

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

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29

30 **Summary**

31 • The impact of long-term N deposition is under-studied in phosphorus (P) limited  
32 subtropical forests, where P limitation may mitigate potential physiological responses  
33 to enhanced N availability. We exploit historically collected herbarium specimens to  
34 investigate these phenomena in three P-limited, subtropical forests representing an  
35 urban-to-rural gradient, across which N deposition likely has varied over the past six  
36 decades. We measured foliar [N] and [P] and  $\delta^{13}\text{C}$ ,  $\delta^{18}\text{O}$  and  $\delta^{15}\text{N}$  in tissue from  
37 herbarium specimens of plant species collected from 1947 to 2014.

38

39 • Foliar [N] and N:P increased, and  $\delta^{15}\text{N}$  and [P] decreased in the two forests close to  
40 urban centers. Recent studies have demonstrated that N deposition in the region is  
41  $^{15}\text{N}$ -deplete; thus, these data suggest that the increased foliar [N] and N:P, and  
42 decreased [P], may be due to atmospheric deposition and associated enhancement of P  
43 limitation.

44

45 • Estimates of intrinsic water use efficiency calculated from foliar  $\delta^{13}\text{C}$  decreased over  
46 time, which contrast with multiple studies conducted in N-limited forests. This effect  
47 may reflect decreased photosynthesis, as suggested by a conceptual model of foliar  
48  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$ .

49

50 • Our work suggests that under long-term N deposition, forests on highly P-limited soils  
51 may not support the projected changes in C stocks driven by elevated  $\text{CO}_2$   
52 concentration due to increased P limitation.

53

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55 **Keywords:**  $\delta^{13}\text{C}$ , herbarium specimens,  $\delta^{15}\text{N}$ , N:P, P-limited soils

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59 **Introduction**

60 Rising atmospheric CO<sub>2</sub> concentrations and enhanced mobilization of reactive nitrogen  
61 (N) have occurred concurrently around the globe for decades (Cui *et al.*, 2013).  
62 Increases in the availability of these resources can result in stoichiometric imbalances  
63 in ecosystem biomass pools on a global scale (Peñuelas *et al.*, 2013), in part because  
64 availability of phosphorus (P) has not increased concurrently. Ecosystem-scale  
65 implications of changes in foliar nutrient content have been explored for decades  
66 (Comins & McMurtrie, 1993, Jonard *et al.*, 2012, Nadelhoffer *et al.*, 1999, Reich *et al.*,  
67 2001), but it remains unclear how increasing C and N availability may influence  
68 terrestrial ecosystem functioning. The ratio of plant productivity to water loss, water  
69 use efficiency (WUE), can increase with atmospheric CO<sub>2</sub> concentration (Keenan *et al.*,  
70 2013) and appears to be occurring at broad spatial scales (van der Sleen *et al.*, 2014).  
71 Because this phenomenon reflects a plant's ability to experience similar diffusion of  
72 CO<sub>2</sub> into the leaf with relatively smaller stomatal aperture as atmospheric CO<sub>2</sub>  
73 concentration increases (CO<sub>2</sub> fertilization effect) (Field *et al.*, 1995), it likely can be  
74 manifest regardless of nutrient limitation, as long as that limitation does not completely  
75 inhibit photosynthesis (De Kauwe *et al.*, 2013, Long *et al.*, 2004). Increased N  
76 deposition also can have a positive influence on WUE even if stomatal conductance  
77 remains constant, via increases in foliar [N] and photosynthetic capacity in N-limited  
78 ecosystems (e.g temperate forests) (Evans, 1989, Guerrieri *et al.*, 2011). However,  
79 several studies report adverse effects of atmospheric N deposition on plant growth and  
80 foliar C gain in forest ecosystems (Aber *et al.*, 1995, Magill *et al.*, 2000), which  
81 suggests that the effect of increased N deposition on WUE in forest ecosystems is less  
82 clear than that of elevated atmospheric CO<sub>2</sub> concentration.

83 Most work evaluating these ideas in specific systems has been performed in  
84 relatively short-term, controlled experiments (Yan *et al.*, 2014), because it is difficult to  
85 assess how decades of increased N availability can influence ecosystem function across  
86 wide regions (Magnani *et al.*, 2007). These problems of spatial and temporal scale can  
87 be addressed in part, however, by exploiting biomass samples formed at distinct time

88 periods such as tree-rings, or foliar samples collected over time and curated in  
89 herbariums (Hietz *et al.*, 2011, McLauchlan *et al.*, 2010, Peñuelas & Matamala, 1990,  
90 Stewart *et al.*, 2002). Though we cannot observe plant functioning directly using such  
91 material, we can invoke the chemical composition of biomass, including its stable  
92 isotopic signatures, to infer how environmental conditions have governed past plant  
93 functioning (Peñuelas & Azcón-Bieto, 1992). For example, foliar  $\delta^{13}\text{C}$  depends mainly  
94 on the  $\delta^{13}\text{C}$  of atmospheric  $\text{CO}_2$  and mole fraction of ambient  $\text{CO}_2$ , at the sites of  
95 carboxylation, and in the intercellular spaces in the leaf. These values, in turn, depend  
96 on photosynthetic rate and conductance of different leaf components (Warren & Adams,  
97 2006). Consequently, foliar  $\delta^{13}\text{C}$  can reveal much about the environment in which the  
98 biomass was generated. Because foliar  $\delta^{13}\text{C}$  is related to the ratio of photosynthetic rate  
99 to stomatal conductance for water vapour (Farquhar *et al.*, 1982), inferences about  
100 intrinsic WUE (iWUE), defined as the rate of C assimilation divided by stomatal  
101 conductance (Helle & Schleser, 2004), can emerge from foliar  $\delta^{13}\text{C}$  and atmospheric  
102  $\text{CO}_2$  concentration. In turn, vegetation WUE can be estimated from inferred iWUE  
103 when the gradient in water-vapour pressure between the leaf and atmosphere remains  
104 constant (Lambers *et al.*, 2008). This approach has enabled inferences of increasing  
105 WUE over multiple decades in the 20<sup>th</sup> century, in diverse systems such as a Sitka  
106 spruce plantation in Scotland (Guerrieri *et al.*, 2011), pine stands in the Iberian  
107 Peninsula (Andreu-Hayles *et al.*, 2011), beech stands in Catalonia (Peñuelas *et al.*,  
108 2008), and North American conifers (Marshall & Monserud, 1996) and oaks (Haavik *et*  
109 *al.*, 2008).

110 In spite of these and related works, the degree to which increasing N availability  
111 may influence iWUE remains unclear, and this is particularly true for systems in which  
112 a nutrient other than N limits ecosystem productivity. Particularly little is known about  
113 the WUE response to enhanced N availability in P-limited ecosystems, where any  
114 effect may be muted or undetectable. Indeed, Liebig's law of the minimum (Chapin,  
115 1980) suggests that in systems already limited by P availability, we might expect that  
116 enhanced mobilization and availability of reactive N may have little effect on plant

117 functioning (Braun *et al.*, 2010, Morecroft *et al.*, 1994), and though the identity of a  
118 limiting resource may change over time, emerging ideas about co-limitation suggest  
119 that limitations on ecosystem productivity likely result from a single resource at a time  
120 (Farrior *et al.*, 2013).

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

145

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

146 **Materials and methods**

147 *Study area and collection of herbarium specimens and plant samples*

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

155 Plant specimens were selected from three forests that were typically composed of  
156 subtropical, evergreen broad-leaved species. The three forests, Guling, Houping, and  
157 Taining, are considered old-growth (the largest trees are >200 years old), and represent  
158 an urban-to-rural gradient (Fig. 1 and Fig.S1) across which the extent of pollution and  
159 N deposition vary: Guling forest is 10 km away from Fuzhou, a city with a population  
160 of 7 million, Houping forest is 15 km from Nanping, a city with a population of 500,000,  
161 and Taining forest is located in a rural area and received less N deposition in the past  
162 decades compared to Guling and Houping forests (Chen *et al.*, 2011, Zheng *et al.*,  
163 2012). To our knowledge, neither fertilizers nor any management practices have been  
164 applied in the selected forests in the past hundred years; indeed, these forests are  
165 considered as “Fengshui” forests at the local status and are protected by the indigenous  
166 residents under traditional Chinese geomancy beliefs (Bruun, 2008).

167 Leaves collected from the Guling forest between 1947 and 1956 (median 1953)  
168 were obtained from 30 herbarium sheets representing 16 tree species and 14 understory  
169 species at the Fujian Agricultural and Forestry University (FAFU) Herbarium, with one  
170 herbarium sheet per species (Table S1). Leaves collected from the Houping forest  
171 between 1951 and 1958 (median 1955), and between June 1975 and July 1983 (median  
172 June 1978) were obtained from 120 herbarium sheets representing 38 tree species and  
173 22 understory species in the FAFU Herbarium, with one herbarium sheet per species  
174 per sampling year (Table S2). Leaves collected from the Taining forest in 1962 and

175 1983 were obtained from 52 herbarium sheets representing 26 plant species at the  
176 FAFU Herbarium, with one herbarium sheet per species per sampling year (Table S3).

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

187

#### 188 *Determination of C, N, and P concentrations and stable isotope analysis*

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

202 Isotopic analyses for C and N were conducted at the Stable Isotope Mass  
203 Spectrometry Laboratory at Fujian Normal University with an isotope ratio mass

204 spectrometer (Finnigan MAT-253, Thermo Electron, San Jose, CA) coupled to an  
205 automatic, online elemental analyzer (Flash EA1112, ThermoFinnigan, San Jose, CA).  
206 Carbon stable isotopic signatures ( $\delta^{13}\text{C}$ ) are expressed (‰) relative to the standard of  
207 Pee Dee Belemnite (PDB). Accuracy, as determined by comparing measured values to  
208 the known value of an internal laboratory standard, was better than 0.12‰ for  $\delta^{13}\text{C}$   
209 across all samples. We also assessed  $\delta^{15}\text{N}$  of the foliage as a means of qualitatively  
210 gauging exposure to N deposition, which has a  $^{15}\text{N}$ -deplete signature in this region  
211 (Chen *et al.*, 2011, Zhao *et al.*, 2009). The  $\delta^{15}\text{N}$  values are reported in per mil (‰),  
212 relative to atmospheric  $\text{N}_2$  concentration. The standard deviation of 10 repeated  
213 samples was  $< 0.2\%$  for  $\delta^{15}\text{N}$ . For stable oxygen isotope ( $\delta^{18}\text{O}$ ) analysis, weighed leaf  
214 samples were combusted in a high temperature conversion/elemental analyzer (1440 °C,  
215 TC/EA Finnigan 165 MAT GmbH, Berman, Germany), and analyzed for  $\delta^{18}\text{O}$  by mass  
216 spectrometry (TC/EA Finnigan 165 MAT GmbH, Berman, Germany) with a ConFlo  
217 interface. Foliar  $\delta^{18}\text{O}$  analyses were conducted at the Laboratory of Stable Isotope  
218 Geochemistry, Institute of Geology and Geophysics, Chinese Academy of Sciences.  
219

#### 220 ***Relationship between plant $\delta^{13}\text{C}$ and $i\text{WUE}$***

221 Foliar  $\delta^{13}\text{C}$  data were used to calculate changes in C isotopic discrimination ( $\Delta$ ):

$$222 \quad \Delta^{13}\text{C} = \frac{\delta^{13}\text{C}_a - \delta^{13}\text{C}_p}{1 + \frac{\delta^{13}\text{C}_p}{1000}} \quad (1)$$

223 where  $\delta^{13}\text{C}_a$  and  $\delta^{13}\text{C}_p$  are the  $\delta^{13}\text{C}$  values of air and plant tissue, respectively. Records  
224 of annual  $\delta^{13}\text{C}_a$  were obtained from Antarctic ice core data (McCarroll and Loader,  
225 2004) and from direct measurements at Mauna Loa, Hawaii (Dlugokencky and Tans,  
226 2013). Assuming that integrated and instantaneous discrimination values can be  
227 substituted for these plant species, the ratio of the  $\text{CO}_2$  concentration inside the leaf ( $c_i$ )  
228 to that of ambient air ( $c_a$ ) can be derived from  $\Delta^{13}\text{C}$  based on:

$$229 \quad \Delta^{13}\text{C} = a + (b - a) \frac{c_i}{c_a} \quad (2)$$

230 where  $a$  is the fraction from diffusion through stomata (4.4‰) and  $b$  is the fraction from



231 carboxylation by ribulose-1,5-bisphosphate carboxylase/oxygenase (27%) (Farquhar  
232 *et al.*, 1982). The  $c_i/c_a$  ratio can then be used to calculate estimated intrinsic water use  
233 efficiency (iWUE) of the plants:

$$234 \text{ iWUE} = \frac{A}{g} = \frac{c_a - c_i}{1.6} \quad (3)$$

235 where 1.6 is the ratio of gaseous diffusivity of CO<sub>2</sub> to water vapor (Ehleringer &  
236 Cerling, 1995). Historical values of  $c_a$  were derived from McCarroll & Loader (2004)  
237 and direct measurements at Mauna Loa, Hawaii (Dlugokencky and Tans, 2013).

238

### 239 *Statistical analysis*

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

257

### 258 **Results**

259 Foliar [N] among samples of the various tree and shrub species varied by over 31.8 mg

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

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

275 Foliar [P] varied between 0.27 and 1.98  $\text{mg g}^{-1}$  in the study forests from 1947 to  
276 2014, with an average of  $0.57 \pm 0.02 \text{ mg g}^{-1}$ . Approximately 86% of the leaves analyzed  
277 exhibited foliar [P]  $< 1 \text{ mg g}^{-1}$  dry weight prior to large-scale N deposition (before  
278 1980), an apparent minimum value for sufficient nutrition (Stefan, 1997). There was a  
279 significant effect of forest site on foliar [P] and a significant interaction effect between  
280 forest site and sampling year (Table 1). On average, foliar [P] was  $0.61 \pm 0.04$ ,  $0.58 \pm$   
281  $0.02$  and  $0.54 \pm 0.02 \text{ mg g}^{-1}$  at Guling, Houping and Taining forest, respectively.  
282 However, these differences were statistically significant only between Guling and  
283 Taining forest ( $P = 0.05$ ). For individual forests, significant declines of foliar [P] were  
284 observed from 1953 to 2014 at Guling forest ( $P = 0.036$ ) and from 1955 to 2014 at  
285 Houping forests ( $P = 0.05$ ), but not at Taining forest ( $P = 0.143$ ) (Fig. 2).

286 There was a significant effect of forest site on foliar N:P ratio and a significant  
287 interaction effect between forest site and sampling year (Table 1). On average, foliar  
288 N:P ratio was  $34.0 \pm 1.3$  at Houping forest and significantly greater than Guling ( $29.4 \pm$

289 1.1) and Taining forest ( $28.7 \pm 0.8$ ). We found significant increases of mean foliar N: P  
290 ratio from 1953 ( $26.7 \pm 1.6$ ) to 2014 ( $33.6 \pm 1.3$ ) at Guling forest ( $P = 0.005$ ) and from  
291 1955 ( $28.6 \pm 0.9$ ) ( $P = 0.001$ ) and 1978 ( $30.9 \pm 1.4$ ) ( $P = 0.003$ ) to 2014 ( $39.0 \pm 1.5$ ) at  
292 Houping forest. There was lack of significant changes in foliar N: P ratio among 1962,  
293 1983 and 2014 at Taining forest ( $P = 0.249$ ) (Fig. 2).

294 Sampling year had significant influences on foliar  $\delta^{13}\text{C}$ , but the sampling year  
295 effect varied by forest site (Table 1). We found significantly more negative foliar  $\delta^{13}\text{C}$   
296 values in 2014 than that in 1953 at Guling forest ( $-32.6\text{‰}$  vs.  $-27.8\text{‰}$ ,  $P = 0.01$ ) and  
297 more negative foliar  $\delta^{13}\text{C}$  in 2014 than in 1955 and 1978 at Houping forest ( $-32.1\text{‰}$  vs.  
298  $-29.2\text{‰}$  and  $-29.4\text{‰}$ , respectively) ( $P = 0.032$  and  $0.041$ , respectively). At Taining  
299 forest the differences in foliar  $\delta^{13}\text{C}$  were not statistically significant across sampling  
300 years, though the mean value of foliar  $\delta^{13}\text{C}$  in 2014 was more negative than in 1962 and  
301 1983 (Fig. 3). Forest site had a significant impact on foliar  $\delta^{18}\text{O}$  (Table 1). Taining  
302 forest had more enriched foliar  $\delta^{18}\text{O}$  than Guling forest ( $P = 0.05$ , Fig.3). In the three  
303 studied forests, sampling year did not significantly affect the foliar  $\delta^{18}\text{O}$  (Table 1). The  
304 foliar  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  data suggested significant declines of foliar photosynthesis from  
305 1953 to 2014 at Guling forest and from 1978 to 2014 at Houping forest (Fig. 3).

306 Sampling year, forest site and the interaction between forest site and sampling  
307 year had significant influences on foliar iWUE (Table 1). At Guling forest, iWUE was  
308 lower in 2014 than in 1953 ( $25.1 \pm 2.0$  vs.  $45.6 \pm 2.5$ ,  $P = 0.001$ ). A similar result was  
309 found at Houping forest, where iWUE was lower in 2014 ( $31.0 \pm 2.1$ ) than in 1978  
310 ( $41.4 \pm 2.2$ ,  $P = 0.009$ ). At Taining forest the differences in foliar iWUE were not  
311 statistically significant across sampling years (Fig. 4). For all samples collected  
312 between 1947 and 2014, we found a significantly positive correlation between foliar [P]  
313 and foliar iWUE and a significantly negative correlation between foliar N: P ratio and  
314 foliar iWUE. There was no significant relationship between foliar iWUE and N in these  
315 forests (Fig. 5).

316

317 **Discussion**

318 *Foliar nutrient status*

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

331 Indices of foliar N status are consistent with increasing N deposition over the past  
332 six decades in forests near urban centers. Foliar [N] of specific plant species are  
333 typically stable in natural forest ecosystems unless environmental processes alter the  
334 availability or source of plant N (Pitcairn *et al.*, 2006), and no evidence of management  
335 from these forests suggests that they are in decline or recovering from a pulse  
336 disturbance. Though we must consider the increasing age of these forests when  
337 interpreting these data, variations in forest foliar [N] with tree age are typically  
338 expressed as declines in [N] (Duquesnay *et al.*, 2000), not the increases we observed.  
339 As such, the most parsimonious explanation of higher foliar [N] in Guling and Houping  
340 forests in 2014 compared to earlier sampling years is an increase in plant N status  
341 caused by cumulative effects of enhanced N deposition. In contrast, the lack of  
342 significant difference in foliar [N] across time at the rural Taining forest suggest that  
343 rates of N deposition there have not been sufficient to lead to a net increase in N  
344 availability in this forest over the past 50 years. The results therefore support the idea  
345 that atmospheric N deposition increases foliar [N], as has been found in a multitude of  
346 studies (Hietz *et al.*, 2011, Liu *et al.*, 2013). The differences in temporal changes in

347 foliar N status among the three forests, in conjunction with their locations along an  
348 urban-to-rural gradient, also suggest that atmospheric N deposition in the region varies  
349 spatially with degree of urbanization in a manner consistent with atmospheric N  
350 deposition measurements conducted during 2004-2005 and 2009-2010 in the same area  
351 (Chen *et al.*, 2011). Consistency between foliar [N] data and knowledge of varying  
352 intensity of N deposition across these sites suggest that our indices of foliar N status are  
353 meaningful, if qualitative, proxies for N deposition.

354 As observed for foliar [N], foliar  $\delta^{15}\text{N}$  was a valuable indicator of a changing N  
355 source in these forests. Temporal constancy in biomass  $\delta^{15}\text{N}$  is consistent with no  
356 change in N source, or isotopic signature of that source (McLauchlan *et al.*, 2010).  
357 Atmospheric N deposition, including  $\text{NO}_x$ ,  $\text{NH}_4^+$  and  $\text{NO}_3^-$ , is more frequently reported  
358 to be  $^{15}\text{N}$  depleted than  $^{15}\text{N}$  enriched, as measured in Europe and North America  
359 (Battipaglia *et al.*, 2010, Jung *et al.*, 1997, Stewart *et al.*, 1995) as well as in subtropical  
360 China near the forests studied in the current work (Chen *et al.*, 2011, Zhao *et al.*, 2009).  
361 Declines in  $\delta^{15}\text{N}$  over time thus are consistent with known  $^{15}\text{N}$ -depleted deposition in  
362 the region and suggest that, in two of these forests, N deposition has had a meaningful  
363 impact on N sources utilized by plants. It should be noted that soil N saturation due to  
364 atmospheric N deposition can also affect plant N isotopic signature by enhancing  
365 nitrification and N leaching (Hietz *et al.*, 2011). However, these phenomena typically  
366 increase plant  $\delta^{15}\text{N}$  values (Hietz *et al.*, 2011) and therefore are likely not the main  
367 reasons for the changes in plant N isotopic signature observed here. That foliar  $\delta^{15}\text{N}$  in  
368 2014 is increasingly depleted with degree of urbanization further suggests that N  
369 deposition varies spatially with degree of urbanization, consistent with the foliar N  
370 concentration data.

371 Plant species sometimes respond to increases in the abundance of N by increasing  
372 P uptake or by increasing the capacity of P re-absorption (Fujita *et al.*, 2010, Phoenix *et al.*,  
373 2004), but anthropogenic increases of N supply can also increase plant N:P ratios,  
374 drive and exacerbate P limitation (Fujita *et al.*, 2010, Vitousek *et al.*, 2010). Declines of  
375 foliar [P] at both Guling and Houping forests from 1950s to 2014 may be attributed to

376 increased N deposition and associated P dilution, as has been demonstrated in multiple  
377 N addition experiments (Aber *et al.*, 1989, Braun *et al.*, 2010, Högberg *et al.*, 2006). In  
378 addition to the dilution effect (Jonard *et al.*, 2015, Sardans *et al.*, 2012), a plausible  
379 explanation for this phenomenon is an inhibition of mycorrhizal activity as N  
380 availability increases (Nilsson & Wallander, 2003), which in turn can limit tree P  
381 uptake (Wallander *et al.*, 2001). Lower soil P availability with enhanced atmospheric N  
382 deposition, as has been demonstrated in some subtropical forests (Huang *et al.*, 2012),  
383 also may have influenced the observed decreases in foliar [P]. This lower P availability  
384 may have emanated from three mechanisms. First, chemical fixation of P with  
385 aluminum in soils can result from increased soil acidification with N deposition,  
386 particularly in already-acidic soils such as these (Ren *et al.*, 2004). Indeed, long-term  
387 records demonstrate a significant decrease of 0.8 units in soil pH in a nearby subtropical  
388 forest over the past three decades, in part due to the increased N deposition (Lu *et al.*,  
389 2014). Second, when soil microbes and fungi are exposed to enhanced N availability,  
390 stoichiometric theory suggests that they may invest more resources in phosphatases  
391 (Sinsabaugh *et al.*, 2009); this, in turn, may exhaust reserves of microbially accessible P,  
392 thus exacerbating soil P-limitation over yearly or decadal timescales (Phoenix *et al.*,  
393 2004). Third, negative effects of deposited N on litter decomposition have been  
394 frequently observed in northern ecosystems (Knorr *et al.*, 2005). A reduction in  
395 decomposition could slow nutrient cycling and further reduce soil P availability  
396 (Peñuelas *et al.*, 2013). These studies, therefore, suggest that both uptake and  
397 availability of P in the soil may be impaired by decades of atmospheric N deposition in  
398 the two forests closest to large urban centers.

399

#### 400 *Inferring forest WUE responses to N deposition*

401 We hypothesized that WUE would increase over time in all forests, but would not vary  
402 with the spatially-dependent degree of N deposition due to P limitation. However, foliar  
403  $\delta^{13}\text{C}$  signatures suggest decreasing iWUE over time in forests receiving the greatest N  
404 deposition. These effects are the opposite of those observed in studies of enhanced N

405 availability in N-limited systems, where iWUE appears to increase (Brooks &  
406 Coulombe, 2009, Jennings, 2010). The decreases in foliar iWUE at Guling and  
407 Houping forest imply a substantial, negative effect of N pollution. The results also  
408 contradict some studies demonstrating invariant foliar iWUE with varying nutrient  
409 availability (Mitchell & Hinckley, 1993, Wong *et al.*, 1979). It is reasonable that plants  
410 maintain stomatal conductance proportional to the photosynthetic capacity of the leaf,  
411 given that plants tend to minimize their rate of transpiration and maximize C  
412 assimilation (Cowan, 1982). However, this feature does not appear ubiquitous in all  
413 species; foliar iWUE can remain constant or near-constant in some species in response  
414 to varied soil nutrient availability and not in others (Ehleringer & Cerling, 1995). Leaf  
415  $\delta^{13}\text{C}$  signatures and decreased iWUE in the current study imply that in these P-limited  
416 forests, the ratio of the  $\text{CO}_2$  concentration inside the leaf to that of ambient atmosphere  
417 increased substantially as N deposition increased (Fig.S2).

418 The increasing ratio of the  $\text{CO}_2$  concentration inside the leaf to that of ambient  
419 atmosphere appears to result from a higher rate of increase in the  $\text{CO}_2$  concentration  
420 inside the leaf than in the  $\text{CO}_2$  concentration of ambient atmosphere. For example, at  
421 Guling forest, estimates of mean  $\text{CO}_2$  concentration inside the leaf suggest an increase  
422 of  $120 \mu\text{mol mol}^{-1}$ , from  $239 \pm 3.9 \mu\text{mol mol}^{-1}$  in 1953 to  $359 \pm 4.1 \mu\text{mol mol}^{-1}$  in 2014.  
423 Concurrently, the  $\text{CO}_2$  concentration of ambient atmosphere increased by only  $87 \mu\text{mol}$   
424  $\text{mol}^{-1}$ , from  $312 \pm 5.0 \mu\text{mol mol}^{-1}$  in 1953 to  $399 \pm 5.3 \mu\text{mol mol}^{-1}$  in 2014. Elevated  
425  $\text{CO}_2$  concentration of ambient atmosphere can, in part, drive increasing the  $\text{CO}_2$   
426 concentration inside the leaf (van der Sleen *et al.*, 2014), but the discrepancy between  
427 changes in the  $\text{CO}_2$  concentration inside the leaf and the  $\text{CO}_2$  concentration of ambient  
428 atmosphere suggest that these forests experienced decreased photosynthetic capacity  
429 across these decades.

430 The idea that forests receiving the most N deposition experiencing a decline in  
431 photosynthetic capacity is also supported by assessing foliar  $\delta^{13}\text{C}$  values in conjunction  
432 with foliar  $\delta^{18}\text{O}$  (Fig.3). The natural abundance of  $^{18}\text{O}$  in plant biomass is influenced by  
433 the  $\delta^{18}\text{O}$  signature of plant water, which varies with air temperature, vapor pressure

434 deficit, stomatal conductance, and water source (Barbour *et al.*, 2000). However,  
435 because plants within the same study site are likely to use similar water sources and  
436 experience similar environmental conditions, any variation across time in plant  $\delta^{18}\text{O}$   
437 values likely would reflect varying stomatal conductance (Scheidegger *et al.*, 2000).  
438 Though Taining forest leaves exhibited generally greater  $\delta^{18}\text{O}$  enrichment than other  
439 forests, we observed no shift in leaf  $\delta^{18}\text{O}$  over time in any forest, suggesting no  
440 temporal trend in average stomatal conductance. Based on the conceptual model of  
441 Scheidegger *et al.* (2000), an increase in the ratio of the  $\text{CO}_2$  concentration inside the  
442 leaf to that of ambient atmosphere can result from increased stomatal conductance if  
443 photosynthetic capacity remains constant, or reduced photosynthetic capacity if  
444 stomatal conductance remains constant. If so, observed increases in the ratio of the  $\text{CO}_2$   
445 concentration inside the leaf to that of ambient atmosphere and decreases in iWUE  
446 across time likely result from decreasing photosynthetic capacity.

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

458 Instead, if photosynthetic capacity declined in the two forests subjected to the  
459 most significant N deposition, this phenomenon may have resulted from a third feasible  
460 mechanism: photosynthetic down-regulation due to long-term, chronic N additions.  
461 Indeed, N saturated ecosystems because long-term additions of inorganic N can lead to  
462 increased production of secondary metabolites and significant changes in foliar N



463 partitioning (e.g. storing more N in the form of nitrate and amino acids), diverting  
464 excess N from carboxylation (Bauer *et al.*, 2000). Photosynthetic capacity may also  
465 have been down-regulated due to enhanced ozone in these forests. Atmospheric  
466 deposition typically contains NO<sub>x</sub> and volatile organic C, which react in the presence of  
467 sunlight to produce tropospheric ozone (Shao *et al.*, 2009). Ozone is a powerful oxidant  
468 that causes both visual and physiological damage to plants, impairing the ability of the  
469 plant to control processes like photosynthesis (Lombardozi *et al.*, 2012).

470 We have no direct means of assessing these forests' patterns of N allocation to  
471 photosynthesis, nor their exposure or response to ozone. However, our data are  
472 consistent with a fifth possible mechanism driving any decline in photosynthetic  
473 capacity at the two forests near urban centers: atmospheric N deposition often leads a  
474 loss of mobile cations (especially of Mg and Ca) (Lu *et al.*, 2014, Minocha *et al.*, 2000)  
475 and a decrease in soil P availability (Huang *et al.*, 2012), frequently through soil  
476 acidification. Limitations of these nutrients have been implicated as a primary cause for  
477 forest decline in Europe experiencing long-term acid deposition (Schulze, 1989). In the  
478 current study, an increase in P limitation due to continual N loadings in recent decades,  
479 reflected in declining foliar [P] and increasing N:P, may underlie any decrease in  
480 photosynthetic capacity. Several studies have reported positive relationships between  
481 foliar [P] and photosynthetic capacity and associated positive correlations between  
482 foliar [P] and WUE, mainly under conditions of P limitation (Graciano *et al.*, 2005,  
483 Singh *et al.*, 2000, Talbi Zribi *et al.*, 2011). Indeed, in our study we also found a  
484 significantly negative correlation between foliar [P] and iWUE, and a significantly  
485 positive correlation between foliar N: P ratio and iWUE. Though foliar [P] and N:P  
486 explained a small fraction of foliar iWUE, the significance of these relationships  
487 implies a potential impact of decreased foliar [P] on plant photosynthetic rate and  
488 increased the CO<sub>2</sub> concentration inside the leaf (Fig. 5).

489 The lack of significant changes in foliar δ<sup>18</sup>O and stomatal conductance in our  
490 study therefore rejects our second hypothesis and contrasts with the study by Medlyn *et*  
491 *al.*, (2001) who performed a meta-analysis and found a significant reduction of stomatal

492 conductance in response to elevated CO<sub>2</sub> concentration. The observations that stomata  
493 of mature trees and trees subject to nutrient stress are generally unresponsive to  
494 elevated atmospheric CO<sub>2</sub> concentration have been invoked to explain the  
495 nonsignificant changes of stomatal conductance in response to elevated CO<sub>2</sub> (Dufrene  
496 *et al.*, 1993; Curtis 1996). Indeed, the studied forests are regarded as mature and subject  
497 to significant P stress as indicated by foliar N:P ratio. Although not significant, the  
498 increasing tendency of foliar iWUE between 1955 and 1978 in Houping forest and  
499 between 1962 and 1983 may therefore be associated with increasing tendency of  
500 light-saturated photosynthesis resulted from elevated atmospheric CO<sub>2</sub> concentration  
501 (Ainsworth and Rogers 2007).

502 Many tropical forest soils are P-limited due to a high degree of soil weathering and  
503 associated low available P contents (Hedin *et al.*, 2003). There is increasing N  
504 deposition in the tropics, and this region may see further increases in the coming  
505 decades (van der Sleen *et al.*, 2014), further exacerbating existing P limitation.  
506 Increases in N deposition might act in concert with projected increases in the  
507 atmospheric CO<sub>2</sub> concentration to increase the growth and productive capacity of many  
508 N-limited forests (Maurer *et al.*, 1999). Phosphorus limitations, however, can  
509 apparently hinder projected increases in growth from N fertilization (Peñuelas *et al.*,  
510 2013), consistent with reports suggesting that only forests with a well-balanced soil  
511 nutrient status are able to increase their growth under higher atmospheric CO<sub>2</sub>  
512 concentration (Fernández-Martínez *et al.*, 2014, Wieder *et al.*, 2015). However, it  
513 should be noted that in addition to increasing P limitation, the decreasing foliar iWUE  
514 resulted from increasing atmospheric N deposition may also be attributed to ozone  
515 damage (Holmes 2014), cation leaching, and soil acidification. Therefore, further  
516 studies are needed to establish the relative importance of these processes in explaining  
517 the observed declines in foliar iWUE and photosynthetic capacity.

518

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525

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Table 1. Effect of forest site and sampling year on foliar nitrogen [N], nitrogen isotopic composition ( $\delta^{15}\text{N}$ ), phosphorus [P], nitrogen to phosphorus ratio (N: P), foliar carbon ( $\delta^{13}\text{C}$ ), oxygen ( $\delta^{18}\text{O}$ ) 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.

		AIC	LogLik	DF		
		1481	-736	296		
		Estimate	SE	t value	P	
Foliar [N]	Intercept	-117.41	63.21	-1.86	0.06	
	Forest site	30.92	32.21	0.96	0.33	
	Sampling year	0.07	0.03	2.15	0.03	
	Site $\times$ Year	-0.04	0.02	-2.02	0.04	
		AIC	LogLik	DF		
		1165	-578	296		
		Estimate	SE	t value	P	
Foliar $\delta^{15}\text{N}$	Intercept	107.71	33.5	3.20	0.001	
	Forest site	6.74	17.07	0.39	0.69	
	Sampling year	-0.05	0.02	-3.21	0.002	
	Site $\times$ Year	-0.02	0.01	-1.93	0.05	
		AIC	LogLik	DF		
		-33	21.5	296		
		Estimate	SE	t value	P	
Foliar [P]	Intercept	996.11	366.89	-2.72	0.007	
	Forest site	386.16	187.78	2.06	0.04	
	Sampling year	284.21	256.51	1.11	0.27	
	Site $\times$ Year	0.52	0.18	2.80	0.005	
		AIC	LogLik	DF		
		1809	-900	296		
		Estimate	SE	t value	P	
Foliar N:P						

	Intercept	996.13	366.90	-2.71	0.007
	Forest site	386.22	187.81	2.05	0.040
	Sampling year	284.24	256.51	1.11	0.269
	Site × Year	0.51	0.22	2.80	0.005
		AIC	LogLik	DF	
		1133	-562	244	
		Estimate	SE	t value	P
Foliar $\delta^{13}\text{C}$	Intercept	170.23	22.5	7.57	0.01
	Forest site	-42.27	11.26	-3.76	0.01
	Sampling year	-0.10	0.01	-8.90	0.01
	Site × Year	0.02	0.01	3.77	0.01
		AIC	LogLik	DF	
		936	-463	244	
		Estimate	SE	t value	P
Foliar $\delta^{18}\text{O}$	Intercept	-44.49	33.43	-1.33	0.18
	Forest site	39.21	16.73	2.34	0.02
	Sampling year	0.03	0.02	1.89	0.07
	Site × Year	-0.02	0.01	-2.28	0.12
		AIC	LogLik	DF	
		2050	-1020	296	
		Estimate	SE	t value	P
Foliar iWUE	Intercept	-592.60	122.20	-4.85	0.003
	Forest site	219.70	62.30	3.53	0.004
	Sampling year	0.31	0.11	5.14	0.001
	Site × Year	-0.11	0.00	-3.57	0.005

826 **Figure legends**

827

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

830 **Fig. 2** Comparisons of foliar N concentrations [N], foliar nitrogen isotopic  
831 composition ( $\delta^{15}\text{N}$ ), foliar phosphorus concentrations [P] and foliar nitrogen to  
832 phosphorus ratio (N:P) between 1953 and 2014 at Guling forest, between 1955, 1978  
833 and 2014 at Houping forest, and between 1962, 1983 and 2014 at Taining forest. The  
834 black and red solid lines, lower and upper edges, bars and circles in or outside the  
835 boxes represent median and mean values, 25th and 75th, 5th and 95th, and < 5th and >  
836 95th percentiles of all data, respectively. Different letters indicate significant  
837 differences at  $P < 0.05$  by one-way analysis of variance and Fisher's LSD  
838 post hoc tests.

839 **Fig. 3** Relationships between  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  in leaves sampled at Guling (2014-filled

840 black circles; 1953- unfilled circles), Houping (2014-filled black circles; 1978- filled  
841 grey circles; 1955- unfilled circles) and Taining (2014-filled black circles; 1983- filled  
842 grey circles; 1962- unfilled circles) forests in subtropical China. Bars are 1.0 SE.  
843 **Lower panel:** Interpretation of the foliar  $\delta^{13}\text{C}$  - $\delta^{18}\text{O}$  trends based on the conceptual  
844 model (Scheidegger *et al.* 2000). According to the model, variations in photosynthetic  
845 capacity (A) were derived from  $\delta^{13}\text{C}$ , while changes in stomatal conductance ( $g_s$ ) were  
846 predicted from  $\delta^{18}\text{O}$ . According to the foliar  $\delta^{13}\text{C}$  - $\delta^{18}\text{O}$  trends, variations of  $\text{CO}_2$   
847 concentration in the intercellular spaces in the leaf and foliar iWUE between 1953 and  
848 2014 at Guling forest and between 1978 and 2014 at Houping forest could be attributed  
849 to significant variations in photosynthetic capacity.

850

851 **Fig. 4** Comparisons of foliar intrinsic water use efficiency (iWUE) between 1953 and  
852 2014 at Guling forest, between 1955, 1978 and 2014 at Houping forest, and between  
853 1962, 1983 and 2014 at Taining forest. The black and red solid lines, lower and upper  
854 edges, bars and circles in or outside the boxes represent median and mean values, 25th  
855 and 75th, 5th and 95th, and < 5th and > 95th percentiles of all data, respectively.  
856 Different letters indicate significant differences at  $P < 0.05$  by one-way analysis of  
857 variance and Fisher's LSD post hoc tests.

858

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

862