



Estimating phosphorus availability for microbial growth in an emerging landscape

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ABSTRACT

Estimating phosphorus (P) availability is difficult—particularly in infertile soils such as those exposed after glacial recession—because standard P extraction methods may not mimic biological acquisition pathways. We developed an approach, based on microbial CO₂ production kinetics and conserved carbon:phosphorus (C:P) ratios, to estimate the amount of P available for microbial growth in soils and compared this method to traditional, operationally-defined indicators of P availability. Along a primary succession gradient in the High Andes of Perú, P additions stimulated the growth-related (logistic) kinetics of glutamate mineralization in soils that had been deglaciated from 0 to 5 years suggesting that microbial growth was limited by soil P availability. We then used a logistic model to estimate the amount of C incorporated into biomass in P-limited soils, allowing us to estimate total microbial P uptake based on a conservative C:P ratio of 28:1 (mass:mass). Using this approach, we estimated that there was <1 µg/g of microbial-available P in recently de-glaciated soils in both years of this study. These estimates fell well below estimates of available soil P obtained using traditional extraction procedures. Our results give both theoretical and practical insights into the kinetics of C and P utilization in young soils, as well as show changes in microbial P availability during early stages of soil development.

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1. Introduction

Phosphorus (P) limitation in terrestrial ecosystems may be widespread (Elser et al. 2007; Wardle et al. 2004), but assessing P limitation is difficult for two main reasons: First, directly assessing ecosystem P limitation can only be achieved by manipulating P inputs and analyzing plant and/or soil responses through time (Vitousek and Farrington 1997). Second, accurately measuring biologically available P pools (available P) is problematic because measurements of “available P” typically utilize operationally-defined extraction procedures. For example, the amount of P that is liberated during an extraction with a particular chemical solution (e.g. Tiessen and Moir 1993) is implicitly assumed to represent available P, but these fractions may contain more or less P than is actually biologically available. Our limited ability to assess available P pools also challenges attempts to assess the extent to which P limits ecosystem processes in general, and to compare P limitation across ecosystems (Vitousek et al. 2010).

In an attempt to overcome these difficulties, we developed a bioassay approach—based on microbial growth and CO₂ production kinetics and constrained microbial carbon:phosphorus (C:P) ratios (Cleveland and Liptzin, 2007). This approach builds on a large body of previous work demonstrating that P can limit both the rate and extent of microbial growth in terrestrial and aquatic systems (Carlsson and Caron, 2001; Cleveland et al., 2002; King et al., 2008; Morris and Lewis, 1992; Souza et al. 2008) and that microbes have high affinity uptake systems that can scavenge P at levels below the detection limits of most methods for measuring P in natural systems (Button, 1985; Voegelé et al., 1997; Zubkov et al., 2007). Our bioassay approach has two primary advantages over traditional methods commonly used to assess P availability and P limitation. First, using short-term laboratory assays, it provides estimates of the amount of P immobilized by microbes during growth, and thus serves as a direct measure of the amount of P available to microorganisms. Second, the method provides information describing relative P limitation in soils, and therefore may provide insight into the overall extent of ecosystem P limitation.

Here, we describe results from two sampling expeditions to a relatively pristine watershed in the High Andes of Peru. Using soil sampled at multiple sites in two years, we used this new technique to assess P availability and relative P limitation across this emerging (recently covered by a glacier) landscape. This remote site has been

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the focus of many recent ecological studies, partly because glaciers in the Sibinacocha valley have receded very rapidly over the past 80 years (Seimon et al., 2007) exposing large areas of soil that can remain devoid of plants for over 20 years (Nemergut et al., 2007; Schmidt et al., 2008). Preliminary studies of the P status of these soils indicated that they are low in available P and that phosphatase activities ($\sim 20 \text{ nmol h}^{-1} \text{ g}^{-1}$) were the same as those in un-vegetated Colorado soils where heterotrophic microbial activity is P limited (King et al., 2008). Using soils collected from these sites, we examined P availability using both traditional methods and our new technique across this potentially P-limited landscape.

2. Methods

2.1. Study sites

We sampled soils in the recently deglaciated valley of the Laguna Sibinacocha Watershed ($13^{\circ}46'20'' \text{ S}$, $71^{\circ}04'37'' \text{ W}$), Cordillera Vilcanota, Peru. Soils were collected during two separate expeditions to the sites in September 2001 and August 2003. At this recently deglaciated site, soils were collected at the edge of the glacial terminus ("0 m" samples) and 100 m from the terminus ("100 m" samples; uncovered for ~ 5 y). In addition, we sampled soil from a set of sites that had been exposed for >20 years ("Spit/Pass" sites) and from a vegetated site that remained ice-free during the Little Ice Age ("Boundary site"). Soil samples were frozen in the field at -10° C (the low night-time soil temperature at the site; Schmidt et al. (2009)) and were transported on ice in thick-walled coolers to the laboratory at the University of Colorado, Boulder. General soil biogeochemical properties of all soils are provided elsewhere (King et al., 2008; Nemergut et al., 2007; Schmidt et al., 2008), and photographs and descriptions of the sites can be found elsewhere (Halloy et al. 2005; Schmidt et al. 2009; Seimon et al. 2009). When we returned to the watershed in 2003, we focused our attention on the rapidly receding Puca glacier and carried out more detailed microbial community structure work as reported elsewhere (Nemergut et al. 2007), as well as repeating the P addition experiments on soils that had been uncovered for <1 year (0 m) and 5 years (100 m). We used 4 spatially distinct replicates for each soil age and added more glutamate than in 2001 in order to enhance the effects of P on respiration kinetics.

2.2. Soil P fractions

Our goal was to compare estimates of available P using a biologically defined method to more traditional, functionally-defined estimates of soil P availability. Therefore, "available P" was assessed using the initial steps of the modified Hedley fractionation procedure of Tiessen and Moir (1993). Briefly, 1 g soil was subjected to a resin extraction in water (Resin P_i) to extract inorganic P_i , followed by a bicarbonate extraction (Bicarb P_i). Organic bicarb extractable P (Bicarb P_o) was determined as the difference between total Bicarb extractable P (Bicarb P_t) and Bicarb P_i following digestion with ammonium persulfate and sulfuric acid (Tiessen and Moir, 1993). These three fractions (resin P_i and Bicarb $P_i + P_o$) are the most labile forms of P, and their sum is often used as a proxy for readily available P (Cross and Schlesinger, 1995). Bowman et al. (1978) and Levy and Schlesinger (1999) have shown that bicarb P ($P_i + P_o$) can be correlated with plant growth. Total P (P_t) in soil samples was determined by digesting 5 g of sieved, air-dried soil in H_2SO_4 and H_2O_2 . Phosphate concentrations in all measured fractions were determined using the ammonium molybdate ascorbic acid method (Kuo 1996) on an AlpKem autoanalyzer (OI Analytical, College Station, TX). Total mineral P was determined using element analyses with a Philips PW1400 Wavelength Dispersive Spectrometer, X-ray fluorescence instrument. Operating conditions for the Rh X-ray tube were 40 Kv and 20 Ma. Samples were first dried and then ground to $<70 \mu\text{m}$. Samples were then mixed with a binder (corn starch) and

pressed into a pellet. Quantitative analyses using USGS rock standards BHVO-1, GSP-1, or GH was performed using the fundamental parameters correction procedure in the Philips X40 V.4.0A software.

2.3. Microbial kinetics

The kinetics of C (glutamate) mineralization (with and without P additions) was measured in soils using previously described approaches (Cleveland et al., 2002; Colores et al., 1996). For each treatment, sieved (2 mm) and homogenized soil samples (5 g dry weight equivalent) were placed in sterile biometer flasks and glutamate (a source of available C and N for soil microbes, Scow et al. 1989) was added in a small amount of sterile water along with tracer concentrations of uniformly ^{14}C -labeled glutamate to yield final concentrations of $50 \mu\text{g C per g of soil}$ (2001) or $128 \mu\text{g C per g of soil}$ (2003). Soils were incubated at 22° C (the high day-time soil temperature at the site; Schmidt et al. 2009) and the evolved CO_2 was trapped in 1 ml of 0.5 M NaOH in the sidearm of the biometer flask. Periodically, the NaOH was removed, mixed with 2.5 ml of scintillation cocktail (Scintiverse II Cocktail, Fisher Scientific, Pittsburgh, PA), and the mixture was counted using a scintillation counter. Fresh NaOH was immediately added back to the sidearm after each sampling. The influence of P on mineralization kinetics was determined by adding $500 \mu\text{g of P per g of soil}$ as 1 M potassium phosphate solution to a subset of the samples. The pH of the phosphate solution (7.8) was approximately the same as the pH of the soil (7.5–7.6; Nemergut et al. 2007) to avoid lowering the soil pH, which can increase the rate of CO_2 efflux from the soil.

2.4. Estimating soil P using microbial kinetics

Our overall approach for estimating microbial available P was to compare respiratory responses of the soil to glutamate additions, with and without added P (all other conditions being equal). Sigmoidal (logistic) CO_2 production kinetics are used to estimate microbial biomass C production under P limitation and subsequently the microbial P uptake can be estimated by assuming a conservative stoichiometry of C:P ratios in the final biomass produced. This overall approach provides a conservative and ecologically relevant estimate of the amount of P that was available to support microbial growth in these soils.

To estimate the biomass produced under P limitation, we invoked the well-established principle that the production of a primary metabolite (e.g. CO_2) by a microbial population is directly proportional to the biomass of organisms producing the metabolite (Anderson and Domsch 1978; Colores et al., 1996; Schlegel, 1992). Furthermore, the growth of microorganisms often follows logistic (sigmoidal) kinetics, indicating an initial growth phase followed by a deceleration and a cessation of growth due to nutrient limitation (Simkins and Alexander, 1984; Zwietering et al., 1990). Thus, we can express the production of CO_2 from a soil sample using the integrated logistic equation (Berman, 1974) in terms of CO_2 production or:

$$C(t) = C_k / \left(1 + e^{-r(t-i)} \right) \quad (1)$$

where $C(t)$ is the total CO_2 produced (with units of $\mu\text{g C g}^{-1}$) at time t , r is the intrinsic rate of increase (equivalent to μ_{max} with units of h^{-1}), i represents inflection point of the CO_2 production curve (with units of h) and C_k is the total CO_2 produced per gram of soil up to the time that growth ceased. In other words, C_k is the total amount of CO_2 produced during the growth of the population up to the carrying capacity (K) of the soil. To estimate C_k , Eq. (1) was fit to curves of CO_2 accumulation over time using the non-linear regression package of Kaliedagraph® (Synergy Software, Reading, PA, USA).

The final biomass of respiring organisms can be estimated by converting C_k to units of biomass C by using (Colores et al., 1996):

$$B_f = C_k(Y_c / 1 - Y_c) \quad (2)$$

where B_f is the final biomass ($\mu\text{gC/g soil}$) and Y_c is the “yield coefficient” or the efficiency of conversion of substrate carbon to microbial biomass. Y_c can be estimated from soils that received excess C, N and P by assuming that the added C not converted to CO_2 during the incubation went into microbial biomass. This assumption probably results in overestimates of Y_c , because it is unlikely that microbes can convert all of the added C to either biomass or CO_2 . Therefore we will assume the lowest Y_c value that we obtained, 0.48 (range = 0.48–0.59, $n = 34$) applies in our calculations of microbial P utilization (see below).

To estimate microbial available P in soils, we assume that any soil in which P amendment stimulates the rate and extent (r and C_k in Eq. (1)) of glutamate mineralization are P limited under the experimental conditions. Therefore we can use the biomass C (B_f in Eq. (2)) and a conservative estimate of a microbial C:P ratio to estimate the amount of P incorporated into biomass in un-amended soil samples. We conservatively assume a C:P ratio of 28:1 (on a mass: mass basis) based on the work of Cleveland and Liptzin (2007) who demonstrated that this ratio is well constrained for soil microbial biomass on a global scale. Thus, we can multiply B_f by 1/28 to obtain an estimate of the amount of P immobilized from the soil by microbes under P limitation in the unamended incubations or:

$$P_a = B_f * 0.0357 \quad (3)$$

where P_a is the estimated concentration of P available to microbes during the incubation.

2.5. Estimating microbial growth rates

It is also useful to compare microbial growth rates (r) from P-amended and control soils. Eq. (1) approximates r , but sensitivity analyses and problems associated with the accumulation of error from using accumulated CO_2 production data to indirectly estimate rates make such estimates less robust than methods that utilize discrete measures of CO_2 flux over time (Colores et al. 1996). Therefore we used plots of rate versus time and the first derivative of Eq. (1) to estimate growth rates:

$$dC/dt = rC_k e^{-r(t-1)} / (1 + e^{-r(t-1)})^2 \quad (4)$$

where all parameters are as defined above. Eq. (4) describes how the instantaneous rate of CO_2 production changes over time. This allows for a statistically valid estimation of r because it is based on the principle that the rate of CO_2 production at any given time is proportional to the population of organisms producing the CO_2 as shown previously (Anderson and Domsch, 1978; Colores et al., 1996; Schlegel, 1992).

2.6. Statistics

A two-way unbalanced ANOVA with interaction (Devore, 2004) was performed on the growth rate data using the program R (version 2.8.1, 12/22/2008, R Foundation for Statistical Computing <http://www.r-project.org/index.html>). Site and treatment were set as categorical variables. The growth rate obtained from the logistic regression was the dependent variable. Tukey's Honestly Significant Difference Test was performed in R 2.8.1 as a post hoc assessment of significant differences (Devore, 2004).

3. Results

During our initial expedition to the Sibinacocha watershed (2001) we sampled soils from five areas: two un-vegetated sites adjacent to receding glaciers (0 and 100 m soils), two older, un-vegetated sites (Pass and Spit sites) and one vegetated soil for reference (“Boundary” soils). These sites are indicated on an aerial photograph in Schmidt et al. (2009). Levels of available P (the sum of resin P_i , bicarb P_i and bicarb P_o) measured using standard approaches in all of the un-vegetated soils were approximately the same (Table 1), whereas the well-developed (vegetated) soils from the Boundary had approximately 40 times higher levels of P (Table 1). P additions stimulated mineralization of added glutamate (a source of available C and N) in the 2001 soils that were recently de-glaciated (Fig. 1), but had no effect on the Pass soils and only a slight effect on soils from the Spit site (data not shown). Using estimates of microbial C production in the control soils (no P addition) from the 0 m, 100 m and Spit sites (e.g. 20.4, 20.7 and 24.4 μgCg^{-1} , respectively), we were able to estimate microbial-available P using Eq. (3). Our estimates of P_a (Table 1) were higher than Bicarb P_i levels and lower than Resin P_i levels and much lower than “available P” as traditionally defined (Table 1).

When we returned to the watershed in 2003, we focused our attention on soils that had been exposed by the rapidly receding Puca glacier (similar to the 0 and 100 m sites from 2001) and carried out more detailed microbial community structure work as reported elsewhere (Nemergut et al. 2007), as well as repeating the P addition experiments on soils that had been uncovered for <1 year (0 m) and 5 years (100 m). All of the soils showed a more accentuated response to P than in 2001, because more glutamate was added in 2003. Control soils (no added P) gave the same kinetic curves in both years (compare Figs. 1 and 2). Estimates of P available for microbial growth (P_a) and traditional P levels reported for these same samples (data from Nemergut et al. 2007) are shown in Table 2.

Fig. 3 shows an example of the effects of P additions on the rate of CO_2 efflux from a P-limited soil (100 m, 2003); these data were used to estimate the rate of microbial growth (r or μ_{max}) with and without the addition of P using Eq. (4). Growth rates for all of the incubations (with and without P) are shown in Fig. 4. Growth rate responded to the increased C additions in the +P treatments in 2003 whereas the growth rates in the control (no P) soils were the same across years.

4. Discussion

Our data show that in these recently deglaciated sites, P additions have a positive effect on the kinetics of glutamate mineralization (Figs. 1, 2 and 3), but this was not the case in soils that had been uncovered for 20 years or more. Thus, P appears to be more limiting in

Table 1

Available P concentrations ($\mu\text{g/g}$) in soils collected from the Sibinacocha watershed in 2001 using the modified Hedley fractionation procedure. Values are means \pm 1 S.E.M. ($N = 3, 3, 5, 5, 2$ for 0 m, 100 m, Spit, Pass and Boundary, respectively). “Available P” represents the sum of bicarb (P_i and P_o) and resin P_i values. P_a represents the estimated amount of P immobilized into microbial cells in soils that were P limited. Total P (X-ray fluorescence) in the 0 m soils was 610 $\mu\text{g/g}$ (SEM = 23, $N = 3$).

	0 m	100 m	Spit	Pass	Boundary
Resin P_i	1.78 (0.43)	0.74 (0.08)	1.40 (0.24)	1.54 (0.35)	36.2 (7.8)
Bicarb P_i	0.44 (0.43)	0.36 (0.28)	0.35 (0.08)	0.91 (0.13)	19.2 (0.7)
Bicarb P_o	3.77 (0.24)	4.91 (0.50)	3.90 (0.23)	5.15 (0.44)	165.46 (1.58)
“Available P” (sum of above)	5.99 (1.04)	6.01 (0.75)	5.60 (0.24)	7.60 (0.57)	221 (7.0)
P_a	0.73 (0.03)	0.74 (0.02)	0.87 (0.02)	ND ^a	ND ^a

^a ND, not determined because these soils were not P-limited for microbial growth.

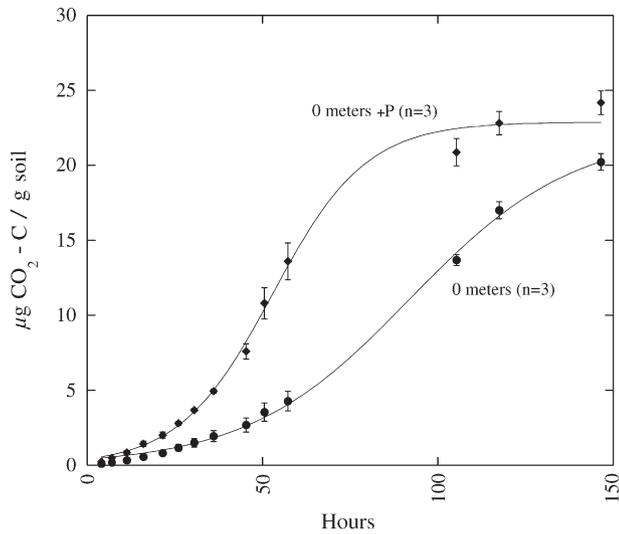


Fig. 1. CO_2 production from glutamate added to soils that had been uncovered for <1 year (0 m soils in 2001) in the presence (+P) or absence of added P. The integrated logistic model (Eq. (1)) was fit to the data using non-linear regression; R^2 values were 0.995 and 0.998 for the +P and -P data, respectively. The estimated asymptote (C_k) for total CO_2 produced in the -P curve was $22.1 \mu\text{gC g}^{-1}$ (SEM = 0.69) and was used to estimate the final biomass using Eq. (2).

soils immediately after deglaciation and then to become less so with time, presumably because P derived from rock P solubilization increases with time as a result of microbial activity and biogeochemical weathering (Walker and Syers, 1976).

These data allowed us to develop a new approach to estimate microbial-available P. Our method takes advantage of the well supported theory that microbial populations with excess C and N will completely scavenge limiting levels of P due to their high surface-to-volume ratios and high-affinity uptake systems for P (Zubkov et al., 2007). Indeed, microbes have uptake systems for P with half-saturation constants (K_m) of sub- μM levels, equivalent to P concentrations in the

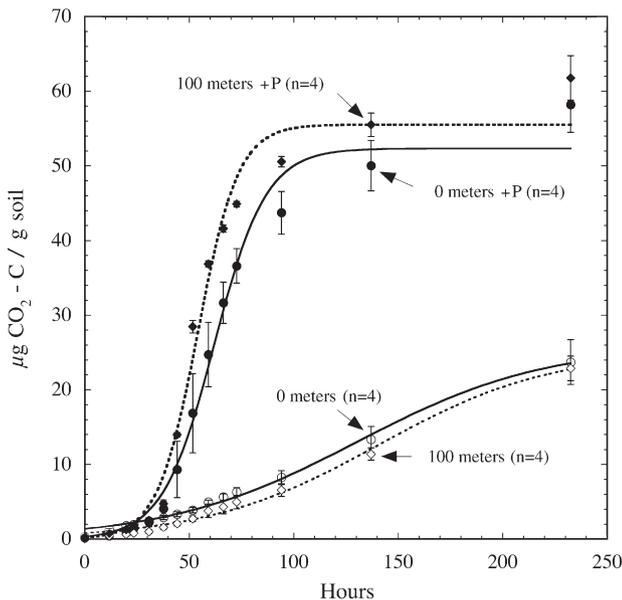


Fig. 2. CO_2 production from glutamate added to soils that had been uncovered for <1 year (0 m soils) or for 5 years (100 m soils) in 2003 in the presence (+P) or absence of added P. In both soils, P strongly stimulated respiration. Eq. (1) was fit to the data and R^2 values were 0.98 or greater for all curve fits. The estimated asymptote (C_k) for total CO_2 produced in the -P curves was 26.0 and $25.3 \mu\text{gC g}^{-1}$ for 0 and 100 m, respectively and were used to estimate the final biomass using Eq. (2).

Table 2

Phosphorus concentrations ($\mu\text{g/g}$) at different distances from the receding Puca glacier in 2003. For each measurement, $N=4$. The resin P_i data are from Nemerugut et al. (2007).

	0 m	100 m
Resin P_i	2.52 (0.85)	1.71 (0.17)
P_a	0.87 (0.09)	0.82 (0.08)

parts per trillion to low parts per billion range (Button, 1985; Voegelé et al., 1997). Thus when provided with a labile source of C and N (such as glutamate) soil microbes can easily scavenge levels of P below the detection limits of traditional methods for measuring available P in soils. Our approach was therefore to measure microbial growth under P limitation (the control soils of sites that were stimulated by P addition in parallel incubations), when C and N availability were abundant, and then estimate the amount of P that was needed to grow that much biomass. The differences in the estimated amount of C mineralized in control versus amended soils form the basis of our approach to estimating how much P was available for microbial growth in these soils. The asymptote of the sigmoidal CO_2 -mineralization curves in the absence of P addition (C_k in Eq. (1)) is proportional to the biomass C produced during the incubation as mathematically described in Eq. (2) and elsewhere (Colores et al., 1996). This relationship allows the estimation of how much P was used to produce this biomass by using well-constrained C:P stoichiometry for microbial biomass (Cleveland and Liptzin, 2007).

Using this approach we estimated that these early successional soils contained about $0.8 \mu\text{g P g}^{-1}$ (see Tables 1 and 2). These estimates are well below traditional estimates of “available P” measured in these same soils. In fact, our estimates range from 12 to 15% of those for “available P” in the same soils (Table 1). Surprisingly, recent work in P-limited ocean waters also indicate that radio-tracer based bioassays show that bio-available P is from 7 to 55% of P in the dissolved phosphate fraction of sea water (Zubkov et al., 2007). Thus, because bioassays represent a biologically-defined measurement of a biologically important pool, they may serve as a better indicator for available P in P-limited systems than traditional measures.

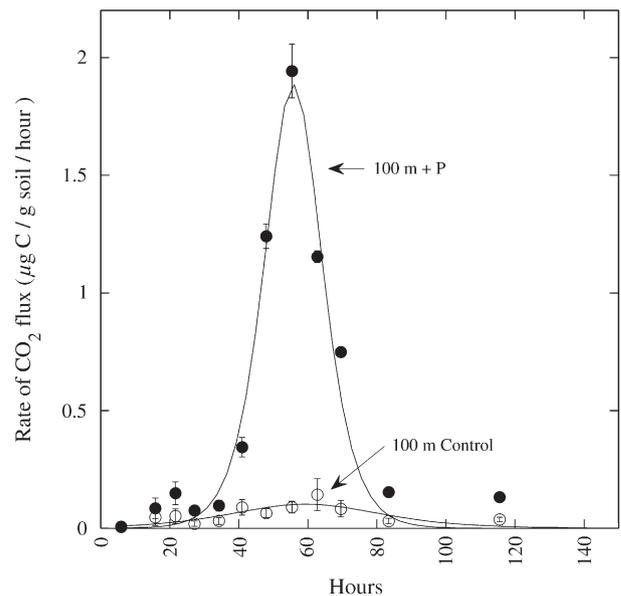


Fig. 3. Effects of P on the rate of CO_2 flux from recently de-glaciated soils. Each curve is the mean of 4 spatially separate soil samples (\pm SEM) collected 100 m from the toe of the receding Puca Glacier. Lines are the best fit of Eq. (4) to the data. Similar curve fits to each of the 4 replicates separately were used to estimate growth rates as depicted in Fig. 4.

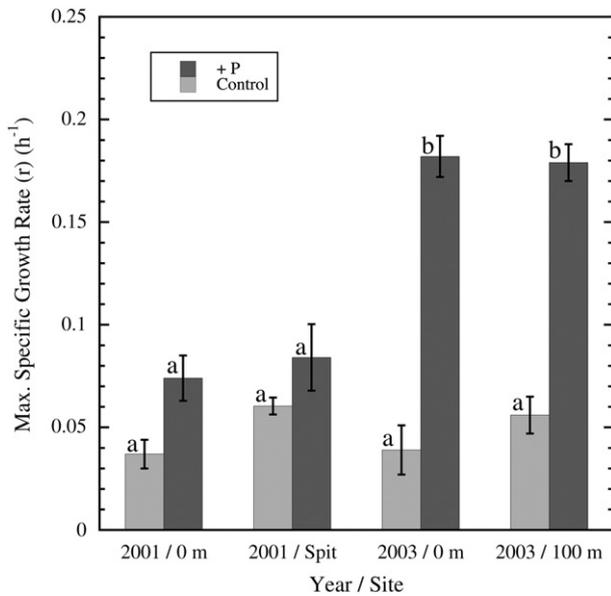


Fig. 4. Maximum specific growth rates (r or μ_{\max}) in incubations with and without added P. Estimates of r were obtained by using Eq. (4) and plots of the change of rate of CO_2 production over time (e.g. Fig. 3) during the soil incubations. $N = 3$ for 2001 and 4 for 2003. Different letters on bars indicate significant differences ($P < 0.001$) among treatments within a site.

This study and our previous work at the same sites point to severe P deficiencies for microbes during the earliest stages of succession. In the Sibinacocha Valley of Perú, the soils that had been ice covered within 5 years of sampling in both 2001 and 2003 (0 and 100 m soils) showed dramatic responses to added P. These are the same soil ages that showed a significant increase in microbial phosphatase activity during the first 5 years of being ice-free (Schmidt et al. 2008). In the present study, the estimated differences in growth rates between the amended and unamended soils are also a strong indicator of P limitation. The 0 and 100 m +P soils exhibited a much higher maximum specific growth rate (r) as compared to the unamended soils in both years of this study. In 2001, r in the +P soils was $0.074 \pm 0.01 \text{ h}^{-1}$ compared to an r of $0.037 \pm 0.007 \text{ h}^{-1}$ in the unamended soils and the difference in growth rates was even more extreme in the 2003 study (Fig. 3) because more C was added in 2003. More importantly for the present study, the unamended soils showed an upper limit in their ability to mineralize added C in the absence of supplementary P (i.e. the unamended CO_2 evolution curves leveled off after only about 20 μg of the added C had been mineralized, even though much more C was added in 2003; Figs. 1 and 2). By contrast, the amount of C mineralized in the +P treatments was proportional to the amount of C added (across both years), indicating that non-P-limited growth was occurring in the +P treatments for the 0 and 100 m soils. These results strongly indicate P limitation is greater in newly de-glaciated soils compared to older soils in the Sibinacocha Valley. Newly exposed soils will have large stocks of P present in primary mineral forms (610 $\mu\text{g/g}$ in the present study) but small stocks of available P (0.8 $\mu\text{g/g}$ in the present study) because biological and physical breakdown of this material has not had time to release the mineral P (Schlesinger et al., 1998; Walker and Syers, 1976). Our data suggest that the microbial communities in these new soils are P limited (Fig. 4) and that, as the soils develop and as P is released through pedogenesis, P limitation declines.

5. Conclusion

While this approach precludes conclusions regarding the extent of P vs. C or N limitations in these soils, we do show that the relative

amount of P that is available to microbes increases during the early stages of microbial succession. Moreover, we suggest that our approach may be a more sensitive indicator of P availability than chemical extractions. Regardless of what the P extractions show, the microbes show that P is relatively more abundant in old soils than in young soils. Thus our results support the hypothesis that the microbial community is initially P limited, but that P constraints relax over the long term. This observed increase in the “functional” P economy may provide insight into why initial plant colonizers are N (and not P) limited; the activity of microbes may drive increases in the relative abundance of P during the earliest stages of primary succession.

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