Reconsidering the phosphorus limitation of soil microbial activity in tropical forests

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Abstract

1. It has long been believed that soil microbial activity in tropical forest ecosystems is limited by phosphorus (P) rather than nitrogen (N) availability. In this study, we reviewed the methods used to determine the limiting nutrients and evaluated the validity of the widespread P-limitation hypothesis in tropical forest soils.

2. The most commonly used analysis method entails testing whether fertilization increased microbial biomass or soil respiration. Fertilization using microbial biomass as an indicator was not a satisfactory method because standing microbial biomass does not always signal microbial activity. An increase in soil respiration after fertilization was also an insufficient indicator because the negative response shown by organic matter decomposition to nutrient addition can also be driven by nutrient shortage (nutrient mining). Nutrient amendment can also shift microbial communities towards more copiotrophic organisms, which may exhibit lower microbial respiration rates.

3. We suggest that P addition may accelerate soil organic matter decomposition compared with N, which is independent of the nutrient limitation of soil microbial activity. The negative response of organic matter decomposition to N addition through nutrient mining is driven by N shortage, which is less likely to occur with P. N addition inhibits microbial activity via chemical reactions, whereas P addition may stimulate activity through replacement by C bound to sorption sites in the soil, improving C availability. Thus, the P-limitation hypothesis must be reconsidered because of the contrasting effects of exogenous N and P addition on soil microbial activity, which could lead to misdetection of P limitation on soil microbial activity in tropical forest ecosystems.

4. We recommend caution in applying the statement that soil microbial activity in tropical forest ecosystems is limited by P until a novel method is established to accurately determine the nutrients limiting soil microbial activity at the ecosystem level. We proposed alternative ways to describe nutrient shortage for soil microbes.

KEYWORDS
fertilization experiments, microbial activities, nitrogen, nutrient limitation, phosphorus, tropical ecosystems

1 | INTRODUCTION

Nutrient limitation is a scientific concept that originated in the field of agricultural chemistry in the 19th century (Perakis, 2002). Since then, several ecologists have sought to identify the nutrients that limit ecosystem production because determining the factors that control production is critical for understanding ecosystem function. The concept of nutrient limitation is one of the organizing principles of modern biogeochemistry. According to Gibson (1971), the term "nutrient limitation" has three possible definitions. First, an organism is limited when it is not growing as fast as it is theoretically able to be. Second, when a factor is in such short supply that no growth is possible, the factor is said to be limiting. Third, if a factor is increased and no effect on growth is observed, then the factor is not limiting. When we consider ecosystem processes that involve various types of species, these definitions can be revised to include shifts in the community species composition (Howarth, 1988). A limiting nutrient can be defined as follows: a nutrient is limiting when the potential rate of net primary production, allowing for possible shifts in the species composition, is suppressed by a lack of the nutrient (following the definition by Howarth, 1988).

Primary production in tropical rainforests established on deeply weathered soils has long been considered phosphorus (P)-limited (Crews et al., 1995; Elser et al., 2007; Kitayama & Aiba, 2002; Sollins, 1998; Vitousek, 1984; Vitousek & Farrington, 1997; Vitousek, Porder, Houlton, & Chadwick, 2010; Vitousek & Sanford, 1986; Walker & Syers, 1976), contrasting with other terrestrial ecosystems, where nitrogen (N) is considered to limit primary production (Hunt, Ingham, Coleman, Elliott, & Reid, 1988; Nilsson & Wiklund, 1992; Sikström, 2002; Tamm, 1956; Tamm, Aronsson, Popovic, & Flower-Ellis, 1999; Vitousek & Howarth, 1991). This determination is made because of the low P availability in tropical soils caused by the binding of a large portion of soil P to aluminium (Al) and iron (Fe) or physico-chemical adsorption to Al and Fe oxides and hydroxides, which then becomes occluded and unavailable to biota (Cross & Schlesinger, 1995). Nitrogen, on the other hand, is expected to be more available due to enhanced N mineralization in a warmer and wetter climate (LeBauer & Treseder, 2008) and due to accumulation through N_2 fixation (Vitousek & Farrington, 1997). However, the results of fertilization experiments, which can directly test nutrient limitation by assuming that addition of the limiting nutrient stimulates primary production, have been inconclusive (Alvarez-Clare, Mack, & Brooks, 2013; Campo & Vazquez-Yanes, 2004; Davidson et al., 2004; Gehring, Denich, Kanashiro, & Vlek, 1999; Lawrence, 2001; Mirmanto, Proctor, Green, & Nagy, 1999; Newbery et al., 2002; Santiago et al., 2012; Wright et al., 2011). Experimental P fertilization did not necessarily stimulate the tree growth (Alvarez-Clare et al., 2013; Newbery et al., 2002). Several studies reported that forest productivity was limited not only by P but also by other nutrients such as N (Campo & Vazquez-Yanes, 2004; Davidson et al., 2004; Mirmanto et al., 1999) and potassium (Wright et al., 2011). One meta-analysis suggested that N frequently limits terrestrial primary production, even in tropical forest ecosystems (LeBauer & Treseder, 2008). Thus, in contrast to the traditional axiom, fertilization experiments have shown that not only P, but also N and other nutrients, limit primary production

in tropical forest ecosystems. The co-limitation of primary production may be explained by several mechanisms. First, the use of substitute chemical compounds (e.g. a replacement of phospholipids by galactolipids or sulfolipids in cellular membranes; Lambers et al., 2012) can reduce P demand under P-depleted conditions. Second, P shortage can be buffered by decreasing stem P concentration, whereas stem N concentration must be maintained due to its role in constructing tree structure, implying that P shortage has a less suppressive effect than N has on tree growth (Aoyagi & Kitayama, 2016). Third, plants can increase P availability at the expense of N by releasing enzymes that decompose P esters in soil organic matter (Houlton, Wang, Vitousek, & Field, 2008; Marklein & Houlton, 2012; Olander & Vitousek, 2000).

Understanding nutrient limitation to soil microbial activity, which is closely related to decomposition, is essential for the prediction of global C cycling because nutrient inputs into environment have been intensively changed by anthropogenic activities in recent decades (Cleveland, Townsend, & Schmidt, 2002; Gallardo & Schlesinger, 1994; Galloway et al., 2004; Ilstedt & Singh, 2005; Mo et al., 2008; Scheu, 1990). In contrast to primary production, where co-limitation by multiple elements has been observed (see above), most studies have come to the conclusion that microbial activity in tropical forest soils is mainly limited by P availability (after C, which is the primary energy source), by demonstrating that P addition stimulated soil heterotrophic microbial respiration (Cleveland, Reed, & Townsend, 2006; Cleveland & Townsend, 2006; Cleveland et al., 2002; Duah-Yentumi, Ronn, & Christensen, 1998; Gnankambary, Ilstedt, Nyberg, Hien, & Malmer, 2008; Ilstedt, Giesler, Nordgren, & Malmer, 2003; Ilstedt & Singh, 2005; Liu, Gundersen, Zhang, & Mo, 2012; Mori, Ohta, Konda, Ishizuka, & Wicaksono, 2010; Mori et al., 2013a,b; Teklay, Nordgren, & Malmer, 2006), and increased microbial biomass (Fanin, Hättenschwiler, Schimann, & Fromin, 2015; Liu et al., 2012, 2015; Mori, Wachrinrat et al., 2016; Turner & Wright, 2014). Those studies reported that N addition had less impact than P addition (Gnankambary et al., 2008) or even had negative effects on microbial activity (Cleveland & Townsend, 2006; Cleveland et al., 2006; Fanin et al., 2015; Mori et al., 2013c). The different responses of primary production and soil microbial activity to the addition of N and P can be explained by the growth rate hypothesis (Elser, Dobberfuhl, Mackay, & Schampel, 1996; Elser et al., 2000), which states that microbes with more rapid growth than plants have a higher demand for P-rich ribosomal RNA, and thus have much lower N:P ratios. Hence, soil microbial activity is more easily limited by P than plant primary production. It seems reasonable that P, rather than N, limits soil microbial activity in tropical forest ecosystems.

Nevertheless, the validity of the hypothesis of P-limited microbial activity in tropical forests has not been well verified. The goal of this paper is to review the methods for determining limiting nutrients and evaluate the validity of the widespread belief that soil microbial activity in tropical forest ecosystems is limited by P and not by N. In the field of soil microbiology, the term nutrient limitation is used frequently without formal definition. In this paper, we defined nutrient limitation of soil microbial activity by modifying the definition for primary production (from Howarth, 1988); a nutrient is limiting when the potential rate of net primary production, allowing for possible shifts in

species composition, is suppressed due to a lack of the nutrient. We replaced the term primary production by microbial growth, and used the term gross microbial growth, rather than net microbial growth, because the much quicker turnover of microbes than that of primary production considerably affects net growth rate and may cause a mis-understanding of microbial activity. Consequently, a nutrient limiting soil microbial activity was defined as a nutrient that stimulates gross microbial growth, allowing for possible shifts in species composition.

2 | LITERATURE REVIEW

2.1 | Literature review and data collection

Studies mentioning nutrient limitation of soil microbial activity (i.e. gross microbial growth) were collected, and the methods used therein were examined. We performed a meta-analysis to obtain response ratios of soil respiration and standing microbial biomass to fertilizing N and P. Because several meta-analyses have already been conducted to test the effects of N or P on standing microbial biomass or soil respiration and microbial respiration (Liu & Greaver, 2010; Yue et al., 2016, 2017), we focused on experiments that applied both N and P fertilizers simultaneously. The search engine Web of Science was used to locate published articles with the following combinations of key words: ("phosph* add*" OR "P add*" OR "phosph* elevat*" OR "P elevat*" OR "phosph* fertiliz*" OR "P fertiliz*" OR "phosph* appl*" OR "P appl*" OR "phosph* enrich*" OR "P enrich*") AND ("nitrogen* add*" OR "N add*" OR "nitrogen elevat*" OR "N elevat*" OR "nitrogen fertiliz*" OR "N fertiliz*" OR "nitrogen appl*" OR "N appl*" OR "nitrogen enrich*" OR "N enrich*") AND ("soil respiration" OR "microbial respiration" OR "CO_2 emission" OR "microbial biomass"). If the data were reported only in figures, the means and standard deviations were extracted using DataThief III version 1.7 (Tummers, 2006). If standard errors were reported, they were converted to standard deviations using replicate numbers.

2.2 | Calculation and data analysis

We calculated the mean effect size and 95% CI of the overall effects of fertilization (N and P) on soil respiration and microbial biomass in the field. We did not include laboratory studies because of the small number of studies available for synthesis (there exist many papers but data are not suitable for the analysis). The effects of fertilization were estimated based on the natural log-transformed response ratio:

$$\ln RR = \ln(\overline{X_t}/\overline{X_c}), \tag{1}$$

where $\overline{X_t}$ is the treatment mean, and $\overline{X_c}$ is the control mean. The variance (v) associated with each value of ln RR was calculated as follows:

$$v = s_t/(n_t \times \overline{X_t^2}) + s_c/(n_c \times \overline{X_c^2}), \tag{2}$$

where n_t and n_c are the sample sizes for the treatment and control groups, respectively; and s_t and s_c are the standard deviations for the treatment and control groups, respectively. This analysis was performed using R version 3.4.0. software (R Core Team, 2017) using random-effect models.

3 | EVALUATION OF METHODS

Several methods have been used to determine the nutrients limiting soil microbial activity; the most common of these is the fertilization method. Changes in microbial respiration and standing biomass have been used as indicators to assess the nutrients limiting soil microbial activity under the assumption that the addition of limiting nutrients increases both microbial respiration and biomass (Cleveland & Townsend, 2006; Cleveland et al., 2002, 2006; Ilstedt & Singh, 2005; Ilstedt et al., 2003; Liu et al., 2012; Mori et al., 2013c; Turner & Wright, 2014). An indirect method using enzymatic stoichiometry was also used by several researchers (Sinsabaugh, Hill, & Follstad Shah, 2009; Sinsabaugh et al., 2008; Waring, Weintraub, & Sinsabaugh, 2014).

3.1 | Fertilization method with microbial biomass as an indicator

Increases in microbial biomass due to P fertilization were reported in several tropical forests (Liu et al., 2012; Mori, Wachrinrat, et al., 2016; Turner & Wright, 2014). Using the data, these researchers suggested that soil microbial activity in tropical forests was limited by P. However, we suggest that using microbial standing biomass can be an inappropriate method to detect limiting nutrients because it does not take microbial turnover into account. For example, boreal and temperate forest soils have much greater microbial biomass than do tropical forest soils (Xu, Thornton, & Post, 2013), even though microbial activity must be much slower in boreal and temperate regions. Similarly, in mountain ecosystems, soils at higher altitudes contain larger amounts of microbial biomass than do those at lower altitudes (Wagai, Kitayama, Satomura, Fujinuma, & Balser, 2011). These observations indicate that higher microbial activity, which leads to faster soil organic matter decomposition, can produce lower standing microbial biomass. Recent studies have suggested that a shift in the microbial community caused by exogenous nutrient addition may lead to the dominance of fast-growing microbes with rapid turnover and small standing biomass (Mori, Yokoyama, & Kitayama, 2016; Riggs & Hobbie, 2016). If this is true, an increase in microbial standing biomass is not a good indicator for determining limiting nutrients. In our meta-analysis, we found that neither N addition nor P addition influenced standing microbial biomass in tropical forests or other ecosystems (Figure 1). This conclusion is in contrast to those of previous studies (Liu et al., 2012; Mori, Yokoyama et al., 2016; Turner & Wright, 2014). Our finding may reflect the difficulty of using soil microbial biomass as an indicator to determine the nutrients limiting soil microbial activity.

3.2 | Fertilization methods using soil respiration as an indicator

An increase in soil respiration following fertilization is a better indicator than microbial standing biomass, because it takes microbial turnover

into account. The widespread belief that soil microbial activity in P-poor tropical forests is limited by P availability largely rests on the finding that P addition stimulated CO_2 emissions in field experiments (Cleveland & Townsend, 2006; Liu et al., 2012; Mori et al., 2013b) and under laboratory conditions (Cleveland et al., 2002; Duah-Yentumi et al., 1998; Gnankambary et al., 2008; Ilstedt & Singh, 2005; Mori et al., 2010, 2013c). However, we also raise the following concerns related to these methods.

If the research is conducted in the field, soil respiration data involve root respiration. Because root respiration can be increased or reduced via fertilization, depending on the balance of stimulated primary production and reduced allocation of photosynthetic products to the roots, it is likely that soil respiration in field experiments will misguide the determination of limiting nutrients. In the present meta-analysis, we found that P addition did not stimulate soil respiration in field experiments performed in tropical forests (Figure 1), in contrast to the stimulatory effects of P fertilization on soil respiration in the field found in previous studies (Cleveland & Townsend, 2006; Liu et al., 2012; Mori et al., 2013b). Although our sample sizes may have been insufficient, the ambiguous results regarding the response of soil respiration to fertilization in our meta-analysis (Figure 1) may have been partly due to uncertainties in the responses of root respiration to fertilizations.

Even if the root respiration was separated from soil respiration (e.g. using trenching method, Mori et al., 2013b), the soil respiration data in the field P-fertilization experiments still have a problem for determining limiting nutrients. Since P addition could increase the plant growth and litterfall of tropical forests, C input into soils could be elevated by P addition through increased litterfall production. Thus

the net increase of soil microbial respiration in field studies could be derived both from (1) the relief of P limitation of microbes and (2) the relief of microbial C limitation through increased C input into soils. We are unable to detect which is the dominant process.

Whether a study is conducted through field or laboratory experiments, microbial community shifts in response to fertilizer application have the potential to confound the determination of the nutrients limiting soil microbial activity. According to the copiotrophic hypothesis (Fierer, Bradford, & Jackson, 2007; Fierer et al., 2012; Fontaine, Mariotti, & Abbadie, 2003; Ramirez, Craine, & Fierer, 2010, 2012), a community shift from more oligotrophic to more copiotrophic conditions may reduce soil respiration because of the decrease in recalcitrant organic matter decomposition by oligotrophs. Because shifts in community composition are greater in nutrient-poor than in nutrient-rich soils (Figure 2), the decrease in soil respiration is larger in nutrient-poor soils. Accordingly, nutrient-rich soils have a larger net increase in soil respiration through nutrient fertilization, that is, nutrient limitation is stronger in nutrient-rich soils. This conclusion is clearly wrong, indicating that fertilization with soil respiration as an indicator is an insufficient method for determining limiting nutrients.

N-mining theory, which accounts for microbial responses to nutrient addition, also suggests the difficulty of employing fertilization experiments (with soil respiration as an indicator) to determine the nutrients limiting soil microbial activity. This theory argues that some microbes use labile C to decompose recalcitrant organic matter to obtain N, and N addition causes microbes to stop mining recalcitrant organic matter to obtain N (Craine, Morrow, & Fierer, 2007). Thus, N limitation (or N shortage) can cause a negative response of soil respiration

(a) Microbial biomass (+N)

(b) Microbial biomass (+P)

(c) Soil respiration (+N)

(d) Soil respiration (+P)
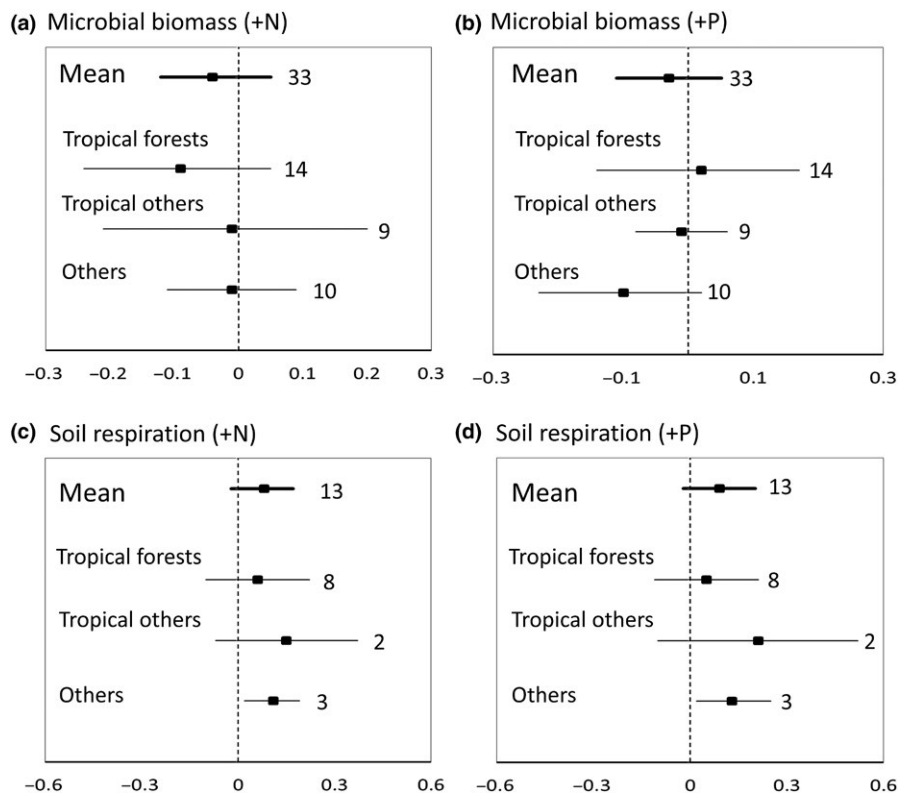
FIGURE 1 Mean and 95% CI of the weighted response ratio of N fertilization on (a) microbial biomass and (c) soil respiration, and of P fertilization on (b) microbial biomass and (d) soil respiration. Tropical forests do not include tropical montane forests. Tropical others are ecosystems in tropical areas excluding tropical forests; tropical montane forests are included in this category because tropical montane forests with cooler weather and less weathered soils are different from typical tropical lowland forests. Others indicate ecosystems not in tropical areas. Numbers beside bars indicate the sample size for each variable
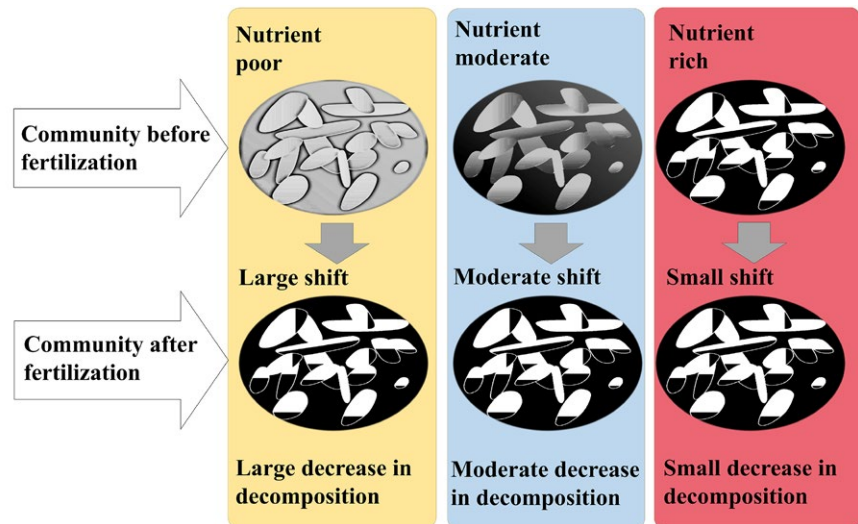
FIGURE 2 Microbial community shift following fertilization. Microbial communities in nutrient-poor soils change their composition more drastically than those in nutrient-rich soils

If a community shift from oligotrophs to copiotrophs causes a decrease in CO_2 emissions (copiotrophic hypothesis), net increase in CO_2 emissions by fertilization is the largest in nutrient rich soils. Accordingly, nutrient limitation is the strongest in the nutrient rich soils. This conclusion is obviously wrong, indicating that fertilization with soil respiration as an indicator is insufficient to determine the limiting nutrients.

to exogenous N addition. In short, it is clear that an increase in soil respiration is not always a good indicator to determine the nutrients limiting soil microbial activity.

Several studies have used incubation experiments with simultaneous labile C addition (Cleveland et al., 2002; Duah-Yentumi et al., 1998; Gnankambary et al., 2008; Ilstedt & Singh, 2005; Ilstedt et al., 2003; Mori et al., 2013c; Teklay et al., 2006). Most of these studies reported the clear stimulatory effects of P addition on soil respiration. However, it is likely that results obtained using this method were biased to determine the nutrient limitations of copiotrophic microbes, which become stronger under resource-rich conditions. Additionally, nutrient mining was observed even after the addition of labile C (Nottingham, Turner, Stott, & Tanner, 2015). Therefore, we conclude that this method is also unsatisfactory.

3.3 | Indirect method using enzymatic stoichiometry

Extracellular enzymatic stoichiometry can be used as an indicator to estimate limiting nutrients. The ratios of β-1,4-glucosidase (BG, hydrolyzing glucose from cellobiose), β-1,4-acetylglucosaminidase (NAG, hydrolyzing glucosamine from chitobiose), leucine aminopeptidase (LAP, hydrolyzing leucine and other hydrophobic amino acids from the N terminus of polypeptide), and acid (alkaline) phosphatase (AP, hydrolyzing phosphate from phosphosaccarides and phospholipids) have been used to assess microbial resource allocation and nutrient demand (Sinsabaugh et al., 2008, 2009; Waring et al., 2014) because extracellular enzymes are produced to mediate microbial nutrient acquisition from organic matter (Caldwell, 2005; Olander & Vitousek, 2000). A meta-analysis by Sinsabaugh et al. (2008) demonstrated that the BG:AP ratio was lower for tropical soils than for higher-latitude soils, indicating the higher microbial investment to enzymes targeting

P in tropical forests than in other ecosystems. Waring et al. (2014) also found BG:AP and NAG:AP ratios in tropical soils lower than global averages, and suggested that microbial activities were mainly limited by P availability in tropical ecosystems. A weakness of this method is that enzymatic stoichiometry does not provide direct evidence for nutrient limitation. Another concern is that it is not clear whether N-limitation can be evaluated by the BG:NAG or BG:(NAG+LAP) ratios. Since both glucosamine and amino acids contain C, NAG and LAP activities can be driven by C-acquisition, rather than by N acquisition. Indeed, a recent meta-analysis demonstrated that experimental N addition did not change the ratios of C-acquiring enzymes and N-acquiring enzymes (Jian et al., 2016), implying that these ratios, including BG:NAG and BG:(NAG+LAP), do not indicate the microbial resource allocation for N acquisition.

4 | EVALUATION OF THE P-LIMITATION HYPOTHESIS

Soil microbial activity in tropical forest ecosystems has been considered limited by P and not by N. After reviewing the literature, we suggest that the P-limitation hypothesis is overestimated due to methodological inadequacy (partly discussed above). In the following section, we show that the contrasting effects of exogenous N and P addition on soil microbial activity can lead to the misidentification of N and P limitation.

As discussed in the previous section, N addition can cause a suppression of organic matter decomposition (N-mining theory), which potentially causes a misunderstanding of N limitation of soil microbial activity. In contrast, this theory has been considered inapplicable to P (Craine et al., 2007), and P addition has been thought

to stimulate organic matter decomposition (Cleveland & Townsend, 2006; Cleveland et al., 2002, 2006; Ilstedt & Singh, 2005; Ilstedt et al., 2003; Kaspari et al., 2008; Liu et al., 2012; Mori et al., 2013b). This is partly explained by the conceptual model of McGill and Cole (1981), which demonstrated that N mineralization is coupled with C respiration due to the binding of C and N in organic matter, whereas P is mineralized by phosphatase independently of C because most of the P contained in organic matter is bound in phosphate esters. This model suggests that P acquisition is less accompanied by C mineralization than N acquisition is, and therefore P addition would not suppress decomposition as strongly as N addition does. Recent studies have revealed that organic P can be also mined (Nottingham et al., 2015; Spohn, Ermak, & Kuzyakov, 2013). Another study suggested that microbes under P-limiting conditions may decompose organic matter to obtain N (i.e. N mining) to synthesize phosphatase; thus, P addition might also reduce organic matter decomposition in the same manner as N addition does (Mori et al., 2015). However, the negative effects of P addition are minor compared with those of N addition (Craine et al., 2007; Nottingham et al., 2015). Therefore, the underestimation of nutrient limitation through nutrient mining is larger for N than for P. Accordingly, fertilization experiments tend to incorrectly support P limitation.
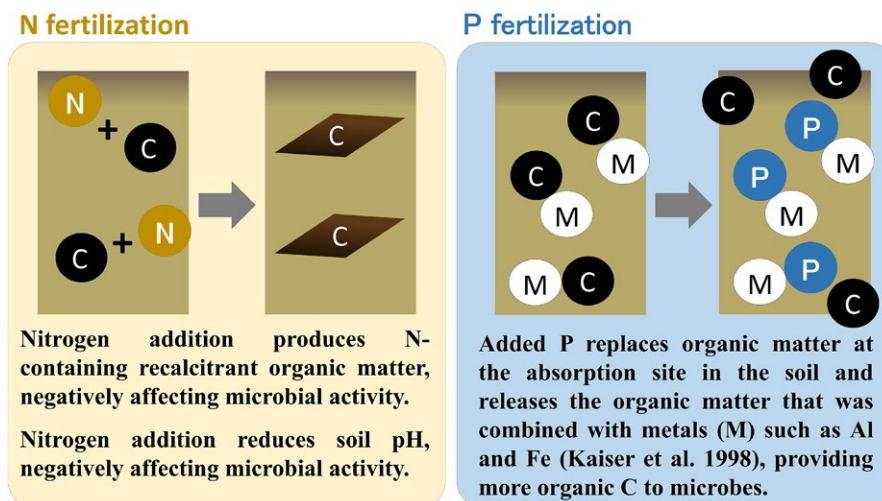
The contrasting effects of N and P addition on soil microbial activity through chemical reactions in soils are an additional concern (Figure 3). In numerous previous studies, N addition generally had a negative effect on microbial organic matter decomposition (Fog, 1988; Hagedorn, Spinnler, & Siegwolf, 2003; Janssens et al., 2010; Knorr, Frey, & Curtis, 2005; Zak, Holmes, Burton, Pregitzer, & Talhelm, 2008). Except for the negative effects of N addition on lignin-degrading fungi (Berg, 1986; Fog, 1988), there have been several attempts to explain this phenomenon using chemical reactions (Fog, 1988); decreases in organic matter decomposition in relation to N addition may have been due to the production of N-containing recalcitrant organic matter (Haider & Martin, 1967; Soderstrom, Baath, & Lundgren, 1983) or a decrease in pH (Treseder, 2008). Negative effects caused by these chemical reactions do not necessarily indicate a lack of N limitation. The microbes may have required more N, but factors other than N

(such as pH or C availability) became unsuitable for microbes following experimental N addition. On the other hand, negative impacts of P addition on microbial organic matter decomposition due to chemical reactions have rarely been reported. Rather, P addition may have an opposite effect. Phosphorus is known to have a higher affinity than labile C for binding to the sorption sites of mineral soils (Kaiser & Zech, 1996), and thus P addition would release organic matter bound to the sorption sites. Organic matter released after P addition would be accessible to microbes, stimulating soil microbial activity by providing C (Figure 3). Thus, the addition of both N and P influences microbial activity indirectly by changing C availability in soils, and these processes are unrelated to whether microbial activity is limited by N or P. We again suggest that fertilization experiments are not an appropriate method to determine the nutrients limiting soil microbial activity. The contrasting effects of N and P addition on microbial activity in these chemical reactions can cause misinterpretation of nutrient fertilization results, such that P limitation is supported when it is not actually occurring.

5 | CONCLUSION AND FUTURE DIRECTIONS

As discussed above, determining the nutrients limiting soil microbial activity is more difficult than primary production because (1) changes in the microbial community occur much more rapidly than those in the plant community, and stimulated gross microbial growth may lead to lower standing biomass; and (2) most microbes are heterotrophs that obtain energy from organic C in the soil, which causes complicated interactions among C, N, and P. As the methods in current use are insufficient, we recommend caution in stating that soil microbial activity in tropical forest ecosystems is limited by P until a novel method is established to accurately determine the nutrients limiting soil microbial activity at the ecosystem level.

We have suggested alternative methods to determine the nutrients limiting soil microbial activity at the ecosystem level. First, by focusing a certain group of microbes, it may be possible to

N fertilization

Nitrogen addition produces N-containing recalcitrant organic matter, negatively affecting microbial activity.

Nitrogen addition reduces soil pH, negatively affecting microbial activity.

P fertilization

Added P replaces organic matter at the absorption site in the soil and releases the organic matter that was combined with metals (M) such as Al and Fe (Kaiser et al. 1998), providing more organic C to microbes.

FIGURE 3 Contrasting effects of N and P on soil organic matter. The availability of organic matter is reduced by N fertilization, but elevated by P fertilization

determine the limiting nutrients if the influence of nutrient manipulation on soil organic matter is reduced. By monitoring the effects of fertilization on the respired amount of targeted substrates that are mixed with a small portion of soils (small enough that the influence of nutrient manipulation on soil organic matter is negligible), the limiting nutrients for certain microbial groups that prefer the targeted substrates can be detected. Combining the substrate utilization profiling method (BIOLOG) and nutrient manipulation has the potential to fulfill this challenge. Second, if the research goal is to understand the strength of the nutrient shortage in a certain ecosystem, indicators other than nutrient limitation are available. For example, the difference between the microbial community composition under the current and nutrient-saturated conditions could be used as an indicator, as this difference represents the distance to the potential nutrient-saturated condition within the ecosystem. It would be possible to determine this distance by clarifying the community composition using molecular biological methods and by calculating the difference in microbial community composition before and after fertilization using multivariable analysis. If the research question is an environmental–scientific one, for example, to determine the net effects of anthropogenic nutrient inputs on the decomposition of soil organic matter, conventional fertilization experiments would be a powerful tool. In this example, the concept of nutrient limitation is not needed because the goal is to determine the net effects of fertilization. One of the most important aims of determining the nutrients limiting soil microbial activity is to predict the effects of anthropogenic nutrient inputs on global C cycling (Cleveland et al., 2002; Gallardo & Schlesinger, 1994; Galloway et al., 2004; Ilstedt & Singh, 2005; Mo et al., 2008; Scheu, 1990). This research question can be answered without considering the concept of nutrient limitation.

AUTHORS' CONTRIBUTION

Mori T and Aoyagi R conceived the research idea. All authors contributed to the discussion and writing of this paper.

DATA ACCESSIBILITY

All data used in our paper are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.2d8c3 (Mori, Lua, Aoyagi, & Moa, 2018).

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