

## Article (refereed) - postprint

---

Xu, Wen; Shang, Bo; Xu, Yansen; Yuan, Xiangyang; Dore, Anthony J.; Zhao, Yuanhong; Massad, Raia-Silvia; Feng, Zhaozhong. 2018. **Effects of elevated ozone concentration and nitrogen addition on ammonia stomatal compensation point in a poplar clone.** *Environmental Pollution*, 238. 760-770. <https://doi.org/10.1016/j.envpol.2018.03.089>

© 2018 Elsevier Ltd.

This manuscript version is made available under the CC-BY-NC-ND 4.0 license <http://creativecommons.org/licenses/by-nc-nd/4.0/>



This version available <http://nora.nerc.ac.uk/id/eprint/519825/>

NERC has developed NORA to enable users to access research outputs wholly or partially funded by NERC. Copyright and other rights for material on this site are retained by the rights owners. Users should read the terms and conditions of use of this material at

<http://nora.nerc.ac.uk/policies.html#access>

NOTICE: this is the author's version of a work that was accepted for publication in *Environmental Pollution*. Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in *Environmental Pollution*, 238. 760-770.

<https://doi.org/10.1016/j.envpol.2018.03.089>

[www.elsevier.com/](http://www.elsevier.com/)

Contact CEH NORA team at  
[noraceh@ceh.ac.uk](mailto:noraceh@ceh.ac.uk)

## Effects of elevated ozone concentration and atmospheric nitrogen deposition on ammonia stomatal compensation point in a poplar clone

Wen Xu<sup>1,2</sup>, Zhaozhong Feng<sup>1,2\*</sup>, Shang Bo<sup>1,2</sup>, Yansen Xu<sup>1,2</sup>, Xiangyang Yuan<sup>1,2</sup>

<sup>1</sup>State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Shuangqing Road 18, Haidian District, Beijing 100085, China.

<sup>2</sup>College of Resources and Environment, University of Chinese Academy of Sciences, Beijing 100049, China.

\*Corresponding author.

E-mail address: fzz@rcees.ac.cn (Z. Feng).

**Abstract:** The stomatal compensation point of ammonia ( $\chi_s$ ) is a key factor controlling plant-atmosphere NH<sub>3</sub> exchange, which is dependent on the nitrogen (N) supply and varies among plant species. However, knowledge gaps remain concerning the effects of elevated atmospheric N deposition and ozone (O<sub>3</sub>) on  $\chi_s$  for forest species, resulting in large uncertainties in the parameterizations of NH<sub>3</sub> incorporated into atmospheric chemistry and transport models (CTMs). Here, we present a leaf-scale  $\chi_s$  for hybrid poplar clone '546' (*Populus deltoides* cv. 55/56 x *P. deltoides* cv. Imperial) growing in two N treatments (N0, no N added; N50, 50 kg N ha<sup>-1</sup> yr<sup>-1</sup> urea fertilizer added) and two O<sub>3</sub> treatments (CF, charcoal-filtered air; E-O<sub>3</sub>, non-filtered air plus 40 ppb) for 126 days. Our results showed that  $\chi_s$  was significantly reduced by E-O<sub>3</sub> (36%) but was not significantly affected by elevated N. Elevated O<sub>3</sub> significantly reduced the light-saturated photosynthetic rate ( $A_{sat}$ ) and chlorophyll (Chl) content and significantly increased intercellular CO<sub>2</sub> concentrations ( $C_i$ ), but had no significant effect on  $g_s$ . By contrast, elevated N significantly influenced  $A_{sat}$  but not the remaining three photosynthetic parameters. Overall,  $\chi_s$  was significantly and positively correlated with  $A_{sat}$ ,  $g_s$  and Chl, whereas a significant and negative relationship was observed between  $\chi_s$  and  $C_i$ . Our results suggest that O<sub>3</sub>-induced changes in  $A_{sat}$ ,  $C_i$  and Chl may affect  $\chi_s$ . Interactions of N and O<sub>3</sub> on  $\chi_s$  as well as all photosynthetic parameters were not significant. Our findings provide a scientific basis for optimizing parameterizations of  $\chi_s$  to respond to environmental change factors (i.e., elevated N deposition and/or O<sub>3</sub>) in the future.

**Keywords:** N deposition; Ozone; Ammonia; Compensation point; Poplar

## 1. Introduction

Atmospheric ammonia ( $\text{NH}_3$ ) is the primary alkaline trace gas in the atmosphere and plays a vital role in many biogeochemical and atmospheric processes (Behera et al., 2013). It neutralizes atmospheric acids to yield ammonium ( $\text{NH}_4^+$ ) aerosols, which resulting in increased mass loadings of fine atmospheric particulate matter ( $\text{PM}_{2.5}$ , aerodynamic diameter  $\leq 2.5$ ) (Xu et al., 2016, 2017), thereby reducing visibility and adversely impacting ecosystem and human health (Gu et al., 2014). By contrast, atmospheric deposition of reduced N ( $\text{NH}_3$  and  $\text{NH}_4^+$ ) can cause soil acidification (Du et al., 2015), eutrophication (Pareman et al., 2016) and loss of biodiversity (Erisman et al., 2007) in sensitive ecosystems.

Plants can be either a source or a sink of atmospheric  $\text{NH}_3$ , depending on the difference between atmospheric  $\text{NH}_3$  concentration and the so-called canopy  $\text{NH}_3$  compensation point (Massad et al., 2010). As a major component of canopy  $\text{NH}_3$  compensation point, Ammonia stomatal compensation point ( $\chi_s$ ) is defined as the atmospheric  $\text{NH}_3$  concentration for which there is no exchange between the leaf and the atmosphere in dry conditions (Flechard et al, 2013). Theoretically,  $\chi_s$  is also the air concentration in the leaf sub-stomatal cavity that is in equilibrium with ammonium concentration in the apoplast (Husted and Schjoerring, 1995). It plays a vital role in controlling the magnitude and the direction of  $\text{NH}_3$  exchange between the canopy and the atmosphere (Sutton et al., 1995). Specifically, if atmospheric  $\text{NH}_3$  concentrations exceed  $\chi_s$  then  $\text{NH}_3$  deposition from the atmosphere to vegetation will occur, while with atmospheric  $\text{NH}_3$  concentrations below  $\chi_s$ , there will be a net uptake of  $\text{NH}_3$  by plants.  $\chi_s$  depends directly on the plant nitrogen (N) status, developmental stage, and environmental conditions (N fertilization or atmospheric N deposition), with larger values generally observed under conditions of N supply and at senescence (Massad et al., 2009; Schjoerring et al., 1998).

$\chi_s$  can be derived from simultaneous measurement of vertical fluxes and concentrations of  $\text{NH}_3$  by using micrometeorological flux techniques over large fields (Hansen et al., 2017; Nemitz et al., 2001; Personne et al., 2015), or in cuvettes by

finding the concentration at which the total flux is zero (Hill et al., 2001; Massad et al., 2009; Wang et al., 2011). In addition, the bioassay approach has also been developed for assessing  $\chi_s$  and it is based on the determination of the leaf apoplastic  $\text{NH}_4^+$  concentration and pH by mean of apoplast extraction (Husted and Schjørring, 1995). These two methods are complementary. Apoplast extraction is more appropriate for leaf and cell scale processes whereas chamber/micrometeorological measurements tend to be more appropriate for flux measurements at an entire plant/canopy scale (Massad et al., 2009; Sutton et al., 2009).

Forests represent a major uncertainty in quantification of regional  $\text{NH}_3$  fluxes and parameterization of bi-directional  $\text{NH}_3$  exchange in atmospheric chemistry and transport models (CTMs) such as AURAMS (A Unified Regional Air-quality Modelling System, Zhang et al., 2010) and CMAQ (Community Multiscale Air-Quality Modeling System, Fu et al., 2015). This is not only due to the large land area of forests but also because of the wide range of forest types and management practices. In conditions of bi-directional  $\text{NH}_3$  exchange, forests are of particular interest. For example, temperate deciduous forests are potentially a natural source of  $\text{NH}_3$  (Hansen et al., 2013, 2017; Neirynek and Ceulemans, 2008), leading to impact of forests on the atmospheric  $\text{NH}_3$  level. In contrast, tropical humid forest and temperate coniferous forest can acts as net  $\text{NH}_3$  sinks (Bertolini et al., 2016; Duyzer et al., 2005), resulting in the impact of atmospheric  $\text{NH}_3$  on the ecological functioning of forests.

$\chi_s$  is one of the key parameters for parameterizations of  $\text{NH}_3$  incorporated into CTMs (Massad et al., 2010). Based on published data on  $\chi_s$  in relation to different plant species, growth stages, N supply etc., Massad et al. (2010) derived a new operational parameterization for integrating bi-directional  $\text{NH}_3$  exchange into CTMs, However, uncertainties still exist for its parameterization, partially due to the following two drawbacks: 1) measurement of  $\chi_s$  for different ecosystems, specific to forests, is very sparse and is only considered for a single growth stage of plants; 2) the relationships established between N fertilizer application and  $\chi_s$  remain uncertain due to a lack of co-measurement of  $\chi_s$  with different organic fertilizer (manure, slurry and urea) application rates. In addition, the actual parameterization of  $\text{NH}_3$  exchange models requires large

databases accounting for the variability of  $\chi_s$ . To our knowledge, there is only one process-based model developed by [Riedo et al. \(2002\)](#) for grasslands which accounts for the plants N nutrition and growth stage in calculating  $\chi_s$ . However, as  $\chi_s$  is not only driven by N input to the ecosystem and plant growth stage, it may be a strongly regulated process that depends on environmental changes such as elevated ground-level O<sub>3</sub>.

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

Poplars are widespread deciduous plants in temperate and boreal forests. In China, poplar is a native species, with a cultivated area of more than 10 million ha (Yuan et al., 2016). We designed an experiment to investigate for the first time the individual effects of elevated N deposition (with controlled application of urea) and O<sub>3</sub> and their interactions on  $\chi_s$  of hybrid poplar clone '546' (*Populus deltoides* cv. 55/56 x *P. deltoides* cv. *Imperial*). In addition, we estimated the relationships between photosynthetic parameters ( $g_s$ ,  $A_{sat}$ ,  $C_i$  and Chl) and  $\chi_s$ , and discussed how N and O<sub>3</sub>, as well as their-driven modifications in photosynthetic parameters ( $g_s$ ,  $A_{sat}$ ,  $C_i$  and Chl), respectively affect  $\chi_s$ .

## 2. Materials and methods

### 2.1. Experimental site and plant materials

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

Rooted cuttings of hybrid poplar clone '546' (*Populus deltoides* cv. 55/56 x *P. deltoides* cv. *Imperial*) were cultivated in individual 20 L circular plastic pots on 7 May 2017. The plots were filled with local light loamy farmland soil, which was excavated at 0-10 cm depth, sieved out by a 0.3 mm pore mesh and then thoroughly mixed for homogeneity. Plants with similar height and basal stem diameter were selected and pre-adapted to open-top chambers (OTCs, octagonal base, 12.5 m<sup>2</sup> of growth space and 3.0 m height, covered with toughened glass) for 10 days before ozone fumigation. All seedlings were manually irrigated at 1-2 day intervals in order to keep moisture to field capacity.

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

The experiment was conducted in six OTCs with two O<sub>3</sub> treatments: charcoal-filtered ambient air (CF) in which ~80% of ambient O<sub>3</sub> was removed, and elevated O<sub>3</sub>

(E-O<sub>3</sub>, non-filtered air with targeted O<sub>3</sub> addition of 40 ppb during fumigation). Each treatment had three OTC replicates, and six potted plants were randomly distributed in each OTC. The O<sub>3</sub> fumigation was performed from 10 June to 22 September 2017 with day length of 10 h (from 08:00 till 18:00), except rainy days. During the fumigation period, the averaged O<sub>3</sub> concentrations in CF and E-O<sub>3</sub> were 24.0 and 80.6 ppb, respectively, and AOT40 (accumulated hourly O<sub>3</sub> concentration above a threshold of 40 ppb) was 2.4 and 41.6 ppm h, respectively (**Fig. 1**). Ozone was generated from pure oxygen using an electrical discharge O<sub>3</sub> generator (HY003, Chuangcheng Co., Jinan, China) and mixed with ambient air using a fan. Ozone concentrations inside the OTCs were continuously monitored at approximately 10 cm above the plant canopy using an ultraviolet (UV) absorption O<sub>3</sub> analyzer (Model 49i-Thermo, Thermo Scientific, Massachusetts, USA). The analyzers were calibrated monthly with a 49iPS calibrator (Thermo Scientific) during the experiment.

In addition to the O<sub>3</sub> treatments, two N treatments were applied with three replicates: control (N0, no N added), and moderate N, (N50, 50 kg N ha<sup>-1</sup> yr<sup>-1</sup>). For N50, N additions were applied five times (13 June, 29 June, 16 July, 1 August, 17 August) to the soil with dilute urea solutions by using a 50 mL plastic bottle and the control pots received equal amounts of pure water. In total, N50 received 0.245 g N (i.e. 0.526 g urea) throughout the experiment.

### 2.3. Measurements of physiological parameters

Measurements of gas exchange, leaf temperature and chlorophyll content were performed during two periods, i.e., at the end of July and August, 2017 (on 30 and 31 July, and on 29 and 31 August, respectively). For all plants, middle leaves were selected as targeted leaves, which were 11th to 13th fully expanded leaves from the apex and comprised the main part of the leaves on each plant. A portable photosynthetic system fitting with a 6400-40 leaf chamber fluorometer (LI-6400-40, LI-COR Co., USA) was used to measure gas exchange and leaf temperature from one middle leaf between 9:00 and 12:00 h. For the measurements, the photosynthetic photon flux density was set at 1200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , the CO<sub>2</sub> concentration of air entering the leaf at 400  $\mu\text{mol mol}^{-1}$  and

the relative humidity at 50-60%. The measured parameters were  $A_{\text{sat}}$ ,  $g_s$ , and  $C_i$ . During the entire experimental period, a total of 72 leaf samples were measured.

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

#### 2.4. Determination of apoplastic $\text{NH}_4^+$ and $\text{H}^+$ concentration

A slightly modified version of the vacuum infiltration technique developed by Husted and Schjoerring (1995) was employed to determine apoplastic  $\text{NH}_4^+$  and  $\text{H}^+$  concentration. Immediately after measurements of physiological parameters, the targeted leaf was cut and washed with high-purity water (18.2  $\Omega$ ), in order to avoid any contamination from air pollutants (e.g., particulate  $\text{NH}_4^+$ ). The leaf was then blotted dry with clean absorbent paper towel and the central petiole was removed. The leaves were separated into three replicates and then weighted, infiltrated with 280 mM sorbitol solution using a 60 mL plastic syringe with a series of vacuum/pressure for 5 min. The vacuum/pressure process was automatically applied with an intercellular fluid extractor (NS-AFE-1, Pulanta Co. Suzhou, China). The infiltrated leaves were quickly rinsed with high-purity water, blotted dry and re-weighted. The leaves were then rolled, inserted into tubes and centrifuged at 9000  $\text{r min}^{-1}$  for 10min at 4°C to collect the apoplastic solution. Cytoplasmic contamination of the apoplast during the extraction procedure was checked by performing the extraction using a buffered solution (0.1 M Ntris[hydroxymethyl]methyl-2-aminoethanesulphonic acid, 2 mM dithiothreitol and 0.2 mM EDTA), and comparing the activity of Malate Dehydrogenase (MDH) in the apoplastic extracts to its activity in bulk tissue extracts as described by Husted and Schjoerring (1995). The contamination was less than  $1.2 \pm 1.1\%$  of MDH activity in apoplast extract relative to bulk tissue extract. The extracted solution was then frozen and stored at -20 °C prior to chemical analysis.

The  $\text{NH}_4^+$  concentrations in the apoplastic extracts were measured with an AA3 continuous-flow analyzer (BranCLuebbe GmbH, Norderstedt, Germany). The

detection limit of  $\text{NH}_4^+$  was  $0.01 \text{ mg N L}^{-1}$ . The pH of the extracted solution was measured with an InLab micro electrode (Mettler Toledo, Udorf, Switzerland). The dilution of the apoplastic solution was determined spectrophotometrically at wavelength 492 nm for the sorbitol, which allowed the calculation of a dilution factor (Hove et al., 2002). The concentration of apoplastic  $\text{NH}_4^+$  and  $\text{H}^+$  was corrected for dilution during the extraction procedure by multiplication with the dilution factor ( $F_{\text{dil}}$ ).

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

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

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

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

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

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

The leaf segments were cut into small pieces, frozen in a ceramic mortar with liquid N ( $-210 \text{ }^\circ\text{C}$ ), and quickly ground into a homogenous powder using a ceramic pestle. The weighed samples (approximately 1.0 g per sample) were put into a 5 mL centrifuge tube with 4 mL of high-purity water, followed by centrifugation at 2000 g ( $4^\circ\text{C}$ ) for 10 min (Loubet et al., 2002). The supernatant was then decanted and filtered through a syringe filter ( $0.45 \text{ }\mu\text{m}$ , Tengda Inc., Tianjin, China) to remove large plant tissues. The filtered solution was frozen and stored at  $-20 \text{ }^\circ\text{C}$  until analysis for  $\text{NH}_4^+$  using an AA3 continuous-flow analyzer as mentioned before.

## 2.6. Calculation of $\text{NH}_3$ stomatal compensation point

The stomatal compensation points were derived using the apoplast pH and  $\text{NH}_4^+$

concentrations (Loubet et al., 2002) according to the equation:

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

where  $\chi_s$  is the stomatal compensation point in  $\mu\text{g NH}_3 \text{ m}^{-3}$ ,  $M_{\text{NH}_3}$  is the molecular mass of  $\text{NH}_3$  in  $\text{g mol}^{-1}$ , The ratio of apoplast  $\text{NH}_4^+$  to apoplast  $\text{H}^+$  concentration, called the emission potential (expressed as  $\Gamma_s$ ), is temperature independent and dimensionless (Massad et al., 2010).  $K_H$  is the Henry constant and  $K_D$  is the dissociation constant. The product  $K_H K_D$  depends on temperature and was calculated following the method of (Hill et al., 2001):

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

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

## 2.7. Statistical analysis

Data of each investigated variable from three plants per OTC were averaged and then used as the statistical unit ( $N=3$ ). Prior to analysis, all data were tested for normality using the Shapiro-Wilk's W-test and for homogeneity of variance using Levene's-test to determine whether data should be transformed to be satisfied in application. A three-way analysis of variance (ANOVA) with a mixed linear model was then conducted to examine the effects of  $\text{O}_3$ , N, measurement dates and their interactions on physiological parameters and  $\chi_s$  as well as other parameters using JMP software (SAS Institute, Cary, NC, USA). Tukey's Honestly Significant Difference (HSD) test was applied to examine the significant differences. Analysis of covariance (ANCOVA) was performed to test the significance of difference in the slopes of the linear relationship between  $\chi_s$  and physiological parameters using SPSS software (version 11.5; SPSS Inc., Chicago, IL, USA). Statistically significant differences were set at  $P < 0.05$ . All the data were shown as mean  $\pm$  standard deviation (SD) of three OTC replicates.

## 3. Results

For all investigated variables, similar and non-significant responses to E- $\text{O}_3$  and N50 were calculated between the two measurement dates, i.e., July and August (**Figs.**

**3 and 4).** Based on integrated analysis of all data from all N and O<sub>3</sub> treatments and the two measurement dates, the main results are presented below.

### 3.1. Photosynthetic parameters

The individual effects of N, O<sub>3</sub> and measurement date, and their interactions on  $A_{\text{sat}}$ ,  $g_s$ ,  $C_i$  and Chl of the poplar clone 546 are shown in **Fig.2** and **Table 1**. For all investigated variables, the interactions of O<sub>3</sub> and N were not significant, but the individual effects of them were significant for most variables (**Table 1**). E-O<sub>3</sub> relative to CF significantly reduced  $A_{\text{sat}}$  by 55%, whereas N50 relative to N0 significantly increased  $A_{\text{sat}}$  by 6% (**Fig. 2a and Table 1**).  $A_{\text{sat}}$  significantly decreased (by 24%) in August relative to that in July, when averaged across all treatments. The effects of N and O<sub>3</sub> on  $g_s$  were both not significant, whereas measurement date significantly affected  $g_s$  (**Table 1, Fig. 2b**). Similar to  $A_{\text{sat}}$ ,  $g_s$  significantly decreased by 30% in August compared with that in July.

Ozone significantly influenced  $C_i$  and Chl whereas N had no significant effects on them (**Table 1, Fig. 2c, d**).  $C_i$  was significantly increased by E-O<sub>3</sub> (+7%) compared with CF, and also was significantly higher (6%) in August than in July. Conversely, Chl was significantly reduced by E-O<sub>3</sub> (-30%), and significantly decreased (by 57%) in August.

### 3.2. Leaf apoplastic NH<sub>4</sub><sup>+</sup> and pH, and leaf tissue NH<sub>4</sub><sup>+</sup>

**Fig. 3a-c** shows the responses of leaf apoplastic NH<sub>4</sub><sup>+</sup> concentration and pH, and leaf tissue NH<sub>4</sub><sup>+</sup> concentration to E-O<sub>3</sub> and N50. The interactions between O<sub>3</sub> and N and/or measurement dates were not significant for all those three variables (**Table 1**). However, individual effects of them on apoplastic NH<sub>4</sub><sup>+</sup> concentrations and pH all reached statistically significant levels. Similarly, leaf tissue NH<sub>4</sub><sup>+</sup> concentration was significantly influenced by both O<sub>3</sub> and measurement dates, but not by N.

Averaged apoplastic NH<sub>4</sub><sup>+</sup> concentration was significant reduced (by 15%) in E-O<sub>3</sub> plants compared with CF plants (**Fig. 3a, Table 1**). By contrast, the mean concentration was significantly increased (by 27%) in N50 plants compared with that

in N0 plants. A significant increase (14%) of the mean occurred in August relative to July. As for apoplastic pH, two small but significant reductions (both 4%) of the mean were found in E-O<sub>3</sub> and N50 plants compared with those in CF and N50 plants, respectively (**Fig. 3c, Table 1**). Also, the mean pH was significantly lower (15%) in plants grown in August than in July.

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

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

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

Similar to  $\Gamma_s$ ,  $\chi_s$  was significantly lower in E-O<sub>3</sub> than in CF ( $0.29 \pm 0.29$  and  $0.48 \pm 0.45 \mu\text{g NH}_3 \text{ m}^{-3}$ ), and also 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. 4**). However, it is noteworthy that the effect of N and its interaction with O<sub>3</sub> on  $\chi_s$  were marginally significant (both  $P=0.059$ ) (**Table 1**).

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

$\chi_s$  was positively and significantly correlated with  $A_{\text{sat}}$ ,  $g_s$  and Chl (**Fig. 5a, b,d**), whereas a negative and significant correlation between  $\chi_s$  and  $C_i$  was observed (**Fig. 5c**). ANCOVA results did not show significant differences in the slope of the regression lines for the individual O<sub>3</sub> or N treatments.

## 4. Discussion

### 4.1. Effect of N application

The application rate of urea fertilizer in the present experiment ( $50 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ ) is approximately 2.3 and 3 times higher than the averages of N deposition over China ( $16 \text{ kg N ha}^{-1} \text{ yr}^{-1}$  in 2008-2012 period, [Zhao et al., 2017](#)) and in China's forests ( $22 \text{ kg N ha}^{-1} \text{ yr}^{-1}$  in 1995-2010 period, [Du et al., 2014](#)), respectively. It is also approximately 1.7-5.0 times greater than reported values during recent years in the N deposition hotspots of western Europe ( $20.0$  to  $28.1 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ , [Vet et al., 2014](#)), and North 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 deposition increased by approximately  $8 \text{ kg N ha}^{-1} \text{ yr}^{-1}$  between the 1980s and the 2000s in China. Also, total N deposition is expected to have a 5%-10% increase in the year 2050 relative to 2005 ([Kanakidou et al., 2016](#)). In view of the above, the level of N addition in this study is sufficient to assess the ecological effects of enhanced N deposition expected in the future.

Apoplastic  $\text{NH}_4^+$  concentration is one of the key factors in controlling  $\chi_s$ , which is associated with the status of leaf N and external N supply (e.g., N fertilization and atmospheric deposition) ([Herrmann et al., 2009](#); [Massad et al., 2008](#)). The apoplastic  $\text{NH}_4^+$  concentrations measured for the N0 and N50 treatments (but without E- $\text{O}_3$ ) were  $0.10 \pm 0.01$  and  $0.13 \pm 0.01$  mM, respectively (**Fig. 3a**). To our knowledge published data on apoplastic  $\text{NH}_4^+$  concentrations in leaves of poplar 546 is unavailable, making a direct comparison with other studies impossible. However, our measured value for N0 treatments were close to that reported for *Fagus sylvatica* growing in July and August ( $0.11$  mM, [Wang et al., 2011](#)). Compared with agricultural crops, our measured value for N0 treatments was higher than that reported for barley ( $0.04$  mM, [Mattsson et al., 1998](#)), and was close to the value of  $0.10$  measured for oilseed rape ([Massad et al., 2009](#)) both growing on N0. The value for plants grown on N50 was similar to that measured for oilseed rape ( $0.18$  mM with  $6 \text{ mM NO}_3^-$ , [Schjoerring et al., 2002](#)), and was lower than the value of  $1.9$  mM observed for barley grown on  $5 \text{ mM NH}_4^+$  ([Mattsson et al., 1998](#)). As expected, enhanced N input (N50) significantly increased the apoplastic  $\text{NH}_4^+$  concentration (**Fig. 2, Table 1**), probably due to increased soil N

availability. This result is similar to the findings of [Massad et al. \(2009\)](#) that the  $\text{NH}_4^+$  concentrations in leaf apoplast of oilseed rape increased significantly with rising N treatments (aside from  $\text{NO}_3^-$  supply).

Besides apoplastic  $\text{NH}_4^+$  concentration, the pH of the apoplast may be the most important factor determining  $\chi_s$ . There is evidence that  $\text{NH}_4^+$ -fed plants have reduced apoplastic pH compared to  $\text{NO}_3^-$ -fed plants, e.g., sunflower ([Hoffmann et al., 1992](#)), soybean ([Kosegarten and Englisch, 1994](#)) and barley ([Mattsson et al., 1998](#)), probably due to a root rather than shoot assimilation of N ([Pearson et al., 1998](#)). Application of urea fertilizer in the present experiment probably enhanced soil  $\text{NH}_4^+$  level via urea hydrolysis, which further significantly increased apoplastic  $\text{NH}_4^+$  concentrations (**Fig. 3a**) via root assimilation and the subsequent transport of  $\text{NH}_4^+$  to the foliar apoplast in the xylem ([Mattsson et al., 1998](#)). This behaviour, along with  $\text{NH}_4^+$  uptake-induced acidification may offer an explanation for lower apoplastic pH (ranging from 4.6 to 6.0, **Fig. 3c**) of poplar 546 compared with those reported for most plant species (between 5.0 and 6.5, [Grignon and Sentenac, 1991](#)).

Enhanced N input significantly increased both apoplastic  $\text{NH}_4^+$  and  $\text{H}^+$  concentration with a similar significant level (**Figs. 3a,c and Table 1**) This may partly explain why N has no significant effect on  $\chi_s$ . This, together with the relatively low calculated  $\chi_s$  throughout, especially in August (**Fig. 4**), suggest that  $\chi_s$  appears to be pH-driven in this study. The apoplastic pH of *Phaseolus vulgaris* has been found to become alkalized during photosynthesis ([Raven and Farquhar, 1989](#)). Similarly, we found that the N load significantly increased  $A_{\text{sat}}$  of poplar 546 (**Fig. 2a, Table 1**), which significantly positively correlated with apoplastic pH (**Fig. 6a**) but had no significant relationship with apoplastic  $\text{NH}_4^+$  concentration ( $R^2=0.09$ ,  $P=0.141$ ). These results provide an explanation for a significant and positive relationship observed between  $A_{\text{sat}}$  and  $\chi_s$  (**Fig. 5a**). Although such an increase in apoplastic pH occurred due to increased  $A_{\text{sat}}$  by N load, it is insufficient to offset  $\text{NH}_4^+$  uptake-induced apoplast acidification, leading to relatively low  $\chi_s$ .

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

According to monitoring results for the 2014-2016 period in 187 Chinese cities, the mean daily 8-h O<sub>3</sub> concentrations peak in summer reached up to 114.30 ± 23.78 ppb (Li et al., 2017). Obviously, the O<sub>3</sub> level in E-O<sub>3</sub> was within the range of current O<sub>3</sub> levels in China and was also in the range of future expected concentrations in warm and sunny areas of the world at the end of this century (The Royal Society 2008; IPCC 2013). The dose of O<sub>3</sub> (41.6 ppm h in AOT40, Fig. 1) in E-O<sub>3</sub> by far exceeded the O<sub>3</sub> exposure limit of 5 ppm h for forest protection (CLRTAP, 2015) and 12 ppm h for poplar protection (Hu et al., 2015). As anticipated, O<sub>3</sub>-induced injuries to poplar 546 were detected in E-O<sub>3</sub> despite N fertilization (e.g., reduction of A<sub>sat</sub> and Chl, Fig. 2a, d), thus confirming that poplar 546 is a very sensitive species to O<sub>3</sub> (Shang et al., 2017).

E-O<sub>3</sub> significantly increased C<sub>i</sub> and decreased A<sub>sat</sub> and Chl, respectively, but did not significantly affect g<sub>s</sub> (Figs. 2 and Table 1). These results are consistent with the findings of Shang et al. (2017) for poplar 546. Similarly, a previous study showed that O<sub>3</sub> can substantially reduce A<sub>sat</sub> in most plants, and also detected an uncoupled relationships between A<sub>sat</sub> and g<sub>s</sub> (Zhang et al., 2012). This can be explained by the fact that the O<sub>3</sub>-induced reduction in A<sub>sat</sub> is largely ascribed to non-stomatal factors, i.e. impaired physiological activity of mesophyll cells (Akhtar et al., 2010; Feng et al., 2016).

We found that E-O<sub>3</sub> significantly reduced the calculated  $\chi_s$  (Fig. 4). This is most likely related to a decline in apoplastic pH resulting from O<sub>3</sub>-induced changes in photosynthetic parameters, i.e., decreased A<sub>sat</sub> and Chl, and increased C<sub>i</sub> (Fig. 2a,c,d, Table 1). This is because in addition to A<sub>sat</sub>, both Chl and C<sub>i</sub> correlated significantly with  $\chi_s$  and apoplastic pH (Figs. 5c,d and 6c,d). In addition, there was a large response of apoplastic NH<sub>4</sub><sup>+</sup> to photosynthesis (Mattsson and Schjoerring, 1996), mainly due to the fact that NH<sub>3</sub> assimilation by plants requires carbon skeletons generated from photosynthesized carbohydrates for the synthesis of amino acids (Huppe and Turpin, 1994). In this regard, a significant reduction in A<sub>sat</sub> can also give rise to declines in apoplastic NH<sub>4</sub><sup>+</sup> concentration. Similar to N, ozone has a greater impact on apoplastic pH than on apoplastic NH<sub>4</sub><sup>+</sup> concentration, as demonstrated by the significant reduction of  $\Gamma_s$  by O<sub>3</sub> (Fig. 3d, Table 1); this therefore led to a significant reduction in  $\chi_s$  (Fig. 4,

**Table 1).**

#### 4.3. Dependence on measurement date

$\chi_s$  can be influenced by plant developmental stage since leaf apoplastic  $\text{NH}_4^+$  concentration and pH varies among leaves of different ages (Hill et al., 2002). Mattsson and Schjoerring (2003) reported that, for ryegrass (*Lolium perenne*), both apoplastic and tissue  $\text{NH}_4^+$  concentrations were 2-3 times higher in intact leaves (with visual symptoms of senescence) compared with green leaves. The present study shows that leaf apoplastic  $\text{NH}_4^+$  concentrations significantly increased in August compared with those in July, whereas a significant reduction was found for leaf tissue  $\text{NH}_4^+$  concentration (**Fig. 3a, b**). In addition, a negative and marginally significant ( $P=0.053$ ) relationship was observed between  $\text{NH}_4^+$  concentrations in apoplast and in tissue (**Fig. 6d**). These results together indicated that  $\text{NH}_4^+$  is actively transported from the leaf tissue to the apoplast. This explanation is supported by evidences from earlier studies showing that apoplastic fluid in leaves constitutes a highly dynamic  $\text{NH}_4^+$  pool, to which  $\text{NH}_4^+$  is constantly supplied via  $\text{NH}_3$  efflux from the mesophyll cells (Nielsen and Schjoerring, 1998; Schjoerring et al. 2000).

Regarding apoplastic pH, the values measured in August were significantly reduced compared to July, and almost all were  $<5$  (**Fig. 3c, Table 1**), for which an explanation is the combined effect of  $\text{NH}_4^+$  uptake-induced acidification (see Sect. 4.1) and  $\text{O}_3$ -accelerated leaf senescence (Gao et al., 2017). As reported by Mattsson and Schjoerring (2003), leaf aging from green leaves to yellow tips resulted in a pronounced decrease of pH by more than 1 unit. Thus, a significantly lower  $A_{\text{sat}}$  in August resulting from accelerated leaf senescence (**Fig. 2a, Table 1**) also contributed to lower apoplastic pH due to the existence of apoplast alkalization during photosynthesis as mentioned earlier.

$\chi_s$  was significantly reduced in August compared with July (**Fig. 4**). This is likely due to the greater impact of measurement date on apoplastic pH than on apoplastic  $\text{NH}_4^+$  concentration as indicated by their respective significant level (**Table 1**). The change in stomatal opening is an important control mechanism for the regulation of

influxes and outfluxes of  $\text{NH}_3$  into or out of the leaves because the conductance for diffusion of  $\text{NH}_3$  is affected (Schjoerrin et al., 1998). We found that  $g_s$  significantly decreased in August (Fig. 2b, Table 1). Also, a positive and significant relationship was observed between  $g_s$  and  $\chi_s$  (Fig. 5b); this is mainly caused by measurement date as both E- $\text{O}_3$  and N did not significantly affect  $g_s$ . These results together suggested that a significant reduction of  $g_s$  also contributed to a reduction in  $\chi_s$ .

#### 4.4. Uncertainty and recommendations

In the present study,  $\chi_s$  is calculated based on direct measurements of leaf apoplastic  $\text{NH}_4^+$  concentration and pH by means of extraction of the apoplastic fluid with successive vacuum infiltration/centrifugation technique (Husted and Schjørring, 1995). Although this technique has been successfully applied to several plant species in the field (Herrmann et al., 2009; Mattsson et al., 2009), it is subject to uncertainties regarding potential regulation of apoplastic  $\text{NH}_4^+$  concentration and pH by the plant during the infiltration and buffering effects (Massad et al., 2009). For example, Nielsen and Schjørring (1998) showed that apoplastic  $\text{NH}_4^+$  concentration in *Brassica napus* L. appeared to be regulated during infiltration. However, Hill et al. (2001) did not detect such a homeostasis for apoplastic  $\text{NH}_4^+$  concentration in *Luzula sylvatica* (Huds.) Gaud. Following the method of van Hove et al. (2002),  $F_{\text{dil}}$ , calculated based on determination of  $V_{\text{air}}$  and  $V_{\text{apo}}$  (Equ. 2), was applied to correct apoplastic concentrations.  $V_{\text{apo}}$  values obtained in the present work varied from 0.06 to 0.23. These values fit well into the range found by other researchers for different plant species (Van Han et al., 2001, 2002). The measured  $V_{\text{air}}$  (0.16 ml  $\text{g}^{-1}$  LFW) was also comparable to values (0.21 mL  $\text{g}^{-1}$  LFW) reported for *Lolium perenne* L. (Van Han et al., 2002). These results indicate that the value for  $F_{\text{dil}}$  determined in the present study was acceptable. However, due to a lack of information regarding infiltration of poplar 546, use of  $F_{\text{dil}}$  might also result in some uncertainties in apoplastic  $\text{NH}_4^+$  concentrations and pH, and probably in  $\chi_s$  if there is a potential difference in the buffering capacity between them.

The vacuum infiltration/centrifugation method is also subject to some uncertainty due to the strong possibility of cytoplasmic contamination of the apoplast during the

extraction procedure (Lohaus et al., 2001). We estimated the error in this method by assaying the contamination of the apoplast by MDH activity. The cytoplasmic contamination in the present work was about 1.2%.

$\chi_s$  obtained in the current study was calculated at the leaf scale and only for the middle leaf position. However,  $\chi_s$  may differ significantly depending on leaf position (e.g., upper, middle and lower) due to difference in the N status of the leaves. Thus, the resulting effects of enhanced N and O<sub>3</sub> on  $\chi_s$  are not enough to represent the characteristics of the entire-plant. Furthermore, the present experiment was designed based on OTC chambers. However, OTCs have effects of their own, such as differences in microclimate (e.g., air temperature and humidity), fixed gas flow and limited space, which could over-estimate or under-estimate the effects of O<sub>3</sub> on plants (Feng et al., 2010). Note that  $\chi_s$  is shown to be influenced by air temperature, partly by affecting the amount of NH<sub>3</sub> dissolved in the apoplast, and partly by affecting the leaf tissue NH<sub>4</sub><sup>+</sup> generation (or assimilation)-associated physiological processes (Schjoerring et al. 1998). To more accurately assess the effects of N, O<sub>3</sub> and/or plant growth stage on  $\chi_s$ , at least the two following developments are recommended in future work: 1) to employ open-air fumigations (O<sub>3</sub>-FACE systems, Paoletti et al., 2016), and 2) to investigate vertical profile of  $\chi_s$  at plant scale

#### 4.5. Implications

For forest ecosystems,  $\chi_s$  is commonly expected to increase considerably with input of the different types of N fertilizer applied (nitrate, ammonium or ammonium nitrate) (Massad et al., 2010). We demonstrate that urea addition did not significantly affect  $\chi_s$  of forest species (i.e., poplar 546) (note however that the resulting reduction reached marginal significant level,  $P=0.059$ ) (**Fig. 4, Table 1**).  $\chi_s$  is affected by the plant's development stage and may peak at senescence, which is especially true for agricultural crops (Hill et al., 2002; Mattsson and Schjoerring, 2003). We find that there is a significant reduction of  $\chi_s$  for forest species (i.e., poplar 546) in the context of O<sub>3</sub>-accelerated senescence. Based on these findings, we propose that current parameterizations of  $\chi_s$  in chemical transport models should be in optimized to partially

respond to changes in environmental conditions (e.g., elevated N and/or O<sub>3</sub>). In addition,  $\chi_s$  is a highly variable parameter, influenced by a range of physiological conditions (Schjoerrin et al., 1998). We found that  $A_{\text{sat}}$ ,  $g_s$  and Chl significantly and positively correlated with  $\chi_s$ , whereas a significant and negative relationship was observed between Ci and  $\chi_s$  (Fig. 5). Understanding these physiological controls of  $\chi_s$  is essential for modeling its dynamic behaviour.

Our selected poplar 546 belongs to deciduous broadleaf species. To preliminarily assess whether Chinese deciduous broadleaf forests (Fig. 7a) act as a source or a sink for atmospheric NH<sub>3</sub>, we compared the calculated  $\chi_s$  for elevated N and O<sub>3</sub> treatment (N50\*E-O<sub>3</sub>) in July (average 0.54  $\mu\text{g NH}_3 \text{ m}^{-3}$ ) and August (average 0.03  $\mu\text{g NH}_3 \text{ m}^{-3}$ ) with modeled atmospheric NH<sub>3</sub> concentration in July (Fig. 7b) and August (Fig. 7c) during 2008-2012 period (which was modeled using the GEOS-Chem model and fitted well with the surface NH<sub>3</sub> measurements, see details in Zhao et al. (2017)). The results of the comparison show that the modeled NH<sub>3</sub> concentrations over approximately 91% and 100% of total land area exceeded the corresponding  $\chi_s$  in July and August, respectively. It should be noted that such percentages are considered to be approximate estimates, as  $\chi_s$  may vary among different forest species, and the current atmospheric NH<sub>3</sub> concentration cannot represent future NH<sub>3</sub> levels. Nevertheless, we may conclude that under the current ambient NH<sub>3</sub> concentrations in China the canopy of deciduous broadleaf forests is unlikely to be a major source of NH<sub>3</sub> emission during summertime.

## 5. Conclusions

This study is the first time to investigate the combined effects of O<sub>3</sub> exposure and N load on  $\chi_s$  of forest species. Our results demonstrated that elevated O<sub>3</sub> significantly reduced  $\chi_s$ , in tandem with  $A_{\text{sat}}$ , Ci and Chl, while use of urea as N load had no significant effect on  $\chi_s$ . The interaction of O<sub>3</sub> exposure and N load on  $\chi_s$  were not significant. These results provide underpinning data for optimizing the parameterizations of  $\chi_s$  in the CTMs, allowing response to global change variables (i.e., atmospheric N deposition and O<sub>3</sub>).

## **Acknowledgments**

This study was supported by the National Key R&D Program of China (2017YFC0210106), the National Natural Science Foundation of China (41705130)

## **References**

- Akhtar, N., Yamaguchi, M., Inada, H., Hoshino, D., Kondo, T., Izuta, T., 2010. Effects of ozone on growth, yield and leaf gas exchange rates of two Bangladeshi cultivars of wheat (*Triticum aestivum L.*). *Environ. Pollut.* 158, 1763–1767.
- Behera, S.N., Sharma, M., Aneja, V.P., Balasubramanian, R., 2013. Ammonia in the atmosphere: a review on emission sources, atmospheric chemistry and deposition on terrestrial bodies. *Environ. Sci. Pollut. R.* 20(11), 8092–8131.
- Bertolini, T., Flechard, C.R., Fattore, F., Nicolini, G., Stefani, P., Materia, S., Valentini, R., Vaglio L.G., Castaldi, S., 2016. DRY and BULK atmospheric nitrogen deposition to a West-African humid forest exposed to terrestrial and oceanic sources. *Agr. Forest Meteor.* 218–219, 184–195.
- Cardoso-Vilhena, J., Barnes, J., 2001. Does nitrogen supply affect the response of wheat (*Triticum aestivum cv. Hanno*) to the combination of elevated CO<sub>2</sub> and O<sub>3</sub>? *J. Exp. Bot.* 52, 1901–1911.
- CLRTAR, 2015. Chapter 3: Mapping critical levels for vegetation. Manual on methodologies and criteria for modelling and mapping critical loads & levels and air pollution effects, risks and trends. Manual on Methodologies and Criteria for Modelling and Mapping Critical Loads and Levels and Air Pollution Effects, Risks and Trends. United Nations Economic Commission for Europe (UNECE)

Convention on Long-range Transboundary Air Pollution, Geneva  
([URL:http://www.icpmapping.org](http://www.icpmapping.org)).

- Cooper, O.R., Parrish, D.D., Ziemke, J., Balashov, N.V., Cupeiro, M., Galbally, I.E., Gilge, S., Horowitz, L., Jensen, N.R., Lamarque, J.F., Naik, V., Oltmans, S.J., Schwab, J., Shindell, D.T., Thouret, V., Wang, Y., Zbinden, R.M., 2014. Global distribution and trends of tropospheric ozone: An observation-based review. *Elementa Science of the Anthropocene* 2, 000029, doi: 10.12952/journal.elementa.000029.
- Du, E., Jiang, Y., Fang, J.Y., de Vries, W., 2014. Inorganic nitrogen deposition in China's forests: Status and characteristics. *Atmos. Environ.* 98(98), 474–482.
- Du, E., de Vries, W., Liu, X., Fang, J., Galloway, J. N., Jiang, Y., 2015. Spatial boundary of urban 'acid islands' in southern China. *Sci. Rep.* 5, 12635.
- Duyzer, J., Pilegaard, K., Simpson, D., Weststrate, H., Walton, S., 2005. A simple model to estimate exchange rates of nitrogen dioxide between the atmosphere and forests. *Biogeosciences Discuss.* 2, 1033–1065.
- Erisman, J.W., Bleeker, A., Galloway, J., Sutton, M.S., 2007. Reduced nitrogen in ecology and the environment. *Environ. Pollut.* 150(1), 140–149.
- Flechard, C. R., Massad, R. S., Loubet, B., Personne, E., Simpson, D., Bash, J. O., Cooter, E. J., Nemitz, E., Sutton, M. A., 2013. Advances in understanding, models and parameterizations of biosphere-atmosphere ammonia exchange. *Biogeosciences* 10(7), 5183–5225.
- Feng, Z.Z., Wang, S.G., Szantoi, Z., Chen, S.A., Wang, X.K., 2010. Protection of plants from ambient ozone by applications of ethylenediurea (EDU): A meta-analytic review. *Environ. Pollut.* 158(10), 3236–3242.
- Feng, Z.Z., Sun, J.S., Wan, W.X., Hu, E.Z., Calatayud, V., 2014. Evidence of widespread ozone-induced visible injury on plants in Beijing, China. *Environ. Pollut.* 193(1), 296–301.
- Feng, Z.Z., Wang, L., Pleijel, H., Zhu, J.G., Kobayashi, K., 2016. Differential effects of ozone on photosynthesis of winter wheat among cultivars depend on antioxidative enzymes rather than stomatal conductance. *Sci. Total Environ.* 572, 404–411.

- Flechard, C.R., Massad, R.S., Loubet, B., Personne, E., Simpson, D., Bash, J.O., Cooter, E.J., Nemitz, E., Sutton, M.A., 2011. Advances in understanding, models and parameterizations of biosphere-atmosphere ammonia exchange. *Biogeosciences* 10(7), 5183–5225.
- Gao, F., Catalayud, V., Paoletti, E., Hoshika, Y., Feng, Z.Z., 2017. Water stress mitigates the negative effects of ozone on photosynthesis and biomass in poplar plants. *Environ. Pollut.* 230, 268–279.
- Grignon, C., Sentenac, H., 1991. pH and ionic conditions in the apoplast. *Annu. Rev. Plant Biol.* 42, 103–128.
- Gu, B.J., Sutton, M.A., Chang, S.X., Ge, Y., Jie, C., 2014. Agricultural ammonia emissions contribute to China's urban air pollution. *Front. Ecol. Environ.* 12(5), 265–266.
- Hansen, K., Sørensen, L.L., Hertel, O., Geels, C., Skjøth, C.A., Jensen, B., Boegh, E., 2013. Ammonia emissions from deciduous forest after leaf fall. *Biogeosciences* 10, 4577–4589.
- Hansen, K., Personne, E., Skjøth, C.A., Loubet, B., Ibrom, A., Jensen, R., Sørensen, L.L., Boegh, E., 2017. Investigating sources of measured forest-atmosphere ammonia fluxes using two-layer bi-directional modeling. *Agr. Forest Meteorol.* 237–238, 80–94.
- Herrmann, B., Mattsson, M., Jones, S. K., Cellier, P., Milford, C., Sutton, M. A., Schjoerring, J. K., Neftel, A., 2009. Vertical structure and diurnal variability of ammonia exchange potential within an intensively managed grass canopy. *Biogeosciences* 6(1), 15–23.
- Hill, P.W., Raven, J.A., Loubet, B., Fowler, D., Sutton, M.A., 2001. Comparison of gas exchange and bioassay determinations of the ammonia compensation point in *Luzula sylvatica*, (Huds.) Gaud. *Plant Phys.* 125, 476–487.
- Hill, P.W., Raven, J.A., Sutton, M.A., 2002. Leaf age-related differences in apoplastic  $\text{NH}_4^+$  concentration, pH and the  $\text{NH}_3$  compensation point for a wild perennial. *J. Exp. Bot.* 53(367), 277–286.
- Hoffmann, B., Planker, R., Mengel, K., 1992. Measurements of pH in the apoplast of

- sunflower leaves by means of fluorescence. *Physiol. Plantarum* 84, 146–153.
- Hu, E.Z., Gao, F., Xin, Y., Jia, H.X., Li, K.H., Hu, J.J., Feng, Z.Z., 2015. Concentration- and fluxbased ozone dose-response relationships for five poplar clones grown in North China. *Environ. Pollut.* 207, 21–30.
- Huppe, H.C., Turpin, D.H., 1994. Integration of carbon and nitrogen metabolism in plant and algal cells. *Annu. Rev. Plant Biol.* 45(1), 577–607
- Husted, S., Schjoerring, J. K., 1995. Apoplastic pH and Ammonium Concentration in Leaves of *Brassica napus* L. *Plant Physiol.* 109(4), 1453–1460.
- IPCC, 2013. *Climate Change 2007: The Physical Science Basis*. In Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Intergovernmental Panel on Climate Change (eds Stocker T.F., Qin D., Plattner G.K., Tignor M.M.B., Allen S.K., Boschung J., et al.), pp. 1552, Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.
- Kanakidou, M., Myriokefalitakis, S., Daskalakis, N., Fanourgakis, G., 2016. Past, present, and future atmospheric nitrogen deposition. *J. Atmos. Sci.* 73 (5), 160303130433005.
- Kosegarten, H., Englisch, G., 1994. Effect of various nitrogen forms on the pH in leaf apoplast and on iron chlorosis of *Glycine max* L. *J. Plant Nutr. Soil Sc.* 157(6), 401–405.
- Li, P., Feng, Z.Z., Catalayud, V., Yuan, X.Y., Xu, Y.S., Paoletti, E., 2017. A meta-analysis on growth, physiological, and biochemical responses of woody species to ground-level ozone highlights the role of plant functional types. *Plant Cell Environ.* 40(10), 2369–2380.
- Li, Y., Schichtel, B.A., Walker, J.T., Schwede, D.B., Chen, X., Lehmann, C.M., Puchalski, M.A., Gay, D.A., Collett, J.L., 2016. Increasing importance of deposition of reduced nitrogen in the United States. *Proc. Natl. Acad. Sci. U.S.A.* 113 (21), 5874.
- Lichtenthaler, H.K., 1987. [34] Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods Enzym.* 148, 350–382.
- Liu, X.J., Song, L., He, C.E., Zhang, F.S., 2010. Nitrogen deposition as an important nutrient from the environment and its impact on ecosystems in China. *J Arid Land*

- 2(2), 137–143.
- Liu, X.J., Duan, L., Mo, J.M., Du, E.Z., Shen, J.L., Lu, X.K., Zhang, Y., Zhou, X.B., He, C.E., Zhang, F.S., 2011. Nitrogen deposition and its ecological impact in China: an overview. *Environ. Pollut.* 159(10), 2251–2264.
- Lohaus, G., Pennewiss, K., Sattelmacher, B., Hussmann, M., Muehling, K.H., 2001. Is the infiltration–centrifugation technique appropriate for the isolation of apoplastic fluid? A critical evaluation with different plant species. *Physiol. Plantarum* 111(4), 457–465.
- Loubet, B., Milford, C., Hill, P.W., Tang, Y. S., Cellier, P., Sutton, M.A., 2002. Seasonal variability of apoplastic  $\text{NH}_4^+$  and pH in an intensively managed grassland. *Plant Soil* 238(1), 97–110.
- Massad, R.S., Loubet, B., Tuzet, A., Cellier, P., 2008. Relationship between ammonia stomatal compensation point and nitrogen metabolism in arable crops: Current status of knowledge and potential modelling approaches. *Environ. Pollut.* 154(3), 390–403.
- Massad, R.S., Loubet, B., Tuzet, A., Autret, H., Cellier, P., 2009. Ammonia stomatal compensation point of young oilseed rape leaves during dark/light cycles under various nitrogen nutritions. *Agr. Ecosyst. Environ.* 133(3), 170–182.
- Massad, R.S., Nemitz, E., Sutton, M.A., 2010. Review and parameterisation of bi-directional ammonia exchange between vegetation and the atmosphere. *Atmos. Chem. Phys.* 10(21), 10359–10386.
- Mattsson, M., Schjoerring, J., 1996. Ammonia emission from young barley plants: influence of N source, light/dark cycles and inhibition of glutamine synthetase. *J. Exp. Bot.* 47(4), 477–484.
- Mattsson, M., Husted, S., Schjoerring, J.K., 1998. Influence of nitrogen nutrition and metabolism on ammonia volatilization in plants. *Nutr. Cycl. Agroecosys.* 51(1), 35–40.
- Mattsson, M., Schjoerring, J. K., 2003. Senescence-induced changes in apoplastic and bulk tissue ammonia concentrations of ryegrass leaves. *New Phytol.* 160(3), 489–499.
- Mattsson, M., Herrmann, B., David, M., Loubet, B., Riedo, M., Theobald, M.R., Sutton,

- M.A., Bruhn, D., Neftel, A., Schjoerring, J.K., 2009. Temporal variability in bioassays of the stomatal ammonia compensation point in relation to plant and soil nitrogen parameters in intensively managed grassland. *Biogeosciences* 6(2), 171–179.
- Neiryneck, J. and Ceulemans, R., 2008. Bidirectional ammonia exchange above a mixed coniferous forest. *Environ. Pollut.* 154, 424–438.
- Nemitz, E., Flynn, M., Williams, P.I., Milford, C., Theobald, M.R., Blatter, A., Gallagher, M.W., Sutton, M.A., 2001. A relaxed eddy accumulation system for the automated measurement of atmospheric ammonia fluxes. *Water Air Soil Pollut. Focus* 1(5–6), 189–202.
- Nielsen, K.H., Schjoerring, J.K., 1998. Regulation of apoplastic  $\text{NH}_4^+$  concentration in leaves of oilseed rape. *Plant Physiol.* 118(4), 1361–1368.
- Pakeman, R.J., Alexander, J., Brooker, R., Cummins, R., Fielding, D., Gore, S., Hewison, R., Mitchell, R., Moore, E., Orford, K., 2016. Long-term impacts of nitrogen deposition on coastal plant communities. *Environ. Pollut.* 212, 337–347.
- Paoletti, E., Materassi, A., Fasano, G., Hoshika, Y., Carriero, G., Silaghi, D., Badea, O., 2016. A new-generation 3D ozone FACE (Free Air Controlled Exposure). *Sci. Total Environ.* 575, 1407–1414.
- Pearson, J., Clough, E.C.M., Woodall, L.J., Havill, D.C., Zhang, X.-H., 1998. Ammonia emissions to the atmosphere from leaves of wild plants and *Hordeum vulgare* treated with methionine sulphoximine. *New Phytol.* 138, 37–48.
- Personne, E., Tardy, F., Générumont, S., Decuq, C., Gueudet, J.C., Mascher, N., Durand, B., Masson, S., Lauransot, M., Fléchar, C., Burkhardt J., Loubet B., 2015. Investigating sources and sinks for ammonia exchanges between the atmosphere and a wheat canopy following slurry application with trailing hose. *Agr. Forest Meteor.* 207, 11–23.
- Raven J.A., Farquhar G.D., 1989. Leaf apoplast pH estimation in *Phaseolus vulgaris*. In: Dainty J, Michelis MI, MarrèE, Rasi-Caldogno F, eds. *Plant membrane transport: the current position*. Amsterdam: Elsevier, 607–610.
- Riedo, M., Milford, C., Schmid, M., Sutton, M.A., 2002. Coupling soil-plant -

- atmosphere exchange of ammonia with ecosystem functioning in grasslands. *Ecol. Model.* 158(1-2), 83–110.
- Schjoerring, J.K., Mattsson, M., Husted, S., 1998. Physiological parameters controlling plant-atmosphere ammonia exchange. *Atmos. Environ.* 32(3): 491-498.
- Schjoerring, J.K., Husted, S.M.G., Nielsen, K., Finnemann, J. Matt, M., 2000. Physiological regulation of plant-atmosphere ammonia exchange. *Plant Soil* 221(1), 95-102.
- Schjoerring, J.K., Husted, S., Mack, G., Mattsson, M., 2002. The regulation of ammonium translocation in plants. *J. Exp. Bot.* 53(370), 883–890.
- Shang, B., Feng, Z.Z., Li, P., Yuan, X.Y., Xu, Y.S., Calatayud, V., 2017. Ozone exposure and flux-based response relationships with photosynthesis, leaf morphology and biomass in two poplar clones. *Sci. Total Environ* 603–604, 185–195.
- Sutton, M.A., Schjoerring, J.K., Wyers, G.P., 1995. Plant atmosphere exchange of ammonia. *Philos. T. Roy. Soc. S-A.* 351, 261–278.
- Sutton, M.A., Nemitz, E., Milford, C., Campbell, C., Erisman, J.W., Hensen, A., Cellier, P., David, M., Loubet, B., Personne, E., Schjoerring, J.K., Mattsson, M., Dorsey, J.R., Gallagher, M.W., Horvath, L., Weidinger, T., Meszaros, R., Dämmgen, U., Neftel, A., Herrmann, B., Lehman, B.E., Flechard, C., Burkhardt, J., 2009. Dynamics of ammonia exchange with cut grassland: synthesis of results and conclusions of the GRAMINAE Integrated Experiment. *Biogeosciences* 6(12), 2907–2934.
- The Royal Society, 2008. Ground-level ozone in the 21st century: future trends, impacts and policy implications. Science Policy Report 15/08. The Royal Society, London.
- Van Hove, L. W. A., Heeres, P., Bossen, M. E., 2002. The annual variation in stomatal ammonia compensation point of rye grass (*Lolium perenne* L.) leaves in an intensively managed grassland. *Atmos. Environ.* 36(18), 2965-2977.
- Vet, R., Artz, R.S., Carou, S., Shaw, M., Ro, C.-U., Aas, W., Baker, A., Bowersox, V.C., Dentener, F., Galy-Lacaux, C., Hou, A., Pienaar, J.J., Gillett, R., Cristina Forti, M., Gromov, S., Hara, H., Khodzher, T., Mahowald, N.M., Nickovic, S., Rao, P.S.P., Reid, N.W., 2014. A global assessment of precipitation, chemistry and deposition of sulfur, nitrogen, sea salt, base cations, organic acids, acidity and pH, and phosphorus.

- Atmos. Environ. 93 (3–4), 3–100.
- Wang, L., Xu, Y.C., Schjoerring, J.K., 2011. Seasonal variation in ammonia compensation point and nitrogen pools in beech leaves (*Fagus sylvatica*). *Plant Soil* 343 (1-2), 51–66.
- Xu, W., Luo, X.S., Pan, Y.P., Zhang, L., Tang, A.H., Shen, J.L., Zhang, Y., Li, K.H., Wu, Q.H., Yang, D.W., Zhang, Y.Y., Xue, J., Li, W.Q., Li, Q.Q., Tang, L., Lu, S.H., Liang, T., Tong, Y.A., Liu, P., Zhang, Q., Xiong, Z.Q., Shi, X.J., Wu, L.H., Shi, W.Q., Tian, K., Zhong, X.H., Shi, K., Tang, Q.Y., Zhang, L.J., Huang, J.L., He, C.E., Kuang, F.H., Zhu, B., Liu, H., Jin, X., Xin, Y.J., Shi, X.K., Du, E.Z., Dore, A.J., Tang, S., Collett, J.L., Goulding, K., Sun, Y.X., Ren, J., Zhang, F.S., Liu, X.J., 2015. Quantifying atmospheric nitrogen deposition through a nationwide monitoring network across China. *Atmos. Chem. Phys.* 15, 12345–12360.
- Xu, W., Wu, Q.H., Liu, X.J., Tang, A.H., Dore, A., Heal, M., 2016. Characteristics of ammonia, acid gases, and PM<sub>2.5</sub> for three typical land-use types in the North China Plain. *Environ. Sci. Pollut. R.* 23(2), 1158–1172.
- Xu, W., Song, W., Zhang, Y., Liu, X., Zhang, L., Zhao, Y., Liu, D., Tang, A., Yang, D., Wang, D., 2017. Air quality improvement in a megacity: implications from 2015 Beijing Parade Blue pollution control actions. *Atmos. Chem. Phys.* 17, 31–46.
- Yuan, X.Y., Calatayud, V., Gao, F., Fares, S., Paoletti, E., Tian, Y., Feng, Z.Z., 2016. Interaction of drought and ozone exposure on isoprene emission from extensively cultivated poplar. *Plant Cell Environ.* 39(10):2276.
- Zhang, L., Wright, P. L., Asman, W. A. H., 2010. Bi-directional air-surface exchange of atmospheric ammonia – A review of measurements and a development of a big-leaf model for applications in regional-scale air-quality models. *J. Geophys. Res.*, 115, D20310, doi:10.1029/2009JD013589.
- Zhang, W.W., Feng, Z.Z., Wang, X.K., Niu, J.F., 2012. Responses of native broadleaved woody species to elevated ozone in subtropical China. *Environ. Pollut.* 163(4), 149–157.
- Zhao, Y., Zhang, L., Chen, Y.F., Liu, X.J., Xu, W., Pan, Y.P., Duan, L., 2017. Atmospheric nitrogen deposition to China: a model analysis on nitrogen budget and

critical load exceedance. *Atmos. Environ.* 153, 32–40.

### Figure captions

Figure 1. The 10 h (8:00-18:00) mean O<sub>3</sub> concentrations (ppb) and AOT40 (ppm h) from 10 June to 22 September 2017 in charcoal-filtered air (CF) and elevated O<sub>3</sub> (E-O<sub>3</sub>) treatments.

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

Figure 3. Effects of ozone (CF, charcoal-filtered ambient air, and E-O<sub>3</sub>, elevated O<sub>3</sub>) 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> concentration, tissue NH<sub>4</sub><sup>+</sup> concentration, apoplastic pH and emission potential ( $\Gamma_s$ ) of hybrid poplar clone '546' (*Populus deltoides* cv. 55/56 x *P. deltoides* cv. *Imperial*). Data shown are the mean  $\pm$  standard deviation of three-OTC measurements. The letters on top of the bars are based on the Tukey test across the two measurements, with different letters indicating significantly different from each other at  $P < 0.05$ .

Figure 4. Effects of ozone (CF, charcoal-filtered ambient air, and E-O<sub>3</sub>, elevated O<sub>3</sub>) and Nitrogen (N0, no N added, and N50, 50 kg N ha<sup>-1</sup> yr<sup>-1</sup>) on the stomatal compensation point ( $\chi_s$ )

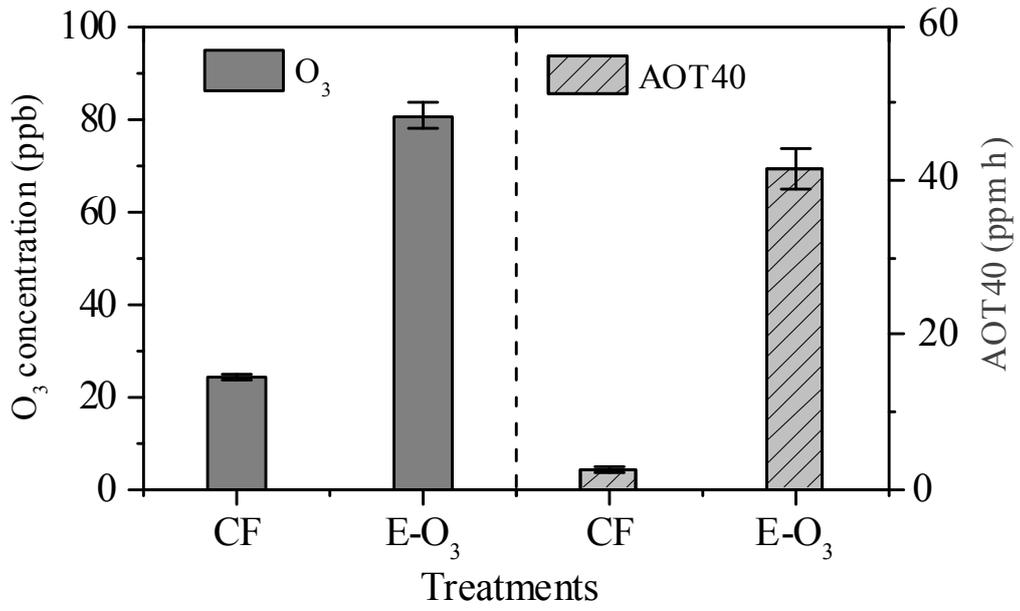
Figure 5. Correlation between the stomatal compensation point ( $\chi_s$ ) and light-saturated photosynthesis ( $A_{\text{sat}}$ ), stomatal conductance ( $g_s$ ), intercellular CO<sub>2</sub> concentration ( $C_i$ ) and chlorophyll (Chl) content across all ozone and nitrogen treatments. Green, red, blue and pink dots represent charcoal-filtered ambient 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-O<sub>3</sub>\*N50 treatment, respectively. As

ANCOVA did not show significant differences in the slope of the regression lines for the individual O<sub>3</sub> or N treatments, one single line is shown.

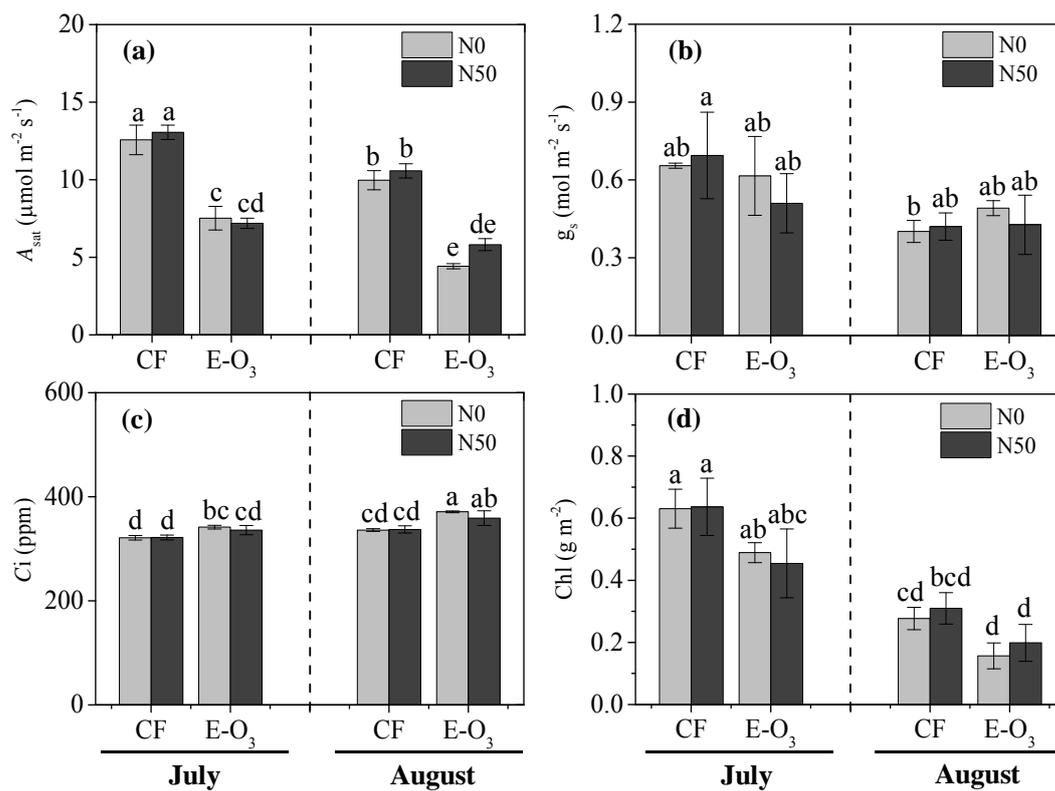
Figure 6. Correlations between apoplastic pH and light-saturated photosynthesis ( $A_{\text{sat}}$ ), chlorophyll (Chl) content, and intercellular CO<sub>2</sub> concentration ( $C_i$ ), and correlation between apoplastic NH<sub>4</sub><sup>+</sup> concentration and leaf tissue NH<sub>4</sub><sup>+</sup> concentration across all ozone and nitrogen treatments. Green, red, blue and pink dots represent charcoal-filtered ambient 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-O<sub>3</sub>\*N50 treatment, respectively. As ANCOVA did not show significant differences in the slope of the regression lines for the individual O<sub>3</sub> or N treatments, one single line is shown.

Figure 7. Actual forest distribution in China (a) (adopting from Li et al. (2017)) and atmospheric NH<sub>3</sub> concentration over deciduous broadleaf forests in July (b) and August (b) modeled using the GEOS-Chem model.

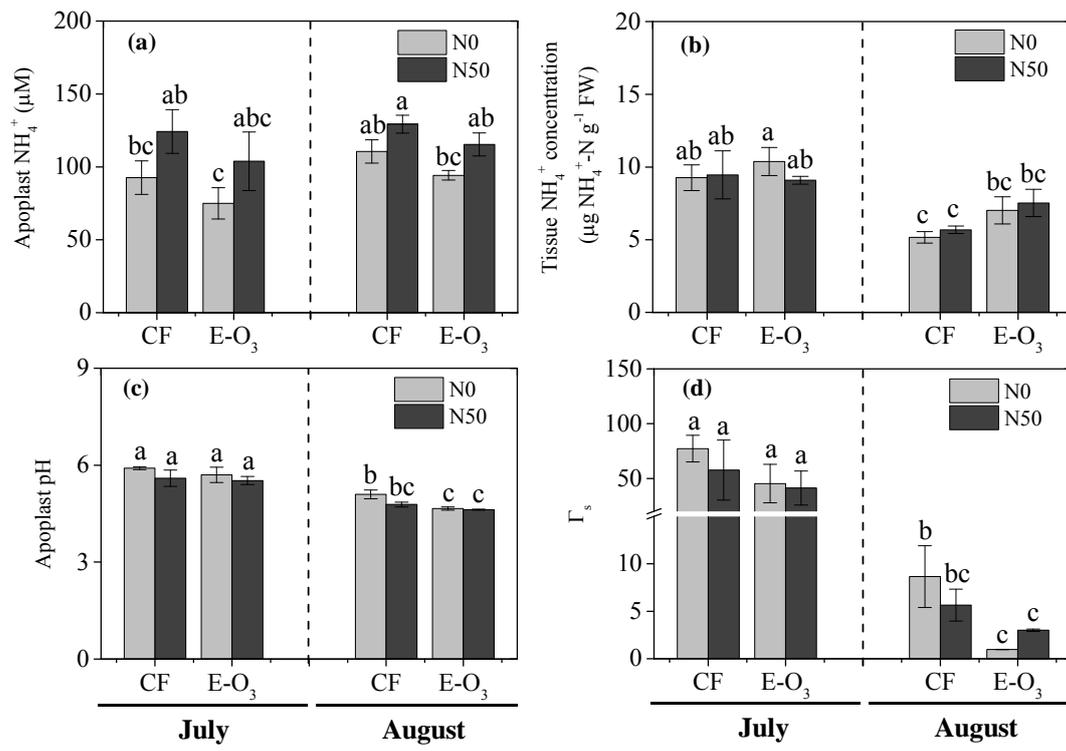
**Figure 1**



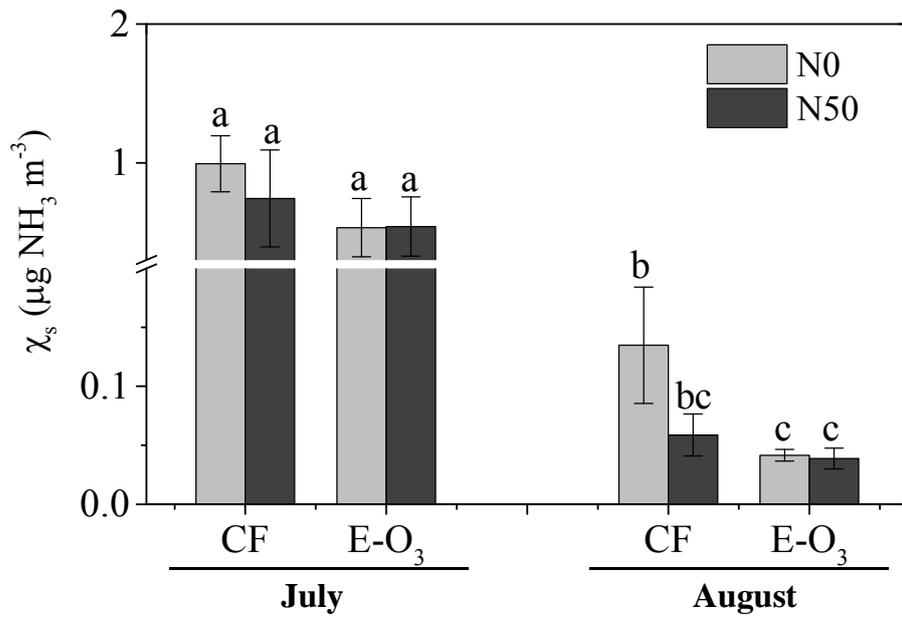
**Figure 2**



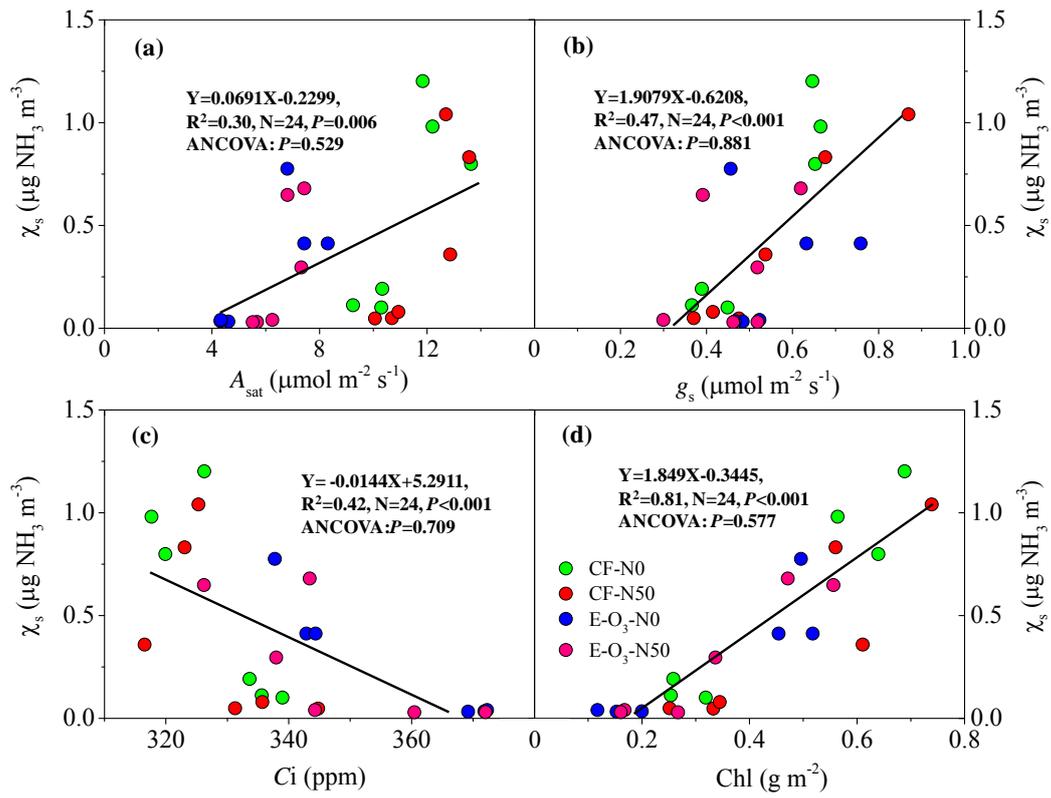
**Figure 3**



**Figure 4**



**Figure 5**



**Figure 6**

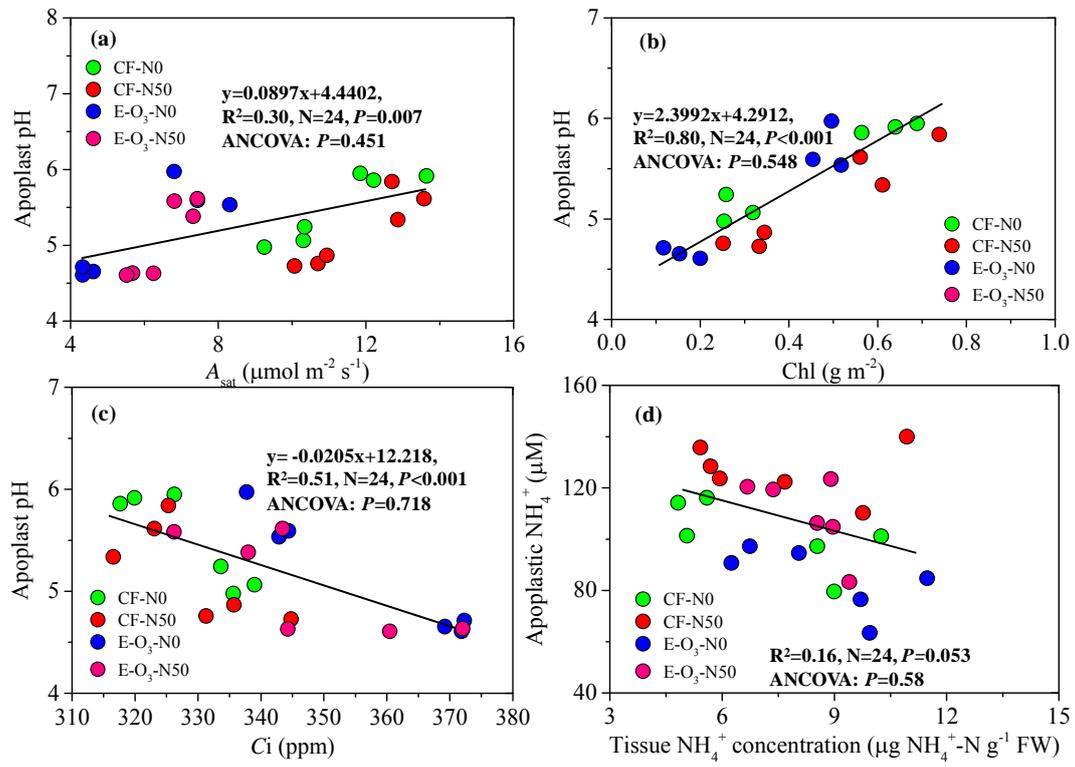
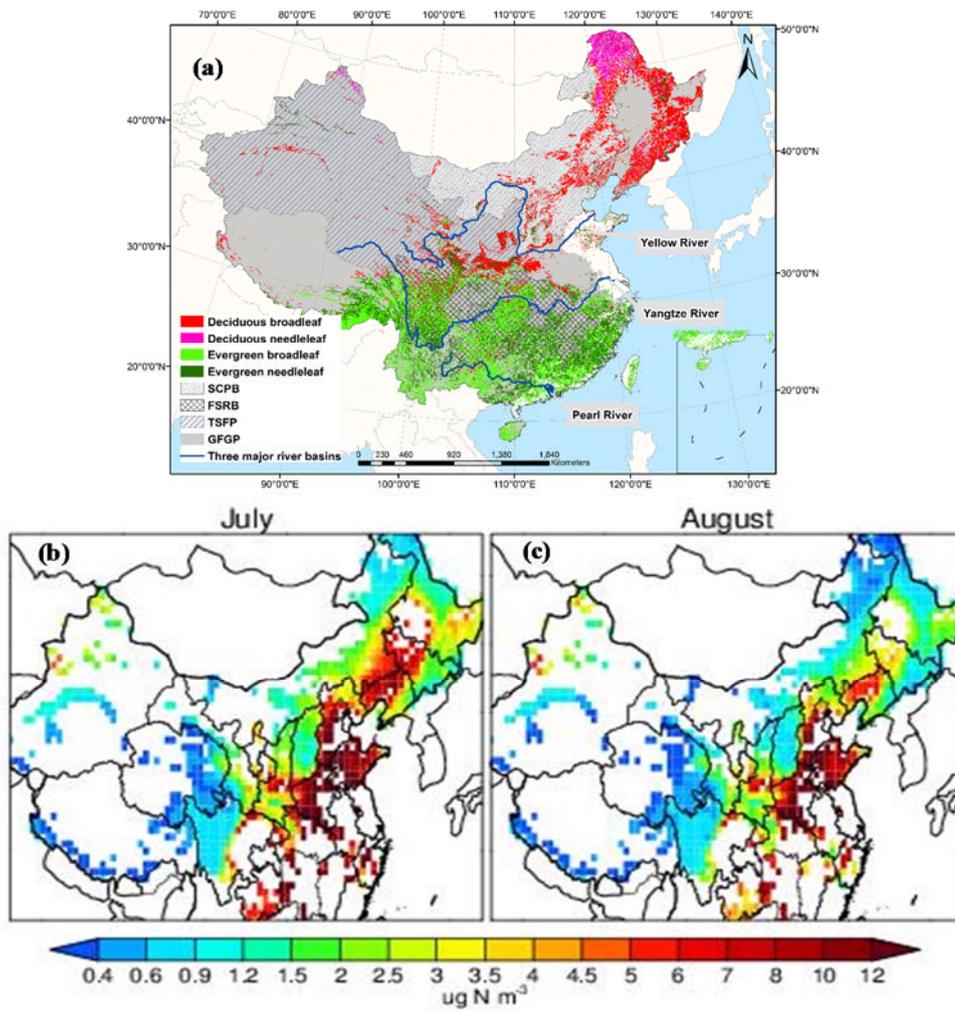


Figure 7



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

	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>Asat</i>	< <b>0.001</b>	<b>0.034</b>	< <b>0.001</b>	0.980	0.518	0.064	0.104
<i>g<sub>s</sub></i>	0.432	0.503	< <b>0.001</b>	0.189	0.071	0.905	0.692
<i>C<sub>i</sub></i>	< <b>0.001</b>	0.150	< <b>0.001</b>	0.099	0.072	0.621	0.542
<i>Chl</i>	< <b>0.001</b>	0.674	< <b>0.001</b>	0.810	0.405	0.373	0.631
Apoplast pH	<b>0.001</b>	<b>0.003</b>	< <b>0.001</b>	0.080	0.103	0.777	0.482
Apoplast NH <sub>4</sub> <sup>+</sup>	<b>0.002</b>	< <b>0.001</b>	<b>0.011</b>	0.958	0.688	0.294	0.820
Leaf tissue NH <sub>4</sub> <sup>+</sup>	< <b>0.001</b>	0.119	< <b>0.001</b>	0.107	0.199	0.848	0.527
<i>Γ<sub>s</sub></i>	<b>0.009</b>	0.982	< <b>0.001</b>	0.347	0.062	0.175	0.326
<i>χ<sub>s</sub></i>	< <b>0.001</b>	0.059	< <b>0.001</b>	0.059	0.103	0.454	0.470

CF: charcoal-filtered ambient air; E-O<sub>3</sub>: elevated O<sub>3</sub>; N0: no N added; N50: 50 kg N ha<sup>-1</sup> yr<sup>-1</sup>; Statistically significant effects (*P*<0.05) are marked in bold.