

1 **Atmospheric nitrogen deposition on petals enhances seed quality of the forest herb**

2 ***Anemone nemorosa***

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40 **Short title:** Seed quality and nitrogen deposition

41 **Abstract**

- 42 • Elevated atmospheric input of nitrogen (N) is currently affecting plant biodiversity and
43 ecosystem functioning. The growth and survival of numerous plant species is known to
44 respond strongly to N fertilization. Yet, few studies have assessed the effects of N
45 deposition on seed quality and reproductive performance, which is an important life-
46 history stage of plants.
- 47 • Here we address this knowledge gap by assessing the effects of atmospheric N deposition
48 on seed quality of the ancient forest herb *Anemone nemorosa* using two complementary
49 approaches.
- 50 • By taking advantage of the wide spatiotemporal variation in N deposition rates in pan-
51 European temperate and boreal forests over two years, we detected positive effects of N
52 deposition on the N concentration (percentage N per unit seed mass, increased from 2.8 to
53 4.1 %) and N content (total N mass per seed, more than doubled) of *A. nemorosa* seeds. In
54 a complementary experiment, we applied ammonium nitrate to aboveground plant tissues
55 and the soil surface to determine whether dissolved N sources in precipitation could be
56 incorporated into seeds. Although the addition of N to leaves and the soil surface had no
57 effect, a concentrated N solution applied to petals during anthesis resulted in increased
58 seed mass, seed N concentration and N content.
- 59 • Our results demonstrate that N deposition on the petals enhances bioaccumulation of N in
60 the seeds of *Anemone nemorosa*. Enhanced atmospheric inputs of N can thus not only affect
61 growth and population dynamics via root or canopy uptake, but can also influence seed
62 quality and reproduction via intake through the inflorescences.

63

64 **Keywords:** latitudinal gradient; nutrient stoichiometry; seed quality; nitrogen deposition; seed
65 provisioning; sexual reproduction; wood anemone.

66

67 **Introduction**

68 An ever-increasing number of anthropogenic stressors is acting on vegetation worldwide (Vellend *et*
69 *al.* 2017). One of the key factors currently affecting plant biodiversity and ecosystem functioning
70 are elevated atmospheric nitrogen (N) inputs (Bobbink *et al.* 2010; Simkin *et al.* 2016). Globally,
71 atmospheric deposition of biologically reactive N more than tripled from 1860 to the early 1990s
72 (Galloway *et al.* 2004), and remains high in many parts of Europe to date (Tørseth *et al.* 2012). The
73 decades-long elevated N input is implicated with already significant losses of terrestrial plant
74 diversity in many ecosystems across the globe (De Schrijver *et al.* 2011; Dirnböck *et al.* 2014; Basto
75 *et al.* 2015; Simkin *et al.* 2016). One of the main reasons for plant species losses by N enrichment is
76 through differential and species-specific N uptake and growth and, as a result, changed competitive
77 interactions (Hautier *et al.* 2009).

78

79 Atmospheric deposition of ammonia (NH₃), ammonium (NH₄⁺), nitrogen oxides (NO_x) and nitrate
80 (NO₃⁻) can occur either as wet (with rainfall and snow) or dry deposition (input of particulate and
81 gaseous compounds). The most widely studied pathway of how the elevated input of atmospheric
82 N is acquired by plants is either through their root systems or through above-ground foliar surfaces
83 (canopy exchange) (Adriaenssens *et al.* 2012). However, much less is known on how other plant
84 organs can contribute to the uptake of atmospherically available N (Carlo & Norris 2012). Recently,
85 nevertheless, it was found that N ion uptake through permeable petal surfaces can be significant
86 (up to 44 % mass recovery rates); N that is subsequently allocated into ovaries and developing seeds
87 (Carlo & Norris 2012). This newly found pathway of petal N absorption and incorporation into the
88 seeds can be especially important for plants with a high petal surface area over (total) seed mass
89 ratio. Seed N concentrations (percentage N per unit seed mass) and contents (total N mass per
90 seed) are key proxies for seed quality: they are positively associated with seedling fitness,
91 establishment and survival, defence against herbivores (e.g. via synthesis of N-containing secondary
92 compounds such as alkaloids) and reproductive success (Hanley *et al.* 2004; Parrish & Bazzaz 1985;

93 Naegle *et al.* 2005; De Frenne *et al.* 2011c). This pathway is thus of potentially high ecological
94 importance though it has never been tested under realistic conditions of N deposition in the field.

95

96 Here we focus on the herbaceous forest understorey plant *Anemone nemorosa*, an important
97 representative of the group of ‘ancient woodland indicator species’ in temperate Europe. The
98 species is renowned for its typical slow colonization rates and associated failure to colonize recently
99 established post-agricultural forests from, even nearby and adjacent, ancient forest patches (Brunet
100 & von Oheimb 1998; Verheyen *et al.* 2003; Baeten *et al.* 2010; De Frenne *et al.* 2011a; Brunet *et al.*
101 2012). *Anemone nemorosa* is also an excellent study species to study the effects of N deposition on its
102 regeneration from seed because: (i) the species has relatively large petals (tepals) compared to its
103 plant size and a relatively large flower surface area to capture both wet and dry deposition; (ii) it
104 flowers during the spring (April-May), which is an active period for farmers spreading fertilizers on
105 grasslands and croplands, with these activities typically resulting in high atmospheric concentrations
106 of ammonia (Cools *et al.* 2016); (iii) being a vernal geophyte, it flowers before canopy tree leaves
107 flush and is thus more exposed to wet N deposition due to the lower precipitation interception by
108 the tree canopy at that time (*cf.* reduced ‘shelter effect’).

109

110 We here report the results of two complementary studies: first, we used a macroecological approach
111 and sampled *Anemone* seeds over two years (2009 and 2013) across its European distributional range
112 thereby taking advantage of the spatiotemporal continental-scale gradients in N deposition rates
113 (typically decreasing from south to north; Fig. 1a). Second, we performed a controlled N addition
114 experiment in which a solution of ammonium nitrate was applied to the petals and leaves of *A.*
115 *nemorosa* and to the soil. We specifically addressed the hypotheses that (i) enhanced N deposition
116 results in increased seed quality as quantified by means of the seed mass, N concentration and N
117 content in the seeds, and (ii) only N addition specifically to the petals, rather than leaves or soil,
118 increases seed quality.

119 **Materials and Methods**

120 *Study species*

121 *Anemone nemorosa* L. (wood anemone; Ranunculaceae) is a perennial vernal forest rhizome geophyte
122 common in deciduous Eurasian woodlands. Within Europe, the species has a latitudinal range from
123 northern Spain to northern Sweden, and longitudinally from Ireland to the Ural (Hultén & Fries
124 1986, Fig. 1b). Most closely associated with ancient woodlands (Verheyen *et al.* 2003; De Frenne *et*
125 *al.* 2011a), *A. nemorosa* grows on not too nutrient-poor and acidic forest soils, relatively dry to wet.
126 Shoots emerge between March and May and flowering starts a few weeks later. Flowers (one per
127 ramet) have typically six white petals (tepals), are hermaphrodite, and mostly self-incompatible and
128 insect pollinated, although selfing occurs (Müller *et al.* 2000). The achenes (mean of 10–30 per
129 flower) contain a single seed and are gravity-, slug- and ant-dispersed between May and June
130 (Delatte & Chabrierie 2008; Türke *et al.* 2010). The indistinct elaiosome (appendix of the fruit, an
131 adaptation to myrmecochory) of *A. nemorosa* is, in comparison to other myrmecochorous species,
132 relatively rich in the fatty acids oleic acid and palmitic acid (Pfeiffer *et al.* 2009), both of which
133 substances contain no N. Achenes (henceforth referred to as ‘seeds’) do not form a persistent seed
134 bank (Eriksson 1995) or only a small short-term persistent seed bank (Plue *et al.* 2012). Vegetative
135 spread through rhizomes is common (Philipp & Petersen 2007), but sexual reproduction is
136 considered to be most important for population spread (Brunet & von Oheimb 1998; Müller *et al.*
137 2000). *Anemone nemorosa* typically has very slow colonization rates (Brunet & von Oheimb 1998;
138 Verheyen *et al.* 2003; De Frenne *et al.* 2011a; Brunet *et al.* 2012).

139

140 *Study 1: Seed sampling along an N deposition gradient - macroecological approach*

141 **Seed sampling.** In 2009 and 2013, seeds were sampled in 11 regions across Europe thereby taking
142 advantage of the spatiotemporal continental-scale variation in N deposition rates (Fig. 1a,b). All
143 populations were large (>10,000 ramets) and situated in deciduous forest (mixed forests dominated
144 by deciduous species, in northern Sweden and Estonia) with 50–95 % canopy cover unaffected by
145 major recent disturbances (thinning, grazing, etc.). In 2009, two populations per region were

146 sampled, each > 1 km apart (8 regions, 15 populations in total; only one population was sampled in
147 northern Sweden due to the rarity in that region). In 2013, one population per region was sampled
148 (11 populations from different regions). This resulted in a set of 26 populations × year
149 combinations. At every collection site, we sampled and pooled the seeds of 30–50 randomly chosen
150 ramets per population. Seed sampling was always performed at seed maturity, i.e., exactly at the
151 moment when natural seed dispersal occurred (May-June).

152

153 **N deposition and climate data.** Since we are not working in sites with high topographic
154 heterogeneity and in close vicinity of important point emission sources, the best available
155 standardized N deposition data at the continental scale were obtained from the European
156 Monitoring and Evaluation Programme database (EMEP) (<http://www.emep.int>). EMEP is the
157 ‘Co-operative Programme for Monitoring and Evaluation of the Long-range Transmission of Air
158 Pollutants in Europe’ and provides scientific information on the emission, transport and deposition
159 of air pollutants in 50 × 50 km grid cells covering Europe (Tørseth *et al.* 2012). Atmospheric
160 deposition rates were calculated by summing the modelled rates of wet and dry oxidised and
161 reduced N and expressed as kg ha⁻¹ year⁻¹. Data from 2009 and 2013 were used for the seeds
162 sampled in the respective years. For simplicity, we assumed a dry deposition factor equal to 1 (that
163 is, the factor by which dry deposition can be increased due to vegetation roughness) (Erisman &
164 Draaijers 2003). Data on N deposition ranged between 1.19 (Trondheim, central Norway) and
165 17.85 kg ha⁻¹ year⁻¹ (Ghent, Belgium) (Fig. 1a). Climate data were obtained from the CRU CL2.0
166 dataset using Fetchclimate 2 (Microsoft Research Cambridge, <http://fetchclimate2.cloudapp.net>).
167 We extracted the mean annual and spring (February-May) temperature and precipitation for each
168 population for 2009 and 2013, depending on the year of seed sampling. Only the mean annual
169 temperature and precipitation were used in the analyses because these were strongly correlated with
170 the spring temperature and precipitation (Pearson $r = 0.951$, $P < 0.001$ and $r = 0.911$, $P < 0.001$,
171 respectively). The effects of soil characteristics (e.g., pH, soil P, Ca, K, Mg, N, P) were not

172 considered here since their effects on seed mass and N concentrations were shown to be negligible
173 in an earlier study (*cf.* De Frenne *et al.* 2011c).

174

175 *Study 2: N addition experiment*

176 **Establishment of the experiment.** The second study was a controlled addition experiment in
177 which an N ion solution was added with a paintbrush to the petals and leaves of *A. nemorosa* and to
178 the soil using a design adapted from Carlo & Norris (2012). Rhizomes with emerging ramets were
179 collected in 2013 before flowering from an ancient deciduous forest dominated by *Fraxinus excelsior*
180 and *Quercus robur* in central Belgium (square in Fig. 1b) (latitude 50.975°N, longitude 3.804°E). The
181 experiment was also performed at this location. Following collection, rhizomes were transplanted
182 immediately into 1.5L-pots on 8 April 2013 using top 10 cm-soil from the collection site. Pots were
183 installed in the forest but below transparent rain shelters (installed *c.* 1 m above the plants) such that
184 no natural precipitation could reach the pots, soil or plants. All pots were arranged randomly and
185 watered throughout the experiment with distilled water as needed, carefully avoiding the plant
186 tissue. We used five replicate pots for each of the nine treatment groups (control and a factorial
187 combination of N application type and concentration, see next section). All individuals were
188 flowering when monitored on 12 April 2013.

189

190 **Treatments.** Plants were treated with aqueous ammonium nitrate solutions using two
191 complementary approaches. First, N was applied with a paintbrush (type Marabu Forte 6) by
192 repeatedly inserting the paintbrush in the N solution and then using a constant number of
193 paintbrush strokes to deposit the solution. Specifically, leaves were treated with five paintbrush
194 strokes randomly spread across the three leaves surrounding each flower. Petals were also treated
195 with five strokes randomly spread across the six petals of each flower (carefully avoiding the carpels
196 and anthers). To also apply the N solution to the soil, a circular filter paper of *c.* 20 cm² was
197 installed on the soil surface immediately surrounding the plant stem and also treated with five
198 paintbrush strokes (after which the filter paper was rinsed with distilled water such that the N was

199 washed into the soil and became potentially available to the plant; cf. Carlo & Norris 2012). Control
200 plants were treated in exactly the same way, but using distilled water on the paintbrush. Secondly,
201 the experiment included a treatment in which whole plants were sprayed with the same N solutions.
202 This whole-plant approach mimicked a natural rainfall event by spraying all aboveground parts of
203 the plants with a handheld sprinkler until water drops were visible on the entire plant. In all
204 treatments, N solutions were applied spread over four occasions (on 15, 16, 23 and 26 April 2013).

205

206 **N solutions and concentrations.** We used ammonium and nitrate as N forms because these are
207 the most abundant N ions in precipitation: the concentration of ammonia and nitrate in bulk open-
208 field precipitation in central Belgium amounts to 0.85 mg N L⁻¹ and 0.50 mg N L⁻¹, respectively
209 (Verstraeten *et al.* 2012), adding up to a total of 1.35 mg N L⁻¹. The long-term average precipitation
210 during the flowering period of *A. nemorosa* (approximately one month) is 48 mm (1981–2010 April
211 average for a weather station at 890 m distance from the experiment) resulting in a deposition of
212 64.8 mg N m⁻² (or 0.648 kg N ha⁻¹). This means that the N dose on the foliage of an average *A.*
213 *nemorosa* ramet (27 cm² leaf area ramet⁻¹ is the mean of this species across Europe, De Frenne *et al.*
214 2011b) amounts to 0.17 mg N per ramet. The N dose on the petals of an average *A. nemorosa* flower
215 (9 cm² surface area flower⁻¹ is the mean for this population) amounts to 0.06 mg N per flower. To
216 determine the exact N dose, we then calculated the amount of water deposited on *A. nemorosa* petals
217 and leaves by five paintbrush strokes. This amounted to 8.5 mg ± 1.0 S.E. (determined from 8
218 replicate leaves) and 6.3 mg ± 1.0 S.E. (determined from 8 replicate petals). To achieve a dose of
219 0.17 mg N per plant, spread over four applications each consisting of five paintbrush strokes, we
220 thus needed to apply a solution with a concentration of 2.859 g NH₄NO₃L⁻¹ (1.0 g N L⁻¹): 8.5 mg
221 H₂O × 4 applications × 5 strokes × 1.10⁻⁶ g N mg⁻¹ H₂O. Our experiment also included a second
222 scenario with a threefold concentration of 8.576 g NH₄NO₃L⁻¹ (3.0 g N L⁻¹) that resulted in a dose
223 of 0.51 mg N per plant (0.38 mg N on the petals). In sum, the average N dose during the entire
224 experiment using the low concentration applied to the leaves and petals was 0.17 mg N and 0.12
225 mg N, respectively, and 0.51 mg N and 0.38 mg N using the highly concentrated solution. This

226 experimental design resulted in a total of 45 plants: 5 control plants (using the paintbrush with
227 distilled water), 30 plants in which either the flowers, leaves or soil were treated with the solution
228 with the low or high concentrations using the paintbrush, and finally 10 plants which were entirely
229 sprayed with the sprinkler with either the low or high concentration. All seeds of each individual
230 were sampled at seed maturity on 24 May 2013.

231

232 *Seed traits*

233 Seeds obtained from both experiments were air-dried to constant mass for \approx 1 week. We then
234 determined mean seed mass by weighing all seeds (to the nearest 0.1 mg) per seed sample and
235 dividing the obtained mass by the number of seeds. A subsample of seeds was then oven-dried to
236 constant mass (50 °C for 72 h) and analysed for C and N using a CNS element analyser (expressed
237 in %) and C:N ratios calculated from these results. The seed N content per seed (mg N seed⁻¹) was
238 calculated by multiplying the N concentration (%) with the seed mass (mg). The response variables
239 for both experiments were the seed mass, seed C and N concentrations and seed C:N ratio and N
240 content since these are key variables reflecting seed quality and provisioning, and potential seedling
241 survival and reproductive success (Parrish & Bazzaz 1985; De Frenne *et al.* 2011c). Illustratively,
242 seed mass is strongly correlated to germination percentages in this species (De Frenne *et al.* 2011c: r
243 = 0.656, $P < 0.05$). We did not consider other seed nutrients such as K, Ca or P for this study
244 because these are unlikely to strongly respond to petal N addition and did not show major variation
245 in this species with latitude (De Frenne *et al.* 2011c). Furthermore, no alkaloids were detected in
246 *Anemone* seeds (De Frenne *et al.* 2011c). This is noteworthy since plants can invest a substantial part
247 of their N in secondary compounds, especially in the Ranunculaceae family. Thus, most probably all
248 N in the seeds is present as free amino acids and/or proteins.

249

250 *Data analyses*

251 To explore whether and to what extent the seed traits varied in response to variation in N
252 deposition rates and climate over time and space in Study 1, we applied linear mixed-effect models

253 using the *lmer*-function from the *lme4*-package in R (R Core Team 2017). Random effect terms
254 'region' and 'population' nested within 'region' were added to the multilevel models to account for
255 the hierarchical nature of the data and the spatiotemporal autocorrelation within populations and
256 regions. We also added a non-nested random effect 'year' to account for the repeated sampling in
257 2009 and 2013 in the same populations (Zuur *et al.* 2009). To avoid multicollinearity issues of the
258 explanatory variables temperature and N deposition within the same model, the effects of the
259 predictors were always tested using separate models (one-by-one). Correlations among predictors
260 were as follows: temperature vs. N deposition: $r = 0.870$, $P < 0.001$; temperature vs. precipitation: r
261 $= 0.202$, $P = 0.284$; N deposition vs. precipitation: $r = 0.0674$, $P = 0.723$.

262

263 The seed quality data from Study 2 were first expressed as response ratios with the natural
264 logarithm of the ratio of the seed traits (x_i) divided by the mean seed traits of the control plants
265 (\bar{x}_{control}) as $\log(x_i/\bar{x}_{\text{control}})$. These response ratios were then analysed with two-way analysis of
266 variance (ANOVA) with the treatment (soil, leaves, flowers or entire plant treated with N) and
267 concentration (low vs. high) as factors using the *aov*-function in R (R Core Team 2017).

268 Results

269 In Study 1, seed traits exhibited strong spatial variation matching closely the estimated N deposition
270 rates across Europe (Fig. 2; Table 1). Seed N concentrations and N contents were strongly
271 positively correlated with N deposition while C:N ratios decreased with N deposition. For instance,
272 N concentrations increased, on average, from *c.* 2.8 % seed N at N deposition levels of 1 kg N ha⁻¹
273 yr⁻¹ to *c.* 4.1 % at N deposition levels of 18 kg N ha⁻¹ yr⁻¹ (Fig. 2b,d; Table 1). By contrast, seed mass
274 and seed C concentrations did not differ significantly in seeds sampled from regions with high vs.
275 low levels of N deposition. Second, temperature was also related to the seed traits along the
276 latitudinal gradient with significant positive effects on N concentration and content and negative
277 effects on seed C:N ratios. Yet, the AIC values of the temperature models were consistently higher
278 for all seed traits than of the models with N deposition as predictor, which demonstrates the higher
279 relative importance of N deposition for seed traits of *A. nemorosa* along the latitudinal transect
280 (Table 1). Precipitation did not demonstrably influence any of the investigated seed traits.
281 Nevertheless, to experimentally disentangle the effects of N deposition from potentially
282 confounding effects of other environmental changes along the latitudinal gradient, we designed the
283 experiment in Study 2.

284 Seed traits also responded significantly to the experimental N treatments in the controlled Study 2,
285 but mostly to the highly concentrated solution (Fig. 3; Table 2). There were significant differences
286 between the mass of seeds, seed N concentration, C:N ratios and N content in the contrasting
287 treatments, with, for instance, seeds containing more N if petals received additional N (Fig. 3). Seed
288 N concentrations varied significantly with treatments, increasing from *c.* 2.8 % to 3.2 % when petals
289 were treated with N compared to the control treatment (Fig. 3b). Nonetheless, the concentration of
290 the solution did not significantly affect this result. Finally, the seed N content responded both to
291 the N addition treatment as well as the applied concentration (Fig. 3d). Seed C concentrations were
292 not significantly affected (Table 2). Addition of N to the leaves and soil did not affect seed N
293 concentrations, but spraying the entire plant resulted in similar responses of seed traits as addition
294 of N to the petals alone (Fig. 3).

295 **Discussion**

296 We detected consistent effects of N deposition on seed traits of *Anemone nemorosa*. Seed N
297 concentrations and total seed N content increased with large-scale N deposition patterns in the
298 observatory across Europe (Study 1) and with experimental N addition (Study 2). We previously
299 established a strong north-south gradient in seed N concentrations in this forest herb, but the
300 causes behind this mechanism remained unknown (De Frenne *et al.* 2011c). With the observational
301 and experimental results reported here, we identify the likely driver for observed latitudinal clines in
302 seed N concentrations as we demonstrate that N deposition on petals during anthesis enhances
303 bioaccumulation of N in seeds. Deposition of N on the leaves and soil alone did not meaningfully
304 change seed N concentrations, but when N was applied to the whole plant, patterns were similar as
305 in the petal treatments. Our results thus corroborate Carlo & Norris (2012) who showed that petals
306 can act as permeable membranes through which N is readily incorporated in the reproductive
307 structures, most likely via N transport to the ovaries and seeds via the phloem (Zhang *et al.* 2010).
308 Additionally, water droplets might flow over the petal surface to reach the ovaries, or dry deposits
309 may be wetted by rainfall and as such transported directly to the ovaries. The lack of significant
310 effects of precipitation on seed traits might indicate non-negligible effects of dry deposition. This is
311 confirmed by the N concentrations obtained in the experiment where we only manipulated wet
312 deposition. N concentrations there reached *c.* 3.2 %, while maximal concentrations of more than 4
313 % were obtained in the field in Study 1.

314

315 Changes in seed size and quality as a result of enhanced atmospheric N deposition are likely to have
316 important ecological implications. Enhanced N accumulation in seeds resulting from N deposition
317 may increase resources available for embryo development, seedling nutrition and establishment.
318 Most probably, this N is stored as free amino acids and proteins (no secondary N compounds such
319 as alkaloids for defence against herbivores are present in *A. nemorosa* seeds, see Materials and
320 Methods). Bioassays on seeds of a wide range of species show that the usable internal N reserves
321 depleted the quickest of all nutrients after germination (Fenner & Thompson 2005). This means

322 that even small differences in seed N provisioning can improve seedling survival and fitness in
323 adverse abiotic and biotic environments such as shady or nutrient-poor habitats, during drought
324 episodes and heat waves, and under intense competition for resources (Parrish & Bazzaz 1985;
325 Moles & Westoby 2004; Naegle *et al.* 2005; De Frenne *et al.* 2011c; but see Hanley *et al.* 2007). These
326 processes can in turn, influence plant population dynamics and community composition.
327 Accordingly, community changes in response to fertilization might not only be due to changes in
328 above- and below-ground competitive interactions for light or nutrients (Hautier *et al.* 2009), but
329 may also result from altered seed provisioning and differential recruitment. Nitrogen uptake
330 through permeable surfaces of petals can also be advantageous to flowering plants in the absence of
331 elevated atmospheric N deposition. For example, arthropod pollinators such as honeybees deposit
332 significant amounts of ammonium and nitrate via their faeces (Mishra *et al.* 2013). Deposition of N
333 via faeces on petals can thus efficiently be incorporated into the seeds. Finally, diaspores of *A.*
334 *nemorosa* and numerous other forest herbs are ingested and dispersed by ants and slugs (Delatte &
335 Chabrerie 2008; Türke *et al.* 2010) and changes in their chemical composition may affect the
336 probability of diaspore removal and ingestion (Reifenrath *et al.* 2012), and consequently species
337 colonization and trophic interactions.

338

339 Scaling up to the population and species level, we expect a positive impact of elevated N deposition
340 on *A. nemorosa* recruitment and population dynamics, especially in the longer run. Nevertheless,
341 previous field studies found mixed results, which varied strongly according to the amount of annual
342 N addition and the sampling period. For instance, in an experiment with addition of not less than
343 180 kg N ha⁻¹ yr⁻¹ in a forest in southern Sweden, Falkengren-Grerup (1993) detected a significantly
344 reduced shoot length, flowering frequency, cover and biomass of *A. nemorosa* relative to the control
345 after six years. Clearly, the applied N level was much higher than the levels of atmospheric N
346 deposition in this study. In a field experiment in Belgium with application of 100 kg N ha⁻¹ yr⁻¹, the
347 percentage ground cover of *A. nemorosa* declined significantly by 32 % over 6 years of N application
348 (unpublished data, experiment described in De Frenne *et al.* 2015). In the last five decades, the

349 frequency of occurrence of *A. nemorosa* in resurveyed semi-permanent vegetation plots within
350 ancient woodlands across Europe has also not changed dramatically (on average this changed from
351 46.3 to 45.2 %; De Frenne *et al.* 2013). Undoubtedly, other global environmental changes such as
352 climate change, land-use change and management change interact with N deposition to affect
353 population dynamics and the species' distribution, and thus play an important role in determining
354 the ultimate response of populations at the continental scale (Baeten *et al.* 2010). The mixed results
355 at the population level between previous N addition field experiments (Falkengren-Grerup 1993,
356 De Frenne *et al.* 2015) and this study are likely due to N addition that was not added exclusively on
357 *A. nemorosa* petals in the other experiments and thus such changes likely reflect negative effects of
358 N addition on adult cover and biomass, and not on recruitment and reproductive success via petal
359 uptake.

360

361 Since N deposition rates across Europe correlate with other environmental changes from north to
362 south (most notably, temperature), it is difficult to distinguish correlation from causation in Study 1.
363 Nonetheless, in the controlled pot experiment of Study 2, only N addition was manipulated; all
364 other biotic and abiotic environmental variables (temperature, soil moisture, soil nutrients, canopy
365 cover, etc.) were kept as constant as possible. Taking our two sub-studies together, our results
366 support a dominant role of N deposition on petals in explaining the observed patterns in seed traits
367 of this species. More generally, we hypothesise that a wide array of plant species (specifically those
368 with relatively large petals and small seeds) in regions with high levels of atmospheric N inputs
369 currently display higher seed mass, N concentrations and contents than before the historical onset
370 of enhanced air pollution and N emissions. Whether enhanced atmospheric inputs of N via direct
371 intake by petals can influence demography, and population and community dynamics clearly
372 warrants further research, for example, by subjecting seedlings resulting from seeds with contrasting
373 N concentrations to stress and/or herbivory experiments and by performing seed dispersal and
374 cafeteria experiments with seeds of contrasting N concentrations.

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484 **Tables**

485 **Table 1.** Effects of N deposition rates, temperature and precipitation on seed traits of *Anemone*
 486 *nemorosa* across Europe. Results from Study 1 (macroecological approach) using linear mixed-effect
 487 models with N deposition rates and climatic variables as predictor variables (see Materials and
 488 Methods).

Predictor	Response variable	Parameter estimate	Chi ² -value	P-value ¹	AIC
N deposition	Seed mass	4.55×10^{-2}	0.84	0.361 (ns)	86.4
	Seed C concentration	-9.8×10^{-2}	2.49	0.114 (ns)	134.7
	Seed N concentration	7.58×10^{-2}	13.85	<0.001 ***	55.4
	Seed C:N ratio	-4.01×10^{-1}	14.13	<0.001 ***	153.7
	Seed N content	3.9×10^{-3}	6.63	0.010 *	-109.6
Temperature	Seed mass	8.41×10^{-2}	0.314	0.575 (ns)	86.9
	Seed C concentration	-1.31×10^{-1}	0.75	0.386 (ns)	136.5
	Seed N concentration	1.63×10^{-1}	10.80	0.0010 **	58.4
	Seed C:N ratio	-8.47×10^{-1}	10.32	0.0013 **	157.5
	Seed N content	7.05×10^{-3}	3.82	0.051 (*)	-106.8
Precipitation	Seed mass	5.41×10^{-5}	0.65	0.999 (ns)	87.9
	Seed C concentration	-9.98×10^{-4}	0.15	0.698 (ns)	137.1
	Seed N concentration	2.29×10^{-4}	0.27	0.606 (ns)	68.9
	Seed C:N ratio	-1.40×10^{-3}	0.30	0.587 (ns)	167.5
	Seed N content	1.06×10^{-5}	0.01	0.999 (ns)	-103.0

489 ¹Significances are indicated: (ns): P > 0.10; (*): P<0.1; *: P<0.05; **: P<0.01; ***: P<0.001.

490 **Table 2.** Effects of the experimental N addition treatments (leaves, flowers, soil or entire plant
 491 treated with N as levels) and concentrations (low vs. high as levels), and their interaction, on seed
 492 traits of *Anemone nemorosa*. Results from the experimental Study 2 using log-response ratios and two-
 493 way ANOVAs (see Materials and Methods).

Response variable	Treatment	Concentration	Treatment: Concentration
Seed mass	0.078 (*)	0.038 *	0.998 (ns)
Seed C concentration	0.637 (ns)	0.339 (ns)	0.323 (ns)
Seed N concentration	0.011 *	0.513 (ns)	0.301 (ns)
Seed C:N ratio	0.085 (*)	0.945 (ns)	0.314 (ns)
Seed N content	0.010 *	0.039 *	0.822 (ns)

494 Significances are indicated: (ns): P > 0.10; (*): P < 0.10; *: P<0.05.

495 **Figure legends**

496 **Fig. 1.** Illustration of the N deposition gradient used in this study: (a) Relationship between latitude
497 ($^{\circ}\text{N}$) and N deposition rates ($\text{kg N ha}^{-1} \text{ yr}^{-1}$) of the study sites. The fitted grey line denotes a mixed-
498 effect model with random-effect term 'region'. (b) Distribution range of *Anemone nemorosa* after
499 Hultén & Fries (1986) (grey shaded area) and sampling sites along the N deposition gradient (circles
500 and square) and location of the experiment (square).

501

502 **Fig. 2.** Relationships between N deposition rates ($\text{kg N ha}^{-1} \text{ yr}^{-1}$) and the measured seed traits of
503 *Anemone nemorosa* seeds sampled along an N deposition gradient in Europe (Study 1:
504 macroecological approach). Fitted regression lines are the linear mixed-effect models shown in
505 Table 1, dashed lines are not significant at $P > 0.1$, solid lines are significant at $P < 0.05$.

506

507 **Fig. 3.** Effects of N addition using a solution with low (open circles) and high (grey circles) N ion
508 concentration to the soil, leaves and petals of the flowers or the entire plant on seed traits of
509 *Anemone nemorosa* (Study 2). The control treatment is shown with a larger black circle. Error bars
510 denote standard errors.