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C:N:P stoichiometry and nutrient limitation of the soil microbial biomass in a grazed grassland site under experimental P limitation or excess

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Abstract

Introduction: The availability of essential nutrients, such as nitrogen (N) and phosphorus (P), can feedback on soil carbon (C) and the soil microbial biomass. Natural cycles can be supplemented by agricultural fertiliser addition, and we determined whether the stoichiometry and nutrient limitation of the microbial biomass could be affected by an unbalanced nutrient supply.

Methods: Samples were taken from a long-term trial (in effect since 1968) with annual applications of 0, 15 and 30 kg P ha^{-1} with constant N and potassium. Soil and microbial biomass CNP contents were measured and nutrient limitation assessed by substrate-induced respiration. Linear regression and discriminant analyses were used to identify the variables explaining nutrient limitation.

Results: Soil and biomass CNP increased with increasing P fertiliser, and there was a significant, positive, correlation between microbial biomass P and biomass C, apart from at the highest level of P fertilisation when the microbial biomass was over-saturated with P. The molar ratios of C:N:P in the microbial biomass remained constant (homeostatic) despite large changes in the soil nutrient ratios. Microbial growth was generally limited by C and N, except in soil with no added P when C and P were the main limiting nutrients. C, N and P, however, did not explain all the growth limitation on the soils with no added P.

Conclusions: Increased soil C and N were probably due to increased net primary production. Our results confirm that C:N:P ratios within the microbial biomass were constrained (i.e. homeostatic) under near optimum soil conditions. Soils with no added P were characterised by strong microbial P limitation and soils under high P by over-saturation of microorganisms with P. Relative changes in biomass C:P can be indicative of nutrient limitation within a site.

Keywords: Carbon, Nitrogen, Nutrient limitation, Phosphorus, Soil microbial biomass, Stoichiometry

Introduction

In terrestrial ecosystems most primary production enters the decomposer pathway (Cebrian 1999), where microorganisms mineralise organic material to simple inorganic compounds and recycle growth-limiting nutrients for autotrophs. This is essential for soil fertility and plant growth. Microorganisms require the nutrients for their own growth and generally the carbon-to-nutrient ratio determines whether nutrients are immobilised in the microbial biomass or mineralised to become available for uptake. The soil microbial biomass therefore acts as both a sink and a source of nutrients which become available during the turnover of microbial biomass. The availability and limitation of essential nutrients, such as nitrogen (N) and phosphorus (P), can thus feed back on soil carbon (C) dynamics and microbial biomass (Wang et al. 2010; Brookes 2001).

In a global-scale meta-analysis of the C:N:P ratio of soil and the soil microbial biomass (Cleveland and Liptzin 2007), the abundance and ratio of elements were constrained. While this was not surprising for C and N, given

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that plants are the major source of both C and N in the soil, relatively fixed C:P and N:P ratios were more unexpected given that organisms do not regulate the total amount of soil P. The amount of P available for plant uptake is related to the total soil P and so indirectly links the abundance of P with that of C and N in soil (Cleveland and Liptzin 2007). Thus, microbial C:N:P ratios are strongly related and not significantly affected by variations in soil element ratios. This has given rise to the suggestion that differences in soil microbial biomass element ratios could provide an insight into nutrient limitation in terrestrial ecosystems (Cleveland and Liptzin 2007). In managed systems nutrient addition is commonly practised, and even in unmanaged systems nutrient deposition occurs, so the objective of this study was to determine how soil and soil microbial biomass elemental ratios responded to nutrient addition.

In agricultural systems the natural biogeochemical cycles can be supplemented by the addition of readily available, inorganic fertilisers. Agricultural productivity in Ireland has historically been limited by P, but the application of fertiliser P, which peaked in the 1970s, has resulted in an increase in levels of available soil P such that about 50% of Irish grassland soils no longer respond to added P (Culleton et al. 2002). Overall fertiliser consumption in Ireland is now falling, from 430,000, 62,000 and 151,000 tonnes N, P, K respectively (N:P 6.9) in 1994 to the latest available figures of 307,000, 20,000 and 52,000 tonnes (N:P 15.2) in 2008 (DAFF 2008). In 2002 Irish grasslands received an average of 123, 11 and 27 kg N, P, K ha^{-1} $year^{-1}$ (N:P 11.2) (Coulter et al. 2004). Even so, the majority of P in typical grassland soil is present as organic P which represents a large pool of potentially available nutrient (Bourke et al. 2008). Although the agronomic objective of the added nutrients is to increase plant production, they will also be available to the soil microbial biomass. The soil microorganisms will see an indirect increase in soil C, from increased rhizodeposition and increased residue incorporation from the extra plant growth, and a direct increase in N and P from the added nutrients. Microorganisms can compete effectively with plants for these nutrients (Schimel et al. 1989; Zak et al. 1990). Applied inorganic N, for example, is rapidly taken up by the soil microbial biomass (Nannipieri et al. 1990) which can be limited by nutrients and not just C (Kaye and Hart 1997; Wang and Bakken 1997), although the outcome of the plant–microorganism competition depends on the spatial heterogeneity of the system (Korsaeth et al. 2001). Previous work on cut, rather than grazed, grassland showed clearly that both microbial biomass P and the biomass C:P ratio were sensitive to long-term (>100 years) fertiliser regimes (He et al. 1997). Growth experiments using environmental isolates of bacteria recently demonstrated that their stoichiometry is very flexible, with a four-fold change in C:P ratio between P-sufficient and P-deficient conditions (Scott et al. 2012). This could result in a less homeostatic variation in C:N:P ratios within a site than suggested by the meta-analysis of Cleveland and Liptzin (2007).

Given the changing inputs of inorganic nutrients to many grassland soils, we felt it timely to determine whether the stoichiometry of the soil microbial biomass could be affected by an unbalanced nutrient supply and if we could experimentally show nutrient limitation of the soil microbial biomass. To do this we sampled plots from a long-term agricultural trial instigated in 1968 (Culleton et al. 2002). Phosphorus had been applied annually at 0, 15 and 30 kg ha^{-1}, with the plots split in 1999 to reduce P applications to half the high-P plots and increase P to half the low-P plots (as detailed below). Soil microbial biomass and its elemental CNP composition were measured and total soil C, N and P determined. In addition, respiration measurements were conducted to assess nutrient constraints at different fertilisation levels. This would test if differences in soil microbial biomass element ratios in an agricultural situation could provide an insight into nutrient limitation in terrestrial ecosystems (Cleveland and Liptzin 2007), or whether microbial biomass element ratios may be responsive to altered fertiliser regimes (Scott et al. 2012).

Methods

Cowlands long-term, grazed field trial

The field site, established on a humic gleysol with a sandy loam texture at Johnstown Castle, County Wexford, Ireland, was described in detail by Culleton et al. (2002) and King-Salter (2008). At the start of the trial, in 1968, the site was ploughed and sown with *Lolium perenne*. Phosphorus (calcium superphosphate) was applied annually at 0 (P0), 15 (P15) and 30 (P30) kg P ha^{-1} to each of twelve 0.45 ha replicate plots. Nitrogen (ammonium nitrate, 240 kg N ha^{-1}) and potassium (potassium chloride, 20 kg K ha^{-1}) were applied annually to all plots, P and K in spring and N between spring and autumn. Six plots of each P treatment were grazed at a low stocking rate (2,200 kg stock ha^{-1}) and six at a high stocking rate (3,300 kg stock ha^{-1}). Each plot was rotationally grazed around six paddocks, with 18- to 24-day intervals between grazing and stocking rates that were progressively reduced as grass growth rates declined through the year. In 1999 the P application and stocking rate were altered, such that all plots now had the same stocking rate (3,300 kg stock ha^{-1}). At the same time, however, on the former low stocking rate plots, P0 now received 30 kg P ha^{-1} $year^{-1}$, P15 received 5 kg P ha^{-1} $year^{-1}$ and P30 received 0 kg P ha^{-1} $year^{-1}$. Thus, since 1999 there were six replicate plots of six P treatments: 0_0, 0_30, 15_15, 15_5, 30_30, 30_0 (kg P ha^{-1} $year^{-1}$ 1968–1999_kg P

ha^{-1} year^{-1} 1999–2009) to determine the rate of response of the sward to altered P fertilisation. See Figure 1.

Soil sampling

The plots were sampled in April 2009. Two separate composite soil samples were collected from the top 10 cm of each plot by taking 20 cores for each composite sample with a gouge auger (1.25 cm diameter) in a stratified random design. The soils were handpicked to remove stones and larger soil fauna, sieved to pass through a 3.35 mm mesh and incubated for 6 days at 25°C to allow respiration to settle down after sieving without letting the soils dry out. Water-holding capacity of each treatment was determined and sub-samples of each plot were oven-dried for 24 h at 105°C for dry weight (d.w.) analysis.

Soil C, N, P

Total C and N in samples were determined using an elemental analyser (Thermo Scientific Flash EA 2000). Total P was extracted in *aqua regia* (ISO 11466: 1995) and measured by ICP.

Soil microbial biomass C, N, P

Microbial biomass C, N and P were measured by the chloroform fumigation-extraction (CFE) technique (Brookes et al. 1984, 1985; Vance et al. 1987): 10 g d.w. equivalent of soil was fumigated for 24 h at 25°C and extracted with 0.5 M K_2SO_4 (for C and N) or 0.5 M $NaHCO_3$ (for P). C, N and P from unfumigated soils were extracted in the same way. In the extracts we determined the following: total organic carbon (TOC) by combustion (Baird 2005) using a Shimadzu TOC-VCPH analyser with ASI-V autosampler; total organic nitrogen

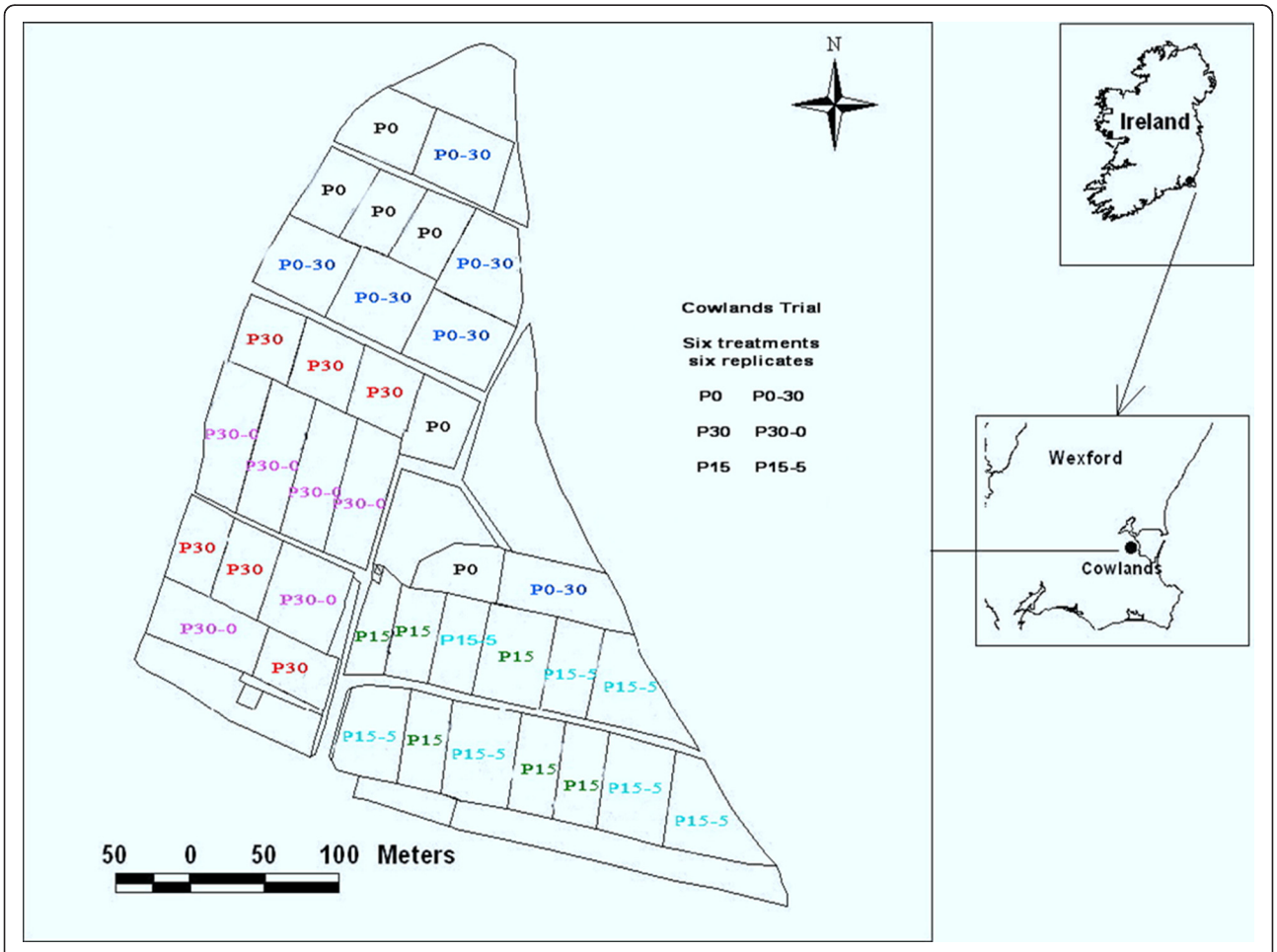

Figure 1 The Cowlands long-term field trial experimental field showing layout of six replicate plots for each of six P treatments. Starting in 1968 P was applied annually at 0 (P0), 15 (P15) and 30 (P30) kg ha^{-1} to each of 12 replicate plots. N (240 kg ha^{-1}) and K (20 kg ha^{-1}) were applied annually to all plots. In 1999 the P application was altered such that half (i.e. 6) of the plots from P0 now received 30 kg P ha^{-1} year^{-1}, half from P15 received 5 kg P ha^{-1} year^{-1} and half from P30 received 0 kg P ha^{-1} year^{-1}. Thus, since 1999 there were six replicate plots of six P treatments: 0_0, 0_30, 15_15, 15_5, 30_30, 30_0 (kg P ha^{-1} year^{-1} 1968–1999 _ kg P ha^{-1} year^{-1} 1999–2009).

(TON) by alkaline persulfate oxidation (Cabrera and Beare 1993); inorganic phosphorus (Pi) by the ammonium molybdate-ascorbic acid method (Watanabe and Olsen 1965). Soil microbial biomass element content was calculated as the difference between the fumigated and unfumigated samples using conversion factors of 0.45 for C (Wu et al. 1990), 0.45 for N (Jenkinson et al. 2004) and 0.40 for P (Hedley and Stewart 1982).

Microbial nutrient limitation

Microbial parameters were determined by a substrate-induced respiration method (Anderson and Domsch 1978) using an automated respirometer based on electrolytic O_2 microcompensation (Scheu 1992). The oxygen consumption rates of 3 g d.w. soil samples were measured at 22°C.

For basal respiration (μl O_2 g^{-1} h^{-1}), the average oxygen consumption was measured 15–20 h after attachment of samples to the respirometer. For SIR measurements, glucose was added to the soil samples in a concentration of 8,000 ppm C, sufficient to induce maximum respiration. The mean of the three lowest measurements during the first 10 h after glucose addition was taken as maximum initial respiratory response (MIRR). Microbial biomass C (C_{mic}, μg g^{-1}) was calculated as 38 × MIRR (Beck et al. 1997). The specific respiration (qO_2, μl O_2 mg^{-1} C_{mic} h^{-1}) was calculated using data on microbial biomass and basal respiration.

In order to detect nutrient limitation of the soil microbial biomass, combinations of nutrients were added in excess (Scheu 1993). Carbon (8,000 ppm glucose), nitrogen ($(NH_4)_2SO_4$) and phosphorus (K_2HPO_4) were added in combination to individual soil samples in a C:N:P ratio of 10:2:1, corresponding to the average element composition of microorganisms in soil. The slope of the microbial growth curves representing microbial growth ability in C, CN, CP and CNP amended samples was taken as a measure of microbial nutrient limitation.

Statistical analysis

Statistical analyses were performed in 'R', version 2.12.2. For stoichiometric analysis data were converted into molar ratios. Data were tested for normal distribution of residuals and homogeneity of variance. Outliers were substituted by mean values, and data were log-transformed if necessary. A one-factorial analysis of variance with the factors 'P-fertilisation treatment' and 'water content' as covariables was conducted. If significant, post-hoc tests using Tukey's test at $P < 0.05$ tested for differences between means. The results are presented as arithmetic means. Linear regression analysis was conducted and the coefficients of determination (r^2) were calculated for soil C and N content, microbial biomass C and P, and log-log plot of total or available soil C:P ratio versus microbial biomass C:P ratio. Discriminant analysis with Tree Classifiers was used to select explanatory variables in multiple regression analysis. Using Akaike's Information Criterion the minimum adequate model in multiple regression analysis was calculated according to Crawley (2007).

Results and Discussion

Results

Soil C, N, P

In general soil C, N and P accumulated with increasing fertiliser P (Table 1). Soil C was 4.6% in 0_0 and rose slightly, but not significantly, in 0_30 and 15_5, before rising significantly in 15_15, reaching a maximum at 5.6% in 30_0 and dropping slightly in 30_30. Soil N showed a strong, positive linear correlation with soil C ($r^2 = 0.92$, $P < 0.0001$) and so responded in a similar manner, rising from 0.44% in 0_0 to 0.55% in 30_0. Soil P rose 2.25-fold from 0.041% in 0_0 to 0.092% in 30_30. Molar nutrient ratios in the soil averaged 219:18:1, and while soil C:N ratios were stable, C:P and N:P ratios declined significantly with added P (Table 2).

Soil microbial biomass C, N, P

Biomass C was least in 0_0 and greatest in 30_0, whether determined by CFE or SIR (Table 1). In general soil microbial biomass C, N and P accumulated with increasing fertiliser P (Table 1). P treatment did not significantly affect the biomass C:N ratio, which ranged from 5.9 to 7.1 (Table 2), but molar element ratios involving P were significantly affected, with the C:P ratio being significantly greater in 0_0 (C:P 16.7) than any other treatment (e.g. C:P 30_30, 10.8) (Table 1), and while the N:P ratio was greatest in 0_0 (2.3), it was only significantly greater than the 0_30 and 30_30 (1.6) treatments (Table 2).

Dissolved organic C and N (i.e. extracted from unfumigated soil during the determination of microbial biomass) behaved differently from P (Table 1). Extractable C and N were significantly greater in the 0_0 treatment and less but stable with added P, whereas extractable P was least in the 0_0 treatment and increased with increasing P.

Microbial nutrient limitation

Basal respiration was fairly constant with an overall mean of 4.6 μl O_2 g^{-1} h^{-1}, which increased slightly but significantly in the 15_15 treatment (Figure 2a). Respiratory quotient was significantly greater in 0_0 than all the other treatments (Figure 2b). Microbial growth was stimulated by the addition of C (Figure 2c) and more so by the addition of CN (Figure 2d), apart from in 0_0 in which microbial growth did not respond to added C or CN. CP enhanced microbial growth in all treatments (Figure 2e), although to a lesser degree in 0_0, while CNP gave maximum microbial growth enhancement

Table 1 Soil and microbial biomass chemistry

	Fertiliser treatment					
	0_0	**0_30**	**15_5**	**15_15**	**30_0**	**30_30**
pH	5.5	5.7	6.1	5.9	6.1	5.8
Soil C (g)	46.1 (2.50)	48.7 (0.9)	47.7 (2.2)	50.4 (4.2)	56.3 (3.1)	53.6 (4.5)
Soil N (g)	4.4 (0.2)	4.6 (0.2)	4.6 (0.2)	4.9 (0.4)	5.5 (0.2)	5.3 (0.5)
Soil P (mg)	408 (20.1)	536 (66.3)	648 (30.7)	662 (53.8)	716 (31.5)	918 (46.5)
Biomass C_CFE (mg)	1.33 (0.13)	1.52 (0.10)	1.52 (0.20)	1.67 (0.37)	1.89 (0.14)	1.76 (0.45)
Biomass C_SIR (mg)	1.34 (0.13)	1.70 (0.20)	1.73 (0.21)	1.76 (0.19)	1.86 (0.11)	1.77 (0.18)
Biomass N (μg)	181 (28.0)	223 (35.8)	255 (21.3)	265 (40.6)	300 (14.4)	263 (28.1)
Biomass P (μg)	79.0 (12.4)	118 (12.0)	113 (17.7)	127 (24.5)	148 (7.6)	160 (26.2)
DOC (μg)	125 (5.4)	105 (3.5)	107 (3.7)	105 (2.5)	101 (5.0)	97.0 (4.7)
NH_4^+-N (μg)	3.65 (0.37)	3.24 (0.49)	2.76 (0.35)	2.48 (0.20)	2.51 (0.27)	2.80 (0.23)
NO_3^--N (μg)	19.6 (2.86)	14.5 (2.04)	16.2 (1.51)	16.0 (2.70)	15.1 (2.1)	14.8 (1.56)
PO_{4+}-P (μg)	8.2 (1.68)	22.0 (2.85)	27.8 (5.21)	31.6 (4.01)	44.8 (4.34)	78.9 (13.29)

pH and C, N and P contents of soils and the soil microbial biomass (in kg^{-1} soil) in a long-term P-fertilisation trial. Soils received 0, 15 or 30 kg P ha^{-1} starting in 1968 (0_, 15_, 30_) and 0, 5, 15 or 30 kg P ha^{-1} since 1999 (_0, _5, _15, _30). See text for details. Microbial biomass was measured by chloroform-fumigation extraction (CFE) or substrate-induced respiration (SIR). DOC is dissolved organic carbon. Data are means, $n = 6$, with standard error *in parentheses*.

with 0_0 still having a significantly lower growth rate (Figure 2f). The addition of NP did not stimulate microbial growth (data not shown).

Multiple regression analysis revealed the significant explanatory variables for microbial nutrient limitation. Thus, microbes were most strongly C-limited with a soil C:P < 90, C_{mic}:C_{org} > 13.8 and N_{mic}:P_{mic} < 4.1 (Table 3). CP-limitation was explained by low CN-limitation, C_{mic}:N_{mic} < 7.4 and a high N_{mic}:P_{mic} (Table 3). CN-limitation was related to a soil N:P < 9.8 (i.e. all P-fertilised soils), C-limitation and an N_{mic}:P_{mic} < 4.6 (Table 3). CNP-limitation was only evident in soils with no P-fertilisation (0_0) and so was positively correlated with CN-limitation (Table 3).

Table 2 Molar C:N:P ratios in soil and microbial biomass

	C:N	C:P	N:P
Total soil pools			
0_0	12.19 (0.26)	301.9 (17.39)	24.62 (1.34)
0_30	12.23 (0.59)	244.7 (29.88)	20.01 (1.72)
15_5	11.97 (0.2)	197.0 (18.35)	16.45 (1.7)
15_15	12.04 (0.23)	204.4 (23.55)	16.97 (1.72)
30_0	11.92 (0.31)	210.1 (11.85)	17.65 (0.79)
30_30	11.88 (0.16)	156.0 (12.91)	13.10 (1.21)
Mean	12.05 (0.35)	219.0 (49.97)	18.13 (3.84)
Microbial biomass			
0_0	8.59 (1.16)	45.57 (6.19)	5.30 (0.86)
0_30	8.15 (1.36)	34.58 (1.94)	4.36 (0.79)
15_5	6.98 (0.82)	36.25 (3.01)	5.27 (0.82)
15_15	7.31 (0.76)	34.99 (3.18)	4.81 (0.47)
30_0	7.37 (0.78)	34.24 (3.15)	4.66 (0.23)
30_30	7.70 (1.28)	29.07 (4.7)	3.80 (0.47)
Mean	7.68 (1.15)	35.77 (6.22)	4.69 (0.82)

Molar element ratios in a long-term P-fertilisation trial. Soils received 0, 15 or 30 kg P ha^{-1} since 1968 (0_, 15_, 30_) and 0, 5, 15 or 30 kg P ha^{-1} since 1999 (_0, _5, _15, _30). See text for details. Data are means, $n = 6$, with standard error *in parentheses*.

Discussion

The Cowlands long-term phosphorus trial

Previous measurements showed that the 15_15 plots were the most productive, having significantly greater live weight gain from the grazed cattle than the 0_0, while the 30_30 plots had a similar live weight gain to the 15_15 but significantly more P loss from overland flow (Culleton et al. 2002). The treatments in the Cowlands long-term field trial were changed in 1999 because the high stocking rate was found to be more productive across all P treatments (Culleton et al. 2002). The comparison then shifted to determining how quickly productivity in the 0_30 plots could be restored, how long accumulated soil P could sustain production in the 30_0 plots and whether reduced application rates in the 15_5 plots were sufficient to maintain optimum productivity (King-Salter 2008). The vegetation composition of the four high P treatments (15_15, 15_5, 30_30, 30_0) was essentially the same, dominated by *Lolium perenne* and *Poa trivialis*, whereas the 0_0 plots were botanically different and dominated by *Agrostis capillaris* and *Holcus lanatus* (King-Salter 2008). The 0_30 plots were intermediate, botanically, and were returning to a high-P-type sward. The treatments, therefore, show a range in P availability, being deficient enough in the 0_0 plots to affect botanical composition, plant productivity and live

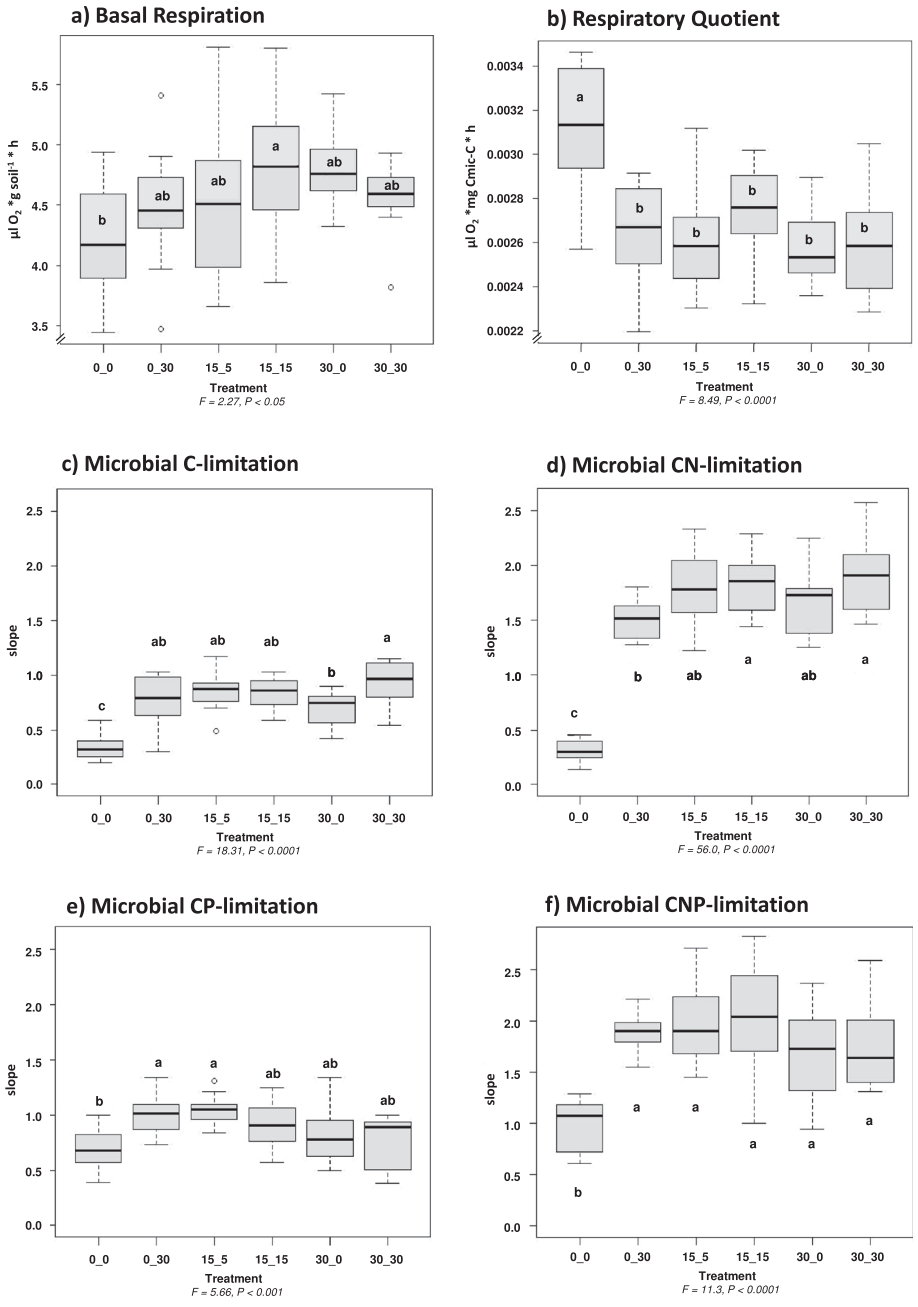

Figure 2 Respiration and microbial nutrient limitation. (a) Basal respiration (µl O_2 g^{-1} soil dry weight h^{-1}), **(b)** respiratory quotient (qO_2 (µl O_2 mg^{-1} C_{mic} h^{-1})) and microbial nutrient limitations, measured by SIR as slope of growth increase following application of **(c)** C, **(d)** CN, **(e)** CP and **(f)** CNP in different soil P-fertilization treatments. Box-plots with upper and lower quartiles. *Different letters* indicate significant differences between means (Tukey's test, $P = 0.05$).

weight gain, and in surplus enough in the 30_30 plots for excess P to be lost by overland flow. It should be noted that even where levels of inorganic P (Pi) were low enough to affect botanical composition (0_0) there were still considerable amounts of organic P (Po) present in the soil, 460 µg Po g^{-1}, but clearly not available for plant uptake (King-Salter 2008).

Phosphorus is relatively immobile in soil and tends to accumulate (Brookes 2001), which would explain the observation that P-fertilisation led to a strong linear increase (2.25-fold) for soil P from unfertilised to medium and high fertilised (0_0 < 15_15 < 30_30) grasslands. Long-term P-application led to increasing soil C and N contents, probably due to increased net primary production of the

vegetation and a subsequent positive feed back by detritus to the soil organic matter (Bever et al. 1997).

The values of Pi extracted from the unfumigated samples were higher than measured in some studies (e.g. 2, 6 and 17 μg Pi g^{-1} for 0_0, 15_15 and 30_30 respectively measured by Culleton et al. (2002) and Fu (2009)), but lower than measured by King-Salter (2008) (e.g. 55, 235 and 435 μg Pi g^{-1}) for the same plots. The differences can be attributed to the different extractants used, as the acetate-acetic acid extraction (Morgan P) used by Culleton et al. (2002), Fu (2009) and King-Salter (2008) is known to give a lower value for Pi than the bicarbonate extraction (Olsen P) used in this study (Foy et al. 1997), while King-Salter (2008) extracted with dilute sulphuric acid. The concentration of Pi in the unfumigated extracts gave the same trend as microbial biomass P and total soil P (i.e. a linear increase as total P application increased), except for the 30_30 treatment. Thus, a total of 900 kg P ha^{-1} had been applied to the 30_0 plots up to 2009 and 1,200 kg P ha^{-1} to the 30_30 plots (an increase of 1.4-fold), similarly P_{mic} increased by 1.1-fold and total soil P by 1.2-fold but extractable Pi almost doubled with a 1.8-fold increase. This indicates oversaturation of P as discussed below.

Table 3 Regression model results for microbial nutrient limitation

Explanatory variable	Estimate	Std. Error	T value	*P* value
C-limitation				
(Intercept)	1.898	0.184	10.33	<0.0001***
$C:P_{soil}$	−0.392	0.074	−5.27	<0.0001***
$N:P_{mic}$	1.088	0.30	3.63	0.0011**
$C:P_{soil} \times C_{mic}$:DOC	0.021	0.005	4.0	0.0004***
$N:P_{mic} \times C_{mic}$:DOC	−0.092	0.023	−4.03	0.0004***
CP-limitation				
(Intercept)	0.622	0.071	8.74	<0.0001***
$CN_{lmt} \times N:P_{mic} \times C:N_{mic}$	0.013	0.003	3.79	0.0003***
CN-limitation				
(Intercept)	13.239	3.534	3.75	0.0009***
$N:P_{soil}$	−1.048	0.339	−3.09	0.0047**
C_{lmt}	−6.749	2.306	−2.93	0.007*
$C_{lmt} \times N:P_{soil}$	0.491	0.192	2.565	0.0164*
$N:P_{mic}$	−1.748	0.635	−2.753	0.0106*
$N:P_{mic} \times C_{lmt}$	0.728	0.304	2.395	0.0241*
$N:P_{mic} \times N:P_{soil}$	0.122	0.055	2.223	0.0351*
CNP-limitation				
(Intercept)	0.842	0.129	6.56	<0.0001***
CN_{lmt}	0.573	0.079	7.26	<0.0001***

Minimum adequate model of a multiple regression on microbial C-, CP-, CN- and CNP-limitation as dependent variables respectively. Explanatory variables are as follows: for C-limitation: soil C:P ratio ($C:P_{soil}$), microbial N:P ratio ($N:P_{mic}$), interactions of these with the ratio of microbial C to dissolved organic C (C_{mic}:DOC) ($r^2 = 0.671$, $F_{[4,29]} = 17.83$, $P < 0.0001$); for CP-limitation: interaction of microbial CN-limitation (CN_{lmt}) × $N.P_{mic}$ × microbial C:N ratio ($C.N_{mic}$) ($r^2 = 0.164$, $F_{[1,67]} = 14.3$, $P = 0.0003$); for CN-limitation: soil N:P ratio ($N:P_{soil}$), microbial C-limitation (C_{lmt}), $N.P_{mic}$, and interactions between these variables ($r^2 = 0.78$, $F_{[6,26]} = 19.58$, $P < 0.0001$); for CNP-limitation: microbial CN-limitation (CN_{lmt}) ($r^2 = 0.429$, $F_{[1,68]} = 52.8$, $P < 0.0001$).

Nutrient limitation of the microbial biomass

We used the CFE technique to determine soil microbial biomass element ratios which, while commonly used, does have limitations which may lead to errors in microbial C, N and P estimation (as discussed by Jenkinson et al. (2004), Cleveland and Liptzin (2007)).

The low amounts of soil C and N in the 0_0 treatment are consistent with low NPP, which is probably accelerated by competition between microbes and plants for P. Soils from the 0_0 treatment had molar $N_{mic}:P_{mic}$ ratios of >5 and were characterised by strong microbial nutrient limitation. The relative microbial growth increase was greatest after CNP addition, illustrating a strong co-limitation by N after P was depleted. These results are consistent with low biomass P content and high C:P and N:P ratios within the 0_0 treatment. Microbial limitation by C was lowest, suggesting that even microbial C-limitation was constrained by nutrients. Constant high P-fertilisation (30_30) led to maximum microbial biomass P and consequently low C_{mic}:P_{mic} and $N_{mic}:P_{mic}$ ratios and strong microbial CN-limitation. However, the correlation of C_{mic} and P_{mic} showed that P_{mic} ratios in 30_30 plot fell well below the regression line (Figure 3) suggesting over-saturation of microorganisms with P, as would also be suggested by the larger than expected concentrations of Pi. The constantly high phosphorus applications led to conditions that were more out of balance than the treatments receiving a medium amount of P fertiliser. There was no additional increase in microbial growth after CNP application compared to the CN addition in the 30_0 treatment and even a decreased microbial growth with added P in 30_30. It can be assumed that in these treatments nutrients other than P and N were limiting and may explain why soil N decreased with decreasing P availability.

The Cowlands trial was grazed, so that even in the no-P fertiliser plots there would have been some return and recycling of organic matter to the sward. All the data indicate that the microorganisms in 0_0 are P limited: the soil microbial biomass had significantly greater C:P than the other treatments. The respiratory quotient indicated a greater degree of stress; microbial response to added nutrients was significantly inhibited and was significantly relieved by the addition of P. The fact that in the respiration experiments with added CNP the 0_0 soil did not respire at the same rate as the other soils implies that this soil is still suffering from limitation by another nutrient, but compared to the effects of adding extra P this is minor. This limitation was removed by the addition

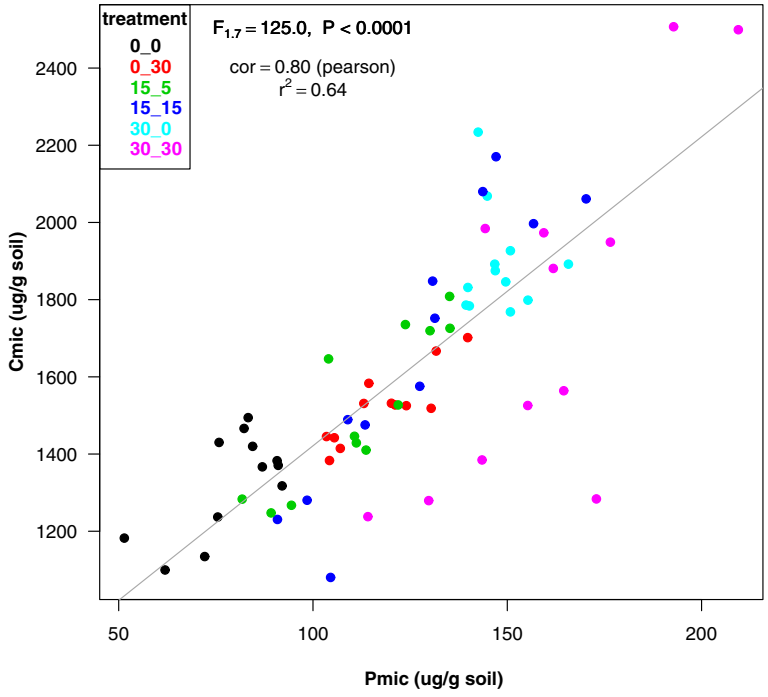

Figure 3 Regression analysis of biomass C (C_{mic} (μg C g^{-1} soil dry weight)) and biomass P (P_{mic} (μg P g^{-1} soil dry weight)) in different soil P-fertilisation treatments. See legend in figure. There are duplicate points for each of six plots per treatment.

of P fertiliser in the 0_30 treatment, which showed no such P limitation 10 years after fertiliser had started to be applied. The opposite effect was less evident in the 30_0 soil from which P fertiliser had been removed for the last 10 years, although there was an indication that the microbial parameters were moving towards signs of P limitation (an increase in C:P ratio and reduced responses to added nutrients compared to the 30_30 soil). In this sense the soil microbial biomass mirrored the response of the vegetation (King-Salter 2008).

In a comparison of forest types in China, Liu et al. (2012) showed that soil respiration measured in situ was significantly enhanced by P fertilisation in an old-growth forest, which was by nature of N-saturation necessarily P-limited, whereas less P-deficient disturbed forests showed no or a lesser respiratory response to P fertilisation.

Microbial community structure

Measuring the gross changes in microbial biomass nutrient contents in response to P fertilisation overlooks any changes in microbial community structure which might explain some of the observed changes. It is likely that the species composition of the microbial biomass would differ in 0_0 from the other treatments due to the change in CNP stoichiometry. Since fungal biomass contains relatively more C than bacterial biomass, it is suggested that fungi might have a higher C-demand than bacteria, while bacteria are more constrained by nutrient ratios (Keiblinger et al. 2010). We found only a weak correlation between the CFE and SIR measurements of C_{mic}, and variation was particularly large in the high P-fertiliser treatments (15_15, 30_0, 30_30). According to Beck et al. (1997) the difference between the two methods is largest when soils contain a disproportionately large component of microorganisms using glucose as an energy source, suggesting a shift in microbial community structure in the high-P soils. The respiratory quotient measurements also indicated a change to the microbial community in the 0_0 soil, with an increase (i.e. more respiration per unit biomass) being indicative of stressed cells or a change in microbial community structure (Anderson and Domsch 1990), although the respiratory quotient showed no variation with reducing P input from the highest level, 30_30 to 30_0. This is supported by measurements at the Cowlands site which indicate an altered microbial community structure (as measured by phospho-lipid fatty acid (PLFA) analysis and nematode community analysis) and decreasing fungal-to-bacterial ratio as P fertilisation increases (Chen, unpublished data). Liu et al. (2012) similarly showed an altered PLFA pattern in the P-limited old-growth forest with added P, but in their case an increased fungal-to-bacterial ratio. Phosphate has recently been shown to be an important driver of microbial community structure in a range of soils (Kuramae et al. 2012).

Response of C:N:P to P fertilisation

The size and elemental composition of the soil microbial biomass in the Cowlands was within the range reported from other temperate grasslands, with biomass C contents of 150–2,800 μg C g^{-1} and biomass P contents of 40–170 μg P g^{-1} with C:P ratios of 11–76 (Brookes et al. 1984; He et al. 1997). However under extreme fertilisation regimes, such as N-only fertiliser since 1897 in a hay meadow (i.e. limited return of organic matter from the cut sward), microbial biomass P can fall to below 10 μg P g^{-1} and the C:P ratio rise to 276 (He et al. 1997). Under managed conditions, therefore, the stoichiometry of the soil microbial biomass can be significantly affected and fall outside the normal range.

The overall soil C:N:P ratio calculated by Cleveland and Liptzin (2007) for grassland soils (166:12:1) is very close to that of our high P 30_30 treatment (156:13:1) but quite far from that of our 0_0 treatment (302:25:1) suggesting that their study might have been based on P-rich soils. The data used by Cleveland and Liptzin partly originated from a study by Turner et al. (2001), who measured soil microbial biomass C, N and P in 29 UK permanent grassland soils by CFE as well as by a UV absorbance procedure. Their soil P contents (0.094 ± 0.032%) were as high as that in our 30_30 treatment (0.091 ± 0.005%). Our microbial C:N ratios were very constant among all treatments supporting the hypothesis of a well constrained ratio within the microbial biomass. Constant C:P ratios occurred predominantly in the medium treatments, but this could be due to a shift in microbial community structure. Our results confirm that C:N:P ratios within the microbial biomass were constrained (homeostatic) as long as soil conditions were not far away from the optimum.

Chemostat experiments using single bacterial species or bacterial communities isolated from aquatic environments have provided a theoretical basis for understanding the stoichiometric response to environmental nutrient ratios (Makino and Cotner 2004). Results have been linked to the growth rate hypothesis that C:P ratios change proportionately to bacterial growth rate because faster growth relates to greater concentrations of RNA and thus P (Elser et al. 1996). The slope of a log-log plot of environmental C:P versus bacterial C:P would show whether the community was constrained (homeostatic) (Makino and Cotner 2004) as has been suggested for the soil microbial biomass (Cleveland and Liptzin 2007). Other studies, however, have indicated aquatic bacterial communities not to be homeostatic, having elemental ratios that varied 1:1 with the environmental ratios (Tezuka 1990). Both results are consistent with the recent findings that bacteria are very flexible in elemental ratios, response to P-limitation, and ability to accumulate P (Scott et al. 2012). A notable difference between the aquatic environments and our edaphic environment is the relative inflexibility of the soil C:P ratio, which only varied by a factor of 2 between the 0_0 and 30_30 soil despite showing clear signs of P-limitation. This may be due to the relatively large and stable background of total P, which varied by a factor of two, compared to the more variable pool of extractable (or readily available) P which varied by a factor of ten (Table 1). Plotting our ratios of soil C:P versus microbial biomass C:P, with the soil C:P calculated either from *total* soil C and P or *available* DOC and PO_4 (data from Table 1, Figure 4), gave slopes that were less than the 1:1 line, indicative of constrained (homeostatic) stoichiometry within the soil microbial community. This is despite the indication of

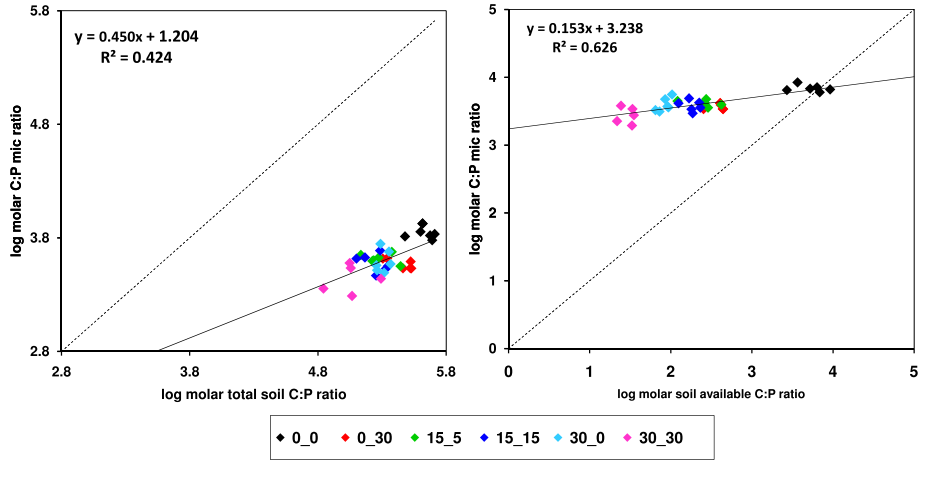

Figure 4 Scatter plots of total soil C:P or soil available C:P ratio versus microbial biomass C:P ratio. Values are shown in comparison to the 1:1 line (dashed) for different soil P-fertilization treatments. See legend in figure. Points represent the mean of duplicate measurements for each of six plots per treatment.

large changes in microbial community structure between the different soils, which is because the community is the highest level of stoichiometric organisation whereas there would be less homeostatic control at the population, cell and macromolecule level (Hall et al. 2010). The molar C:P ratio of the soil microbial biomass in the 0_0 soil was 45:1, very close to the global grassland average of 47:1 (Brookes et al. 1984; Cleveland and Liptzin 2007), which reduced significantly to 29:1 in the 30_30 soil. A study of the stoichiometry of exoenzymes concluded that increased C:P ratios and decreased microbial growth efficiencies are indicators of nutrient limitation (Sinsabaugh et al. 2009). On a global scale the 0_0 soil microbial biomass has an average C:P ratio and would not be considered nutrient limited. However, when compared with the other treatments at the Cowlands site the significant increase in soil microbial biomass C:P, together with the respiration data and corroborating data on vegetation, livestock and microbial community structure, does indicate P limitation and suggests that relative changes in biomass C:P can be indicative of nutrient limitation within a site.

Conclusions

Long-term P-application led to increasing soil and microbial C, N and P contents, with C and N probably reflecting increased net primary production. There were also indications of an altered microbial community structure. Our results confirm that C:N:P ratios within the microbial biomass were constrained (homeostatic) under near optimum soil conditions. Thus, molar nutrient ratios in the soil averaged 219:18:1 and in the microbial biomass the average was 36:5:1. Soils with no added P were characterised by strong microbial nutrient limitation (P and minor nutrients) and soils under high P by over-saturation of microorganisms with P. Relative changes in soil microbial biomass C:P can be indicative of nutrient limitation within a site.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BSG and MB conceived the project, oversaw the experimental plan and drafted the manuscript. BSG organised and assisted with field sampling. AS was responsible for field sampling and laboratory analyses in Ireland and Germany. MB conceived and organised the respiration studies. All authors read and commented on the manuscript.

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