

Predicting microbial nutrient limitations from a stoichiometry-based threshold framework

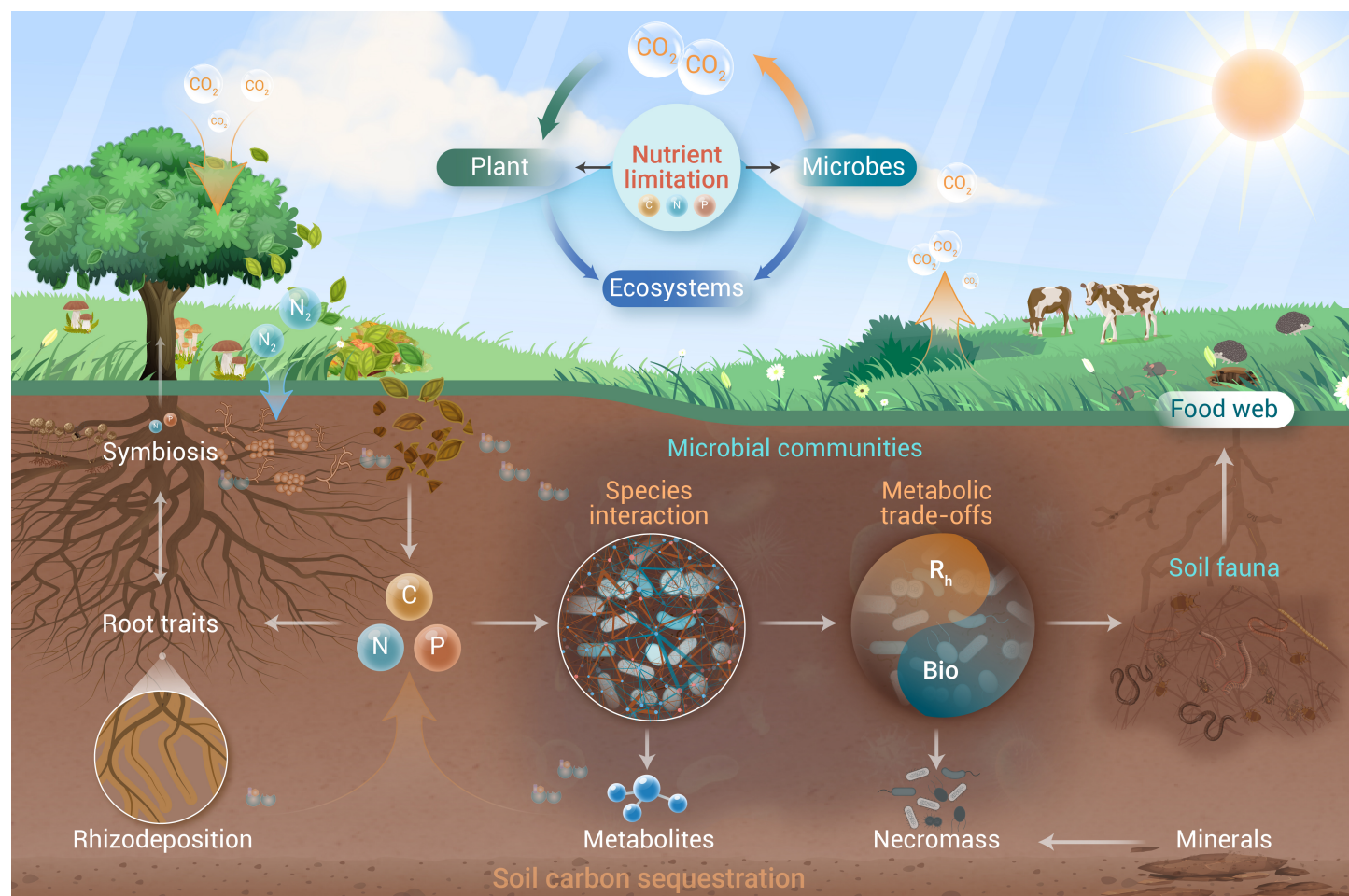
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Received: July 12, 2023; Accepted: December 30, 2023; Published Online: January 26, 2024; <https://doi.org/10.59717/j.xinn-geo.2024.100048>

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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- A threshold framework is developed to predict soil microbial nutrient limitations.
- The theoretical thresholds are validated by global dataset-based empirical thresholds.
- Vector angle of 45° in the ecoenzymatic vector model overestimates microbial phosphorus limitation.
- Vector length of 0.61 and angle of 55° can be used as thresholds to identify microbial nutrient limitations.

Predicting microbial nutrient limitations from a stoichiometry-based threshold framework

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Citation: Cui Y., Moorhead D. L., Peng S., et al., (2024). Predicting microbial nutrient limitations from a stoichiometry-based threshold framework. *The Innovation Geoscience* **2**(1): 100048.

While ecological stoichiometry-based theories and methodologies have been successfully applied to identify nutrient limitations in plankton and plants (e.g., leaf nutrient resorption ratios), determining nutrient limitations in soil microorganisms by them has great uncertainties. Establishing stoichiometric thresholds that define specific nutrient limitations for soil microbial communities has been a major challenge. Here, we present a threshold framework that predicts the limitations of carbon (C), nitrogen (N), and phosphorus (P) for microbial communities by leveraging the convergence of elemental stoichiometry from soils to microorganisms. Combined with a widely used model of microbial nutrient limitations (i.e., ecoenzyme vector model), the theoretical thresholds derived from this framework are validated by empirical thresholds identified through analysis of a comprehensive global dataset ($n = 3277$). Notably, we find that the commonly assumed threshold of 45° in the ecoenzyme vector model overestimates microbial P limitation and underestimates microbial N limitation. By contrast, a vector length of 0.61 (unitless) and an angle of 55° in the vector model are more reliable thresholds identifying microbial C and N/P limitations, respectively. This framework, with a fundamental understanding of microbially-driven stoichiometric convergence in plant-soil-microbe systems, offers a practical and general avenue for identifying microbial nutrient limitations in soils.

INTRODUCTION

Microbial catabolism in soil, as the basis of food webs, is widely constrained by nutrient availability across ecosystems.^{1–4} A fundamental characteristic of microbial metabolism is the allocation of extracellular enzymes (ecoenzymes) to acquire nutrients from organic polymers, which mediates the mineralization and sequestration of soil organic matter.^{1,3,5} Relative to the constraints on primary production imposed by plant nutrient limitations,⁶ microbial nutrient limitations determine the retention of detrital organic carbon (C) and ultimately the size of soil C pools.^{5,7–10} The topic has thus gained increasing attention in the fields of global change ecology and soil ecology.^{11–15} Identifying microbial nutrient limitations, however, is intrinsically difficult due to microscopic scales, overwhelming species diversity, rapid reproduction and biomass turnover as well as elusive adaptive strategies in microbial communities.

Several approaches have been used to infer microbial nutrient limitations, including laboratory substrate-added incubations, field fertilization experiments, and stoichiometry-based models.^{3,11,13,16} Laboratory incubations typically add soluble substrates (e.g., glucose) that can be acquired by microorganisms without ecoenzyme catalysis to highly disturbed soil samples.^{17,18} It thus measures uptake potential favoring copiotrophs and circumventing catalytic relationships within the natural saprophytic community.¹⁰ Fertilization experiments can directly test nutrient limitations to microbial respiration or growth,^{11,19} but altering the availability of any one nutrient can induce community shifts and metabolic responses to other resources.^{5,20} Also, Meta-analyses conducted on substrate additions or fertilization experiments have not revealed significant large-scale spatial patterns in microbial nutrient limitations.^{11,21} This lack of clear patterns could be attributed to variations in the

conditions and methodologies employed across these studies. Those aspects limit the suitability of the two approaches for identifying real limitations of native communities as well as application to large-scale surveys of natural ecosystems.

Alternatively, approaches based on ecological stoichiometry can identify microbial nutrient limitations indirectly without altering existing ecosystem status.^{7,13,16} A clear advantage of the stoichiometry approach is estimating microbial nutrient limitations based on their ecoenzymatic characteristics *in situ* rather than their responses to pulse manipulations of anthropogenically disturbed systems.³ Specifically, most of the energy and nutrients required by microorganisms in bulk soils of natural ecosystems are originally derived from organic polymers (i.e., plant litter) through ecoenzyme catalysis.^{3,10,22} Because microorganisms secrete ecoenzymes to catalyze specific reactions that generate specific products for microbial uptake, the stoichiometry of ecoenzymatic activities reflects the relative nutrient limitations on microbial community.^{3,23} However, determining microbial nutrient limitations using ecoenzyme-based stoichiometric approaches remains highly uncertain, in part because we do not know how to precisely determine the thresholds of ecoenzymatic stoichiometry that correspond to the limitations of specific nutrients.

The ecoenzyme vector model is one of the most widely used approaches to interpret ecoenzymatic stoichiometry.^{13,16,24} It includes vector length and angle for estimating microbial nutrient limitations, and defines a vector angle of 45° representing the threshold of ecoenzymatic activities distinguishing microbial phosphorus (P) limitation ($> 45^\circ$) and nitrogen (N) limitation ($< 45^\circ$). This definition is based on the observed pattern of ecoenzymatic stoichiometry for C:N:P-acquiring activities near 1:1:1 at a global scale, so the slopes of their regression relationships are close to 45° .^{7,25} A reasonable and mechanistic explanation for the angle of 45° representing the threshold of microbial N/P limitation, however, has never been clearly elucidated, which is an important source of uncertainty to the approach. Indeed, some recent studies have identified potentially large uncertainties to infer microbial N or P limitation using this threshold.^{17,18,26,27} This model also lacks a definitive threshold for microbial C limitation because the vector length only represents the potential C acquisition relative to both N and P acquisition. Mechanistically evaluating the thresholds of microbial C, N and P limitations for the ecoenzyme vector model is therefore a priority for accurately predicting microbial nutrient limitations using patterns of ecoenzymatic activities.

FRAMEWORK FOR PREDICTING MICROBIAL NUTRIENT LIMITATIONS

From the perspective of food webs, the widespread phenomenon of elemental stoichiometric convergence from plants to microorganisms is observed in plant-soil-microbe systems.^{1,28} This convergence heavily relies on the resource-use efficiency of consumers across trophic levels.² Within microbial communities, microbial elemental homeostasis determines that resource stoichiometry, which can be highly variable, converges toward a more stable stoichiometry aligning with microbial demands. Specifically, the interplay among soil resource stoichiometry, microbial elemental stoichiometry, and resource-use efficiencies dictates microbial resource demand and investment in essential ecoenzymes (Figure 1).²³ The ecoenzymatic activities

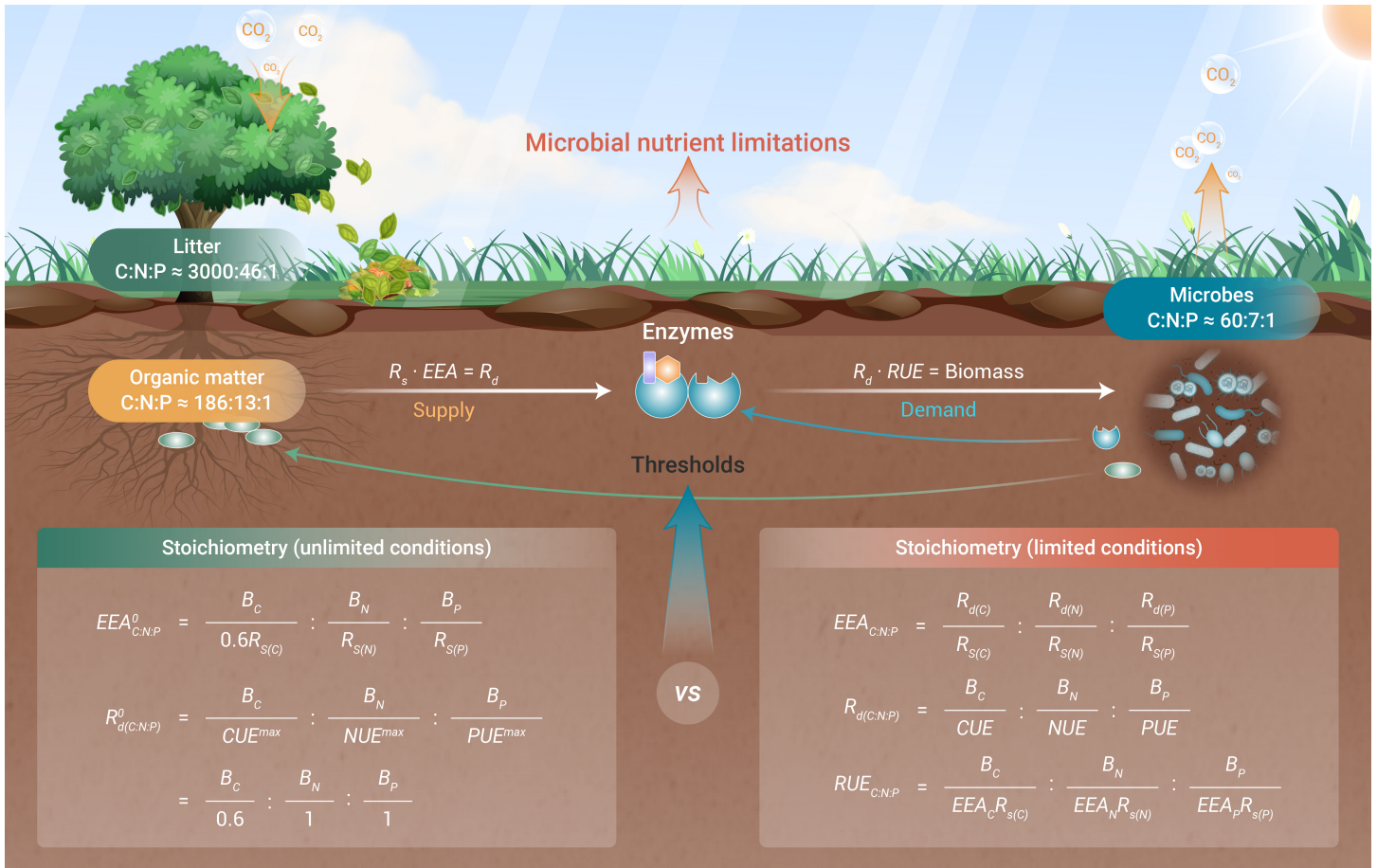


Figure 1. Operational framework predicting microbial nutrient limitations from supply-and-demand stoichiometry The processes of supplying substrates to meet microbial demand can be expressed as two fundamental functions, $R_s \times EEA = R_d$ and $R_d \times RUE = \text{Biomass}$; R_s , resource supply from the environment; EEA , eoenzymatic activities used to decompose polymeric organic substrates; R_d , microbial resource demand; RUE , microbial resource-use efficiency; Biomass , microbial biomass. Two scenarios of the relationships of supply and demand are distinguished: unlimited and limited conditions. Under unlimited conditions, eoenzymatic stoichiometry is defined as $EEA_{C:N:P}^0$, where B_C , B_N and B_P are microbial biomass C, N and P concentrations, respectively, and $R_{s(C)}^0$, $R_{s(N)}^0$ and $R_{s(P)}^0$ are the optimal supplies of soil C, N and P, respectively. R_d stoichiometry can be defined as $R_{d(C:N:P)}^0$ given microbial resource-use efficiencies, with $CUE^{\max}$ as the upper limit of the microbial growth efficiency (0.6) based on thermodynamic constraints, and $NUE^{\max}$ and $PUE^{\max}$ as 1.0. Under limited conditions, EEA and RUE depend on both the actual, usually suboptimal supply of resources and the microbial demand. The differences between $EEA_{C:N:P}^0$ and $EEA_{C:N:P}$ thus dictates the thresholds for identifying microbial C, N and/or P limitations in combination with specific approaches (e.g., the eoenzyme vector model). Under limited conditions, the ratios of actual resource-use efficiencies ($RUE_{C:N:P}$) can also be determined using eoenzymatic activities, resource concentrations ($R_{s(C)}$, $R_{s(N)}$, and $R_{s(P)}$) and microbial biomass, which are generally easy to measure.

reflect the mismatches between soil resource supply and microbial resource demand. Higher resource supply corresponds to lower eoenzymatic activities, and both collectively define microbial resource demand (or actual resource acquisition). Further, resources acquired by microorganisms together with resource-use efficiency determine microbial growth (i.e., microbial biomass). Based on these relationships, we establish two fundamental expressions:

$$R_s \times EEA = R_d \quad (1)$$

$$R_d \times RUE = \text{Biomass} \quad (2)$$

where R_s , EEA , R_d , RUE and Biomass represent resource supply from the environment, eoenzymatic activities used to decompose polymeric organic substrates, microbial resource demand, microbial resource-use efficiency and microbial biomass, respectively.

Any environmental condition with variable relationships between resource supply and microbial demand can be distinguished as two scenarios: unlimited and limited (Figure 1). Both metabolic and stoichiometric theories of ecology suggest that microorganisms could maximize growth efficiencies (i.e., maximum efficiencies of C, N and P use ($CUE^{\max}$, $NUE^{\max}$ and $PUE^{\max}$, respectively)) when neither energy nor nutrients were limiting.^{13,29,30} R_d stoichiometry under unlimited conditions can thus be defined as $R_{d(C:N:P)}^0$ and expressed as:

$$R_{d(C:N:P)}^0 = \frac{B_C}{CUE^{\max}} : \frac{B_N}{NUE^{\max}} : \frac{B_P}{PUE^{\max}} \quad (3)$$

$CUE^{\max}$ is the theoretical upper limit for the efficiency of microbial growth (0.6) based on thermodynamic constraints, and $NUE^{\max}$ and $PUE^{\max}$ are close to 1.0.^{29,30} Eq. 3 can thus be converted as:

$$R_{d(C:N:P)}^0 = \frac{B_C}{0.6} : \frac{B_N}{1} : \frac{B_P}{1} \quad (4)$$

where B_C , B_N and B_P are microbial biomass C, N and P concentrations, respectively.

The ratios of eoenzymatic stoichiometry under unlimited conditions can thus be identified by:

$$EEA_{C:N:P}^0 = \frac{B_C}{0.6R_{s(C)}^0} : \frac{B_N}{R_{s(N)}^0} : \frac{B_P}{R_{s(P)}^0} \quad (5)$$

where $R_{s(C)}^0$, $R_{s(N)}^0$ and $R_{s(P)}^0$ are the optimal supplies of soil C, N and P, respectively.

Under limited conditions that are common in natural ecosystems, however, EEA and RUE depend on both actual resource supply and microbial demand, and vary widely across microbial habitats. The differences between $EEA_{C:N:P}^0$ and $EEA_{C:N:P}$ can thus be used to define the stoichiometric thresholds identifying the limitations of microbial C, N and/or P in combination with other specific methods (e.g., the eoenzyme vector model) (Figure 1). In addition, the ratios of resource-use efficiencies ($RUE_{C:N:P}$) can be determined using eoenzymatic activities, resource supplies and microbial biomass that are

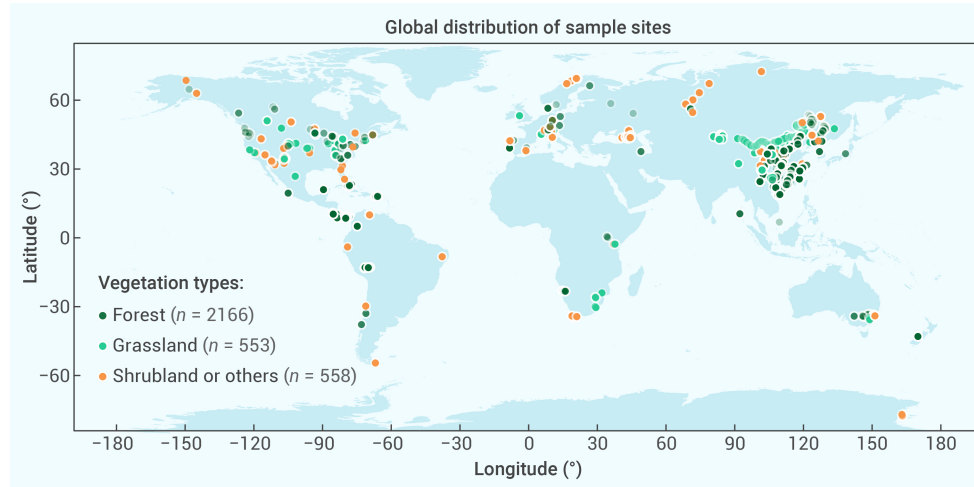


Figure 2. Global distribution of sample sites ($n = 3277$) All sample sites were in natural ecosystems. Main vegetation types included forest, grassland, and shrubland or others (e.g., tundra).

generally easy to measure:

$$RUE_{C:N:P} = \frac{B_C}{EEA_C R_{s(C)}} : \frac{B_N}{EEA_N R_{s(N)}} : \frac{B_P}{EEA_P R_{s(P)}} \quad (6)$$

IDENTIFYING THRESHOLDS OF MICROBIAL NUTRIENT LIMITATIONS BY THIS FRAMEWORK

Using the ecoenzyme vector model as a test item, we adopted a global mean molar C:N:P ratio of soil microbial biomass ($B_{C:N:P}$) of 60:7:1,²⁸ a global mean microbial C-use efficiency of 0.30³¹ and global empirical N and P efficiencies of 0.8^{30,32} to estimate the resource stoichiometry that meets microbial demand ($R_{C:N:P}$):

$$R_{C:N:P} = \frac{60}{0.3} : \frac{7}{0.8} : \frac{1}{0.8} = 160 : 7 : 1 \quad (7)$$

The global mean molar C:N:P ratio of soil organic matter, as the stoichiometry of resource supply, is 186:13:1.²⁸ If only considering nutrients released by extracellular enzymes from soil organic matter to be used by microorganisms,^{3,22} then the stoichiometry of ecoenzymatic activity ($EEA_{C:N:P\text{-required}}$) required to meet microbial demand is calculated by:

$$EEA_{C:N:P\text{-required}} = \frac{160}{186} : \frac{7}{13} : \frac{1}{1} = 0.86 : 0.54 : 1 \quad (8)$$

The proportional activity of ecoenzymes seeking $\frac{C}{C+P}$ (x-axis) and $\frac{C}{C+N}$ (y-axis) in the ecoenzyme vector model meets the microbial nutrient demand when:

$$x = \frac{C}{C+P} = \frac{0.86}{0.86+1} = 0.46 \quad (9)$$

$$y = \frac{C}{C+N} = \frac{0.86}{0.86+0.54} = 0.62 \quad (10)$$

where x is the proportional activity of C- vs. C+P-acquiring enzymes, and y is the proportional activity of C- vs. C+N-acquiring enzymes.

In the ecoenzyme vector model,¹⁶ vector length, representing the relative C limitation of microbial community, is calculated as the square root of the sum of x^2 and y^2 . Vector angle, representing microbial N or P limitation, is calculated as the arctangent of the line extending from the plot origin to point (x , y).

Based on the values of $EEA_{C:N:P\text{-required}}$ (Eq. 8), the corresponding vector length and angle were 0.77 and 53°, respectively (see Supporting Information for more details). These two values represent the average theoretical thresholds for the ecoenzyme vector model at a global scale. By contrast, under unlimited conditions (as a reference), the $R_{C:N:P}$ was 100:7:1 using these maximum values of $CUE^{\max}$, $NUE^{\max}$ and $PUE^{\max}$, and the corresponding vector length and angle were 0.61 and 55°, respectively (see Supporting Information for more details). The two values can be deemed as the optimal theoretical thresholds identifying the limitations of microbial C and N/P via the ecoenzyme vector model. Specifically, vector lengths > 0.61 indicate microbial C limitation, with larger values indicating stronger C limitation. Vector angles > 55° indicate microbial P limitation, with larger values indicating stronger P

limitation, whereas values < 55° indicate microbial N limitation, with smaller values indicating stronger N limitation.

A lower apparent C limitation necessarily implies to a higher N or P limitation due to microbial trade-offs in the relative allocations of a limited ecoenzyme pool.^{3,22} Therefore, the slope of regression relationships between vector length and angle should be positive when microorganisms are N-limited and negative when microorganisms are P-limited. In other words, these regression relationships

could be utilized to identify the empirical thresholds of microbial nutrient limitations for the ecoenzyme vector model. We hypothesized that the relationships between vector length and angle would first be positive and then turn negative as angle increases. The value of vector length corresponding to the inflection point can be considered as the threshold distinguishing whether the microbial community is C-limited or not. Similarly, the value of vector angle corresponding to the inflection point can be considered as the threshold distinguishing microbial N vs. P limitations.

EMPIRICAL EVIDENCE TESTING THE EFFECTIVENESS OF THIS FRAMEWORK

To test our hypothesis and the effectiveness of this framework by the ecoenzyme vector model, we compiled a global dataset of soil ecoenzymatic activities from natural ecosystems (Figure 2; $n = 3277$; see Supporting Information for more details). Vector lengths and angles calculated from these data confirmed our hypothesis that vector angle was positively correlated with vector length when it is low, whereas this relationship was negative when vector angle is high (Figure 3). Correspondingly, we identified the empirical thresholds of microbial C and N/P limitations of 0.74 and 47° for length and angle, respectively. The two values are similar to the average theoretical thresholds (0.77 and 53°, respectively) we derived using global mean nutrient-use efficiencies. Moreover, the empirical and average theoretical thresholds are comparable to the optimal theoretical thresholds (0.61 and 55°, respectively) derived from the maximum resource-use efficiencies. These results of the two independent methods suggested that our stoichiometry-based framework would be valid for identifying the thresholds of microbial nutrient limitations.

Soil depth and vegetation types could influence the estimates of empirical thresholds for microbial nutrient limitations due to generally higher organic matter concentration in topsoil compared to subsoil, as well as the variations in plant litter among ecosystem types.^{33,34} Therefore, we further explored the possible effects of soil depth and vegetation types on the empirical thresholds using this global dataset (Figures S3 and S5). We found that, however, the thresholds neither had significant differences between the two layers and among the three vegetation types nor differed significantly from the thresholds for the overall soil dataset ($p > 0.05$; Figures S4 and S6). The empirical and theoretical thresholds consistently indicated that the threshold of microbial N/P limitation exceeded the previously defined 45°. The threshold of 45° thus overestimates microbial P limitation and underestimates microbial N limitation. This discrepancy could explain anomalous results of many studies that identified microbial P limitation with the threshold of 45° for alkaline soils at high latitudes and even in P-addition experiments.^{4,5,35} In addition, both the empirical and theoretical thresholds of vector length in our study provide a consistent baseline for identifying microbial C limitation.

UNCERTAINTIES AND IMPLICATIONS

The operational framework offers valuable insights and a tangible pathway for identifying microbial nutrient limitations. Our proposed approach, which

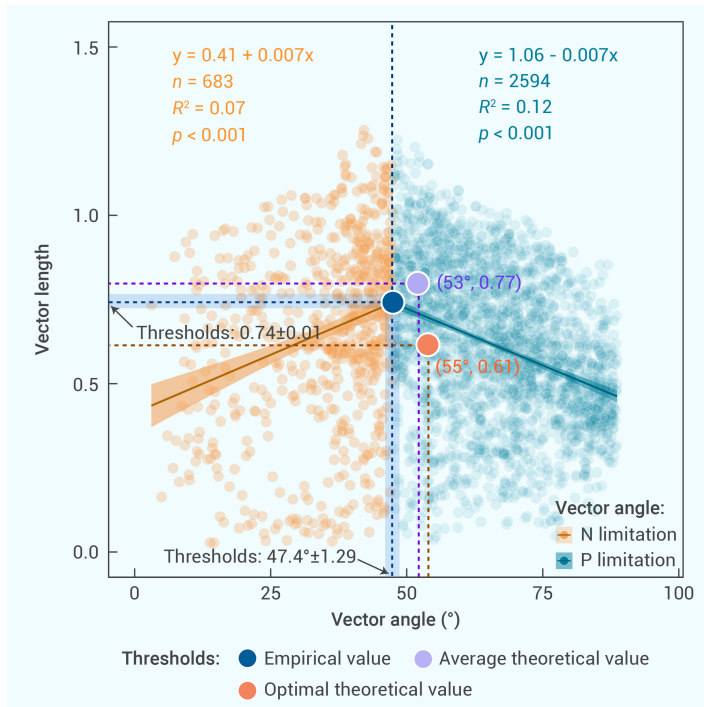


Figure 3. Theoretical and empirical thresholds of the microbial C and N/P limitations using the ecoenzyme vector model on a global dataset of soil ecoenzymatic activities ($n = 3277$) The empirical threshold of microbial N/P limitation (vector angle) was estimated using piecewise regression analyses, and the relationships between vector length and angle below and above the threshold were identified using generalized linear models. The results indicated an adjusted R^2 of 0.111 and $p < 0.001$ for the piecewise regression analyses. The blue circle indicates the threshold in vector angle with shaded areas as the 97.5% confidence intervals of the threshold. The solid black lines indicate the fits of the generalized linear model between vector length and angle. The purple and orange circles indicate the average and optimal theoretical thresholds, respectively.

predicts microbial nutrient limitations by considering the stoichiometry of supply and demand, holds significant potential for application in various ecoenzyme-based models of microbial nutrient limitations. Through the integration of this framework with empirical evidence at a global scale, we have established a vector length of 0.61 and an angle of 55° as reliable thresholds of microbial C and N/P limitations, respectively, in the ecoenzyme vector model.

However, it is essential to acknowledge that ecoenzymatic activities serve merely as proxies for microbial metabolism based on the utilization of polymeric resources.^{3,36} The presence of soluble resources in soil, not requiring enzymatic catalysis for acquisition, may influence these apparent thresholds.^{10,36} Additionally, C released by N-acquiring enzymes could impact the relationship between vector angle and length,²⁷ particularly at low vector angles where high N-acquiring enzyme activities may contribute to substantial release of available C. Furthermore, thresholds were greatly variable among ecosystem types (i.e., forests, shrublands, and grasslands; Figures S5 and S6) especially in grassland ecosystems, which could be due to differences in plant litter inputs, environmental conditions and microbial communities. Abiotic processes, such as gaseous N losses or the fixation of available P by soil cations, could further differentiate these thresholds among ecosystems.¹¹ Unfortunately, we only tested the two most likely contributing factors, specific thresholds for different ecosystems were not explored in this study due to data limitations.

Given these uncertainties, we recommend that future studies measure necessary indicators for specific ecosystems, and identify and compare ecosystem-specific thresholds against our framework. It is also crucial to explore and incorporate alternative proxies representing microbial metabolic demands, such as the abundances of functional genes,³⁷ to enhance predictive accuracy. Additionally, integrating resource-food web relations could help link microbial nutrient limitations to plant nutrient limitations. For instance, exploring comparable thresholds for microbial and plant nutrient limitations from resource flows within plant-soil-microbe systems could provide valu-

able insights into nutrient competition and cooperation between plants and microorganisms, advancing our understanding of terrestrial C cycling.

Despite these considerations, our study fills a longstanding knowledge gap by elucidating how to comprehend and identify thresholds within patterns of ecoenzymatic activities for predicting microbial nutrient limitations. This contribution represents a significant advancement in the field of ecological stoichiometry and microbial ecology.

MATERIALS AND METHODS

See supplemental information for details.

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ACKNOWLEDGMENTS

We are thankful to three anonymous reviewers for their constructive comments on this manuscript. We also grateful to more than twenty scientists for providing their data on our work. This work was financially supported by the National Natural Science Foundation of China (32101378). JP was supported by the TED2021-132627B-I00 grant, funded by the Spanish MCIN and the European Union NextGeneration EU/PRTR.

AUTHOR CONTRIBUTIONS

Y.C. conceived the research idea. Y.C. compiled the database and performed analysis of the data. Y.C., D.L.M., S.P., R.L.S., and J.P. contributed to the interpretation of the results. Y.C., D.L.M., and J.P. wrote the manuscript. Y.C., D.L.M., S.P., R.L.S., and J.P. reviewed and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

It can be found online at <https://doi.org/10.59717/j.xinn-geo.2024.100048>

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