Revision Notes

Blue text = authors' reply. Line numbers mentioned here correspond to those in the revised manuscript.

Anonymous Referee #1

General comments:

1. I revised the paper "Stronger microbial nutrient limitations in subsoil along the precipitation gradient of agroecosystem: Insights from soil enzyme activity and stoichiometry". The topic of the paper could be interesting for soil scientists, but the paper showed some issues.

Much information in Materials and methods are absent or not complete. I suggest including the analysis of available P in soil, in particular to better discuss the effect of P concentration on enzyme activities.

The authors analysed different soil sampled in different site in a climate transect, in my opinion the authors should better describe the soil type and the different soil properties. The biological parameters as enzyme activities are strictly related to soil physio-chemical properties, the weather, the temperature and the season during the soil sampling, therefore the authors should better highlight these parameters.

The authors showed the results of different parameters putting together the results obtained in different soils. I suggest the authors show the results of different soils to highlight the effect of climate transect on soil properties (chemical and biological parameters).

Consequently, the discussion is affected by the results presentation. In many cases, the discussion of their results is not completely clear. In particular, the authors should consider that the enzyme activities are strongly affected by the soil depth and this aspect should be more stressed in the text.

I suggest a strong revision of the paper, including the analysis of available P in soil, considering the physio-chemical properties of soils, sampled in the different climate transect sites, and the soil depth effect on soil enzyme activities.

I greatly appreciate and agree with your comments. We describe the experimental methods and the weather, temperature, and season during soil sampling in more detail in Materials and methods. Both available phosphorus and total phosphorus are important environmental factors that affect soil enzyme activity. In addition, there was a consistent distribution pattern of soil available phosphorus and total phosphorus in the NECT. We will focus on the relationship between available phosphorus and soil enzymes in our future studies. Furthermore, we analyzed the patterns of soil properties and soil enzyme activity with soil depth in each site to study the effect of the climate transect on soil properties. Although soil properties and enzyme activity in one site are important, we were more interested in highlighting the patterns of soil enzyme activity along the precipitation gradient in different soil layers. We showed that stronger microbial nutrient limitation in the subsoil along the precipitation gradient of agroecosystem, which is rarely studied at large scales. Large-scale studies conducted through NECT (one of 15 international standard transects established as part of the Global Change and Terrestrial Ecosystems initiative) are more important than analysis of one site.

Specific comments:

Materials and methods

2. L128: please explain the acronym MAP the first time you use it.

I'm sorry the acronym is not explicit. "MAP" means mean annual precipitation (see lines 128).

3. L 128: please replace 6.2 °C to 4.1 °C with 6.2°C to 4.1°C

We have revised the same problems in the whole manuscript.

4. L136-138: The basic information reported are not sufficient to understand the potential interaction between the enzymes and soil particles. The soil texture (clays %, sand % etc.) should be included in the soil analysis. Additional information as cation exchange capacity could be another suitable information to explain the results of the experiment.

I greatly agree with your comments. Soil enzyme activity is affected by a combination of environmental factors. We analyzed the factors influencing soil enzyme activity and microbial nutrient limitation by selecting the most common and important soil properties based on the extensive literature on large scale soil enzyme research. The relationship between other soil properties such as soil texture and cation exchange capacity and soil enzyme activity will continue to be investigated in later work.

5. L149: "during the maize harvest period", the authors should clarify if they sampled the soil immediately after harvest or before the harvest. The agricultural equipment induces a disturbance in soil, and it should be considered. Moreover, did you perform the soil sampling simultaneously? The temperature and the weather affect the biological parameters as enzyme activities.

Thanks for your advice. We sampled before maize harvest and completed all sampling within two days of sunny weather to ensure consistent weather and temperature. Please see lines 155-158.

6. L152: Soil moisture (SM)

Thanks. We have added it in line 161.

7. L152-153: the protocol the authors used to measure the soil moisture is not clear.

Soil moisture was determined by oven-drying technique using 10 g of fresh soil samples dried at 105°C for 48 h to a constant weight. The amount of water in the sample can be determined and the moisture content calculated and expressed as a percentage of the dry soil weight (Schmugge et al., 1980). We have provided detailed information in lines 161-164.

8. L154: You should replace with "the analysis of edaphic properties"

Many thanks for your suggestion. We have changed the “edaphic property analyses” to “the analysis of edaphic properties” (line 165).

9. L156-158: please briefly describe the methods you used to determine TN, TC, TP or report the proper references. Moreover, the P availability in soi in general is very low in comparison to total P, therefore the available P (e.g. Olsen P)

Thanks for your suggestion. Total C (TC) and N (TN) contents were determined by combustion using an automatic elemental analyzer (Zhang et al., 2022). Total P was first digested using H_2SO_4-$HClO_4$, and then the total P (TP) concentration was measured using a continuous flow analyzer (Wang et al., 2022). Please see lines 167-170. Both available phosphorus and total phosphorus are important environmental factors that affect soil enzyme activity. In this NECT belt, it has been shown that soil available phosphorus has a highly significant positive correlation with total soil phosphorus with a correlation coefficient of $R = 0.892^{**}$. Furthermore, there was a consistent distribution pattern of soil available phosphorus and total phosphorus in the NECT, both showing a trend of higher in the east and lower in the west (Wang et al., 2002). Therefore, we can use total phosphorus for the analysis. We will focus on the relationship between available phosphorus and soil enzymes in our future studies.

10. L163: why did you modify the protocol proposed by Saiya-Cork et al. 2002? You should include some words about that.

We did not express it clearly. We only made minor adjustments based on the samples. For example, the mass of the soil sample was increased from 1 g to 1.5 g. The incubation temperature of the sample was adjusted from 20°C to 25°C according to the pre-experiment. To avoid ambiguity, we modify it to “based on the methods described by Saiya-Cork et al. (2002) and German et al. (2011)”. Meanwhile, we add the details in lines 176-187.

11. L165: Did you measure the acetate buffer pH? The buffer pH is a crucial parameter for enzyme activities because the efficiency of enzyme activity measured could be affected by buffer pH.

We measured the acetate buffer pH at 7.7. In order to estimate potential enzyme activities in environmental samples, enzyme assays should be run at a pH appropriate for that sample. Thus, soil pH is the desirable pH to use for the estimation of potential digestive enzyme activities in soil samples (German et al., 2011). We used glacial acetic acid to adjust acetate buffer pH to the pH of this study site (lines 178-179).

12. L166: Indicate the method you used to homogenize the soil samples

Thanks. The samples were homogenized by blending on highest speed for 2 minutes to make a slurry with a magnetic stirrer (lines 179-180).

13. L 167: The figure S1 did not improve the fundamental information to understand the protocol the authors used. Therefore, I suggest the authors delete the S1 figure and better describe the protocol used in the text. In particular, the preliminary experiment

should be described to understand the information the authors achieved by this experiment. Moreover, the specific substrates for each enzyme activities should be clearly indicated.

Thanks. We deleted the S1 figure and described the protocol in detail in our manuscript. Blank wells received 250 µl acetate buffer. Standard wells received 50 µl of standard substrate and 200 µl acetate buffer. Substrate wells received 50 µl of 200 µM enzyme substrate solution in 200 µl of acetate buffer. Sample wells received 50 µl of acetate buffer and 200 µl of soil sample suspension. Quench Control wells received 50 µl of the standard substrate (10 µM 4-methylumvelliferyl or 7-amino-4-methylcoumarin) plus 200 µl of soil sample suspension. Assay wells received 50 µl of 200 µM enzyme substrate solution and 200 µl of soil sample suspension. Please see lines 180-187.

We also indicated the specific substrates for each enzyme activities in Table 1.

Table 1. Names, Abbr. (abbreviations), functions, substrate and groups of soil enzymes that were measured in the current study.

Group	Enzyme name	Abbr.	Function	Substrate
C acquisition	β-1,4-glucosidase	BG	Cellulose degradation	4-MUB-β-D-glucosidase
	β-D-cellobiohydrolase	CBH	Cellulose degradation	4-MUB-β-D-cellobioside
N acquisition	β-1,4-N-acetylglucosaminidase	NAG	Chitin degradation	4-MUB-N-acetyl-β-D-glucosaminide
	L-leucine aminopeptidase	LAP	Peptide breakdown	L-leucine-7-amino-4-methylcoumarin
P acquisition	Phosphatase	AP	Mineralizes organic P into phosphate	4-MUB-phosphate

14. L215-220: the authors could report the soil pH, TC, TN and TP for each site in a table to clearly show the effect of climate transect on pH and soil nutrients, the average and standard deviation of the soil properties. In the text it's not clear the soil type analysed and the weather in the site during the soil sampling. These information are fundamental to understand the real effect of precipitation. Otherwise, you could relate the soil pH and nutrient to the climate transect and not to the precipitation. The soil pH and soil nutrient cannot be related only to precipitation because the soil physio-chemical properties affect the soil pH, too. The authors showed a strong acidification of soil, and the explanation cannot be related only to precipitation. I suggest reporting the data for each site analysed, considering the effect also to the soil depth.

I greatly appreciate and agree with your comments. We report the soil properties for each site in Table 2. Meanwhile, we analyzed the vertical patterns of soil physical and chemical properties at each site in Figure S1. We made a detailed description of the soil type and the sampling weather (see lines 137-145, 156-158). Northeast China Transect (NECT) represents a precipitation gradient and is one of fifteen international standard transects established as part of the Global Change and Terrestrial Ecosystems initiative, and a key component of the International Geosphere-Biosphere Programme (IGBP). Therefore, the precipitation gradient represents the climate transect. We related soil properties to precipitation in order to show the pattern of soil properties with precipitation gradient. We strongly agree that soil pH is not only related to precipitation, but also to historical events and pedogenic factors, etc. This is determined by a combination of multiple factors. We have added a discussion of soil properties to the Discussion. Please see lines 302-314.

Table 2. Soil properties and their stoichiometry from 0-50 cm at each sampling site.

Site	**SM**	**pH**	**TC**	**TN**	**TP**	**TC:TN**	**TC:TP**	**TN:TP**	**Soil type**
CL	0.07 ± 0.00	8.26 ± 0.04	8.20 ± 0.09	0.78 ± 0.09	0.21 ± 0.01	10.70 ± 2.19	39.74 ± 1.94	3.80 ± 0.68	Salt-alkali
SJF	0.15 ± 0.00	7.81 ± 0.23	14.88 ± 0.15	1.27 ± 0.21	0.47 ± 0.07	11.92 ± 1.66	32.12 ± 4.60	2.70 ± 0.22	Light chernozem
NOA	0.17 ± 0.02	5.30 ± 0.28	9.20 ± 0.35	1.22 ± 0.04	0.33 ± 0.04	7.53 ± 0.28	28.55 ± 3.62	3.80 ± 0.60	Chernozem
CC	0.19 ± 0.00	5.76 ± 0.18	13.39 ± 0.32	1.41 ± 0.02	0.35 ± 0.03	9.52 ± 0.17	38.51 ± 3.18	4.05 ± 0.31	Black soil
DL	0.23 ± 0.04	5.57 ± 0.08	11.68 ± 4.14	1.39 ± 0.31	0.43 ± 0.06	8.20 ± 1.19	26.36 ± 6.22	3.18 ± 0.33	Dark brown soil
LW	0.37 ± 0.02	5.73 ± 0.17	36.96 ± 1.04	3.72 ± 0.09	1.88 ± 0.04	9.94 ± 0.10	19.69 ± 0.12	1.98 ± 0.01	Dark brown soil
BH	0.27 ± 0.02	5.27 ± 0.13	12.56 ± 2.62	1.50 ± 0.21	0.53 ± 0.03	8.31 ± 0.55	23.52 ± 3.44	2.82 ± 0.22	Dark brown soil

Note: SM: soil moisture; TC: total carbon; TN: soil nitrogen; TP: soil phosphorus. Values were presented as means ± SD.

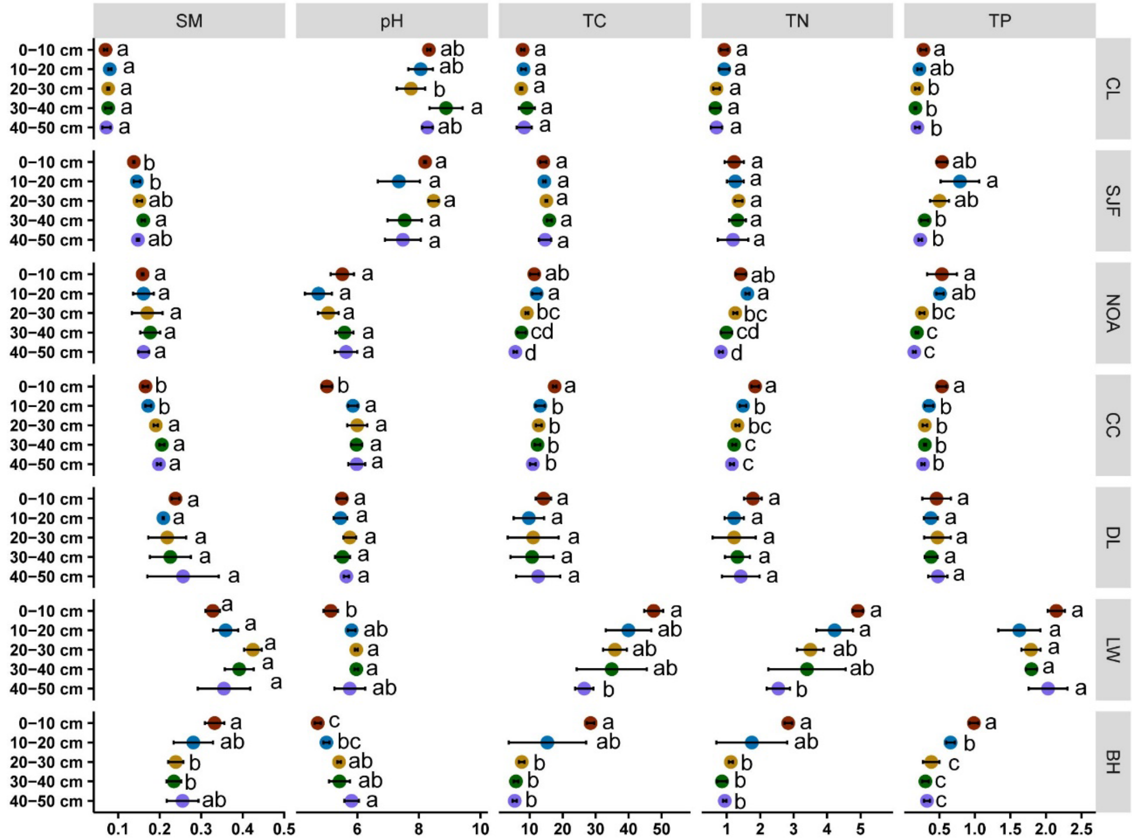

Figure S1. Variations in soil properties with soil depth at seven sites. Lowercase letters indicate different levels of one-way ANOVA among different depths ($P < 0.05$). All values were expressed in means ± standard deviation.

15. L226-272: the same observations previously reported for soil pH and nutrients can be considered also for soil enzyme activities: the soil properties and not only the precipitations affect the soil enzyme activities and they have to be considered in the text. Moreover, why did you report the results of 0-50 cm layer? The 0-50 cm layer are not included in soil pH and nutrients section.

I absolutely agree that soil properties and not only precipitation affect soil enzyme activities. We related soil enzyme activities to precipitation in order to show the pattern of soil enzyme activities with precipitation gradient. We considered the relationship between soil properties and enzyme activity, and found that soil pH and moisture were the most important factors affecting enzyme activity by RDA analysis in Figure 3. The results of 0-50 cm layer can give a more comprehensive picture of the study site than a single soil layer. We have added the results of soil properties for the 0-50 cm layer in Figure 1.

16. The enzyme activities are strongly affected by the soil depth therefore in general the enzyme activities analysis is related to 0-20 cm. I suggest considering this aspect.

Thanks for your advice. We analyzed the vertical patterns of soil enzyme activities at each site in Figure S2. We took into careful consideration the changes in enzyme activity and enzyme stoichiometry of the soil from 0-20 cm. Especially in agroecosystems, the 0-20 cm as topsoil is the tillage layer, which is also quite different from the subsoil, and this is one of the highlights of our research.

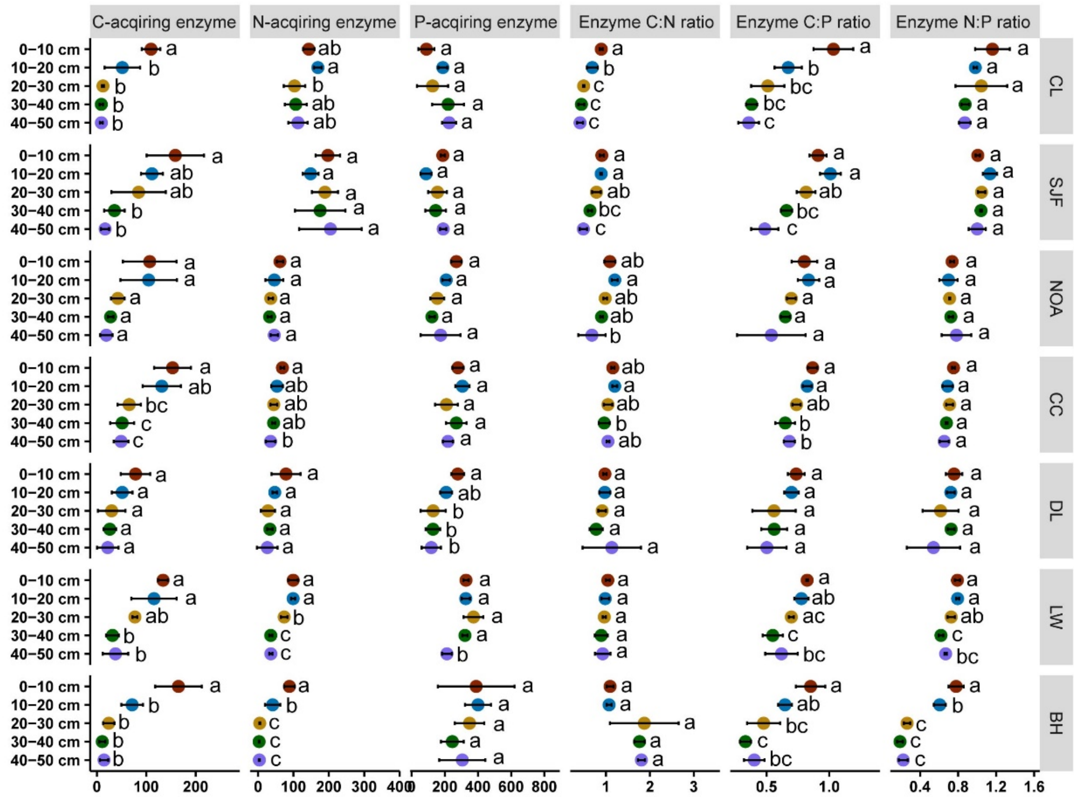

Figure S2. Variations in soil enzyme activities and stoichiometry with soil depth at seven sites. Lowercase letters indicate different levels of one-way ANOVA among different depths ($P < 0.05$). All values were expressed in means ± standard deviation.

17. The discussion: the discussion is necessarily affected by the results presentation. The discussion of soil chemical properties should be insert before the discussion of enzyme activities. Moreover, I suggest considering the climate transect effect on soil properties and consequently the authors can discuss the effect of precipitations. The description of the soil properties in different site can help to understand the effect of climate transect and consequently the effect of precipitation. The figure 5 is interesting and it should be better discussed and explained in the text.

Many thanks for your suggestion. It has been done. Please see lines 302-314. Soil moisture, total carbon, total nitrogen, total phosphorus increased as the MAP increased along the NECT, but the soil pH decreased as the MAP increased. These results are consistent with the results at the regional scale (Cui et al., 2019; Shu-Ping et al., 2002). Due to the steep moisture gradient, the virgin vegetation changes gradually from mixed deciduous broad-leaved forest to meadow steppe along the transect (Prentice et al., 2011). Forests can improve the availability of soil nutrients through nitrogen fixation and phosphorus strategies (Nasto et al., 2014; Nasto et al., 2017). In addition, soil properties are related to the interaction of precipitation and

soil parent materials (Huston, 2012). The high pH of the soil in west of the transect is due to the fact that the soil is salt-alkali and the evaporation is three to four times higher than the precipitation (Wang and Ba, 2008; Yang et al., 2021). Therefore, less precipitation, high surface evaporation and parent salinity are the major reasons for high soil pH. Understanding soil properties at the regional scale is fundamental to predicting climate changes at the global scale.
Figure 5 is discussed in lines 225-228, 322-325, 339-340, 363-369.

18. L301-303: this sentence is not clear. The authors analysed the total P and not the available P in soil. The amount of available P in soil is very lower than total P and strictly related to soil type. The amount of available P could help the authors to better explain their results.
I'm sorry this sentence is not explicit. Microbial metabolic limitation in soil is attributed to an imbalance in nutrient stoichiometry. “If the C and N accumulated in the soil dilute the soil P content, the microorganisms may be limited by P due to the elemental stoichiometric balance of the microorganisms” means that the soil carbon and nitrogen accumulate and the phosphorus content needs to be higher to sustain the microbial metabolism. If carbon and nitrogen accumulate and there is no more phosphorus to sustain microbial metabolism, this can lead to phosphorus limitation. Microbial resource limitation is a relative concept that cannot be judged simply by the abundance of the resource. Therefore, "dilution of soil P content" is not a real reduction of soil phosphorus content, but a relative concept. Both total phosphorus and available phosphorus are consistent with this rule. We improved this sentence in lines 342-344.

19. L305-307: this sentence is not clear. Please explain why the C and N limitation should affect the phosphatases and not the other enzymes.
This sentence does not include the C and N limitation. As proteins, carbon is an important component of phosphatase and phosphatases have relatively high N concentrations (between 8% and 32%), and may represent a significant investment of C and N (Treseder and Vitousek, 2001). This sentence has been improved in lines 346-348.