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Differential Effects of Oxidised and Reduced Nitrogen on Vegetation and Soil Chemistry of Species-Rich Acidic Grasslands

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Abstract Emissions and deposition of ammonia and nitrogen oxides have strongly increased since the 1950s. This has led to significant changes in the nitrogen (N) cycle, vegetation composition and plant diversity in many ecosystems of high conservation value in Europe. As a consequence of different regional pollution levels and of the increased importance of reduced N in the near future, determining the effect of different forms of N is an important task for understanding the consequences of atmospheric N inputs. We have initiated three replicated N addition experiments in species-rich, acidic grasslands spanning a climatic gradient in the Atlantic

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biogeographic region of Europe in Norway, Wales and France at sites with low levels of pollution. N was added in two doses (0 and 70 kg N ha^{-1} year^{-1} above background) and in three forms (oxidised N, reduced N and a 50–50 combination). After 2.5 years of N additions, the effects of these treatments on plant biomass, plant nutritional status, soil pH and soil nutrient availability were determined. Impacts of the N additions were observed within the 2.5-year research period. In some cases, the first signs of differential effects of N form could also be demonstrated. In the French site, for example, grass biomass was significantly increased by the oxidised N treatments but decreased by the reduced N treatments. In the Norwegian site, the reduced N treatments significantly reduced soil pH, whereas oxidised N did not. Effects on nutrient availability were also observed. These experiments will be continued to elucidate the longer term impacts of N deposition on these grasslands.

Keywords European acidic grasslands · Nitrogen deposition · Nitrogen supply rate · Oxidised nitrogen · Reduced nitrogen · Species richness

1 Introduction

Emissions of ammonia (NH_3) and nitrogen oxides (NO_x) have strongly increased since the 1950s (Galloway et al. 2008). Ammonia is volatilised from intensive agricultural systems, such as dairy farming and intensive animal husbandry, whereas nitrogen oxides originate mainly from burning of fossil fuel by traffic, industry and households. Because of short- and long-range transport of these nitrogenous pollutants, atmospheric nitrogen (N) deposition has clearly increased in many natural and semi-natural ecosystems across the world (Bobbink et al. 2010). Areas with currently high atmospheric N deposition (20–100 kg N ha^{-1} year^{-1}) are central and western Europe, eastern USA and, since the 1990s, eastern Asia and India. Estimated background inputs (pre-1900s) range between 1 and 3 kg N ha^{-1} year^{-1} (e.g. Asman et al. 1998; Dentener et al. 2006; Galloway and Cowling 2002).

Nutrient availability is one of the major factors that determine the plant species composition in ecosystems. N is the primary limiting nutrient for plant growth in many natural and semi-natural ecosystems, especially under oligotrophic and mesotrophic conditions in temperate and boreal regions (Bobbink et al. 2010; Sala et al. 2000). Many plant species in such ecosystems are adapted to nutrient-poor conditions and can only survive or compete successfully on soils with low N availability (Aerts and Chapin 2000; Tamm 1991). The series of events which occur when N inputs increase in an area with originally low background deposition rates is complex. Many ecological processes interact and operate at different temporal and spatial scales. As a consequence, large variations in sensitivity to atmospheric N deposition have been observed between different natural and semi-natural ecosystems (e.g. Maskell et al. 2010). Despite this diverse sequence of events, the impacts of increased N deposition are (a) direct foliar toxicity; (b) changes in structure and function by eutrophication; (c) soil-mediated effects of acidification; (d) negative impacts of reduced N (NH_y) such as stunted root growth, acidification of plant cells and toxicity effects; and (e) increased sensitivity for stress and disturbances. Because of these processes, significant changes in the N cycle, in vegetation composition and in plant diversity have been observed in many ecosystems of high nature conservation value in Europe (Bobbink et al. 2010, 1998).

Species-rich grasslands are an important component of European biodiversity, supporting a wide range of plant, invertebrate and bird species. Many of these semi-natural grasslands are listed in the Conservation of Natural Habitats and of Wild Fauna and Flora Directive (92/43/EEC) (the "Habitats Directive") and are thus a major part of the European nature conservation network. Species-rich acidic grasslands are present throughout Europe on soil of intermediate pH (4.5–6.0) in both lowland and mountainous regions. They were originally widespread but have been heavily affected by land-use changes (e.g. abandonment) or intensification of agricultural use (Ellenberg 1996). Species-rich *Nardus* grasslands (*Violion caninae* alliance; Schwickerath 1944) are a characteristic form of these acidic grasslands in the Atlantic biogeographic region of Europe. These species-rich, acidic grasslands are today highly threatened in parts of Europe and are protected for nature conservation under the Habitats Directive (habitat 6230; Directive 92/43/EEC).

As in many other species-rich grasslands, increased N deposition can have profound impacts on the structure and function of these acidic and oligotrophic systems. A decline of plant species richness in Britain and in the Atlantic zone of continental Europe was significantly correlated with increasing ambient atmospheric N deposition in the range 2–44 kg N ha^{-1} year^{-1} (Stevens et al. 2004, 2010). Forb richness in particular was negatively correlated to N deposition, while the more competitive graminoids increased (Stevens et al. 2011b). The same negative correlations between N deposition and species richness were also detected by a temporal comparison of vegetation data spanning a period of almost 70 years (Duprè et al. 2010). Furthermore, correlative field studies in the Netherlands showed the importance of high soil NH_4^+ concentrations and/or high soil NH_4^+ to NO_3^- ratios for the absence of many Red List plant species of these *Violion* grasslands (De Graaf et al. 2009; Kleijn et al. 2007). Experiments in water culture, containers and mesocosms have also revealed the sensitivity of characteristic endangered species of these grasslands for high NH_4^+ or high soil NH_4^+ to NO_3^- ratios (De Graaf et al. 1998; van den Berg et al. 2005, 2008). However, few N addition field studies have been done in these species-rich acidic grasslands to unravel the mechanisms of the possible decline of diversity (Bobbink and Hettelingh 2011). Berlin (2000) found an increase in graminoids within a 3-year N addition experiment on a *Festuca ovina* grassland but not on an *Agrostis capillaris* grassland. A longer term experiment at Wardlow Hay Cop (UK) has shown impacts on vegetation species composition (Carroll et al. 2003), vegetation chemistry (Arroniz-Crespo et al. 2008) and soil chemistry (Horswill et al. 2008; Phoenix et al. 2003), but this experiment is on a more acidic soil (pH 4.4) with high ambient N deposition. In addition, both experiments have focussed on the impact of N dose, and not on N form. As a result of different regional pollution climates and potential shifts in emission ratios toward reduced N in the near future, determining the effect of the different N forms is now considered as main task in understanding the consequences of atmospheric N inputs (Achermann and Bobbink 2003; Bobbink and Hettelingh 2011).

The main aim of this research is to determine the processes behind the susceptibility of biodiversity to N deposition by carrying out replicated N manipulation experiments in species-rich acidic grasslands in regions with low levels of pollution. More specifically, we pose the following research questions:

(a) What is the effect of N addition on the biomass and species richness of the vegetation and on soil chemistry?
(b) What is the difference between adding N as oxidised N (NO_3^-) or as reduced N (NH_4^+)?
(c) Are there any signs of negative effects of NH_4^+ on certain plant groups?

To address these questions, a set of three replicated N addition experiments were initiated spanning a climatic gradient in the Atlantic biogeographic region of Europe, in Norway, Wales and France. In this paper, we report the findings after 2.5 years of these field experiments contrasting the impacts of NH_4^+ and NO_3^- in species-rich acidic (pH 5–5.5) grasslands. Biomass, species richness and soil chemistry are described.

2 Materials and Methods

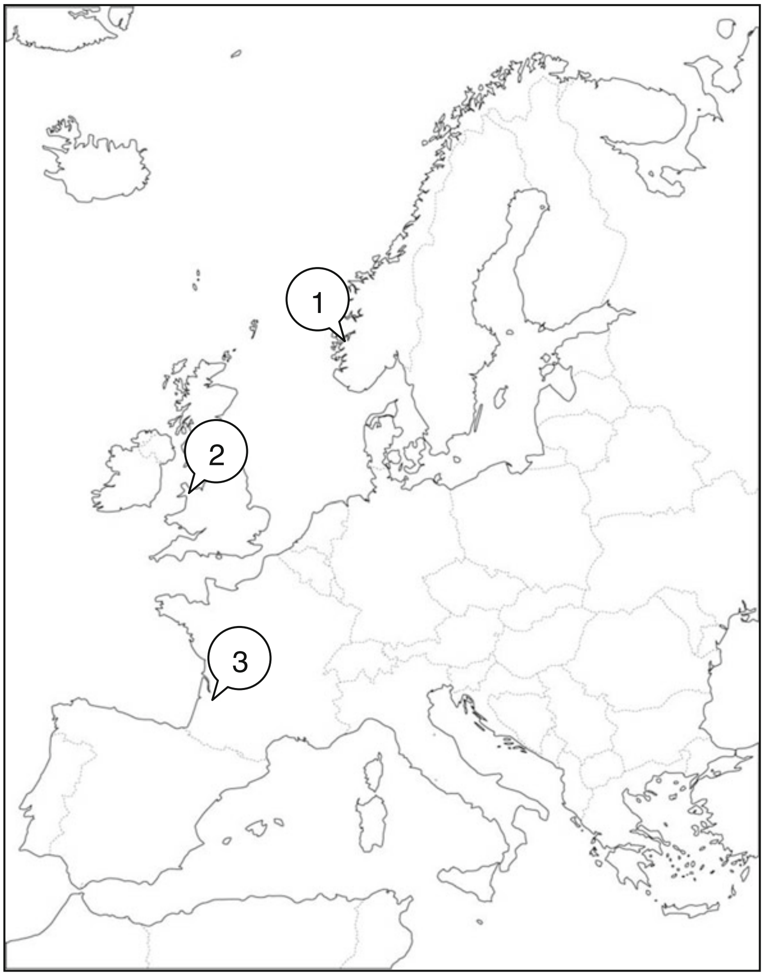
2.1 Site Descriptions

Nitrogen addition experiments were carried out in acidic grasslands (EUNIS code 1.7) in three countries along the Atlantic climate gradient: Norway, Wales (UK) and France (Fig. 1). All sites belonged to the *V. caninae* alliance (Schwickerath 1944) and had, for Europe, low background N deposition (6–10 kg N ha^{-1} $year^{-1}$). Site meteorological and soil characteristics are summarised in Table 1.

The experimental site in Norway is located at Revne, in the Fusa municipality (Hordaland). Bedrock consists of green schist/mica schists overlain by a shallow soil. The site was traditionally managed for haymaking with annual mowing and grazing by sheep in spring and autumn (grazing was discontinued in 2005). Low levels of fertiliser were applied up to 2000, although neither the type nor amounts of fertiliser is known. Part of the area has been ploughed and was probably used for arable farming prior to ca. 1940 (potato, grains). The vegetation is dominated by grasses such as *Anthoxanthum odoratum*, *Festuca rubra* and *Agrostis capillaris*. *Rhytidiadelphus squarrosus* is the dominant bryophyte. Several sedges (*Carex nigra*, *Carex panicea*) and forbs (*Viola palustris*, *Leontodon autumnalis*, *Plantago lanceolata*, *Prunella vulgaris*) are also present. Initial species richness was 14.9 species per 2×2 m plot for vascular plants and 2.7 for bryophytes.

The experimental site in the UK is situated at Trefor, North Wales. The site sits on a slight slope at the top of sea cliffs. Although formerly heathland, the site has been managed for extensive grazing of sheep for many years and consists of a short grassland sward with scattered shrubs. The grass sward is dominated by *Agrostis capillaris*, *F. rubra* and *Potentilla erecta*. Initial species richness was 16.6 species per 2×2 m plot for vascular plants and 3.0 for bryophytes.

Fig. 1 Locations of the experimental sites. *1*, Revne, Norway; *2*, Trefor, Wales (UK); *3*, Léognan, France



The experimental site in France is located at Léognan, near Bordeaux. The soil is an organic podzol overlaying a quaternary deposition of sand on top of limestone. Situated on the edge of an airfield, the site has been maintained as grassland with four to five mowings (with clippings removed) between April and September. The vegetation is dominated by grasses (*Agrostis capillaris*, *Agrostis curtisii*, *Danthonia decumbens* and *Pseudarrhenatherum longifolium*) and includes dwarf shrubs such as *Calluna vulgaris*, *Erica* spp. and *Ulex minor*; sedges (*Carex pilulifera*, *Carex binervis*) and forbs (*Potentilla erecta* and *Polygala* spp.). Initial species richness was 21.7 species per 2×2 m plot for vascular plants and 2.3 for bryophytes.

2.2 Experimental Design

At each of the three sites, 40 experimental plots of 2×2 m in a randomised block design with five blocks were set up in early spring 2007. Eight treatments were applied; four of these were used for the analysis presented in this paper (20 plots).

Nitrogen was applied in solution at a rate of 70 kg N ha^{-1} year^{-1} in three different forms: reduced N (as NH_4Cl, referred to as N70-red), oxidised N (as $NaNO_3$, N70-ox) or in combination (as NH_4NO_3, N70). These treatments provided the opportunity to analyse the effects of reduced and oxidised N separately and in combination. Control plots (N0) received corresponding amounts of deionised water only. The yearly number of applications depended on the length of the growing season at each site (eight additions in Trefor and Léognan and five in Revne). Intensive sampling of vegetation and soil was carried out in early spring 2007 to establish an experimental baseline and again in 2009 after 2.5 years of N application.

The distance between replicate blocks was at least 2 m. One metre wide buffer zones were laid out between

Table 1 Overview of site and meteorological characteristics of the experimental sites. The meteorological data are averages over the period 1971–2000 unless otherwise stated

	Revne (Norway)[a]	Trefor (Wales)[b]	Léognan (France)[c]
Site characteristics			
Coordinates	60° 09 29 N 5° 44 31.5 E	52° 59 56 N 4° 26 04 W	44° 42 11 N 0° 35 54 W
Altitude (m asl)	160	40	53
Slope (°)	13	6	–
Aspect	North–west	West	0
Average background N deposition[d]	6.1	9	8.8
Climate characteristics			
Mean annual temperature (°C)	6–8	9.5–10.5	13.3
Mean maximum daily temperature (°C)	9.6	13.1	18.1
Mean minimum daily temperature (°C)	4.2	7.5	8.5
Mean annual rainfall (mm)	1,773.4	827.9	984
Mean annual sun hours	1,186[e]	1,621.4	1,992
Mean number of rainfall days (>1 mm)	202[e]	140.9	128
Soil characteristics			
Soil depth (cm)	13	15–65	>150
Soil pH	5.4	5.1	5.2
Extractable NH_4^+ (mg kg^{-1} dry soil, NaCl extract)	1.5	0.5	3.0
Extractable NO_3^- (mg kg^{-1} dry soil, NaCl extract)	16.6	0.8	7.4
Extractable PO_4^{2-} (mg kg^{-1} dry soil, NaCl extract)	2.9	0.1	0.1
Exchangeable $Ca^{2+}/K^+/Mg^{2+}$ (mg kg^{-1} dry soil, NaCl extract)	419.4/13.7/53.5	159.7/69.3/177.6	62.1/4.9/14.4

[a] Average values at weather station at Bergen (http://retro.met.no)

[b] Average values for Station de Bordeaux, Merignac (1991–2000 for mean annual sun hours; http://climat.meteofrance.com)

[c] Average values for weather station Valley (http://www.metoffice.gov.uk)

[d] CBED (RoTAP 2012) was used for the UK; EMEP-based IDEM models were used for Norway and France (Pieterse et al. 2007)

[e] Data from http://www.climatedata.eu

plots to avoid edge effects and interactions between treatments. In Trefor and Revne, plots were fenced to exclude grazing by sheep and red deer, respectively. Grazing by large herbivores did not occur in Léognan.

2.3 Vegetation Sampling and Analysis

In each 2×2 m plot, all higher plants and bryophytes were identified to a species level and visual estimates of percentage cover were made in July 2009. Species richness and Shannon diversity index and evenness were calculated per plot. Biomass of each plot was collected in 50×50 cm subplots cut to 3 cm above soil level and separated into five functional groups (grasses, forbs, legumes, shrubs and mosses). Dry weight of biomass was measured after drying at 55 °C for 72 h.

N, P and K contents were determined in subsamples of the collected biomass for forbs and grasses. Dried plant material was ground to <1 mm using a ball mill and digested using a modified Kjeldahl method (Bremner and Mulvaney 1982). Nutrient contents were analysed colorimetrically (N and P) and flame photometrically (K) on a Skalar SA-40 continuous flow analyser (Skalar BV, Breda, the Netherlands).

2.4 Soil Sampling and Analysis

In July 2009, two topsoil samples (0–10 cm) were collected with an auger (diameter 2.5 cm) from opposing corners of each plot. Samples from each plot were pooled, homogenised by hand removing stones and large roots, stored on ice and transported to the laboratory. Two extractions were performed, both used 15 g of fresh soil extracted on a rotary shaker (100 rpm) for 1 h. One extraction used 100 ml 0.4 M NaCl and the second used the same amount of demineralised water. The soil suspensions were centrifuged for 5 min at 4,000 rpm and a subsample of 20 ml of the supernatant was filtered through a Whatman GF/C filter. The NaCl extracts were stored at −20 °C until analysis of NH_4^+ and NO_3^-. Soil pH-H_2O was determined in the demineralised water extract. Extractable concentrations of NH_4^+ and NO_3^- were analysed colorimetrically using a continuous flow analyser (Skalar SA-40, Skalar Analytical BV, Breda, the Netherlands). Soil inorganic P availability was measured by Olsen P extraction and colorimetric analysis (MAFF, 1986). Soil moisture content was measured after drying 15 g of fresh soil at 105 °C for 24 h.

Plant N (both NH_4^+-N and NO_3-N) supply rates were assessed by 1-month incubations of Plant Root Simulator probes (PRS^{TM}, Western AG Innovations Inc., Saskatoon, Canada). The ion exchange resins in the probes give a measure of N in the soil that is available to plants (nutrient surplus rather than net mineralisation due to competition from plant roots) integrated over 10 cm^2 for the duration of the burial (Western Ag Innovations, 2008). Probes were incubated in July 2009. After incubation, the probes were cleaned in deionized water and returned to Western Ag Innovations for analysis. Probes were eluted with HCl and analysed for NO_3^--N and NH_4^+-N colorimetrically using an autoanalyser.

Potential NH_4^+ oxidising activities (PAA) were determined for each plot. Soil slurries consisting of 15 g fresh, sieved (4 mm) soil in 100 ml buffered medium with 2 mM $(NH_4)_2SO_4$ were kept in 250 ml glass Erlenmeyer flasks. The buffer solution was composed of 2 mM phosphate buffer (an equimolar mixture of KH_2PO_4 and K_2HPO_4, adapted to the prevailing soil pH of 5). During the PAA measurements, the slurries were permanently shaken on a rotary shaker (RO 20, Gerhardt, Bonn, Germany; 100 rpm) in the dark at a temperature of 25 °C. Subsamples of 3 ml were taken at t=0.5, 2, 4, 6, 24 and 96 h, and centrifuged for 5 min at 13,000 rpm (Biofuge pico, Heraeus instruments, South Plainfield, USA), decanted and stored frozen (−20 °C) until further analysis. At each sampling time, the pH of the incubation medium was checked and set to its original value with 0.1 N NaOH or 0.1 N HCl, if necessary. Concentrations of NO_3^- and NO_2^- in the subsamples were measured on a continuous flow analyser (Skalar 40, Skalar Analytical BV, Breda, the Netherlands). PAA rates were determined from the changes in NO_3^- and NO_2^- concentrations in time, based on the slopes of the linear parts of the regression lines. The R^2 values of the linear regressions were all 0.99 (±0.01).

2.5 Data Analysis

All soil, nutrient and vegetation variables were analysed for differences between treatments within sites using analysis of variance (SPSS 15, Microsoft Corporation, release 15.0.1., 2006) with N treatment as fixed factor and block as random factor. When necessary, data were logarithmically or square root transformed to obtain normality and homogeneity of variances. Post hoc pair-wise comparisons between all treatments were made using Tukey's method. For calculations of post hoc statistics, interaction terms (treatment x block) were excluded. Differences between sites were tested using analysis of variance. When it was not possible to normalise the data with transformations, data were analysed using a Mann–Whitney *U* test. The level of significance was set at p=0.05.

3 Results

Outcomes of data analysis for all results are summarised in supporting electronic material Table 1.

3.1 Vegetation

The average species numbers of the control treatments in 2009 was 16.4 (s.d.±2.2), 20.4 (standard deviation 0.9) and 23.8 (standard deviation 3.1) for Revne, Trefor and Léognan, respectively, and did not differ significantly from those in 2007. None of the experimental sites showed significant effects of the N treatments on average species numbers per 2×2 m sample after 2.5 years of N addition. The Shannon index and evenness were also not affected by the N treatments (data not shown). However in Revne, there was a trend of declining Shannon index in the N70-ox addition compared to the control treatment (p=0.065).

Total biomass production differed considerably between the experimental sites. In Léognan and Trefor, the mean total biomass in 2009 in control plots was 358 and 431 g m^{-2}, respectively. In the Revne control plots, the total biomass of the vegetation was only 239 g m^{-2}

(Fig. 2). Due to the relatively high variability in biomass, significant effects of N treatments on total biomass of the vegetation were not found, but some trends could be observed. In Trefor, all N treatments resulted in a non-significant increase in total biomass production.

Forb biomass in Revne was decreased by the N treatments compared to the control, with a significant decrease for the N70-red treatment (*p*=0.037, Fig. 2). An opposite effect, although non-significant, was found in Léognan and Trefor, where forb biomass tended to be higher following N additions (Léognan *p*=0.244; Trefor *p*=0.081).

Grass biomass in Léognan was increased by the N70-ox treatment, whereas it was decreased by the N70-red treatment resulting in a significant difference (*p*=0.015; Fig. 2). However, treatment effects did not differ from control.

In Trefor and Revne, grass biomass showed a non-significant increase with N additions, irrespective of the N form applied (Trefor *p*=0.547 and Revne *p*=0.201).

Although significant differences were not found in Léognan, the N treatments resulted in a non-significant decrease the grass to forb ratio, with the lowest ratios found in the N70-red treatments. In Trefor, no effects were found, but in Revne the N treatments resulted in increased grass/forb ratios with N70-red being significantly higher than control (*p*=0.037).

All N treatments led to increased plant N contents of both grasses and forbs in Revne (legumes were not measured due to insufficient biomass), although these differences were only significantly higher compared to the control treatments for reduced N (Grass N70 *p*=0.170, N70-ox *p*=0.118, N70-red *p*=0.029; Forb N70 *p*=0.118, N70-ox *p*=0.060, N70-red *p*=0.015; Table 2). In Trefor, only grasses seemed to have (non-significant; *p*=0.72) higher N contents compared to the control, whereas no effects were found in forbs or legumes. In Léognan, there was a non-significant trend (*p*=0.06) of increasing N content of legumes with the N treatments, but no effects of the N treatments were found in the other functional groups. Plant P content was not affected by the treatments (Table 2). Average plant N/P ratios of both grasses and forbs in the control plots were lowest in Revne, intermediate in Trefor and highest in Léognan (Table 2). The N treatments resulted in a non-significant trend of increased plant N/P ratios for grasses in Revne and a significant increase in Trefor (*p*=0.02), and for forbs in Revne (*p*=0.009). In Léognan, an opposite, non-significant trend was found, with the N treatments leading to a trend of decreased plant N/P ratios of all functional groups (*p*=0.07). Average C/N ratios of grasses in the control plots did not differ between sites (between 42.5 and 47.7, Table 2). In Revne, the average C/N ratios of grasses and

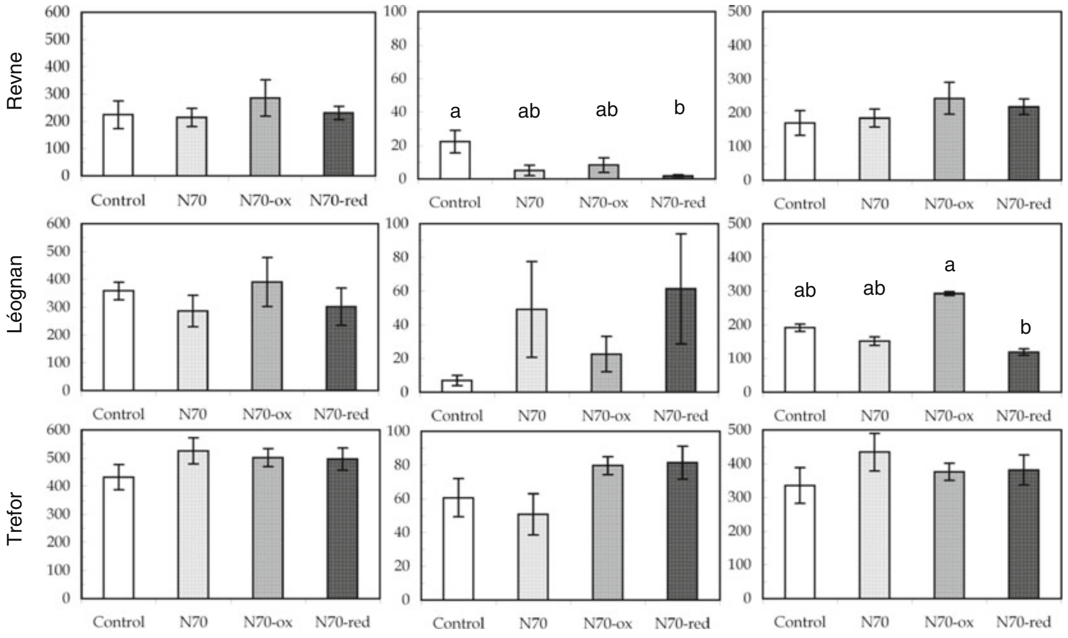


Fig. 2 Average (±s.e) total biomass production (*left*), and that of forbs (*middle*) and grasses (*right*) in the three experimental sites (gram per square metre). Significant differences between groups are indicated by *different letters*

Table 2 Average (±s.e.) plant N and P contents (milligram per square metre), N/P and C/N ratios in aboveground tissues for functional groups (grasses (G), forbs (F), and legumes (L)) ($n=5$ replicates per treatment)

N treatment	Revne[a]		Trefor			Léognan		
	Grasses	Forbs	Grasses	Forbs	Legumes	Grasses	Forbs	Legumes
N content								
Control	9.5±1.7	22.6±5.1	9.8±1.4	12.8±1.2	23.9±0.5	10.3±0.4	15.4±1.2	13.2±0.5
N70	13.4±1.4	31.7±6.5	12.2±1.6	13.4±0.7	24.0±1.5	11.3±0.7	15.3±1.1	16.4±1.1
N70-ox	13.8±1.8	32.3±4.9	13.7±2.0	13.8±1.0	23.4±1.2	10.6±0.6	15.4±1.8	15.2±1.0
N70-red	15.3±2.0	38.8±5.5	10.2±1.0	11.7±1.2	22.1±1.1	10.3±0.5	14.7±1.1	16.1±1.0
P content								
Control	2.2±0.3	4.6±0.5	1.2±0.1	1.1±0.1	1.5±0.2	0.5±0.0	0.8±0.0	0.5±0.0
N70	2.6±0.1	5.0±0.3	1.2±0.1	1.1±0.1	1.4±0.1	0.5±0.0	0.9±0.1	0.7±0.1
N70-ox	2.2±0.2	4.6±0.3	1.3±0.1	1.3±0.2	1.3±0.1	0.5±0.0	0.9±0.1	0.6±0.1
N70-red	2.9±0.3	5.3±0.2	1.1±0.1	1.1±0.1	1.5±0.1	0.6±0.1	0.8±0.1	0.7±0.1
N/P ratio								
Control	4.3±0.3	4.7±0.6	8.9±0.5	10.6±0.0	16.5±1.5	21.1±1.0	19.4±0.7	27.5±1.6
N70	5.2±0.4	6.1±0.9	10.8±0.2	11.1±0.5	17.5±1.2	21.1±0.7	17.6±1.6	24.5±0.4
N70-ox	6.2±0.7	6.9±0.8	10.3±1.2	10.6±0.3	18.0±0.8	19.7±1.0	18.2±1.6	24.5±0.4
N70-red	5.2±0.3	7.2±0.8	9.5±0.5	10.5±0.1	15.0±0.6	18.3±2.6	18.6±2.0	25.7±2.9
C/N ratio								
Control	43.3±7.0	21.5±3.7	42.5±2.1	31.6±0.5	17.7±1.1	47.7±3.2	29.3±2.2	33.5±1.2
N70	30.5±2.8	13.8±2.7	38.2±1.1	31.7±3.4	17.4±0.9	42.2±2.6	30.8±2.3	27.2±1.5
N70-ox	30.8±4.4	13.6±2.6	40.7±2.0	29.6±0.9	16.8±0.7	45.9±2.9	30.0±3.4	35.4±0.1
N70-red	28.0±3.4	12.5±1.9	37.5±1.8	31.3±1.4	17.7±2.5	49.0±1.9	32.0±2.4	29.2±1.4

[a] Legumes are absent in Revne

forbs were decreased by all N treatments, but differences were only significant for grasses (Table 2). In Trefor and Léognan, the N treatments did not affect the average C/N ratios in grasses and forbs, but in Léognan, legumes did show a significant decrease ($p=0.02$).

3.2 Soil

Average soil pH-H$_2$O values of control treatments were not significantly different between sites: Trefor (5.1), Revne (5.4) and Léognan (5.2; Table 3). In Trefor, no effects of N treatments were found on soil pH. In Léognan, all N treatments seemed to increase soil pH slightly (but not significantly), but differential effects were not observed. In Revne, the N70-red treatment significantly decreased soil pH compared to the control and N70-ox treatments ($p=0.039$). This effect was not observed when NH_4^+ was added in combination with NO_3^- (N70).

Average soil NO$_3$-N concentrations in the control treatments were highest in Revne, lowest in Trefor and intermediate in Léognan (Table 3; $p<0.05$). Results for NH$_4$-N showed a similar pattern but showed a greater within site variability and the difference between sites was not significant ($p=0.14$). Soil extractable NO$_3$-N more than doubled in Revne due to the N treatments ($p=0.03$). No effects of N addition on extractable NH$_4$-N concentrations were found at this site. In Trefor and Léognan, no effects of treatments on extractable N concentrations were found.

Soil P concentrations (Olsen P) were highest in Revne (average in control plots of 149 mg kg^{-1} dry soil, indicative of its former agricultural use). Soil P concentrations were more than tenfold lower in Trefor and even lower in Léognan (average in control plots of 12.5 and 5.5 mg kg^{-1} dry soil, respectively). No significant effects of N treatments on soil P concentrations were found in any of the experimental sites (Table 3).

Table 3 Average (±s.e.) values for soil parameters. Soil nutrients are all expressed in milligram per kilogram dry soil. Potential nitrification is NO_3^- concentrations (mg kg^{-1} dry soil) after 96 h of incubation. Plant N supply rates are in micrograms 10 per square centimetre per burial length (*n*=5 replicates per treatment)

Site	No.	pH	Extractable NO_3-N	Extractable NH_4-N	PO_4-P (Olsen P)	Plant NO_3-N supply rates	Plant NH_4-N supply rates	Potential nitrification
Revne	Control	5.4±0.1 ab	2.3±1.2	2.4±0.5	149.3±10.6	5.4±0.9 a	5.6±0.3 ab	141.7±40.5
	N70	5.2±0.2 bc	6.3±2.0	2.4±0.1	127.7±15.7	275.0±82.7 b	10.0±1.4 ab	158.9±32.4
	N70-ox	5.8±0.4 a	5.6±1.8	3.7±0.9	130±16.2	264.4±76.4 b	5.1±0.6 a	163.8±41.3
	N70-red	4.9±0.2 c	5.1±2.2	2.2±0.1	143.8±11.3	24.7±16.5 a	14.1±3.7 b	156.2±37.0
Trefor	Control	5.1±0.1	0.4±0.1	1.5±0.2	12.5±1.1	1.1±0.3 a	14.1±2.1	[a]
	N70	5.1±0.1	0.9±0.3	4.0±1.3	12.4±2.3	5.1±1.6 b	14.8±2.5	[a]
	N70-ox	5.2±0.1	0.4±0.1	1.6±0.2	10.8±0.5	3.1±0.9 ab	13.6±1.7	[a]
	N70-red	5.2±0.1	0.4±0.1	1.7±0.3	13.4±1.1	0.8±0.3 a	21.5±5.7	[a]
Léognan	Control	5.2±0.2	0.9±0.4	0.6±0.2	5.5±2.2	74.0±38.5	56.0±18.8	11.2±4.5
	N70	5.4±0.4	1.0±0.5	0.9±0.3	4.2±0.8	74.7±27.4	54.5±16.4	12.9±6.6
	N70-ox	5.4±0.4	0.9±0.2	0.5±0.1	5.3±1.1	123.6±51.9	29.4±6.1	13.4±5.2
	N70-red	5.4±0.6	0.6±0.2	0.6±0.1	5.6±2.1	34.4±17.0	62.9±17.8	8.4±2.8

Significant differences between treatments are indicated by different letters

[a] In Trefor no nitrification could be measured

Large differences in plant N supply rates in control plots were found between sites (Table 3). Both NO_3-N and NH_4-N supply rates were ten times higher in Léognan than those in Revne and Trefor (significant differences for total N, NO_3-N and NH_4-N; $p<0.001$). In Trefor, N was predominantly present as NH_4-N, whereas in Léognan and Revne, NO_3-N and NH_4-N supply rates were more or less equal. The effects of N additions on N supply rates differed between countries. In Revne, the N70 and N70-ox treatments resulted in significantly increased supply rates of total N ($p<0.05$ for both) and NO_3-N ($p<0.05$ for both), but not of NH_4-N (N70 $p=0.47$; N70-ox $p=0.99$). The N70-red treatment significantly increased NH_4-N supply rates compared to the N70-ox treatment ($p<0.05$), but not compared to the control or N70 treatment ($p=0.06$; $p=0.54$). In Trefor, the N70 treatment resulted in significantly higher NO_3-N rates than the control ($p<0.05$), the N70-red treatment was also significantly lower than the N-70 treatment ($p<0.05$), and there were no effects of N treatment on total N or NH_4-N ($p=0.29$; $p=10$). In Léognan, no effects of N treatment were found (total N $p=0.60$; NO_3-N $p=0.9$; NH_4-N $p=0.24$).

In Trefor, nitrification rates were below detection limit (Table 3). Potential nitrification rates in the control plots of Revne were considerably higher than those in Léognan ($p<0.001$). None of the N treatments had significant effect on potential nitrification.

4 Discussion

4.1 Differences Between Sites

The three N addition experiments were replicated spanning a climatic gradient in the Atlantic biogeographic region of Europe. This resulted in differences between the three experimental sites in terms of climate, soil conditions (Table 1) and, to some degree, species composition. Soil depth also varied considerably between sites, and the deep soils at Léognan may have reduced the impact of the warmer drier climate, resulting in higher water availability than if soils had been shallow. Total biomass was highest in Trefor and lowest in Revne ($p<0.001$) and did not reflect nutrient availability (Table 3). The differences between biomass at the sites are most likely to be driven by climate: Revne has a much shorter growing season and lower temperatures than the other sites and Léognan experienced a drought in 2009.

There were also some differences in the soil pH between the sites (Table 1). There was no significant difference in pH between sites ($p=0.14$) but there was a significant site × treatment interaction for soil pH ($p<0.01$). Combined with climate, these differences appear to have an impact on N processing. Revne is the least acidic site and this is also where we observed highest levels of nitrification as we would expect since nitrification is hampered by low soil pH (e.g. Ste-Marie and Parè 1999). We also saw a significant reduction in soil pH in Revne as a consequence of the reduced N treatment, this is probably because the high rates of nitrification release more H^+ and thus have greater potential to acidify the soil than with oxidised N input (Johnston et al. 1986). Remarkably, in Trefor, nitrification was below the detection limit. Although this is the site with the lowest pH in 2009 (5.1 in control plots), we would still have expected to see nitrifying activity because nitrification is known to be hampered strongly below soil pH values of 4.5 (Roelofs 1986). Smits et al. (2010) found similarly low levels of nitrification in an acidic matgrass sward in the Netherlands. They hypothesised that that the nitrifying bacteria are strongly suppressed, possibly by root exudates of the dominant *Violion* plants (Smits et al. 2010). A number of studies have identified the exudation of biological nitrification inhibitors from crop species (e.g. Subbarao et al. 2007; Zakir et al. 2008). As the grasslands in this study are a similar grassland type to that used in the study of Smits et al., it is possible that the same phenomenon is being observed here.

Sites responded differently to the N treatments as a consequence of varying conditions, and over time we would expect differences between sites to become more apparent.

4.2 Impacts of N Addition and Form

Several gradient studies have shown clear negative correlations between species richness and N deposition in this habitat (Duprè et al. 2010; Maskell et al. 2010; Stevens et al. 2010) but, consistent with other controlled field experiments (Carroll et al. 2003), we did not observe a strong effect in the first 2.5 years. This is not surprising in areas with low background deposition as impacts on species composition may take many years to become apparent and are likely to be cumulative, especially with respect to the accumulation of N in the system and with soil acidification (Duprè et al. 2010). We did observe a non-significant decreasing trend in the species diversity in Revne which indicates that some early phase changes are starting to occur in species composition of the vegetation.

Plant biomass was quite variable but non-significant trends in biomass, especially for grasses were observed. Several experiments have reported increased plant biomass as a consequence of N addition (e.g. Clark and Tilman 2008; Jones et al. 2004; Mountford et al. 1993) and this is a typical response in vegetation where N is the limiting nutrient (LeBauer and Treseder 2008). Grasses typically respond more favourably to N addition than forbs (Bobbink 1991; Stevens et al. 2006), although somewhat surprisingly, we saw an increasing trend in forb biomass in Trefor and Léognan. These results suggest there may be N limitation on biomass production, especially in Trefor where all forms of N addition increased biomass. Reduced forb biomass in Revne with the N70-red treatment but not the N70 or N70-ox treatments may suggest that this group is more sensitive to reduced N inputs. This is in line with the N/P ratios of the plant tissues; at Trefor and Revne, the low N/P ratios indicate N limitation but in Léognan higher values could indicate co-limitation or P limitation (Güsewell and Koerselman 2002). Over time we would typically expect a changed species composition as a consequence of increased biomass production as small stature plants (e.g. forbs) cannot compete for light with tall grass species (Hautier et al. 2009) and as soils become more acidic (Stevens et al. 2011b). In Léognan, the reduced N treatment resulted in a non-significant reduction in total and grass biomass. This may have been related to an interaction with the drought in Bordeaux in the summer of 2009. Experiments investigating the interactions between N addition and drought have shown that N can exacerbate the impacts of drought (Friedrich et al. 2012). Tissue N content showed a non-significant trend towards being higher at Revne and in grasses at Trefor, but was unchanged at other sites. Tissue N content has been suggested as an indicator of N deposition (Pitcairn et al. 2001). However, in sites such as Trefor, where background levels of N are low and there has never been fertiliser addition, we may expect additional N resources to be used for growth rather than luxury storage (when plants take up more nutrients than they need storing the excess in plant tissues (Barraclough 1993)). Although a number of N addition experiments have found impacts on tissue N, including in comparable plant communities (Carroll et al. 2003; Morecroft et al. 1994), Stevens et al. (2011a) did not find any relationship between N inputs and tissue N concentration of vascular plants in a European survey of acidic grasslands. At Revne, where soil nutrient status is typically a bit higher due to past fertilisation, we see slight increases in tissue N concentration.

Most of the soil variables remained unchanged by the N addition but, given the low background levels of deposition, and the relatively short period of N addition, we would only expect to see the first signs of impact on soil chemistry. Soil pH showed a significant reduction with the addition of reduced N at Revne and a slight increase with N treatments at Léognan. We would typically expect N addition to reduce soil pH (Skiba et al. 1989) but, depending on the buffering capacity of the soil, changes in soil pH may take many years to become apparent. Extractable soil NO_3^- and NH_4^+ showed small, non-significant increases at Revne, but not at other sites. Since both NO_3^- and, to a lesser extent, NH_4^+ are relatively mobile in soil and both are readily utilised by plants (Matson et al. 2002), it is not surprising that we do not see clear trends. However, plant N supply rates did show significant results. Increases in plant N supply rates were observed with the N70 and N70-ox treatments in Revne and with the N70-red treatment in Trefor. This shows differential impacts of N form and, if this continues, is likely to result in changes in plant biomass and/or tissue nutrient concentrations, both of which frequently respond positively to experimental N additions (Arroniz-Crespo et al. 2008; Hautier et al. 2009; Phoenix et al. 2012). Nitrification did not increase at any of the sites in response to N addition and was not measureable at Trefor (see Section 4.1). Morecroft et al. (1994) found similar results in an acid grassland experiment with low nitrification rates, which did not increase significantly at lower N addition treatments (35 and 70 kg N ha^{-1} $year^{-1}$), but were increased by additions of 140 kg N ha^{-1} $year^{-1}$. We may expect to see larger changes as N additions are applied for a longer time period and a cumulative effect became apparent.

5 Conclusion

We find the first effects of N addition in three species-rich acidic grasslands in low pollution regions have become apparent within 2.5 years. There were some changes in the vegetation biomass apparent although many of these were not significant and were not consistent across sites. There were few significant changes in vegetation nutrient contents. Over time, these differences may become more apparent. In some cases, the first signs of differential effects of N form could be demonstrated, forb biomass decreased significantly in Revne with the reduced N treatment indicating a negative effect of ammonium. Other differences in the impact of N form were observed for soil response variables. These experiments will be continued to elucidate the longer term impacts of N deposition in formerly unaffected European grasslands.

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