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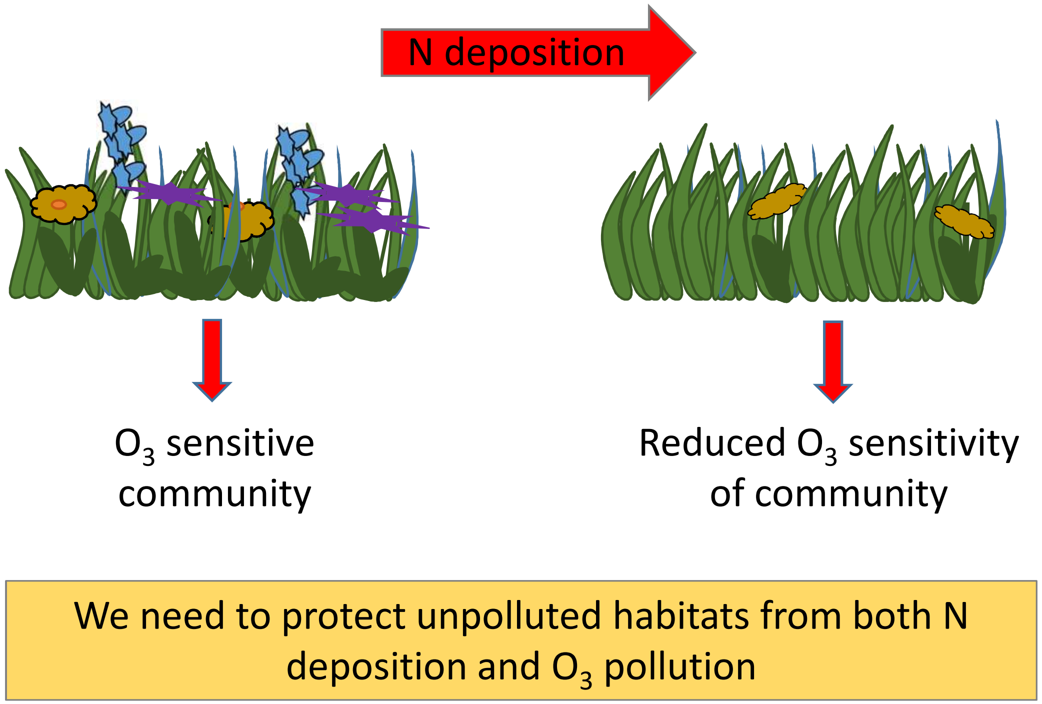
N deposition
O_3 sensitive community
Reduced O_3 sensitivity of community
We need to protect unpolluted habitats from both N deposition and O_3 pollution

Impact of long-term nitrogen deposition on the response of dune grassland ecosystems to elevated summer ozone

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Abstract

Nitrogen deposition and tropospheric ozone are important drivers of vegetation damage, but their interactive effects are poorly understood. This study assessed whether long-term nitrogen deposition altered sensitivity to ozone in a semi-natural vegetation community. Mesocosms were collected from sand dune grassland in the UK along a nitrogen gradient (5 to 25 kg N/ha/y, including two plots from a long-term experiment), and fumigated for 2.5 months to simulate medium and high ozone exposure. Ozone damage to leaves was quantified for 20 ozone-sensitive species. Soil solution dissolved organic carbon (DOC) and soil extracellular enzymes were measured to investigate secondary effects on soil processes.

Mesocosms from sites receiving the highest N deposition showed the least ozone-related leaf damage, while those from the least N-polluted sites were the most damaged by ozone. This was due to differences in community-level sensitivity, rather than species-level impacts. The N-polluted sites contained fewer ozone-sensitive forbs and sedges, and a higher proportion of comparatively ozone-resistant grasses. This difference in the vegetation composition of mesocosms in relation to N deposition conveyed differential resilience to ozone.

Mesocosms in the highest ozone treatment showed elevated soil solution DOC with increasing site N deposition. This suggests that, despite showing relatively little leaf damage, the ‘ozone resilient’ vegetation community may still sustain physiological damage through reduced capacity to assimilate photosynthate, with its subsequent loss as DOC through the roots into the soil.

We conclude that for dune grassland habitats, the regions of highest risk to ozone exposure are those that have received the lowest level of long-term nitrogen deposition. This highlights the importance of considering community- and ecosystem-scale impacts of pollutants in addition to impacts on individual species. It also underscores the need for protection of ‘clean’ habitats from air pollution and other environmental stressors.

Capsule

For dune grassland habitats, the regions of highest risk to ozone exposure are those that have received the lowest level of long-term nitrogen deposition

Introduction

Excess nitrogen deposition and elevated tropospheric ozone are two of the most important pollutants driving vegetation damage and community composition change. There are many studies on the impacts of these pollutants individually, but few on their combined effects, and a particular knowledge gap is the in-combination responses of intact communities or species mixes (Mills et al., 2016).

Atmospheric nitrogen deposition impacts on vegetation

Atmospheric deposition of reactive nitrogen (‘N’) has greatly increased in the UK over the last century (Fowler et al., 2004). Nitrogen is emitted to the atmosphere in gaseous form both as reduced nitrogen (NH_3, ammonia, and related forms) for which the sources are predominantly agricultural (livestock and fertilizer), and as oxidized nitrogen (NO and NO_2) from a variety of combustion processes including road transport. The gases NO_2, and NH_3 as well as the aerosol nitric acid (HNO_3) can be deposited directly to vegetation (‘dry deposition’) over relatively short distances, within tens of kilometers. In addition, long-range transport of air pollutants can also occur when gaseous nitrogen and sulphur compounds react to form particulate matter, that is washed out of the atmosphere by precipitation (‘wet deposition’), sometimes thousands of kilometers from the source. Atmospheric emissions of both NH_3 and NO_x peaked in western Europe and the UK around 1990 (NAEI, 2012). During recent decades there have been significant decreases in NOx emissions, which have fallen to approximately half of the 1990 level, and a more modest decrease of 20% in NH_3 emissions. However, the atmospheric deposition of N has declined at a slower rate and whereas NO_x deposition decreased by approximately 22%, the total deposition of N changed very little over the period 1987-2006, due to the non-linearity of atmospheric chemistry including the influence of climate variability, particularly temperature (RoTAP, 2012; Tang et al, 2018). In addition, observations of atmospheric NH_3 mixing ratios have been shown to increase over recent decades in large parts of Europe (Warner et al., 2007). Effective reductions of NO_x and SO_2 emissions lead to a lower abundance of acids for NH_3 to react with and form particulate matter, with the resulting higher NH_3 mixing ratios leading to higher NH_3 deposition rates and therefore a lower decline in N deposition than expected.

Nitrogen is an essential nutrient for plants: it is a component of amino acids and proteins and is needed for growth and repair of tissue. However, excess nitrogen deposition has been identified as an important driver of vegetation change by processes including competitive exclusion of species characteristic of nutrient-poor communities, soil acidification, increased susceptibility to environmental stressors, and direct foliar damage (Dise et al 2011; De Schrijver et al., 2011, Maskell et al., 2010). Field experiments have shown that the abundance of sensitive forbs and bryophytes declines when exposed to long-term excess nitrogen deposition, with nutrient- or acid-tolerant grasses and shrubs increasing (Cunha et al., 2002, Throop and Lerdau, 2004, Jones et al. 2014, Phoenix et al. 2012). Changes in species composition of plant communities in relation to nitrogen deposition have also been demonstrated through spatial gradient surveys and temporal re-surveys in many habitats, including nutrient-poor sand dune and other grasslands, bog, heathland, and forest floor communities (Stevens et al. 2004; Jones et al. 2004; Dupre et al. 2010; Field et al. 2014). Nitrogen deposition over many sensitive habitats in Europe and other densely populated global regions exceeds the critical levels and loads set for those habitats (Matejko et al, 2009; RoTAP, 2012).

Tropospheric ozone impacts on vegetation

Tropospheric ozone is created and destroyed through a series of photochemical reactions involving precursor molecules including nitrogen oxides, methane, carbon monoxide and non-methane volatile organic carbons (Royal Society, 2008). Ozone concentrations in Europe have been rising since the Industrial Revolution from 10-15 ppb to current levels of 30-40 ppb (Stich et al., 2007, Schultz et al., 2017, Cooper et al., 2014). More recently, the size of ozone peaks has been decreasing over much of Europe (Schultz et al., 2017, Cooper et al., 2014), but background concentrations in Europe and throughout the northern hemisphere have been rising due to increased emissions of precursor molecules, particularly from sources in Asia (Granier et al., 2011).

Ozone affects plants in a variety of ways including reduced photosynthesis rate, impaired stomatal control, accelerated leaf senescence, reproductive damage, a reduction in the supply of photosynthate to roots, other changes in carbon allocation, and impaired root respiration (Yue and Unger, 2014; Wagg et al, 2013; Emberson et al., 2018). Responses of vegetation to ozone can vary greatly between species. Reasons for differential sensitivity include differences in the ability to exclude ozone by stomatal regulation (Hoshika et al, 2013), the rate at which plants can detoxify reactive oxygen species to protect the photosynthetic apparatus (Di Baccio et al, 2008), and the plasticity of resource partitioning to replace damaged leaves (Grantz et al, 2006). However, unlike nitrogen, ozone is chemically unstable and does not accumulate in the vegetation or the soil. Therefore, although its impacts can be long-term (e.g. changes in community composition or below-ground carbon cycling) ozone itself does not remain in the ecosystem. Ozone damage to individual plants can often be detected over periods of days (VanderHeyden et al., 2001), although impacts on higher-level characteristics such as plant community composition may take years to manifest. Physiological damage can reduce the capacity of plants to assimilate carbon, which is then lost as DOC through the roots. Soil enzymes respond to changes in root exudates and plant litter quality and quantity, which are in turn governed by rates of plant growth, litter production and root decomposition (Henry et al., 2005; Allison and Treseder 2008). Thus measuring these soil components can give an indication of the functioning of the community as a whole.

Nitrogen-ozone interactions

While numerous studies have been conducted separately on the impacts of ozone or nitrogen on semi-natural and cultivated vegetation, far fewer experiments have investigated the interactions between these two pollutants in combination. The studies to date have shown a wide range of vegetation responses, with nitrogen ameliorating (Yendrek et al., 2013; Jones et al. 2010; Häikiö et al., 2007), exacerbating (Wanatabe et al., 2012, Wyness et al. 2011, Hayes et al., 2007), or not affecting sensitivity to ozone (Bassin et al, 2013; Harmens et al 2017).

Some of the variation in vegetation responses can be explained by differing physiological responses. For example, a plant may respond to an increase in available N by increasing photosynthetic rate, opening stomata to take in more CO_2 which would then also increase the passive uptake of ozone, causing N to exacerbate ozone damage. Conversely, a plant may react to ozone stress by allocating additional N to protect or repair photosynthetic apparatus, with an amelioration of ozone damage (Jones et al. 2010). Intrinsic differences in species' metabolic and growth rates can also explain differences in rates of response to N and ozone, as well as the relative importance of other drivers such as climate and hydrology. Responses of individual species, and interactions between and among species may then be reflected in different responses to N and ozone at the population and community levels (e.g. Payne et al., 2011). Both nitrogen and ozone can affect plant community composition and species richness, but the few studies considering both pollutants together have not demonstrated interactive effects (Payne et al. 2011, Bassin et al. 2013).

In this study we assessed whether chronic long-term N deposition affects the sensitivity of dune grassland vegetation to acute short-term ozone pollution. We address this question by experimentally elevating the tropospheric ozone concentrations to sand dune ecosystem mesocosms collected from sites along a range of long-term nitrogen deposition in the UK, and measuring species- community- and ecosystem-level responses. We chose dune grassland because it is a well-studied community with documented sensitivity to both nitrogen deposition (Field et al., 2014, Plassmann et al., 2009) and ozone enrichment (Mills et al., 2007). The UK is well documented for both N and ozone impacts, has strong N gradients across the country, and previous studies have shown impacts on plant communities across this gradient after accounting for climate and other drivers (e.g. Payne et al., 2011). Ozone is a more transient pollutant, the location of highest impact can vary between and within years (Hewitt et al., 2016). Typically there is a gradient of ozone fluxes across the UK, but is less strong than for N, particularly in the northern half of the UK, from where we collected our mesocosms. Since the impact of N on an ecosystem can take decades to manifest, we use the N gradient of deposition as our N-addition 'experiment'. Thus this study uniquely combines a gradient and an experimental approach to investigate the combined long-term effects of N and the acute effects of ozone on a habitat vulnerable to both stressors.

Specifically, we address the research questions 1) Does N deposition change the ozone sensitivity of individual species, and does this alter the sensitivity of the community to ozone via changes in plant community composition? 2) Does the combined impacts of N and ozone affect plant community functioning, specifically changes in dissolved organic carbon (DOC) in soil pore-water, and soil extracellular enzyme activity?

Methods

<u>Habitat and site selection</u>

Dune grasslands in Europe are distributed around the coastal fringes and are most extensive around the north-Eastern Atlantic, North Sea and Baltic Sea regions (Doody, 2001). Although often sites of conservation status, dune grassland are threatened by a range of factors such as land use change (e.g. grazing), sedimentation, sea level rise, and air pollution (Jones et al, 2011). Grassland habitats in general have a high proportion of ozone-sensitive species (Mills et al., 2007) which may be in part due to the low leaf mass area (LMA) of these plants, giving a relatively high leaf surface area for ozone uptake (Feng et al., 2018). Since sandy soils are generally poor in nutrients with a low acid neutralising capacity, dune grassland communities are also potentially sensitive to nutrient enrichment and acidification from atmospheric nitrogen pollution (Bobbink et al., 2003). Changes in species composition or abundance in dune grassland have been demonstrated in N-addition studies (van den Berg et al. 2005, Plassmann et al. 2009), in national- or local-scale N-gradient studies (Jones et al. 2004, Field et al. 2014) and in re-surveys (Pakeman et al., 2016). These have shown evidence of eutrophication above 4-6 kg N ha^{-1} yr^{-1} in fixed dune vegetation in the UK, with a shift towards species with higher Ellenberg N indicator values, indicating a change towards component species with increased nutrient tolerance.

From a previous N-gradient survey of dune grassland (Jones et al, 2004, Field et al, 2014), we selected a subset of seven sites, ranging in N deposition from 5.4 to 16.7 kg N/ha/yr, and with relatively constant long-term background ozone exposure of approximately 30 ppb (Figure 1, Table S1). Site selection was designed to maximise the N deposition gradient within the existing survey whilst keeping as constant as possible other drivers such as rainfall and temperature, although we ackowledge that the two sites with the lowest N deposition had the lowest temperature and the highest rainfall. We also included two 11-year nitrogen addition experiments at one of the sites, Newborough in Wales (Plassmann et al., 2009). In these experiments, N deposition was increased from background levels of 10 kg N/ha/yr to 17.5 and 25 kg N/ha/yr by monthly additions of NH_4NO_3. During that time period, soil pH remained around 6.5, indicating some soil buffering, possibly from soil carbonates.

The mesocosm sites are a subset of a larger survey of 24 dune grassland habitats studied in 2009, in which the species richness of forbs and mosses was significantly negatively related to nitrogen deposition after accounting for other drivers such as precipitation, temperature, soil chemistry, and altitude (Field et al. 2014). In choosing our sub-sites we took advantage of a large amount of background information from the full survey, such as community composition, species richness, soil chemistry, land use, temperature and precipitation (Table S1). Analysis of the larger survey data identified N deposition and soil pH as the major correlates to species richness and composition.

Site-specific nitrogen deposition and ozone exposure modelling

The Concentration Based Emissions and Deposition model (CBED, Smith et al., 2000) was used to estimate total inorganic N deposition to the sites (Figure 1A). The CBED model uses a network of measured ionic concentrations in precipitation interpolated with annual precipitation to generate national-scale estimates of wet deposition of NH_4^+ and NO_3^- at a 5 km spatial resolution. Annual dry deposition of NH_3 and NO_x is similarly calculated as the product of network-based annual average gas concentration and modelled concentrations and deposition velocities (Sutton et al., 2001, Smith et al., 2000).

The EMEP MSC-W model (www.emep.int; Simpson et al., 2012), an atmospheric chemistry transport model that simulates atmospheric composition and deposition of pollutants including ozone, was used to estimate ozone flux for 2015 (Figure 1B). Data are presented as POD_3IAM, which is the Phytotoxic Ozone Dose above a threshold of 3 nmol m^{-2} s^{-1} accumulated during daylight hours, and although parameterised based on the response by wheat, indicates the potential ozone uptake by semi-natural vegetation.

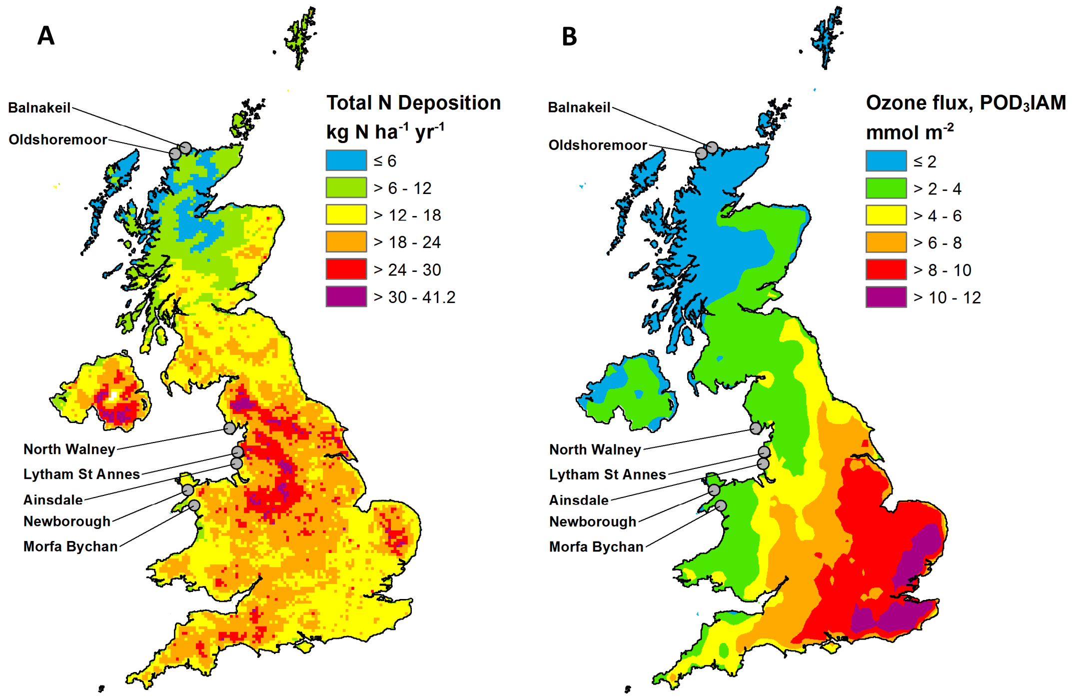

Figure 1: Modelled A) total N deposition averaged over the years 2012-2014, using CBED and B) ozone fluxes (POD$_3$IAM) for the year 2015 for the UK, using EMEP. Sites used in this study are indicated.

Mesocosm extraction and preparation

Between 10th April and 6th June 2014, nine intact mesocosms of size 30 cm diameter, 25 cm deep were collected from each site and the two field experiments, choosing areas where the organic layer of the soil was 5 to 10 cm deep. A perforated plastic base was added to each mesocosm and they were transported to our field facility in Abergwyngregyn, North Wales, UK (Latitude 53.2389, Longitude -4.0185). In June, cover estimates of all vascular plants were made for each mesocosm, and the vegetation composition of each mesocosm was photographed, after which the vegetation was cut back to 3 cm for standardisation. Supplementary watering was given to all mesocosms during dry periods.

Ozone exposure system

Mesocosms were exposed to ozone using a Free Air Ozone Enrichment (FAOE) facility. The FAOE system uses nine 4 m diameter rings to supply ozone at a height of 30 cm. The rings were arranged in a 3 × 3 matrix, with 10 m between the centres of each ring (Figure S1). Treatments were an ambient air (AA) control, 'AA+' with an addition of approximately 10 ppb O_3 to ambient, and 'AA++', with an addition of approximately 20 ppb O_3 to ambient air. There were three replicate FAOE rings per treatment.

After a 2-week acclimation period in ambient air, ozone fumigation started on 17th July and ended on 13 October (Figure 2). Ozone was supplied using an ozone generator (G11, Pacific Ozone) which utilised oxygen concentrated from ambient air (Integra 10, SeQual). Ozone delivery was via computer-controlled (LabView version 2012) solenoid valves operating using pulse width modulation. Small fans (200 mm, Xpelair) were used to push the ozone through the delivery pipe (65 mm diameter, with 3 mm holes every 20cm; Figure S2) at a rate of 0.17 m^3/s per FAOE ring. Wind speed was monitored continuously (WindSonic, Gill Instruments Ltd, UK) and was used to instantaneously adjust solenoid operation and thus ozone delivery. Ozone release was reduced at wind speeds below 16 m/s and stopped below 2 m/s and, therefore, the ozone mixing ratio was dependant on windspeed.

Ozone was sampled adjacent to the plants in each ring at a height of 30 cm for approximately 3.5 minutes in every half-hour using an ozone analyser (Thermo 49i). During the period of ozone exposure of the mesocosms, the ozone concentration in the AA control remained fairly constant with a mean concentration of 28 ppb (±1.2), the AA+ treatment had a mean concentration of 36 ppb (±4.0), and the AA++ treatment had a mean concentration of 48 ppb (±5.6) (Figure 2B; Table 1). Over this period the mean daytime temperature was 17.5 °C, and mean N deposition at the site estimated using the CBED model (Smith et al., 2000) was approximately 20 kg/ha/yr. We recognise that this represented an increase in N deposition for all but one of the mesocosms, but was negligible compared with the previous N deposition history for these mesocosms, and N impacts on vegetation composition of intact communities tend to act over timescales of years to decades (Dise et al, 2011).

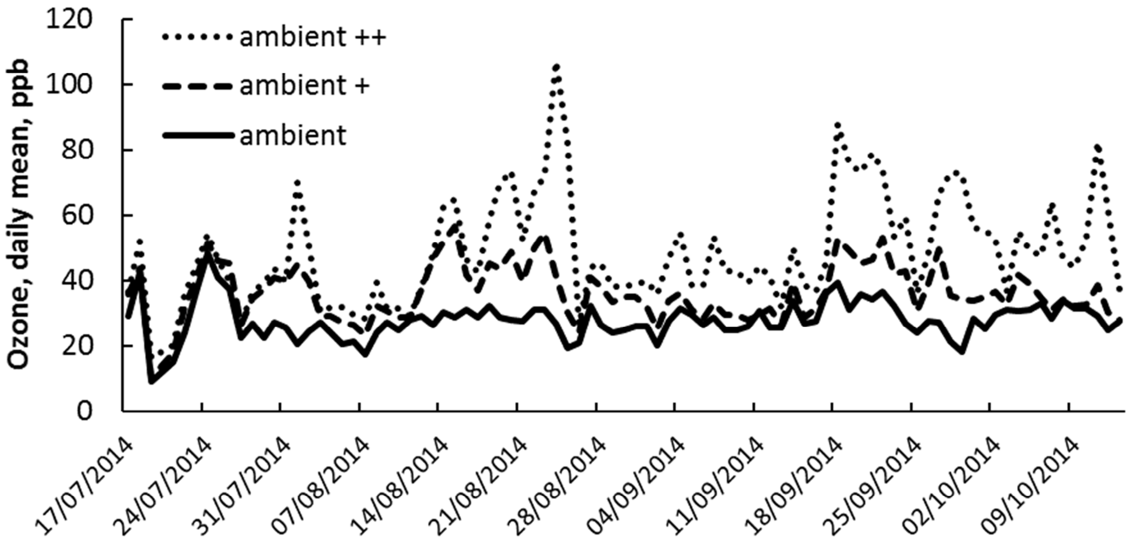

Figure 2: Daily mean ozone concentration for the ambient, ambient + and ambient ++ treatments for the duration of the exposure period.

Table 1: Season ozone exposure of the ambient air, ambient air + and ambient air ++ treatments. Standard errors are shown.

Ozone treatment	24h mean (ppb)	Daylight mean (ppb)	Mean daily maximum (ppb)	AOT40 (ppm.h)
Ambient air (AA)	27.8 (± 1.2)	29.9 (± 1.5)	39.0 (± 1.4)	1.2 (± 0.3)
AA+	36.3 (± 4.1)	38.7 (± 3.6)	66.6 (± 11.1)	8.9 (± 4.1)
AA++	48.9 (± 5.7)	48.9 (± 4.5)	97.7 (± 12.4)	21.8 (± 7.5)

Ozone injury assessment.

On 5th August, after exposure of all mesocosms to the ozone regime for three weeks, an assessment of visible leaf injury was undertaken, as visible leaf damage was widely occurring and clearly identifiable at this time. Twenty species exhibited signs of leaf injury or senescence: 6 grasses, 11 forbs and 3 sedges/rushes. These 20 target species were subsequently assessed in each mesocosm in the ambient and high ozone treatments after exposure to the ozone regime for six weeks. For each target species we counted the number of damaged leaves and the total number of leaves per mesocosm. For forbs, full leaves were classified as either damaged or healthy. For grasses and sedges, a leaf was classified as damaged if >25% of the leaf blade was affected, otherwise it was classified as healthy.

Porewater DOC extraction and analysis

Water samples were collected from each mesocosm every two weeks between 14th August and 22nd October using Rhizon MOM samplers (Rhizosphere Research Products, The Netherlands). All samples were filtered immediately (filter pore size 0.45 µm) and stored at 5 ^{o}C in the dark until analysis. Samples were analysed for DOC using a TOC and TN analyser (Thermalox® Analytical Sciences). Samples were first acidified with 45µL of 1M HCl for samples from Newborough, Ainsdale, Morfa Bychan and North Walney, and 75 µL of 1M HCl for Lytham St Annes, Balnakiel and Oldshoremore, based on the concentration of total inorganic carbon in the samples. All standards were also acidified to the same level.

Soil enzyme extraction and assay

We also measured the activity of the soil-based enzymes B-D-glucosidase (which degrades carbohydrates, particularly cellulose) and N-acetyl-beta-D-glucosaminidase (which converts complex organic molecules to simpler amino-sugars) at the end of the ozone exposure period; these enzymes are important for the microbially-mediated cycling of carbon and nitrogen, respectively, in the soil.

Soil samples (approximately 10 g) were collected from each mesocosm on the 20th of October 2014 and stored at 4 ^{o}C. The samples were homogenised by hand, removing any stones and/or large roots. Three 1 g (+/- 0.05) sub-samples of each soil sample were placed into reinforced stomacher bags (Seward, UK) and stored at 4 ^{o}C overnight. 7 ml of substrate (4-MUF beta-D-glucopyranoside for Beta-D-glucosidase, or 4-MUF N-acetyl-beta-D-glucosaminide for N-acetyl-beta-D-glucosaminidase) was added to one 1 g of each soil sample. Each bag was homogenised for 30 then incubated at 18 ^{o}C for 55 minutes, after which they were removed and 1.5 ml was transferred from each bag and centrifuged at 10,000 rpm for 5 minutes. 250 microliters of the supernatant from each enzyme sample was extracted and added to 50 µL of ultrapure water in Sterilin® Microplate wells which were analysed using a plate reader (Spectramax M2e) to determine the fluorescence at 450 and 330 nm excitation and then emission. Fluorescence was converted into enzyme activity according to Dunn et al. (2014).

Statistical analyses

Stepwise multiple linear regression was used to identify predictive relationships from the potential driver variables (total N deposition, wet NO_3 deposition, mean annual precipitation, growing degree days, total mineralisable N, soil pH, and % soil organic matter, Table S1), and the response variables of total number of species, grass species number, sedge species number, forb species number, and bryophyte species number. We employed a combination of forwards and backwards selection, with variables included if they explained significant variation in addition to those already included in the model. Analysis of the distribution of residuals was made to confirm that the overall assumptions of the regression were met.

Results

Pre-ozone treatment

Species richness relationships with long-term N deposition

In the pre-treatment assessment of the mesocosms, 93% of the variability ($p < 0.001$) in total species richness was explained by a model combining soil pH (65%) and total nitrogen deposition (28%), although the single best predictor was growing degree days (72%; $p = 0.002$). When these three variables were included in the regression, the remaining variables of annual precipitation, wet NO_3 deposition, total mineralisable N, and % soil organic matter were not significant. Annual precipitation, wet NO_3 deposition, and % soil organic matter were also not significant explanatory variables in linear regression relationships using single predictors (Table 2, Table S2). There was no single species group that dominated this relationship, as soil pH was one of the significant predictors for the forb (67%; $p = 0.033$), grass (47%; $p = 0.033$) and sedge (82%; $p = 0.001$) richness. The relationships between nitrogen deposition and growing degree days with species richness were negative, whilst the relationship between pH and species richness was positive.

There was a significant negative relationship ($p = 0.031$) between the number of vascular plant species and the nitrogen deposition at a site (Figure 3A), with species number declining from 15-20 in mesocosms from the least polluted sites to 5-10 for the sites with the highest N deposition. The change in species number was most pronounced for forbs, which declined from 8-10 at low-N sites to 0-2 at high-N sites ($p = 0.006$; Figure 3B). Both relationships were best fitted with an exponential curve ($r^2 = 0.57$ for all species; $r^2 = 0.50$ for forbs), indicating a greater reduction in species number per kg N as nitrogen deposition increased from the least polluted sites. The number of sedge species per mesocosm showed a non-significant decline with increasing N deposition, whereas the number of grass species and the number of moss species showed no significant trend. The relationship between species number and nitrogen deposition in the mesocosms was similar to that found in the larger survey of 24 sites (Field et al. 2014), although there were more species found in the survey quadrats, which at 2×2 m were over four times the area of the mesocosms.

Table 2: P-values based on linear regressions between species richness per mesocosm and driver variables. Significant relationships ($p<0.05$) are shown in bold, and the response direction is indicated. Corresponding r^2 values are shown in Supplementary Material Figure S2.

	N deposition	Wet NO_3 deposition	Annual precipitation	Growing degree days	Total mineralisable N	Soil pH	% soil organic matter
Grasses	0.743	0.648	0.278	0.381	0.827	**0.033**	0.534
Sedges	0.441	0.585	0.314	**0.033**	0.158	**0.001**	0.538
Forbs	**0.038**	0.929	0.281	**0.006**	**0.007**	**0.033**	0.202
Bryophytes	0.221	0.113	0.342	0.997	0.641	0.471	0.501
Total species	**0.046**	0.887	0.132	**0.002**	**0.032**	**0.006**	0.383
Response direction	↘		↗	↘	↘	↗	↗

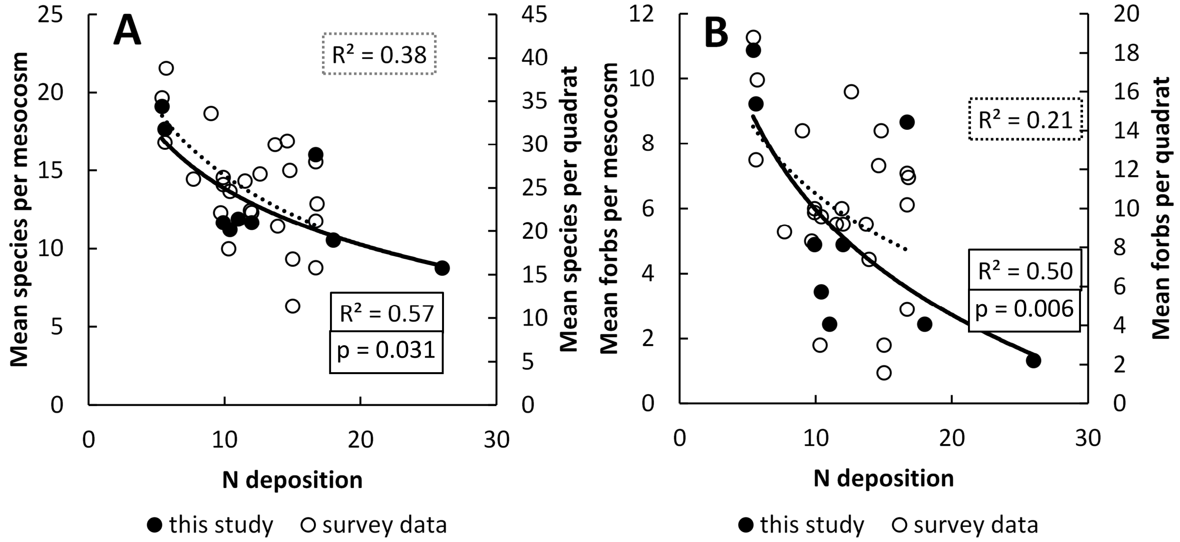

Figure 3: Species richness in relation to modelled N deposition of mesocosms for A) all species, and B) forbs only. Filled symbols are mesocosms, this study; open circles are survey field data from a larger survey of 2×2 m quadrats from sand dunes (Field et al. 2014), including some of the same sites, shown for comparison.

With increasing site nitrogen deposition and soil pH there were changes in the cover of the different species groups (Figure 4). The cover of forbs and sedges in the mesocosms showed a decline with increasing nitrogen deposition ($p=0.028$ for combined forb + sedge cover, Figure 4e), with an increasing but non-significant trend for the cover of grasses (Figure 4a). There was also a decrease in the forb:grass ratio of mesocosms with increasing N deposition ($p = 0.081$, Figure 4g). However, with increasing soil pH there was a significant decline in grass cover ($p = 0.031$, Figure 4b) and an increasing but non-significant trend for the cover of forbs, giving an increase in the forb:grass ratio of mesocosms with increasing soil pH ($p = 0.015$, Figure 4h). A model combining nitrogen deposition and soil pH explained 62% of the variability in forb cover ($p = 0.021$) and 37% of the variability in grass cover ($p = 0.115$).

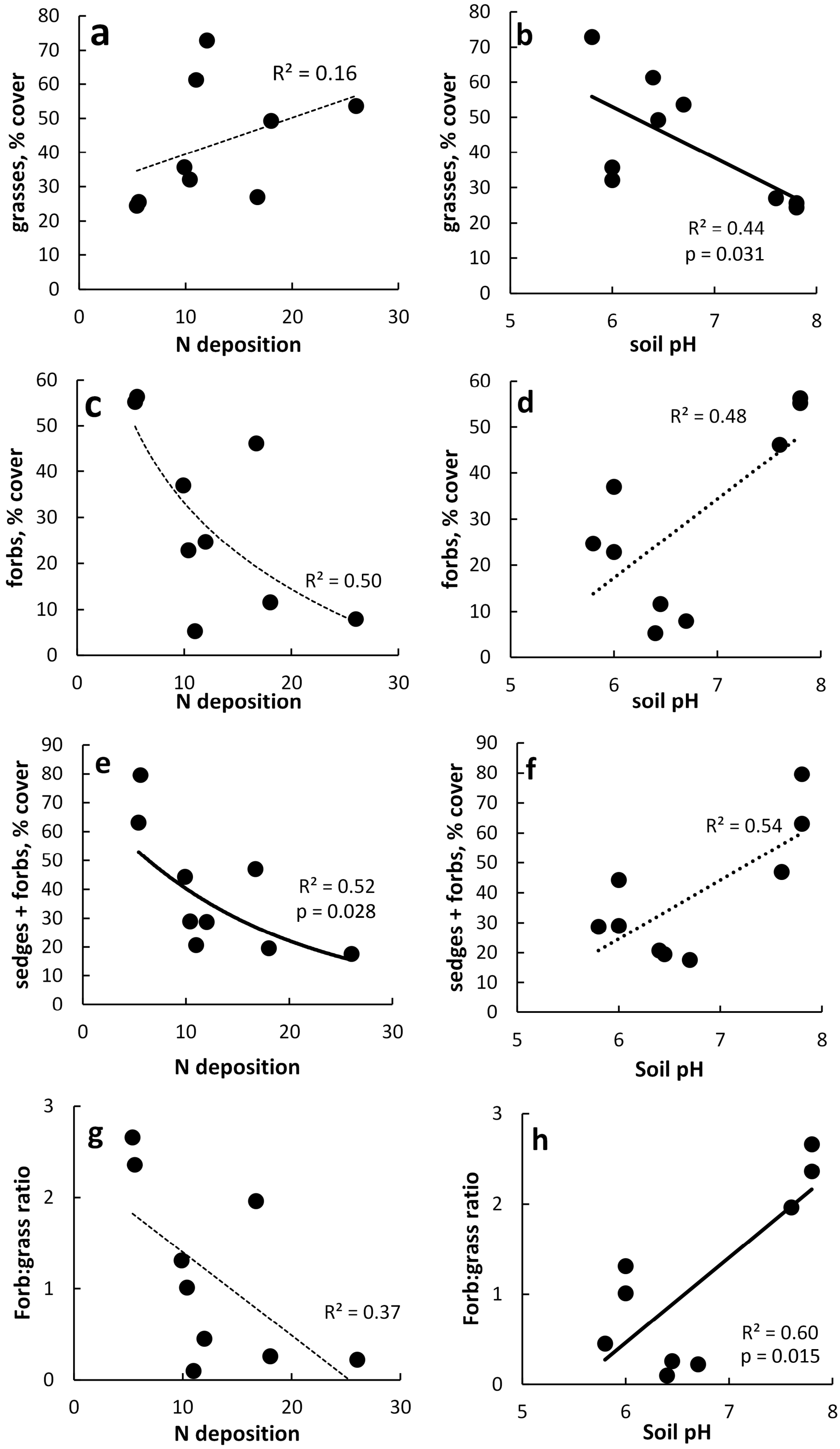

Figure 4: Average % cover of grasses (a, b), forbs (c, d) and sedges+forbs (e, f) in the mesocosms in relation to site nitrogen deposition and soil pH. Forb:grass ratio in the mesocosms in relation to site nitrogen deposition (g) and soil pH (h). Solid trendlines indicate statistically significant relationships (p<0.05).

Post-Ozone treatment

After six weeks of the 2.5 month ozone fumigation, we found that the highest ozone treatment, AA++, caused damage to some individuals from all of the 20 target species. The AA+ ozone treatment also caused damage, but less severely and to fewer individuals and species. For each of the target species in each mesocosm of the control and AA++ treatments, we identified the number of leaves showing ozone damage or senescence, and the number of healthy leaves, and calculated the proportion of damaged or senesced leaves. We used the mean proportion of leaf damage or senescence in the unfumigated mesocosms as the baseline, and subtracted the mean values from the treatment mesocosms to give an average damage estimate.

We found that the mean proportion of damaged leaves in each mesocosm declined with increasing site N deposition (r^2 for logarithmic curve = 0.40; p = 0.042, Figure 5). In other words, vegetation from the mesocosms receiving higher N deposition was in aggregate less sensitive to ozone. This could be due to a direct physiological effect: exposure to elevated N imparting increased ozone resilience to individual plants by, for example, the allocation of additional N to protect or repair tissues from ozone damage. Alternatively, it could be due to a community composition shift at elevated N to more ozone-resistant species. Further investigation of all species present in cores from at least three different N-deposition sites supports the latter hypothesis. The site N deposition had no additional effect for any species on the proportion of damaged leaves at a given level of ozone exposure, with one exception (the forb *Leontodon spp*, which showed a reduced response to elevated ozone with increasing site N deposition). Thus it appears that 'ozone resilience' in mesocosms from sites receiving higher N deposition is a result of a community-level difference in species composition.

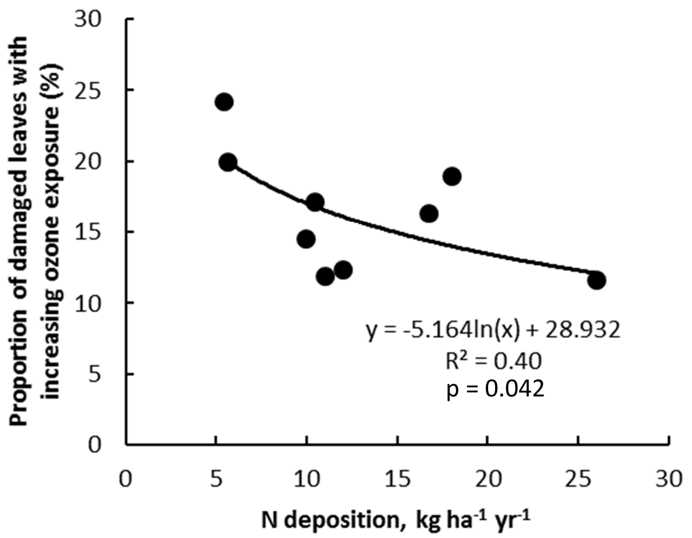

Figure 5: Community-level ozone sensitivity in relation to long-term nitrogen deposition based on the aggregate response of 20 potentially ozone-sensitive dune grassland species, and the difference between the % damaged leaves in the AA++ compared to AA ozone treatment.

At the end of the 2.5 month ozone treatment the mean DOC concentration in soil pore water showed a positive relationship with long-term N deposition (p = 0.008 across all ozone treatments). There was, however, a non-significant interaction between the two treatments (p = 0.058), with no relationship between DOC and N deposition for the ambient mesocosms, an increasing (non-significant) trend for the AA+ mesocosms, and a significant increase in DOC with increasing long-term N deposition for the mesocosms receiving the highest ozone dose AA++ ($p = 0.023$; Figure 6). There were no significant differences in the activity of either the soil-based enzymes B-D-glucosidase or N-acetyl-beta-D-glucosaminidase in relation to site N deposition or ozone treatment and no interactive effects detected (data not shown).

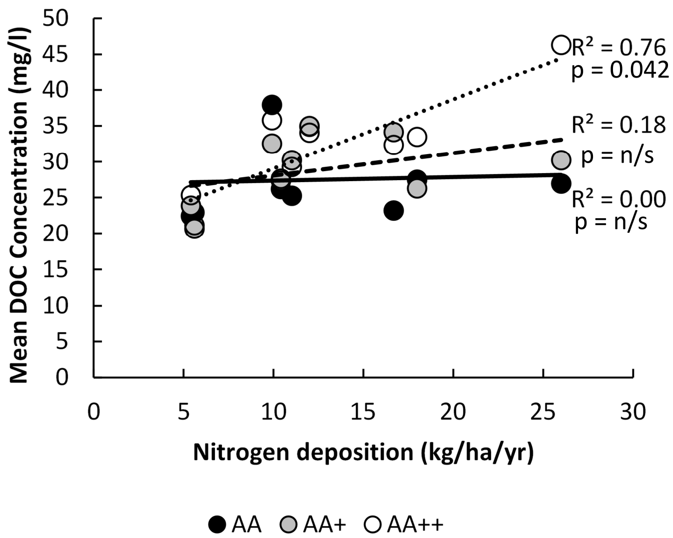

Figure 6: DOC of soil solution in relation to site N deposition ($p=0.008$) and ozone treatment. The slope of the regression line for the highest ozone treatment is significant ($p = 0.023$).

Discussion

Nitrogen deposition and ozone pollution can both affect semi-natural vegetation, with effects including vegetation damage, species composition shifts, and changes in soil biology and chemistry. Our study has supported these findings for dune grassland vegetation, and provided new evidence of interactions between the two pollutants. We found that the sites that are the least damaged by nitrogen deposition are also the most sensitive to ozone pollution. However, for all but one of the 20 species investigated, there was no change in the sensitivity to ozone of an individual species with increasing long-term N deposition. Together with the decline in forb species and cover with increasing nitrogen deposition, this implies that it is the change in species composition that is driving the change in ozone sensitivity of the mesocosms. Although some grasses are sensitive to ozone pollution, the dominant grasses in the mesocosms in this study (*Festuca rubra, Agrostis capillaris, Anthoxanthum odoratum*) are classified as resistant (Hayes et al., 2007) and did not have any additional leaf damage with increasing of ozone exposure.

Because of its short duration, we are unable to say from the experiment if ozone exposure alone alters vegetation community composition. Multi-year ozone exposure studies have shown few changes in species community composition in intact communities (Thwaites et al, 2006; Bassin et al 2007). This may be because, as in other pollution exposure studies (including nitrogen), the experiments were not long enough to detect a community shift. It also may be due to the fact that ozone does not accumulate in the ecosystem as nitrogen does.

On a regional scale, ozone is a more spatially and temporally variable pollutant than nitrogen and, although there are broad-scale trends across large areas such as the UK (see Figure 1), areas of high or low ozone exposure can vary greatly within and between years (Hewitt et al, 2016). This makes it difficult to identify an ozone gradient to investigate species richness or cover trends in the same way as has been done for nitrogen. Payne et al. (2011), however, attempted this by relating the species composition and richness of acid grassland in Great Britain to modelled 5-year annual average tropospheric ozone exposure (AOT40, from the UK Air Pollution Information System – APIS), modelled annual N deposition (from CBED, as with our study) and a number of other potential drivers. They found nitrogen deposition and ozone exposure to be associated with different plant community parameters: N deposition was most strongly associated with species richness and diversity indices, and ozone exposure with overall community composition, but not necessarily the richness or diversity of the community. Despite year-to-year variability in ozone levels, the relative crudeness of the AOT40 calculation used, and the uncertainty inherent in applying regional-scale modelled data to specific localities, ozone exposure was a significant predictor of plant community composition, illustrating the potential importance of ozone on a national scale.

In this study, the cores receiving the highest ozone treatment had significantly increased soil DOC with increasing site N deposition, despite showing no additional visual damage to aboveground tissues. Whereas elevated N deposition can increase the capacity of vegetation to assimilate carbon (Dise et al., 2011), elevated ozone reduces photosynthetic capacity and, through early senescence or leaf death, can lead to increased release of stored carbon as root exudates (McCrady and Andersen, 2000). Root exudates are mostly comprised of low molecular weight compounds such as sugars, organic acids and amino acids (van Hees et al., 2005) and these have a fast turnover in the soil (Boddy et al., 2007). Carbon can leave the plant via root exudate only a few hours after being fixed by the plant, and it is estimated that 70-80% of the carbon exuded is cycled through the microbial biomass (Boddy et al., 2007). Thus the interactions between N and ozone could affect the structure and composition of the microbial community, thereby affecting C and N cycling (Manninen et al., 2009). These ecosystem-level changes may be apparent well before, or even in the absence of, apparent damage to vegetation or community composition shifts. Despite the increase in DOC concentration in the high-N cores, we found no evidence of changes in the activity of either of the carbon- or nitrogen- cycling enzymes we studied, in line with changes in low molecular weight substrates that can be directly assimilated, rather than long chain polymers requiring enzymic cleavage before microbial uptake.

An important finding of our study is that the ‘cleanest’ habitats, those that have been the least damaged by nitrogen pollution, are the most vulnerable to ozone damage. Conversely, those that have been the most damaged by nitrogen pollution are the most resilient to ozone. In both cases, the impact is at the level of the community rather than the species. The dune grasslands in this study are most similar to those of the Baltic, North Sea, English Channel and northern Atlantic regions (EUNIS category B1.41; EUNIS habitat classification 2007). Over much of this area, both ozone flux and nitrogen deposition are elevated due to regional-scale pollution, and for some of the areas of the English Channel and North Sea coastal regions, nitrogen deposition is higher than that of our study sites (Figure 7). It is likely that dune grasslands over this region have already been impacted by nitrogen deposition, and our study would predict that they are relatively resilient to ozone damage. However, this ‘resilience’ is because they have shifted to a more grass-dominated vegetation composition, having lost forb species richness. The return of a diverse forb community to these habitats would require a long-term reduction of nitrogen pollution, may take many years, and, depending on the level of damage, may require active restoration.

Dune grassland receiving low nitrogen deposition in Europe occurs in the northern UK, Ireland, and Scandinavia. These are likely to be more forb-rich than more N-polluted habitats, and therefore more sensitive to ozone. Unlike the polluted habitats, they have retained a high species-richness and require no intervention other than the prevention of new sources of pollution, although they could still be impacted by stressors such as climate change or changes in land use. We therefore suggest that protection of ‘clean’ habitats from any increases in nitrogen or ozone pollution should be the first priority for policymakers and managers. Since ozone and nitrogen interactions are driven by community level species-change, these findings are likely to be applicable to a wider range of vegetation communities and global regions which are known to respond in a similar way to nitrogen deposition (Midolo et al. 2019), and potentially to different combinations of pollutants. This highlights the need for awareness that habitats in the real world are exposed to numerous interacting environmental drivers, including multiple pollutants, which may combine with, enhance, or negate the effects of each other. Determining the net long-term effect on habitats of drivers that are changing in space and time, and complexly interacting, is a major challenge in environmental science.

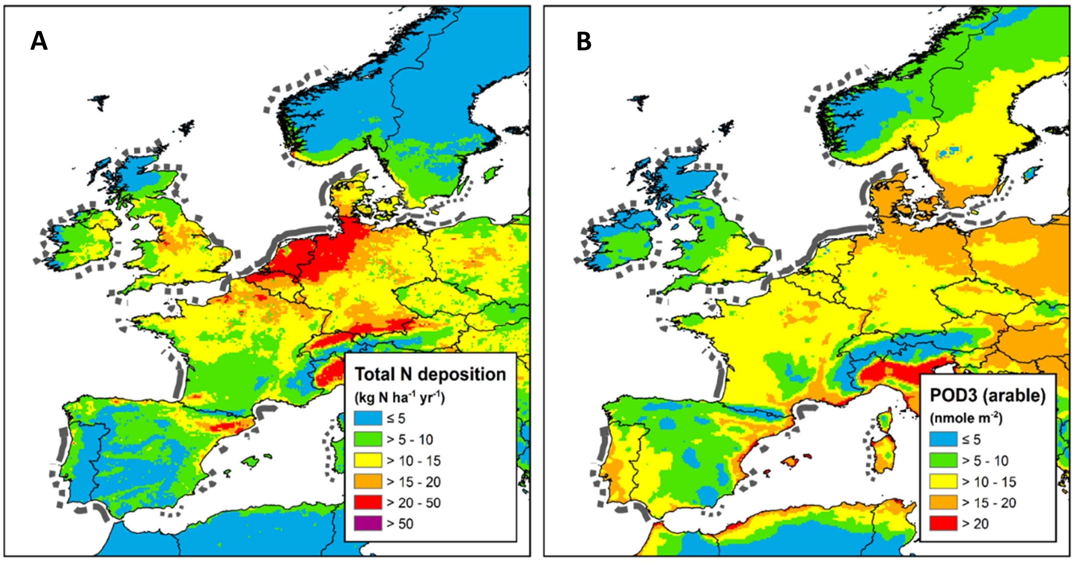

Figure 7: a) Nitrogen deposition) and b) ozone flux (POD_3IAM for arable crops) to coastal western European regions. Both calculated with the EMEP model (Simpson et al, 2012) for the year 2014. Areas where sand dune grassland is prevalent are indicated in grey (based on data from Doody, 2001).

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Highlights

- The N-polluted sites contained fewer ozone-sensitive forbs and sedges
- Vegetation composition in relation to N deposition conveyed differential ozone-resilience.
- Mesocosms in the highest ozone treatment showed elevated soil solution DOC.

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: