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### ► To cite this version:

Wen Xu, Bo Shang, Yansen Xu, Xiangyang Yuan, Anthony J Dore, et al.. Effects of elevated ozone concentration and nitrogen addition on ammonia stomatal compensation point in a poplar clone. *Environmental Pollution*, 2018, 238, pp.760-770. 10.1016/j.envpol.2018.03.089 . hal-04353491

**HAL Id: hal-04353491**

**<https://hal.science/hal-04353491>**

Submitted on 19 Dec 2023

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1 MS Number: ENVPOL\_2018\_333\_R1

2 **Effects of elevated ozone concentration and fertilization on ammonia stomatal**  
3 **compensation point in a poplar clone**

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20 **Abstract:** The stomatal compensation point of ammonia ( $\chi_s$ ) is a key factor controlling  
21 plant-atmosphere NH<sub>3</sub> exchange, which is dependent on the nitrogen (N) supply and  
22 varies among plant species. However, knowledge gaps remain concerning the effects  
23 of elevated atmospheric N deposition and ozone (O<sub>3</sub>) on  $\chi_s$  for forest species, resulting  
24 in large uncertainties in the parameterizations of NH<sub>3</sub> incorporated into atmospheric  
25 chemistry and transport models (CTMs). Here, we present leaf-scale measurements of  
26  $\chi_s$  for hybrid poplar clone ‘546’ (*Populus deltoides* cv. 55/56 x *P. deltoides* cv. Imperial)  
27 growing in two N treatments (N0, no N added; N50, 50 kg N ha<sup>-1</sup> yr<sup>-1</sup> urea fertilizer  
28 added) and two O<sub>3</sub> treatments (CF, charcoal-filtered air; E-O<sub>3</sub>, non-filtered air plus 40  
29 ppb) for 105 days. Our results showed that  $\chi_s$  was significantly reduced by E-O<sub>3</sub> (41%)  
30 and elevated N (19%). The interaction of N and O<sub>3</sub> were significant, and N can mitigate  
31 the negative effects of O<sub>3</sub> on  $\chi_s$ . Elevated O<sub>3</sub> significantly reduced the light-saturated  
32 photosynthetic rate ( $A_{sat}$ ) and chlorophyll (Chl) content and significantly increased  
33 intercellular CO<sub>2</sub> concentrations ( $C_i$ ), but had no significant effect on stomatal  
34 conductance ( $g_s$ ). By contrast, elevated N did not significantly affect all measured  
35 photosynthetic parameters. Overall,  $\chi_s$  was significantly and positively correlated with  
36  $A_{sat}$ ,  $g_s$  and Chl, whereas a significant and negative relationship was observed between  
37  $\chi_s$  and  $C_i$ . Our results suggest that O<sub>3</sub>-induced changes in  $A_{sat}$ ,  $C_i$  and Chl may affect  $\chi_s$ .  
38 Our findings provide a scientific basis for optimizing parameterizations of  $\chi_s$  in  
39 atmospheric chemistry and transport models to respond to environmental change  
40 factors (i.e., elevated N deposition and/or O<sub>3</sub>) in the future.

41 **Keywords:** Ammonia; Ozone; Apoplast; Compensation point; Forest species

42 **Capsule:** Both elevated O<sub>3</sub> and fertilization can significantly reduced NH<sub>3</sub> stomatal  
43 compensation point ( $\chi_s$ ) of poplar clone ‘546’, and fertilization can mitigate the negative  
44 effects of O<sub>3</sub> on  $\chi_s$ .

45

## 46 **1. Introduction**

47 Atmospheric ammonia (NH<sub>3</sub>) is the primary alkaline trace gas in the atmosphere  
48 and plays a vital role in many biogeochemical and atmospheric processes (Behera et al.,  
49 2013). It neutralizes atmospheric acids to yield ammonium (NH<sub>4</sub><sup>+</sup>) aerosols,

50 which results in increased mass loadings of fine atmospheric particulate matter (PM<sub>2.5</sub>,  
51 aerodynamic diameter  $\leq 2.5$ ) (Xu et al., 2016, 2017), thereby reducing visibility and  
52 adversely impacting ecosystem and human health (Gu et al., 2014). By contrast,  
53 atmospheric deposition of reduced N (NH<sub>3</sub> and NH<sub>4</sub><sup>+</sup>) can cause soil acidification (Du  
54 et al., 2015), eutrophication (Pareman et al., 2016) and loss of biodiversity (Erisman et  
55 al., 2007) in sensitive ecosystems.

56 Plants can be either a source or a sink of atmospheric NH<sub>3</sub>, depending on the  
57 difference between atmospheric NH<sub>3</sub> concentration and the so-called canopy NH<sub>3</sub>  
58 compensation point (Massad et al., 2010). As a major component of canopy NH<sub>3</sub>  
59 compensation point, ammonia stomatal compensation point ( $\chi_s$ ) is defined as the  
60 atmospheric NH<sub>3</sub> concentration for which there is no exchange between the leaf and the  
61 atmosphere under dry conditions (Flechard et al., 2013). Theoretically,  $\chi_s$  is also the  
62 gaseous NH<sub>3</sub> concentration in the leaf sub-stomatal cavity that is in equilibrium with  
63 ammonium concentration in the apoplast (Husted and Schjoerring, 1995). It plays a  
64 vital role in controlling the magnitude and the direction of NH<sub>3</sub> exchange between the  
65 canopy and the atmosphere (Sutton et al., 1995). Specifically, if atmospheric NH<sub>3</sub>  
66 concentrations exceed  $\chi_s$  then NH<sub>3</sub> deposition from the atmosphere to vegetation will  
67 occur, while with atmospheric NH<sub>3</sub> concentrations below  $\chi_s$ , there will be a net emission  
68 of NH<sub>3</sub> by plants.  $\chi_s$  depends directly on the plant nitrogen (N) status, developmental  
69 stage, and environmental conditions (e.g., N fertilization), with larger values generally  
70 observed under conditions of high N supply to the soil-plant system and at senescence  
71 (Massad et al., 2009; Schjoerring et al., 1998).

72  $\chi_s$  can be derived from simultaneous measurement of vertical fluxes and  
73 concentrations of NH<sub>3</sub> by using micrometeorological flux techniques over large fields  
74 (Hansen et al., 2017; Nemitz et al., 2001; Personne et al., 2015), or in chambers by  
75 finding the concentration at which the total flux is zero (Hill et al., 2001; Massad et al.,  
76 2009; Wang et al., 2011). In addition, the bioassay approach has also been developed  
77 for assessing  $\chi_s$  and it is based on the determination of the leaf apoplastic NH<sub>4</sub><sup>+</sup>  
78 concentration and pH by means of apoplast extraction (Husted and Schjoerring, 1995).  
79 These two methods are complementary. Apoplast extraction is more appropriate for leaf

80 and cell scale processes whereas chamber/micrometeorological measurements tend to  
81 be more appropriate for flux measurements at an entire plant/canopy scale (Massad et  
82 al., 2009; Sutton et al., 2009).

83 Forests represent a major uncertainty in quantification of regional NH<sub>3</sub> fluxes and  
84 parameterization of bi-directional NH<sub>3</sub> exchange in atmospheric chemistry and  
85 transport models (CTMs) such as AURAMS (A Unified Regional Air-quality  
86 Modelling System, Zhang et al., 2010) and CMAQ (Community Multiscale Air-Quality  
87 Modeling System, Fu et al., 2015). This is not only due to the large land area of forests  
88 but also because of the wide range of forest types and management practices. In  
89 conditions of bi-directional NH<sub>3</sub> exchange, forests are of particular interest. For  
90 example, temperate deciduous forests are potentially a natural source of NH<sub>3</sub> (Hansen  
91 et al., 2013, 2017; Neirynek and Ceulemans, 2008), leading to impact of forests on the  
92 atmospheric NH<sub>3</sub> level. In contrast, tropical humid forest and temperate coniferous  
93 forest can acts as net NH<sub>3</sub> sinks (Bertolini et al., 2016; Duyzer et al., 2005), resulting in  
94 the impact of atmospheric NH<sub>3</sub> on the ecological functioning of forests.

95  $\chi_s$  is one of the key parameters for parameterizations of NH<sub>3</sub> incorporated into  
96 CTMs (Massad et al., 2010). Based on published data on  $\chi_s$  in relation to different plant  
97 species, growth stages, N supply etc., Massad et al. (2010) derived a new operational  
98 parameterization for integrating bi-directional NH<sub>3</sub> exchange into CTMs, However,  
99 uncertainties still exist for its parameterization, partially due to the following two  
100 drawbacks: 1) measurement of  $\chi_s$  for different ecosystems, specific to forests, is very  
101 sparse and is only considered for a single growth stage of plants; 2) the relationships  
102 established between N fertilizer application and  $\chi_s$  remain uncertain due to a lack of co-  
103 measurement of  $\chi_s$  with different organic fertilizer (manure, slurry and urea) application  
104 rates. In addition, the actual parameterization of NH<sub>3</sub> exchange models requires large  
105 databases accounting for the variability of  $\chi_s$ . To our knowledge, there is only one  
106 process-based model developed by Riedo et al. (2002) for grasslands which accounts  
107 for the plants N nutrition and growth stage in calculating  $\chi_s$ . However, as  $\chi_s$  is not only  
108 driven by N input to the ecosystem and plant growth stage, it may be a strongly  
109 regulated process that depends on environmental changes such as elevated ground-level

110 O<sub>3</sub>.

111 Ground-level O<sub>3</sub> can be considered as the most phytotoxic air pollutant due to  
112 visible injury to a variety of plants and the rising concentrations in different regions of  
113 the world (Cooper et al., 2014; Feng et al., 2014). It affects photosynthetic parameters  
114 (e.g., stomatal conductance ( $g_s$ ), light-saturated CO<sub>2</sub> assimilation rate ( $A_{sat}$ ),  
115 intercellular CO<sub>2</sub> concentration ( $C_i$ ) and chlorophyll (Chl) content) of forest species to  
116 a varying extent (Li et al., 2017). In contrast, atmospheric N deposition represents an  
117 important nutrient from the environment for plants (Liu et al., 2010). In N-limited  
118 ecosystems (e.g., forest) N deposition might enhance photosynthetic activity (i.e.  
119 photosynthetic enzyme activity) and net primary productivity (N fertilization effect)  
120 (Liu et al., 2011). In the context of an N-saturation ecosystem, however, N deposition  
121 may render plants more susceptible to pollutants and natural environmental stressors  
122 (Cardoso-Vilhena and Barnes, 2001). Ozone and N induced changes in the growth and  
123 metabolism of plants may affect the  $\chi_s$  of plants due to a clear link between  $\chi_s$  and  
124 photosynthetic parameters. For example, Mattsone and Schjoerring (1996) showed  
125 that leaf NH<sub>3</sub> emission from *Hordeum vulgare L. cv. Golf* plants showed a consistent  
126 diurnal pattern with photosynthesis but the opposite trend with  $g_s$ . Furthermore,  
127 Schjoerring et al. (1998) found that NH<sub>3</sub> emission from leaves of *Brassica napus L.*  
128 plants increased with Chl degradation. Such results demonstrate that there are  
129 corresponding influences of those parameters on  $\chi_s$ , which positively impacts leaf NH<sub>3</sub>  
130 emission (Massad et al., 2010). In this context, understanding the effects of elevated O<sub>3</sub>  
131 and N as well as their influence on the plant physiological parameters controlling  $\chi_s$  is  
132 important for prediction of  $\chi_s$ . Unfortunately, the relevant information for different  
133 forest species is still unknown, significantly restricting the optimization of the  $\chi_s$   
134 parameter in CTM models.

135 Poplars are widespread deciduous plants in temperate and boreal forests. In China,  
136 poplar is a native species, with a cultivated area of more than 10 million ha (Yuan et al.,  
137 2016). We designed an experiment to investigate for the first time the individual effects  
138 of elevated N deposition (with controlled application of urea) and O<sub>3</sub> and their  
139 interactions on  $\chi_s$  of hybrid poplar clone '546' (*Populus deltoides cv. 55/56 x P. deltoides*

140 *cv. Imperial*). In addition, we estimated the relationships between photosynthetic  
141 parameters ( $g_s$ ,  $A_{sat}$ ,  $C_i$  and Chl) and  $\chi_s$ , and discuss how N and O<sub>3</sub>, as well as their-  
142 driven modifications in the aforementioned photosynthetic parameters, respectively  
143 affect  $\chi_s$ .

## 144 **2. Materials and methods**

### 145 *2.1. Experimental site and plant materials*

146 The study was conducted in Yanqing Field and Experimental Basin, Tangjiapu  
147 village, Yanqing District (40°29'N, 115°59'E, 500 m.a.s.l.), about 74 km northwest of  
148 Beijing city centre. When the winds come from the north or northwest, this basin is  
149 located upwind of the Beijing urban area. The site is characterized by a continental  
150 monsoon climate, with mean annual temperature of 9 °C and mean annual precipitation  
151 of 400-500 mm.

152 Rooted cuttings of hybrid poplar clone '546' (*Populusdeltoides cv. 55/56 x P.*  
153 *deltoides cv. Imperial*) were planted on 7 May 2017 and cultivated in individual 20 L  
154 circular plastic pots when they were about 31 days old. The plots were filled with local  
155 light loamy farmland soil, which was excavated at 0-10 cm depth, sieved out by a 0.3  
156 mm pore mesh and then thoroughly mixed for homogeneity. Plants with similar height  
157 (ca. 27.2 cm) and basal stem diameter (ca. 4.5 mm) were selected and pre-adapted to  
158 open-top chambers (OTCs, octagonal base, 12.5 m<sup>2</sup> of growth space and 3.0 m height,  
159 covered with toughened glass) for 10 days before O<sub>3</sub> fumigation. All seedlings were  
160 manually irrigated at 1-2 day intervals in order to keep moisture at a similar level to  
161 that in farm fields.

### 162 *2.2. O<sub>3</sub> and N treatments*

163 The experiment was conducted in six OTCs with two O<sub>3</sub> treatments: charcoal-  
164 filtered ambient air (CF), and elevated O<sub>3</sub> (E-O<sub>3</sub>, non-filtered air with targeted O<sub>3</sub>  
165 addition of 40 ppb during fumigation). Each treatment had three OTC replicates, and  
166 six potted plants were randomly distributed in each OTC. The O<sub>3</sub> fumigation was  
167 performed from 10 June to 22 September 2017. The daily fumigation time was 10 h  
168 (from 08:00 till 18:00), except on rainy days. During the fumigation period, the

169 averaged O<sub>3</sub> concentrations in CF and E-O<sub>3</sub> were 24.0 and 80.6 ppb, respectively, and  
170 AOT40 (accumulated hourly O<sub>3</sub> concentration above a threshold of 40 ppb) was 2.4  
171 and 41.6 ppm h, respectively. Ozone was generated from pure oxygen using an  
172 electrical discharge O<sub>3</sub> generator (HY003, Chuangcheng Co., Jinan, China) and mixed  
173 with ambient air using a fan. Ozone concentrations inside the OTCs were continuously  
174 monitored at approximately 10 cm above the plant canopy using two ultraviolet (UV)  
175 absorption O<sub>3</sub> analyzers (Model 49i-Thermo, Thermo Scientific, Massachusetts, USA,  
176 one for three OTCs). The analyzers were calibrated with a 49iPS calibrator (Thermo  
177 Scientific) before the experiment and once a month during the experiment.

178 In addition to the O<sub>3</sub> treatments, two N treatments were applied with three  
179 replicates: control (N0, no N added), and moderate N, (N50, 50 kg N ha<sup>-1</sup> yr<sup>-1</sup>). For N50,  
180 N additions were applied five times (13 June, 29 June, 16 July, 1 August, 17 August) to  
181 the soil with dilute urea solutions by using a 50 mL plastic bottle and the control pots  
182 received equal amounts of pure water. In total, N50 received 0.245 g N per pot (i.e.  
183 0.526 g urea per pot) throughout the experiment. This application rate (0.245 g N per  
184 pot) can be projected to a dose of 50 kg N ha<sup>-1</sup> based on the area (0.049 m<sup>2</sup>) of each pot.  
185 The concentrations of NH<sub>4</sub><sup>+</sup>-N and NO<sub>3</sub><sup>-</sup>-N in the soil of N0 treatment were 6.5 ± 2.2  
186 and 4.6 ± 1.4 mg N kg<sup>-1</sup> wet soil, respectively (unpublished data).

### 187 2.3. *Measurements of physiological parameters*

188 Measurements of gas exchange, leaf temperature and chlorophyll content were  
189 performed during two periods, i.e., at the end of July and August, 2017 (on 30 and 31  
190 July, and on 29 and 31 August, respectively). For all plants, middle leaves were selected  
191 as targeted leaves, which were 11th to 13th fully expanded leaves from the apex and  
192 comprised the main part of the leaves on each plant. A portable photosynthetic system  
193 fitting with a 6400-40 leaf chamber fluorometer (LI-6400-40, LI-COR Co., USA) was  
194 used to measure gas exchange and leaf temperature from one middle leaf between 9:00  
195 and 11:00 h. For the measurements, the photosynthetic photon flux density was set at  
196 1200 μmol m<sup>-2</sup> s<sup>-1</sup>, the CO<sub>2</sub> concentration of air entering the leaf at 400 μmol mol<sup>-1</sup> and  
197 the relative humidity at 50-60%. The measured parameters were  $A_{\text{sat}}$ ,  $g_s$ , and  $C_i$ . It

198 should be noted that the 2-h measurement duration has only a small influence on  $g_s$ ,  
199 since the influencing factors on  $g_s$  (e.g., leaf temperature, vapor pressure deficit,  
200 ambient CO<sub>2</sub> concentration, Hu et al., 2015) have a small fluctuation during the  
201 corresponding time period (data not shown), and the photosynthetic photon flux density  
202 leaf was fixed. During the entire experimental period, a total of 72 leaf samples were  
203 measured.

204 Immediately after measurements of gas exchange and leaf temperature, two leaf  
205 discs were sampled from the targeted leaf and then extracted with 2 mL 95% ethanol  
206 solution in the dark for at least 72 h at 4 °C. The Chl content in the extract was  
207 determined using the specific absorption coefficients (Lichtenthaler, 1987).

#### 208 2.4. Determination of apoplastic NH<sub>4</sub><sup>+</sup> and H<sup>+</sup> concentration

209 A slightly modified version of the vacuum infiltration technique developed by  
210 Husted and Schjoerring (1995) was employed to determine apoplastic NH<sub>4</sub><sup>+</sup> and H<sup>+</sup>  
211 concentrations. Immediately after measurements of physiological parameters, the  
212 targeted leaf was cut and washed with high-purity water (18.2 Ω), in order to avoid any  
213 contamination from air pollutants (e.g., particulate NH<sub>4</sub><sup>+</sup>). The leaf was then blotted dry  
214 with clean absorbent paper towel and the central petiole was removed. The leaves were  
215 separated into three replicates and then weighted, infiltrated with 280 mM sorbitol  
216 solution using a 60 mL plastic syringe with a series of vacuum/pressure for 5 min. The  
217 vacuum/pressure process was automatically applied with an intercellular fluid extractor  
218 (NS-AFE-1, Pulanta Co. Suzhou, China). The infiltrated leaves were quickly rinsed  
219 with high-purity water, blotted dry and re-weighted. The leaves were then rolled,  
220 inserted into tubes and centrifuged at 9000 r min<sup>-1</sup> for 10 min at 4°C to collect the  
221 apoplastic solution. Cytoplasmic contamination of the apoplast during the extraction  
222 procedure was checked by performing the extraction using a buffered solution (0.1 M  
223 Ntris[hydroxymethyl]methyl-2-aminoethanesulphonic acid, 2 mM dithiothreitol and  
224 0.2 mM EDTA), and comparing the activity of Malate Dehydrogenase (MDH) in the  
225 apoplastic extracts to its activity in bulk tissue extracts as described by Husted and  
226 Schjørring (1995). The contamination was less than  $1.2 \pm 1.1\%$  of MDH activity in

227 apoplast extract relative to bulk tissue extract. The extracted solution was then frozen  
228 and stored at -20 °C prior to chemical analysis.

229 The  $\text{NH}_4^+$  concentrations in the apoplastic extracts were measured with an AA3  
230 continuous-flow analyzer (BranCLuebbe GmbH, Norderstedt, Germany). The  
231 detection limit of  $\text{NH}_4^+$  was 0.01 mg N  $\text{L}^{-1}$ . The pH of the extracted solution was  
232 measured with an InLab micro electrode (Mettler Toledo, Udorf, Switzerland). The  
233 dilution of the apoplastic solution was determined spectrophotometrically at  
234 wavelength 492 nm for the sorbitol, which allowed the calculation of a dilution factor  
235 (Hove et al., 2002). The concentration of apoplastic  $\text{NH}_4^+$  and  $\text{H}^+$  was corrected for  
236 dilution during the extraction procedure by multiplication with the dilution factor ( $F_{\text{dil}}$ ).

237 The aqueous volume of the apoplast ( $V_{\text{apo}}$ , mL  $\text{g}^{-1}$  leaf fresh weight (LFW)) was  
238 estimated using the equation (Hove et al., 2002):

$$239 \quad V_{\text{apo}} = \frac{V_i(F_{\text{d,sorb}}-1)}{\text{LFW}} \quad (1)$$

240 where  $V_i$  is the infiltration volume which was calculated by assuming a leaf density  
241 of 1  $\text{g cm}^{-3}$  the difference in weight before and after infiltration,  $F_{\text{d,sorb}}$  is the dilution  
242 factor of sorbitol.

$$243 \quad F_{\text{dil}} = \frac{V_{\text{apo}}+V_{\text{air}}}{V_{\text{apo}}} \quad (2)$$

244 where  $V_{\text{air}}$  is the air volume inside the leaf ( $\text{cm}^3 \text{g}^{-1}$  LFW) which was measured by  
245 infiltrating leaves with low-viscosity silicone oil (10 mPa s). Based on the increase in  
246 weight and oil density (0.93  $\text{g cm}^{-3}$ ), the  $V_{\text{air}}$  was estimated to be approximately 0.16  
247 mL  $\text{g}^{-1}$  throughout the experiment.

## 248 2.5. Determination of the leaf tissue $\text{NH}_4^+$ concentration

249 The leaf segments were cut into small pieces, frozen in a ceramic mortar with  
250 liquid N (-210 °C), and quickly ground into a homogenous powder using a ceramic  
251 pestle. The weighed samples (approximately 1.0 g per sample) were put into a 5 mL  
252 centrifuge tube with 4 mL of high-purity water, followed by centrifugation at 2000 g  
253 (4°C) for 10 min (Loubet et al., 2002). The supernatant was then decanted and filtered  
254 through a syringe filter (0.45  $\mu\text{m}$ , Tengda Inc., Tianjin, China) to remove large plant

255 tissues. The filtered solution was frozen and stored at -20 °C until analysis for NH<sub>4</sub><sup>+</sup>  
256 using an AA3 continuous-flow analyzer as mentioned before.

## 257 2.6. Calculation of NH<sub>3</sub> stomatal compensation point

258 The stomatal compensation points were derived using the apoplast pH and NH<sub>4</sub><sup>+</sup>  
259 concentrations (Loubet et al., 2002) according to the equation:

$$260 \quad \chi_s = M_{\text{NH}_3} \times K_H \times K_D \times \frac{[\text{NH}_4^+]}{[\text{H}^+]} \times 10^9 \quad (3)$$

261 where  $\chi_s$  is the stomatal compensation point in  $\mu\text{g NH}_3 \text{ m}^{-3}$ ,  $M_{\text{NH}_3}$  is the molecular  
262 mass of NH<sub>3</sub> in  $\text{g mol}^{-1}$ , The ratio of apoplast NH<sub>4</sub><sup>+</sup> to apoplast H<sup>+</sup> concentration, called  
263 the emission potential (expressed as  $\Gamma_s$ ), is temperature independent and dimensionless  
264 (Massad et al., 2010).  $K_H$  is the Henry constant and  $K_D$  is the dissociation constant. The  
265 product  $K_H K_D$  depends on temperature and was calculated following the method of  
266 (Hill et al., 2001):

$$267 \quad K_H K_D = \frac{161512}{T_{\text{leaf}}} \times 10^{\frac{-4507.11}{T_{\text{leaf}}}} \quad (4)$$

268 where  $T_{\text{leaf}}$  is leaf temperature in Kelvin.

## 269 2.7. Statistical analysis

270 Data of each investigated variable from three plants per OTC were averaged and  
271 then used as the statistical unit (N=3). Prior to analysis, all data were tested for  
272 normality using the Shapiro-Wilk's W-test and for homogeneity of variance using  
273 Levene's-test to determine whether data should be transformed to satisfy statistical  
274 assumptions. A split-plot design was used, with O<sub>3</sub> as the main plot, N level as sub-plot  
275 and measurement dates as repeat-measurement. A repeated measure analysis of  
276 variance (ANOVA) with a mixed linear model was then conducted to examine the  
277 effects of O<sub>3</sub>, N, measurement dates and their interactions on physiological parameters  
278 and  $\chi_s$  as well as other parameters. Tukey's Honestly Significant Difference (HSD) test  
279 was applied to examine the significant differences. Analysis of covariance (ANCOVA)  
280 was performed to test the significance of difference in the slopes of the linear  
281 relationship between  $\chi_s$  and physiological parameters. All statistical analyses were  
282 performed in R version 3.3.3 (<http://www.r-project.org/>), with significant level set at

283  $P < 0.05$ . All the data were shown as mean  $\pm$  standard deviation (SD) of three OTC  
284 replicates.

### 285 3. Results

286 For all investigated variables, similar and non-significant responses to E-O<sub>3</sub> and  
287 N50 were found between the two measurement dates, i.e., July and August (**Figs. 1 and**  
288 **2**). Based on integrated analysis of all data from all N and O<sub>3</sub> treatments and the two  
289 measurement dates, the main results are presented below.

#### 290 3.1. Photosynthetic parameters

291 For all investigated variables ( $A_{\text{sat}}$ ,  $g_s$ ,  $C_i$  and Chl), the interactions of O<sub>3</sub> and N  
292 were not significant, but the individual effects of them were significant for most  
293 variables (**Table 1**). Relative to CF, E-O<sub>3</sub> significantly reduced  $A_{\text{sat}}$  by 55%, whereas  
294  $A_{\text{sat}}$  has a small non-significant increase (6%) in NF50 relative to N0 (**Fig. 1a, Table 1**).  
295  $A_{\text{sat}}$  significantly decreased (by 24%) in August relative to that in July, when averaged  
296 across all treatments. The effects of N and O<sub>3</sub> on  $g_s$  were both not significant, whereas  
297 measurement date significantly affected  $g_s$  (**Fig. 1b, Table 1**). Similar to  $A_{\text{sat}}$ ,  $g_s$   
298 significantly decreased by 30% in August compared with that in July.

299 Ozone significantly influenced  $C_i$  and Chl, whereas N had no significant effects  
300 on them (**Fig. 1c d, Table 1**).  $C_i$  was significantly increased by E-O<sub>3</sub> (+7%) compared  
301 with CF, and also was significantly higher (6%) in August than in July. Conversely, Chl  
302 was significantly reduced by E-O<sub>3</sub> (-30%), and significantly decreased (by 57%) in  
303 August.

#### 304 3.2. Leaf apoplastic $\text{NH}_4^+$ and pH, and leaf tissue $\text{NH}_4^+$

305 **Fig. 2a-c** shows the responses of leaf apoplastic  $\text{NH}_4^+$  concentration and pH, and  
306 leaf tissue  $\text{NH}_4^+$  concentration to E-O<sub>3</sub> and N50. The interactions between O<sub>3</sub> and N  
307 and/or measurement dates were not significant for all those three variables (**Table 1**).  
308 However, individual effects of them on apoplastic  $\text{NH}_4^+$  concentrations and pH all  
309 reached statistically significant levels. Similarly, leaf tissue  $\text{NH}_4^+$  concentration was  
310 significantly influenced by both O<sub>3</sub> and measurement dates, but not by N.

311 Averaged apoplastic  $\text{NH}_4^+$  concentration was significantly reduced (by 15%) in E-

312 O<sub>3</sub> plants compared with CF plants (**Fig. 2a, Table 1**). By contrast, the mean  
313 concentration was significantly increased (by 27%) in N50 plants compared with that  
314 in N0 plants. A significant increase (14%) of the mean occurred in August relative to  
315 July. As for apoplastic pH, two small but significant reductions (both 4%) of the mean  
316 were found in E-O<sub>3</sub> and N50 plants compared with those in CF and N50 plants,  
317 respectively (**Fig. 2c, Table 1**). Also, the mean pH was significantly lower (15%) in  
318 plants grown in August than in July.

319 In contrast to apoplastic NH<sub>4</sub><sup>+</sup>, the mean tissue NH<sub>4</sub><sup>+</sup> concentration significantly  
320 increased (by 15%) in E-O<sub>3</sub> plants relative to that in CF plants, whereas a small and  
321 non-significant difference in the mean was found between N0 and N50 plants (**Fig. 2b,**  
322 **Table 1**). The mean concentrations decreased significantly (on average by 6%) in  
323 August relative to July.

### 324 3.3. Emission potential ( $\Gamma_s$ ) and stomatal compensation point ( $\chi_s$ )

325 As presented in **Table 1**, both O<sub>3</sub> and measurement dates significantly affected  $\Gamma_s$ ,  
326 whereas N had no significant effect on it. Also, no significant interactions between O<sub>3</sub>,  
327 N and/or measurement dates were observed. E-O<sub>3</sub> treatment relative to CF significantly  
328 reduced  $\Gamma_s$  by 39% (**Fig. 2d**).  $\Gamma_s$  was significantly reduced (by 85%) in August  
329 compared with that in July.

330  $\chi_s$  was significantly lower in E-O<sub>3</sub> than in CF ( $0.29 \pm 0.29$  and  $0.48 \pm 0.45 \mu\text{g NH}_3$   
331  $\text{m}^{-3}$ ), and also in N50 than N0 ( $0.34 \pm 0.36$  and  $0.42 \pm 0.41 \mu\text{g NH}_3 \text{m}^{-3}$ ) (**Fig. 3, Table**  
332 **1**). A significant interaction of O<sub>3</sub> and N was found (**Table 1**).  $\chi_s$  was significantly lower  
333 in August than in July ( $0.07 \pm 0.05$  and  $0.70 \pm 0.29 \mu\text{g NH}_3 \text{m}^{-3}$ ) (**Fig. 3, Table 1**).

### 334 3.4. Correlations between stomatal compensation point ( $\chi_s$ ) and photosynthetic 335 parameters

336  $\chi_s$  was positively and significantly correlated with  $A_{\text{sat}}$  and  $g_s$  (**Fig. 4a, b**), whereas  
337 a negative and significant correlation between  $\chi_s$  and  $C_i$  was observed (**Fig. 4c**). The  
338 correlation between  $\chi_s$  and Chl was positive and significant in July, but was not  
339 significant in August (**Fig. 4d**).

## 340 4. Discussion

### 341 4.1. Effect of N application

342 The application rate of urea fertilizer in the present experiment ( $50 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ )  
343 is approximately 2.3 and 3 times higher than the averages of N deposition over China  
344 ( $16 \text{ kg N ha}^{-1} \text{ yr}^{-1}$  in 2008-2012 period, [Zhao et al., 2017](#)) and in China's forests ( $22 \text{ kg}$   
345  $\text{N ha}^{-1} \text{ yr}^{-1}$  in 1995-2010 period, [Du et al., 2014](#)), respectively. It is also approximately  
346 1.7-5.0 times greater than reported values during recent years in the N deposition  
347 hotspots of western Europe ( $20.0$  to  $28.1 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ , [Vet et al., 2014](#)), and North  
348 America ( $10.0$  to  $20.0 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ , [Li et al., 2016](#)). According to [Liu et al. \(2013\)](#), N  
349 deposition increased by approximately  $8 \text{ kg N ha}^{-1} \text{ yr}^{-1}$  between the 1980s and the 2000s  
350 in China. Also, total N deposition is expected to have a 5-10% increase in the year 2050  
351 relative to 2005 ([Kanakidou et al., 2016](#)). In view of the above, the level of N addition  
352 in this study is sufficient to assess the ecological effects of enhanced N deposition  
353 expected in the future. However, it should be pointed out that application of N to the  
354 soil is not able to fairly reflect the process of N deposition in the real-world, in which  
355 natural deposited N would at least partially be captured by the canopy. As reported by  
356 [Du et al. \(2014\)](#), for example, canopy captured dry deposition of total inorganic N in  
357 China's forest was estimated at  $6.9 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ .

358 Apoplastic  $\text{NH}_4^+$  concentration is one of the key factors in controlling  $\chi_s$ , which is  
359 associated with the status of leaf N and external N supply (e.g., N fertilization and  
360 atmospheric deposition) ([Herrmann et al., 2009](#); [Massad et al., 2008](#)). The apoplastic  
361  $\text{NH}_4^+$  concentrations measured for the N0 and N50 treatments (but without E-O<sub>3</sub>) were  
362  $0.10 \pm 0.01$  and  $0.13 \pm 0.01 \text{ mM}$ , respectively (**Fig. 2a**). To our knowledge published  
363 data on apoplastic  $\text{NH}_4^+$  concentrations in leaves of poplar clone 546 is unavailable,  
364 making a direct comparison with other studies impossible. However, our measured  
365 value for N0 treatments were close to that reported for *Fagus sylvatica* growing in July  
366 and August ( $0.11 \text{ mM}$ , [Wang et al., 2011](#)). Compared with agricultural crops, our  
367 measured value for N0 treatments was higher than that reported for barley ( $0.04 \text{ mM}$ ,  
368 [Mattsson et al., 1998](#)), and was close to the value of  $0.10$  measured for oilseed rape  
369 ([Massad et al., 2009](#)) both growing on N0. The value for plants grown on N50 was  
370 similar to that measured for oilseed rape ( $0.18 \text{ mM}$  with  $6 \text{ mM NO}_3^-$ , [Schjoerring et al.,](#)  
371 [2002](#)), and was lower than the value of  $1.9 \text{ mM}$  observed for barley grown on  $5 \text{ mM}$

372  $\text{NH}_4^+$  (Mattsson et al., 1998). As expected, enhanced N input (N50) significantly  
373 increased the apoplastic  $\text{NH}_4^+$  concentration (**Fig. 2, Table 1**), probably due to increased  
374 soil N availability. This result is similar to the findings of Massad et al. (2009) that the  
375  $\text{NH}_4^+$  concentrations in leaf apoplast of oilseed rape increased significantly with rising  
376 N treatments (aside from  $\text{NO}_3^-$  supply).

377 Besides apoplastic  $\text{NH}_4^+$  concentration, the pH of the apoplast may be the most  
378 important factor determining  $\chi_s$ . There is evidence that  $\text{NH}_4^+$ -fed plants have reduced  
379 apoplastic pH compared to  $\text{NO}_3^-$ -fed plants, e.g., sunflower (Hoffmann et al., 1992),  
380 soybean (Kosegarten and Englisch, 1994) and barley (Mattsson et al., 1998), probably  
381 due to a root rather than shoot assimilation of N (Pearson et al., 1998). Application of  
382 urea fertilizer in the present experiment probably enhanced soil  $\text{NH}_4^+$  level via urea  
383 hydrolysis, which further significantly increased apoplastic  $\text{NH}_4^+$  concentrations (**Fig.**  
384 **2a**) via root assimilation and the subsequent transport of  $\text{NH}_4^+$  to the foliar apoplast in  
385 the xylem (Mattson et al., 1998). This behaviour, along with  $\text{NH}_4^+$  uptake-induced  
386 acidification may offer an explanation for lower apoplastic pH (ranging from 4.6 to 6.0,  
387 **Fig. 2c**) of poplar 546 compared with those reported for most plant species (between  
388 5.0 and 6.5, Grignon and Sentenac, 1991). However, it is still a little controversial how  
389 the type on mineral N supply ( $\text{NO}_3^-$  versus  $\text{NH}_4^+$ ) affects apoplastic pH in leaves.  
390 Nitrate type mineral N ( $\text{NO}_3^-$ ) has been shown to an alkalization while  $\text{NH}_4^+$  induced  
391 an acidification (Hoffmann et al., 1992; Mengel et al., 1994). Sattelmacher. (2001)  
392 suggested that nitrate effectively leads to higher pH levels but only depending on its  
393 concentration in the xylem sap. However,  $\text{NH}_4^+$  nutrition didn't show any effect on leaf  
394 apoplast pH at low concentrations. Finnemann and Schjoerring (1999) questioned this  
395 result and show a relation between root N supply type and  $\text{NH}_4^+$  concentrations in the  
396 xylem sap and in the leaf apoplast. They explain this result by high levels of  
397 photorespiration (Kronzucker et al., 1998; Nielsen and Schjoerring, 1998). However  
398 there is a clear effect on apoplastic pH due to foliar application of  $\text{NH}_4^+$  which leads to  
399 lowed pH values (Peuke et al., 1998). On the other hand, fumigation with  
400  $\text{NH}_3$  decreases  $\text{H}^+$  activity (Hanstein et al., 1999).

401 Enhanced N input significantly increased both apoplastic  $\text{NH}_4^+$  and  $\text{H}^+$

402 concentration with a similar significant level (**Figs. 2a,c and Table 1**). Conversely,  $\chi_s$   
403 was significantly reduced (by 19%) by N50 (**Fig. 3**). These results, together with the  
404 relatively low calculated  $\chi_s$  throughout, especially in August (**Fig. 3**), suggest that  $\chi_s$   
405 appears to be pH-driven in this study. The apoplastic pH of *Phaseolus vulgaris* has been  
406 found to become alkalized during photosynthesis ([Raven and Farquhar, 1989](#)).  
407 Similarly, we found that the N load marginally significantly ( $P=0.057$ ) increased  $A_{\text{sat}}$  of  
408 poplar 546 (**Fig. 1a, Table 1**), which significantly positively correlated with apoplastic  
409 pH (**Fig. 5a**) but had no significant relationship with apoplastic  $\text{NH}_4^+$  concentration  
410 ( $R^2=0.09$ ,  $P=0.141$ ). These results provide an explanation for a significant and positive  
411 relationship observed between  $A_{\text{sat}}$  and  $\chi_s$  (**Fig. 4a**). Although such an increase in  
412 apoplastic pH occurred due to non-significant increased  $A_{\text{sat}}$  by N load, it is insufficient  
413 to offset  $\text{NH}_4^+$  uptake-induced apoplast acidification, leading to relatively low  $\chi_s$ .

#### 414 4.2. Effect of $\text{O}_3$ application

415 According to monitoring results for the 2014-2016 period in 187 Chinese cities,  
416 the mean daily 8-h  $\text{O}_3$  concentrations peak in summer reached up to  $114.30 \pm 23.78$  ppb  
417 ([Li et al., 2017](#)). Obviously, the  $\text{O}_3$  level in E- $\text{O}_3$  was within the range of current  $\text{O}_3$   
418 levels in China and was also in the range of future expected concentrations in warm and  
419 sunny areas of the world at the end of this century ([The Royal Society 2008](#); [IPCC](#)  
420 [2013](#)). The dose of  $\text{O}_3$  (41.6 ppm h in AOT40) in E- $\text{O}_3$  by far exceeded the  $\text{O}_3$  exposure  
421 limit of 5 ppm h accumulated over the growing season for forest protection ([CLRTAP,](#)  
422 [2015](#)) and 12 ppm h for poplar protection ([Hu et al., 2015](#)). As anticipated,  $\text{O}_3$ -induced  
423 injuries to poplar 546 were detected in E- $\text{O}_3$  despite N fertilization (e.g., reduction of  
424  $A_{\text{sat}}$  and Chl, **Fig. 1a, d**), thus confirming that poplar 546 is a very sensitive species to  
425  $\text{O}_3$  ([Shang et al., 2017](#)).

426 E- $\text{O}_3$  significantly increased  $C_i$  and decreased  $A_{\text{sat}}$  and Chl, respectively, but did  
427 not significantly affect  $g_s$  (**Figs. 1 and Table 1**). These results are consistent with the  
428 findings of [Shang et al. \(2017\)](#) for polar clone 546. Similarly, a previous study showed  
429 that  $\text{O}_3$  can substantially reduce  $A_{\text{sat}}$  in most plants, and also detected an uncoupled  
430 relationships between  $A_{\text{sat}}$  and  $g_s$  ([Zhang et al., 2012](#)). This can be explained by the fact  
431 that the  $\text{O}_3$ -induced reduction in  $A_{\text{sat}}$  is largely ascribed to non-stomatal factors, i.e.

432 impaired physiological activity of mesophyll cells (Akhtar et al., 2010; Feng et al.,  
433 2016). Although each investigated photosynthetic parameter was significantly  
434 influenced by O<sub>3</sub>, interactions of O<sub>3</sub> and N were not significant (Table 1). This is similar  
435 to the findings of Harmens et al. (2017) that no any interactions between O<sub>3</sub> and N  
436 regarding photosynthetic parameters, chlorophyll content, g<sub>s</sub>, N content in senesced  
437 leaves and leaf number for *Betula pendula* saplings.

438 We found that E-O<sub>3</sub> significantly reduced the calculated  $\chi_s$  (Fig. 3). This is most  
439 likely related to a decline in apoplastic pH resulting from O<sub>3</sub>-induced changes in  
440 photosynthetic parameters, i.e., decreased  $A_{\text{sat}}$  and Chl, and increased  $C_i$  (Fig. 1a,c,d,  
441 Table 1). This is because in addition to  $A_{\text{sat}}$ , both Chl and  $C_i$  correlated significantly  
442 with  $\chi_s$  and apoplastic pH (Figs. 4c,d and 5b,c). In addition, there was a large positive  
443 response of ammonia emission (from young barely) to photosynthesis (Mattsson and  
444 Schjoerring, 1996), mainly due to the fact that NH<sub>3</sub> assimilation by plants requires  
445 carbon skeletons generated from photosynthesized carbohydrates for the synthesis of  
446 amino acids (Huppe and Turpin, 1994). In this regard, a significant reduction in  $A_{\text{sat}}$  can  
447 also give rise to declines in apoplastic NH<sub>4</sub><sup>+</sup> concentration. Similar to N, ozone has a  
448 greater impact on apoplastic pH than on apoplastic NH<sub>4</sub><sup>+</sup> concentration, as  
449 demonstrated by the significant reduction of  $\Gamma_s$  by O<sub>3</sub> (Fig. 2d, Table 1); this therefore  
450 led to a significant reduction in  $\chi_s$  (Fig. 3, Table 1).

#### 451 4.3. Combined effects of N and O<sub>3</sub> application

452 The present results showed that N and O<sub>3</sub> interacted significantly in  $\chi_s$  (Table 1).  
453 Further analysis showed that  $\chi_s$  in N0\*E-O<sub>3</sub> ( $0.28 \pm 0.30 \mu\text{g NH}_3 \text{ m}^{-3}$ ) was 50% lower  
454 than that in N0\*CF ( $0.56 \pm 0.49 \mu\text{g NH}_3 \text{ m}^{-3}$ ), suggesting a negative effect of E-O<sub>3</sub> on  
455  $\chi_s$ . This, together with a 28% decrease in  $\chi_s$  when comparing  $\chi_s$  in N50\*E-O<sub>3</sub> ( $0.29 \pm$   
456  $0.31 \mu\text{g NH}_3 \text{ m}^{-3}$ ) with that in N50\*CF ( $0.40 \pm 0.44 \mu\text{g NH}_3 \text{ m}^{-3}$ ), also indicate that N  
457 application can mitigate the negative effects of O<sub>3</sub> on  $\chi_s$ .

458 Our selected poplar 546 belongs to deciduous broadleaf species. To make a  
459 preliminary assessment of whether Chinese deciduous broadleaf forests (Fig. 6a) act as  
460 a source or a sink for atmospheric NH<sub>3</sub>, we compared the calculated  $\chi_s$  with mean  
461 atmospheric NH<sub>3</sub> concentration in July (Fig. 6b) and August (Fig. 6c) during 2008-

462 2012 period. The concentrations were modeled using the GEOS-Chem model and fitted  
463 with the surface NH<sub>3</sub> measurements, see details in [Zhao et al. \(2017\)](#)). The results of  
464 the comparison show that the modeled NH<sub>3</sub> concentrations over approximately 91%  
465 and 100% of total land area exceeded the calculated  $\chi_s$  for elevated N and O<sub>3</sub> treatment  
466 in July (average 0.54  $\mu\text{g NH}_3 \text{ m}^{-3}$ ) and August (average 0.03  $\mu\text{g NH}_3 \text{ m}^{-3}$ ), respectively.  
467 It should be noted that such percentages are considered to be approximate estimates, as  
468  $\chi_s$  may vary among different forest species, and the current atmospheric NH<sub>3</sub>  
469 concentration cannot represent future NH<sub>3</sub> levels. Nevertheless, we may conclude that  
470 under the current ambient NH<sub>3</sub> concentrations in China the canopy of deciduous  
471 broadleaf forests is unlikely to be a major source of NH<sub>3</sub> emission during summertime.

472

#### 473 *4.4. Dependence on measurement date*

474  $\chi_s$  can be influenced by plant developmental stage since leaf apoplastic NH<sub>4</sub><sup>+</sup>  
475 concentration and pH varies among leaves of different ages ([Hill et al., 2002](#)). [Mattsson  
476 and Schjoerring \(2003\)](#) reported that, for ryegrass (*Lolium perenne*), both apoplastic  
477 and tissue NH<sub>4</sub><sup>+</sup> concentrations were 2-3 times higher in senescing or injured leaves  
478 (with visual symptoms of senescence) compared with green leaves. The present study  
479 shows that leaf apoplastic NH<sub>4</sub><sup>+</sup> concentrations significantly increased in August  
480 compared with those in July, whereas a significant reduction was found for leaf tissue  
481 NH<sub>4</sub><sup>+</sup> concentration (**Fig. 2a, b**). In addition, a negative relationship was observed  
482 between NH<sub>4</sub><sup>+</sup> concentrations in apoplast and in tissue (**Fig. 5d**). These results together  
483 indicated that NH<sub>4</sub><sup>+</sup> is actively transported from the leaf tissue to the apoplast. This  
484 explanation is supported by evidences from earlier studies showing that apoplastic fluid  
485 in leaves constitutes a highly dynamic NH<sub>4</sub><sup>+</sup> pool, to which NH<sub>4</sub><sup>+</sup> is constantly supplied  
486 via NH<sub>3</sub> efflux from the mesophyll cells ([Nielsen and Schjoerring, 1998](#); [Schjoerring et  
487 al. 2000](#)).

488 Regarding apoplastic pH, the values measured in August were significantly  
489 reduced compared to July, and almost all were <5 (**Fig. 2c, Table 1**), for which an  
490 explanation is the combined effect of NH<sub>4</sub><sup>+</sup> uptake-induced acidification (see Sect. 4.1)  
491 and O<sub>3</sub>-accelerated leaf senescence ([Gao et al., 2017](#)). As reported by [Mattsson and](#)

492 [Schjoerring \(2003\)](#), leaf aging from green leaves to yellow tips resulted in a pronounced  
493 decrease of pH by more than 1 unit. Thus, a significantly lower  $A_{\text{sat}}$  in August resulting  
494 from accelerated leaf senescence (**Fig. 1a, Table 1**) also contributed to lower apoplastic  
495 pH due to the existence of apoplast alkalinization during photosynthesis as mentioned  
496 earlier.

497  $\chi_s$  was significantly reduced in August compared with July (**Fig. 3**). This is likely  
498 due to the greater impact of measurement date on apoplastic pH than on apoplastic  
499  $\text{NH}_4^+$  concentration as indicated by their respective significant level (**Table 1**). The  
500 change in stomatal opening is an important control mechanism for the regulation of  
501 influxes and outfluxes of  $\text{NH}_3$  into or out of the leaves because the conductance for  
502 diffusion of  $\text{NH}_3$  is affected ([Schjoerring et al., 1998](#)). We found that  $g_s$  significantly  
503 decreased in August (**Fig. 1b, Table 1**). Also, a positive and significant relationship was  
504 observed between  $g_s$  and  $\chi_s$  (**Fig. 4b**); this is mainly caused by measurement date as  
505 both E- $\text{O}_3$  and N did not significantly affect  $g_s$ . These results together suggested that a  
506 significant reduction of  $g_s$  also contributed to a reduction in  $\chi_s$ .

#### 507 4.5. Uncertainty and recommendations

508 In the present study,  $\chi_s$  is calculated based on direct measurements of leaf  
509 apoplastic  $\text{NH}_4^+$  concentration and pH by means of extraction of the apoplastic fluid  
510 with successive vacuum infiltration/centrifugation technique ([Husted and Schjørring.,](#)  
511 [1995](#)). Although this technique has been successfully applied to several plant species in  
512 the field ([Herrmann et al., 2009](#); [Mattsson et al., 2009](#)), it is subject to uncertainties  
513 regarding potential regulation of apoplastic  $\text{NH}_4^+$  concentration and pH by the plant  
514 during the infiltration and buffering effects ([Massad et al., 2009](#)). For example, [Nielsen](#)  
515 [and Schjørring \(1998\)](#) showed that apoplastic  $\text{NH}_4^+$  concentration in *Brassica napus* L.  
516 appeared to be regulated during infiltration. However, [Hill et al. \(2001\)](#) did not detect  
517 such a homeostasis for apoplastic  $\text{NH}_4^+$  concentration in *Luzula sylvatica* (Huds.) Gaud.  
518 Following the method of [van Hove et al. \(2002\)](#),  $F_{\text{dil}}$ , calculated based on determination  
519 of  $V_{\text{air}}$  and  $V_{\text{apo}}$  (**Equ. 2**), was applied to correct apoplastic concentrations.  $V_{\text{apo}}$  values  
520 obtained in the present work varied from 0.06 to 0.23. These values fit well into the  
521 range found by other researchers for different plant species ([Van Han et al., 2001, 2002](#)).

522 The measured  $V_{\text{air}}$  ( $0.16 \text{ ml g}^{-1} \text{ LFW}$ ) was also comparable to values ( $0.21 \text{ mL g}^{-1} \text{ LFW}$ )  
523 reported for *Lolium perenne* L. (Van Han et al., 2002). These results indicate that the  
524 value for  $F_{\text{dil}}$  determined in the present study was acceptable. However, due to a lack of  
525 information regarding infiltration of poplar 546, use of  $F_{\text{dil}}$  might also result in some  
526 uncertainties in apoplastic  $\text{NH}_4^+$  concentrations and pH, and probably in  $\chi_s$  if there is a  
527 potential difference in the buffering capacity between them.

528 The vacuum infiltration/centrifugation method is also subject to some uncertainty  
529 due to the strong possibility of cytoplasmic contamination of the apoplast during the  
530 extraction procedure (Lohaus et al., 2001). We estimated the error in this method by  
531 assaying the contamination of the apoplast by MDH activity. The cytoplasmic  
532 contamination in the present work was about 1.2%.

533  $\chi_s$  obtained in the current study was calculated at the leaf scale and only for the  
534 middle leaf position. However,  $\chi_s$  may differ significantly depending on leaf position  
535 (e.g., upper, middle and lower) due to difference in the N status of the leaves. Thus, the  
536 resulting effects of enhanced N and  $\text{O}_3$  on  $\chi_s$  are not enough to represent the  
537 characteristics of the entire-plant. Furthermore, the present experiment was designed  
538 based on OTC chambers. However, OTCs have effects of their own, such as differences  
539 in microclimate (e.g., air temperature and humidity), fixed gas flow and limited space,  
540 which could over-estimate or under-estimate the effects of  $\text{O}_3$  on plants (Feng et al.,  
541 2010). Note that  $\chi_s$  is shown to be influenced by air temperature, partly by affecting the  
542 amount of  $\text{NH}_3$  dissolved in the apoplast, and partly by affecting the leaf tissue  $\text{NH}_4^+$   
543 generation (or assimilation)-associated physiological processes (Schjoerring et al.  
544 1998). To more accurately assess the effects of N,  $\text{O}_3$  and/or plant growth stage on  $\chi_s$ ,  
545 at least the two following developments are recommended in future work: 1) to employ  
546 open-air fumigations ( $\text{O}_3$ -FACE systems, Paoletti et al., 2016), and 2) to investigate  
547 vertical profile of  $\chi_s$  at plant scale

#### 548 4.6. Conclusions and Implications

549 In summary, the current study is the first time to investigate the combined effects  
550 of  $\text{O}_3$  exposure and use of urea as N load on  $\chi_s$  of forest species (i.e., poplar clone 546).  
551 Our results demonstrated that elevated  $\text{O}_3$  significant reduced  $\chi_s$ , in tandem with  $A_{\text{sat}}$ ,

552 Ci and Chl. Also, urea addition significantly reduced  $\chi_s$ , and can mitigate the negative  
553 effects of O<sub>3</sub> on  $\chi_s$ (**Fig. 3, Table 1**). Our findings filled knowledge gaps in the influence  
554 of O<sub>3</sub> on  $\chi_s$ , and provided valuable and additional information on effect of N addition  
555 on  $\chi_s$ , which is commonly expected to increase considerably with input of the different  
556 types of inorganic N fertilizer applied (Massad et al., 2010). In addition, we  
557 demonstrated a significant reduction of  $\chi_s$  in the context of O<sub>3</sub>-accelerated senescence,  
558 in contrast to the common view that  $\chi_s$  is affected by the plant's development stage  
559 and may peak at senescence (Hill et al., 2002; Mattsson and Schjoerring, 2003).  
560 Based on these findings, we propose that current parameterizations of  $\chi_s$  in chemical  
561 transport models should be optimized to partially respond to changes in environmental  
562 conditions (e.g., elevated N and/or O<sub>3</sub>). In addition,  $A_{sat}$ ,  $g_s$  and Chl significantly and  
563 positively corrected with  $\chi_s$ , whereas a significant and negative relationship was  
564 observed between Ci and  $\chi_s$  (**Fig. 5**). Understanding these physiological controls of  $\chi_s$   
565 is essential for modeling its dynamic behaviour.

566

### 567 **Acknowledgments**

568 This study was supported by the National Natural Science Foundation of China  
569 (41705130), Key Research Program of Frontier Sciences, CAS (QYZDB-SSW-  
570 DQC019), the National Key R&D Program of China (2017YFC0210106), the Hundred  
571 Talents Program, Chinese Academy of Sciences.

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837 **Figure captions**

838 Figure 1. Effects of ozone (CF, charcoal-filtered ambient air, and E-O<sub>3</sub>, elevated O<sub>3</sub>)  
839 and Nitrogen (N0, no N added, and N50, 50 kg N ha<sup>-1</sup> yr<sup>-1</sup>) on light-saturated  
840 photosynthesis ( $A_{\text{sat}}$ ), stomatal conductance ( $g_s$ ), intercellular CO<sub>2</sub> concentration ( $C_i$ )  
841 and chlorophyll (Chl) content of hybrid poplar clone ‘546’ (*Populus deltoides* cv. 55/56  
842  $\times$  *P. deltoides* cv. *Imperial*). Data shown are the mean  $\pm$  standard deviation of three-  
843 OTC measurements. The letters on top of the bars are based on the Tukey test across  
844 the two measurements, with different letters indicating significantly different from each  
845 other at  $P < 0.05$ .

846 Figure 2. Effects of ozone (CF, charcoal-filtered ambient air, and E-O<sub>3</sub>, elevated O<sub>3</sub>)  
847 and Nitrogen (N0, no N added, and N50, 50 kg N ha<sup>-1</sup> yr<sup>-1</sup>) on apoplastic NH<sub>4</sub><sup>+</sup>  
848 concentration, tissue NH<sub>4</sub><sup>+</sup> concentration, apoplastic pH and emission potential ( $\Gamma_s$ ) of  
849 hybrid poplar clone ‘546’ (*Populus deltoides* cv. 55/56  $\times$  *P. deltoides* cv. *Imperial*). Data  
850 shown are the mean  $\pm$  standard deviation of three-OTC measurements. The letters on  
851 top of the bars are based on the Tukey test across the two measurements, with different

852 letters indicating significantly different from each other at  $P < 0.05$ .

853 Figure 3. Effects of ozone (CF, charcoal-filtered ambient air, and E-O<sub>3</sub>, elevated O<sub>3</sub>)  
854 and Nitrogen (N0, no N added, and N50, 50 kg N ha<sup>-1</sup> yr<sup>-1</sup>) on the stomatal  
855 compensation point ( $\chi_s$ )

856 Figure 4. Correlation between the stomatal compensation point ( $\chi_s$ ) and light-saturated  
857 photosynthesis ( $A_{sat}$ ), stomatal conductance ( $g_s$ ), intercellular CO<sub>2</sub> concentration ( $C_i$ )  
858 and chlorophyll (Chl) content across all ozone and nitrogen treatments. Green and red  
859 dots represent charcoal-filtered ambient air (CF)\*N0 (no N added), CF\*N50 (50 kg N  
860 ha<sup>-1</sup> yr<sup>-1</sup>), elevated O<sub>3</sub> (E-O<sub>3</sub>)\*N0 and E-O<sub>3</sub>\*N50 treatment in July and August,  
861 respectively. While ANCOVA did not show significant differences in the slope of the  
862 regression lines for the relationships between the two measurement dates (i.e., July and  
863 August), one single line is shown; otherwise, two lines are shown.

864 Figure 5. Correlations between apoplastic pH and light-saturated photosynthesis ( $A_{sat}$ ),  
865 chlorophyll (Chl) content, and intercellular CO<sub>2</sub> concentration ( $C_i$ ), and correlation  
866 between apoplastic NH<sub>4</sub><sup>+</sup> concentration and leaf tissue NH<sub>4</sub><sup>+</sup> concentration across all  
867 ozone and nitrogen treatments. Green and red dots represent charcoal-filtered ambient  
868 air (CF)\*N0 (no N added), CF\*N50 (50 kg N ha<sup>-1</sup> yr<sup>-1</sup>), elevated O<sub>3</sub> (E-O<sub>3</sub>)\*N0 and E-  
869 O<sub>3</sub>\*N50 treatment in July and August, respectively. While ANCOVA did not show  
870 significant differences in the slope of the regression lines for the relationships between  
871 the two measurement dates (i.e., July and August), one single line is shown; otherwise,  
872 two lines are shown.

873 Figure 6. Actual forest distribution in China (a) (adopting from Li et al. (2017)) and  
874 atmospheric NH<sub>3</sub> concentration over deciduous broadleaf forests in July (b) and August  
875 (c) modeled using the GEOS-Chem model.

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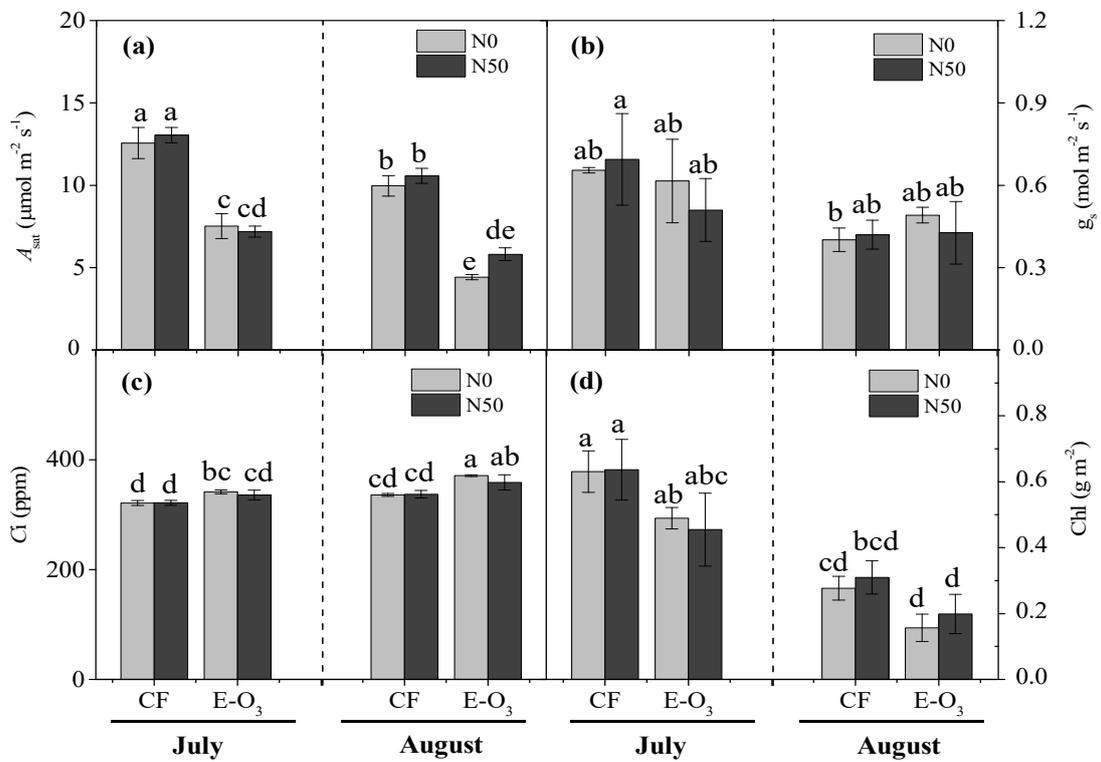
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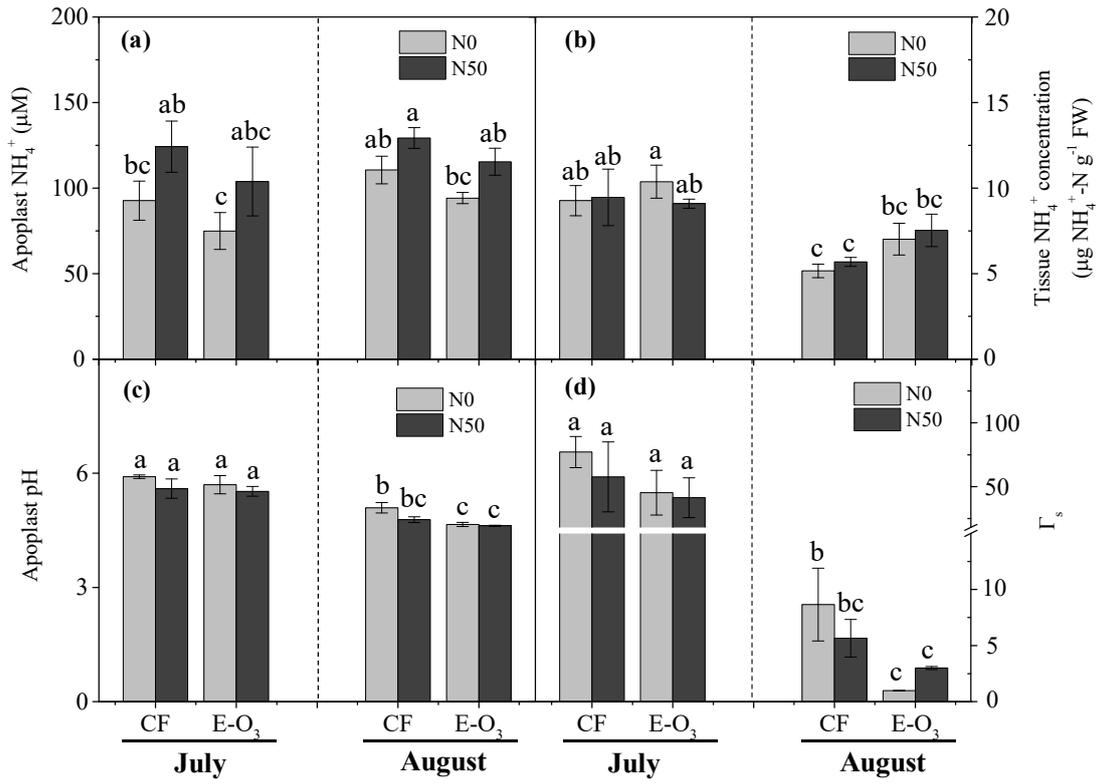
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 897 **Figure 1**

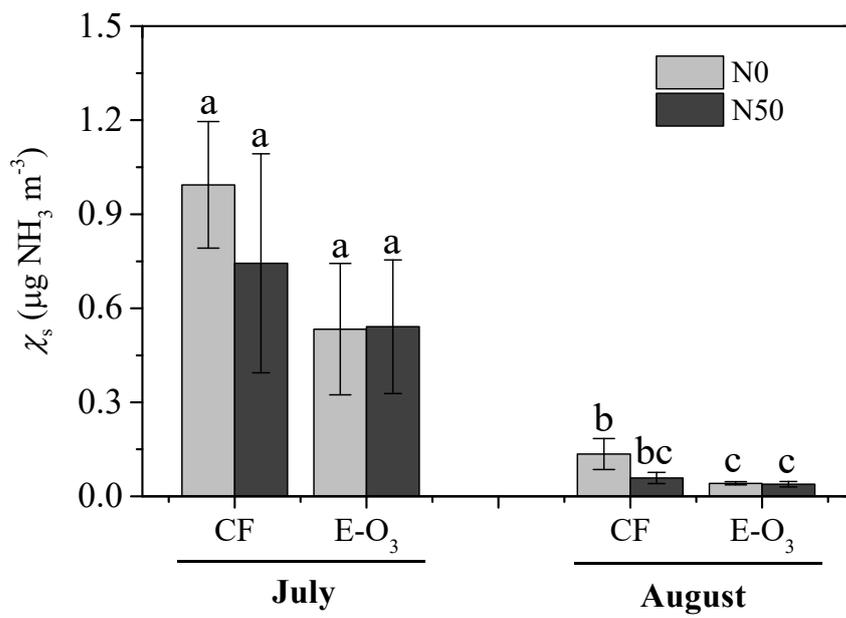


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 899 **Figure 2**



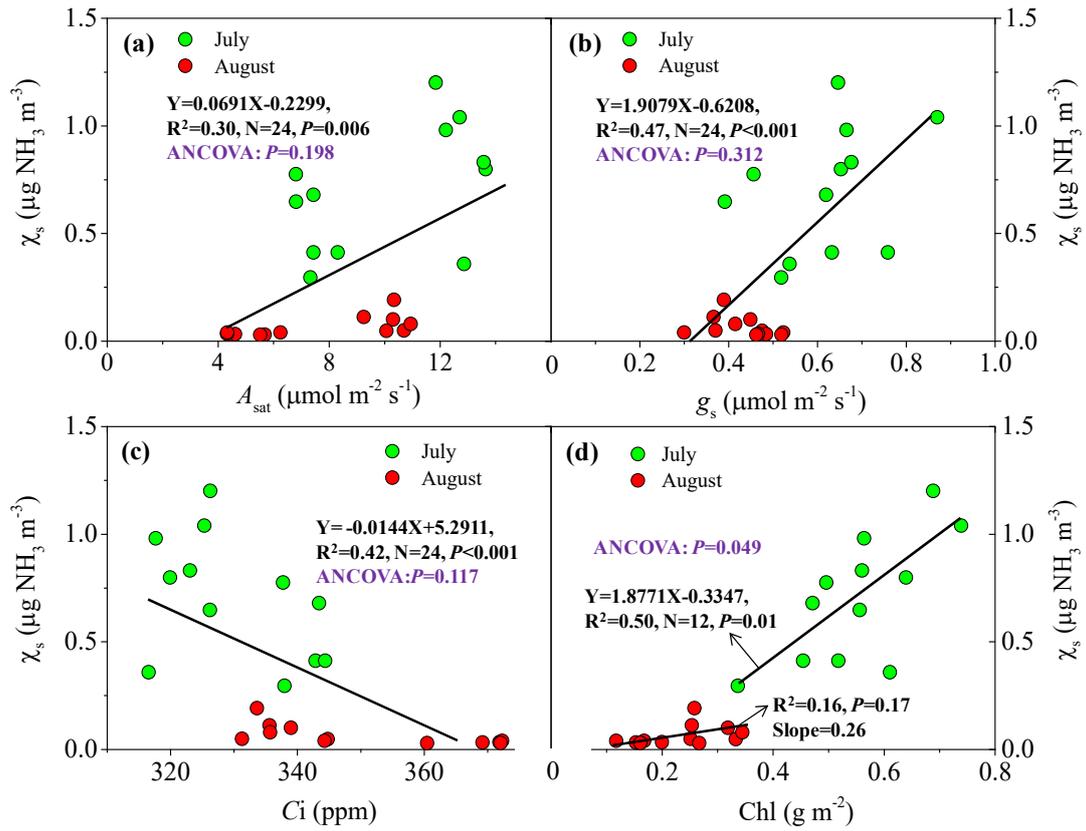
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**Figure 3**



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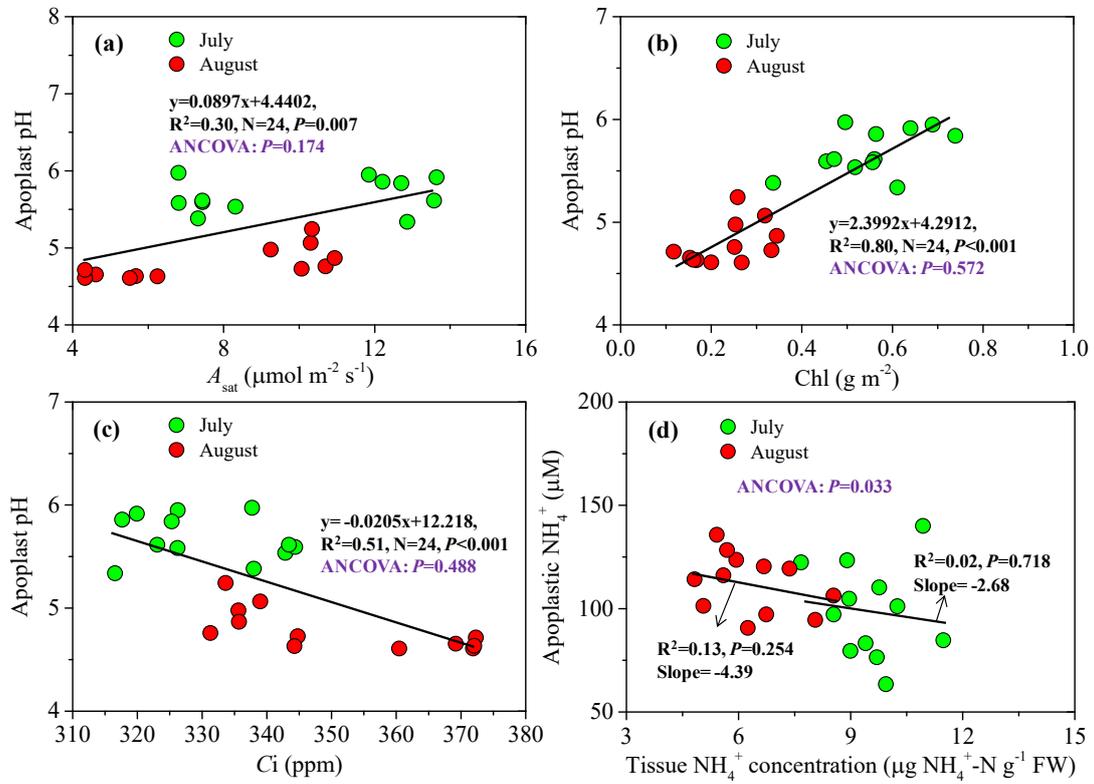
**Figure 4**



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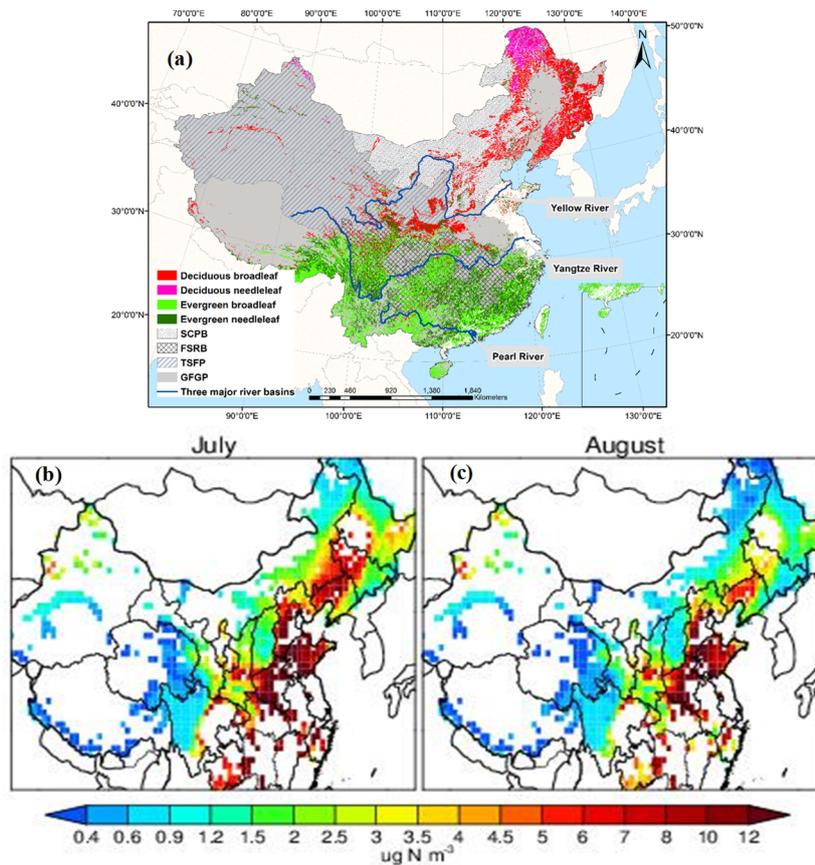
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910 **Figure 5**



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915 **Table 1.** ANOVA results (*P* values) for the individual effects of interactions of O<sub>3</sub> (CF  
 916 and E-O<sub>3</sub>), N (N0 and N50), and sampling data (July and August) on light-saturated rate  
 917 of CO<sub>2</sub> assimilation (*A*<sub>sat</sub>), stomatal conductance (*g*<sub>s</sub>), intercellular CO<sub>2</sub> concentration  
 918 (*C*<sub>i</sub>), chlorophyll content (Chl), apoplastic pH, apoplastic NH<sub>4</sub><sup>+</sup>, leaf tissue NH<sub>4</sub><sup>+</sup>,  
 919 potential emission ( $\Gamma_s$ ), and stomatal compensation point ( $\chi_s$ ).

	O <sub>3</sub>	N	Date (D)	O <sub>3</sub> *N	O <sub>3</sub> *D	N*D	O <sub>3</sub> *N*D
<i>A</i> <sub>sat</sub>	<b>&lt;0.001</b>	0.057	<b>0.001</b>	0.977	0.553	<b>0.042</b>	0.063
<i>g</i> <sub>s</sub>	0.610	0.579	<b>0.003</b>	0.285	<b>0.048</b>	0.843	0.564
<i>C</i> <sub>i</sub>	<b>0.007</b>	0.257	<b>&lt;0.001</b>	0.192	<b>0.002</b>	0.404	0.295
Chl	<b>0.006</b>	0.612	<b>0.001</b>	0.727	0.527	0.375	0.658
Apoplast pH	<b>0.015</b>	<b>0.018</b>	<b>&lt;0.001</b>	0.118	0.240	0.657	0.391
Apoplast NH <sub>4</sub> <sup>+</sup>	<b>0.039</b>	<b>0.017</b>	<b>0.004</b>	0.996	0.431	0.172	0.698
Leaf tissue NH <sub>4</sub> <sup>+</sup>	<b>0.041</b>	0.965	<b>0.003</b>	0.265	0.219	0.097	0.210
$\Gamma_s$	<b>0.026</b>	0.067	<b>&lt;0.001</b>	0.062	0.318	0.777	0.369
$\chi_s$	<b>0.019</b>	<b>0.034</b>	<b>&lt;0.001</b>	<b>0.032</b>	0.203	0.322	0.337

920 CF: charcoal-filtered ambient air; E-O<sub>3</sub>: elevated O<sub>3</sub>; N0: no N added; N50: 50 kg N

921  $\text{ha}^{-1} \text{yr}^{-1}$ ; Statistically significant effects ( $P < 0.05$ ) are marked in bold.

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