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Is microbial biomass measurement by the chloroform fumigation extraction method biased by experimental addition of N and P?

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The chloroform fumigation extraction (CFE) method determines microbial biomass carbon (MBC) or nitrogen (MBN) by calculating the increase in extractable carbon (C) or nitrogen (N) due to microbial lysis during chloroform fumigation. In China, many studies have focused on the impacts of N and phosphorus (P) addition on soil MBC and MBN in forest ecosystems, where substantial atmospheric N deposition has strongly acidified soils. The addition of nutrients may alter the extraction process applied in the CFE method, potentially influencing the MBC and MBN determined by the CFE method independently of the actual microbial biomass. In this study, we tested whether the MBC and MBN determined by the CFE method were biased by the experimental addition of N and P in strongly acidified Chinese forest soils by adding N and P to the soils immediately before chloroform fumigation, which should not affect the actual microbial biomass. P addition significantly elevated the dissolved organic carbon (DOC) content, especially after fumigation, while N addition significantly reduced the dissolved nitrogen (DN) content. The added N was subtracted using blank samples without soil. However, the altered DOC and DN contents did not affect the MBC and MBN contents determined by the CFE method. In conclusion, our study suggests that the CFE is a relatively robust method to test the impacts of nutrient addition on microbial biomass in the strongly acidified soils of Chinese forests. We also suggested that: (i) even if a fertilization experiment results in an elevated DOC content following P addition, it does not necessarily indicate a stimulation of DOC production by microbes; and (ii) the soil adsorption capacity or the strength of microbial N uptake during the extraction procedure applied in the CFE method may affect the determination of MBN by influencing the DN extraction efficiency.

Keywords: Chloroform Fumigation Extraction, Microbial Biomass, Nitrogen, Phosphorus, Soil, Tropical Forest

Introduction

The chloroform fumigation extraction (CFE) method (Brookes et al. 1985, Vance et al. 1987) is widely used in ecological studies (Xu et al. 2013) to determine microbial biomass *via* elements such as microbial biomass carbon (MBC) or microbial biomass nitrogen (MBN). The CFE method determines microbial biomass by calculating the increase in soil extractable fractions, such as dissolved organic carbon (DOC) or dissolved nitrogen (DN) extracted by 0.5 M K_2SO_4, during chloroform fumigation. During fumigation, microbial cells lyse and a portion of the dead microbial constituents are transformed into extractable components through enzymatic autolysis. The CFE method assumes that the increases in DOC or DN are in proportion to the soil microbial biomass, and therefore the microbial biomass can be calculated using conversion factors (Jenkinson et al. 2004). This method enables a relatively accurate and quick determination of soil microbial biomass.

Researchers have investigated the impacts of nutrient addition, such as nitrogen (N) and phosphorus (P), on soil microbial biomass. This has enabled an evaluation of the impacts of anthropogenic nutrient loading into soils, such as atmospheric nutrient deposition or nutrient fertilization, on soil microbial biomass (Treseder 2008, Liu & Greaver 2010). Additionally, microbial nutrient limitations have been determined (Liu et al. 2012, Liu et al. 2015, Turner & Wright 2014, Mori et al. 2018). In China, many studies have focused on the impacts of the substantial increases in atmospheric N deposition, which have strongly acidified forest soils (Lu et al. 2014), and the subsequent imbalanced input of N and P into ecosystems that is consequently occurring (Du et al. 2016). The CFE method has been generally used to examine the effects of N and P addition on soil microbial biomass (Treseder 2008, Liu & Greaver 2010, Liu et al. 2012, Turner & Wright 2014, Fanin et al. 2015, Mori et al. 2016).

Several studies have reported methodological weaknesses of the CFE method.

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For example, Alessi et al. (2011) suggested that chloroform can adsorb onto the soil during fumigation, especially onto the clay minerals, causing an increase in DOC and hence an overestimation of the MBC in clay-rich soils (Rotbart et al. 2017). It has also been demonstrated that soil moisture can affect the determination of MBC (Ross 1989). Despite these methodological problems can under- or overestimate soil MBC and MBN, it is assumed that the impacts of nutrient addition on soil microbial biomass can be tested relatively robustly, because both nutrient-amended soils and a non-amended control are similarly influenced by these methodological problems.

However, the robustness of the CFE method for testing the impacts of nutrient addition on soil microbial biomass has not been fully examined. Added nutrients may alter the extraction process applied in the CFE method, potentially influencing the MBC and MBN determined by the method independently of the actual microbial biomass. It is possible that the changes in soil pH through nutrient addition affect the microbial biomass determined by the CFE method, because soil pH can affect the extraction efficiency of DOC by K_2SO_4 (Haney et al. 1999, 2001). Müller et al. (2003) reported that NH_4^+ immobilization may occur during fumigation. The determination of MBN could then be affected by nutrient addition through changes in DN immobilization (Hall & Matson 1999, 2003) during fumigation. Changes in soil adsorption capacity may also affect the determination of MBC or MBN. It has been reported that the extracted DOC content can be physico-chemically affected by P fertilization (Mori et al. 2018), because P has a higher affinity with soil surfaces than DOC (Kaiser & Zech 1996) and P can desorb DOC at the mineral surfaces (Hobara et al. 2016). If a portion of the DOC flushed out by chloroform fumigation (*i.e.*, increase in DOC during fumigation) is adsorbed by soil (and 0.5 M K_2SO_4 does not extract all of the adsorbed DOC), P addition could cause larger MBC values by desorbing the adsorbed DOC.

The aim of this study was to test whether the MBC and MBN determined by the CFE method are biased by the experimental addition of N and P in strongly acidified Chinese forest soils. By adding N and P immediately before fumigation, we evaluated the CFE method-dependent impacts of nutrient addition on microbial biomass determination. Because the addition of N and P immediately before fumigation should not affect the actual microbial biomass (*i.e.*, microbes have no time to change their biomass), any changes in the MBC and MBN should be caused by the impacts of N and P addition on the CFE method (*e.g.*, alterations to the DOC and DN extraction process).

Materials and methods

Study sites

Soil samples for the experiment were collected from six forests in China. Three of the six forest sites were located in the Dinghushan Biosphere Reserve (DHS; 23° 10′ N, 112° 10′ E – Mo et al. 2003, 2006): a primary monsoon evergreen broadleaf forest (BF), a secondary mixed pine/broadleaf forest (MF), and a planted *Pinus massoniana* forest (PM). Two forest sites were located in the Heshan National Field Research Station (HS; 22° 34′ N, 112° 50′ E – Zhang et al. 2012): a planted *Acacia auriculiformis* forest (AA) and a planted *Eucalyptus urophylla* forest (EU). The final site was a mixed deciduous forest (MDF) located in the Jigongshan National Nature Reserve (JGS; 31° 46′ - 31° 52′ N, 114° 01′ - 114° 06′ E – Zhang et al. 2015). The annual average temperature is 21.0 °C, 22.5 °C, and 15.2°C and the annual average precipitation is 1580, 1927, and 1119 mm in DHS, HS, and JGS, respectively (Huang & Fan 1982, Zhang et al. 2015, Shao et al. 2017). The soils in DHS, HS, and JGS are a lateritic red earth formed from sandstone (Mo et al. 2003, Zhou et al. 2018), an Acrisol (Zhu et al. 2015), and a yellow brown soil (Zhang et al. 2015), respectively. The basic characteristics of the study sites are summarized in Tab. 1. All six forests have received large amounts of atmospheric N deposition (Mo et al. 2006, Zhang et al. 2012, 2015, Mao et al. 2017, Zhou et al. 2018).

Experimental setup and chloroform fumigation

Surface soil samples (0-10 cm) were collected from subplots at the six forest sites using soil cores (three, three, and four subplots in DHS, HS, and JGS, respectively). Soil samples taken from the same site were combined, and six replicates were prepared for the experiment. We sieved the soil samples through a 2-mm sieve after removing the fine roots and coarse organic matter. Sieved soil samples (6 g) were placed in bottles (glass bottles for fumigated soils and plastic bottles for unfumigated soils) and 1.5 mL N (500 µg N per 1.5 mL solution in the form of NH_4NO_3) or P (500 µg P per 1.5 mL solution in the form of KH_2PO_4) were added. The final concentrations of the added N and P (around 100 µg N and P per g soil) were in a similar range to that of previous laboratory experiments where nutrient concentrations were decided based on the nutrient doses in the field (Duah-Yentumi et al. 1998, Cleveland et al. 2002, Ilstedt & Singh 2005, Mori et al. 2013a, 2013b). Controls without N or P addition were prepared in the same manner by adding pure water. Blanks without soils were also prepared for both fumigated and unfumigated samples. The DOC and DN before the fumigation (DOC_{bef} and DN_{bef}) were extracted immediately after the addition of the N or P solutions by shaking the soils with 30 mL 0.5 M K_2SO_4 for 30 min. Fumigated samples were placed in a vacuum desiccator and exposed to chloroform vapor for 24 h (Vance et al. 1987). The DOC and DN after the fumigation (DOC_{aft} and DN_{aft}) were extracted in the same manner as DOC_{bef} and DN_{bef}. The MBC and MBN were then calculated by subtracting DOC_{bef} from DOC_{aft} and DN_{bef} from DN_{aft}, respec-

Tab. 1 - Selected basic characteristics (mean ± standard error) of the six forest sites investigated. Data collection and source: (a) measured in 2015 (from Zheng et al. 2018); (b) measured in 2015 (from Zheng et al. 2017); (c) measured in Dec 2011 (from Zhu et al. 2015); (d) measured in Jul 2018 (from Liu et al. 2020); (e-f) measured in the present study. Sample size: (a-d) n = 3; (e) n = 5; (f) n = 1. Sites: (BF) primary monsoon evergreen broadleaf forest; (MF) secondary mixed pine/broadleaf forest; (PM) planted *Pinus massoniana* forest; (AA) planted *Acacia auriculiformis* forest; (EU) planted *Eucalyptus urophylla* forest; (MDF) mixed deciduous forest. Variables: (DOC) dissolved organic carbon; (DN) dissolved nitrogen. Soil samples for chemical analysis were taken from 0-10 cm depths.

Characteristics	Sites					
	BF	MF	PM	AA	EU	MDF
Soil organic C (g kg^{-1})	40.0 ± 4.2 [a]	32.1 ± 4.0 [b]	23.3 ± 1.6 [b]	23.8 ± 1.7 [c]	18.5 ± 0.4 [c]	63 ± 13 [d]
Soil total N (g kg^{-1})	2.9 ± 0.5 [a]	2.0 ± 0.3 [b]	1.4 ± 0.2 [b]	2.0 ± 0.1 [c]	1.5 ± 0.2 [c]	3.4 ± 0.5 [d]
Soil available P (mg kg^{-1})	2.1 ± 0.4 [a]	1.0 ± 0.3 [b]	1.5 ± 0.3 [b]	2.5 ± 0.2 [c]	2.1 ± 0.1 [c]	6.9 ± 2.0 [d]
DOC (µg C g^{-1})	220.7 ± 3.6 [e]	154.4 ± 2.5 [e]	208.0 ± 4.4 [e]	275.6 ± 3.5 [e]	297.9 ± 3.6 [e]	135.0 ± 2.0 [e]
DN (µg C g^{-1})	29.9 ± 1.9 [e]	17.9 ± 1.0 [e]	25.9 ± 1.8 [e]	38.5 ± 1.1 [e]	41.8 ± 2.2 [e]	25.4 ± 0.8 [e]
Soil pH (H_2O)	3.53 [f]	3.68 [f]	3.6 [f]	3.4 [f]	3.65 [f]	4.25 ± 0.10 [d]
Soil water content	0.24 [f]	0.18 [f]	0.22 [f]	0.23 [f]	0.27 [f]	0.13 [f]

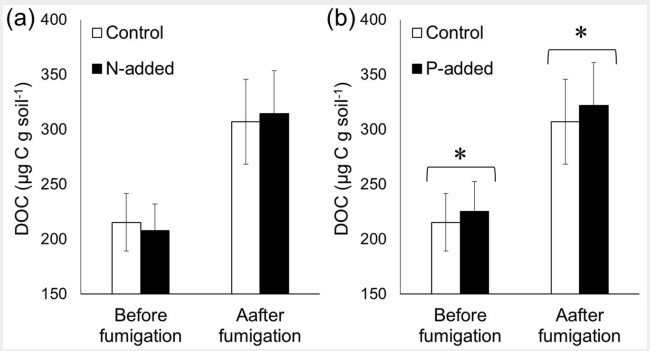

Fig. 1 - Effects of experimental (a) N addition and (b) P addition on the dissolved organic carbon (DOC) content before and after chloroform fumigation. The DOC was extracted by 0.5 M K_2SO_4 after 30 min of shaking. Error bars indicate the standard error of data from six sites. Statistical significance was determined by a paired *t*-test. (*): $p < 0.05$; (**): $p < 0.01$.

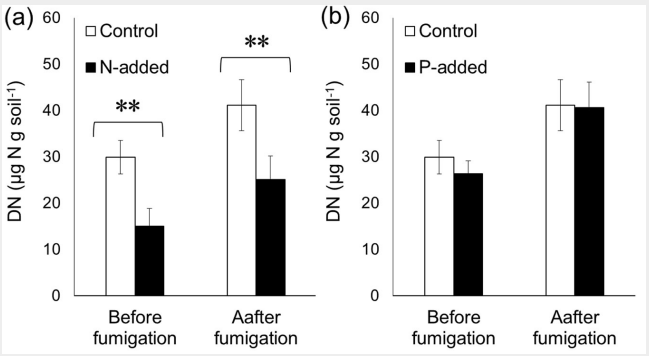

Fig. 2 - Effects of experimental (a) N addition and (b) P addition on the dissolved nitrogen (DN) content before and after chloroform fumigation. The DN was extracted by 0.5 M K_2SO_4 after 30 min of shaking. Error bars indicate the standard error of data from six sites. Statistical significance was determined by a paired *t*-test. (*): $p < 0.05$; (**): $p < 0.01$.

tively. A conversion factor of 0.45 was used for calculating both MBC and MBN (Jenkinson et al. 2004). The DOC and DN were measured with a total organic carbon analyzer (TOC-5000®, Shimadzu, Japan). Soil pH(H_2O) (soil to water ratio 1:2.5) was measured using unfumigated soils. We had only five replicates for the pH analysis because we failed to analyze JGS soils.

Statistics

A paired *t*-test was used to determine the statistical significance of differences between control soils and N- or P-amended soils. This statistical analysis was used because our main purpose was to confirm that N and P addition did not affect the MBC and MBN determination by the CFE method (*i.e.*, repeating a *t*-test increases type 1 errors, and therefore a more robust result could be obtained if there were no statistical differences). If the paired *t*-test revealed any significant differences, an additional analysis was performed using a linear mixed effect model. All statistical analyses were performed using R version 4.0.2 (R Core Team 2020).

Results

Effects of N and P addition on DOC and DN

The DOC and DN contents in our study sites ranged from 130 to 300 μg C g $soil^{-1}$ and from 15 to 45 μg N g $soil^{-1}$, respectively. Chloroform fumigation caused a large elevation in both the DOC and DN contents (by ~100 μg C g $soil^{-1}$ and 10 μg N g $soil^{-1}$, respectively – Fig. 1, Fig. 2). N addition did not show any impact on the DOC content (DOC_{bef} or DOC_{aft}) extracted by 0.5 M K_2SO_4 ($p > 0.05$ – Fig. 1a). By contrast, a paired *t*-test demonstrated that P addition significantly elevated the DOC content ($p < 0.05$ – Fig. 1b). According to a linear mixed effect model analysis, the impact of P addition was statistically significant on the DOC_{aft} content ($p < 0.01$), but not on the DOC_{bef} content ($p = 0.26$). Both the paired *t*-test and linear mixed effect model analysis demonstrated that N addition significantly decreased both the DN_{bef} and DN_{aft} contents ($p < 0.01$ – Fig. 2a). Note that the amount of added N was subtracted using the blank samples. P addition did not affect the DN content ($p > 0.05$ – Fig. 2b).

Effects of N and P addition on MBC, MBN, and pH

N addition did not affect the MBC content determined by the CFE method ($p > 0.05$ – Fig. 3a), which was consistent with the fact that neither DOC_{bef} nor DOC_{aft} were affected by N addition. Similarly, the MBN content was not influenced by P addition ($p > 0.05$ – Fig. 4b). Despite the altered DN content by N addition, the MBN content was not affected by N addition ($p > 0.05$ – Fig. 4a). P addition did not affect the MBC content ($p > 0.05$ – Fig. 3b), although P addition significantly increased the DOC_{aft} content.

Both the paired *t*-test and linear mixed effect model analyses demonstrated that N addition significantly decreased soil pH ($p <$

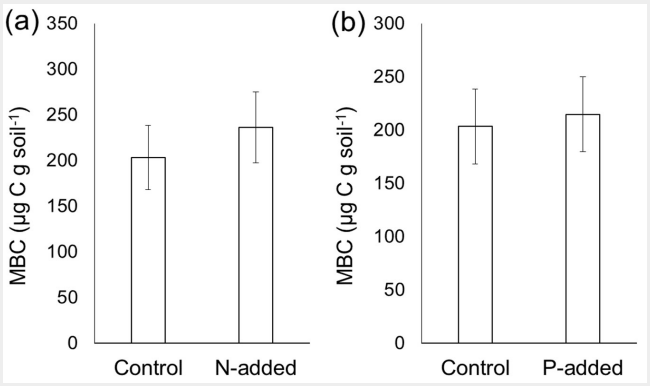

Fig. 3 - Effects of experimental (a) N addition and (b) P addition on the microbial biomass carbon (MBC) content before and after chloroform fumigation. Error bars indicate the standard error of data from six sites. A paired *t*-test did not reveal significant differences ($p > 0.05$).

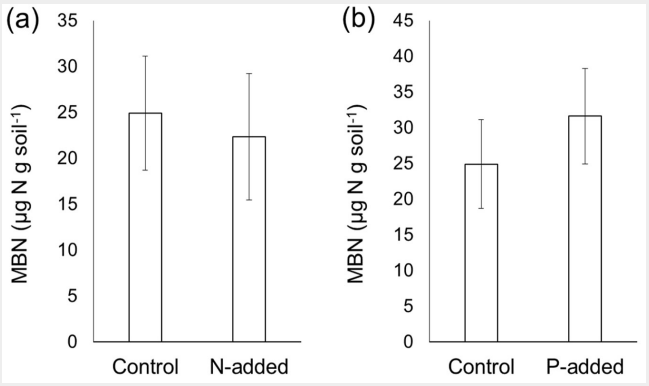

Fig. 4 - Effects of experimental (a) N addition and (b) P addition on the microbial biomass nitrogen (MBN) content before and after chloroform fumigation. Error bars indicate the standard error of data from six sites. A paired *t*-test did not reveal significant differences ($p > 0.05$).

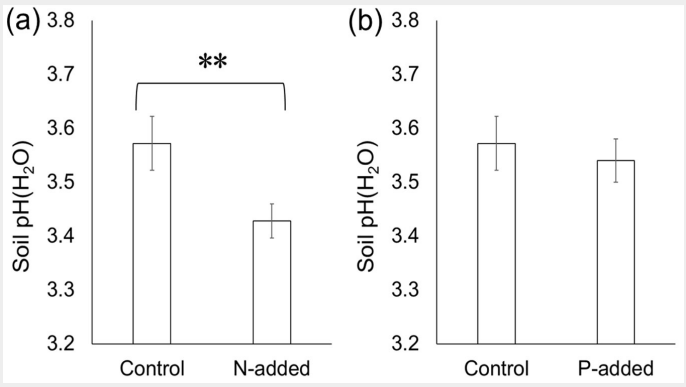

Fig. 5 - Effects of experimental (a) N addition and (b) P addition on soil pH. Error bars indicate the standard error of data from five sites (BF, MF, PM, AA, and EU). A paired *t*-test did not reveal significant differences ($p > 0.05$).

0.01 – Fig. 5a). Meanwhile, P addition did not have a significant influence ($p > 0.05$ – Fig. 5b).

Discussion

Effects of N and P addition on DOC and DN

The decrease in the DN content following N addition in both the unfumigated and fumigated soils indicated that a portion of the added N (NH_4NO_3) was adsorbed by the soil or immobilized by microbes (Fig. 2a). It has been suggested that microbes could reduce the DN content during the extraction process (Rousk & Jones 2010). This is important because it indicates that the soil adsorption capacity or the strength of microbial N immobilization may affect the DN extraction efficiency during the extraction procedure applied in the CFE method, influencing the MBN calculation. If this is the case, the recovery efficiency of the flush of DN during fumigation should be taken into account when calculating the MBN. Further studies are required to investigate this.

We also found that P addition elevated the DOC content (especially DOC_{aft} – Fig. 1b). This was probably because the DOC adsorbed at the mineral surface (part of which was not extracted by 0.5 M K_2SO_4) was desorbed by P (Mori et al. 2018). Kaiser & Zech (1996) performed sorption experiments where $H_2PO_4^-$ had a higher affinity to soil than DOC. Hobara et al. (2016) demonstrated that extracting organic C using a phosphate solution provided around 10 times more C than the use of KCl or water as an extractant, indicating that P has a strong ability to extract organic C from soils. Thus, we attributed the increased DOC content to the desorption of DOC by the added P. Changes in soil pH following P addition may have also caused the higher DOC content in P-amended soils (Haney et al. 2001), but this was less likely because P addition did not change soil pH(H_2O) significantly (Fig. 5b), and the decrease in soil pH in N-amended soils (Fig. 5a) did not affect the DOC content (Fig. 1a). The higher DOC content in P-amended soils in our study suggested that the impacts of P fertilization on DOC content should be carefully interpreted in fertilization studies, because the elevated DOC content following P addition does not necessarily indicate stimulated DOC production by microbes.

Effects of N and P addition on MBC and MBN

By adding N and P immediately before fumigation, we conducted tests to determine whether the MBC and MBN measured by the CFE method were biased by the experimental addition of N and P in strongly acidified Chinese forest soils. Our results demonstrated that the MBC and MBN contents were not significantly biased. Despite the increase in the DOC content following P addition (Fig. 1b) and reduced DN content following N addition (Fig. 2a), neither MBC (calculated as the differences between DOC_{bef} and DOC_{aft}) nor MBN (calculated as the differences between DN_{bef} and DN_{aft}) was affected by N or P addition. The reduced DN_{aft} content following N addition did not differ from the reduced DN_{bef} content (Fig. 2a), which consequently caused the MBN content to be unaffected by N addition (Fig. 4a). Similarly, the elevated DOC_{aft} content following P addition did not differ from the elevated DOC_{bef}, resulting in an insignificant difference in the MBC between P-amended soils and the control without P addition (Fig. 3b). Overall, our results suggest that the CFE method is a relatively robust method to test the impacts of nutrient addition on microbial biomass in the strongly acidified soils of Chinese forests. However, to generalize our results, more studies are needed, especially in soils with a high pH.

Conclusions

By testing whether the N or P addition immediately before fumigation affected the results of a microbial biomass determination by the CFE method, we evaluated the robustness of the CFE method for determining the impacts of N and P addition on microbial biomass C and N in the strongly acidified soils of Chinese forests. We found that P addition significantly elevated the DOC content (especially DOC_{aft}); N addition significantly reduced the DN content; and the altered DOC and DN contents did not change the MBC or MBN contents determined by the CFE method. We concluded that CFE is a relatively robust method to determine the impacts of nutrient addition on microbial biomass in the strongly acidified soils of Chinese forests. The results also suggest that even if a fertilization experiment revealed an elevated DOC content after P addition, it does not necessarily indicate DOC production by microbes. The soil adsorption capacity or strength of microbial N uptake during the extraction procedure applied in the CFE method may affect the determination of MBN by influencing the DN extraction efficiency.

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