



Nitrogen availability does not affect ozone flux-effect relationships for biomass in birch (*Betula pendula*) saplings

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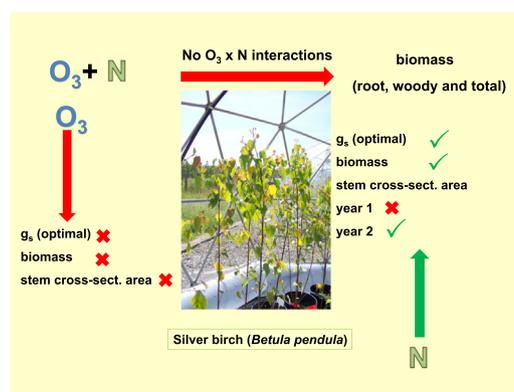
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HIGHLIGHTS

- O₃ flux-effect relationships investigated for birch at 7 O₃ profiles and 4 N loads
- g_s under optimal conditions was stimulated by N but not affected by O₃.
- O₃ flux-effect relationships for biomass were not affected by N supply.
- O₃ reduced stem cross-sectional area after the first season only.
- N stimulation of stem cross-sectional area increased in the second season.

GRAPHICAL ABSTRACT



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ABSTRACT

To investigate whether nitrogen (N) load affects the ozone (O₃) stomatal flux-effect relationship for birch biomass, three-year old birch saplings were exposed to seven different O₃ profiles (24 h mean of 35–66 ppb) and four different N loads (10, 30, 50 and 70 kg ha⁻¹ yr⁻¹) in precision-controlled hemispherical glasshouses (solardomes) in 2012 and 2013. Stomatal conductance (g_s) under optimal growth conditions was stimulated by enhanced N supply but was not significantly affected by enhanced O₃ exposure. Birch root, woody (stem + branches) and total biomass (root + woody) were not affected by the Phytotoxic Ozone Dose (POD₁SPEC) after two seasons of O₃ exposure, and enhanced N supply stimulated biomass production independent of POD₁SPEC (i.e. there were no POD₁SPEC × N interactions). There was a strong linear relationship between the stem cross-sectional area and tree biomass at the end of the experiment, which was not affected by O₃ exposure or N load. Enhanced N supply stimulated the stem cross-sectional area at the end of season 2, but not at the end of season 1, which suggests a time lag before tree biomass responded to enhanced N supply. There was no significant effect of POD₁SPEC on stem cross-sectional area after either the first or second growing season of the experiment. Contrasting results reported in the literature on the interactive impacts of O₃ and N load on tree physiology and growth are likely due to species-specific responses, different duration of the experiments and/or a limitation of the number of O₃ and N levels tested.

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1. Introduction

Tropospheric ozone (O_3) is the most important air pollutant in terms of adverse effects on (semi-)natural vegetation and cultivated crops and it is of primary interest due to its strict relation with climate change (Lefohn et al., 2018). Rising tropospheric O_3 concentration is an important air pollution problem in northern mid-latitudes, with levels rising since the Industrial Revolution, when O_3 concentrations were approximately 10 ppb (Cooper et al., 2014; Hartmann et al., 2013). In Europe, ground-level O_3 concentrations have roughly doubled between 1950 and 2000, and the present day annual average background O_3 concentrations are ca. 30–40 ppb (Cooper et al., 2014; Royal Society, 2008). Although there is some evidence that the peaks (Simpson et al., 2014) and daytime mean concentrations of O_3 (EMEP/CCC, 2016; Mills et al., 2018) are decreasing across parts of Europe in rural areas, potentially plant-damaging O_3 episodes are still occurring when climatic conditions conducive to O_3 formation (hot, dry and sunny days) coincide with abundance of precursors (Mills et al., 2016). Effects of O_3 on vegetation are dependent on O_3 uptake (Mills et al., 2011a, b), which is also affected by climate conditions. At the same time, in the mid-20th century, the emissions of anthropogenic reactive nitrogen to the atmosphere accelerated because of increased fossil fuel combustion and intensification of agricultural activities. This resulted in a large increase in nitrogen (N) deposition to ecosystems through dry and wet processes that has approximately doubled since 1900 (Li et al., 2016b; Sutton et al., 2011). In Europe, the total deposition of N remains high (typically 5–30 kg N ha⁻¹ yr⁻¹, depending on locality and vegetation type) and control of ammonia emissions is hard to achieve, with European emissions stable since 2000 and predicted to remain stable at current levels in the 2020s and 2030s (Winiwarter et al., 2011).

Tropospheric O_3 pollution is known to cause damage to vegetation and effects have been reported on a wide range of species. There are numerous studies showing effects of O_3 on trees and reported impacts include leaf level responses such as decreased photosynthesis, visible leaf damage, accelerated senescence and weakened defence against biotic/abiotic stress (e.g. Krupa et al., 2001; Ainsworth et al., 2012; Li et al., 2016a; Dai et al., 2017). At the whole tree level, biomass reductions induced by O_3 have been reported on seedlings, for example *Populus* (Hu et al., 2015) and *Quercus ilex* L. (Gerosa et al., 2015) and on mature trees such as *Betula pendula* (Kontunen-Soppela et al., 2007). Two quantitative meta-analyses indicated that ambient O_3 concentrations with an average of 40 ppb significantly decrease net tree photosynthesis by 11% and total tree biomass by 7% compared to preindustrial O_3 levels in the northern hemisphere (approx. 10 ppb) (Wittig et al., 2007; Wittig et al., 2009).

In contrast, N usually tends to have a fertilization effect on plant growth and photosynthesis in areas where N is a limiting nutrient, although tree growth response to elevated N varies between species and is not related to successional status (Tripathi and Raghubanshi, 2014). Furthermore, N supply above a certain critical load might not result in further biomass stimulation, it might even result in a decline in biomass (Braun et al., 2017).

Enhanced O_3 concentrations and N deposition often co-occur as NO_2 emission and O_3 formation are chemically linked (Simpson et al., 2014). However, relatively few studies have investigated the interactive impacts of both O_3 and N on trees using 3 or more O_3 concentrations (Mills et al., 2016). Evidence shows that the interaction effects between O_3 and N on tree species can be either antagonistic or synergistic (Harmens et al., 2017). Previously, Harmens et al. (2017) showed that the sensitivity of leaf traits (including photosynthetic parameters) of silver birch (*Betula pendula*) to O_3 are not affected by N supply. Therefore, the interaction between O_3 and N is complex and may depend on the tree species, exposure concentrations, duration of exposure and soil/climate conditions. The nature and the extent of the interaction between O_3 and N on a long-term time scale are still uncertain (Mills et al., 2016).

In recent decades, different metrics have been used to assess adverse O_3 effects on vegetation (e.g. Emberson et al., 2007; Mills et al., 2007, 2011a, 2011b, 2018; Yuan et al., 2017). Among them, the Phytotoxic Ozone Dose (POD_y, accumulated stomatal O_3 flux above a threshold Y nmol O_3 m⁻² s⁻¹ of projected leaf area (PLA) over the growing season) has been recommended as the preferred metric as it is well suited to evaluate O_3 effects in a changing climate (Mills et al., 2011b; Hayes et al., 2012). POD_y's have been defined for specific plant species or a group of species (POD_ySPEC) or in a simplified form defined for application in large scale and integrated assessment modelling (POD_yIAM; LRTAP Convention, 2017). POD_y takes into account the effects of environmental conditions such as air temperature, vapour pressure deficit (VPD) and photosynthetic active radiation (PAR), soil moisture content and plant growth stage on stomatal opening and subsequent O_3 flux into the leaves (LRTAP Convention, 2017). Although some studies have developed O_3 -flux-effect functions under controlled experimental conditions based on tree biomass (Büker et al., 2015; Hu et al., 2015; Gerosa et al., 2015; LRTAP Convention, 2017) and physiological parameters e.g. chlorophyll content or photosynthetic rate (Bagard et al., 2015; Shang et al., 2017), most of them are limited to the single stress of O_3 . Few studies have investigated the effect of N deposition on O_3 flux-effect relationships, and those that did, studied N effects at only two levels (control and elevated), e.g. (Bagard et al., 2015). For example, Marzuoli et al. (2016, 2018) showed that enhanced N supply increased the biomass and partially mitigated the O_3 negative impact on the growth of *Quercus robur* (L.) and *Carpinus betulus* L., respectively. Considering the frequent co-occurrence between O_3 and N deposition, it is important to verify that O_3 flux-effect relationships are valid under a range of N deposition. To address the issue, we developed O_3 flux-effect relationships for silver birch based on seven O_3 regimes (24 h mean of 35–66 ppb) and four levels of N load (10, 30, 50 and 70 kg ha⁻¹ yr⁻¹).

In this study, stomatal conductance (g_s), O_3 stomatal fluxes, biomass and stem cross-sectional area were measured or calculated (O_3 fluxes) in silver birch (*B. pendula*). The objectives of this study were to: (1) determine the impact of O_3 and N load on the g_s of birch saplings; (2) assess whether N load affects the O_3 stomatal flux-effect relationship for birch biomass; (3) investigate if stem cross-sectional area could be used as a proxy for changes in woody biomass and (4) determine changes in the biomass response to O_3 and N load with time. We tested the hypothesis that (1) elevated O_3 negatively and N load positively affect the growth of birch, and that the effects are additive, i.e. there are no significant interactions; and (2) the O_3 flux-effect relationship for birch is independent on the N load.

2. Materials and methods

2.1. Plant materials

Three-year-old birch (*Betula pendula*) trees were purchased from Cheviot Trees (<http://www.cheviot-trees.co.uk/>) and planted in 6.5 litre pots (diameter 21 cm, height 25 cm) filled with John Innes No.1 compost (J. Arthur Bowers) on 13th April 2012 (Harmens et al., 2017). Pots were lined with perforated plastic to discourage roots from growing outside the pot. The trees were 'bare root' and inoculated with mycorrhiza (obtained from Buckingham Nurseries, UK) before planting in pots and watered as required. Trees of similar size (initial average height of 68 cm) were selected for use in the experiment.

2.2. Experiment site and treatments

The experiment was carried out in solardomes (hemispherical glass-houses; 3 m diameter, 2.1 m height) located at Abergwyngregyn nr. Bangor, North Wales, United Kingdom (53° 23' N, 4° 01' W). Plants were exposed to O_3 in seven solardomes during two growing seasons. After being acclimated in the solardomes, exposure began on the 30th

April and finished on 12th October in 2012, and for the second growing season, began on 17th May and finished on 16th September in 2013, when the trees were harvested. Between the two growing seasons, the trees were kept outside under ambient conditions. The two growing seasons were slightly different due to a later spring and subsequent bud-burst in 2013 than in 2012.

We used an O₃ generator (G11, Dryden Aqua, UK) to generate O₃ from oxygen concentrated from air (Sequal 10, Pure O₂ Ltd., UK). Ozone was distributed to each solardome via Poly Tetra Fluoro Ethylene (PTFE) tubing and delivered to each solardome using solenoid valves (Kinesis, UK) controlled by computer software (Lab VIEW version 2012, National Instruments, Texas, US). Charcoal-filtered air was injected with controlled amounts of O₃, with the amount of O₃ injected in each 5 minute interval determined according to the target O₃ profile. In all solardomes, the O₃ concentration was measured for 5 min every 30 min using two O₃ analysers (Envirotech API 400A) of matched calibration. The seven O₃ concentration treatments were based on an episodic profile recorded at a rural O₃ monitoring site during July 2006 (Aston Hill, Wales, UK, 52°50'N, 30°3'W). The Aston Hill profile was applied as the highest treatment, and for the other treatments the O₃ concentration was reduced by 10 ppb during the peaks (when ozone concentrations were above 45 ppb) and 3 ppb during periods with background O₃ (all other times). The weekly averaged O₃ profiles in 2012 and 2013 are shown in Hewitt et al. (2016) and Harmens et al. (2017), respectively. The weekly averaged O₃ profiles for the 2012 and 2013 are shown in Fig. 1. Night-time and day-time values are similar, as the daily O₃ concentrations above 45 ppb ('peaks') extended beyond midnight in many cases, as can occur in rural areas. The environmental conditions for 2012 and 2013 are shown in Table 1.

All trees received a nutrient solution containing water, ammonium nitrate, macronutrients (P, K, S, Ca, Mg) and various micronutrients (Fe, Mn, Mo, Cu, Zn, B). The base solution of macronutrients (excluding N) and micronutrients was ¼ strength Long Ashton nutrient solution (Hewitt, 1966). Different nitrogen regimes equivalent to 10, 30, 50 and 70 kg N ha⁻¹ yr⁻¹ (N10, N30, N50, and N70) were achieved by varying the amount of ammonium nitrate only. N and nutrient additions were started at the same time as the O₃ exposure and continued for the duration of the experiment. The nutrient treatment was applied in solutions of 200 ml per tree, applied weekly during the growing season and once every three weeks during the winter. Plants were well-watered to maintain soil moisture content near field capacity every one or two days. There were 24 pots in each dome, i.e. six pots per N treatment. O₃ profiles were randomly assigned to the solardomes.

Ambient air temperature, photosynthetic active radiation (PAR), temperature and relative humidity were continuously measured in one solardome, using an automatic weather station (Skye Instruments Ltd., Llandridod Wells, UK). Theta Probes (Delta-T Devices Ltd.,

Cambridge, UK) was used to continuously monitor soil moisture content in a total of 8 pots in 2 domes.

While O₃ treatments are not replicated in this system, numerous previous studies (Hayes et al., 2012; Hewitt et al., 2014, 2016; Harmens et al., 2017) have been published using data from this facility. Rather than replicating a limited number of O₃ treatments, this system allows for the development and assessment of O₃ flux-effect relationships. Hewitt et al. (2016) found no detectable solardome effects in independent measurements of air or leaf temperature. Also, the statistical methods used in this study control for any pseudo-replication in the data.

2.3. Stomatal conductance (g_s) measurements

We used a porometer (AP4, Delta-T, UK) to measure abaxial g_s on the third youngest fully expanded leaf from the top of the canopy at approximately four-weekly intervals during the growing seasons of 2012 and 2013. The maximum stomatal conductance (g_{max}) was determined as the 95-percentile of the data, with data assessed separately under different N loads. To investigate the effects of O₃ and N on g_s under optimal conditions, a subset of the 2012 and 2013 g_s data was taken, using thresholds for the variables temperature, light, soil water potential (SWP) and vapour pressure deficit (VPD) (Table 2). Optimal conditions were defined as temperature, light, SWP and VPD values when g_s (expressed in relative terms, as a proportion of g_{max}) was ≥0.75 (Fig. S1).

2.4. O₃ stomatal fluxes

We used the DO₃SE model (Deposition of O₃ for Stomatal Exchange, version 3.0.5; <https://www.sei-international.org/do3se>) to calculate O₃ stomatal fluxes for sunlit leaves at the top of the tree canopy, following the method of Emberson et al. (2000). Response functions relating to photosynthetic photon flux density (f_{light}), temperature (f_{temp}), water vapour pressure deficit (f_{VPD}), and soil water potential (f_{SWP}) were also parameterized and expressed in relative terms (i.e. varying between 0 and 1 as a proportion of g_{max}). The modifying function related to soil water potential (f_{SWP}) was set to 1, because the soil in the pots was well-watered so as not to be limiting for the duration of the experiment. Response functions relating to phenological stage (f_{phen}), O₃ (f_{O3}) were also set to 1 as there was no evidence that these varied for the duration of the experimental treatments. These parameterizations were applied to the DO₃SE model (Emberson et al., 2000; LRTAP Convention, 2017) to calculate g_s:

$$g_s = g_{max} \times \left[\min(f_{phen}, f_{O3}) \times f_{light} \times \max[f_{min}, (f_{Temp} \times f_{VPD} \times f_{SWP})] \right]. \quad (1)$$

A conversion factor of 0.663 was used to calculate the stomatal flux of O₃ (F_{stO3}) from Eq. (1) to account for the difference in the molecular diffusivity of O₃ compared to that of water vapour (LRTAP Convention, 2017):

$$F_{stO3} = [O_3] \times 0.663 \times g_s. \quad (2)$$

All calculations were performed using hourly averages of all the variables included in the model.

The Phytotoxic Ozone Dose above a threshold flux of 1 nmol m⁻² s⁻¹ (POD_YSPEC) was calculated from the hourly stomatal O₃ fluxes for the duration of O₃ exposure as described in LRTAP Convention (2017). This threshold was used as it was selected by 'expert judgement' in the determination of flux-based critical levels of O₃ for trees within the LRTAP Convention.

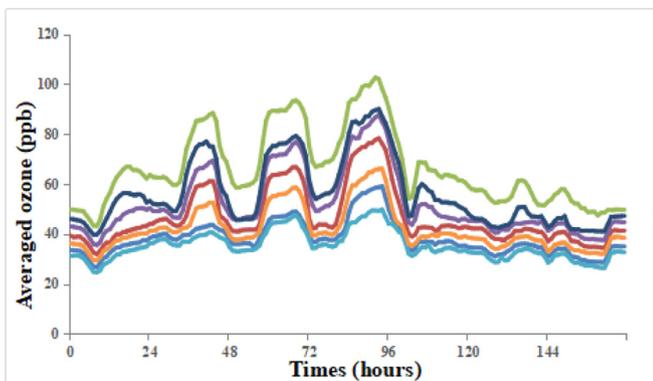


Fig. 1. Weekly averaged O₃ profile for the seven O₃ treatments in 2012 and 2013. The green line (topmost) is the Aston Hill profile (July 2006).

$$POD_YSPEC = \sum \left[(F_{st} - Y) \cdot (3600/10^6) \right] \text{ (mmol m}^{-2} \text{ PLA)} \quad (3)$$

Table 1Summary of O₃ treatments and environmental conditions in 2012 and 2013 (averaged values over the two growing seasons). Daylight was defined as PAR > 200 μmol m⁻² s⁻¹.

O ₃ treatment	1	2	3	4	5	6	7
24 h mean (ppb)	35.2	38.0	41.9	46.6	52.2	56.5	66.2
Daylight mean (ppb)	35.1	38.4	42.5	47.8	53.3	59.4	68.9
AOT40 (ppm h ⁻¹)	1.8	3.4	6.5	11.1	16.5	23.4	33.6
Season hourly maximum (ppb)	69.7	77.0	83.7	98.2	107.3	118.0	125.0
Air temperature (°C)			VPD (kPa)			PAR (μmol m ⁻² s ⁻¹)	
24 h mean	Daylight mean	Mean daily maximum	24 h mean	Daylight mean	Mean daily maximum	Daylight mean	Mean daily maximum
19.1	22.8	30.0	0.61	1.25	2.3	528.5	1065

The value Y (nmol m⁻² PLA s⁻¹) is subtracted from each hourly averaged F_{st} (nmol m⁻² PLA s⁻¹) value only when $F_{st} > Y$, during daylight hours (when global radiation is >50 W m⁻²). The value is then converted to hourly fluxes by multiplying by 3600 and to mmol by dividing by 10⁶ to get the stomatal O₃ flux in mmol m⁻² PLA. In statistical analyses the mean ozone flux for the two growing seasons was used.

2.5. Biomass and stem cross-sectional area

All trees were harvested at the end of the second season in September 2013, and separated into leaves, branches, stems and roots. All plant material was oven-dried at 65 °C for a minimum of 7 days before weighing. As some leaves had dropped off towards the end of the growing season and therefore could not be collected per pot, leaf weights at final harvest were not included in the assessment of treatment effects. The stem base diameter was measured using digital callipers, with two measurements per tree (at 90°) taken at a height of 30 cm above the soil of each pot at the beginning of the experiment in 2012 and at the end of each growing season in 2012 and 2013. The cross-sectional area was calculated based on the two base diameters. The relationship between the stem cross-sectional area and woody biomass (i.e. stem + branches) was determined at harvest to assess whether stem cross-sectional area could be used as a proxy for changes in woody biomass responses to treatments with time.

2.6. Statistical analyses

Sets of linear mixed models (with normal error) were run using the lme4 package (Bates et al., 2015) of R (R Core Team, 2016) to test each hypothesis. To investigate if g_s (under optimal conditions) varied with O₃ and N, ozone (24 h mean for each year) and N were included in the model as fixed, continuous predictor variables, with a covariate to control for changes in g_s with time (month).

To investigate the effect of the O₃ and N treatments (and their interaction) on the biomass of the birch trees after harvest at the end of the second season, models included N and mean annual stomatal O₃ flux (POD₁SPEC; mean for 2012 and 2013) as continuous fixed effects. A covariate of the initial stem cross-sectional area of each tree was added to control for any small initial differences in tree size at the beginning of

the experiment. Models were run with the response variables a) woody; b) root and c) total biomass (woody + root biomass).

To investigate the relationship between the total woody biomass and the stem cross-sectional area at the end of the experiment (at varying levels of O₃ and N), models included interactions between the continuous fixed effect variables of cross-sectional area (2013), stomatal O₃ flux and added N.

To determine the effect of O₃ and N on stem cross-sectional area, stem measurements from the end of the first and second growing seasons were pooled and analysed using linear mixed models. O₃ flux (values for the 2012 and 2013 growing season) and N were included as fixed continuous variables, with a covariate of initial stem cross-sectional area, to control for any differences in initial plant size at the start of the experiment. The models were also re-run for each year separately to test if the slopes for the O₃ and N relationships differed from zero for each season.

For all linear mixed models, a random effect of dome (and pot where needed) were included to control for any pseudoreplication in the data. A model set was created for each analysis and the optimal model chosen using top down selection, by examining values of the Akaike Information Criterion corrected for small sample size (AICc), following Zuur et al. (2009). Likelihood ratio tests were used to obtain p -values for the key variables of interest. Model residual plots were examined for even spread of residuals and normality, and response variables were transformed if necessary (e.g. square root transformation). Where predictor variables were on different scales, variables were standardised before the model was run.

3. Results

3.1. Stomatal conductance (g_s) under optimal conditions

There was a significant positive relationship ($p < 0.01$) between increasing N supply and g_s under optimal conditions (Fig. 2a). O₃ treatment (24 h mean) did not affect g_s ($p = 0.48$) (Fig. 2b). Conditional (fixed and random effects) R² for the optimal mixed model (including nitrogen and month) was 0.336, which increased only slightly with the addition of 24 h mean O₃ to 0.340. Analysis of the g_s measurements made during the exposure showed that the parameterisations of light (f_{light}), temperature (f_{temp}), water vapour pressure deficit (f_{VPD}), and soil water potential (f_{SWP}) for the DO₃SE model were not affected by either N dose (Fig. S2) or O₃ treatment (Fig. S3).

3.2. Biomass dose–response relationships based on POD₁

The stomatal O₃ flux did not affect birch biomass (Fig. 3, $p > 0.05$ for POD₁SPEC in all models). The same result was found when biomass was plotted against the mean O₃ concentrations for 2012 and 2013 (Fig. S4, Supplement). On the other hand, N supply stimulated root, woody and total biomass of birch ($p < 0.001$ in all models). There was no interaction between N and O₃ flux for any of the measures of birch biomass ($p > 0.05$ in all models). When the O₃ and N interaction was added to the

Table 2Thresholds for the variables temperature, light, soil water potential (SWP) and vapour pressure deficit (VPD), used to define optimal conditions for g_s .

Variable	Threshold for optimal conditions
Temperature	10.5–22.5 °C
Light	>127 μmol m ⁻² s ⁻¹
SWP	>−0.25 MPa
VPD	<1.52 kPa

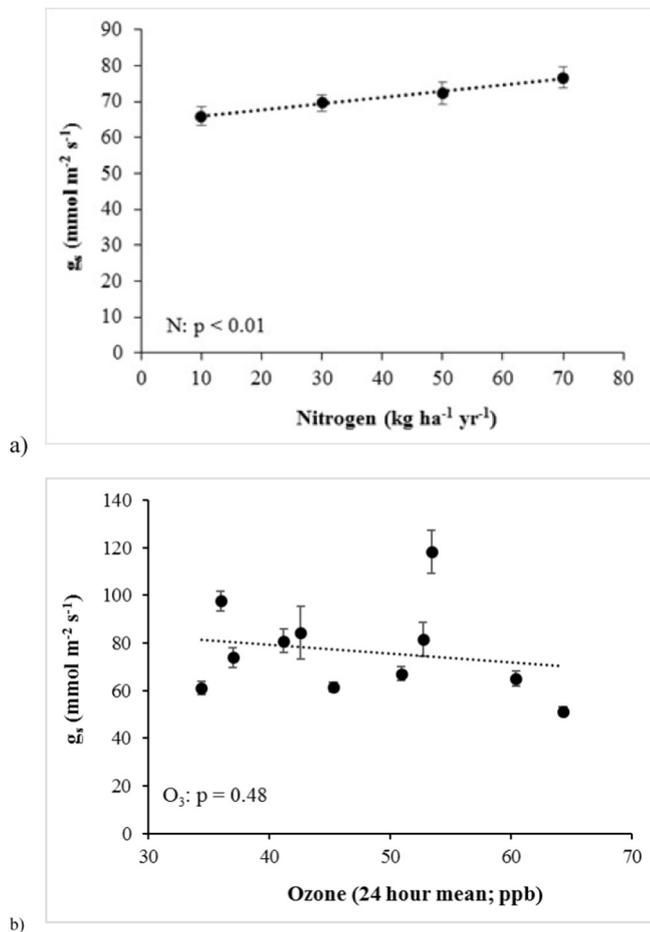


Fig. 2. The effect of a) N supply and b) O_3 (24 h mean for the measurement year) on stomatal conductance (g_s ; for O_3 , Projected Leaf Area) under optimal conditions, measured on silver birch leaves in the growing seasons (May to August) of 2012 and 2013. Plots show raw data and error bars are \pm one standard error. Total number of data points = 621. P -values are from mixed model results.

optimal model for each measurement of biomass, the R^2 of the model increased but minimally (see Table S1).

3.3. Impacts on stem cross-sectional area: Implications for woody biomass changes with time

There was a strong positive relationship between cross-sectional area and woody biomass ($p < 0.001$, conditional $R^2 = 0.88$) after two years of exposure, which was not significantly affected by O_3 flux ($p = 0.4$) or N treatment ($p = 0.6$) (Fig. 4). When the two seasons of data were pooled, mean stem cross-sectional area was not significantly affected by O_3 flux ($p = 0.15$) and the slope of the O_3 flux effect did not change with year of exposure ($p = 0.89$; Fig. 5a). The range of POD_1SPEC values varied between years, with 12–35 $\text{mmol m}^{-2} \text{PLA}$ for 2012 and 9–26 $\text{mmol m}^{-2} \text{PLA}$ for 2013. This variation between years was due to slight differences in O_3 and climatic conditions for the duration of the experiment. When the data was split by year, there was no effect of POD_1SPEC for either season ($p > 0.05$). Enhanced N load significantly ($p < 0.001$) stimulated the mean stem cross-sectional area, and there was a significant difference in the slope of the N effect between the two growing seasons ($p < 0.001$) (Fig. 5b). Added N had no effect on cross-sectional area after the first season ($p = 0.33$) but showed a strong positive relationship after the second growing season ($p < 0.001$). The optimal mixed model (containing the N interaction with season) had a conditional R^2 of 0.91.

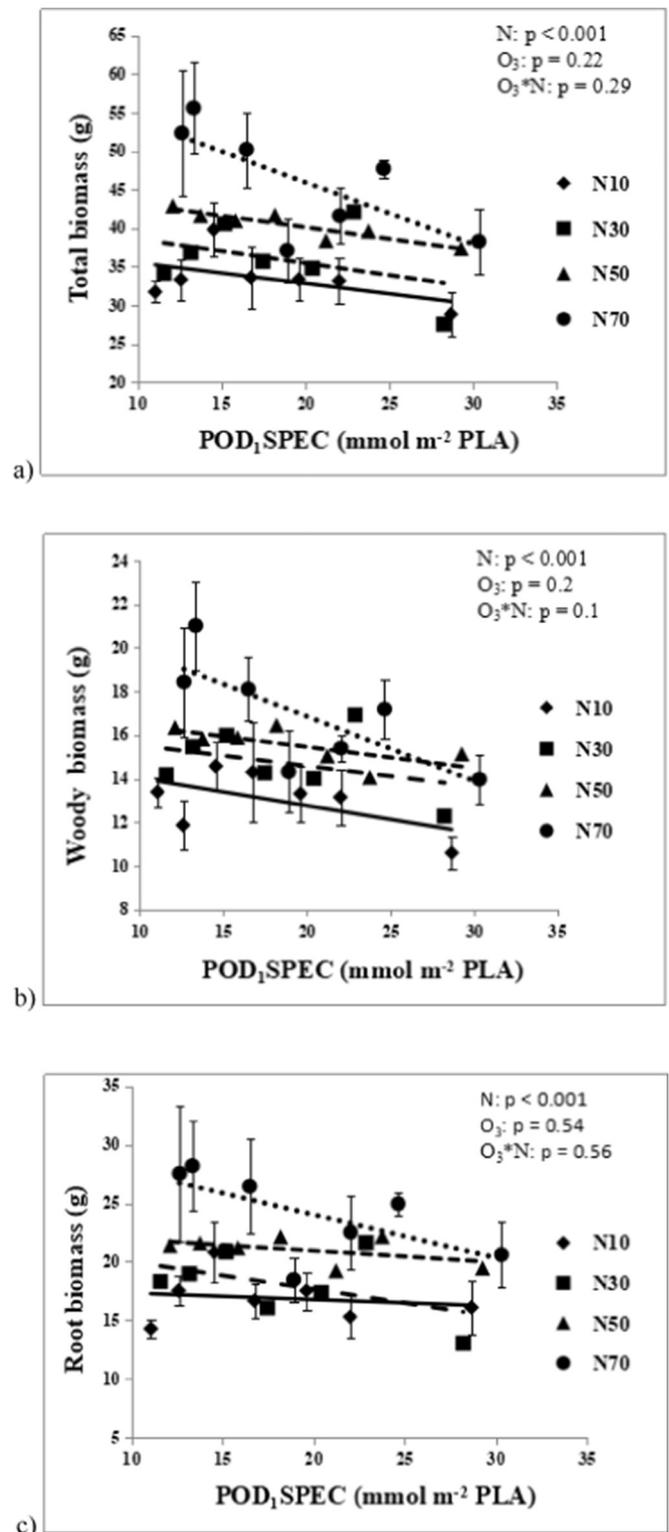


Fig. 3. Mean total (a), woody (b) and root (c) biomass of silver birch plotted against the Phytotoxic Ozone Dose (POD_1SPEC) at different N loads (N10, N30, N50 and N70: 10, 30, 50 and 70 $\text{kg N ha}^{-1} \text{yr}^{-1}$ respectively). Plots show raw data, and error bars are for \pm one standard error. Error bars are only shown for the lowest and highest treatments (N10 and N70) for clarity of the graphs. Total number of data points = 167. P -values are from mixed model results.

4. Discussion

The results presented here extend our understanding of the interactions between O_3 and N on the growth of silver birch and the Phytotoxic

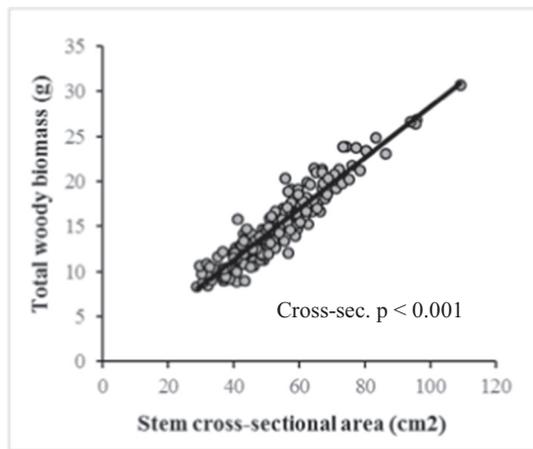


Fig. 4. Relationship between stem cross-sectional area and mean woody biomass for silver birch trees after two years of exposure to O_3 and N treatments. Number of data points = 169. The plot shows raw data and the P-value is from mixed model results.

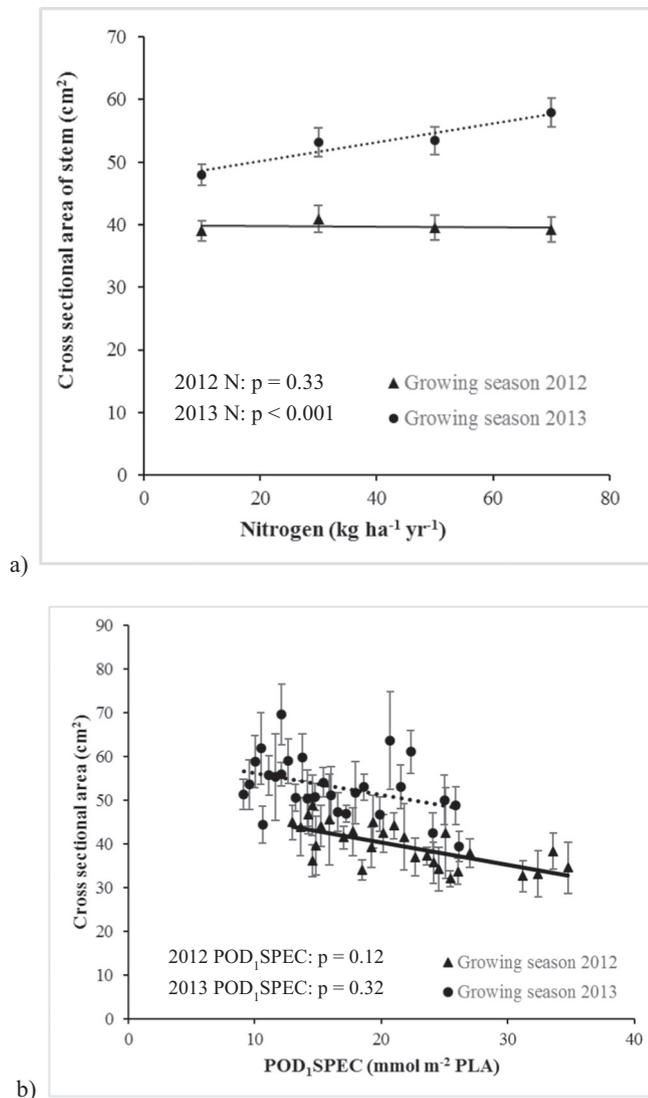


Fig. 5. The effect of a) stomatal O_3 flux (POD_1SPEC) and b) N on cross-sectional area of silver birch trees over 2 growing seasons (2012 and 2013). Plots show raw data, and error bars are for \pm one standard error. Number of data points = 331 (2012: 162 points, 2013: 168 points). P-values are from mixed model results.

Ozone Dose (POD_1SPEC) response based on mean biomass after two years of exposure. So far, few studies have investigated the combined effects of varying O_3 and N depositions on trees using a wide range of O_3 concentrations and N load (Marzuoli et al., 2018; Marzuoli et al., 2016). Our study includes the influence of N on stomatal O_3 fluxes and flux-effect relationships.

4.1. Ozone effects

There was no significant O_3 flux effect found on birch biomass after two seasons of O_3 exposure, and on the stem cross-sectional area after the first or second growing season. For both biomass and stem cross-sectional area (season 1 and 2), mean values declined with O_3 flux, but there was considerable variation between replicate trees. One main reason for a decrease in biomass induced by elevated O_3 could be that elevated O_3 negatively affects leaf photosynthetic parameters. This has previously been reported for several tree species, for example, *Betula pendula* (Wittmann et al., 2007; Harmens et al., 2017) and *Fagus crenata* (Yamaguchi et al., 2007). The duration of O_3 exposure and the O_3 concentration applied in experiments is likely to affect the impact of O_3 on *Betula pendula*. A compilation of data for *Fagus sylvatica* L and *Betula pendula* for different experiments conducted in Europe indicated a high variation in the total tree biomass response to O_3 up to a POD_1SPEC of ca. $30 \text{ mmol m}^{-2} \text{ yr}^{-1}$ (as applied in the current study), with a reduction in total biomass becoming more pronounced at higher POD_1SPEC (LRTAP Convention, 2017). Other studies using controlled conditions reported that growth and/or biomass of *Betula pendula* is increased by elevated O_3 treatments based on ozone concentration data (e.g. Oksanen and Holopainen, 2001; Yamaji et al., 2003), and increased for other species as well, for example, *Fagus sylvatica* and *Quercus robur* (Pollastrini et al., 2010) and *Liriodendron tulipifera* L (Rebeck and Scherzer, 2002). In contrast, Oksanen (2003) found that elevated O_3 did not affect the growth of potted *Betula pendula* saplings exposed to $1.6 \times$ ambient O_3 for one growing season, whereas the open-soil-grown *Betula pendula* trees fumigated with elevated O_3 for six growing seasons showed a 3–38% reduction in shoot growth. A lack of significant impacts of elevated O_3 concentrations on growth parameters were also reported for other tree species, for example, *Fagus sylvatica* (Bortier et al., 2000), *Picea abies* seedlings (Thomas et al., 2005) and *Cinnamomum camphora* (Feng et al., 2011). In agreement with our study, Marzuoli et al. (2018) observed no statistical significant impact of O_3 on growth of *Carpinus betulus* L based on an O_3 flux assessment.

For mature trees under field conditions, Braun et al. (2017) found that O_3 flux was significantly and negatively correlated with basal area increment both in beech (*Fagus sylvatica* L.) and Norway spruce (*Picea abies*), using observational data of mature forests for a 30-year time series. It is suggested that tree age may make a difference on the O_3 -flux effect on growth, therefore, we recommend to investigate the interactive effects of O_3 and N further in long-term free air O_3 exposure studies with mature birch trees. For example, Matyssek et al. (2010) reported a reduction of 44% in whole-stem growth of adult beech trees exposed for eight years to elevated O_3 in a free-air O_3 fumigation study. Our study indicates a strong positive relationship between stem cross-sectional area and woody biomass, suggesting that O_3 -effect on stem cross-sectional area can be used as a proxy for O_3 -effect on biomass.

In our study, elevated 24 h mean O_3 concentration did not affect g_s (under optimal conditions) for birch grown in 2012 and 2013, which is consistent with the g_s response in the same experiment measured only in 2013 (Harmens et al., 2017). Wittig et al. (2007) reported that no significant effects of elevated O_3 concentrations on g_s were detected for *Abies*, *Betula*, *Prunus*, *Picea* and *Pinus* species. Dai et al. (2017) also reported that elevated O_3 concentrations did not affect g_s among 13 *Betula pendula* cultivars. However, other studies have found that enhanced O_3 concentrations decreased g_s for the different tree species (e.g. Paoletti and Grulke, 2005; Calatayud et al., 2011; Feng et al., 2011; Marzuoli et al., 2016). The different g_s response to elevated O_3 concentrations

may be due to variations in O₃ exposure time/concentrations, species/cultivars applied in the experiments, leaf/tree age tree and environmental factors such as soil moisture. These factors may also influence stomatal sluggishness in response to O₃, causing a delay in the stomatal response to environmental factors and a potential increase in O₃-sensitivity (Paoletti and Grulke, 2010). The effects of elevated O₃ concentrations on g_s, especially for trees grown in open air conditions in summer are difficult to interpret due to large temporal changes in g_s (Oksanen, 2003).

4.2. Nitrogen effects

High N supply enhanced the growth of birch saplings as shown by the increase in mean woody, root and total biomass at high N regardless of O₃ treatment. In the current study, the highest biomass was found at 70 kg N ha⁻¹ yr⁻¹ and at low O₃ exposure. The stimulation of biomass by high N supply is in agreement with the enhanced chlorophyll content and photosynthetic capacity (V_{cm} and J_{max}) of birch leaves in the same study (Harmens et al., 2017). The stimulation of g_s by high N may result from the changes in hydraulic conductivity (Pivovarov et al., 2016), or nitric oxide (NO) emission as a side-reaction of the nitrate assimilation process (Wildt et al., 1997). Hence, the increase in total N deposition may lead to an enhancement of C sequestration by plants, as reported for other tree species (Feng et al., 2011; Marzuoli et al., 2016).

Interestingly, when we used the cross-sectional area as a proxy for the biomass, enhanced N loads stimulated the mean stem cross-sectional area significantly only in 2013, hence there seemed to be a lag-time or cumulative effect in the growth response of the birch saplings to elevated N. Such a lag time has not been reported in other studies that investigated the interactive impacts of O₃ and N on the biomass of trees (Thomas et al., 2005; Marzuoli et al., 2016, 2018).

This study showed an increase in g_{max} with increasing N load. This is in agreement with O₃ and N interaction studies in trees by Marzuoli et al. (2018) and has important implications when modelling O₃ impacts and performing risk assessments at a regional or global scale. The O₃ uptake could be affected by N load, however, the combined impact of O₃ and N is less clear (see Section 4.3 below).

4.3. Ozone and nitrogen interactions

Our results confirmed the hypothesis that there were no significant interactions between the impacts of O₃ flux and N on *Betula pendula* biomass. The conclusion is consistent with the results of Harmens et al. (2017), who found no significant interactions between N and O₃ on photosynthetic parameters of *Betula pendula*. However, it should be noted that in the current study young trees were only exposed to elevated O₃ and N for two years. Longer-term studies might show different results, particularly if there would be a delayed response to treatment. Feng et al. (2011) also reported no significant effects of N loads (30 and 60 kg ha⁻¹ yr⁻¹) on the O₃ sensitivity of *Cinnamomum camphora*. However, Pääkkönen and Holopainen (1995) found that high N loads might reduce O₃ sensitivity in *Betula pendula*, with experiments conducted at two O₃ exposure levels (ambient and ca. 1.6 × ambient) and three N loads (37, 74 and 150 kg N ha⁻¹ yr⁻¹). For other tree species, different results of the interaction between O₃ concentrations and N load were reported. High N supply ameliorated (e.g. Landolt et al., 1997; Handley and Grulke, 2008) or exacerbated (e.g. Utriainen and Holopainen, 2001; Watanabe et al., 2012) the negative impact of O₃. For *Pinus sylvestris*, a significant interaction between O₃ and N was found only in the third experimental year (Utriainen and Holopainen, 2001), suggesting that interactive impacts due to O₃ and N are dynamic and change over time. A delayed response could potentially also be due to a lag effect as found for N in our study. The variation in results reported for different tree species and experiments may therefore be

due to differences in O₃-sensitivity of tree species, O₃ and/or N exposure concentrations and soil/climate conditions (Mills et al., 2016) and/or duration of exposure. Variations in exposure between experiments result in differences in accumulated O₃ exposure and therefore effects. It remains unclear whether high N deposition might increase O₃ sensitivity of tree species by stimulating g_s and/or specific leaf area (Bassin et al., 2009) or decrease O₃ sensitivity of trees species through enhanced detoxification of O₃.

4.4. Extrapolation to field conditions

In this study *B. pendula* was exposed to combined O₃ and N treatments in controlled conditions in pots. This provides clear indications for improved ecological understanding of these impacts, however, it remains important to validate the magnitude of these impacts in field conditions and using mature trees to further assess the risks to forest ecosystems. Epidemiological analysis of mature trees across O₃ and N gradients have previously been used to confirm the results from controlled studies using young trees (Braun et al., 2014), although these studies also have to be interpreted with care due to confounding factors. Thus a combination of controlled studies and field studies are required to further improve understanding of combined impacts of O₃ and N.

5. Conclusion

The results indicate that birch root, woody and total biomass were not affected by the Phytotoxic Ozone Dose (POD₁SPEC), and enhanced N supply stimulated biomass production independently of POD₁SPEC (i.e. there were no POD₁SPEC × N interactions). Enhanced N supply increased stomatal conductance under optimal growth conditions, but elevated O₃ exposure (24 h mean) did not affect stomatal conductance. Effects of stem cross-sectional area can be used as a proxy for the biomass response to the treatments with time. The effect of added N was found to vary with growing season, with no effect after the first growing season and a strong positive effect after two years. This suggests that there may be a lag-time in the growth response of the birch saplings to elevated N, which has not been reported before. There was no negative effect of O₃ flux (POD₁SPEC) on stem cross-sectional area after either the first or second growing season. High levels of variation were seen between tree replicates. Further studies, including epidemiological studies, are needed to explore the interactive effects between elevated O₃ and N supply, particularly in long-term studies with mature trees under field conditions.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2019.01.092>.

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