing organic acid
170 exudates³¹ and arbuscular mycorrhizal fungal (AMF) activity³⁴. In contrast, in the acid
171 grassland, Ca is 95% less abundant, and inorganic P predominantly bound to Fe and Al¹⁰, to
172 which plant and microbial access may be extremely limited³⁵. Greater microbial biomass P
173 will thus promote stronger competition for organic P, and may facilitate adsorption and
174 occlusion of microbial biomass-derived P into the low-availability soil P pools. As well as
175 lysing microbial cells, chloroform fumigation may liberate some organic P from soil pools.
176 Therefore, the increase in ‘chloroform-labile P’ in the acidic grassland soils reflects greater
177 amounts of P immobilised in live microbial biomass and potentially some P immobilised by
178 microbes into organic matter pools. In the longer-term, the latter could become a substantial
179 sink for P further reducing plant productivity under eCO₂. In summary, the limestone and
180 acidic soils represent early and later stages of ecosystem retrogression respectively through
181 loss of mineral P (especially Ca-P), acidification, and enrichment of highly recalcitrant Fe
182 and Al secondary P minerals^{11,35}. Our findings are likely to be applicable to other P-limited
183 ecosystems at these different stages of ecosystem retrogression. Furthermore, because the
184 contrasting eCO₂ responses of the two grasslands are consistent across the different nutrient
185 treatments, this suggests the mechanisms hold true irrespective of nutrient status, suggesting
186 a very strong controlling role of soil microbes and chemistry.

187 *P-limited ecosystem responses to eCO₂*

188 Grasslands represent 20% of global terrestrial net primary productivity³⁶ and make major
189 contributions to soil C stocks. They are also the most spatially extensive P-limited

190 ecosystem in temperate regions⁷. More broadly, greater than 40% of terrestrial ecosystems
191 globally may be P-limited, highlighting the need to understand the impact of eCO₂ on the
192 productivity of these ecosystems. Overall, the quantity, pathways, function and fate of C
193 below ground controls the future C gain in plants and soils under eCO₂. We have shown that
194 two P-limited grasslands show directly opposing above-ground biomass responses to eCO₂,
195 and that these responses may be driven by competition for the limiting P-resource between
196 plants and soil microbes. These contrasting responses appear to be controlled by differences
197 in soil chemistry, giving new insight into how P-limited ecosystems of contrasting stages of
198 pedogenesis may respond to eCO₂. Below-ground interactions between plants, microbes and
199 soil strongly dictate the fate of the C and potential sequestration, therefore current climate
200 models which do not consider plant-microbe competition for limiting resources may under-
201 or over-estimate terrestrial C sequestration dependent on the outcome of the competition³⁷.
202 Multiple studies of long-term nutrient and CO₂ manipulations have been invaluable for our
203 understanding of N-limited productivity responses to eCO₂³⁸, and we hope that more studies
204 will now do the same regarding P limitation. It is essential that plant microbial competition,
205 and their interactions with soil chemistry, are more clearly understood to better predict how
206 ecosystems will respond to eCO₂ and climate change³⁹.

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222 **Author Contributions Statement**

223

224 IPH, GKP, JBK and JRL designed the eCO₂ experiment. GKP, IPH, FM, JBK and CRT
225 installed the FACE system and the mesocosms. JBK and CRT oversaw the operation and
226 maintenance of the experiment. Lab analyses were undertaken by JBK and CRT and JBK
227 performed the data analyses. JBK, GKP and IPH wrote the original draft of the manuscript
228 and all authors contributed to subsequent revisions.

229

230 **Competing Interests Statement**

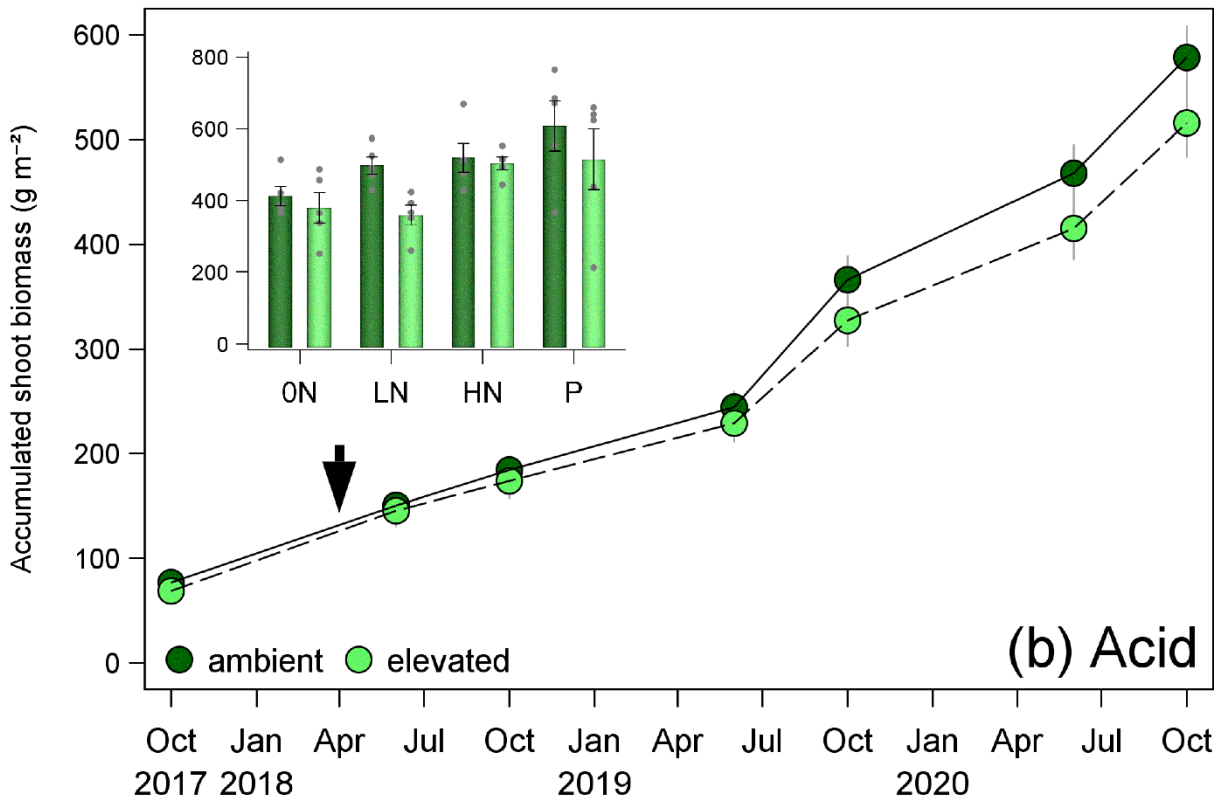
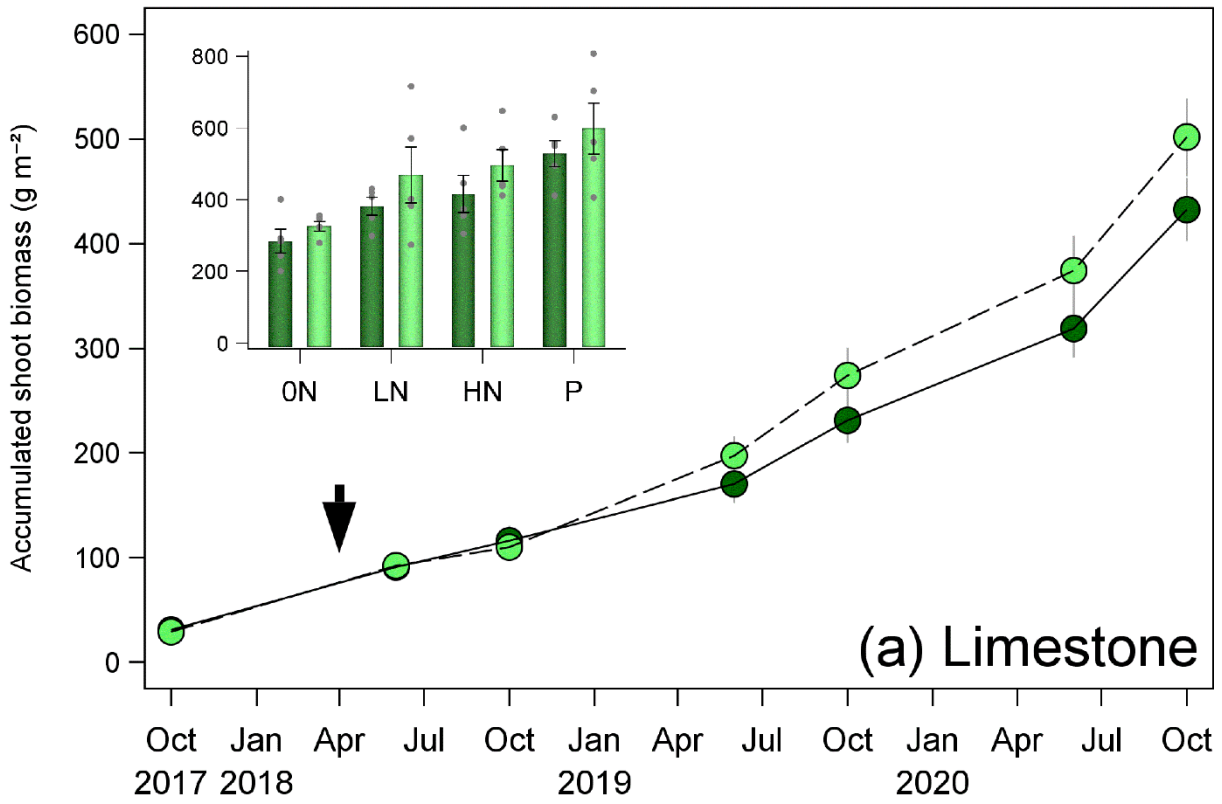
231 The authors declare no competing interests

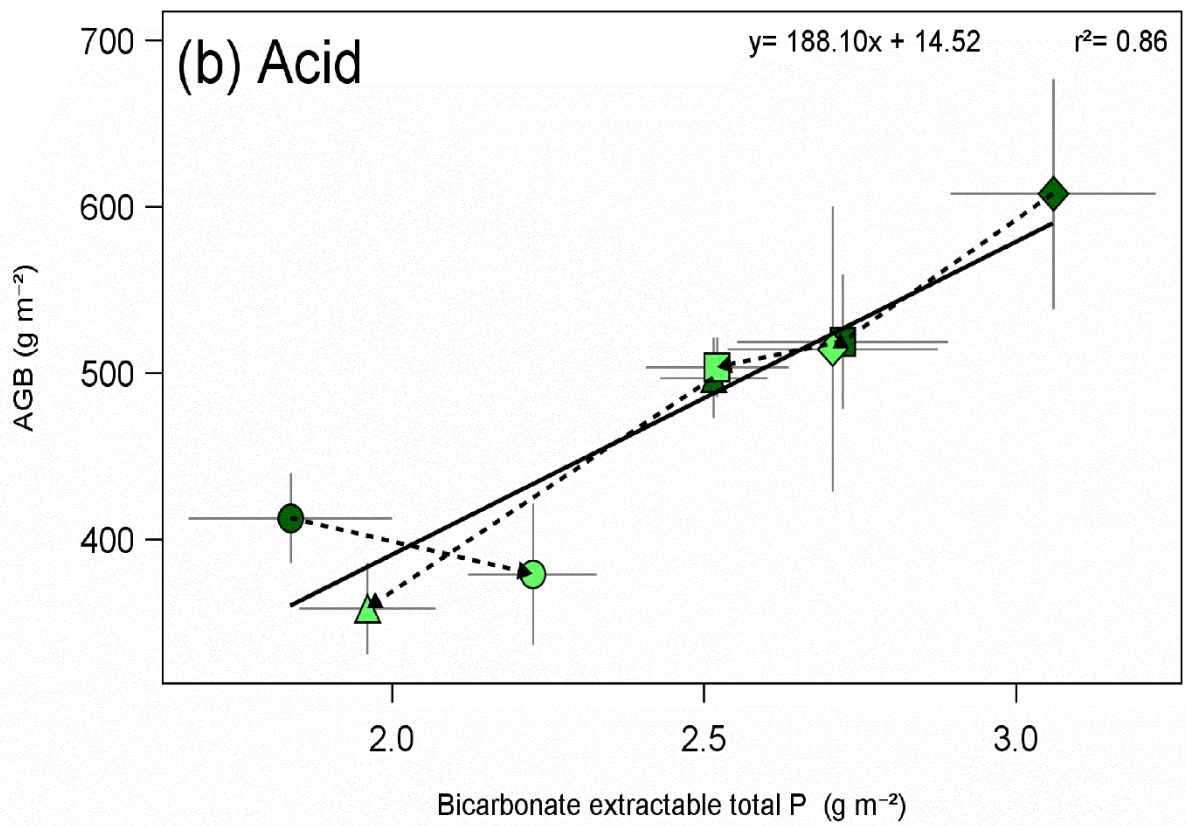
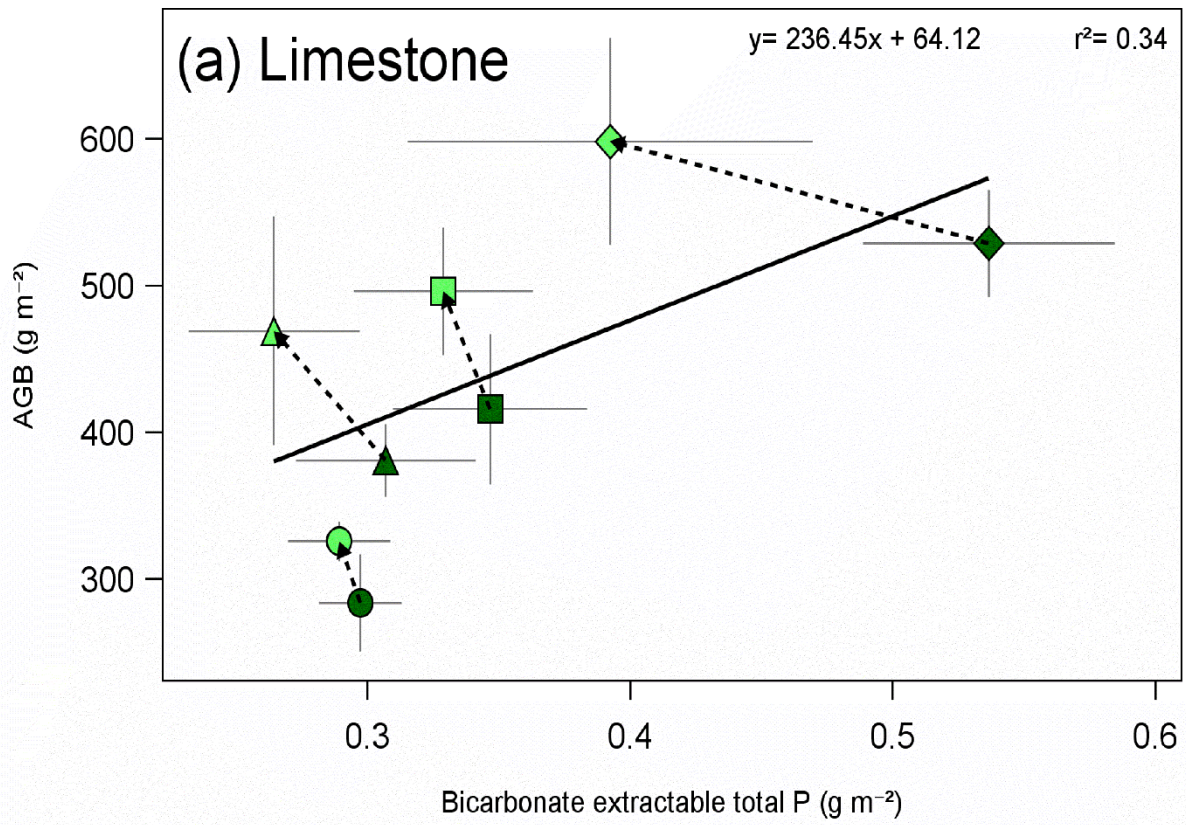
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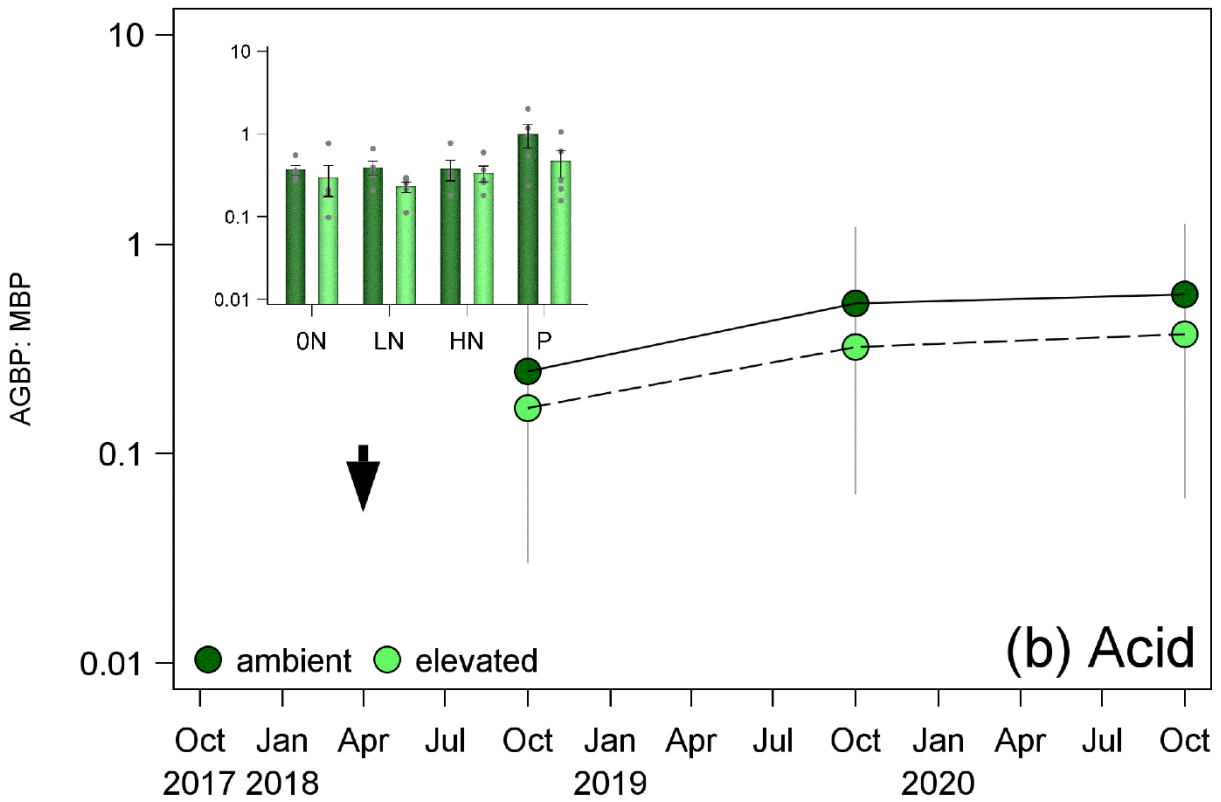
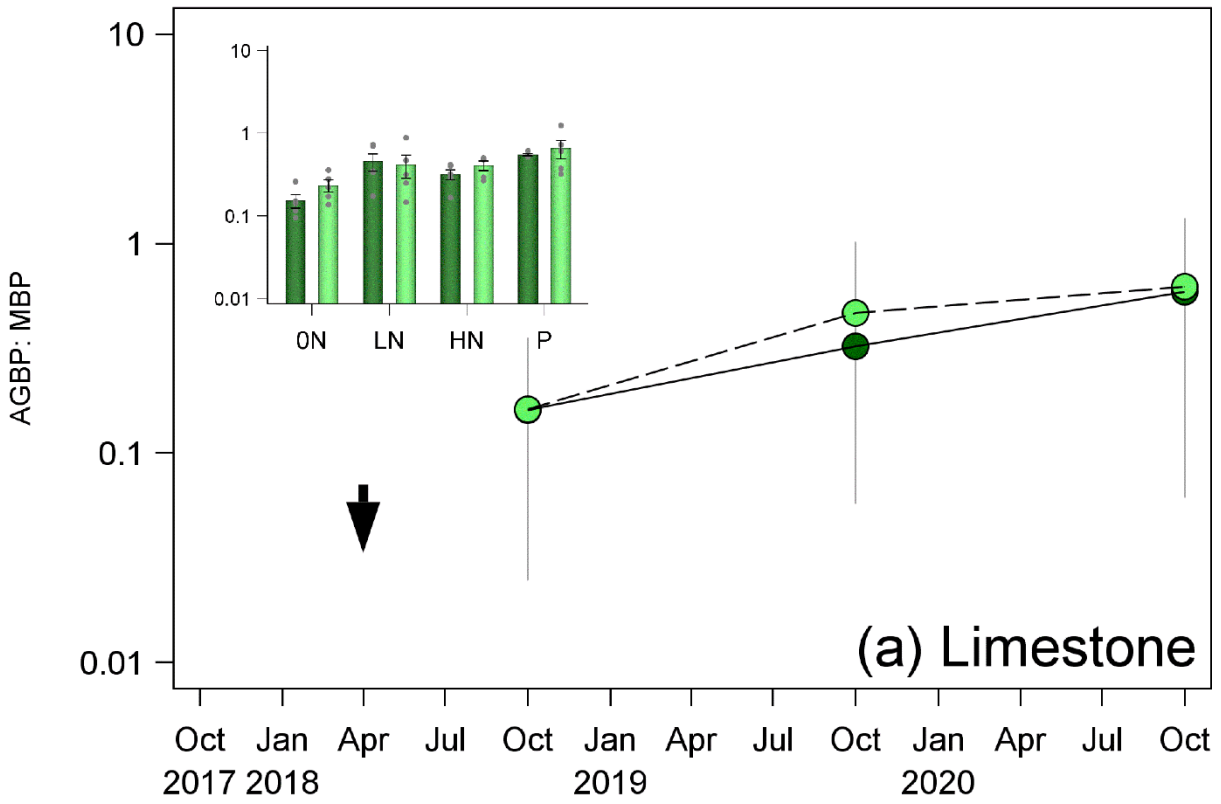
233 **Tables**

234 **Table 1 Summary of productivity and soil and microbial phosphorus pools. The mean**
235 **(*n*= 5, with standard errors in parentheses) annual net primary production (NPP),**
236 **aboveground biomass phosphorus (AGBP) and microbial biomass phosphorus (MBP)**
237 **stocks from three years of CO₂ fumigation (a= ambient, e= elevated) from the acid and**
238 **limestone grasslands, subjected to four nutrient amendment treatments (0N= control,**
239 **LN= 3.5 g N m⁻² y⁻¹, N m⁻² y⁻¹, HN= 14 g N m⁻² y⁻¹, P= 3.5 g P m⁻² y⁻¹)**

Grassland	Nutrient	CO ₂	NPP (g m ⁻²)								AGB P (mg m ⁻²)								MBP (mg m ⁻²)								
			2018		2019		2020		Mean		2018		2019		2020		Mean		2018		2019		2020		Mean		
Limestone	ON	a	41.00	(7.98)	80.42	(11.49)	162.37	(21.82)	94.60	(13.76)	39.21	(7.39)	74.59	(23.46)	243.22	(49.52)	119.01	(26.79)	577.78	(51.62)	524.55	(61.93)	600.37	(37.57)	567.57	(50.37)	
		e	41.44	(6.72)	104.13	(11.80)	180.05	(7.00)	108.54	(8.51)	38.71	(5.24)	114.26	(17.75)	221.54	(9.41)	124.83	(10.80)	494.60	(77.40)	440.00	(90.15)	468.03	(70.11)	467.54	(79.22)	
	LN	a	73.26	(13.10)	120.23	(10.76)	187.38	(10.08)	126.96	(11.31)	66.94	(12.18)	123.27	(13.90)	249.11	(21.74)	146.44	(15.94)	366.02	(58.10)	245.94	(67.36)	434.02	(68.86)	348.66	(64.77)	
		e	86.55	(21.86)	154.43	(38.62)	227.98	(29.17)	156.32	(29.88)	69.38	(17.45)	128.08	(27.39)	268.20	(36.12)	155.22	(26.99)	374.35	(52.80)	390.25	(104.79)	410.56	(69.94)	391.72	(75.84)	
	HN	a	84.51	(20.81)	126.92	(26.74)	204.24	(18.58)	138.56	(22.04)	73.37	(18.18)	102.52	(10.76)	252.21	(29.91)	142.70	(19.62)	404.50	(59.69)	346.63	(53.36)	430.24	(34.83)	393.79	(49.29)	
		e	81.47	(7.92)	171.53	(26.56)	243.07	(10.36)	165.36	(14.95)	66.47	(5.64)	172.39	(29.79)	294.52	(15.66)	177.79	(17.03)	392.39	(36.15)	319.62	(49.93)	467.89	(61.35)	393.30	(49.14)	
	P	a	144.73	(6.36)	131.58	(20.68)	252.24	(32.67)	176.19	(19.91)	343.03	(17.31)	373.18	(89.99)	825.90	(103.02)	514.04	(70.11)	937.27	(71.73)	713.78	(65.88)	895.57	(114.38)	848.87	(83.99)	
		e	113.53	(7.85)	225.78	(33.61)	258.96	(40.35)	199.42	(27.27)	207.57	(11.84)	559.72	(87.36)	669.42	(122.43)	478.91	(73.88)	705.89	(93.14)	595.64	(41.28)	1060.08	(347.06)	787.20	(160.49)	
	Acid	ON	a	90.20	(13.09)	134.38	(15.51)	188.36	(16.54)	137.65	(15.05)	85.12	(17.87)	148.99	(22.42)	262.85	(35.59)	165.65	(25.29)	435.19	(85.24)	485.16	(152.21)	610.47	(79.42)	510.27	(105.62)
			e	96.98	(12.64)	107.51	(16.15)	174.86	(20.73)	126.45	(16.51)	95.73	(15.72)	103.50	(21.53)	223.99	(22.06)	141.08	(19.77)	563.06	(125.73)	435.28	(172.00)	1155.20	(209.33)	717.84	(169.02)
		LN	a	121.84	(17.18)	182.69	(8.30)	192.69	(21.90)	165.74	(15.80)	121.74	(17.14)	187.39	(32.63)	268.91	(37.96)	192.68	(29.25)	680.99	(135.02)	413.75	(79.57)	563.57	(177.19)	552.77	(130.59)
			e	69.94	(5.60)	134.20	(18.96)	154.50	(5.72)	119.55	(10.09)	52.89	(3.78)	130.57	(20.66)	175.64	(4.14)	119.70	(9.53)	584.39	(149.11)	682.07	(398.24)	614.30	(114.72)	626.92	(220.69)
HN		a	106.64	(19.77)	200.05	(26.85)	212.33	(16.58)	173.01	(21.06)	99.91	(19.31)	157.98	(20.40)	244.30	(24.20)	167.40	(21.31)	655.00	(154.56)	357.14	(111.15)	701.90	(108.13)	571.35	(124.61)	
		e	107.69	(11.18)	190.56	(12.13)	205.34	(19.68)	167.86	(14.33)	97.55	(13.59)	183.81	(14.81)	224.46	(23.64)	168.61	(17.35)	753.36	(94.60)	701.90	(225.59)	487.49	(192.65)	647.58	(170.94)	
P		a	142.68	(10.32)	208.33	(38.07)	256.70	(23.71)	202.57	(24.03)	421.38	(38.37)	515.33	(173.14)	767.59	(63.17)	568.10	(91.56)	674.55	(89.92)	691.65	(273.81)	733.60	(198.73)	699.93	(187.49)	
		e	113.32	(32.51)	181.27	(26.33)	219.97	(34.94)	171.52	(31.26)	318.34	(66.20)	434.07	(104.78)	561.94	(89.93)	438.12	(86.97)	1129.69	(256.07)	1140.56	(138.16)	1286.02	(147.50)	1185.43	(180.58)	







243

244

245 **Figure Legends**

246 **Fig. 1** Contrasting aboveground shoot productivity responses to CO₂. Limestone (a) and
247 acidic (b) grasslands exposed to ambient (dark green circles and bars) or 600 ppm CO₂ (light
248 green circles and bars), from April 2018 (denoted by vertical arrows). The main panels show
249 the cumulative productivity over the study period, averaging across nutrient treatments
250 (mean ± 1 SE, n= 20). Inset bar charts show the final accumulated shoot biomass (mean ± 1
251 SE, n=5) in relation to the four multi-decadal nutrient treatments (0N= control, LN= 3.5 g N
252 m⁻² y⁻¹, HN= 14 g N m⁻² y⁻¹, P= 3.5 g P m⁻² y⁻¹).

253 **Fig. 2:** Relationship between bicarbonate-extractable total P (as determined by ICP-
254 OES) and aboveground biomass. Please note this measure of available P differs from
255 Olsen P by including bicarbonate-extractable organic P in addition to inorganic P. Two
256 grasslands, limestone (a) and acidic (b) were exposed to ambient CO₂ (dark green
257 symbols) or eCO₂ (600 ppm, light green symbols) under four nutrient manipulations
258 (0N= control (circles), LN= 3.5 g N m⁻² y⁻¹ (triangles), HN= 14 g N m⁻² y⁻¹ (squares),
259 P= 3.5 g P m⁻² y⁻¹ (diamonds)). Dashed arrows show the directional change in soil P
260 availability and aboveground productivity under eCO₂ within each grassland-nutrient
261 combination. The overall positive relationship between aboveground productivity and
262 soil available P for each grassland is described by a linear regression (solid black line)
263 and the formula is displayed in the top right of each panel. Data points are means for
264 each nutrient-grassland-CO₂ combination (n= 5 ± 1SE).

265
266 **Fig. 3:** Contrasting ratio (on a log scale) of aboveground plant biomass P (AGBP) to
267 soil microbial biomass P (MBP). A limestone (a) and acidic grassland (b) were exposed
268 to ambient (dark green circles and bars) or 600 ppm CO₂ (light green circles and bars),
269 from April 2018. The main panels show time series measurements (mean ± 1 SE, n=
270 20), inset bar charts giving overall means (± 1 SE, n=5) for each nutrient treatment

271 **(0N= control, LN= 3.5 g N m⁻² y⁻¹, HN= 14 g N m⁻² y⁻¹, P= 3.5 g P m⁻² y⁻¹). Vertical**
272 **arrows denote start of CO₂ fumigation.**
273

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411 **Corresponding author:** please direct correspondence to Ben Keane, ben.keane@york.ac.uk

412

413 **Methods**

414 *Nutrient manipulation*

415 Sets of 10 intact soil-turf monoliths of area 0.35 x 0.35 m, were taken from each treatment
416 plot of a long-term grassland nutrient manipulation experiment that was established in 1995
417 at Wardlow, Peak District National Park, UK²⁰. The limestone grassland (NVC
418 classification

419 *Festuca-Avenula* CG2d) occurs on a shallow ranker (~ 10 cm A horizon down to limestone
420 parent material) that is transitioning from a humic rendzina due to leaching¹⁰. The acidic
421 grassland (*Festuca-Agrostis-Galium* U4e) is on a cryptic podzol, with an organic-rich A
422 horizon ca. 10 cm deep above a loessic mineral horizon extending to at least 70 cm depth
423 (though rooting and biological activity is largely limited to the top 15 cm). On each
424 grassland, replicate 9 m² experimental plots have received either no treatment (natural P
425 limitation, application of distilled water only (0N)), monthly applications of P at 35 kg P ha⁻¹
426 y⁻¹ (P), or N at 35 and 140 kg N ha⁻¹ y⁻¹ (LN and HN, respectively)) and were continued
427 throughout the study.

428 The monoliths were taken during February and March 2017, being excavated to the bedrock
429 (~10 cm) in the limestone grassland and to below the main rooting depth in the acidic
430 grassland (~20 cm). They were transported in polypropylene boxes to the Bradfield
431 Environment Laboratory research station, also in the Peak District National Park. Since
432 roots readily reach the underlying limestone in shallow rendzina soils, a base of limestone
433 chippings, sourced from a quarry on the same limestone as Wardlow (Bee Low limestone,
434 Dove Hole Quarry, Derbyshire UK), was applied to the bottom of the limestone mesocosms.
435 At the Bradfield research station, the mesocosms were set flush with the ground surface in
436 holes dug out of the soil to ensure they were thermally buffered, and the surface of the turfs
437 matched the surrounding vegetation. The mesocosm sides were solid so there was no direct

438 contact with the surrounding soils, and the base freely drained through holes covered with a
439 mesh voile (to stop particulate loss and root outgrowth, or ingrowth of roots from
440 surrounding plants). The Bradfield research station (ca. 390 m asl) is less than 20 km from
441 Wardlow (ca. 350 m asl) and has similar climate.

442 *CO₂ enrichment*

443 Mesocosms were assigned to form groups of 8, comprising one of each of the four nutrient
444 treatments from both grasslands, and these were placed within five miniFACE or five
445 control rings of 1.6 m diameter that were supported ca. 20 cm above the ground surface. The
446 mesocosms in control rings experienced ambient CO₂ concentrations, whilst the miniFACE
447 system⁴⁰ Each ring consisted of PVC tubes with laser drilled micro-holes. The processors in
448 the FACE ring control units received CO₂ information from sensors (GTM222, Vaisala,
449 Finland) installed in the centre of each ring. Microprocessors, linked to automated pressure
450 regulators, controlled the pressure inside the releasing pipes and therefore delivery of CO₂ to
451 the rings. Fumigation started in 2018, with fossil fuel-derived CO₂ supplied by BOC
452 Cryospeed (BOC Limited, Manchester, UK). During the hours of daylight, CO₂ enrichment
453 was set to a target of 600 ppm, which continued from the beginning of April until the end of
454 October for three year (2018-2020). The FACE system achieved a mean of 598 ± 0.07 ppm
455 across all elevated plots over the experiment, with the 5% and 95% quantiles being 555 and
456 643 ppm respectively.

457 *Biomass harvesting*

458 Aboveground biomass was harvested twice each year, at the end of June and during
459 September. Plant biomass was cut at 2.5 cm and 5 cm height in the limestone and acidic
460 grasslands respectively and oven dried at 70 °C until constant weight. Dried plant material
461 was first homogenised using a food processor then milled to a fine powder (IKA 10 Mill,
462 IKA®-Werke GmbH & Co.KG, Staufen, Germany).

463 *Soil collection*

464 Soil was sampled once a year from each mesocosm contemporaneously with the September
465 aboveground biomass harvest. Triplicate 2 cm diameter soil cores were taken from random
466 locations within each mesocosm and in the acid grassland these were divided into the A and
467 B horizons. Soil was passed first through a 10 mm sieve and then roots were removed by
468 hand. The remaining soil was then passed through a 2 mm sieve in preparation for chemical
469 analyses, with a subsample placed in the oven at 105 °C to determine soil moisture content.

470 *Determination of soil and plant biomass P content*

471 Plant biomass underwent hydrogen peroxide acid digest modified from Grimshaw (1987)⁴¹
472 and Leake (1988)⁴². Dried plant material (20– 50 mg) was added to a glass digest tube and 1
473 cm³ of 100% H₂SO₄ was added. The tubes were gently shaken and left overnight in a fume
474 cupboard to ‘pre-digest’. Custom-made glass ‘cold fingers’ were used to cover the tubes,
475 allowing acid condensate to safely reflux with the reagents. Two digest blanks per 30
476 samples were prepared for colorimetry, which consisted of 1 cm³ H₂SO₄ which underwent
477 the same subsequent procedure as the plant sample digests. Samples were heated in a
478 heating block (Grant heat block, BT5D model) to 350 °C. Before reaching the target
479 temperature (and at approximately 250 °C) samples were briefly removed from the block
480 and swirled to mix the digestate. The tubes were allowed to reach 350 °C and remained at
481 temperature for 15 minutes. Tubes were removed from the blocks, swirled again and
482 allowed to partially cool.

483 Samples were clarified by addition of H₂O₂ while solutions were still hot, following a delay
484 of 30 –60 seconds to prevent loss of sample by spitting. An initial volume of 800 µl H₂O₂
485 was added to each tube and boiled off in the heating block until most visible evaporation
486 had ceased. Samples were cooled again and another 200 µl of hydrogen peroxide was added
487 and boiled off. This latter addition was repeated once more to ensure all samples were clear

488 and no more plant sample remained adhered to the tubes. The digest solutions were diluted
489 to 10 ml by adding 9 ml of ultra-high purity (UHP) water. Total P in the digest solutions was
490 then determined using an adapted version of the molybdate blue reaction^{43,44}.

491 *Determination of soil microbial biomass P*

492 Soil microbial P (MBP) was determined using the chloroform-fumigation method of
493 Brookes et al (1982)⁴⁵. Gravimetric water content of soil was determined from oven drying a
494 4 g fresh weight subsample for 48 hours at 105°C. Two further 4 g fresh weight aliquots
495 were weighed into acid washed (HCl) 50 cm³ beakers: one for fumigation and one for non-
496 fumigation. One aliquot was placed in a vacuum desiccator with a beaker containing boiling
497 chips and 20 ml chloroform (CHCl₃). The desiccator was evacuated until chloroform boiled
498 three times, venting between the first two times and left in darkness for 24 hours. After
499 incubation, CHCl₃ was removed by drawing a vacuum and venting 5 times.

500 Both fumigated and unfumigated soils were extracted in 50 ml of 0.5 M sodium bicarbonate
501 (NaHCO₃; pH 8.5). Extracts were shaken for 1 hour on a rotary shaker and filtered through
502 pre-leached Whatman 44 filter paper. Extracts were stored at -18 °C until quantification of P
503 was determined using Inductively Coupled Plasma - Optical Emission Spectrometry
504 (ICPOES; Thermo Fisher iCAP PRO). Soil MBP was calculated by subtracting the
505 concentration of P in the non-fumigated aliquot from the fumigated aliquot.

506 *Statistical analyses*

507 Linear mixed effects models were used to test for fixed effects of eCO₂, nutrient treatment,
508 and grassland, with block as a random factor, on aboveground biomass, cumulative
509 aboveground biomass, aboveground biomass P, soil microbial P and AGB:MBP ratio. A log
510 transformation was performed on AGBP:MBP before analysis. Differences between groups
511 were assessed using least square means. All figures and analyses were performed using SAS
512 9.4 (SAS Institute, Cary, NC, USA).

513 **Data availability**

514 Data used to produce this paper are available via the EIDC data repository

515 <https://doi.org/10.5285/35921c93-2d9e-4e35-8de5-adbfc37641b4>

516

517 **Methods References**

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