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Science of the Total Environment

DOI:
[10.1016/j.scitotenv.2023.168288](https://doi.org/10.1016/j.scitotenv.2023.168288)

Published: 15/01/2024

Publisher's PDF, also known as Version of record

[Cyswllt i'r cyhoeddiad / Link to publication](#)

Dyfyniad o'r fersiwn a gyhoeddwyd / Citation for published version (APA):
Brewster, C., Fenner, N., & Hayes, F. (2024). Chronic ozone exposure affects nitrogen remobilization in wheat at key growth stages. *Science of the Total Environment*, Article 168288. <https://doi.org/10.1016/j.scitotenv.2023.168288>

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Chronic ozone exposure affects nitrogen remobilization in wheat at key growth stages

Clare Brewster^{a,b,*}, Nathalie Fenner^{b,**}, Felicity Hayes^a

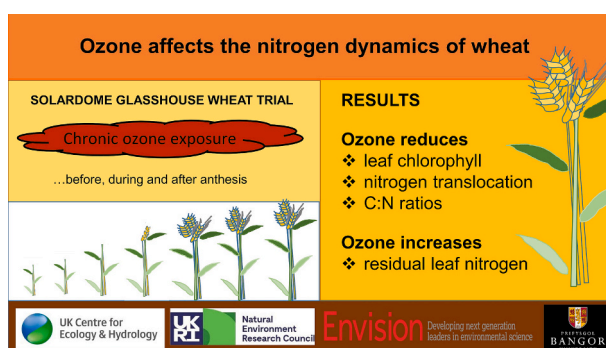
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HIGHLIGHTS

- The effect of ozone stress on nitrogen remobilization in wheat is not well understood.
- Four ozone and two nitrogen treatments led to differences in nitrogen remobilization.
- Chronic ozone reduced chlorophyll in older leaf cohorts at reproductive growth stages.
- Chronic ozone reduced nitrogen remobilization and increased residual nitrogen levels.
- Additional nitrogen fertilization at anthesis ameliorated the effect of chronic ozone.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Pavlos Kassomenos

Keywords:

Abiotic stress
Chlorophyll
Senescence
Nitrogen translocation
Nitrogen fertilization
Wheat yields

ABSTRACT

The interaction between nitrogen storage and translocation, senescence, and late phase photosynthesis is critical to the post-anthesis grain fill period in wheat, but ozone's effect on nitrogen dynamics within the wheat plant is not well understood. This study used solar domes to expose a widely grown elite spring wheat cultivar, cv. Skyfall, to four levels of ozone (30 ppb, 45 ppb, 70 ppb, 85 ppb) for 11 weeks, with two levels of nitrogen fertilization, 140 kg ha⁻¹ and 160 kg ha⁻¹, the higher rate including an additional 20 kg N ha⁻¹ at anthesis. Chronic ozone exposure triggered earlier senescence in the 4th, 3rd and 2nd leaves but not the flag leaf, with a similar pattern of reduced chlorophyll content in the lower, older leaf cohorts, which started before senescence became visible. At anthesis there was no evidence of any effect of ozone on nitrogen storage in upper plant parts. However, high ozone increased levels of residual nitrogen found within plant parts at harvest, with concomitant reductions in C:N ratios and Nitrogen Remobilization Efficiency. Extra nitrogen fertilization applied at anthesis appeared to ameliorate the effect of ozone on nitrogen content and nitrogen translocation. The application of ¹⁵N ammonium nitrate at anthesis confirmed that the majority of post-anthesis nitrogen uptake had been translocated to the ear/grain by harvest, with no effect of ozone on the translocation of nitrogen around the plant. These data can inform future modelling of ozone's effect on nitrogen dynamics and global wheat yields.

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<https://doi.org/10.1016/j.scitotenv.2023.168288>

Received 27 August 2023; Received in revised form 27 October 2023; Accepted 31 October 2023

Available online 2 November 2023

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1. Introduction

1.1. Ozone, wheat and nitrogen

Ground level ozone is a secondary air pollutant and abiotic stress which continues to have a detrimental effect on global wheat yields (Mills et al., 2018b). With ozone concentrations predicted to continue at levels which are phytotoxic to wheat across many wheat-growing areas of the world (Revell et al., 2015; Mills et al., 2018a), efforts to more effectively model potential yield losses (Emberson et al., 2018; Feng et al., 2022), and to develop more ozone tolerant cultivars (e.g. Ainsworth, 2017), are more important than ever.

Ozone concentrations are highest in the northern hemisphere, where higher levels of ozone's precursor gases are emitted and circulate (Fleming et al., 2018). Annual mean concentrations range from <30 ppb in the most northerly latitudes of Europe and North America, to >85 ppb in parts of western USA, southern Europe, and east Asia, with mid-range concentrations of 40–70 ppb commonly occurring, including across the wheat-growing areas of the UK, Europe, the USA and Asia (Fleming et al., 2018; Emberson et al., 2018). Reports relating to ozone's effects on wheat in Europe and North America (Pleijel et al., 2019), India (Mukherjee et al., 2021), China (Wang et al., 2021) and Africa (Hayes et al., 2020) suggest ozone causes 4–15 % annual yield reductions.

Although nitrogen fertilization is essential to plant growth and to maintaining higher wheat yields, only about a third of applied nitrogen fertilizer is retained in the grain at harvest (Hawkesford and Riche, 2020). This, in combination with the increasing costs of nitrogen fertilization, mean that efforts to improve Nitrogen Use Efficiency (NUE), and to avoid reductions in NUE due to abiotic stress, are now a priority for wheat breeders (Hawkesford, 2017). Developing a better understanding of how ozone affects the nitrogen dynamics of wheat will help to identify mechanisms of yield reduction, as well as key factors for maintaining NUE and improving ozone tolerance in wheat (Broberg et al., 2017).

1.2. Effect of ozone on senescence, the C:N balance and nitrogen partitioning

When ozone enters a wheat plant through its stomata it rapidly reacts to form multiple Reactive Oxygen Species (ROS) which, if not reduced by antioxidants, lead to a cascade of harmful effects within leaf cells and ultimately to reduced functioning of plant processes (see reviews e.g. Emberson et al., 2018; Ainsworth, 2017). Correlations between elevated ozone, earlier senescence, reduced photosynthetic capacity, and subsequent wheat yield reductions, have been extensively researched (Amundson et al., 1987; Pleijel et al., 1997; Gelang et al., 2000; Xu et al., 2009; Osborne et al., 2019; Feng et al., 2022). The reproductive growth phase of wheat appears to be the most sensitive to the effect of ozone (Pleijel et al., 1998; Soja et al., 2000), with reductions in photosynthate during anthesis and grain fill particularly evident, although not fully understood (Emberson et al., 2018). In addition, whilst the impact of ozone on the flag leaf has been widely researched, there is also an acknowledgement of the need to take the effect of ozone on all cohorts of leaves more effectively into account (Emberson et al., 2018; Osborne et al., 2019; Feng et al., 2022).

There is a growing consensus that premature and accelerated senescence is one of the primary factors driving yield reductions, and that this needs to be incorporated into modelling work being undertaken to estimate the effects of ozone on global wheat yields (Emberson et al., 2018). As senescence plays an important role in balancing carbon and nitrogen within the whole plant, it therefore also interacts with multiple external factors which can generate a C:N or source:sink imbalance, including nutrient supply and abiotic stress (Paul and Foyer, 2001; Winger et al., 2006). In stress-affected plants accelerated senescence results in both carbon and nitrogen use efficiency being compromised (Gregersen et al., 2013; Sade et al., 2018). Research in tree species has

found that ozone can increase the residual nitrogen concentrations in leaves, stems and roots, with related reductions in C:N ratios (Shang et al., 2018). However, ozone's effect on C:N ratios within the plant, is also not well understood, and there is a lack of data on C:N stoichiometry in ozone-affected wheat (Emberson et al., 2018).

The nitrogen content of plant parts at the start of grain filling is crucial, for both efficient nitrogen remobilization as well as ongoing photosynthesis for carbon assimilation (Kong et al., 2016). Chlorophyll is a good indicator of nitrogen levels within a plant because nitrogen is mainly stored in organic form, mostly as proteins and amino acids within chlorophyll. The senescence process then breaks down chlorophyll and translocates the nitrogen compounds to the ear and grain (Avila-Ospina et al., 2014; Maillard et al., 2015). Whilst the majority of wheat's grain starch is generated directly by post-anthesis photosynthesis, up to 90 % of the grain protein comes from the nitrogen stored prior to anthesis in leaves, stems, ear and root (Kichey et al., 2007; Bogard et al., 2010; Kong et al., 2016). In addition, a lesser but still important proportion of grain nitrogen can also be taken up via the roots in the post-anthesis growth phases (Kichey et al., 2007; Kong et al., 2016). When assessing the effect of ozone on levels of nutrients in wheat, Broberg et al. (2021) found that ozone reduced the harvest index for the majority of essential nutrients including nitrogen, whilst the total pool of each nutrient was unaffected, suggesting that ozone affected remobilization and translocation efficiency rather than nutrient uptake. However, there has been limited research into whether, and to what extent, ozone affects levels of nitrogen stored prior to anthesis, its subsequent translocation to the grain, and nitrogen uptake in the post-anthesis period.

1.3. Aims of the study

This study therefore aimed to assess the effect of ozone on senescence, the C:N balance, and subsequent nitrogen partitioning in a widely grown elite spring wheat cultivar, cv. Skyfall. This cultivar was selected due to its ozone sensitivity, as found in previous research (Harmens et al., 2018; Brewster et al., 2019).

The hypotheses being tested were that:

- ozone would reduce the levels of chlorophyll and the timing of senescence in multiple plant parts, particularly during the key growth stages of anthesis and grain fill;
- ozone would alter the C:N ratio in plant parts at anthesis and at harvest;
- ozone would alter the levels of nitrogen found in plant parts both at anthesis and at harvest, and also alter the remobilization of nitrogen between anthesis and harvest;
- ozone would affect the uptake of extra nitrogen fertilizer applied at anthesis;
- the addition of the extra nitrogen would alter the effects of ozone in the post-anthesis period.

2. Materials and methods

2.1. Plant establishment

The trial took place in 2021 at the UK Centre for Ecology & Hydrology's air pollution facility at Abergwyngregyn North Wales (53.2°N, 4.0°W). One line of a high yielding (winter/spring) cultivar (*T. aestivum* L., cv. Skyfall) released in 2014 (RAGT Semences, France) was selected for the trial.

Seeds were germinated (15–18 April) in petri dishes at room temperature, and sown into modular plug trays containing low nutrient sandy loam topsoil (J.A. Bowers). Seedlings were grown on in an unheated glasshouse without supplementary lighting or temperature control (19 April–4 May, Bangor, UK), with one balanced NPK nutrient feed (23 April).

At 3-leaf stage (GS13, 5 May) seedlings were transplanted, into 6.3 L round black plastic pots (25 cm deep, 20 cm in diameter at the rim) with individual drip trays, two seedlings per pot, containing well mixed low nutrient sandy loam top soil (J.A. Bowers). The topsoil contained 2.1 kg ha⁻¹ of available nitrogen, 5.6 mg litre⁻¹ of phosphate and 28 mg litre⁻¹ of potassium, at pH 7.2. To ensure adequate plant nutrition for spring wheat grown in sandy loam soil (AHDB RB209, 2022) each pot was fertilized with phosphate (110 kg ha⁻¹ equivalent), and potassium (95 kg ha⁻¹ equivalent) at 3-leaf stage (Zadoks et al., 1974), and with ammonium nitrate (140 kg ha⁻¹ equivalent, in two split doses of 70 kg ha⁻¹) added at 3-leaf and 1st node (GS31, 9 June) growth stages. Micronutrients and soil bacteria were added to all pots via the addition of 30 g per pot of farm field sandy loam soil (Henfaes Research Station, Abergwyngregyn, North Wales).

Plants were established under glass (5 May–2 June), with pots rotated randomly every 7 days. All pots were manually watered daily, or as required, to maintain adequate soil moisture levels throughout the trial. To maintain plant health, applications of insecticide (4th June, GS31–32) and fungicide (16th June, GS41–43) treatments were made as required (Table S1).

2.2. Additional nitrogen fertilization, ¹⁵N tracer experiment and ozone exposures

Six replicate pots per treatment were randomly selected, placed in (1 June, GS30), and rotated weekly within, four hemispherical glasshouses (solar domes, 2.1 m high, 3 m diameter). Within each of the four ozone treatments/solar domes there were four nitrogen treatment sub-sets (Table 1): NØX plants had no additional ammonium nitrate and were harvested at mid-anthesis (GS65, 2 July); NØ plants also received no additional ammonium nitrate at mid-anthesis but were grown to full term (GS93, 15–17 Aug), whilst the N+ and 15N+ plants were also harvested at full term (GS93, 15–17 Aug) but with the addition of 20 kg ha⁻¹ ammonium nitrate at mid-anthesis. The 15N+ replicates received a solution of unlabeled ammonium nitrate combined with ¹⁵N dual labelled ammonium nitrate (48.23 atom% ¹⁵N) at a combined rate of 20 kg ha⁻¹. In all cases the solution was applied evenly across the pre-wetted soil surface of each pot.

Ozone treatments (3 June–15 Aug, 11 weeks) achieved peak concentrations of 30 ppb (low), 45 ppb (medium), 70 ppb (high), and 85 ppb (very high) (Fig. 1) using Pulse Width Modulation (PWM), reduced to ~20 ppb at night-time (~30 ppb in very high ozone treatment dome) and for two days per week to mimic natural ozone fluctuations. 24 h means and AOT40 values for each treatment level are as shown in Fig. 1. Charcoal-filtered air ventilated the solar domes approximately twice per minute, with ozone pumped into the solar domes via PTFE tubing using a controlled injection system (G11 ozone generator, Pacific O3, USA) and AirSep NewLife Intensity 10 Litre oxygen concentrator (CAIRE Inc., USA), with computer-controlled concentrations (Lab VIEW version 2015, National Instruments, USA) which were monitored every half hour by two calibrated automatic ozone analyzers (400a, Enviro Technology Services, Stroud, UK, and Thermo Scientific Model 49i Electron O3 Analyser, Fischer Scientific, Waltham, MA, USA).

Ozone concentrations were maintained throughout the plant trial

Table 1

Summary of amount and timing (growth stage - GS) of nitrogen fertilization for each subset of plants (NØX, NØ, N+ and 15N+) along with growth stage at harvest.

	NØX	NØ	N+	15N+
Nitrogen (kg ha ⁻¹)	+70 GS13 +70 GS31	+70 GS13 +70 GS31	+70 GS13 +70 GS31 +20 GS65	+70 GS13 +70 GS31 +20 GS65 + ¹⁵ N
Growth stage at harvest	GS65	GS93	GS93	GS93

until plants were harvested. The average temperature inside the solar domes was 20.05 °C. Environmental conditions within and between the solar domes have been found not to vary (Hewitt et al., 2016; Harmens et al., 2019).

2.3. Senescence and chlorophyll indices

The extent of senescence was assessed weekly in NØ and N+ plants from early stem elongation growth stages (GS31, 9 June) until ripening (GS90s, 10 Aug) using an index of 0–10 (Further detail is shown in Fig. 4). Relative chlorophyll levels (SPAD units) in the flag, 2nd, 3rd, and 4th leaves of the NØ and N+ plants were measured weekly from stem elongation (GS31, 8 June) until mid-grain-fill (GS80s, 27 July) using non-destructive measurements (CCM 200, ADC Biosciences, UK) of the upper surface of the central section of each leaf from a randomly selected shoot from either of the two plants in each replicate pot.

2.4. Biomass and yield measurements at anthesis and post-harvest

All NØX replicates were harvested at mid-anthesis (2 July), and the other sub-sets (NØ, N+ and 15N+), whose final ripening stages became synchronized, were harvested when all spikes had fully senesced and grains had fully ripened (GS93, 15–16 Aug). The harvesting of all replicates from each sub-set was necessary to provide sufficient material for analysis. Shoots were cut at the base of the stem and ears cut at the top of the peduncle. NØ, N+ and 15N+ plants had grains extracted from ears manually and grain was then weighed. All shoots, ears and grain were dried in drying cabinets (65 °C for 14+ days) before shoots and ears (minus their grain) were weighed. Roots of the low and very high treatment plants were kept in situ in pots and left to dry for at least 3 days prior to wet sieving, drying (65 °C for 14+ days) and weighing to derive the dry root biomass. After drying NØX, NØ, N+ and 15N+ shoots had flag leaves and 2nd leaves stripped from stems and the segment of upper stem (including leaf sheath), between the flag leaf node and 2nd leaf node was cut just above the 2nd leaf node. Flag, 2nd leaf and upper stem samples from NØX, and N+ plants were weighed to derive actual (and proxy for NØ and 15N+) dry biomass weights for flag leaf, 2nd leaf, and upper stem.

2.5. C:N and ¹⁵N analysis

All plant parts (flag leaf, 2nd leaf, upper stem, ear, root and grain) were ground and homogenized using a dry ball mill (Retsch MM200, Haan, Germany), with a dried root sub-sample taken from the upper, middle and lower part of the root. In all cases, 15N+ samples were processed after all others, and in the order of 'least to most' expected ¹⁵N concentration. Samples of NØX, NØ, and N+ plants were then analyzed to derive N%, C%, and C:N ratio (Leco TruSpec CN, Michigan, USA). N+ and 15N+ samples were also analyzed separately to derive N% and ¹⁵N atom% values (Elementar vario MICRO cube elemental analyser (Elementar Analysensysteme GmbH, Langenselbold, Germany) interfaced to a Sercon Europa 20-20 IRMS (Sercon Ltd., Cheshire, United Kingdom)) using internal standards calibrated against international reference materials. Total nitrogen per plant part was derived from dry biomass and N% values.

2.6. Nitrogen remobilization efficiency (NRE)

For NØ and N+ plants, NRE was calculated (Bogard et al., 2010) as follows:

$$NRE = \frac{N\% \text{ at anthesis} - N\% \text{ at harvest}}{N\% \text{ at anthesis}}$$

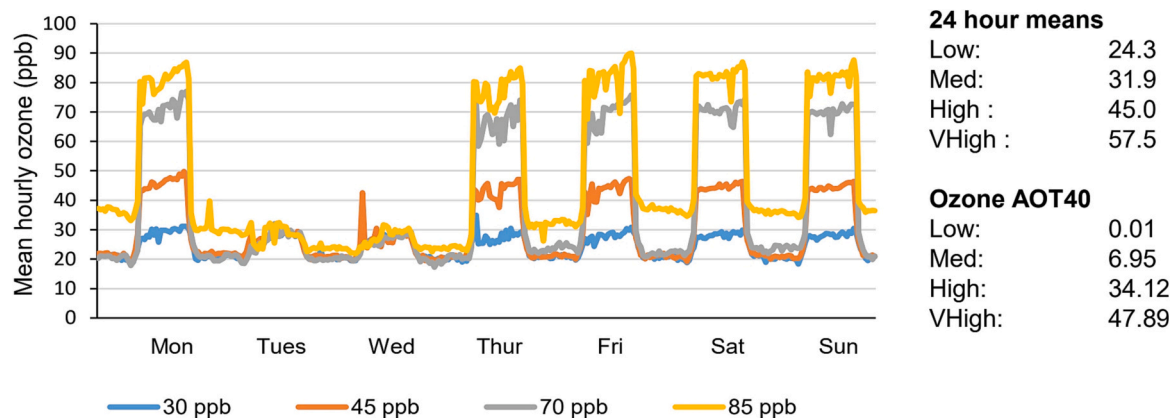


Fig. 1. Mean hourly ozone profiles (replicated each week) over the course of ozone treatment (3 June–15 Aug 2021) with 24 h mean and AOT40 values, where 24 h mean represents the mean ozone concentrations (ppb) across the whole treatment period, and the AOT40 value (ppb.h) shows the extent of ozone exposure above 40 ppb during daylight hours.

2.7. ^{15}N trace experiment – calculation of $^{15}\text{N}\%$ recovery

$^{15}\text{N}\%$ recovered represents the percentage of ^{15}N applied (per pot) which was recovered in the plant part (pool), and was calculated (Rowe et al., 2001) as follows:

- ^{15}N excess atom% = ^{15}N atom% – pool natural abundance
- ^{15}N excess $\mu\text{g g}^{-1}$ = $\frac{\text{N}\% \times ^{15}\text{N}$ excess atom%}{10,000} \times 1,000,000
- ^{15}N excess μg (total μg in pool) = ^{15}N excess $\mu\text{g g}^{-1}$ \times pool dry biomass (g)
- ^{15}N excess g (total g in pool) = $\frac{^{15}\text{N}$ excess (total μg in pool)}{1,000,000}
- $^{15}\text{N}\%$ recovered = $\frac{^{15}\text{N}$ excess g (total g in pool)}{\text{total excess g } ^{15}\text{N applied}} \times 100

2.8. Statistical analysis

R (version 4.0.2, R Core Team, 2020) was used to undertake all tests for statistical significance apart from simple regression analysis which was undertaken in Excel (Microsoft Corporation, 2016). All error margins show standard error. Significance was assessed at $p < 0.05$. Linear mixed effect models (package ‘nlme’, Pinheiro et al., 2017) were used to assess, in each nitrogen fertilization sub-set, the effect of ozone and plant part, and any interaction between them, on total pool N, N%, $^{15}\text{N}\%$ recovered, and C:N ratio, with all plant parts (flag, upper stem, second leaf, ear, and grain) included in the model except root. Ozone treatment level and plant part were used as factors, with replicate ID as a random effect variable. A linear mixed effect model was also used for repeated measures data (chlorophyll index), with data first separated by each sub-set (NØ, N+) and leaf part (flag, second leaf, third leaf, fourth leaf). Growth phase and ozone were used as factors to assess any effect and interaction, and replicate ID also included as a random effect variable. Results were summarized using ANOVA ‘type 3’ (package ‘car’, Fox and Weisberg, 2011) including a Wald chi-square test (χ^2). In addition, linear models (package ‘lsmeans’, Lenth, 2016) were used to test for statistically significant differences between ozone treatment levels in each plant part subset within each parameter (total pool nitrogen (Total N), carbon percentage (C%), nitrogen percentage (N%), percentage of ^{15}N recovered ($^{15}\text{N}\%$ recovered), and the C:N ratio). In all models, residuals and plots were checked for the appropriateness of each model and where necessary data were log transformed (Table S2). Tukey HSD post-hoc tests (package ‘multcomp’, Hothorn et al., 2008) and pairwise comparisons/contrasts of means (package ‘emmeans’, Lenth et al., 2020) were carried out where appropriate.

3. Results

3.1. Senescence index

Ozone exposure began at stem elongation, and prior to booting there were almost no signs of visible senescence and no differences between the ozone treatments in both the N+ (Fig. 2) and NØ (Fig. S1) plants. After booting, the stages of senescence were reached earlier with each increase in ozone concentration. Both NØ and N+ plants senesced progressively from the lower leaves up to the spike until complete senescence of the whole plant took place. Senescence was triggered earlier, and therefore lasted longer, in plants exposed to higher ozone, and was also therefore at a more advanced stage during anthesis and grain fill growth stages, with the same pattern found in both N+ and NØ plants.

At ear emergence (two weeks after the start of ozone exposure) plants in the very high ozone treatment already had visible senescence in all 4th leaves (Index 3), whilst in the high ozone treatment Index 3 was not reached until the start of anthesis, and not until the end of anthesis in both the low and medium ozone treatments. By the start of milk development in early grain fill (five weeks after the start of ozone exposure), plants under the very high ozone treatment already had all 2nd leaves senescing (Index 5), those under high ozone were between Index 4 (all 3rd leaves senescing) and Index 5, whilst the plants in both low and medium treatments were still only between Index 3 and Index 4. By dough development (7 weeks after the start of ozone exposure), all plants had reached Index 7 (all flag leaves fully senesced), and all then senesced through to Index 10, although the exact end of ripening was not identified in each treatment level.

3.2. Chlorophyll index

Chlorophyll levels varied by leaf, growth stage, and ozone concentration (Figs. 3 & 4). The decline in chlorophyll content started earliest in the 4th leaf, followed in sequence by the 3rd, 2nd and finally the flag leaf. In response to ozone exposure, the overall pattern was of lower chlorophyll content and/or an earlier decline occurring with increasing duration of exposure to, and concentration of, ozone. The leaves most affected were the 2nd, 3rd, and 4th leaves, with little or no effect in the flag leaf.

In the NØ plants the differences in chlorophyll levels between low and high treatments were most apparent at ear emergence in the 4th leaf, at the start of anthesis in the 3rd leaf, at the end of anthesis in the 2nd leaf, and at the start of milk development in the flag leaf. At low to moderate ozone concentrations the additional nitrogen supplied to the N+ plants at anthesis allowed higher chlorophyll levels to be maintained during early grain fill in the flag, 2nd and 3rd leaves compared to the NØ

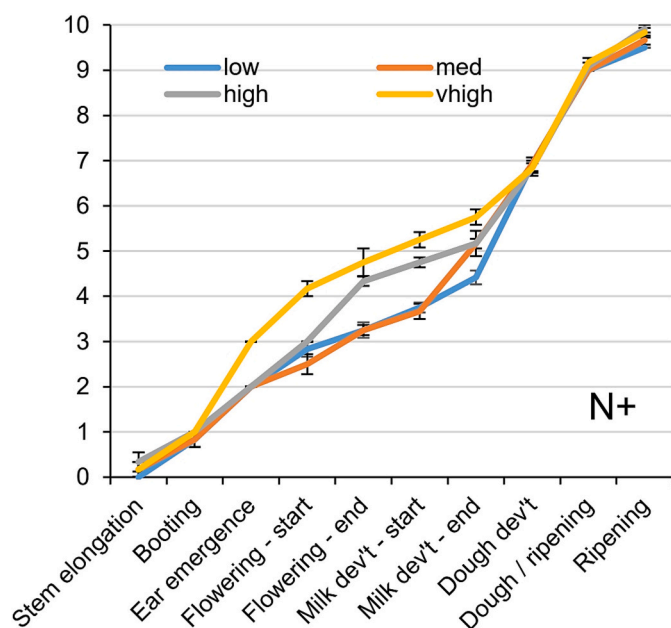


Fig. 2. Senescence index: N+ plants. Index ranges from 0 (no senescence) to 10 (fully senesced), mapping the progression of senescence at successive growth stages under low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatments. Error bars show standard error of the mean (n = 6).

plants. However, this protective effect was lost at the highest ozone concentrations.

In the 4th leaves in all ozone treatments the levels of chlorophyll peaked at booting and declined at similar rates, and were below 10 SPAD units at the start of anthesis in both the NØ and N+ plants (Figs. 3 & 4). In the NØ plants the effect of ozone overall on the fourth leaves was not significant, although there was a significant interaction between ozone and growth stage ($p = 0.032$), with significant reductions in chlorophyll levels under very high ozone found at ear emergence (low/vhigh $p = 0.028$) and at the start of anthesis (low/vhigh $p = 0.039$). This effect was smaller in the 4th leaves of the N+ plants, with only a trend towards an effect of ozone ($p = 0.069$).

In the 3rd leaves of both the NØ and N+ plants (Figs. 3 & 4) there was a highly significant effect of ozone ($p < 0.0001$), and interaction between ozone and growth stage ($p < 0.0001$). In both the NØ and N+ plants, levels of chlorophyll peaked at booting and then declined, dropping below 20 SPAD units in all ozone treatments from the end of anthesis, and below 10 under very high ozone from the start of anthesis. In the NØ plants, although the levels were lower under very high ozone from ear emergence onwards, the only significant difference between treatment levels was found at the start of anthesis (low/vhigh $p = 0.02$). In the N+ plants there were similar significant reductions under the very high ozone treatments at the start of anthesis (med/vhigh $p = 0.032$) but also in the early milk development growth stage (low/vhigh $p = 0.029$; med/vhigh $p = 0.025$). This suggests that the additional nitrogen fertilizer applied at anthesis had maintained chlorophyll levels in the 3rd leaves in the low/medium treatments but not in the higher ozone concentrations.

In the 2nd leaves of the NØ plants (Figs. 3 & 4) there was a highly significant negative effect of ozone overall on chlorophyll levels ($p < 0.0001$), and also a significant interaction between ozone and growth stage ($p < 0.0001$). At the end of anthesis there were significant reductions in chlorophyll levels under the higher ozone treatments (low/vhigh $p = 0.0042$; med/vhigh $p = 0.021$), and also in early milk development (low/vhigh $p = 0.0054$; med/vhigh $p = 0.0055$). In comparison, in the N+ plants, there was no significant effect of ozone overall on the 2nd leaves, although there was a significant interaction between ozone and growth stage ($p = 0.0059$), with the levels of chlorophyll significantly reduced in the early milk development phase under very

high ozone (low/vhigh $p = 0.048$). The addition of nitrogen appeared to maintain chlorophyll levels in the 2nd leaves under lower ozone treatment levels, especially during early grain fill.

In both the NØ and N+ plants, there was no significant effect of ozone on the flag leaf chlorophyll levels at any growth stage, although in the NØ plants there was a non-significant reduction in levels under very high ozone at the start of milk development (high/vhigh $p = 0.074$).

3.3. Nitrogen percentage (N%) in plant parts

At anthesis in the NØX plants, N% levels were highest in the flag (~3.5 %) and 2nd leaves (~3 %) (Fig. 5). There was no apparent effect of ozone on N% levels and no interaction between ozone and plant part. Similar N% levels were found in the roots of the NØX, NØ and N+ plants (Fig. 5).

In the N+ plants at harvest (Fig. 5), the highest N% levels were found in the grain (~3.5 %) and the ear (~1.5 %). The translocation of nitrogen from leaves to ear and grain appeared similar under the different treatment levels, and there was only a trend towards an effect of ozone overall ($p = 0.094$). Although mean N% levels in the N+ flag, 2nd leaf, upper stem and ear were all slightly higher under the highest ozone exposure compared to the low treatment, there was only a significant increase in the 2nd leaf (med/vhigh $p = 0.039$).

Whilst the NØ plants at harvest (Fig. 5) demonstrated a similar redistribution and level of nitrogen within plant parts, N% levels overall were significantly affected by ozone ($p = 0.032$), with a highly significant interaction between ozone and plant part ($p = 0.001$). Under the highest ozone exposures higher N% levels were found in the flag, 2nd leaf, stem and ear, with significant differences between the lower and higher treatment levels in the 2nd leaves (low/vhigh $p = 0.001$; med/vhigh $p = 0.003$), and ears (low/vhigh $p = 0.01$; med/vhigh $p = 0.017$). These data again suggest that the addition of nitrogen ameliorated the effect of ozone in the N+ plants.

In contrast to N% levels, there was minimal variation in C%, both between plant parts and between flowering and harvest, as well as between the different ozone and nitrogen treatment levels (Fig. S2), with overall levels ranging from ~41 % in second leaves, ~42–43 % in flag leaves and stems, with ~43 % in the grain and ~44 % in the ears.

Index	
0	no senesced leaves
1	1-4 small leaves
2	4th leaves started to senesce
3	all 4th leaves senescing
4	all 3rd leaves senescing
5	all 2nd leaves senescing
6	all flag leaves senescing
7	all flag leaves fully senesced
8	all stems & peduncles senescing
9	all ears and awns fully senescing
10	all leaf, stems, spikes, awns senesced

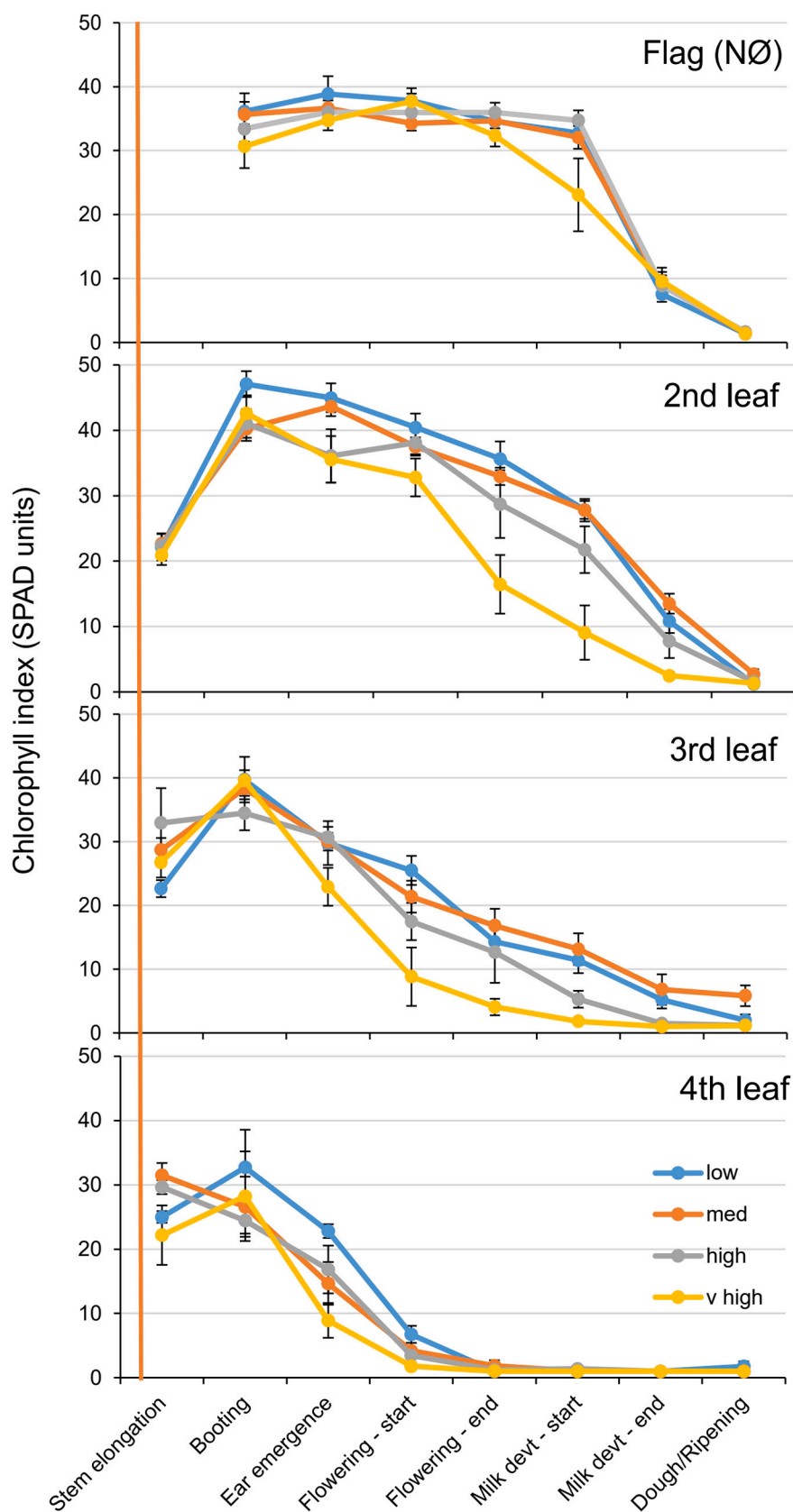


Fig. 3. Chlorophyll Index (SPAD units) in the flag, 2nd, 3rd, and 4th leaves of the NØ plants under low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatments, measured weekly at each growth stage, from stem elongation to ripening, following onset of ozone exposure (vertical orange line) at stem elongation. Error bars show standard error of the mean (n = 6).

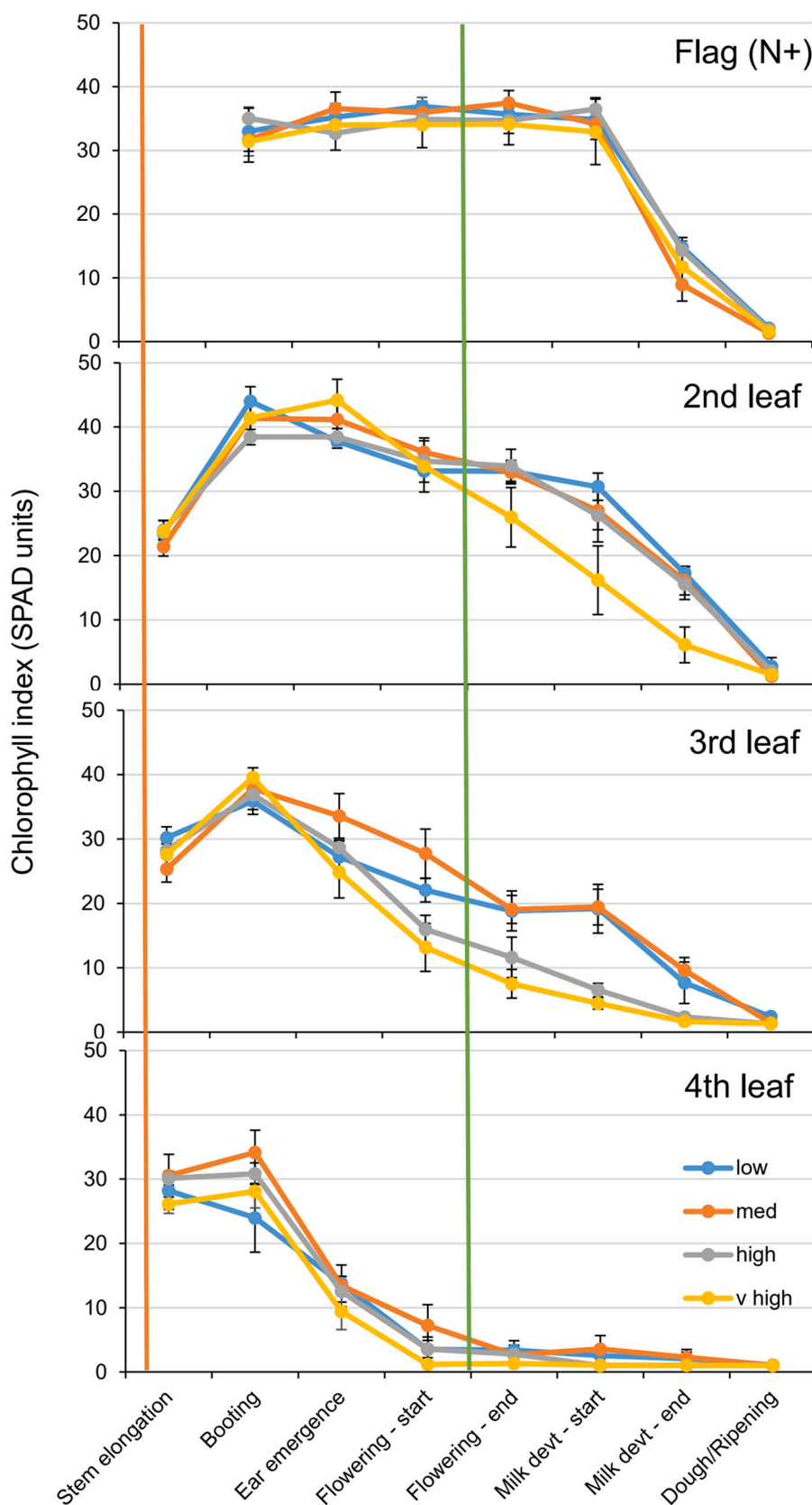


Fig. 4. Chlorophyll Index (SPAD units) in the flag, 2nd, 3rd, and 4th leaves of the N+ plants under low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatments, measured weekly at each growth stage, from stem elongation to ripening, following onset of ozone exposure (vertical orange line) at stem elongation. N+ plants received an extra nitrogen fertilization addition (20 kg ha⁻¹) at mid-anthesis (vertical green line). Error bars show standard error of the mean (n = 6).

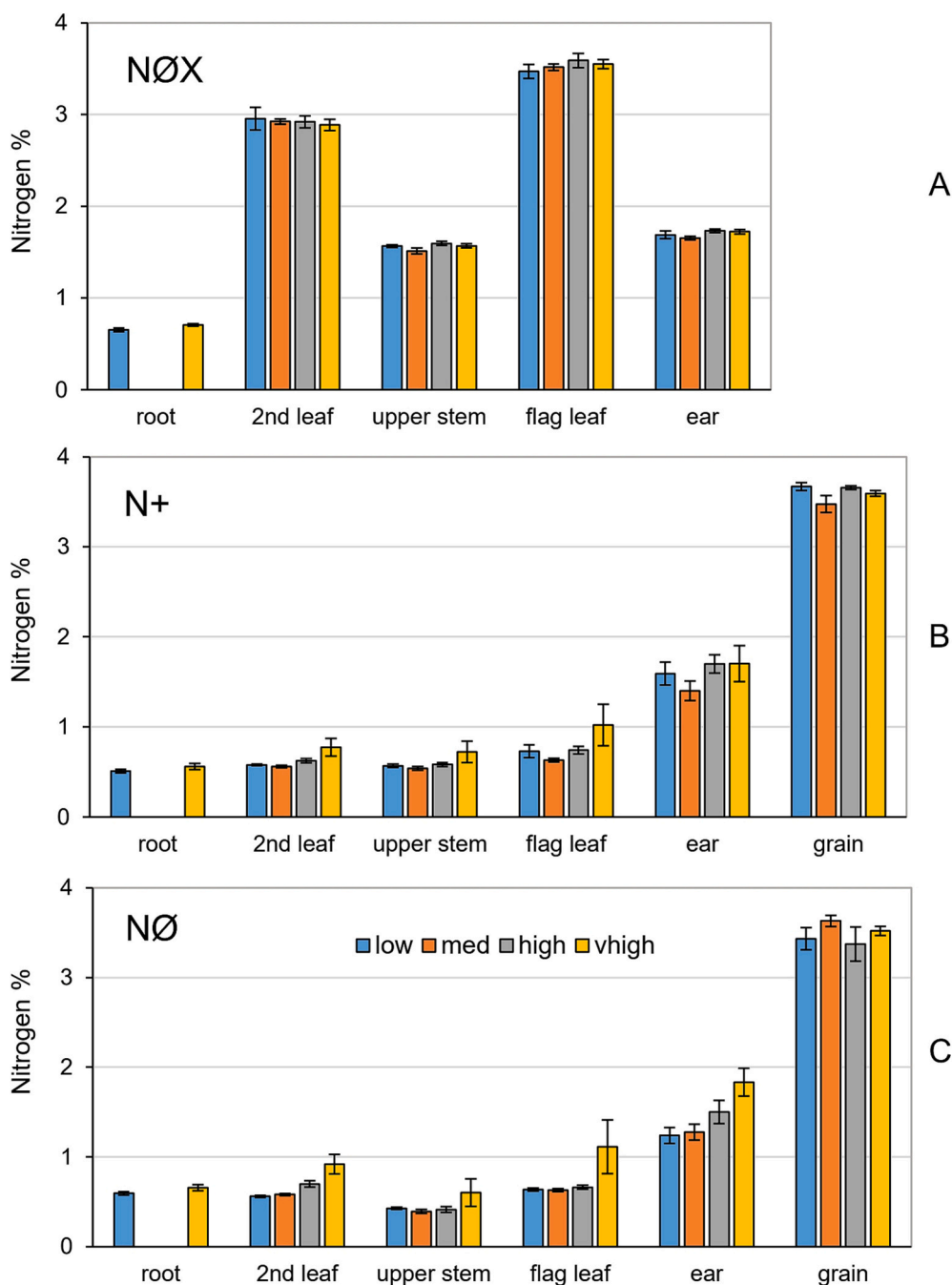


Fig. 5. Nitrogen percentage (N%) in plant parts under low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatments, in NØX plants at anthesis (A) in root, 2nd leaf, upper stem, flag leaf, and ear; at harvest in N+ plants (B) and in NØ plants (C) in root, 2nd leaf, upper stem, flag leaf, ear and grain. All NØX replicates were harvested at mid-anthesis. Error bars show standard error of the mean (n = 6).

3.4. C:N ratio in plant parts

At anthesis, in the NØ plants, C:N ratios showed no effect of ozone overall because both C% and N% levels were relatively stable across all treatments, and there were no significant differences between treatment levels in the flag, 2nd leaf, stem, ear or root (Fig. 6).

However by harvest, in the NØ plants, there was a highly significant effect of ozone overall on C:N ratios ($p = 0.0014$) (Fig. 6), and a significant interaction between ozone and plant part ($p = 0.022$). Ozone significantly reduced C:N ratios in the higher ozone treatments at harvest, in the flag leaves (low/vhigh $p = 0.003$; med/vhigh $p = 0.002$; high/vhigh $p = 0.009$), 2nd leaves (low/vhigh $p < 0.0001$; med/vhigh $p = 0.0003$), and ear (low/vhigh $p = 0.023$; med/vhigh $p = 0.046$).

Although in the N+ plants at harvest ozone had significantly affected the C:N ratio overall ($p = 0.015$) (Fig. 6), there were significant reductions only under the highest ozone treatment, in the C:N ratio of 2nd leaves (low/vhigh $p = 0.025$; med/vhigh $p = 0.014$) and upper stem (med/vhigh $p = 0.047$), suggesting the effect of ozone was ameliorated by the addition of N at anthesis.

Although slightly reduced by ozone, there was no significant difference in the root C:N ratio between the low and very high treatments in either the N+ or NØ plants, and no effect of ozone found on grain C:N.

3.5. Total nitrogen in plant parts

At anthesis the largest pool of nitrogen in the NØX plants was found

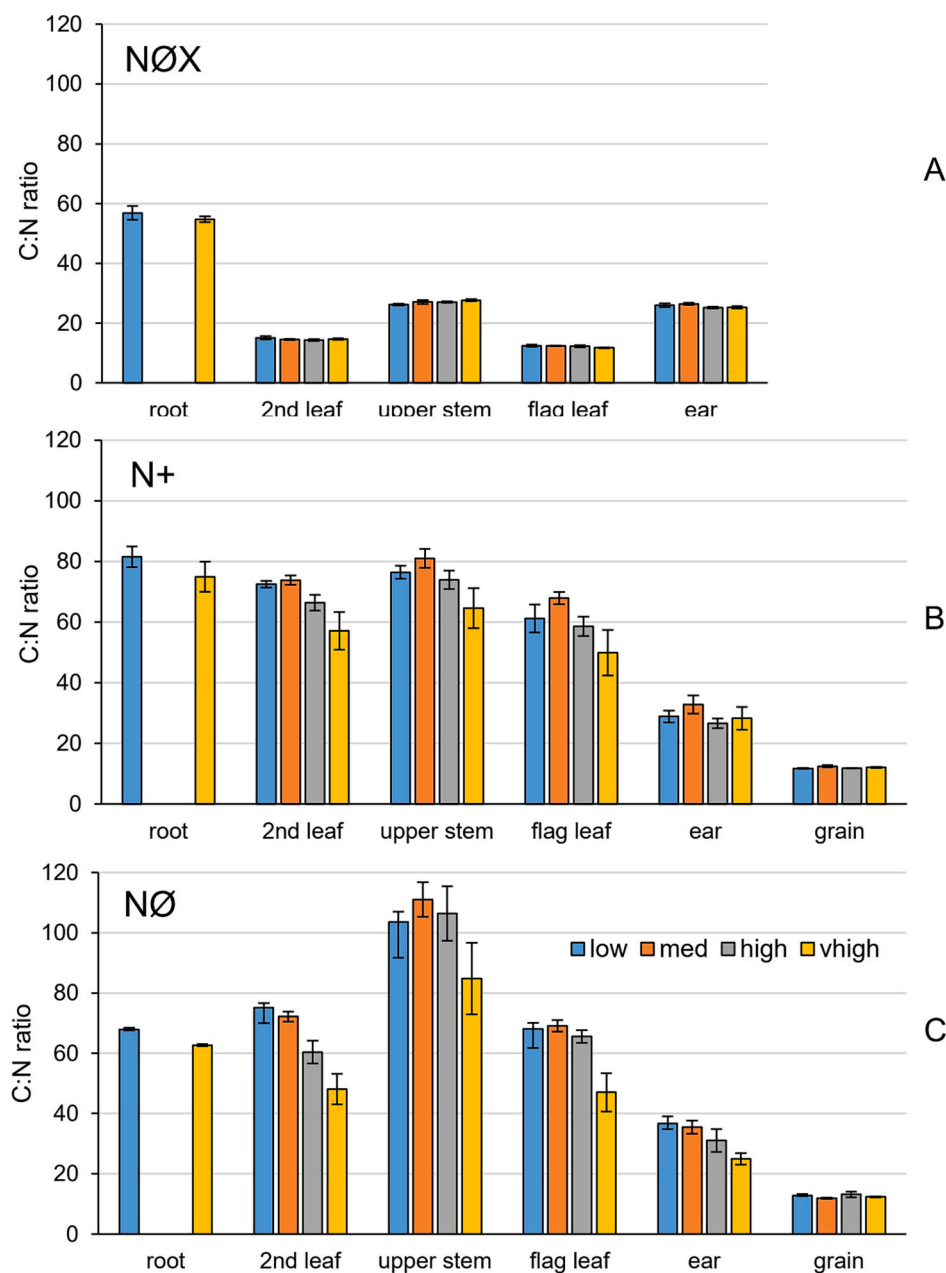


Fig. 6. C:N ratio in plant parts in NØX plants at anthesis (A) and at harvest in N+ plants (B) and NØ plants (C) under low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatments. All NØX replicates were harvested at mid-anthesis. Error bars show standard error of the mean ($n = 6$).

in the ears, followed by the roots and then the stems, with the lowest levels in the flag and 2nd leaves (Fig. S3). There was only a slight and insignificant increase in total nitrogen in aboveground plant parts in response to increasing ozone ($p = 0.078$), and analysis of individual parts found no significant differences between treatments.

At harvest in both N+ and NØ plants the total nitrogen in the ear had increased from >80 mg at anthesis to >120 mg (Fig. S3), reflecting a greater biomass as well as higher N% levels, with this increase driven by a substantial translocation of nitrogen from both the flag and 2nd leaves and the upper stems.

In N+ plants there was a significant effect of ozone ($p = 0.013$), with a pattern of total nitrogen increasing in plant parts under the higher ozone treatments. There were significant increases found in the 2nd leaf (med/vhigh $p = 0.005$) and stem (med/vhigh $p = 0.029$).

Without the addition of extra nitrogen at anthesis, total nitrogen in the NØ plants showed a highly significant effect of ozone overall ($p =$

0.00015), with significant increases under higher ozone exposures found in the flag leaf (med/vhigh $p = 0.037$), 2nd leaf (low/vhigh $p = 0.0015$; med/vhigh $p = 0.0009$), and ear (low/vhigh $p = 0.041$; med/vhigh $p = 0.018$).

3.6. Nitrogen remobilization efficiency (NRE)

In both NØ and N+ plants, NRE was highest in the flag leaf and 2nd leaf, followed by the upper stem, with smaller values for the ear, and very small negative values in the root (Table 2). In both NØ and N+ plants, in all plant parts apart from the N+ flag leaf, NRE was lowest under very high ozone exposure compared to the low ozone treatment. The largest reductions in the NRE of each plant part (between low and very high ozone) occurred in the NØ plants, especially in the ear but also in the 2nd leaf and upper stem. In both N+ and NØ plants NRE increased in the flag leaf under both medium and high ozone, before declining

Table 2

Nitrogen remobilization efficiency between NØX plants at anthesis and both N+ and NØ plants at harvest in low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatment levels, along with the difference between the low and very high treatment levels.

NØX to N+	Root	2nd leaf	Upper stem	Flag leaf	Ear
Low	-0.124	2.759	1.204	3.261	0.747
Med		2.733	1.154	3.337	0.807
High		2.707	1.230	3.383	0.752
Very high	-0.089	2.620	1.108	3.263	0.734
Difference low-vhigh	0.035	-0.139	-0.096	0.002	-0.013

NØX to NØ	Root	2nd leaf	Upper stem	Flag leaf	Ear
Low	-0.256	2.765	1.294	3.288	0.955
Med		2.726	1.252	3.338	0.882
High		2.681	1.336	3.406	0.865
Very high	-0.227	2.569	1.185	3.238	0.658
Difference low-vhigh	0.029	-0.196	-0.109	-0.050	-0.297

again under the highest ozone exposure. The flag leaf in both nitrogen treatments, and the ear in the N+ treatment, showed the least difference between the low and very high treatment suggesting the plant parts with the shortest exposure to ozone were least affected.

3.7. ¹⁵N percentage recovery in plant parts

Measurement of the ¹⁵N% levels recovered in the different plant parts of 15N+ plants at harvest (Fig. S4), following application of ¹⁵N at mid-anthesis, showed that, as expected, the majority of the recovered nitrogen was translocated to the grain (4–10 %) and ear (3–4 %), with <1 % remaining in the root, <0.4 % in the stem, and <0.1 % in both flag leaves and 2nd leaves. There was no significant effect of ozone, and there were also no significant differences between ozone treatments in any of the individual plant parts, so although the flag leaf, 2nd leaf, upper stem and ear all reflected a pattern of increasing N% with increasing ozone concentration, none of the differences between treatments were significant. What the trace experiment did show was that the nitrogen added at anthesis travelled to, or through, all parts of the plant and that this translocation was unaffected by ozone.

3.8. Grain yield

In all subsets and treatments grown to harvest (NØ, N+ and 15N+) there were low grain yields, (Fig. S5). The cause is unknown as the plants developed and flowered normally.

4. Discussion

Effective grain fill is determined by multiple factors: whole canopy photosynthesis throughout the life of the plant, effective pre-anthesis storage of surplus photosynthate, the timing and rate of senescence, and the efficiency of post-anthesis translocation of nutrients to the developing grain (Gaju et al., 2014; Araus et al., 2021). This discussion focuses on how this study informs our understanding of the effect that chronic ozone exposure has on each of these factors.

4.1. Chronic ozone affects whole canopy chlorophyll during critical growth stages

The pattern of increasing ozone damage, both as each leaf aged and as the ozone concentration increased, was apparent in all leaf cohorts below the flag leaf, with effects particularly noticeable in the 2nd, 3rd and 4th leaves during anthesis and at the start of grain fill. Whilst ozone sensitivity has previously been found to increase as the age of the leaf increases (Sandelius et al., 1995) making the lower leaves and later

growth stages more vulnerable to ozone (Pleijel et al., 1998; Gelang et al., 2000; Xu et al., 2009), few studies have measured the effect of chronic ozone on all leaf cohorts through all key growth stages. Xu et al. (2009) found an increase in visible leaf damage in the 2nd, 3rd and 4th leaves in two wheat cultivars after just three days of elevated ozone exposure applied shortly after the end of anthesis, with damage increasing both with increasing leaf age and with the ozone sensitivity of the cultivar. Although not measured in this study, reductions in chlorophyll levels inevitably also reduce photosynthetic capacity, and Soja and Soja (1995), measuring the effect of ozone on photosynthesis efficiency via Fv/Fm chlorophyll fluorescence at ear emergence in the flag, 2nd, 3rd and 4th leaves, found a similar pattern of response in each leaf cohort to this trial at this growth stage.

These effects are likely linked to changes in the capacity of plant tissue to detoxify and reduce ROS as the leaf ages and as senescence progresses (Khanna-Chopra, 2012; Bender et al., 1994; Taiz et al., 2015, p.680). The increase in ozone sensitivity during anthesis and grain fill, as found in this study, has clear implications for wheat yields and grain quality, particularly as there is evidence that modern cultivars rely as much on post anthesis photosynthesis as on pre-anthesis stored carbohydrates during grain fill (Maydup et al., 2012; Sanchez-Bragado et al., 2016; Tambussi et al., 2021).

4.2. Less impact on flag leaf compared to lower canopy

In this study, the reductions in chlorophyll levels under the higher ozone treatments were most marked in the 2nd and 3rd leaves, but notably had little effect on the flag leaf which maintained similar levels of chlorophyll in all ozone treatments until normal senescence began in this leaf at the start of grain fill. Although the flag leaf plays a key role in supplying photosynthate to the ear/grain during grain fill, the contributions of the lower leaves in combination are arguably as important as the flag. For example, research into nitrogen remobilization in 20 elite wheat cultivars (Barraclough et al., 2014) found that, at anthesis, nitrogen was apportioned as follows: stem (28 %), ear (23 %), flag leaf (15 %), leaf sheaths (14 %), 2nd leaf (11 %), 3rd leaf (6 %), and remaining leaves (3 %), with 80–85 % remobilization efficiency from each plant part to the grain by harvest. Although many studies have measured the effect of ozone on chlorophyll levels in the flag leaf (e.g. Finnan et al., 1998; Osborne et al., 2019), it is clear from the chlorophyll data from all four leaf cohorts in this trial that modelling based solely on flag leaf data will obscure the effect of ozone on whole canopy chlorophyll levels and whole canopy carbon/nitrogen assimilation, confirming previous recommendations that physiology data from all leaf cohorts needs to be incorporated into crop modelling in future (Emberson et al., 2018; Osborne et al., 2019).

4.3. Ozone affects the timing of nitrogen remobilization and senescence

Nitrogen compounds released from the breakdown of leaf chlorophyll by the senescence process contribute a substantial proportion of final grain proteins (Avila-Ospina et al., 2014; Maillard et al., 2015). Under optimal conditions, nitrogen remobilization in wheat starts immediately after anthesis is completed and before senescence is visible in the canopy (Kong et al., 2016). This prior onset of nitrogen remobilization is linked to the subsequent initiation of full senescence (Gрегersen et al., 2008; Bogard et al., 2011). In this study the concurrent measurements of chlorophyll levels and senescence in all four leaf cohorts across all key growth stages clearly show that chronic elevated ozone induced a premature start to this sequence, especially in the lower leaf cohorts. As a result, under the higher ozone treatments, visible senescence – and nitrogen translocation - was already well advanced in the lower half of the plant during anthesis and in the early- and mid-grain fill growth stages, with clear implications for levels of post anthesis photosynthesis and final grain yield. Although previous studies have varied in the length and timing of ozone exposure, in all cases elevated

ozone exposure led to reductions in green leaf duration and caused earlier and longer periods of senescence (e.g. Grandjean and Fuhrer, 1989; Finnan et al., 1998), with the resulting reduction in the duration of grain fill being linked to reduced yields (Gelang et al., 2000), as is commonly found in wheat in response to many abiotic stress (Sade et al., 2018).

4.4. Senescence is slower in ear/awns during grain fill

In this study it was observed that senescence in the peduncle, ear and awns was less rapid than the leaves, and the differences between treatments was much reduced in the final growth stages, leading to the synchronizing of the senescence index in the different treatment levels at the start of dough development. Few studies report on the effects of ozone on the photosynthetic capacity of ears of wheat, but Sild et al. (2002) found that ozone had no effect on non-structural carbohydrate levels in ears. Although the cause is not fully understood, ear photosynthesis has been found to have greater tolerance to abiotic stress than the flag, and to contribute proportionately more to grain fill when the plant is subject to stress (Maydup et al., 2010; Wang et al., 2016; Tambussi et al., 2021). As the proportion of photosynthate contributed by the ear during grain fill has also steadily increased in cultivars throughout the 20th and 21st centuries (Maydup et al., 2012; Wang et al., 2016) further research is needed to assess the effect of ozone on ear photosynthesis in the post anthesis period, and the role this may play in ozone sensitivity and tolerance.

4.5. Ozone affects N% and C:N partitioning at harvest but not at anthesis

At anthesis, the stable C:N ratios in all plant parts across all treatments suggested that C% and N% levels in the upper half of the plant, represented by the 2nd leaf, upper stem, flag and ear, were still yet to be noticeably affected by ozone. Further research is required to clarify if ozone affects the C:N ratio in the lower half of the plant (as might be inferred from the reduced chlorophyll levels in the 3rd and 4th leaves in this trial), and in what way these C:N changes are linked to the triggering of early senescence.

At harvest, the slight increase in residual N% and related reductions in C:N ratios are similar to the effects of ozone on C:N stoichiometry found in other studies undertaken mostly on tree leaf material. These studies revealed that whilst 'total C' levels largely remained stable, nitrogen levels increased by ~5 %, resulting in reduced C:N ratios (Wittig et al., 2009; Cao et al., 2016). Few studies have assessed this issue in crop plants exposed to ozone, but an increase in residual N% has also been found in rice straw in a meta-analysis of the effects of various abiotic stress on a wide range of rice cultivars (Yulong et al., 2021).

Several explanations have been proposed for these increases in residual nitrogen concentrations. Broberg et al. (2021) found that ozone reduced Nitrogen Use Efficiency (NUE), reducing the rate of remobilization of nitrogen to the grain, and concluded the shortened grain fill period arising from the early onset of senescence could then lead to more nitrogen remaining in 'source' plant parts. Although the efficiency of nitrogen remobilization (NRE) was slightly reduced by very high ozone in this study, particularly from the 2nd leaf and upper stem, NRE from the flag leaf was largely unaffected. Despite this, there were still increased residual N% levels found in the flag leaf, as well as the 2nd leaf and stem, suggesting that reduced NRE is not the only cause of the increase in residual nitrogen. It is also important to note that although this study had low levels of grain set, N% data collected in related studies of ozone-affected wheat where yields were normal (Brewster, 2023, PhD thesis chapter 5) still showed that the same pattern of increased residual leaf nitrogen occurs, irrespective of the sink demand from the grain. Others have suggested that the increase in N% may also be linked to a defence response at the whole plant level related to structural proteins which protect cell membranes (Shang et al., 2018), possibly the amino acid, proline, (Nikolaeva et al., 2010; Hayat et al., 2012; Boubliin et al.,

2022) which has been found to accumulate in plant tissues in response to many abiotic stress including ozone (Verbruggen and Hermans, 2008; Sarkar et al., 2010; Li et al., 2016). However, further research is needed to identify the exact makeup of the residual nitrogen found in ozone-affected wheat, and the underlying mechanisms which cause the increase.

4.6. Extra nitrogen fertilization ameliorated effect of ozone

Whilst abiotic stress is known to decrease levels of cytokinin (the plant hormone also involved in delaying senescence in plants), increasing the levels of nitrogen available to a plant can lead to an increase in both cytokinins (Taiz et al., 2015, p.682–3) and antioxidants (Ma et al., 2022), enabling the plant to reduce levels of ROS and oxidative damage. This may have taken place in this study, with the slight but noticeable differences between the N0 and N+ plants suggesting that the extra nitrogen applied at anthesis protected chlorophyll levels during grain fill in the flag, as well as in the 2nd and 3rd leaves in the lower ozone treatments, delaying senescence and maintaining photosynthesis in these leaves at critical reproductive growth stages. This addition of nitrogen also reduced levels of residual nitrogen left in the leaves and stem at harvest, ameliorating the effect of ozone on C:N ratios and the efficiency of translocation of stored nitrogen. Whilst antioxidant levels were not measured in this study, increased antioxidant levels in response to higher nitrogen fertilization have been found in ozone-affected wheat (Pandey et al., 2018). As the timing and triggering of early senescence is partly dependent upon nitrogen supply future research could investigate the effect of different levels of nitrogen fertilization regimes on ozone sensitivity.

Interestingly, despite the differences between the nitrogen treatments, the ¹⁵N trace experiment revealed that only a small amount of nitrogen applied at anthesis was taken up by the plants. Although the very low grain yield will have substantially reduced the sink strength, the majority of the nitrogen was still translocated either to the ear or the grain, and only small residual amounts were left in the root, upper stem, 2nd leaf and flag leaf. However, it is important to note that there were no differences found between any of the treatments, indicating that the uptake by the root and subsequent translocation of nitrogen to the shoot was unaffected by ozone.

5. Conclusion

This study found that when a widely grown modern spring wheat cultivar was exposed to chronic ozone for 11 weeks it triggered an earlier and extended senescence under the higher ozone treatments, preceded by an earlier start to nitrogen translocation, represented by reductions in chlorophyll. These reductions in chlorophyll, particularly evident in the older leaves of the lower leaf cohorts during anthesis and grain fill, will inevitably reduce the plant's total photosynthetic capacity. This is a critical issue due to modern wheat's greater reliance on post-anthesis photosynthesis to maximize grain fill and starch content. Notably, the flag leaf was largely unaffected by ozone, and therefore not representative of lower – and total - canopy chlorophyll levels, confirming that lower leaf cohorts need to be accounted for in work to model the effects of ozone on global wheat yields.

Although nitrogen storage in the upper plant parts prior to anthesis appeared to be unaffected by ozone, nitrogen remobilization efficiency between anthesis and harvest was reduced by the highest ozone treatment, particularly in the 2nd leaf and upper stem but, notably, not in the younger flag leaf. The resulting higher N% and total nitrogen levels found in plant parts at harvest under the highest ozone treatment, also led to reduced C:N ratios. An ameliorating effect of the additional nitrogen fertilization applied at anthesis was revealed by the chlorophyll, N%, C:N and NRE data, highlighting the key role that nitrogen fertilization levels could play in tolerance mechanisms in ozone-affected wheat.

Further research is needed to clarify the effect of ozone on nitrogen dynamics within the wheat plant, in terms of whole plant nitrogen storage prior to anthesis, nutrient partitioning between the shoot and root, especially during anthesis and grain fill, and the role that levels of nitrogen fertilization may play in any ozone tolerance, linked to delaying senescence and maintaining chlorophyll levels and photosynthetic capacity during anthesis and grain fill in all leaf cohorts.

Funding

This work was supported by the Natural Environment Research Council (NERC), grant number 500491042 NERC Envision DTP Studentship (UKCEH).

CRediT authorship contribution statement

Conceptualization, Methodology, Writing - review & editing: CB, NF, FH; Data curation, Formal analysis, Visualization, Writing - original draft: CB; Supervision: NF & FH; Funding acquisition, Project administration, Resources: FH. Additional technical support: Ed Rowe* (Stable Isotope calculations). *UK Centre for Ecology & Hydrology

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors wish to thank Aled Williams (Aled Williams Mechatronics) for technical support for the ozone exposure facility, and Ed Rowe (UKCEH) for his input and advice regarding staple isotope calculations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2023.168288>.

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