Title: Long-term nitrogen deposition linked to reduced water use efficiency in forests with low phosphorus availability

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Summary

• The impact of long-term N deposition is under-studied in phosphorus (P) limited subtropical forests, where P limitation may mitigate potential physiological responses to enhanced N availability. We exploit historically collected herbarium specimens to investigate these phenomena in three P-limited, subtropical forests representing an urban-to-rural gradient, across which N deposition likely has varied over the past six decades. We measured foliar [N] and [P] and δ¹³C, δ¹⁸O and δ¹⁵N in tissue from herbarium specimens of plant species collected from 1947 to 2014.

• Foliar [N] and N:P increased, and δ¹⁵N and [P] decreased in the two forests close to urban centers. Recent studies have demonstrated that N deposition in the region is ¹⁵N-deplete; thus, these data suggest that the increased foliar [N] and N:P, and decreased [P], may be due to atmospheric deposition and associated enhancement of P limitation.

• Estimates of intrinsic water use efficiency calculated from foliar δ¹³C decreased over time, which contrast with multiple studies conducted in N-limited forests. This effect may reflect decreased photosynthesis, as suggested by a conceptual model of foliar δ¹³C and δ¹⁸O.

• Our work suggests that under long-term N deposition, forests on highly P-limited soils may not support the projected changes in C stocks driven by elevated CO₂ concentration due to increased P limitation.

Keywords: δ¹³C, herbarium specimens, δ¹⁵N, N:P, P-limited soils
Introduction

Rising atmospheric CO$_2$ concentrations and enhanced mobilization of reactive nitrogen (N) have occurred concurrently around the globe for decades (Cui et al., 2013). Increases in the availability of these resources can result in stoichiometric imbalances in ecosystem biomass pools on a global scale (Peñuelas et al., 2013), in part because availability of phosphorus (P) has not increased concurrently. Ecosystem-scale implications of changes in foliar nutrient content have been explored for decades (Comins & McMurtrie, 1993, Jonard et al., 2012, Nadelhoffer et al., 1999, Reich et al., 2001), but it remains unclear how increasing C and N availability may influence terrestrial ecosystem functioning. The ratio of plant productivity to water loss, water use efficiency (WUE), can increase with atmospheric CO$_2$ concentration (Keenan et al., 2013) and appears to be occurring at broad spatial scales (van der Sleen et al., 2014).

Because this phenomenon reflects a plant’s ability to experience similar diffusion of CO$_2$ into the leaf with relatively smaller stomatal aperture as atmospheric CO$_2$ concentration increases (CO$_2$ fertilization effect) (Field et al., 1995), it likely can be manifest regardless of nutrient limitation, as long as that limitation does not completely inhibit photosynthesis (De Kauwe et al., 2013, Long et al., 2004). Increased N deposition also can have a positive influence on WUE even if stomatal conductance remains constant, via increases in foliar [N] and photosynthetic capacity in N-limited ecosystems (e.g temperate forests) (Evans, 1989, Guerrieri et al., 2011). However, several studies report adverse effects of atmospheric N deposition on plant growth and foliar C gain in forest ecosystems (Aber et al., 1995, Magill et al., 2000), which suggests that the effect of increased N deposition on WUE in forest ecosystems is less clear than that of elevated atmospheric CO$_2$ concentration.

Most work evaluating these ideas in specific systems has been performed in relatively short-term, controlled experiments (Yan et al., 2014), because it is difficult to assess how decades of increased N availability can influence ecosystem function across wide regions (Magnani et al., 2007). These problems of spatial and temporal scale can be addressed in part, however, by exploiting biomass samples formed at distinct time.
periods such as tree-rings, or foliar samples collected over time and curated in herbariums (Hietz et al., 2011, McLauchlan et al., 2010, Peñuelas & Matamala, 1990, Stewart et al., 2002). Though we cannot observe plant functioning directly using such material, we can invoke the chemical composition of biomass, including its stable isotopic signatures, to infer how environmental conditions have governed past plant functioning (Peñuelas & Azcón-Bieto, 1992). For example, foliar δ\(^{13}\)C depends mainly on the δ\(^{13}\)C of atmospheric CO\(_2\) and mole fraction of ambient CO\(_2\), at the sites of carboxylation, and in the intercellular spaces in the leaf. These values, in turn, depend on photosynthetic rate and conductance of different leaf components (Warren & Adams, 2006). Consequently, foliar δ\(^{13}\)C can reveal much about the environment in which the biomass was generated. Because foliar δ\(^{13}\)C is related to the ratio of photosynthetic rate to stomatal conductance for water vapour (Farquhar et al., 1982), inferences about intrinsic WUE (iWUE), defined as the rate of C assimilation divided by stomatal conductance (Helle & Schleser, 2004), can emerge from foliar δ\(^{13}\)C and atmospheric CO\(_2\) concentration. In turn, vegetation WUE can be estimated from inferred iWUE when the gradient in water-vapour pressure between the leaf and atmosphere remains constant (Lambers et al., 2008). This approach has enabled inferences of increasing WUE over multiple decades in the 20\(^{th}\) century, in diverse systems such as a Sitka spruce plantation in Scotland (Guerrieri et al., 2011), pine stands in the Iberian Peninsula (Andreu-Hayles et al., 2011), beech stands in Catalonia (Peñuelas et al., 2008), and North American conifers (Marshall & Monserud, 1996) and oaks (Haavik et al., 2008).

In spite of these and related works, the degree to which increasing N availability may influence iWUE remains unclear, and this is particularly true for systems in which a nutrient other than N limits ecosystem productivity. Particularly little is known about the WUE response to enhanced N availability in P-limited ecosystems, where any effect may be muted or undetectable. Indeed, Liebig’s law of the minimum (Chapin, 1980) suggests that in systems already limited by P availability, we might expect that enhanced mobilization and availability of reactive N may have little effect on plant
functioning (Braun et al., 2010, Morecroft et al., 1994), and though the identity of a limiting resource may change over time, emerging ideas about co-limitation suggest that limitations on ecosystem productivity likely result from a single resource at a time (Farrior et al., 2013).

To investigate how increasing N availability in P-limited ecosystems may influence WUE, we assessed the influence of decades of enhanced availability of reactive N on historical WUE in multiple P-limited, subtropical forests of southeastern China, as inferred from multiple measures of plant N status (leaf [N], N:P, and N stable isotopic signatures ($\delta^{15}N$)), and $\delta^{13}C$ and $\delta^{18}O$ of archived foliar samples. Low P availability in these forests is evidenced by relatively high foliar N:P ratios (Huang et al., 2013, Liu et al., 2012, Liu et al., 2010, Zhang et al., 2008), with mean N:P ratio (in mass basis) of 27 prior to large-scale N deposition. In forest ecosystems, the foliar N:P ratio above 16 often indicates P limitation (Tessier & Raynal, 2003). Güsewell (2004) proposed a broader range of ratios of co-limitation and suggested that N:P ratios >20 correspond to P-limited biomass production. The combination of $\delta^{13}C$ and $\delta^{18}O$ may provide insights into which physiological traits, i.e., the rate of C assimilation and stomatal conductance, was the dominant contributor to variations in iWUE (Guerrieri et al., 2011, Scheidegger et al., 2000, Siegwolf et al., 2001). Even more so than most forests in China, some of these forests have been subjected to rising atmospheric N deposition since the 1980s due to rapid urbanization (Liu et al., 2013, Lu et al., 2014, Yu et al., 2014). We hypothesized that though temporal trends in foliar $\delta^{13}C$ would be consistent with studies demonstrating enhanced WUE with increasing atmospheric CO$_2$ concentration (Keenan et al., 2013, van der Sleen et al., 2014), the degree to which WUE increased over time would not depend on foliar [N], given the vegetation’s P-limited status. We also hypothesized that stomatal conductance of leaves would decrease, but the foliar $\delta^{18}O$ would increase as atmospheric CO$_2$ concentration increases. The increased WUE is linked to the reduced stomatal conductance and increased foliar $\delta^{18}O$.  

Comentari [j1]: I suppose in mass basis and not in molar basis such as is frequent in terrestrial ecosystems studies!!
Materials and methods

Study area and collection of herbarium specimens and plant samples

Vegetation was sampled in Fujian Province, southeastern China. The province has a humid subtropical climate. Mean precipitation between 2002 and 2012 for the Province is 1670 mm annually and concentrated in spring and summer. Trees in this region are often subject to seasonal water shortage between October and February. Soils are Ferric Acrisols according to the FAO/UNESCO classification (Bohan et al., 1998), and are broadly similar across the three forests. Soils are iron and clay-rich, with pH values ranging from 4.0 to 5.0.

Plant specimens were selected from three forests that were typically composed of subtropical, evergreen broad-leaved species. The three forests, Guling, Houping, and Taining, are considered old-growth (the largest trees are >200 years old), and represent an urban-to-rural gradient (Fig. 1 and Fig.S1) across which the extent of pollution and N deposition vary: Guling forest is 10 km away from Fuzhou, a city with a population of 7 million, Houping forest is 15 km from Nanping, a city with a population of 500,000, and Taining forest is located in a rural area and received less N deposition in the past decades compared to Guling and Houping forests (Chen et al., 2011, Zheng et al., 2012). To our knowledge, neither fertilizers nor any management practices have been applied in the selected forests in the past hundred years; indeed, these forests are considered as “Fengshui” forests at the local status and are protected by the indigenous residents under traditional Chinese geomancy beliefs (Bruun, 2008).

Leaves collected from the Guling forest between 1947 and 1956 (median 1953) were obtained from 30 herbarium sheets representing 16 tree species and 14 understory species at the Fujian Agricultural and Forestry University (FAFU) Herbarium, with one herbarium sheet per species (Table S1). Leaves collected from the Houping forest between 1951 and 1958 (median 1955), and between June 1975 and July 1983 (median June 1978) were obtained from 120 herbarium sheets representing 38 tree species and 22 understory species in the FAFU Herbarium, with one herbarium sheet per species per sampling year (Table S2). Leaves collected from the Taining forest in 1962 and
1983 were obtained from 52 herbarium sheets representing 26 plant species at the
FAFU Herbarium, with one herbarium sheet per species per sampling year (Table S3).

Contemporary foliar specimens of the same tree and understory species were
collected between May and July 2014 from the Guling, Houping, and Taining forests,
from five individuals of each species whenever possible. One south-facing branch in
the middle of the canopy was randomly selected from the targeted plants following the
standard collection procedure recorded at the FAFU Herbarium. Three mature leaves
from the current year were collected from the branch. We bulked the samples from the
five individuals to make one sample per species. The leaves from the five individuals
were similar in mass, so that each individual contributes the same amount of influence
to the value for that species. A total of 298 fresh and herbarium specimens were
collected from the three forests.

Determination of C, N, and P concentrations and stable isotope analysis
All contemporary leaves were oven dried at 60 °C for 48 h, while drying temperature
for herbarium sheets was less controlled. We therefore measured leaves of 20 species
dried at 30, 60 and 80°C and found no effect of drying temperature on leaf nutrients and
foliar isotopic compositions (data not shown). All contemporary and herbarium leaves
were entirely ground to a fine powder with a mortar and pestle before analyses. The C
and N concentrations in the plant tissues were determined on sub-samples using a
LECO EPS-2000 CNS thermal combustion furnace (LECO Corp., St Jose, MI). Total P
analysis of plants was performed as outlined in Chen et al. (1956). Briefly, 0.5 gram
ground samples were digested with heated concentrated nitric acid followed by
concentrated perchloric acid in 50 mL porcelain crucibles. Samples were then
quantitatively transferred into 100 mL volumetric flasks and diluted with distilled water.
The concentration of P \([P]\) in samples was read at 660 nm with a visible
spectrophotometer.

Isotopic analyses for C and N were conducted at the Stable Isotope Mass
Spectrometry Laboratory at Fujian Normal University with an isotope ratio mass
spectrometer (Finnigan MAT-253, Thermo Electron, San Jose, CA) coupled to an automatic, online elemental analyzer (Flash EA1112, Thermofinnigan, San Jose, CA).

Carbon stable isotopic signatures (δ^{13}C) are expressed (‰) relative to the standard of Pee Dee Belemnite (PDB). Accuracy, as determined by comparing measured values to the known value of an internal laboratory standard, was better than 0.12‰ for δ^{13}C across all samples. We also assessed δ^{15}N of the foliage as a means of qualitatively gauging exposure to N deposition, which has a ^{15}N-deplete signature in this region (Chen et al., 2011, Zhao et al., 2009). The δ^{15}N values are reported in per mil (‰), relative to atmospheric N₂ concentration. The standard deviation of 10 repeated samples was < 0.2‰ for δ^{15}N. For stable oxygen isotope (δ^{18}O) analysis, weighed leaf samples were combusted in a high temperature conversion/elemental analyzer (1440 °C, TC/EAA Finnigan 165 MAT GmbH, Berman, Germany), and analyzed for δ^{18}O by mass spectrometry (TC/EAA Finnigan 165 MAT GmbH, Berman, Germany) with a ConFlo interface. Foliar δ^{18}O analyses were conducted at the Laboratory of Stable Isotope Geochemistry, Institute of Geology and Geophysics, Chinese Academy of Sciences.

**Relationship between plant δ^{13}C and iWUE**

Foliar δ^{13}C data were used to calculate changes in C isotopic discrimination (Δ):

\[
\Delta^{13}C = \frac{\delta^{13}C_a - \delta^{13}C_p}{1 + \delta^{13}C_p/1000}
\]  

(1)

where δ^{13}C_a and δ^{13}C_p are the δ^{13}C values of air and plant tissue, respectively. Records of annual δ^{13}C_a were obtained from Antarctic ice core data (McCarroll and Loader, 2004) and from direct measurements at Mauna Loa, Hawaii (Dlugokencky and Tans, 2013). Assuming that integrated and instantaneous discrimination values can be substituted for these plant species, the ratio of the CO₂ concentration inside the leaf (c_i) to that of ambient air (c_a) can be derived from Δ^{13}C based on:

\[
\Delta^{13}C = a + (b - a) \frac{c_i}{c_a}
\]  

(2)

where a is the fraction from diffusion through stomata (4.4‰) and b is the fraction from
carboxylation by ribulose-1,5-bisphosphate carboxylase/oxygenase (27‰) (Farquhar et al., 1982). The c/Ca ratio can then be used to calculate estimated intrinsic water use efficiency ($iWUE$) of the plants:

$$iWUE = \frac{\Delta}{R} = \frac{Ca-Ci}{1.6}$$

(3)

where 1.6 is the ratio of gaseous diffusivity of CO$_2$ to water vapor (Ehleringer & Cerling, 1995). Historical values of $c_a$ were derived from McCarroll & Loader (2004) and direct measurements at Mauna Loa, Hawaii (Dlugokencky and Tans, 2013).

Statistical analysis

For leaf data collected from the three targeted forests (Guling, Houping and Taining), we tested the effect of forest site, median sampling year and site × year interactions with linear mixed models fitted by maximum likelihood with individual species as a random factor. For foliar [N], δ$^{15}$N, δ$^{13}$C, δ$^{18}$O, [P], N: P and $iWUE$, we selected the most parsimonious model from a series of competing models by first evaluating the significance of the fixed effects terms with Wald tests and likelihood-ratio tests, and then the significance of the random-effects terms and error autocorrelation using likelihood ratio tests. Finally, we compared models using Akaike’s information criteria (Schielzeth, 2010). Prior to calculating the interaction effects, interactive parameters were mean-centered to avoid problems associated with collinearity. If a significant effect of forest site, sampling year, or their interaction was observed, we used one way ANOVA and Fisher’s LSD post hoc tests to assess that effect. Non-normally distributed data were log-transformed prior to statistical analyses to satisfy assumptions of normality. We assessed potential correlations between foliar $iWUE$ and foliar [N], [P] and N:P ratios using Pearson correlation coefficients. Significance was determined using a $P$ value of 0.05; we report means and standard errors. All statistical analyses were carried out in R Version 2.11.1 (R Development Core Team 2010).

Results

Foliar [N] among samples of the various tree and shrub species varied by over 31.8 mg
g\(^{-1}\) (from 7.2 to 39.0 mg g\(^{-1}\)) across the investigated forests during the studied period. Sampling year and the interaction between sampling year and forest site significantly affected foliar [N] (Table 1). At Guling, the forest closest to a large city, mean foliar [N] significantly increased by 1.8 mg g\(^{-1}\) when comparing leaves sampled in 1953 to those sampled in 2014 \((P = 0.049)\). At Houping forest, near a mid-sized city, foliar [N] was significantly higher in 2014 than in 1978 \((P = 0.01)\) and 1955 \((P = 0.05)\). At the Taining forest, in the most rural area assessed, there was no significant difference in mean foliar [N] among the sampling years (Fig. 2).

Sampling year and the interaction between sampling year and forest site significantly affected foliar δ\(^{15}\)N (Table 1). Mean foliar δ\(^{15}\)N in 2014 was -4.3 ± 0.43‰ at Guling forest, significantly lower than in 1953 (2.1 ± 0.48‰) \((P = 0.001)\). At Houping forest, foliar δ\(^{15}\)N in 2014 was -1.2 ± 0.49‰, significantly decreased by 1.5‰ and 2.2‰ compared to those in 1978 \((P = 0.002)\) and 1955 \((P = 0.001)\), respectively. At Taining forest, foliar δ\(^{15}\)N did not vary across time, and averaged -0.50 ± 0.22‰ across all years (Fig. 2).

Foliar [P] varied between 0.27 and 1.98 mg g\(^{-1}\) in the study forests from 1947 to 2014, with an average of 0.57 ± 0.02 mg g\(^{-1}\). Approximately 86% of the leaves analyzed exhibited foliar [P] < 1 mg g\(^{-1}\) dry weight prior to large-scale N deposition (before 1980), an apparent minimum value for sufficient nutrition (Stefan, 1997). There was a significant effect of forest site on foliar [P] and a significant interaction effect between forest site and sampling year (Table 1). On average, foliar [P] was 0.61 ± 0.04, 0.58 ± 0.02 and 0.54 ± 0.02 mg g\(^{-1}\) at Guling, Houping and Taining forest, respectively. However, these differences were statistically significant only between Guling and Taining forest \((P = 0.05)\). For individual forests, significant declines of foliar [P] were observed from 1953 to 2014 at Guling forest \((P = 0.036)\) and from 1955 to 2014 at Houping forests \((P = 0.05)\), but not at Taining forest \((P = 0.143)\) (Fig. 2).

There was a significant effect of forest site on foliar N:P ratio and a significant interaction effect between forest site and sampling year (Table 1). On average, foliar N:P ratio was 34.0 ± 1.3 at Houping forest and significantly greater than Guling (29.4 ±
1.1) and Taining forest (28.7 ± 0.8). We found significant increases of mean foliar N: P ratio from 1953 (26.7 ± 1.6) to 2014 (33.6 ± 1.3) at Guling forest (P = 0.005) and from 1955 (28.6 ± 0.9) (P = 0.001) and 1978 (30.9 ± 1.4) (P = 0.003) to 2014 (39.0 ± 1.5) at Houping forest. There was lack of significant changes in foliar N: P ratio among 1962, 1983 and 2014 at Taining forest (P = 0.249) (Fig. 2).

Sampling year had significant influences on foliar δ¹³C, but the sampling year effect varied by forest site (Table 1). We found significantly more negative foliar δ¹³C values in 2014 than that in 1953 at Guling forest (-32.6‰ vs. -27.8‰, P = 0.01) and more negative foliar δ¹³C in 2014 than in 1955 and 1978 at Houping forest (-32.1‰ vs. -29.2‰ and -29.4‰, respectively) (P = 0.032 and 0.041, respectively). At Taining forest the differences in foliar δ¹³C were not statistically significant across sampling years, though the mean value of foliar δ¹³C in 2014 was more negative than in 1962 and 1983 (Fig. 3). Forest site had a significant impact on foliar δ¹⁸O (Table 1). Taining forest had more enriched foliar δ¹⁸O than Guling forest (P = 0.05, Fig.3). In the three studied forests, sampling year did not significantly affect the foliar δ¹⁸O (Table 1). The foliar δ¹³C and δ¹⁸O data suggested significant declines of foliar photosynthesis from 1953 to 2014 at Guling forest and from 1978 to 2014 at Houping forest (Fig. 3).

Sampling year, forest site and the interaction between forest site and sampling year had significant influences on foliar iWUE (Table 1). At Guling forest, iWUE was lower in 2014 than in 1953 (25.1 ± 2.0 vs. 45.6 ± 2.5, P = 0.001). A similar result was found at Houping forest, where iWUE was lower in 2014 (31.0 ± 2.1) than in 1978 (41.4 ± 2.2, P = 0.009). At Taining forest the differences in foliar iWUE were not statistically significant across sampling years (Fig. 4). For all samples collected between 1947 and 2014, we found a significantly positive correlation between foliar [P] and foliar iWUE and a significantly negative correlation between foliar N: P ratio and foliar iWUE. There was no significant relationship between foliar iWUE and N in these forests (Fig. 5).

**Discussion**
Foliar nutrient status

The foliar N:P ratios from this study, ranging from 25 to 40 on a mass basis, are higher than the averages reported in global data sets (Reich and Oleksyn, 2004) and in the data sets of tropical forests (mean N:P of 19.5) where the productivity is often regarded to be limited by P (Townsend et al., 2007). The relatively high values reported here also are consistent with prior knowledge of these systems being P limited (Huang et al., 2012, Liu et al., 2012, Zhang et al., 2008). The increases in foliar N:P observed in the two forests closest to large cities are consistent with an enhancement in P limitation associated with increasing N deposition. The story these data suggest about forest response to increasing N deposition across time is thus valuable, as it gives a hint of how P limited forests may respond; thought N deposition’s effects on multiple forest features are generally well-studied (Guerrieri et al., 2011, Jennings, 2010, Nadelhoffer et al., 1999), comparable knowledge in P limited forests is limited (Wieder et al., 2015).

Indices of foliar N status are consistent with increasing N deposition over the past six decades in forests near urban centers. Foliar [N] of specific plant species are typically stable in natural forest ecosystems unless environmental processes alter the availability or source of plant N (Pitcairn et al., 2006), and no evidence of management from these forests suggests that they are in decline or recovering from a pulse disturbance. Though we must consider the increasing age of these forests when interpreting these data, variations in forest foliar [N] with tree age are typically expressed as declines in [N] (Duquesnay et al., 2000), not the increases we observed. As such, the most parsimonious explanation of higher foliar [N] in Guling and Houping forests in 2014 compared to earlier sampling years is an increase in plant N status caused by cumulative effects of enhanced N deposition. In contrast, the lack of significant difference in foliar [N] across time at the rural Taining forest suggest that rates of N deposition there have not been sufficient to lead to a net increase in N availability in this forest over the past 50 years. The results therefore support the idea that atmospheric N deposition increases foliar [N], as has been found in a multitude of studies (Hietz et al., 2011, Liu et al., 2013). The differences in temporal changes in...
foliar N status among the three forests, in conjunction with their locations along an urban-to-rural gradient, also suggest that atmospheric N deposition in the region varies spatially with degree of urbanization in a manner consistent with atmospheric N deposition measurements conducted during 2004-2005 and 2009-2010 in the same area (Chen et al., 2011). Consistency between foliar [N] data and knowledge of varying intensity of N deposition across these sites suggest that our indices of foliar N status are meaningful, if qualitative, proxies for N deposition.

As observed for foliar [N], foliar δ^{15}N was a valuable indicator of a changing N source in these forests. Temporal constancy in biomass δ^{15}N is consistent with no change in N source, or isotopic signature of that source (McLauchlan et al., 2010). Atmospheric N deposition, including NO_x, NH_4^+ and NO_3^−, is more frequently reported to be ^{15}N depleted than ^{15}N enriched, as measured in Europe and North America (Battipaglia et al., 2010, Jung et al., 1997, Stewart et al., 1995) as well as in subtropical China near the forests studied in the current work (Chen et al., 2011, Zhao et al., 2009). Declines in δ^{15}N over time thus are consistent with known ^{15}N-depleted deposition in the region and suggest that, in two of these forests, N deposition has had a meaningful impact on N sources utilized by plants. It should be noted that soil N saturation due to atmospheric N deposition can also affect plant N isotopic signature by enhancing nitrification and N leaching (Hietz et al., 2011). However, these phenomena typically increase plant δ^{15}N values (Hietz et al., 2011) and therefore are likely not the main reasons for the changes in plant N isotopic signature observed here. That foliar δ^{15}N in 2014 is increasingly depleted with degree of urbanization further suggests that N deposition varies spatially with degree of urbanization, consistent with the foliar N concentration data.

Plant species sometimes respond to increases in the abundance of N by increasing P uptake or by increasing the capacity of P re-absorption (Fujita et al., 2010, Phoenix et al., 2004), but anthropogenic increases of N supply can also increase plant N:P ratios, drive and exacerbate P limitation (Fujita et al., 2010, Vitousek et al., 2010). Declines of foliar [P] at both Guling and Houping forests from 1950s to 2014 may be attributed to
increased N deposition and associated P dilution, as has been demonstrated in multiple N addition experiments (Aber et al., 1989, Braun et al., 2010, Högberg et al., 2006). In addition to the dilution effect (Jonard et al., 2015, Sardans et al., 2012), a plausible explanation for this phenomenon is an inhibition of mycorrhizal activity as N availability increases (Nilsson & Wallander, 2003), which in turn can limit tree P uptake (Wallander et al., 2001). Lower soil P availability with enhanced atmospheric N deposition, as has been demonstrated in some subtropical forests (Huang et al., 2012), also may have influenced the observed decreases in foliar [P]. This lower P availability may have emanated from three mechanisms. First, chemical fixation of P with aluminum in soils can result from increased soil acidification with N deposition, particularly in already-acidic soils such as these (Ren et al., 2004). Indeed, long-term records demonstrate a significant decrease of 0.8 units in soil pH in a nearby subtropical forest over the past three decades, in part due to the increased N deposition (Lu et al., 2014). Second, when soil microbes and fungi are exposed to enhanced N availability, stoichiometric theory suggests that they may invest more resources in phosphatases (Sinsabaugh et al., 2009); this, in turn, may exhaust reserves of microbially accessible P, thus exacerbating soil P-limitation over yearly or decadal timescales (Phoenix et al., 2004). Third, negative effects of deposited N on litter decomposition have been frequently observed in northern ecosystems (Knorr et al., 2005). A reduction in decomposition could slow nutrient cycling and further reduce soil P availability (Peñuelas et al., 2013). These studies, therefore, suggest that both uptake and availability of P in the soil may be impaired by decades of atmospheric N deposition in the two forests closest to large urban centers.

Inferring forest WUE responses to N deposition

We hypothesized that WUE would increase over time in all forests, but would not vary with the spatially-dependent degree of N deposition due to P limitation. However, foliar δ¹³C signatures suggest decreasing IWUE over time in forests receiving the greatest N deposition. These effects are the opposite of those observed in studies of enhanced N
availability in N-limited systems, where iWUE appears to increase (Brooks & Coulombe, 2009, Jennings, 2010). The decreases in foliar iWUE at Guling and Houping forest imply a substantial, negative effect of N pollution. The results also contradict some studies demonstrating invariant foliar iWUE with varying nutrient availability (Mitchell & Hinckley, 1993, Wong et al., 1979). It is reasonable that plants maintain stomatal conductance proportional to the photosynthetic capacity of the leaf, given that plants tend to minimize their rate of transpiration and maximize C assimilation (Cowan, 1982). However, this feature does not appear ubiquitous in all species; foliar iWUE can remain constant or near-constant in some species in response to varied soil nutrient availability and not in others (Ehleringer & Cerling, 1995). Leaf δ¹³C signatures and decreased iWUE in the current study imply that in these P-limited forests, the ratio of the CO₂ concentration inside the leaf to that of ambient atmosphere increased substantially as N deposition increased (Fig.S2).

The increasing ratio of the CO₂ concentration inside the leaf to that of ambient atmosphere appears to result from a higher rate of increase in the CO₂ concentration inside the leaf than in the CO₂ concentration of ambient atmosphere. For example, at Guling forest, estimates of mean CO₂ concentration inside the leaf suggest an increase of 120 µmol mol⁻¹, from 239 ± 3.9 µmol mol⁻¹ in 1953 to 359 ± 4.1 µmol mol⁻¹ in 2014. Concurrently, the CO₂ concentration of ambient atmosphere increased by only 87 µmol mol⁻¹, from 312 ± 5.0 µmol mol⁻¹ in 1953 to 399 ± 5.3 µmol mol⁻¹ in 2014. Elevated CO₂ concentration of ambient atmosphere can, in part, drive increasing the CO₂ concentration inside the leaf (van der Sleen et al., 2014), but the discrepancy between changes in the CO₂ concentration inside the leaf and the CO₂ concentration of ambient atmosphere suggest that these forests experienced decreased photosynthetic capacity across these decades.

The idea that forests receiving the most N deposition experiencing a decline in photosynthetic capacity is also supported by assessing foliar δ¹³C values in conjunction with foliar δ¹⁸O (Fig.3). The natural abundance of ¹⁸O in plant biomass is influenced by the δ¹⁸O signature of plant water, which varies with air temperature, vapor pressure
deficit, stomatal conductance, and water source (Barbour et al., 2000). However, because plants within the same study site are likely to use similar water sources and experience similar environmental conditions, any variation across time in plant $\delta^{18}$O values likely would reflect varying stomatal conductance (Scheidegger et al., 2000).

Though Taining forest leaves exhibited generally greater $\delta^{18}$O enrichment than other forests, we observed no shift in leaf $\delta^{18}$O over time in any forest, suggesting no temporal trend in average stomatal conductance. Based on the conceptual model of Scheidegger et al. (2000), an increase in the ratio of the CO$_2$ concentration inside the leaf to that of ambient atmosphere can result from increased stomatal conductance if photosynthetic capacity remains constant, or reduced photosynthetic capacity if stomatal conductance remains constant. If so, observed increases in the ratio of the CO$_2$ concentration inside the leaf to that of ambient atmosphere and decreases in iWUE across time likely result from decreasing photosynthetic capacity.

There are multiple, non-mutually exclusive mechanisms that could have driven a decline in photosynthetic capacity. Drought can affect plant photosynthetic capacity (Matyssek et al., 1995) but drought frequency and severity did not change during recent decades at the weather station closest to the three forests (data not shown). Forest aging may decrease photosynthetic capacity (Drake et al., 2010), but foliar samples in this study were not collected on specific trees across the years, and thus do not necessarily reflect older trees at later sampling dates. Furthermore, if forest aging is the main mechanism driving declining photosynthetic capacity in these forests, the foliar photosynthetic capacity should respond in the same way in the three forests. Therefore, we believe both drought and forest aging are implausible as potential reasons driving a decline in photosynthetic capacity.

Instead, if photosynthetic capacity declined in the two forests subjected to the most significant N deposition, this phenomenon may have resulted from a third feasible mechanism: photosynthetic down-regulation due to long-term, chronic N additions. Indeed, N saturated ecosystems because long-term additions of inorganic N can lead to increased production of secondary metabolites and significant changes in foliar N
partitioning (e.g. storing more N in the form of nitrate and amino acids), diverting excess N from carboxylation (Bauer et al., 2000). Photosynthetic capacity may also have been down-regulated due to enhanced ozone in these forests. Atmospheric deposition typically contains NOx and volatile organic C, which react in the presence of sunlight to produce tropospheric ozone (Shao et al., 2009). Ozone is a powerful oxidant that causes both visual and physiological damage to plants, impairing the ability of the plant to control processes like photosynthesis (Lombardozzi et al., 2012).

We have no direct means of assessing these forests’ patterns of N allocation to photosynthesis, nor their exposure or response to ozone. However, our data are consistent with a fifth possible mechanism driving any decline in photosynthetic capacity at the two forests near urban centers: atmospheric N deposition often leads a loss of mobile cations (especially of Mg and Ca) (Lu et al., 2014, Minocha et al., 2000) and a decrease in soil P availability (Huang et al., 2012), frequently through soil acidification. Limitations of these nutrients have been implicated as a primary cause for forest decline in Europe experiencing long-term acid deposition (Schulze, 1989). In the current study, an increase in P limitation due to continual N loadings in recent decades, reflected in declining foliar [P] and increasing N:P, may underlie any decrease in photosynthetic capacity. Several studies have reported positive relationships between foliar [P] and photosynthetic capacity and associated positive correlations between foliar [P] and WUE, mainly under conditions of P limitation (Graciano et al., 2005, Singh et al., 2000, Talbi Zribi et al., 2011). Indeed, in our study we also found a significantly negative correlation between foliar [P] and iWUE, and a significantly positive correlation between foliar N: P ratio and iWUE. Though foliar [P] and N:P explained a small fraction of foliar iWUE, the significance of these relationships implies a potential impact of decreased foliar [P] on plant photosynthetic rate and increased the CO2 concentration inside the leaf (Fig. 5).

The lack of significant changes in foliar δ18O and stomatal conductance in our study therefore rejects our second hypothesis and contrasts with the study by Medlyn et al., (2001) who performed a meta-analysis and found a significant reduction of stomatal
conductance in response to elevated CO$_2$ concentration. The observations that stomata of mature trees and trees subject to nutrient stress are generally unresponsive to elevated atmospheric CO$_2$ concentration have been invoked to explain the nonsignificant changes of stomatal conductance in response to elevated CO$_2$ (Dufrene et al., 1993; Curtis 1996). Indeed, the studied forests are regarded as mature and subject to significant P stress as indicated by foliar N:P ratio. Although not significant, the increasing tendency of foliar iWUE between 1955 and 1978 in Houping forest and between 1962 and 1983 may therefore be associated with increasing tendency of light-saturated photosynthesis resulted from elevated atmospheric CO$_2$ concentration (Ainsworth and Rogers 2007).

Many tropical forest soils are P-limited due to a high degree of soil weathering and associated low available P contents (Hedin et al., 2003). There is increasing N deposition in the tropics, and this region may see further increases in the coming decades (van der Sleen et al., 2014), further exacerbating existing P limitation. Increases in N deposition might act in concert with projected increases in the atmospheric CO$_2$ concentration to increase the growth and productive capacity of many N-limited forests (Maurer et al., 1999). Phosphorus limitations, however, can apparently hinder projected increases in growth from N fertilization (Peñuelas et al., 2013), consistent with reports suggesting that only forests with a well-balanced soil nutrient status are able to increase their growth under higher atmospheric CO$_2$ concentration (Fernández-Martínez et al., 2014, Wieder et al., 2015). However, it should be noted that in addition to increasing P limitation, the decreasing foliar iWUE resulted from increasing atmospheric N deposition may also be attributed to ozone damage (Holmes 2014), cation leaching, and soil acidification. Therefore, further studies are needed to establish the relative importance of these processes in explaining the observed declines in foliar iWUE and photosynthetic capacity.

Acknowledgments

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Table 1. Effect of forest site and sampling year on foliar nitrogen [N], nitrogen isotopic composition (δ^{15}N), phosphorus [P], nitrogen to phosphorus ratio (N: P), foliar carbon (δ^{13}C), oxygen (δ^{18}O) isotopic composition and intrinsic water use efficiency (iWUE) in subtropical forests in China. We selected the most parsimonious model from a series of competing models by first evaluating the significance of the fixed effects terms with Wald tests and likelihood-ratio tests, and then the significance of the random-effects terms and error autocorrelation using likelihood ratio tests.

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Figure legends

**Fig. 1** Collection sites (stars) within Fujian Province, China, for the herbarium specimens used in this study. The circles indicate major cities.

**Fig. 2** Comparisons of foliar N concentrations [N], foliar nitrogen isotopic composition (δ¹⁵N), foliar phosphorus concentrations [P] and foliar nitrogen to phosphorus ratio (N:P) between 1953 and 2014 at Guling forest, between 1955, 1978 and 2014 at Houping forest, and between 1962, 1983 and 2014 at Taining forest. The black and red solid lines, lower and upper edges, bars and circles in or outside the boxes represent median and mean values, 25th and 75th, 5th and 95th, and < 5th and > 95th percentiles of all data, respectively. Different letters indicate significant differences at P < 0.05 by one-way analysis of variance and Fisher's LSD post hoc tests.

**Fig. 3** Relationships between δ¹³C and δ¹⁸O in leaves sampled at Guling (2014-filled
black circles; 1953- unfilled circles), Houping (2014-filled black circles; 1978- filled grey circles; 1955- unfilled circles) and Taining (2014-filled black circles; 1983- filled grey circles; 1962- unfilled circles) forests in subtropical China. Bars are 1.0 SE. **Lower panel**: Interpretation of the foliar $\delta^{13}C - \delta^{18}O$ trends based on the conceptual model (Scheidegger et al. 2000). According to the model, variations in photosynthetic capacity ($A$) were derived from $\delta^{13}C$, while changes in stomatal conductance ($g_s$) were predicted from $\delta^{18}O$. According to the foliar $\delta^{13}C - \delta^{18}O$ trends, variations of CO$_2$ concentration in the intercellular spaces in the leaf and foliar iWUE between 1953 and 2014 at Guling forest and between 1978 and 2014 at Houping forest could be attributed to significant variations in photosynthetic capacity.

**Fig. 4** Comparisons of foliar intrinsic water use efficiency (iWUE) between 1953 and 2014 at Guling forest, between 1955, 1978 and 2014 at Houping forest, and between 1962, 1983 and 2014 at Taining forest. The black and red solid lines, lower and upper edges, bars and circles in or outside the boxes represent median and mean values, 25th and 75th, 5th and 95th, and < 5th and > 95th percentiles of all data, respectively. Different letters indicate significant differences at $P < 0.05$ by one-way analysis of variance and Fisher's LSD post hoc tests.

**Fig. 5** Correlations between foliar intrinsic water use efficiency (iWUE) and foliar N (No significant correlation), $P (r = 0.26, P < 0.01)$ and N:P ratio ($r = -0.30, P < 0.01$) with all the data across sites.