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Title: Microbial competition for phosphorus determines grassland responses to elevated CO_2

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Rising atmospheric CO_2 has stimulated plant productivity, with terrestrial ecosystems currently absorbing nearly one-third of anthropogenic CO_2 emissions[1]. There is growing evidence that nitrogen (N) availability constrains eCO_2 responses[1-4], yet we know much less about the role of phosphorus (P). This is important because P-limited ecosystems are globally widespread, and the biogeochemical cycles of N and P differ fundamentally. Using a free-airCO_2-enrichment (FACE) experiment on two contrasting P-limited grasslands, we show that competition between plants and microbes for P can determine plant productivity responses to eCO_2. In a limestone grassland, aboveground productivity increased (16%) and microbial biomass P remained unchanged, whereas in an acidic grassland, aboveground productivity and P uptake declined (11% and 20%, respectively), but P immobilisation into microbial biomass increased (36%). Our results demonstrate that strong competition with microbes can cause plant P uptake to decline under eCO_2, with implications for the future productivity of P-limited ecosystems.

The ability of terrestrial ecosystems to sequester more C as CO_2 levels rise represents potentially the single most important biogeochemical feedback limiting climate change[1]. This ability can be constrained substantially by nutrient availability[1-4], with Earth system models indicating that nitrogen (N) limitation reduces this capacity for greater C sequestration by as much as 58%[5]. However, between a third and a half of terrestrial ecosystems are limited by P, not N[6,7]. Despite the spatial extent of P limitation, we know very little about how P limitation affects ecosystem responses to eCO_2, thus significantly limiting our ability to predict future rates of C uptake by the terrestrial biosphere[8,9].

Crucially, understanding of eCO_2 responses in N-limited ecosystems does not transfer to P-limited ecosystems because the mechanisms differ so greatly. While C can be utilised by soil microbes to fix atmospheric N, there is no biological equivalent that may increase ecosystem P stock, which is reliant principally on weathering of mineral P. Thus, total ecosystem P stocks will only increase if weathering of P-containing minerals is enhanced. Where the primary minerals have already been weathered, and secondary mineral P is insoluble, competition between plants and microbes for P may control productivity responses. Similarly, in acidic soils inorganic P is likely bound to abundant iron (Fe) and aluminium (Al)[10], to which plant and microbe access may be extremely limited and thus competition for organic P is more intense. Furthermore, unlike the N cycle, soil microbial biomass (MBP) is typically a larger store of P than above ground plant biomass (AGBP), especially during ecosystem retrogression[11], where ecosystems become increasingly P-limited during soil development as they age. Thus, changes in competition between plants and microbes could potentially control the response of P limited ecosystems to eCO_2, but this has not been investigated to date.

Many of the strategies employed by plants to acquire P require C, either through mycorrhizal symbiosis, or the production of C-rich compounds such as organic acids to mobilise organic- and mineral-bound P, or root surface enzymes such as phytases and phosphatases to mineralise organic P sources[12]. Therefore, the expected increase in C entering ecosystems via photosynthesis under eCO_2 may then provide a mechanism by which P limited productivity is alleviated, consequently increasing C sequestration. However, it has been shown that changes in C input can shift investment by soil microbes away from C acquisition to N and P acquisition under eCO_2[13], and the consequences for competition between plants and microbes is unclear[14].

Currently, only one FACE experiment has been established on an ecosystem of proven P-limitation. Importantly, this study in Eucalypt forest[15] in Australia, found that net primary productivity did not increase in response to eCO_2, with forest maturity or P-limitation proposed as potential explanations[16,17]. Meta-analysis of eCO_2 productivity responses indicated that P availability is an important constraint[18], but without direct experimental evidence from P-limited ecosystems, major uncertainty surrounds eCO_2 responses of P-limited ecosystems that are widespread and prevalent globally. This holds especially true for grasslands, which represent 20% of terrestrial net primary productivity[19] and are the most spatially extensive P-limited ecosystem in temperate regions[7].

To address this, we established a Free Air Carbon dioxide Enrichment (FACE) experiment, where intact soil-turf monoliths were exposed to either ambient CO_2 (ca. 410 ppm) or eCO_2 of 600 ppm. The monoliths used for the experiment were extracted from a long-term N and P manipulation study established in 1995 on two adjacent naturally P-limited grasslands[20]: a limestone grassland and an acidic grassland, which represent different stages of ecosystem retrogression. The limestone soil sits over a potential source of readily weatherable mineral P in calcium phosphates (Ca; Ca-P). This contrasts with the acidic soil, where Ca is 95% less abundant, and inorganic P is likely bound to abundant Fe and Al, to which plant and microbe access may be extremely limited, and thus competition for organic P is more intense[10]. In addition to control (no nutrient addition) plots, nutrient inputs have been manipulated through long-term (ca. 25 years) additions of P (35 kg P ha^{-1} 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 of P alleviates the P-limitation of productivity and N additions simulate atmospheric N deposition: in line with the latest understanding of N loading impacts[27], these are now also alleviating Plimitation, potentially by more weathering of Ca-P in the limestone soil, and possible stimulation of organic P mineralisation by microbial phosphatases[13,21]. CO_2 fumigation began in 2018 with the FACE system supplying eCO_2 during the hours of daylight from April until November in each year for three years (2018-2020).

Aboveground biomass responses to eCO_2

There were consistent, contrasting responses to eCO_2 of aboveground primary productivity between the two ecosystems (grassland x CO_2 interaction; $F_{1,60}= 8.60$, $p< 0.01$; Fig. 1). This was due to cumulative above ground biomass production in the limestone grassland increasing 16% in response to eCO_2, ($p= 0.03$; Fig. 1) whilst in the acidic grassland it declined by 11% ($p= 0.05$, Fig. 1). These contrasting responses were consistent through years 2-3 (2019-2020) and multiple harvests within these years (Fig. 1; $F_{1,449}= 14.67$ $p< 0.0001$, and Supp Fig. 3). The increased productivity in the limestone grassland is broadly in line with previously reported eCO_2 responses[22,23], where swards increased by 7- 20% in the first 3-4 years of fumigation. The decline in productivity in the acidic grassland was unexpected but not unprecedented: of 139 studies in a meta-analysis, two grassland systems showed significant declines in productivity under eCO_2[17]. The mechanisms for driving these previously reported reductions are not understood, but our outdoor study of grasslands supplied with eCO_2 after several decades of different nitrogen and phosphorus addition treatments, provide this mechanistic understanding of the contrasting plant responses for the first time.

Nutrient treatment response of biomass

Aboveground biomass increased with both nitrogen and phosphorus additions in both ecosystems ($F_{1,60}$= 23.15, p< 0.0001, Supp Fig.3). Total productivity was in the order 0N < LN < HN < P across both grasslands, though the acidic grassland was less sensitive to N addition (grassland x nutrient, $F_{3,60}$= 1.32, p> 0.2). Productivity only increased under HN (by 29%) in the acidic grassland relative to controls (p<0.005), whereas LN increased productivity in the limestone (by 39%; p<0.007), but did not further increase under HN. In both grasslands, P addition gave the highest shoot productivity, confirming this as the most limiting nutrient in these ecosystems. While N-loading may exacerbate P-demand, the increase in productivity arising from the N treatments is in line with current understanding that N addition can alleviate P-limitation in the long-term, by enhancing weathering of any Ca-P, and mineralisation of organic P by microbial phosphatases[13,21].

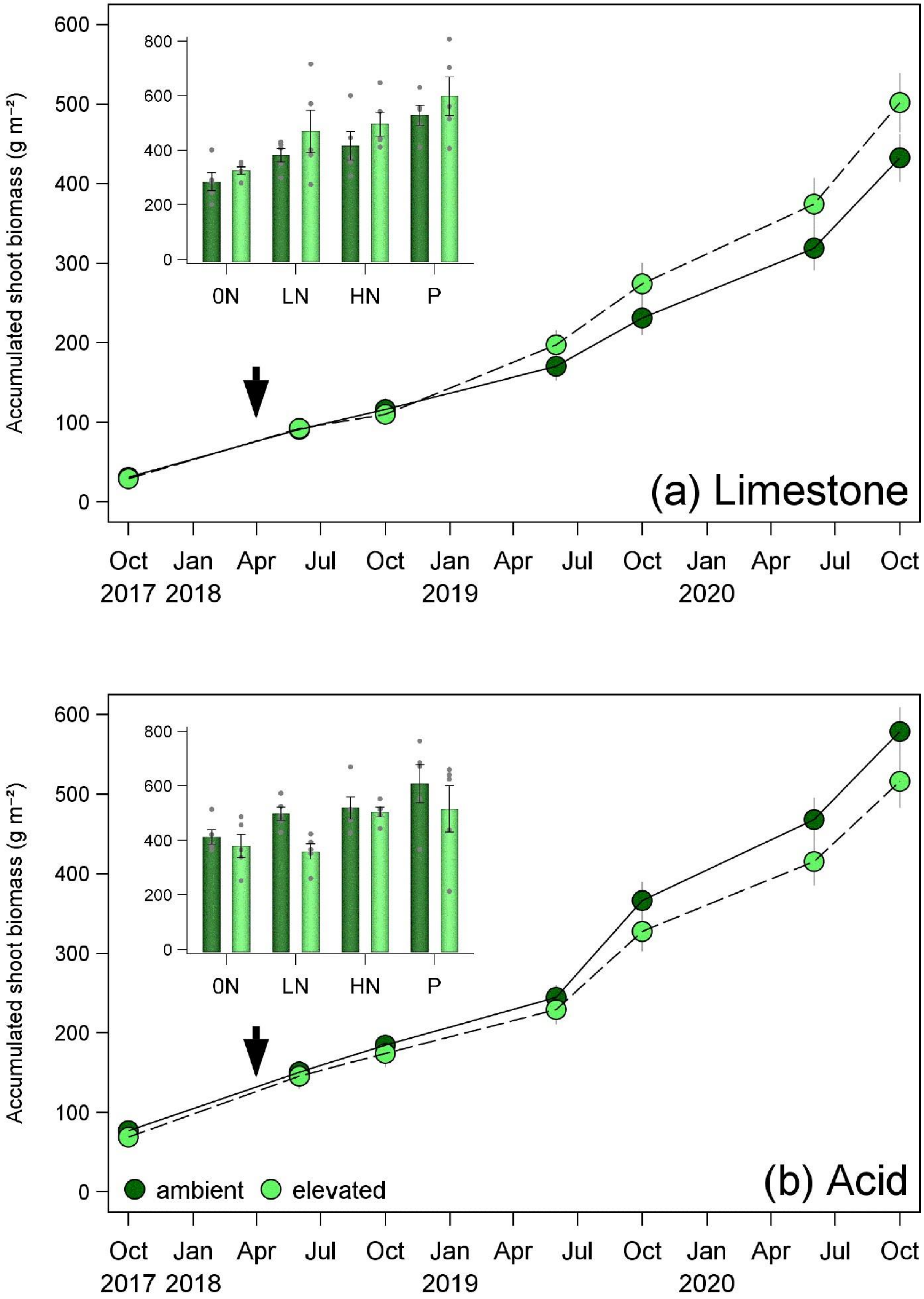
Accumulated shoot biomass (g m⁻²)
0
100
200
300
400
500
600
800
0N
LN
HN
P
(a) Limestone
Oct 2017
Jan 2018
Apr
Jul
Oct
Jan 2019
Apr
Jul
Oct
Jan 2020
Apr
Jul
Oct
(b) Acid
ambient
elevated

Fig. 1 Contrasting aboveground shoot productivity responses in (a) limestone and (b) acidic grasslands exposed to ambient CO_2 (dark green circles and bars) or 600 ppm CO_2 (light green circles and bars), supplied from April 2018 (denoted by vertical arrows). The main panels for each grassland show the cumulative productivity over the study period, averaging across nutrient treatments (mean ± SE, n= 20). Inset bar charts show the final accumulated shoot biomass (mean ± SE, n=5) in relation to the four multi-decadal nutrient treatments (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}).

Plant-microbe competition for P as a driver of contrasting productivity responses

Three growing seasons of eCO_2, caused no change in the limestone grassland MBP ($p> 0.5$), contrasting with the 36% increase in MBP in the acidic grassland across all nutrient treatments ($p< 0.03$, Supplementary Fig. 4). These responses were reflected in strong eCO_2 effects on the ratios of above ground plant biomass P (AGBP) to MBP in the two grasslands ($F_{1,185}= 6.78$, $p= 0.01$). Whilst eCO_2 tended to increase AGBP:MBP in the limestone grassland this was not significant, in the acidic grassland it highly significantly decreased it ($p< 0.0001$). These contrasts gave a significant interaction between CO_2 treatment and grassland type ($F_{1, 185}= 11.21$, $p= 0.001$, Fig. 2). The AGBP:MBP responses to eCO_2 were apparent from the end of the first year of enrichment and persisted throughout the experiment (Fig. 2).

Increases in microbial P pools causing immobilisation, can reduce plant productivity[24]. The decline in the AGBP:MBP under eCO_2 in the acidic grassland implies that competition between soil microbes and plants for P intensified, increased plant growth limitation by P, and reduced shoot productivity. It has long been thought that, in the short term at least, microbes are the better competitors[25] for limiting nutrients, and microbial N uptake can be an order of magnitude greater than by plants in grasslands[26]. None-the-less, while competition for P can be intense[27], it is less well-understood[28] and our work suggests an important role for this in mediating eCO_2 productivity responses. The contrasting, lack of decline in AGBP:MBP under eCO_2 in the limestone grassland shows no evidence of microbial-driven exacerbation of plant P-limitation and allowed the positive plant productivity and shoot P content response to eCO_2 in this grassland (see NPP and AGBP, Table 1).

Table 1 The mean (*n*= 5, with standard errors in parentheses) annual net primary production (NPP), aboveground biomass phosphorus (AGBP) and microbial biomass phosphorus (MBP) stocks from three years of CO_2 fumigation (a= ambient, e= elevated) from the acid and limestone grasslands, subjected to four nutrient amendment treatments (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}).

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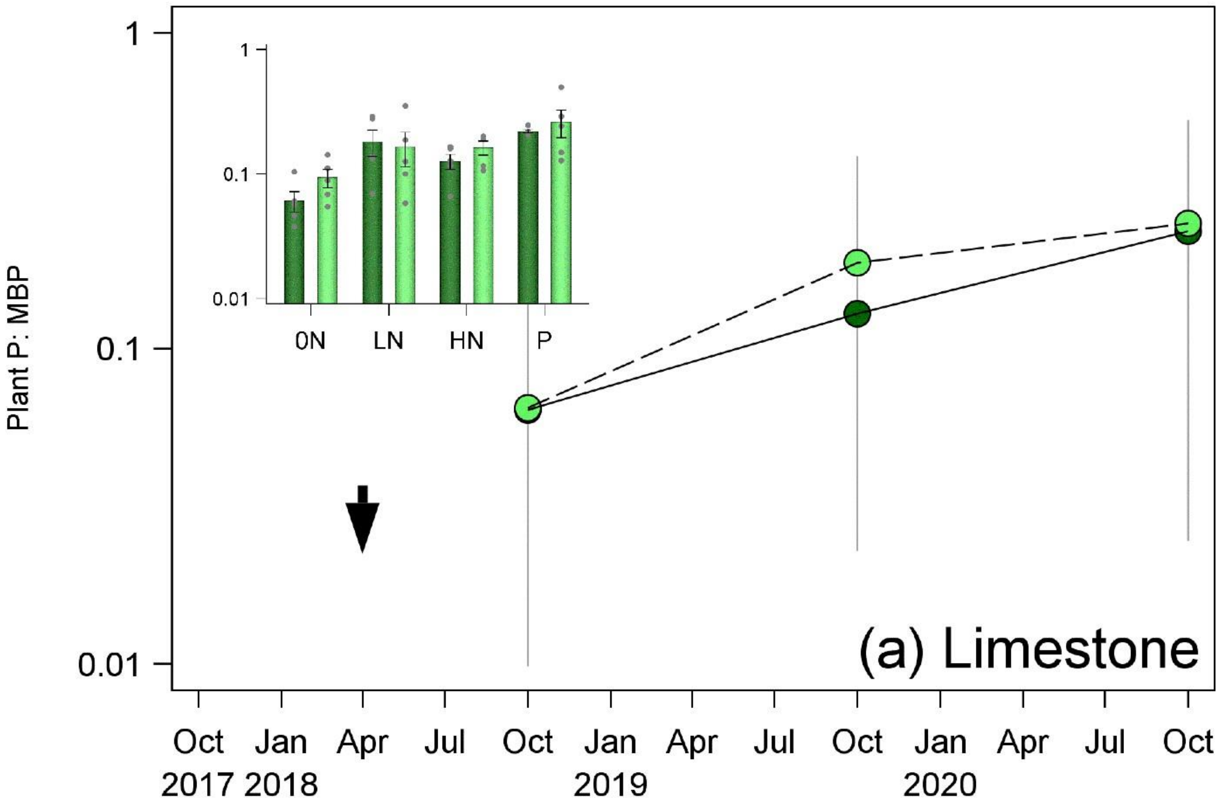
Grassland	Nutrient	CO_2	NPP (g m-2)								AGBP (mg m^{-2})								MBP (mg m^{-2})							
			2018		2019		2020		Mean		2018		2019		2020		Mean		2018		2019		2020		Mean	
Acid	0N	a	90.20	(13.09)	134.38	(15.51)	188.36	(16.54)	137.65	(15.05)	85.12	(17.87)	148.99	(22.42)	262.85	(35.59)	165.65	(25.29)	1087.97	(213.10)	1212.89	(380.52)	1526.17	(198.55)	1275.68	(264.06)
		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)	1407.64	(314.34)	1088.19	(430.01)	2887.99	(523.32)	1794.61	(422.56)
	LN	a	121.84	(17.18)	182.69	(8.3)	192.69	(21.9)	165.74	(15.79)	121.74	(17.14)	187.39	(32.63)	268.91	(37.96)	192.68	(29.24)	1702.48	(337.56)	1034.37	(198.92)	1408.92	(442.97)	1381.92	(326.48)
		e	69.94	(5.6)	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)	1460.98	(372.79)	1705.17	(995.59)	1535.74	(286.80)	1567.30	(551.73)
	HN	a	106.64	(19.77)	200.05	(26.85)	212.33	(16.58)	173.01	(21.07)	99.91	(19.31)	157.98	(20.4)	244.30	(24.2)	167.40	(21.30)	1637.49	(386.40)	892.86	(277.86)	1754.76	(270.32)	1428.37	(311.53)
		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)	1883.40	(236.50)	1754.74	(563.96)	1218.73	(481.62)	1618.96	(427.36)
	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)	1686.37	(224.79)	1729.13	(684.53)	1834.00	(496.83)	1749.84	(468.72)
		e	113.32	(32.51)	181.27	(26.33)	219.97	(34.94)	171.52	(31.26)	318.34	(66.2)	434.07	(104.78)	561.94	(89.93)	438.12	(86.97)	2824.24	(640.19)	2851.40	(345.40)	3215.05	(368.74)	2963.56	(451.44)
Limestone	0N	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)	1444.45	(129.06)	1311.37	(154.83)	1500.93	(93.92)	1418.92	(125.94)
		e	41.44	(6.72)	104.13	(11.8)	180.05	(7)	108.54	(8.51)	38.71	(5.24)	114.26	(17.75)	221.54	(9.41)	124.83	(10.80)	1236.49	(193.49)	1099.99	(225.38)	1170.08	(175.28)	1168.86	(198.05)
	LN	a	73.26	(13.1)	120.23	(10.76)	187.38	(10.08)	126.96	(11.31)	66.94	(12.18)	123.27	(13.9)	249.11	(21.74)	146.44	(15.94)	915.04	(145.24)	614.84	(168.40)	1085.06	(172.16)	871.65	(161.93)
		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)	935.88	(131.99)	975.62	(261.97)	1026.40	(174.85)	979.30	(189.61)
	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)	1011.25	(149.22)	866.56	(133.39)	1075.59	(87.08)	984.47	(123.23)
		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)	980.97	(90.36)	799.06	(124.83)	1169.74	(153.37)	983.26	(122.85)
	P	a	144.73	(6.36)	131.58	(20.68)	252.24	(32.67)	176.19	(19.90)	343.03	(17.31)	373.18	(89.99)	825.90	(103.02)	514.04	(70.11)	2343.18	(179.32)	1784.44	(164.71)	2238.94	(285.94)	2122.19	(209.99)
		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)	1764.72	(232.86)	1489.09	(103.20)	2650.19	(867.64)	1968.00	(401.23)


(a) Limestone
Plant P: MBP
1
0.1
0.01
Oct 2017
Jan 2018
Apr
Jul
Oct
Jan 2019
Apr
Jul
Oct
Jan 2020
Apr
Jul
Oct
1
0.1
0.01
0N
LN
HN
P

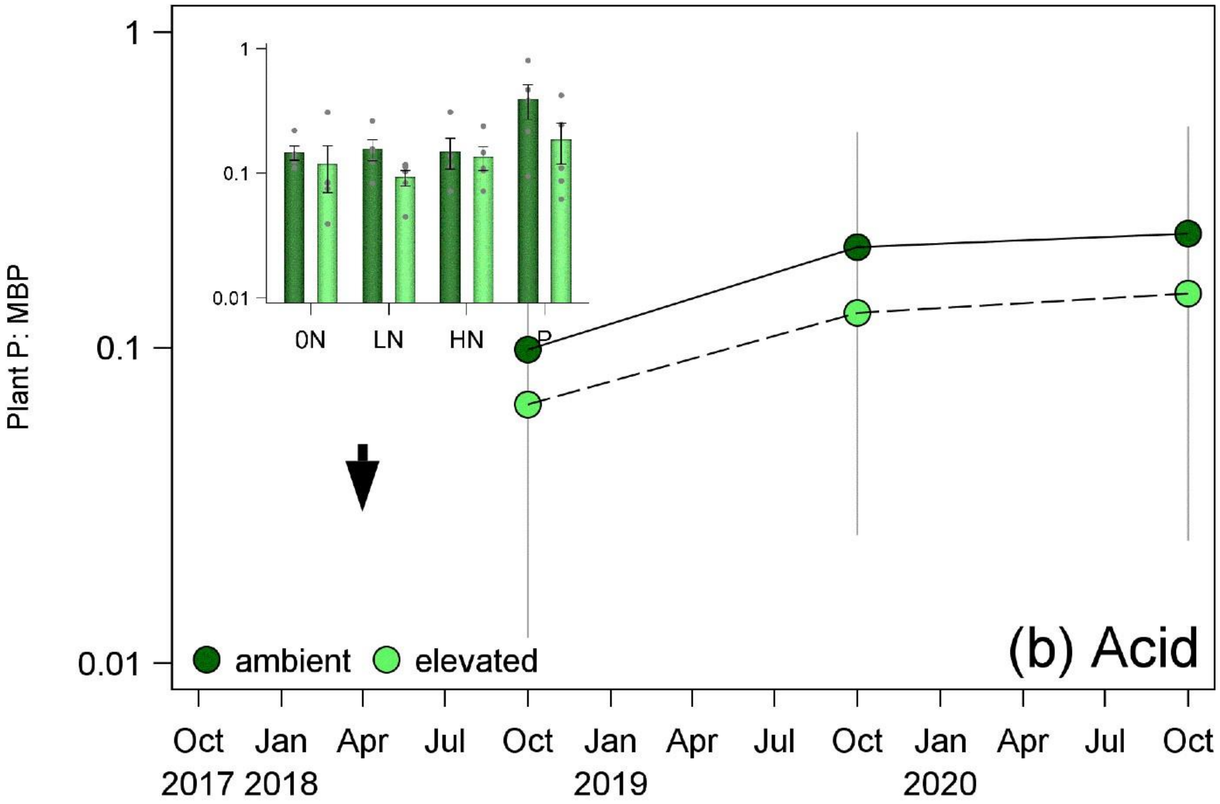

(b) Acid
Plant P: MBP
1
0.1
0.01
● ambient
● elevated
Oct 2017
Jan 2018
Apr
Jul
Oct
Jan 2019
Apr
Jul
Oct
Jan 2020
Apr
Jul
Oct
1
0.1
0.01
0N
LN
HN
P

Fig. 2 Contrasting ratio (on a log scale) of aboveground plant biomass P (AGBP) to soil microbial biomass P (MBP) in (a) limestone and (b) acidic grassland (b) exposed to ambient CO_2 (dark green circles and bars) or 600 ppm CO_2 (red circles and bars), supplied from April 2018. The main panels for each grassland show the time series measurements (mean ± SE, n= 20), with the inset bar charts giving overall means (± SE, n=5) for each nutrient treatment (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 arrows denote start of CO_2 fumigation.

There was no evidence of CO_2 × nutrient treatment interactions on plant productivity (Supp Fig 3; $p< 0.7$). Thus, the contrasting responses of the two grasslands appear to be driven by differences in microbial immobilisation even in plots receiving P additions (see below). In the acidic grassland eCO_2 still reduced plant productivity in plots receiving P, probably because the increase in immobilisation was greatest in this treatment; with microbial biomass P increased by a factor of 1.7 (Table 1). In this context, and in contrast to previous studies[9,29], the ecosystems had undergone nutrient manipulation for > 25 years, thus the CO_2 effects on each grassland-nutrient combination represented responses of grasslands that have largely stabilised to their new nutrient status rather than responding to a recent step-change in nutrient loading.

The key to the differences in the outcome of microbial-plant competition may lie in differences in soil P chemistry between the two grasslands. In both cases, recently-fixed C that enters soil via roots and associated mycorrhizal fungi[30] includes enzymes and organic acids, that liberate P needed to sustain a positive plant growth response[31] to eCO_2. However, in the limestone grassland soil, abundant calcium phosphates (Ca-P) offer potential new P inputs, the weathering of which may be enhanced by eCO_2 increasing organic acid exudates[31] and arbuscular mycorrhizal fungal (AMF) activity[32]. In contrast, in the acid grassland, Ca is 95% less abundant, and inorganic P predominantly bound to Fe and Al[10], to which plant and microbial access may be extremely limited[33]. Greater microbial biomass P will thus promote stronger competition for organic P, and may facilitate adsorption and occlusion of microbial biomass-derived P into the low-availability soil P pools. In this context, the limestone and acidic soils represent early and later stages of ecosystem retrogression respectively through loss of mineral P (especially Ca-P), acidification, and enrichment of highly recalcitrant Fe and Al secondary P minerals[11,33]. Our findings are likely to be applicable to other P-limited ecosystems at these different stages of ecosystem retrogression. Furthermore, because the contrasting eCO_2 responses of the two grasslands are consistent across the different nutrient treatments, this suggests the mechanisms hold true irrespective of nutrient status, suggesting a very strong controlling role of soil microbes and chemistry.

P-limited ecosystem responses to eCO_2

Grasslands represent 20% of global terrestrial net primary productivity[34] and make major contributions to soil C stocks. They are also the most spatially extensive P-limited ecosystem in temperate regions[7]. More broadly, greater than 40% of terrestrial ecosystems globally may be P-limited, highlighting the need to understand the impact of eCO_2 on the productivity of these ecosystems. Overall, the quantity, pathways, function and fate of C below ground controls the future C gain in plants and soils under eCO_2. We have shown that two P-limited grasslands show directly opposing above-ground biomass responses to eCO_2, and that these responses may be driven by competition for the limiting P-resource between plants and soil microbes. These contrasting responses appear to be controlled by differences in soil chemistry, giving new insight into how P-limited ecosystems of contrasting stages of pedogenesis may respond to eCO_2. Below-ground interactions between plants, microbes and soil strongly dictate the fate of the C and potential sequestration, therefore current climate models which do not consider plant-microbe competition for limiting resources may under- or over-estimate terrestrial C sequestration dependent on the outcome of the competition[35]. It is essential that plant microbial competition, and their interactions with soil chemistry, are more clearly understood to better predict how ecosystems will respond to eCO_2 and climate change[36].

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Data availability

All data are available via the EIDC data repository (https://eidc.ac.uk/).

Author contributions

IPH, GKP, JBK and JRL designed the eCO_2 experiment. GKP, IPH, FM, JBK and CRT installed the FACE system and the mesocosms. JBK and CRT oversaw the operation and maintenance of the experiment. Lab analyses were undertaken by JBK and CRT and JBK performed the data analyses. JBK, GKP and IPH wrote the original draft of the manuscript and all authors contributed to subsequent revisions.

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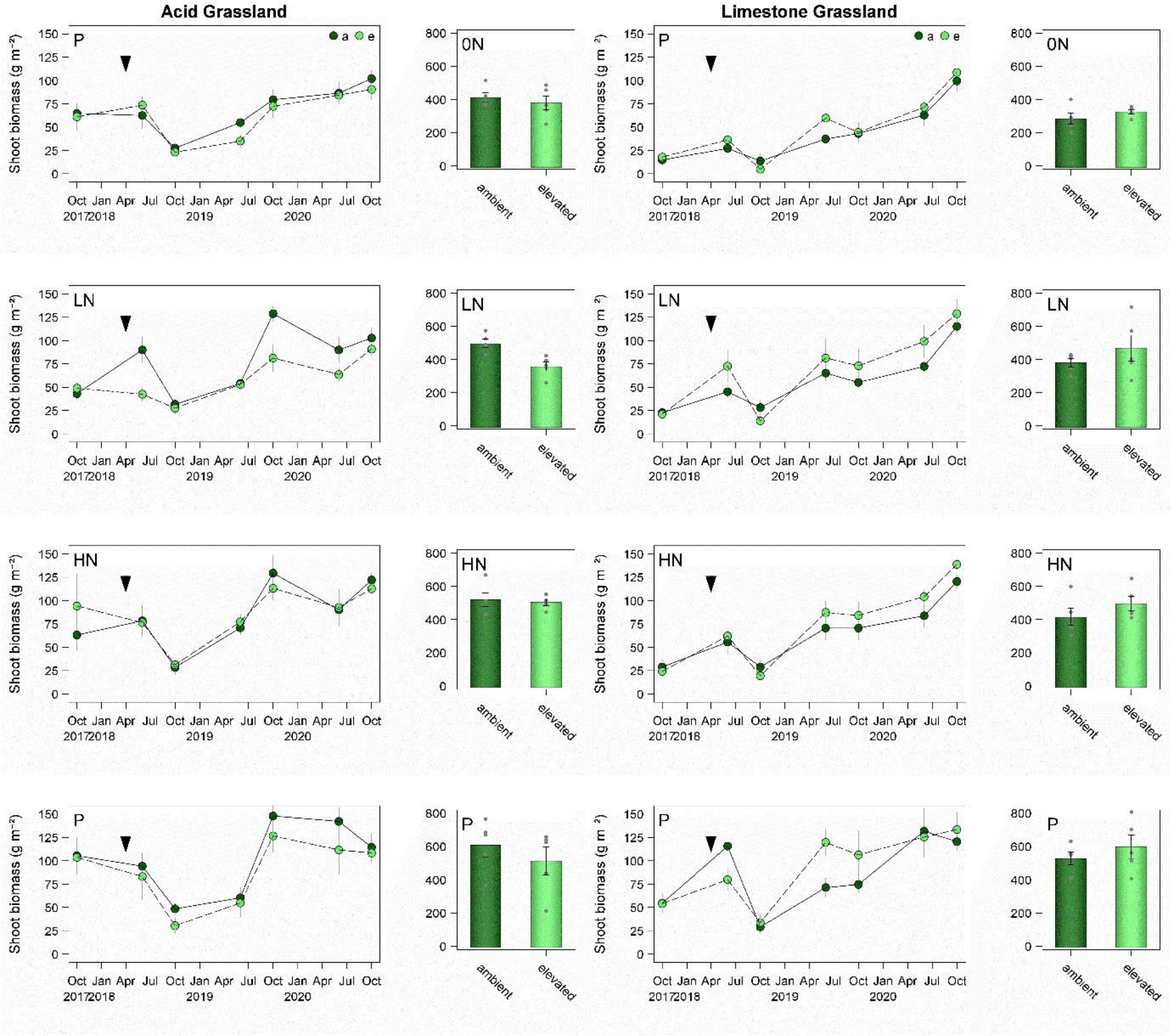
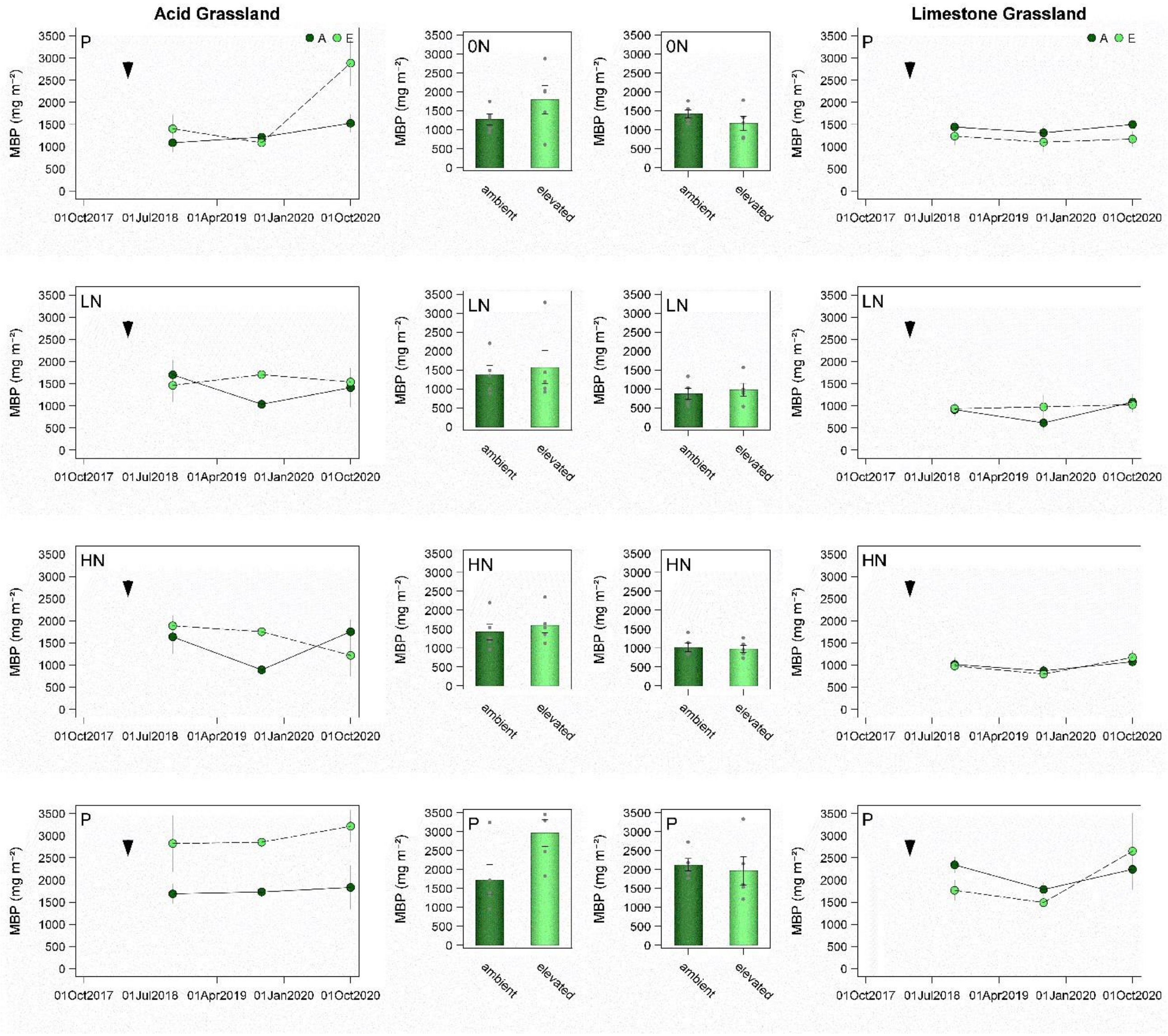
Supplementary Information



Supplementary Fig. 3 Contrasting aboveground productivity responses to ambient CO_2 (a- dark green bars and filled circles) or elevated CO_2 at 600 ppm (e-light greenbars and filled circles), in acidic, (left hand column) and limestone grasslands, (right hand column). Data show mean (± SE, n= 5) in time series and cumulative productivity (vertical bars, mean ± SE, n= 5) over the study period. Vertical arrows denote the start of CO_2 fumigation.



Supplementary Fig. 4. Soil microbial biomass P (MBP) responses to ambient CO_2 (a- dark green bars and filled circles) or elevated CO_2 at 600 ppm (e-light green bars and filled circles), in acidic, (left hand column) and limestone grasslands, (right hand column). Data show means (± SE) in time series (n= 5) and cumulative productivity (n= 5) over the study period. Vertical arrows denote start of CO_2 fumigation.

Methods

Nutrient manipulation

Sets of 10 intact soil-turf monoliths of area 0.35 x 0.35 m, were taken from each treatment plot of a long-term grassland nutrient manipulation experiment that was established in 1995 at Wardlow, Peak District National Park, UK[20]. The limestone grassland (NVC classification

Festuca-Avenula CG2d) occurs on a shallow ranker (~ 10 cm A horizon down to limestone parent material) that is transitioning from a humic rendzina due to leaching[10]. The acidic grassland (*Festuca-Agrostis-Galium* U4e) is on a cryptic podzol, with an organic-rich A horizon ca. 10 cm deep above a loessic mineral horizon extending to at least 70 cm depth (though rooting and biological activity is largely limited to the top 15 cm). On each grassland, replicate 9 m^2 experimental plots have received either no treatment (natural P limitation, application of distilled water only (0N)), monthly applications of P at 35 kg P ha^{-1} y^{-1} (P), or N at 35 and 140 kg N ha^{-1} y^{-1} (LN and HN, respectively)].

The monoliths were taken during February and March 2017, being excavated to the bedrock (~10 cm) in the limestone grassland and to below the main rooting depth in the acidic grassland (~20 cm). They were transported in polypropylene boxes to the Bradfield Environment Laboratory research station, also in the Peak District National Park. Since roots readily reach the underlying limestone in shallow rendzina soils, a base of limestone chippings, sourced from a quarry on the same limestone as Wardlow (Bee Low limestone, Dove Hole Quarry, Derbyshire UK), was applied to the bottom of the limestone mesocosms.

At the Bradfield research station, the mesocosms were set flush with the ground surface in holes dug out of the soil to ensure they were thermally buffered, and the surface of the turfs matched the surrounding vegetation. The mesocosm sides were solid so there was no direct contact with the surrounding soils, and the base freely drained through holes covered with a mesh voile (to stop particulate loss and root outgrowth, or ingrowth of roots from surrounding plants). The Bradfield research station (ca. 390 m asl) is less than 20 km from Wardlow (ca. 350 m asl) and has similar climate.

CO_2 enrichment

Mesocosms were assigned to form groups of 8, comprising one of each of the four nutrient treatments from both grasslands, and these were placed within five miniFACE or five control rings of 1.6 m diameter that were supported ca. 20 cm above the ground surface. The mesocosms in control rings experienced ambient CO_2 concentrations, whilst the miniFACE system[37] Each ring consisted of PVC tubes with laser drilled micro-holes. The processors in the FACE ring control units received CO_2 information from sensors (GTM222, Vaisala, Finland) installed in the centre of each ring. Microprocessors, linked to automated pressure regulators, controlled the pressure inside the releasing pipes and therefore delivery of CO_2 to the rings. Fumigation started in 2018, with fossil fuel-derived CO_2 supplied by BOC Cryospeed (BOC Limited, Manchester, UK). During the hours of daylight, CO_2 enrichment was set to a target of 600 ppm, which continued from the beginning of April until the end of October for three year (2018-2020). The FACE system achieved a mean of 598 ± 0.07 ppm across all elevated plots over the experiment, with the 5% and 95% quantiles being 555 and 643 ppm respectively.

Biomass harvesting

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

Soil collection

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

Determination of soil and plant biomass P content

Plant biomass underwent hydrogen peroxide acid digest modified from Grimshaw (1987)[38] and Leake (1988)[39]. Dried plant material (20– 50 mg) was added to a glass digest tube and 1 cm^3 of 100% H_2SO_4 was added. The tubes were gently shaken and left overnight in a fume cupboard to ‘pre-digest’. Custom-made glass ‘cold fingers’ were used to cover the tubes, allowing acid condensate to safely reflux with the reagents. Two digest blanks per 30 samples were prepared for colorimetry, which consisted of 1 cm^3 H_2SO_4 which underwent the same subsequent procedure as the plant sample digests. Samples were heated in a heating block (Grant heat block, BT5D model) to 350 °C. Before reaching the target temperature (and at approximately 250 °C) samples were briefly removed from the block and swirled to mix the digestate. The tubes were allowed to reach 350 °C and remained at temperature for 15 minutes. Tubes were removed from the blocks, swirled again and allowed to partially cool.

Samples were clarified by addition of H_2O_2 while solutions were still hot, following a delay of 30 –60 seconds to prevent loss of sample by spitting. An initial volume of 800 µl H_2O_2 was added to each tube and boiled off in the heating block until most visible evaporation had ceased. Samples were cooled again and another 200 µl of hydrogen peroxide was added and boiled off. This latter addition was repeated once more to ensure all samples were clear and no more plant sample remained adhered to the tubes. The digest solutions were diluted to 10 ml by adding 9 ml of ultra-high purity (UHP) water. Total P in the digest solutions was then determined using an adapted version of the molybdate blue reaction[40,41].

Determination of soil microbial biomass P

Soil microbial P (MBP) was determined using the chloroform-fumigation method of Vance et al (1987)[42]. Gravimetric water content of soil was determined from oven drying a 4 g fresh weight subsample for 48 hours at 105°C. Two further 4 g fresh weight aliquots were weighed into acid washed (HCl) 50 cm^3 beakers: one for fumigation and one for non-fumigation. One aliquot was placed in a vacuum desiccator with a beaker containing boiling chips and 20 ml chloroform ($CHCl_3$). The desiccator was evacuated until chloroform boiled three times, venting between the first two times and left in darkness for 24 hours. After incubation, $CHCl_3$ was removed by drawing a vacuum and venting 5 times.

Both fumigated and unfumigated soils were extracted in 50 ml of 0.5 M sodium bicarbonate ($NaHCO_3$; pH 8.5). Extracts were shaken for 1 hour on a rotary shaker and filtered through pre-leached Whatman 44 filter paper. Extracts were stored at -18 °C until quantification of P was determined using Inductively Coupled Plasma - Optical Emission Spectrometry (ICPOES; Thermo Fisher iCAP PRO). Soil MBP was calculated by subtracting the concentration of P in the non-fumigated aliquot from the fumigated aliquot and dividing by an adjustment factor of 0.4 after Brookes et al. (1982)[43].

Statistical analyses

Linear mixed effects models were used to test for fixed effects of eCO_2, nutrient treatment, and grassland, with block as a random factor, on aboveground biomass, cumulative aboveground biomass, aboveground biomass P, soil microbial P and AGB:MBP ratio. A log transformation was performed on AGBP:MBP before analysis. Differences between groups were assessed using least square means. All figures and analyses were performed using SAS 9.4 (SAS Institute, Cary, NC, USA).