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Contact CEH NORA team at
noraceh@ceh.ac.uk

1 **Leaf traits and photosynthetic responses of *Betula pendula* saplings to a range of**

2 **ground-level ozone concentrations at a range of nitrogen loads**

3

4 Harry Harmens[1,*], Felicity Hayes[1], Katrina Sharps[1], Gina Mills[1], Vicent Calatayud[2]

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6 [1] Centre for Ecology & Hydrology, Environment Centre Wales, Deiniol Road, Bangor,

7 Gwynedd LL57 2UW, UK. hh@ceh.ac.uk; fhay@ceh.ac.uk; katshar@ceh.ac.uk;

8 gmi@ceh.ac.uk

9 [2] Fundación CEAM, c/Charles R. Darwin 14, Parque Tecnológico, 46980 Paterna, Valencia,

10 Spain. vicent@ceam.es

11

12 * Corresponding Author: Harry Harmens

13   E-mail address: hh@ceh.ac.uk

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15

16 **Summary**

17 Ground-level ozone (O_3) concentrations and atmospheric nitrogen (N) deposition rates have

18 increased strongly since the 1950s. Rising ground-level O_3 concentrations and atmospheric N

19 deposition both affect plant physiology and growth, however, impacts have often been

20 studied in isolation rather than in combination. In addition, studies are often limited to a

21 control treatment and one or two elevated levels of ozone and/or nitrogen supply. In the

22 current study, three-year old *Betula pendula* saplings were exposed to seven different O_3

23 profiles (24 hour mean O_3 concentration of 36 – 68 ppb in 2013, with peaks up to an average

24 of 105 ppb) in precision-controlled hemispherical glasshouses (solardomes) and four different

25 N loads (10, 30, 50 or 70 kg N ha^{-1} y^{-1}) in 2012 and 2013. Here we report on the effects of

26 enhanced O_3 concentrations and N load on leaf traits and gas exchange in leaves of varying

27 age and developmental stage in 2013. The response of leaf traits to O_3 (but not N) vary with

28 leaf developmental stage. For example, elevated O_3 did not affect the chlorophyll content of

29 the youngest fully expanded leaf, but it reduced the chlorophyll content and photosynthetic

30 parameters in aging leaves, relatively more so later than earlier in the growing season.

31 Elevated O_3 enhanced the N content of senesced leaves prior to leaf fall, potentially affecting

32 subsequent N cycling in the soil. Enhanced N generally stimulated the chlorophyll content

33 and photosynthetic capacity. Whilst elevated O_3 reduced the light-saturated rate of

34 photosynthesis (A_{sat}) in aging leaves, it did not affect stomatal conductance (g_s). This

35 suggests that photosynthesis and g_s are not closely coupled at elevated O_3 under-light

36 saturating conditions. We did not observe any interactions between O_3 and N regarding

37 photosynthetic parameters ($V_{c,max}$, J_{max}, A_{sat}), chlorophyll content, g_s, N content in senesced

38 leaves and leaf number. Hence, the sensitivity of these leaf traits to O_3 in young silver birch

39 trees is neither reduced nor enhanced by N load.

40

41 **Keywords**: Air pollution; chlorophyll content; leaf age; nitrogen content; photosynthetic

42 capacity; stomatal conductance.

43

44 **Abbreviations**

45 A_{sat} = light-saturated rate of photosynthesis at ambient CO_2 (390 ppm)

46 A/Ci curve = plot of net photosynthesis (A) against leaf internal CO_2 concentration (Ci)

47 AICc = Akaike Information Criterion, corrected for small sample size

48 g_s = stomatal conductance

49   ID = identity

50   J_{max} = maximum rate of electron transport

51   LMM = linear mixed model

52   ppb = parts per billion

53     $V_{c,max}$ = maximum rate of carboxylation

54

55   **Introduction**

56   Tropospheric ozone (O_3) concentrations have been increasing from a background of ca. 10-15

57   ppb in the early 20th century, due to increased emissions of O_3 precursors from anthropogenic

58   sources (Cooper et al., 2014; Royal Society, 2008; Solberg et al. 2005; Volz and Kley, 1988).

59   In Europe, background concentrations have roughly doubled between 1950 – 2000, followed

60   by a decade with no further rise or even a reduction at some sites (Cooper et al., 2014; Parrish

61   et al., 2012). Present day annual average background concentrations are levelling off at ca. 30

62   – 40 ppb in Europe and North America, (Cooper et al., 2014; Royal Society, 2008), but are

63   still rising significantly in East Asia (Cooper et al., 2014). In addition, atmospheric

64   concentration and deposition of reactive nitrogen (N) has increased strongly since the 1950s

65   with the application of the Haber-Bosch process to produce N fertilizers on an industrial scale

66   (Sutton et al., 2011). Despite the rise in both ground-level O_3 concentrations and atmospheric

67   N deposition since the 1950s, impacts on vegetation have often been studied in isolation

68   rather than in a combination of both factors. The two pollutants are closely interlinked as a

69   major pathway for the formation of tropospheric O_3 is the photochemical reaction with N

70   oxides (Royal Society, 2008).

71 Generally, O_3 tends to have a negative impact on sensitive plant species, resulting in a decline

72 in photosynthesis, biomass production or crop yield, and accelerated senescence (Ainsworth

73 et al., 2012). A meta-analysis by Wittig et al. (2007) showed that the increase in O_3 since the

74 industrial revolution has been responsible for a reduction in photosynthesis of approximately

75 11% in trees, which reduced tree productivity by approximately 7% (Wittig et al. 2009).

76 Further reductions were observed with a rise in O_3 concentration above the current ambient

77 level (Wittig et al., 2007, 2009). Partly due to the successful implementation of air pollution

78 abatement policies in Europe, reductions in O_3 precursor emissions have resulted in a decline

79 in peak O_3 concentrations in the summer in recent years at some sites across Europe

80 (Simpson et al., 2014; Torseth et al., 2012). However, mean annual O_3 concentrations have

81 hardly changed in recent decades (EMEP, 2016) due to a rise in the frequency of low-range

82 O_3 concentrations (Simpson et al., 2014). Background O_3 concentrations are currently at

83 levels where they chronically affect sensitive plant species (Mills et al., 2011a).

84 In contrast, N tends to have a fertilising effect on plant growth and photosynthesis in areas

85 where N is a limiting nutrient. However, above the critical load or level, the impact of N on

86 sensitive species will become negative in most species (Bobbink and Hettelingh, 2011; Cape

87 et al., 2009). For example, forest observations in Switzerland suggested an increase in

88 phosphorus limitation with increasing N deposition, resulting in a decline in growth in *Fagus*

89 *sylvatica* (Braun et al., 2010). N pollution is now a major threat to terrestrial biodiversity

90 (Bobbink et al., 2010; Dise et al., 2011).

91 Relatively few studies have investigated the combined impacts of both O_3 and N on

92 vegetation at realistic ozone concentrations (Mills et al., 2016). Evidence suggests that O_3 and

93 N can have both synergistic and antagonistic effects on tree species. Pääkkönen and

94 Holopainen (1995) and Landolt et al. (1997) showed that high N supply ameliorates the

95 negative impact of O_3 in *Betula pendula*. Similar responses were reported for *Larix kaempferi*

96 (Watanabe et al., 2006), *Populus tremula x Populus tremuloides* (Häikiö et al., 2007) and

97 *Quercus kelloggii* (Handley and Grulke, 2008). However, enhanced N supply increased

98 growth sensitivity to O_3 of *Pinus sylvestris* (Utriainen and Holopainen, 2001b) and *Fagus*

99 *crenata* (Wanatabe et al., 2012; Yamaguchi et al., 2007) seedlings. The effect of N supply on

100 the sensitivity to O_3 was not significant for *Picea abies* (Thomas et al., 2005; Utiainen and

101 Holopainen, 2001a) or *Quercus robur* seedlings (Marzuoli et al., 2016). Complex interactions

102 were observed between O_3 and N impacts on growth of young trees of *Fagus sylvatica*

103 (Thomas et al., 2006). In a recent review, Mills et al. (2016) showed that the beneficial effect

104 of N on root development was lost at high O_3 exposure whilst the effects of increasing O_3 on

105 root biomass became more pronounced as N supply increased.

106 Many previous studies investigating the interactive impacts of O_3 and N on trees have been

107 limited to two to three O_3 and N exposure levels. In the current study, three year old silver

108 birch (*Betula pendula*) seedlings were exposed to seven O_3 regimes (24 h mean of 36 – 68

109 ppb) and four levels of N load (10 – 70 kg N ha^{-1} y^{-1}) for two years (2012 and 2013). Silver

110 birch is known to be sensitive to O_3 (LRTAP Convention, 2015; Mills et al., 2011b) and has a

111 widespread distribution in Europe, from northern Finland to Italy and from Ireland to

112 Kazakhstan. We investigated whether O_3 sensitivity of leaf traits was affected by N supply.

113 We report in detail on the impacts of O_3 and N on chlorophyll content, stomatal conductance

114 (g_s), leaf photosynthetic traits, N concentration in senesced leaves and leaf number during the

115 second year of exposure. Based on the cumulative nature of O_3 impacts on plants (LRTAP

116 Convention, 2015), we hypothesised that negative effects of O_3 on leaf traits will increase

117 with time of exposure, with older leaves being more affected than younger leaves. Based on

118 previously reported results (Landolt et al., 1997; Pääkkönen and Holopainen, 1995) for silver

119 birch, we hypothesised that enhanced N will reduce negative effects of O_3.

120

121

Material and methods

Plant material, experimental site and treatments

On 13th April 2012, three-year old trees of silver birch (*Betula pendula*, UK provenance) were planted in 6.5 litre pots (diameter 21 cm, height 25 cm) filled with John Innes No. 1 compost (J. Arthur Bowers). Mean height of the trees when planted was 68 cm and they had not branched yet. All plants were inoculated with mycorrhiza (obtained from Buckingham Nurseries, UK). The pots were randomly distributed between seven hemispherical glasshouses (solardomes; 3 m diameter, 2.1 m height) at Abergwyngregyn nr. Bangor, North Wales, United Kingdom. After an acclimation period in the solardomes, treatments were started on 30th April 2012. Plants were exposed to O_3 based on an episodic profile recorded at a rural O_3 monitoring site during July 2006 (Aston Hill, Wales, UK, 52°50'N, 3°03'W). The Aston Hill profile was applied as the highest treatment, and for the other treatments the O_3 concentration was reduced by 10 ppb during the peaks and 3 ppb during periods with background O_3 (Figure 1; Hewitt et al., 2016). The profiles cover a range of concentrations found in Europe and this is the only facility that has sufficiently sophisticated control of O_3 to be able to deliver O_3 treatments that increase in small increments. The 24 hour mean concentration was between 36 and 68 ppb in 2013, with peaks up to on average 105 ppb (Figure 1). Plants were exposed to O_3 during the growing season, starting on 30th April and finishing on 12th October in 2012, and starting on 17th May and finishing on 16th September in 2013, when the trees were harvested. At harvest, the roots didn't show any signs of being pot-bound. Between 17th October 2012 and 14th May 2013, the trees were kept outside under ambient conditions. The solardomes were ventilated at a rate of two air changes per minute and charcoal-filtered air was injected with controlled amounts of O_3. O_3 was provided by a G11 O_3 generator (Ozone Industries, UK) equipped with oxygen concentrator (Dryden Aqua,

146   UK). Concentrations were determined by a computer-controlled O_3 injection system (Lab

147   VIEW version 8.6, National Instruments, Texas, US). O_3 was distributed to each solardome

148   via polytetrafluoroethylene (PTFE) tubing, with the concentration inside each solardome

149   measured for 5 min every 30 minutes using two O_3 analyzers (400a, Enviro Technology

150   Services, Stroud, UK) of matched calibration. Four N treatments were applied weekly in each

151   solardome as ammonium nitrate, in 200 ml water containing other macronutrients (P, K, S,

152   Ca, Mg) and various micronutrients (Fe, Mn, Mo, Cu, Zn, B). N addition rates were 10, 30,

153   50 and 70 kg N ha^{-1} yr^{-1}. N and nutrient additions were started at the same time as the O_3

154   exposure (see above) and continued for the duration of the experiment, including in the

155   winter. Plants were watered twice a week or as required to maintain soil moisture content

156   near field capacity. Each dome contained 24 pots, i.e. six pots per N supply. The O_3 profiles

157   were assigned randomly to the solardomes. The weekly O_3 profiles for 2012 were described

158   by Hewitt et al. (2014), the weekly O_3 profiles for 2013 were very similar and are shown in

159   Figure 1. A summary of O_3 treatments and climate conditions for 2013 is provided in Table 1.

160   In one solardome, ambient air temperature, photosynthetically active radiation (PAR),

161   temperature and relative humidity were continuously monitored by an automatic weather

162   station (Skye Instruments Ltd, Llandridod Wells, UK) and soil moisture content was

163   continuously monitored using Theta Probes (Delta-T Devices Ltd, Cambridge, UK).

164   Although O_3 treatments were not replicated, numerous previous studies (Hayes et al., 2012;

165   Hewitt et al., 2014, 2016; Mills et al., 2009) have established the statistical validity of

166   conducting un-replicated experiments using the solardome facility, and no solardome effects

167   were detectable in independent measurements of air or leaf temperature (Hewitt et al., 2016).

168

169   *Chlorophyll content and stomatal conductance (*g_s*) measurements*

170   Chlorophyll content (determined non-destructively using CCM200, ADC, UK, and expressed

171   as an index) and g_s (AP4 Porometer, Delta T, UK) measurements were conducted at about

172   four-weekly intervals on the third leaf (youngest fully expanded leaf) from the top of the

173   canopy throughout the growing season in five O_3 treatments. The maximum g_s for each

174   treatment was determined as the 95-percentile. Silver birch is heterophyllous (Clausen and

175   Kozlowski, 1965), i.e. has early and late leaves which started to flush in April and July

176   respectively in our study. In addition, the chlorophyll content was determined on leaves used

177   for leaf gas exchange measurements (including g_s), i.e. a designated leaf throughout the

178   growing season (see below), and determined near the end of the growing season on the eight

179   leaves on the highest branch of trees (six replicates per treatment).

180

181   *Leaf gas exchange measurements*

182   Youngest fully expanded leaves were identified on 21 May 2013. Gas exchange

183   measurements were conducted on these designated leaves exposed to the lowest (24 hr mean

184   = 36 ppb) and highest O_3 concentration (24 hr mean = 68 ppb) and supplied with the lowest

185   (10 kg N ha^{-1} yr^{-1}) and highest N rate (70 kg N ha^{-1} yr^{-1}). Measurements on the designated

186   leaves were conducted at 6-weekly intervals during the following periods: 10 - 14 June, 22 -

187   25 July and 2 - 5 September 2013. To determine photosynthetic capacity of birch leaves, the

188   response curve of the net photosynthetic rate (A) to the intercellular CO_2 concentration (Ci),

189   i.e. the A/Ci curve, was constructed using a portable gas analyser with PLC6 universal leaf

190   cuvette (Ciras-2, PPsystems). The measurements were conducted at light saturation

191   (photosynthetic photon flux density = 1500 μmol m^{-2} s^{-1}; LED light source), a leaf

192   temperature of 22 °C (determined by built-in infrared temperature sensor), and a leaf-to-air

193   vapour pressure deficit of 0.6 – 1.1 kPa. The A/C_i curve was constructed by measuring A at

194   11 air CO_2 concentrations between ca. 50 and 1900 μmol mol^{-1}. A_{sat} and associated g_s were

195 determined at ambient CO_2 (390 µmol mol^{-1}). Leaves were inserted into a broad leaf chamber

196 (area = 2.5 cm^2) and allowed to stabilise for 15 min at growth CO_2. At each CO_2

197 concentration, gas exchange parameters were recorded after readings had stabilised (ca. 2 – 3

198 min). The maximum rate of carboxylation ($V_{c,max}$) and the maximum rate of electron

199 transport (J_{max}) were calculated from the A/Ci curve according to the methodology described

200 by Sharkey et al. (2007). $V_{c,max}$ and J_{max} were calculated for a leaf temperature of 25 ^{o}C, using

201 the measurement leaf temperature and atmospheric pressure as input parameters. The ratio

202 between J_{max} and $V_{c,max}$ was also determined. In addition to the A_{sat} measurements from the

203 A/Ci curve, A_{sat} was also measured in late August/early September at all six O_3

204 concentrations at low and high N supply (4 - 5 replicates per treatment).

205

206 *Leaf number and N content of leaves*

207 Leaf number was counted on four days during the 2013 growing season: 14 May, 31 July, 2

208 and 24 September (day before harvest). Throughout September, leaves that were ready to

209 drop off were collected for N analysis. The N content of mature, green leaves was also

210 determined in the middle of July. The leaves were dried at 70 ^{o}C, ground with a (Cyclotec)

211 and 10 mg was weighed in tin capsules for analysis. Leaves were analysed for N content at

212 the Chemical Analysis Laboratory, Forest Research, UK. The total N content was determined

213 by dry combustion (ISO 13878, using Carbo Erba CN analyser, Flash1112 series).

214

215 *Statistical analyses*

216 The precise O_3 control system used in the solardomes allowed relatively small changes in O_3

217 profile to be simulated, facilitating dose-response analyses rather than just comparison of the

218 impacts at low and high ozone exposure. The large number of O_3 treatments allowed for

219 effects of N on the slope of dose-response functions to be determined. We note that the lack

220   of treatment replication may raise concerns, however, we believe that the benefit of using

221   more treatments outweighs this limitation, as published previously (Hayes et al., 2012;

222   Hewitt et al., 2014, 2016; Mills et al., 2009). Air flow rates were matched between

223   solardomes, and recorded climatic conditions did not vary significantly from solardome to

224   solardome (Hewitt et al., 2016). For all linear mixed models (LMMs; normal error) applied

225   below, a model set was created using the package lme4, v1.1-7 (Bates et al., 2015) in R

226   version 3.2.3 (R core team, 2014), sequentially removing interactions and predictor variables

227   from the global model, and model selection was carried out by examining the change in

228   Akaike Information Criterion, corrected for small sample size (AICc). The model with the

229   lowest AICc value is optimal, with models differing in 2 - 7 AICc units from the best model

230   having little empirical support (Burnham and Anderson, 2002). Bayesian Information

231   Criterion (BIC) values, which penalise the number of model parameters more strictly, were

232   also examined. For the optimal model selected, p-values were obtained for each term in the

233   model using the R package lmerTest, v2.0-20 (Kuznetsova et al., 2014). For all models,

234   statistical assumptions (normality and even spread of residuals) were checked using residual

235   plots. Response variables were transformed where necessary (log or square root).

236

237   <u>Chlorophyll content and stomatal conductance (g_S)</u>

238   To investigate factors influencing chlorophyll content of the youngest fully expanded leaf, the

239   LMM included added N, 24 hour mean O_3 concentration (2013, n = 5 levels) and

240   measurement period as fixed, continuous variables and the random effects of dome and plant

241   identity (ID). As there was a suggestion of a quadratic relationship between the chlorophyll

242   content and time, a quadratic time term was included. For eight leaves on the top branch (near

243   the end of the growing season), a further LMM with chlorophyll content as the response

244   variable, the continuous fixed effects of 24 hour mean O_3 (2013, n = 4) and leaf number, N

245   (factor: high and low), and a random effect of plant ID was also run. A quadratic O_3 term was

246   included to allow for the suggested non-linear relationship between chlorophyll content and

247   O_3. The LMM for g_s included added N, 24 hour mean O_3 concentration (2013, n = 5 levels)

248   and measurement period as fixed, continuous variables. A quadratic term for measurement

249   period and random effects of dome and plant ID were also included. Extreme outliers in the

250   g_s data (thought to be due to measurements from damp leaves) were removed using the

251   median absolute deviation (mad) method (using a rejection criteria of median $\pm$ 5.2 mad).

252

253   <u>Leaf gas exchange measurements</u>

254   For each parameter (e.g. $V_{c,max}$, J_{max}) of the designated leaf, a LMM including the fixed

255   effects of time, N and O_3 (as factors, low and high), and the random effect of plant ID, was

256   applied. The relationship between A_{sat} and chlorophyll content and A_{sat} and g_s was tested

257   using LMMs, including the fixed effects of O_3 and N and the random effect of plant ID. The

258   A_{sat} measurements taken in late August were analysed using a LMM with a continuous fixed

259   effect for 24 hour mean O_3 (2013, n = 6 levels), N as a factor (high and low), and a random

260   effect of dome. Two further A_{sat} model sets were also run, with chlorophyll content and g_s

261   included as continuous fixed predictors.

262

263   <u>N content of leaves and leaf number</u>

264   The effect of O_3 and added N on the N content of mature (mid July) and senesced leaves

265   (September) was analysed using a general linear model (glm) containing an interaction

266   between the continuous variables of added N and 24 hour mean O_3 concentration (2013, n = 7

267   levels). As the variance explained by the random effect of dome was zero, a mixed effects

268   model was not used. To investigate changes in leaf number, a generalised linear mixed effect

269   model (GLMM), with leaf count as the response variable and a Poisson error distribution,

270   was run using the glmmPQL function in package MASS (Venables and Ripley, 2002) to deal

271   with over-dispersion in the data. Fixed effects included 24 hour mean O_3 concentration

272   (2012-2013, n = 7 levels) and added N as continuous variables and month as a factor, while

273   dome and plant ID were included as random effects. As the glmmPQL function does not

274   provide AIC values, p-values and estimated standard errors were used to determine which

275   fixed effects were needed in the model.

276

277

278   **Results**

279   *Chlorophyll content and* g_s *of the youngest fully expanded leaf*

280   The chlorophyll content of the third leaf (youngest fully expanded leaf) declined in the first

281   part of the growing season but then increased again between 23 July and 23 August due to

282   new growth of late leaves ($p < 0.001$ for 'Time'; Figure 2, Table 2). The third leaf developed

283   from new growth (late leaf) had a similar chlorophyll content as the third leaf near the start of

284   the season (early leaf). Remobilisation of N within the trees is likely to have started as older

285   leaves began to drop off from the middle of July onwards as new leaves developed.

286   Throughout the season, the chlorophyll content was higher at 70 than at 10 kg N ha^{-1} y^{-1} ($p <$

287   0.001; Figure 2, Table 2), with the difference being lowest at the end of July and highest at

288   the end of August. O_3 concentration did not affect the chlorophyll content of the youngest

289   fully expanded leaf. While visual examination of the raw data suggested that stimulation of

290   the chlorophyll content at high N supply was diminished at the highest O_3 exposure at the end

291   of May, two or three-way interactions were not observed (Table 2). The g_s changed with time

292   and was highest at the end of August (late leaves) compared to earlier in the year (early

293   leaves); g_s was not affected by O_3 or N treatment and there were no significant two or three-

294   way interactions (Figure 3; Table 2). The maximum g_s reached its highest value at 50 kg N

295 ha^{-1} y^{-1}, and was 256, 307, 339 and 337 mmol H$_2$O m^{-2} s^{-1} at 10, 30, 50 and 70 kg N ha^{-1} y^{-1}

296 respectively.

297

298 *Chlorophyll content of leaves along the first top branch near the end of the growing season*

299 To assess the response to treatment of the chlorophyll content of leaves of different

300 developmental stages, the chlorophyll content along a branch was measured late in the season

301 (4th September) when a wide range of chlorophyll content was present. The chlorophyll

302 content was higher at 70 kg N ha^{-1} y^{-1} than at 10 kg N ha^{-1} y^{-1} ($p < 0.001$) and decreased with

303 increasing leaf age (i.e. leaf number; $p < 0.001$; Figure 4, Tables 3 and S1). The effect of O$_3$

304 was dependent on leaf age, with young leaves showing stimulation in the chlorophyll content

305 (although the highest chlorophyll content was already reached in the youngest leaf at 39 ppb

306 O$_3$) and medium-aged leaves showing no clear O$_3$ effect. In the oldest leaves (leaf 8), the

307 chlorophyll content was highest at 36 ppb O$_3$ and 70 kg N ha^{-1} y^{-1} and lowest at 68 ppb O$_3$

308 and 10 kg N ha^{-1} y^{-1}. At 68 ppb O$_3$ the chlorophyll content declined faster with increasing leaf

309 age than at 36 ppb O$_3$.

310

311 *Photosynthetic capacity and other leaf traits of a leaf aging throughout the season*

312 The photosynthetic capacity (V$_{c,max}$ and J$_{max}$) of an aging leaf was reduced by elevated O$_3$ and

313 stimulated by high N supply, with no significant interaction being observed between O$_3$ and

314 N (Figure 5, Table 4 and S2). Whilst elevated O$_3$ increased the J$_{max}$/V$_{c,max}$ ratio (ca. 7% on

315 average), enhanced N supply reduced the J$_{max}$/V$_{c,max}$ ratio (ca. 8% on average; Table S2).

316 Although the photosynthetic capacity declined with time, this was primarily due to a

317 significant decline between June and July, with no further decline being observed between

318 July and September. It should be noted that some of the designated leaves had already

319 dropped off the trees in September, hence their photosynthetic capacity could not be assessed

320 in September. In addition, variation for all measured parameters was highest near the end of

321 the growing season. The negative effect of O_3 on photosynthetic capacity increased with time

322 (Table S2), resulting in an O_3 x time interaction for J_{max} (for $V_{c,max}$ p = 0.08 for the interaction

323 term in the model). The response of light-saturated photosynthesis (A_{sat}) to treatment was

324 similar to that of J_{max}, except that the response to N was not significant (p= 0.12). The

325 response of the chlorophyll content to treatment was also similar to J_{max} (a measure of the

326 maximum rate of electron transport in the photosystems). The g_s of leaves under ambient CO_2

327 and light saturation was not affected by time, O_3 exposure or N supply. There was a linear

328 relationship between A_{sat} and chlorophyll content (p < 0.001; Figure 6a), indicating that non-

329 destructive measurement of the chlorophyll content provides a good estimate of A_{sat}. This

330 relationship was not affected by O_3 and N treatment. There was also a linear relationship

331 between photosynthetic capacity ($V_{c,max}$ and J_{max}) and chlorophyll content (p < 0.001; Figure

332 S1). There was a weak relationship (p = 0.038) between A_{sat} and g_s, with a lot of scatter in the

333 data, and the relationship was not affected by O_3 or N treatment (Figures 6b, S2). At the end

334 of August, A_{sat} was also measured at additional O_3 treatments at an N supply of 10 and 70 kg

335 $ha^{-1} y^{-1}$. These measurements showed a linear decline in A_{sat} with O_3 exposure later in the

336 season (p < 0.001; Figure 7). Enhanced N supply stimulated A_{sat} (p = 0.012): the average A_{sat}

337 (+/- one SE) was 4.8 (+/- 0.3) and 6.0 (+/- 0.4) $\mu mol\ m^{-2}\ s^{-1}$ at 10 and 70 kg N $ha^{-1}\ y^{-1}$,

338 respectively. There was no interaction between O_3 exposure and N supply. Again, there was a

339 strong linear relationship between A_{sat} (p < 0.001) and chlorophyll content and a weak linear

340 relationship (p = 0.033) between A_{sat} and g_s (Figure S3).

341

342 *N content of leaves*

343 The N content of green, mature leaves in the middle of July was not affected by treatment;

344 the mean N content of these leaves was 1.2% on average. Near the end of the growing season,

345   the N content of leaves that were ready to drop off had declined to $0.5 - 0.7\%$. An increase in

346   O_3 exposure resulted in a significant ($p < 0.001$) increase in N content of these leaves (Figure

347   8). N supply did not significantly affect the N content of senesced leaves and there was no

348   significant O_3 x N interaction.

349

350   *Leaf number*

351   Independent of time of the season, enhanced N supply stimulated the number of leaves on

352   silver birch ($p < 0.001$; Figure 9). On the other hand, while there was no effect of O_3 on leaf

353   number at the start of the season, leaf number decreased with higher O_3 at the end of the

354   season ($p < 0.001$). There was no evidence of an interaction between O_3 and nitrogen.

355

356   **Discussion**

357   In the current study, conducted at a range of O_3 and N exposure levels, no significant

358   interactions between the impacts of O_3 and N on photosynthesis and other leaf traits were

359   observed, either for young leaves or aging leaves. This suggests that the sensitivity of these

360   traits to O_3 in young silver birch trees is neither ameliorated (as shown for some trees species,

361   e.g. Häikiö et al., 2007; Handley and Grulke, 2008; Watanabe et al., 2006) nor enhanced by

362   N fertilization (as shown for some other tree species, e.g. Utriainen and Holopainen, 2001b;

363   Wanatabe et al., 2012; Yamaguchi et al., 2007). Although Pääkkönen and Holopainen (1995)

364   suggested that sufficient N supply might reduce O_3-sensitivity in silver birch, interactions

365   between O_3 and N supply were not consistent between experiments and only observed

366   consistently for certain leaf parameters (e.g. leaf area and leaf dry mass per area). They had

367   conducted experiments at only two O_3 exposure levels (ambient and ca. 1.6 x ambient) and

368   up to three N loads (37, 74 and 150 kg N ha^{-1} y^{-1}). Our results, however, confirmed that the

369   response of leaf traits to O_3 is dependent on the developmental stage and age of the leaf

370　studied, in agreement with various other studies (Bagard et al., 2008; Hoshika et al., 2013;

371　Mäenpää et al., 2011; Oksanen et al., 2003). As the magnitude of the impact of O_3 is related

372　to the accumulated O_3 flux (Phytotoxic Ozone Dose) entering the stomata (Mills et al.,

373　2011b), relatively little impact was expected early in the growing season, with impacts

374　increasing with leaf age and longer-term exposure. Indeed, O_3 x time interactions were

375　observed for most photosynthetic parameters and the chlorophyll content of aging leaves. In

376　contrast, the g_s of aging leaves was not affected by O_3 and no O_3 x time interactions were

377　found.

378

379　*Traits of a young leaf throughout the growing season*

380　Although the leaf primordia for early leaves were developed during the previous year, there

381　did not seem to be a carry-over effect of ozone on the number of leaves developed in 2013.

382　Enhanced N, however, seems to have stimulated the number of leaves present at the start of

383　2013, before O_3 and N treatments were started again (Figure 8). The late leaves started to

384　develop in July and the first older leaves started to drop off near the end of July. The

385　heterophyllous character of silver birch explains why the chlorophyll content index of the

386　third leaf (youngest fully expanded leaf) declined first between the end of May and 23rd July,

387　and subsequently rose again by the 23rd of August to a level slightly higher than at the end of

388　May (Figure 2). It is likely that remobilisation of N occurred during this period from the older

389　to the young, newly grown leaves. As to be expected, the chlorophyll content of the young

390　leaves increased significantly with increasing N supply, and this increase was independent of

391　the Julian day. O_3, however, did not affect the chlorophyll content of the young leaves, which

392　might be due to the relatively short time of exposure to O_3 (as discussed below, elevated O_3

393　reduced the chlorophyll content of aging leaves). Linear relationships between chlorophyll

394　content and $V_{c,max}$, J_{max} and A_{sat} (see Figures 6, S1 and S2) suggest that a similar response to

395 O_3 and N can be expected for these photosynthetic traits for the third leaf when developing

396 throughout the season. Indeed, Riikonen et al. (2009) did not find an effect of O_3 on $V_{c,max}$,

397 J_{max} and A_{sat} in *Betula pendula* saplings on the youngest fully expanded leaf, however, the

398 level of O_3 exposure was very low in their study (mean O_3 concentration of $32 - 34$ ppb). On

399 the other hand, g_s was not affected by O_3 exposure or N supply in our study. Although many

400 studies have reported a decline in g_s in trees with increasing O_3 (Wittig et al., 2007, and

401 references therein), including for *Betula pendula* (Riikonen et al., 2009), other studies have

402 reported no change or even an increase in g_s in response to O_3 exposure (Mills et al., 2016,

403 and references therein). The varying responses might well be explained by inherent

404 differences in responses of species/genotypes to O_3, variation in exposure facilities,

405 magnitude and duration of O_3 exposure. For example, Wittig et al. (2007) observed no

406 change at low to medium O_3 concentrations ($30 - 59$ ppb), a decline at medium to high O_3

407 concentration ($60 - 119$ ppb) and no change at very high O_3 concentrations (>120 ppb).

408

409 *Traits of an aging leaf throughout the growing season*

410- Leaf photosynthetic capacity ($V_{c,max}$, J_{max}) and A_{sat} declined between $10 - 14^{th}$ June and $22 -$

411 25th July (Figure 5), but did not decline any further between $22 - 25^{th}$ July and $2 - 5^{th}$

412 September, apart from at 10 kg N ha^{-1} y^{-1} and 68 ppb O_3, where the decline continued. The

413 halt in decline later in the season may be influenced by the fact that older, senesced leaves

414 started to drop off with some new growth and remobilisation of N occurring (see above).

415 High O_3 exposure reduced the photosynthetic capacity and A_{sat} of leaves, and more so later

416 than early in season. A significant interaction between O_3 and time (although not significant

417 for $V_{c,max}$) confirms that effects of O_3 are dependent on the accumulated phytotoxic O_3 dose

418 (LRTAP Convention, 2015; Mills et al., 2011b). Noormets et al. (2010) also found that

419 elevated O_3 decreased photosynthetic parameters and leaf chlorophyll content, particularly in

420   the lower canopy, and that the negative impact of O_3 increased with time. A similar response

421   to O_3 was found for A_{sat} in *Fagus crenata* seedlings, with a significant interaction between O_3

422   and N only observed in July (Yamaguchi et al., 2007). However, it is not clear for which

423   leaves A_{sat} was determined in *Fagus crenata*. Whilst Marzuoli et al. (2016) reported a

424   significant decline in $V_{c,max}$ due to elevated O_3 in *Quercus robur* seedlings in the first year, no

425   significant effect of elevated O_3 on $V_{c,max}$ was observed in the second year of O_3 exposure. In

426   contrast to Yamaguchi et al. (2007), we did not find any evidence that N impacts dominate

427   early in the season and that O_3 impacts dominate later in the season. Although elevated N

428   supply stimulated photosynthetic capacity ($V_{c,max}$ and J_{max}) as to be expected, N supply did

429   not significantly affect A_{sat}. Whilst high N supply seems to have alleviated to some extent the

430   impact of O_3 on photosynthetic capacity observed at low N supply later in the growing

431   season, there was no significant interaction between O_3 and N treatment, not even when the

432   data for September were analysed separately. The latter might be due to the low number of

433   replication (four instead of six as some leaves had already dropped off the tree) and the

434   higher variability later in the season. The higher decline in A_{sat} compared to photosynthetic

435   capacity in September at high O_3 exposure and high N supply can be explained by a decline

436   in g_s (Figure 5). Nevertheless, O_3 exposure, N supply and time did not affect g_s in the aging

437   leaf in our study when measured at light saturation. In contrast, Yamaguchi et al. (2007)

438   reported a significant increase in g_s in September due to high O_3 exposure in *Fagus crenata*,

439   with no O_3 effect observed earlier in the year, however, they did not define clearly which leaf

440   was measured. We observed a significant and strong linear relationship between A_{sat} and the

441   chlorophyll content of leaves. Although significant, the linear relationship between A_{sat} and g_s

442   was weak (p = 0.038), with the data showing a lot of scatter. This suggests that impacts of

443   treatment on A_{sat} were mainly driven by impacts on photosynthetic capacity rather than

444   changes in g_s. Indeed, previous studies have shown that O_3-induced reductions in A_{sat} are

445   mainly driven by reductions in photosynthetic capacity in the chloroplasts (Farage et al.,

446   1991; Hoshika et al., 2013; Niu et al., 2014; Yamaguchi et al., 2007). In agreement with

447   Noormets et al. (2010) and Bagard (2008), the biochemical component of photosynthesis

448   ($V_{c,max}$) was damaged more by O_3 than electron transport (J_{max}), as the ratio of J_{max} to $V_{c,max}$

449   was enhanced at elevated O_3 exposure. Decreases of both Rubisco content and activation

450   state have been reported for trees and crops (Ainsworth et al., 2012), often associated with

451   down regulation of photosynthetic genes and upregulation of genes involved in programmed

452   cell death and/or tissue senescence (Bohler et al., 2007; Ernst, 2013; Heath, 2008; Pell et al.,

453   1997). In general, terrestrial plants allocate as much as 50% of their leaf N content to Rubisco

454   because of inefficient catalytic activity of the enzyme (Spreitzer and Salvucci, 2002). If A_{sat}

455   is mainly determined by the amount of Rubisco in birch leaves, then the O_3-induced

456   reduction in A_{sat} might well be due to a decrease in allocation of leaf N to Rubisco

457   (Yamaguchi et al., 2007). Because photosynthetic measurements are often made on the most

458   recently fully expanded upper canopy leaves, it is often difficult to demonstrate differences in

459   net photosynthesis due to O_3 until near or after the time when production of new upper

460   canopy leaves has ceased. The growth of new leaves observed in July might have enabled

461   birch to compensate for some of the O_3 damage to the photosynthetic apparatus occurring in

462   older leaves later in the growing season.

463

464   *Developmental stage of the leaves affects the response to treatments*

465   The results discussed above indicate that the response of leaf traits to treatments is affected

466   by the developmental stage of leaves, with different responses being observed in young

467   leaves compared to aging leaves (Bagard et al., 2008; Hoshika et al., 2013; Mäenpää et al.,

468   2011; Oksanen et al., 2003). This was further confirmed when the chlorophyll content of

469   different leaves along a branch was determined. Whereas the chlorophyll content declined

470   with O_3 concentration in older leaves, in younger leaves the highest chlorophyll content was

471   found at intermediate O_3 concentrations at high N supply (Table 2, Figure 4). For medium-

472   aged leaves, there was no effect of O_3 on the chlorophyll content. Hoshika et al. (2013)

473   reported differences in stress resistance to O_3 between early and late leaves in white birch

474   (*Betula platyphylla* var. *japonica*), with early leaves not showing an O_3-induced reduction in

475   photosynthesis, and an O_3-induced decline in photosynthesis and early defoliation occurring

476   in late leaves. Hence, late leaves were more sensitive to O_3 than early leaves in white birch.

477   However, our results indicate that differences in response to O_3 are primarily determined by

478   the age/developmental stage of leaves and exposure time to O_3 rather than the type of leaf.

479   Responses to treatments are further confounded when nearer the end of the growing season N

480   is remobilised from old leaves to young leaves, maintaining the photosynthetic capacity of

481   the young leaves. The various responses to O_3, and the interaction between O_3 and N,

482   reported in the literature might well be due to measurements on leaves at different

483   developmental stages, which confounds comparisons between experiments. In agreement

484   with Uddling et al. (2005), we observed that enhanced O_3 impairs the resorption of N in

485   leaves of silver birch near the end of the growing season. Similar results were also reported

486   for *Fagus crenata* (Yamaguchi et al., 2007). An increase in leaf N, partly due to the release of

487   NH_4^+ from both protein degradation and enhanced activity of the shikimate pathway, is

488   known to occur at high ozone dose (early senescence) and a part of this N could be

489   transiently re-assimilated via an increased activity of the cytosolic glutamine synthetase

490   (Bohler et al., 2007; Dizengremel et al., 2012; Galant et al., 2012) or stored in gamma-

491   aminobutyric acid (Dizengremel et al., 2012). The enhanced N content in litter at elevated O_3

492   is likely to affect the subsequent cycling of N in the soil.

493

494   **Conclusions**

495   At the leaf level, we did not observe any interactions between O_3 and N regarding

496   photosynthetic parameters, chlorophyll content, N content in senesced leaves and leaf

497   number. This suggests that the sensitivity of these leaf traits to O_3 in young silver birch trees

498   was neither reduced nor enhanced by N fertilization under our experimental conditions. Our

499   results suggest that the response of leaf traits to O_3 is dependent on the developmental

500   stage/age of the leaf studied. Whilst O_3 reduced the net rate of photosynthesis in aging birch

501   leaves later in the season, O_3 did not affect the g_s of those leaves. This suggests that there was

502   only a weak coupling between photosynthesis and g_s in aging silver birch leaves under light-

503   saturating conditions. When studying the impacts of O_3 on leaf traits, it is important to define

504   the developmental stage of the leaves, as this will affect the measured response to O_3.

505

512

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745   biochemistry: a quantitative meta-analysis. Global Change Biology 15, 396-424.

746

747   Yamaguchi, M., Watanabe, M., Iwasaki, M., Tabe, C., Matsumura, H., Kohno, Y., Izuta, T.,

748   2007. Growth and photosynthetic responses of *Fagus crenata* seedlings to O_3 under different

749   nitrogen loads. Trees 21, 707–718.

750

751 **Table 1.** Summary of ozone treatments and climate conditions in 2013.

Ozone treatment	1	2	3	4	5	6	7
24 hr mean (ppb)	35.7	39.3	42.3	47.8	53.1	59.1	67.9
Daylight mean (ppb)	36.1	40.0	43.6	50.0	54.7	63.9	71.5
AOT40 (ppm h^{-1})	2.4	4.2	7.1	12.1	16.1	25.1	32.5
Season max. (ppb)	62.4	71.3	81.3	100.4	111.3	113.5	126.3

Air temperature (^{o}C)			VPD (kPa)			PAR (umol m^{-2} s^{-1})	
24 h mean	*Daylight mean*	*Daily max. mean*	*24 h mean*	*Daylight mean*	*Daily max. mean*	*Daylight mean*	*Daily max. mean*
19.8	24.0	25.6	0.67	1.24	1.55	515	808

752

753

754 **Table 2.** Summary statistics of effects of treatments and time[1] on the chlorophyll content and
755 stomatal conductance (G_s) of the third leaf (youngest fully expanded) measured throughout
756 the growing season.

Variable	Chl. content	G_s
O_3	n.e.	n.e.
N	$p<0.001$	n.e.
Time	$p<0.001$	$p<0.01$
Time^2	$p<0.001$	$p<0.01$

757
758 [1] No two or three-way interactions were found.
759 n.e. = no significant effect at p = 0.05.
760 Time^2 = time squared.
761

762 **Table 3.** Summary statistics of effects of treatments and leaf number on the chlorophyll
763 content of silver birch leaves of different age along the first top branch near the end of the
764 growing season (on 4 September 2013).
765

| Variable	Chl. content
O_3	n.e.
N	$p<0.001$
Leaf number	$p<0.001$
O_3 * N	n.e.
O_3 * Leaf number	$p<0.001$
N * Leaf number	n.e.
O_3 * N * Leaf number	n.e.

766
767 n.e. = no significant effect at p = 0.05.
768

769

Table 4. Summary statistics of effects of treatments and time[1] on photosynthetic parameters, stomatal conductance (G_s) and chlorophyll (Chl.) content (index) of the same birch leaves measured throughout the season, i.e. aging with time.

Variable	$V_{c,max}$	J_{max}	$J_{max}/V_{c,max}$	A_{sat}	G_s (at A_{sat})	Chl. content
O_3	$p<0.01$	$p<0.01$	$p=0.05$	$p<0.01$	n.e.[2]	$(p=0.055)$
N	$p<0.01$	$p=0.02$	$p=0.01$	n.e.	n.e.	$p=0.03$
Time	$p<0.001$	$p<0.001$	$(p=0.09)$	$p<0.001$	n.e.	$p<0.001$
O_3 * Time	$(p=0.08)$	$p<0.01$	n.e.	$p=0.001$	n.e.	$p=0.04$

[1] No O_3 x N, N x Time or O_3 x N x Time interactions were found.

[2] n.e. = no significant effect at $p = 0.05$.
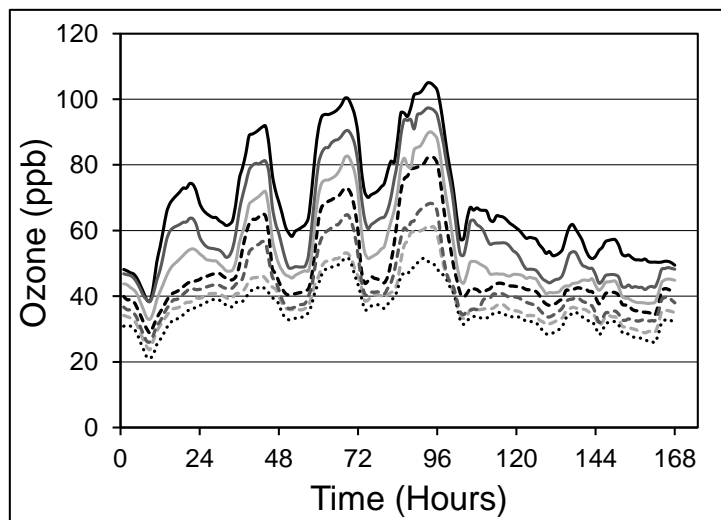
Figure 1. Weekly ozone profile in seven solardomes at Abergwyngregyn, nr. Bangor, North Wales in 2013.
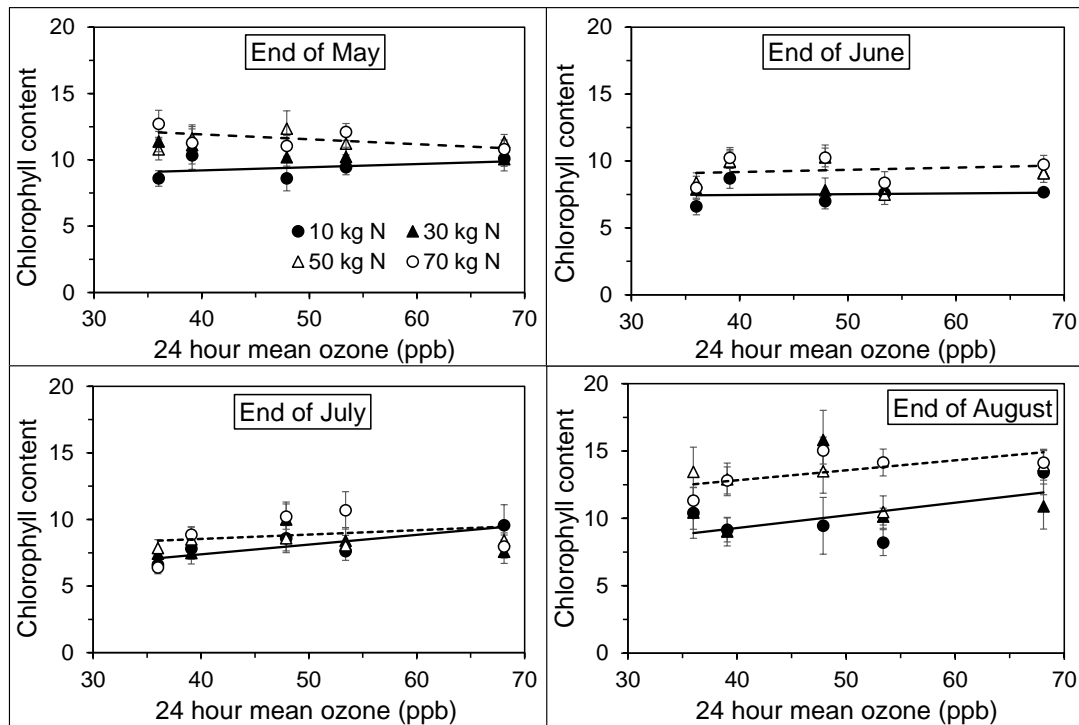
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786
Figure 2. Effect of ozone exposure and nitrogen supply on the chlorophyll content (index) of
silver birch leaves (third leaf on main stem, fully expanded) from late May to late August
2013. Values are means ± one SE (n = 5 − 6). Solid line: 10 kg N ha^{-1} y^{-1}, broken line: 70 kg
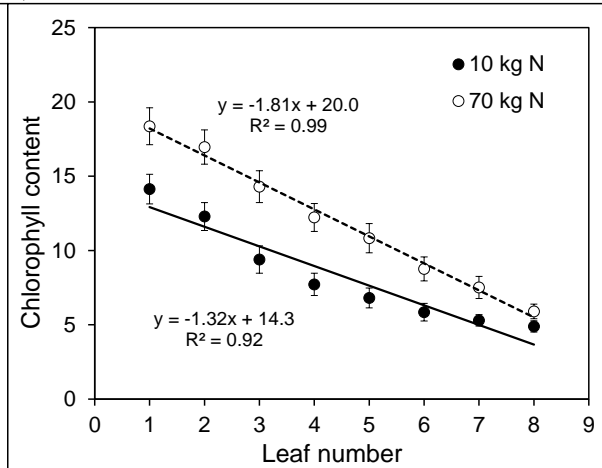N ha^{-1} y^{-1}.

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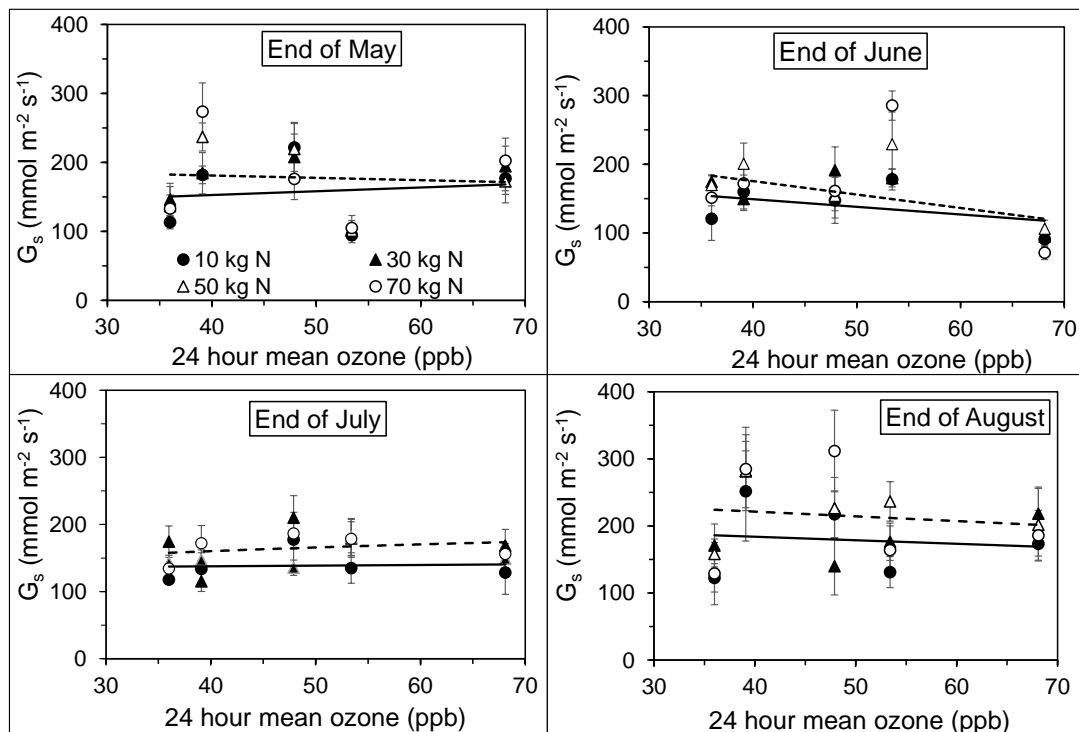
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Figure 3. Effect of ozone exposure and nitrogen supply on the stomatal conductance (G$_s$) of
silver birch leaves (third leaf on main stem, fully expanded) from late May to late August
2013. Values are means ± one SE (n = 5 − 6). Solid line: 10 kg N ha^{-1} y^{-1}, broken line: 70 kg
N ha^{-1} y^{-1}.
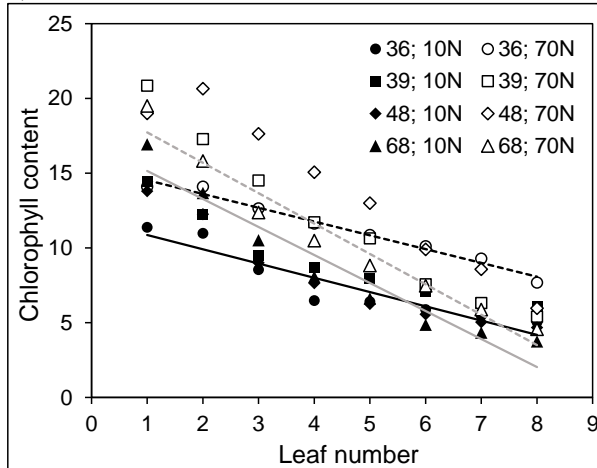
Figure 4. Effect of ozone exposure and nitrogen supply on the chlorophyll content (index) of silver birch leaves of different age along the first top branch near the end of the growing season (4 September 2013). a) Data are means (n = 6); the first number in the legend refers to the 24 hr mean ozone concentration (ppb) and the second number to the nitrogen supply (kg N ha^{-1} y^{-1}); trend lines are shown for the lowest (black lines) and highest (grey lines) ozone exposure for low (solid lines) and high (broken lines) nitrogen supply. See Table S1 for values and standard errors. b) Data are means ± one SE (n = 24), with ozone exposure data averaged for low (filled circles, solid line) and high (open circles, broken line) nitrogen supply. Leaf number one is the youngest leaf near the top of the branch, whereas leaf number eight is the oldest measured leaf situated near the origin of the branch.
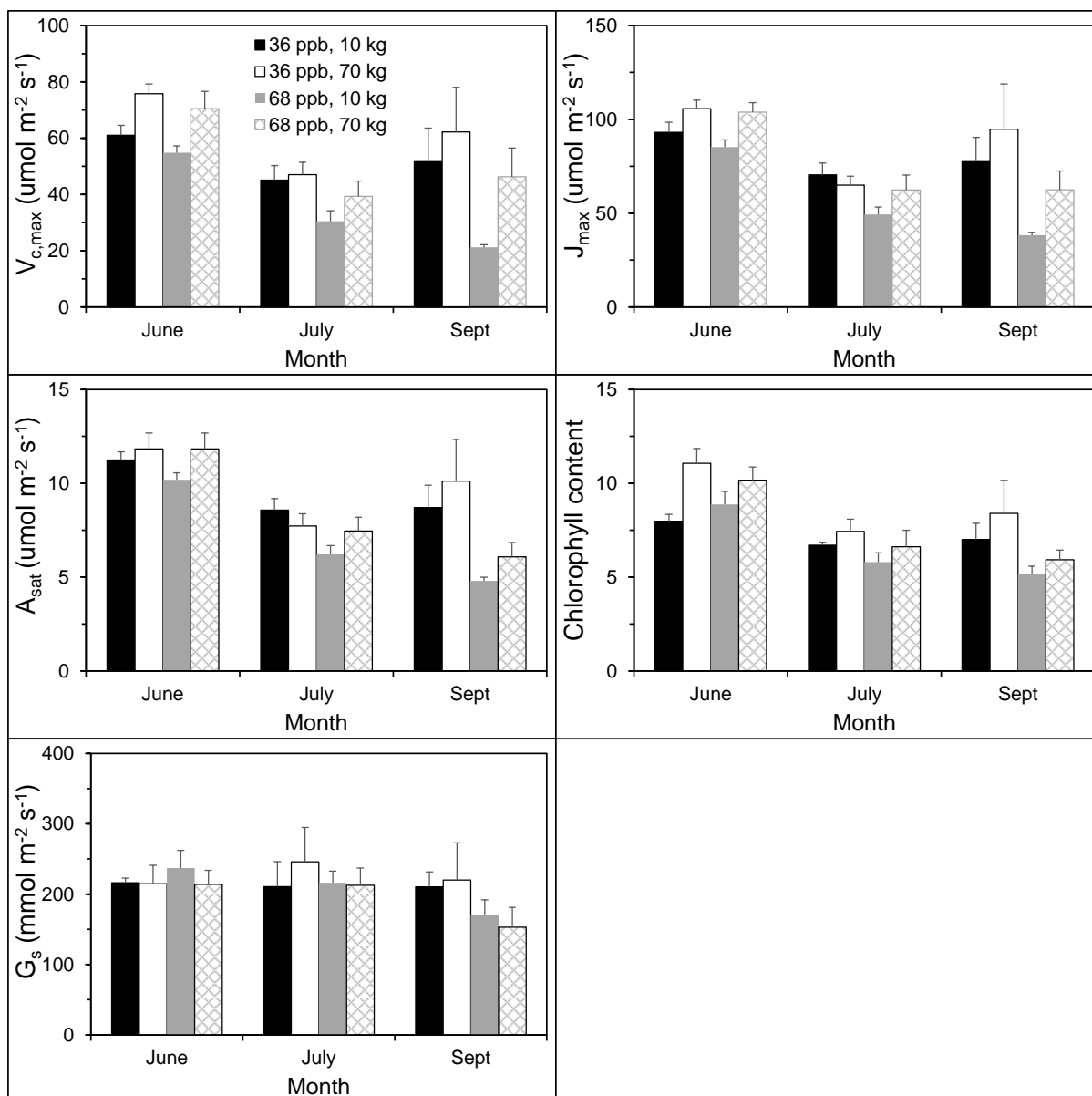
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Figure 5. Photosynthetic capacity ($V_{c,max}$ and J_{max}), light-saturated rate of photosynthesis
(A_{sat}), chlorophyll content (index), and stomatal conductance (G_s; at ambient CO_2) of aging
silver birch leaves, i.e. the same leaves were measured throughout the growing season. Trees
were exposed to a 24 hr mean ozone concentration of 36 or 68 ppb and supplied with 10 or
70 kg N ha^{-1} yr^{-1} in 2013. Data are means ± one SE (n = 4 - 6).
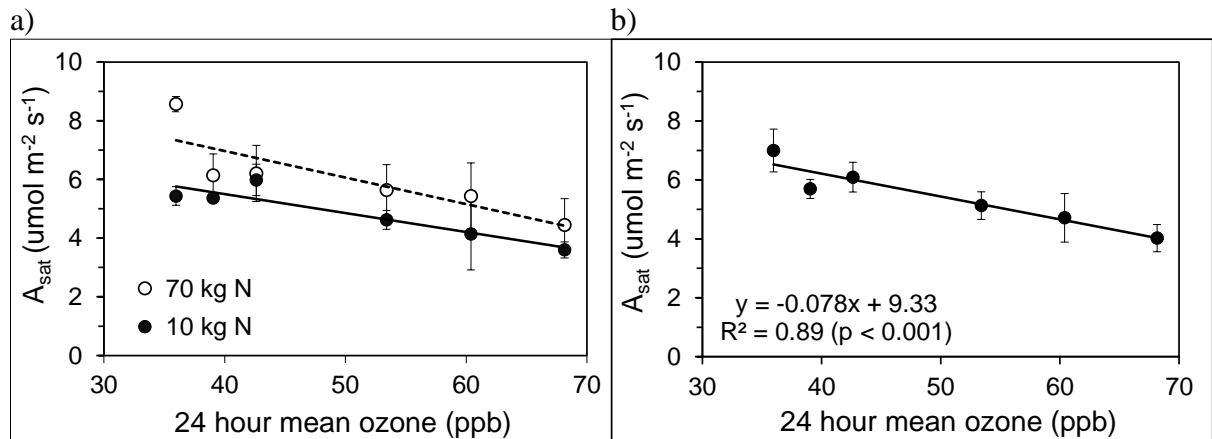819
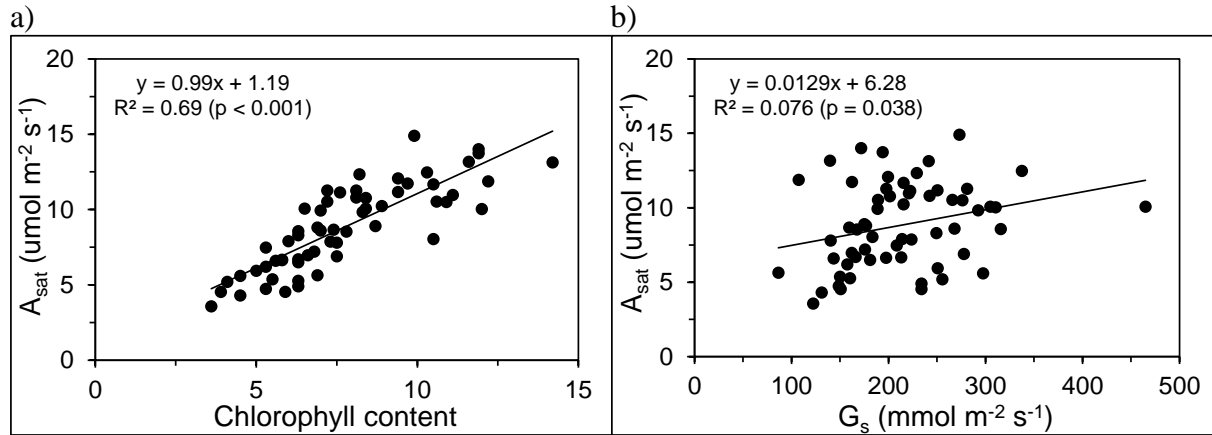
Figure 6. Relationship between light-saturated rate of photosynthesis (A_{sat}) and a) chlorophyll content (index) and b) stomatal conductance (G_s) of aging silver birch leaves (see Figure 5). Trees were exposed to a 24 hr mean ozone concentration of 36 or 68 ppb and supplied with 10 or 70 kg N ha^{-1} yr^{-1} in 2013.


Figure 7. Light-saturated rate of photosynthesis (A_{sat}) of aged silver birch leaves near the end of the growing season (late August, early September). Trees were exposed to a 24 hr mean ozone concentration of 36 - 68 ppb and supplied with 10 or 70 kg N ha^{-1} yr^{-1} in 2013. In a) mean values (± one SE; n = 4 – 5) are shown for different nitrogen supply rates, whereas in b) mean values (± one SE; n = 8 – 10) for different nitrogen supply rates were pooled.
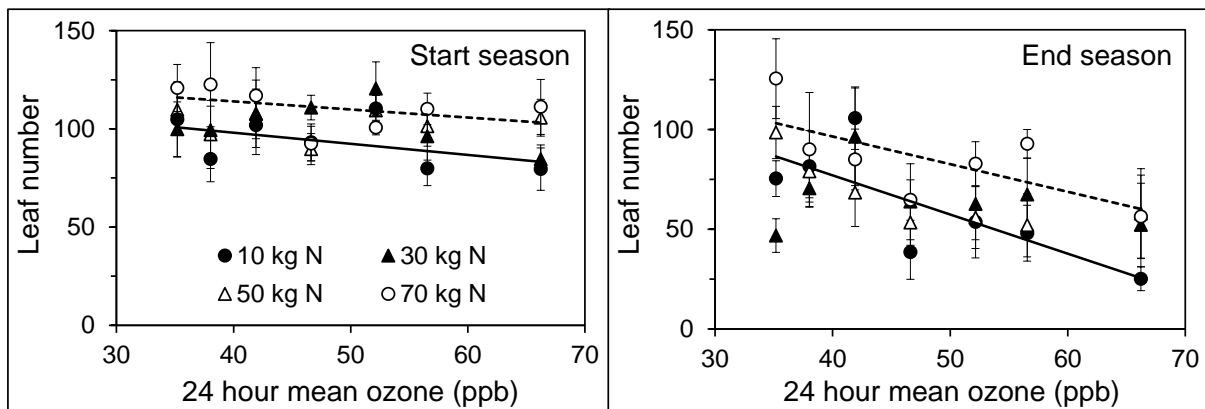
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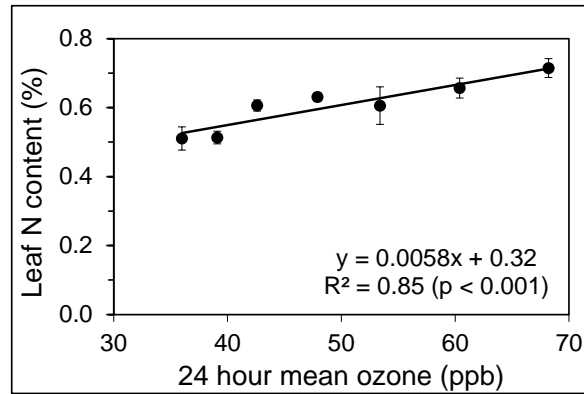
Figure 8. Impacts of ozone exposure on the leaf nitrogen concentration of silver birch leaves just before they fell of the tree. Values are the mean of four different nitrogen supply rates (± one SE).

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Figure 9. Impact of ozone and nitrogen on leaf number near the start (5th May) and end of the 2013 growing season (24th September). Data are means ± one SE (n = 5 - 6). Any effect early in the season reflect a carry-over effect from the previous growing season. Trend lines are shown for the lowest (solid line) and highest (broken line) nitrogen supply.

1 **Supplement**

2

3 **Table S1.** Effect of ozone exposure and nitrogen supply on the chlorophyll content of silver
4 birch leaves of different age along the first top branch near the end of the growing season (4
5 September 2013). Leaf number one is the youngest leaf near the top of the branch.

6

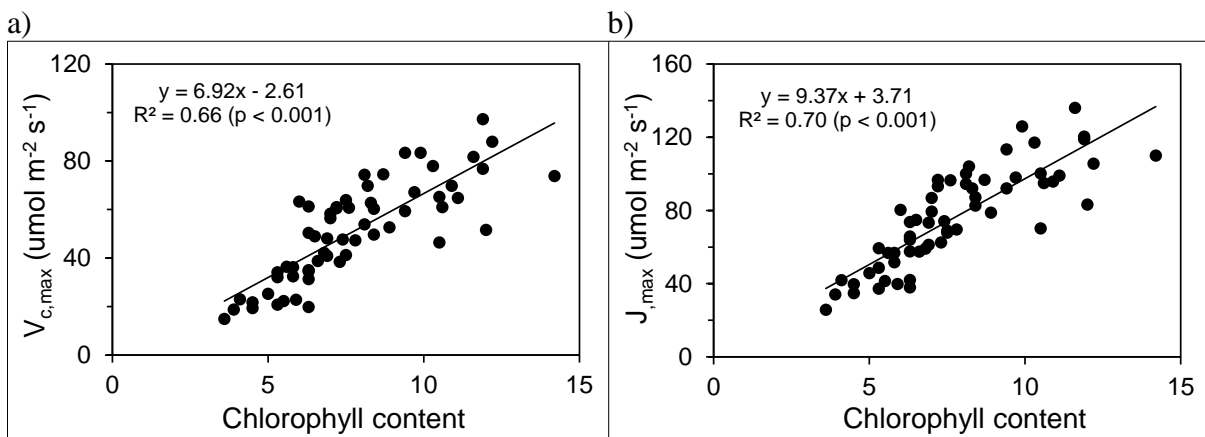|			Chlorophyll content	
Ozone (ppb)	N (kg ha^{-1} y^{-1})	Leaf no.	Mean	Standard error
35.7	10	1	11.4	2.2
		2	11.0	2.1
		3	8.6	1.2
		4	6.5	1.0
		5	6.4	0.8
		6	5.9	0.9
		7	5.5	0.8
		8	5.0	0.8
	70	1	14.1	2.9
		2	14.1	2.7
		3	12.7	1.9
		4	11.6	1.6
		5	10.9	1.6
		6	10.1	1.7
		7	9.3	1.8
		8	7.7	1.0
39.3	10	1	14.4	1.1
		2	12.2	1.3
		3	9.5	1.8
		4	8.7	1.8
		5	8.0	1.8
		6	7.1	1.7
		7	6.4	1.1
		8	6.1	1.0
	70	1	20.9	2.0
		2	17.3	1.2
		3	14.5	1.0
		4	11.7	1.0
		5	10.6	1.7
		6	7.6	0.8
		7	6.3	0.8
		8	5.4	0.9
47.8	10	1	13.8	2.3
		2	12.3	2.9
		3	9.0	2.5
		4	7.7	1.6
		5	6.3	1.4
		6	5.6	1.2
		7	5.0	0.2
		8	4.7	0.4
	70	1	19.0	2.4
		2	20.7	2.6
		3	17.6	2.5
		4	15.1	2.3
		5	13.0	2.5
		6	9.9	1.9
		7	8.6	1.8
		8	6.0	0.7
67.9	10	1	16.9	2.0
		2	13.7	1.6
		3	10.5	2.0
		4	8.1	1.7
		5	6.6	1.5
		6	4.9	0.9
		7	4.3	0.5
		8	3.8	0.4
	70	1	19.5	2.3
		2	15.8	2.1
		3	12.4	2.6
		4	10.5	2.3
		5	8.8	2.1
		6	7.5	1.9
		7	5.9	1.1
		8	4.6	1.1

7

Table S2. Relative difference (%) in photosynthetic parameters, chlorophyll (Chl.) content and stomatal conductance (g_s) at high (24 hr mean of 68 ppb) compared to low ozone (24 hr mean of 36 ppb) at different times during the growing season. Replace Chlorophyll index with Chlorophyll content in table.

| Month	N (kg ha^{-1} y^{-1})	A_{sat}	$V_{c,max}$	J_{max}	$J_{max}/V_{c,max}$	Chl. index	g_s
June	10	9.4	10.2	8.5	-2.0	-11.2	-9.8
	70	-0.1	7.1	1.8	-7.0	8.1	0.3
July	10	27.4	32.4	30.0	-5.5	13.4	-2.5
	70	3.6	16.5	4.0	-14.8	11.0	13.6
September	10	45.0	59.0	50.7	-14.9	26.4	18.8
	70	39.9	25.7	34.0	7.7	29.5	30.4
June	Combined[1]	5.0	10.4	6.1	-5.3	3.2	-4.8
July	Combined[1]	15.3	24.6	16.8	-11.2	13.0	7.6
September	Combined[1]	42.2	40.8	41.5	-3.6	28.1	24.8

[1] Data for low and high N were combined as there was no significant O_3 x N interaction.
Negative values indicate a higher value of the variable at higher compared to low O_3 exposure.

a)

b)

Figure S1. Relationship between a) $V_{c,max}$ and b) J_{max} and chlorophyll content of aging silver birch leaves (see Figure 5). Trees were exposed to a 24 hr mean ozone concentration of 36 or 68 ppb and supplied with 10 or 70 kg N ha^{-1} yr^{-1} in 2013. See also Figure 5.
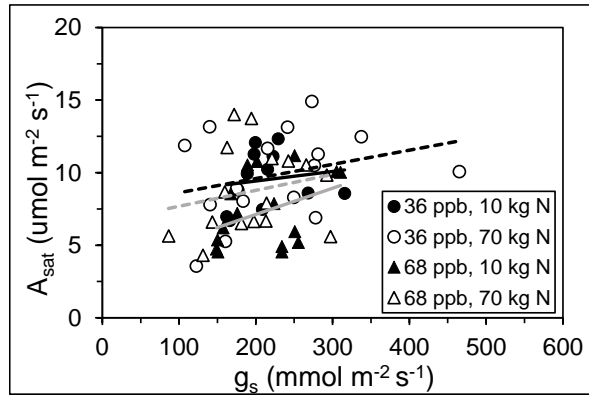
Figure S2. Relationship between light-saturated rate of photosynthesis (A_{sat}) stomatal conductance of aging silver birch leaves (see Figure 6b). Trees were exposed to a 24 hr mean ozone concentration of 36 (black lines) or 68 ppb (grey lines) and supplied with 10 (solid lines) or 70 kg N ha^{-1} yr^{-1} (broken lines) in 2013.
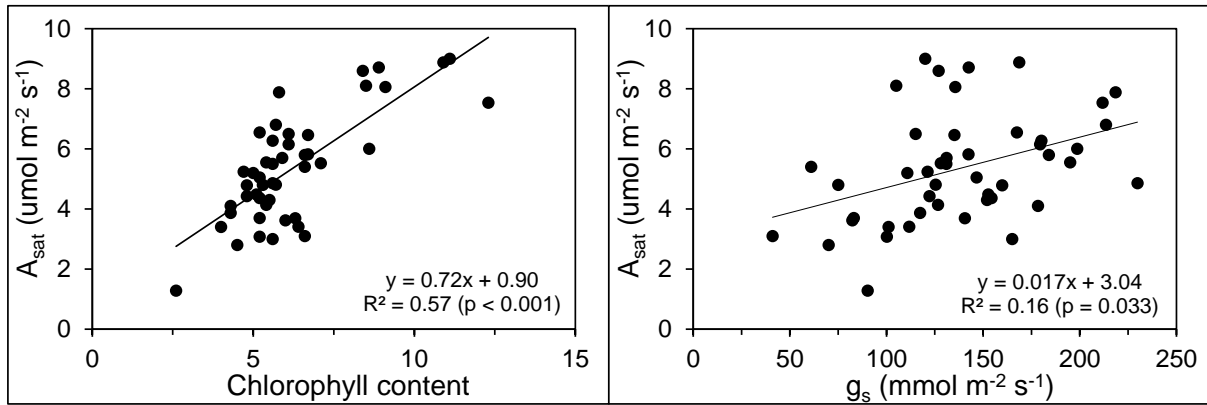
Figure S3. Relationship between light-saturated rate of photosynthesis (A_{sat}) and and a) chlorophyll content and b) stomatal conductance of aged silver birch leaves near the end of the growing season (late August, early September). Trees were exposed to a 24 hr mean ozone concentration of 36 - 68 ppb and supplied with 10 or 70 kg N ha^{-1} yr^{-1} in 2013. See also Figure 7.