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Effects of simulated nitrogen deposition on the nutritional and physiological status of beech forests at two climatic contrasting sites in Italy

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Abstract

Anthropogenic activities have resulted in a significant increase of reactive nitrogen (N) compounds in the atmosphere and a rise in N deposition on forest ecosystems. Increasing N loads impact forest productivity and health, altering tree physiological status and nutrient balance with possible beneficial and detrimental consequences. The impact of N deposition has received considerable attention by scientific research, covering medium and high latitudes, while experimental studies in the Mediterranean area are almost absent. The present study used a manipulative approach, through replicated N additions (background deposition, 30, 60 kg N ha^{-1} yr^{-1}) to simulate the cumulative effects of N deposition in two beech (Fagus sylvatica L.) forests located in contrasting climatic regions of Italy. Leaf nutrients and photosynthetic pigments were tested as monitoring indicators after four years of N fertilization. Foliar N and pigment concentrations indicated not limiting N conditions at both forest sites, although changes in chlorophylls and carotenoids showed an early response of the canopy to N additions. N-to-phosphorus (P) and sulphur (S) ratios increased under elevated N fertilization, which could be partly related to the relative enhancement of foliar N concentration, and partly associated with the reduction of foliar P and S. The two eutrophic beech forests monitored were not severely affected by chronic N addition, not showing critical nutritional and physiological impairments over the short to medium period. However, the modifications in leaf nutrient and pigment compositions showed an incipient stress response and accentuated the differences induced by climatic and soil characteristics at the two sites. The potential use of nutrients and photosynthetic pigments in monitoring forest N deposition under contrasting climatic conditions and the eventual limits of manipulative experiments are discussed.

Keywords: Nutrients; Chlorophyll; Carotenoids; Nitrogen deposition.

Highlights:

- ✓ Foliar nutrient concentrations are slightly decreased by N deposition.
- ✓ N addition affected significantly foliar N:P and N:S ratios.
- ✓ The two climatic contrasting sites differently respond to N addition.

Introduction

Human activities over the last decades have drastically altered the natural nitrogen (N) cycle, as fertilizer manufacturing and fossil-fuel combustion have generated and released in the atmosphere an increasing amount of N reactive compounds such as nitric oxides (NO_x) and ammonia (NH_3), which has then deposited onto managed and natural ecosystems.

Sulfur (S) deposition, often associated with N deposition in the past, has now dropped dramatically due to environmental regulations (Berger et al., 2016; Cecchini et al., 2021; Johnson et al., 2018), bringing N deposition to the forefront of current environmental concerns. Indeed, atmospheric N deposition to forests has reached globally 187 million tonnes in 2005 and has been estimated to almost double by 2050 (Galloway et al., 2008; Law, 2013). N deposition in Italy is among the highest in Europe (Cecchini et al., 2021). The highest rate of N deposition occurred in Northern Italy (up to a maximum of 28.8 kg N ha^{-1} y^{-1}, Ferretti et al., 2014), around the Po valley, one of the most urbanized and industrialized areas in Europe (Cecchini et al., 2021). Relatively lower N deposition rates (4.5 kg N ha^{-1} y^{-1}) were found in Southern Italy (Ferretti et al., 2014), as measured by the permanent monitoring network ICP Forests (International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests). Even if the N deposition rate in Southern Italian regions is not as high as in the North of Europe, chronic N inputs to forest ecosystems are not negligible, and they could cause a cumulative effect in the long term (Cecchini et al., 2021; De Marco et al., 2014; Gentilesca et al., 2013).

Atmospheric deposition is an essential driver of various changes in forest ecosystem productivity and health. The long-term N addition to forest ecosystems could result in a cascade of different, often contrasting, consequences. Although excess N have a detrimental physiological effect on the plant (Pearson and Stewart, 1993), N often stimulates growth, in particular in temperate and boreal ecosystems, which are often limited by its availability. On the other hand, on non-N-limited sites (e.g., in tropical climate regions, or already fertile sites) atmospheric N deposition could also cause ecosystems N saturation, increased nitrification rate and N leaching. The latter, in turn, could induce soil acidification and nutrient losses, as well as the mobilization of other minerals (Galloway et al., 2003). The main effects on plants are nutrient imbalances in leaf tissues, especially due to the decreasing availability of Ca^{2+} and Mg^{2+}, with relative deficiency symptoms such as chlorosis and necrosis of tips (Taiz et al., 2015; Carter et al., 2017); such effects may, in turn, increase plant sensitivity to pest or pathogens (Braun et al., 1999), and induce growth reduction (Etzold et al., 2020). From a monitoring point of view, the evaluation of the foliar data collected at ICP Forests plots over a 20-year period has highlighted a critical decreasing trend in the foliar concentrations of some nutrients (Jonard et al., 2014). In particular a significant decline in foliar phosphorus (P), magnesium (Mg), and calcium (Ca) concentrations has been observed in beech (*Fagus sylvatica* L.) forests across Europe over the last ten years, while the foliar contents of N and S have shown a significant increase (Jonard et al., 2014). This trend has often led to unbalanced foliar nutrient ratios in forest trees, with a consistent increase of N:P ratios across all species in Europe (Penuelas et al., 2020; Talkner et al., 2015). In many European beech stands, in particular, an excess of N nutrition has been detected (Jonard et al., 2014), raising the concern that these forests could be exposed to the ecological and growth impairments often found in N saturated ecosystems (Aber et al., 1998). The response of beech biochemistry and stoichiometry to increasing atmospheric N, however, is far from clear, influenced as it is by differences in soil and climate across the ICP monitoring range, which have a profound effect on associated biota and on soil biogeochemical processes involved in the N cycle (e. g. Braun et al., 2020; Clausing et al., 2021; Dziedek et al., 2016).

Changes in leaf nutrients could affect the content of key biochemical compounds, in particular photosynthetic pigments. The leaf content of photosynthetic pigments reflects the plant's light capture performance and, consequently, its functional ecology and productivity (Poorter et al., 2009; Scartazza et al., 2016). Chlorophylls and carotenoids play essential functional and protective roles in plant photosynthetic processes, and therefore they are often used as biomarkers in monitoring forest health conditions at different scales, from the leaf to the canopy level (Nestola et al., 2018; Niinemets et al., 2015; Scartazza et al., 2016).

Leaf biochemical parameters, such as the content of elements and photosynthetic pigments, are a valuable tool to assess nutrient limitations status and health of plants, commonly adopted in nutrient manipulative experiments designed to establish the effects of fertilization in forest ecosystems (Ågren, 2008; Sullivan et al., 2014). Indeed, foliar biochemistry and elemental stoichiometry can appropriately reflect the availability of nutrients in the forest soil (Cleveland et al., 2011; Han et al., 2005). These leaf traits respond over a relatively short timescale to natural deposition or fertilization, in contrast with tree growth and biometric parameters which often require more than a decade of treatments to manifest a shift (Wright, 2011). At the same time, nutrients and photosynthetic pigments are characterized by relatively simple collecting techniques, relative to physiological parameters such as photosynthesis. Despite dynamic nature of biochemical traits requires careful planning and standardization (e.g., to avoid the effects of phenological changes; Grassi et al., 2005), their analysis can provide a powerful tool for the early detection of plant responses to environmental drivers.

Several replicated manipulative studies have been carried out over the last decades to forecast N deposition impacts on ecosystems, but they have mainly focused on coniferous forests (de Schrijver et al., 2011; Hyvönen et al., 2008), for which N fertilization has also been widely applied with productive purposes under boreal and temperate climates (e.g., Billow et al., 1994; Kaakinen et al., 2009). More limited information is available for deciduous forests in the Mediterranean area, where summer drought could constrain soil processes, plant function, and growth irrespective of N availability. European beech is one of the most dominant tree species in Europe (Poetzelsberger et al., 2015), as well as in Italy, where beech forests are broadly distributed and represent about 12% of the national forest coverage, extending from the Alps to Sicily's southernmost sites (INFC et al., 2007). Due to the species sensitivity to water supply (Granier et al., 2007), and to chronic N deposition (Braun et al., 2020; Jonard et al., 2014; Talkner et al., 2015), the future of beech at a southern distribution limit is uncertain under expected global change. In countries like Italy, in particular, a long history of integration between forests and agricultural activities has created relevant environmental and socio-economic concerns for the preservation of carbon stocks and mitigation capacity, sustainable wood production and maintenance of biodiversity, in the framework of traditional and sustainable forest management (Cullotta et al., 2016).

Against this backdrop, the addition of chronic N inputs under field conditions can be a useful tool to test and predict the potential long-term N cumulative effects on forests exposed to elevated atmospheric N loads, especially keeping in mind that a multidecadal perspective is needed in monitoring due to the inherently slow processes involved (Bortolazzi et al., 2021; Braun et al., 2020).

In this work, a replicated long-term soil N addition experiment was set up in two monospecific beech forests without understory and similar in age, both close to ICP Forest Level 2 permanent monitoring plots historically exposed to different N depositions levels and located at two contrasting geographical sites (Cansiglio and Collelongo, Northern and Central Italy, respectively).

The main questions explored in the present paper were: i) if leaf nutrient and photosynthetic contents are suitable indicators for beech forest monitoring under N deposition, simulating N cumulative effects; ii) how beech differently responds to N addition in two climatic contrasting Mediterranean sites. We expected higher soil N availability to enhance foliar N and photosynthetic pigment contents and induce a decline in foliar P, Mg, and Ca concentrations. Moreover, we expected the magnitude of the impact of chronic N addition on foliar nutrient imbalances to be higher at the Cansiglio site, which is already subjected to high natural N deposition and likely closer to N saturation.

Material and methods

Site description

The study was carried out in two Italian beech forests located on the Eastern Alps (Cansiglio; 46° 3' 19'' N 12° 22' 51'' E) and on the Central Apennines (Collelongo; 41° 50' 59'' N 13° 35' 6'' E). Both experimental sites are close to ICP Forest Level 2 permanent monitoring plots (VEN1 - Pian del Cansiglio, Northern Italy and ABR1 - Selva Piana-Collelongo, Central Italy, respectively; Marchetto et al., 2014). The vicinity of the two sites to long-term ICP monitoring stations (1996 to present) allowed the availability of key meteorological variables, forest growth rates, atmospheric N deposition data, leaf and soil biochemical characteristics (Table S1 and Fig. S1). This has made it possible to place the results of the present N fertilization study in a broader perspective.

At both sites, the forest is a pure single-layer beech stand; the stands were selected to have similar age and geological substrate but with marked differences in climate and background N deposition. The Cansiglio area (1200 m a.s.l.) is characterized by the presence of an oceanic climate, and a period of summer dryness is generally absent (Fig. S2). On the other side, the Collelongo area (1560 m a.s.l) is subjected to mild summer drought, characteristic of the typical Mediterranean-mountain climate (Fig. S2; Scartazza et al., 2016). Cansiglio forest grows on a Calcareous pedogenetic substrate (Haplic Luvisol), while Collelongo on a calcareous substrate with sizeable stoniness and clay components (Humic Alisols). The natural atmospheric N deposition exceeds the estimated critical N load (Ferretti et al., 2014) only in the Northern site (Cansiglio). The main characteristics of the two forest stands are summarized in Table 1.

Experimental design and N treatments

Three N addition treatments were compared at each site: untreated control (Control), soil addition of 30 (Medium), and 60 kg N ha^{-1} yr^{-1} (High). While the Medium treatment falls within the range of predicted N deposition scenarios for 2050 (~ 12-24 kg N ha^{-1} yr^{-1}; Phoenix et al., 2006) and the range of N deposition measured in other Mediterranean areas (up to 45 kg ha^{-1} yr^{-1}; Fenn et al., 2003), the High-level treatment was chosen to achieve N saturation in both forest ecosystems. The experiment was arranged in a completely randomized design with three replicate plots for each treatment, located at a suitable distance from ICP monitoring sites. The plots have dimensions of 30 x 30 m and are adjacent to each other. In order to avoid any lateral contamination effects, measurements were carried out on plants (*n* ≥ 3) in the central area of each plot (15 x 15 m). N fertilization has been applied at the two sites since the start of the experiment in 2015. N has been applied as an ammonium nitrate solution (NH_4NO_3, 35.1 g L^{-1}) and distributed on the ground using a backpack sprayer. The fertilizer has been equally distributed in three annual applications (June, July, and September), performed simultaneously at the two sites. Because of the small amount of solution added (< 1 mm yr^{-1}), no water has been added to control plots.

Sample collection and analysis

Leaf samples were collected in 2016 and 2018 just after the peak of the vegetative period (late July / early August; Nolè et al., 2009) at both sites by the tree-climbing technique, following the ICP Forests manual (ICP Forests, 2016). Two branches developed in full light were sampled from 3 plants per plot. Leaves were collected from each branch between 11:00 and 13:00 h from healthy trees (i.e. with no sign of stress due to pathology or photobleaching) at the top of the canopy to obtain two paired samples of about 20 leaves each. Based on their position within the canopy, all sampled leaves can be classified as “sun leaves” adapted to high solar radiation (Scartazza et al., 2016). To be used for nutrient determination, a first sample was stored in a cooler bag during fieldwork, then kept at 4 °C in the laboratory until analysis. The second sample for photosynthetic pigments was immediately packed in aluminum foil, frozen in liquid N, and kept at -80 °C until analysis.

Leaves were washed, oven-dried at 60 °C until constant weight, ground, and analyzed for C, N, P, K, Ca, Mg, Mn, Fe, Zn, Cu, and B concentrations. The percentage of foliar N was measured with a C/N elemental analyzer (Model NA 1500, Carlo Erba, Milan, Italy). The other nutrients were determined by an Ion Coupled Plasma Atomic Emission Spectrometer (Inductively Coupled Plasma-ICP; Ametek Spectro Arcos, Kleve, Germany) on samples mineralized (US EPA Methods 3052; Kingston 1988) in a microwave mineralizer (Ethos TC, Mileston, Bergamo, Italy).

Photosynthetic pigments (chlorophyll *a*, Chl *a*, chlorophyll *b*, Chl *b,* and total carotenoids) were determined on liquid N_2 homogenized leaf samples according to the method of Wellburn (1994), with some modifications. Briefly, 30-50 mg of leaf material were extracted in 80% (w/v) cold acetone, centrifuged at 3000×g for 10 min at 4°C, filtered, if necessary (0.2-µm, Sartorius Stedim Biotech, Göttingen, Germany), and the absorbance of the supernatant was measured at 663.2, 646.8, and 470.0 nm using an UV-vis spectrophotometer (UV-1800 Spectrophotometer, Shimadzu).

Statistical analysis

All data were averaged within the plot to avoid pseudo-replication. Data were analyzed separately for each sampling year (2016 and 2018) in a complete randomized factorial design with two factors: site (2 levels: Cansiglio and Collelongo) and N treatment (3 levels: Control, Medium and High). For each combination of treatments, three replicate plots (*n* = 3) were considered. The normality and homoscedasticity of data were assessed by the Shapiro-Wilk and Bartlett tests, respectively. Since their distribution of the data respected the ANOVA assumptions, no transformation was applied. When the two-way analysis of variance (2-way-ANOVA) showed a significant ($p \leq 0.05$) interaction between factors, *a posteriori* separation of means was performed by the Student Newman-Keuls test (SNK). An analysis of covariance was also performed to highlight any differences in the relationship between leaf chlorophylls and N content in response to N treatments at the two sites for each sampling date, utilizing the leaves collected on tree branches as sampling units (*n* = 5-10). A mixed-effect model was not applied, since the specific time and site were of interest, therefore were considered as fixed effects. A Canonical Discriminant Analysis (CDA, Friendly M, Fox J (2021) was also performed to identify the most relevant factors explaining the variance between and within sites. CDA is a multivariate statistical technique that can identify differences among groups of individuals, taking into account the group structure of the data

(Cruz-Castillo et al., 1994). All statistical analyses were carried out with the R open-source software (R Core Team 2017) using the *easyanova*, *agricolae,* and *candisc* libraries.

Results

A significant interannual variability in leaf nutrient and photosynthetic pigment concentrations was observed in Cansiglio and Collelongo beech forests, independently of N addition levels. For this reason, foliar data from 2016 and 2018 were analyzed separately.

In both sampling sites and years, the concentration of most macro- and meso-nutrients in leaves from the control plots fell within the optimal range for the species. In both 2016 and 2018, leaf N concentration differed significantly between sites, with higher values in Collelongo than Cansiglio, but was not affected by fertilization (Table 2). On the contrary, leaf P concentration was affected by N additions in 2016, showing a slight decrease in both beech forests with increasing N addition levels (on average more than 4% and 9% at Medium and High N treatment, respectively; Table 2). Remarkably different P concentrations were measured at both sites between the two years, with values consistently above the optimum range in 2016 but close to its minimum value in 2018. Moreover, in 2016 also leaf K concentration was influenced by both site and N treatment, with generally higher values at Collelongo than Cansiglio and a slight but significant decrease ($p = 0.045$) in response to the higher N dose. In 2018, on the contrary, neither the site nor N addition had a significant effect on the parameter (Table 2). Also leaf Ca concentration was consistently higher in Collelongo than in Cansiglio, in particular in the 2016 growing season ($p < 0.001$), while the concentration of Mg was not significantly affected by site, N fertilization, or their interaction in either year of sampling (Table 2). Finally, the foliar S concentration was consistently lower in Cansiglio than in Collelongo (-12.5% and -10.3% in 2016 and 2018, respectively) and was significantly affected by N treatment, although only in 2016 (Table 2). In this year, the addition of N caused a progressive S reduction of -8.5 % and -16.41 % with the Medium and High N fertilization treatments, respectively, compared to control (Table 2).

Considering the leaf concentration of micronutrients (Table 3), only Mn and B consistently differed between sites in both growing seasons. Mn was more elevated at Cansiglio than

Collelongo, while the opposite was true for B. Differences between sites in Fe, Zn, and Cu concentrations were only observed in either one or the other of the two campaigns (Table 3). An effect of fertilization was only observed in 2016, when foliar Fe and Zn concentrations at both sites showed a significant and progressive decline with increasing N doses, which did not persist, however, in 2018. Finally, a significant increase in Mn concentration at the Cansiglio site was observed in 2018.

The N treatments investigated had a significant effect on foliar N:P and N:S ratio in both years of sampling (Fig. 1). During 2016, at both sites, N addition led to a general increase of the leaf N:P ratio of +6.0 and +8.7 % for Medium and High N treatments, respectively (Fig. 1A, B). In 2018, on the contrary, the response to fertilization differed between sites, with a marked increase in Cansiglio (+14.3% and +16.5% for Medium and High N treatments, respectively) but rather stable N:P values in Collelongo (Fig. 1B, C). In both years, N:P ratios were consistently higher in Collelongo than Cansiglio.

The N:S ratio was also influenced in both sites and study years by N fertilization. While a similar increase was observed at both sites in 2016, in 2018, N addition resulted in a 14% increase in High N plots at the Cansiglio site while the increase at Colleongo was not statistically significant (Fig. 1B and Fig. 1C). N treatments did not significantly affect the N to Ca, K, and Mg ratios in either site or year (Table S2), except for N:Fe ratio, which increased significantly in treated plots during the first year of sampling (p=0.006), primarily due to a significant decrease in foliar Fe concentration (p=0.005, Table 3).

In order to better understand the functional significance of the observed changes in leaf nutrients, these have been complemented by paired measurements of leaf pigment concentration. The content of photosynthetic pigments in beech leaves was generally higher in 2018 than in 2016 (Fig. 2 A, B). In 2016, Chl (*a*+*b*) was influenced neither by site nor by N application or their interaction (Fig.2 E). This was largely due to a relative decrease of both Chl *a* and Chl *b* in response to Medium and High N fertilization levels at Cansiglio, and to a parallel increase at the Collelongo site (1-way ANOVA within site, $p \leq 0.05$; Table S4); the interaction between site and N addition, however, was not statistically significant. The Chl *a*/*b* ratio, on the contrary, was significantly affected in 2016 by both site and treatment, but not by their interaction (Table S3). In 2018, Chl (*a*+*b*) was significantly different between sites, with a higher Chl *a*/*b* ratio in Collelongo than Cansiglio (Fig. 2 B, E and Table S3). At both sites, chlorophylls tended to increase with the addition of N: at Cansiglio, Chl (*a*+*b*) progressively increased with N fertilization (1.5-fold with Medium N and 1.7-fold with High N); in Collelongo Chl (*a*+*b*) increased about 1.2-fold with both Medium and High N fertilization (1-way ANOVA within site, $p \leq 0.05$; Table S5).

As for the leaf carotenoid content, in the 2016 growing season a contrasting effect of N fertilization was observed at the two sites (Fig. 2 E), with a decrease at Cansiglio in response to both Medium and High N fertilization (~ -18%) and, on the contrary, an increase at Collelongo in response to the High N fertilization level (+52%, Fig. 2C). In 2018, on the contrary, total carotenoids were positively affected by N at both forest sites (Fig. 2 E), increasing with the Medium N dose at Collelongo (+36%) and with the High N dose at both Cansiglio (+33%) and Collelongo sites (+48%, Fig. 2 D). However, the ratio of total chlorophyll to carotenoids (Chls/Car ratio) was not affected by fertilization either in 2016 or in 2018 (Table S3).

The expected correlation between leaf N content and Chl (*a*+*b*) was weak and negative at the Cansiglio site in 2016 (r = 0.34, $p = 0.014$), while the two variables were positively related in 2018 (r = 0.65; $p < 0.001$, Fig. 3A, B). In Collelongo, on the contrary, the relationship was positive and significant in both 2016 (r = 0.64; $p < 0.001$) and 2018 (R = 0.63; $p < 0.001$, Fig. 3C, D).

The results of the canonical discriminant analysis (CDA), performed for the foliar nutrient and photosynthetic pigment concentrations separately for the two study years (2016 and 2018), are presented in Fig. 4A and 4B, respectively. The CDA finds the canonical coordinates (linear combinations of independent continuous variables, i.e., leaf concentrations of macro-, meso- and micro-nutrients, chlorophylls, and carotenoids) that best separate the plots belonging to different factor-groups (sites and N treatments). In both years, the first and second canonical variable components accounted for more than 90% of total variation

(95.5% and 95.8%, referring to 2016 and 2018, respectively). Along the primary gradient of variance (Can1), in both 2016 and 2018, there was an evident separation of data between the two sites, while N treatments within site were poorly discriminated. The first canonical coordinate, however, markedly differed between the two years (as apparent from an analysis of the blue arrows in Fig. 4). The N fertilization treatments, on the contrary, were better discriminated along the second gradient of variance (Can2), which explains, however, less than 20 % of total variation in both years. In the first study year, the second canonical coordinate determined a clear separation among N treatment groups (C, Medium, and High), with the same sequence at both sites (Fig. 4A), but explained only 16.1 % of the overall system variability. On the contrary, in 2018 the discrimination of N treatments by foliar nutritional status was not confirmed at the Collelongo site, for which all different groups of N fertilization appeared clustered together (Fig. 4B). In Cansiglio, on the contrary, the discrimination of N fertilization treatments observed in 2016 persisted in 2018, but the Medium and High N treatments clustered together and only separated from the control plots. Also, in this case, the factors contributing to the second canonical coordinate differed between 2016 and 2018.

Discussion

Last decades' reports on N deposition in forest ecosystems describe different and sometimes contrasting effects, from the marked increase in biomass production to the lack of growth stimulation, possibly also as a result of differences in simulated or natural N deposition, timing and mode of exposure, canopy structure (de Schrijver et al., 2011; Liu et al., 2020; Reay et al., 2008; Y. Zhang et al., 2017). In the present study, after four years of N treatments neither a significant increase in radial growth nor in leaf production was detected in the two mature beech stands in northern (Cansiglio) and central Italy (Collelongo) (Teglia et al., in preparation). Two Mediterranean forests of similar composition (pure old-growth beech forest stands without understory species) had been chosen for the study, so as to better compare the effects of N fertilization at two different sites. Nevertheless, apart from the different soil and climatic characteristics (Fig. S1, Table 1, and Table S1), each site presents a distinct pattern of environmental conditions, with a combination of independent parameters that differ in intensity during the year (Pierce et al., 2005; Rezaie et al., 2018). In particular, during spring 2016 Collelongo site was hit by an exceptional late frost, causing complete defoliation, which altered some tree phenological traits and non-structural carbohydrate dynamics (D'Andrea et al., 2019).

In forest ecosystems, where the primary limiting factors are light and soil nutrient availability, leaf biochemical traits such as nutrient content and photosynthetic pigments directly reflect the plant capacity of using these resources (Niinemets, 2007; Sardans and Peñuelas, 2013). In particular, leaf mineral content and stoichiometry are standardized parameters widely used in forest monitoring programs, while the content of photosynthetic pigments is less used in this context for its relatively higher determination costs, but more exploited for their spectral properties in remote sensing monitoring systems (Jonard et al., 2010; Nestola et al., 2018). In the current trend of climate change and environmental pollution, verifying the efficiency of leaf nutritional elements and photosynthetic pigments as indicators of forest nutritional status and photosynthesis rates can have widespread interest in monitoring applications at local and regional scale, respectively.

Foliar nutrient concentrations were found to be adequate in both Cansiglio and Collelongo forests, as indicated by a comparison with foliar nutritional thresholds (Mellert and Göttlein, 2012; Stefan et al., 1997). In this context, the foliar N concentration of the control plots at the Collelongo site was above the adequate range for beech leaves in both years of sampling, while at Cansiglio foliar N in control plots approached the upper reference limit. In the two years investigated, both stands showed foliar N levels typical of forest ecosystems close to N saturation despite the different N deposition backgrounds. If, on the one hand, N is likely not a limiting element for the primary production of trees in both sites (Ferretti et al., 2014), on the other hand it was observed that in the short period, additional inputs of N did not induce severe nutritional imbalances at leaf level. The differences observed between the two sites for almost all nutrients analysed could be related to differences in soil characteristics, as the

Collelongo site showed an average higher soil cation exchange capacity and base saturation than Cansiglio (Table S1). This appears to confirm that foliar stoichiometry can be a valuable tool to assess the supply of nutrients in the forest soil (Cleveland et al., 2011). Leaf P concentrations measured agree with other monitoring and manipulative studies (Braun et al., 2010; Gradowski and Thomas, 2008, 2006; Prietzel and Stetter, 2010), which observed that soil acidification due to N deposition and climate change negatively affect P availability in forest ecosystems. A low P availability, a decrease of this macronutrient in leaves, or a high N:P ratio were also found in Swiss (Braun et al., 2010), French, Belgian, and Luxemburg beech forests (Jonard et al., 2010) under conditions of high N deposition. In our study, foliar P concentrations significantly decreased with increasing doses of N applied in 2016, when the P availability for plants was more elevated, judging from the high foliar content of this nutrient even in control plots of both sites (Table 2). The depletion of foliar P with high N addition was also found in one experiment with maple trees treated with chronic N additions at the Harvard Forest (Minocha et al., 2000). In 2018, on the contrary, the foliar P concentration was lower than in 2016, regardless of the N treatments, and was not significantly influenced by the N supply. This might be explained by the *F. sylvatica* attempt to maintain a certain degree of leaf homeostasis, trying to preserve the foliar nutrient concentrations within the optimum range. This mechanism might allow the plant to maintain the leaf physiological functions, compensating for the limited availability of nutrients in the soil by re-mobilizing the stored P in the reserve organs, such as the root system (Zavišić and Polle, 2018). If this hypothesis were true, the general decrease in the P foliar concentration in European forests could be a symptom both of the decline in soil P availability and of the consequent depletion of this nutrient content in the plant reserve organs.

Foliar S concentrations during 2016 decreased significantly in the plots treated with N fertilization. Chen et al. (2018) observed that N addition had inhibitory effects on the activity of soil arylsulfatase (an enzyme involved in the mineralization of organic S) and suggested that N deposition may decelerate soil S cycling. At Collelongo, the leaf S reduction could also be explained by the physiological unbalances that occurred to the canopy after the foliar depletion and the reserve remobilization caused by an exceptional late frost (D’Andrea et al., 2021, 2019), although this would not be the case at Cansiglio.

The marked differences between the two years could also be explained by other factors, such as fruit production, which was significantly higher than in 2016 (Table S6). Elevated fruit production is thought to be a high energy-demanding process for trees, leading to a consistent depletion of individuals' stored resources due to substantial investment in sexual reproduction (Fernández-Martínez et al., 2017; Sala et al., 2012; Yasumura et al., 2006). This might have consequences on the foliar nutrient stoichiometry (Güsewell, 2004), resulting in a consistent reduction in the majority of foliar macro- and meso-nutrients (especially P, K, and Ca) and significant higher N:P ratios compared to the 2016 growing season.

The increase of N:P and N:S ratios in both sites and sampling years can be partially related to the relative increase of foliar N concentration (although not significant in any experimental conditions), and partially associated with the reduction of foliar P and S content, which showed a decreasing trend in both sampling years, albeit significantly so only for 2016. As well as N:P and N:S ratios, also the N:Fe ratio was significantly altered by N fertilization in 2016. A pot experiment on N fertilization of young beech plants (Flückiger and Braun, 1998) caused an increase in the ratio of N to all other nutrients; it should be mentioned, however, that a considerable amount of N (200 kg ha^{-1} yr^{-1}) was applied, which hardly reflects the actual effect of N deposition in forest ecosystems, contrary to the aim of our research. In our case, the observed leaf N:P and N:S variations could indicate that the beech nutrient demand can be almost totally supplied by the soil nutrient pool available at both experimental sites and that N deposition mainly influences the S and P availability (Berger et al., 2016). For what concerns the N:Fe ratio, a similar response was also found by Liu et al. (2021) in grassland ecosystems after three years of chronic N additions. The addition of N could reduce soil pH, which involves an enhanced Fe and Mn mobility (Haynes and Swift, 1985; Thomson et al., 1993). Despite this, we found a significant decrease in foliar Fe in 2016, while we detected increases in Mn concentration in 2018 in the Cansiglio site. Increased foliar Mn concentrations were also found by Flückiger and Braun (1998) in trees subjected to high N deposition. The concentration of the other foliar micronutrients did not vary in function of N treatments, except for Zn, which showed a significant reduction in treated plots only in 2016. The micronutrients Mn, Fe, Zn, and Cu, are redox-active metals involved in photosynthetic processes, and Fe is also fundamental in chlorophyll biosynthesis (Schmidt et al., 2020; Shahid et al., 2016). In any case, Fe concentration was more consistent in the beech leaves of Collelongo than Cansiglio, reflecting the relatively higher content of Chl a and Chl b at this site. On the contrary, most of the other microelements seem to be more available in Cansiglio, probably due to the higher soil acidification (Table S1).

An increase of leaf chlorophyll content (and photosynthetic rates) is usually associated with high N availability. However, this appears to strictly depend on forest type and physiological status (species, age, and nutrient condition) and climatic conditions (Liu et al., 2019; Tavarini et al., 2016; Yan et al., 2013). In our study, leaf N content was higher in the Collelongo beech forest than in Cansiglio, independently of N addition; the same was true for Chl (*a*+*b*), especially in the 2018 growing season. These results are consistent with the positive and significant correlation found between foliar N concentration and Chl (*a*+*b*), except at the Cansiglio site in the first year of sampling (Fig. 3A). The late frost event of 2016, which induced the remobilization of C reserves and a re-growth of the canopy after defoliation at Collelongo (D'Andrea et al., 2021), probably impaired the N assimilation and allocation in the photosynthetic apparatus (chlorophylls and proteins) and other metabolic pathways. Nevertheless, a similar unchanged or reduced value of Chl (*a*+*b*) was observed in other experiments of N deposition (Arróniz-Crespo et al., 2008; M. Li et al., 2018). The relatively slow response of the beech forest at Cansiglio site to three years-N fertilization with regard to the leaf concentration of chlorophylls might be related to the different climatic conditions and plant stand characteristics (density, age, canopy architecture, management, etc.) of the two sites (R. Li et al., 2018; Phoenix et al., 2012; R. Zhang et al., 2017). However, if in some cases, total leaf chlorophylls content cannot be an appropriate indicator of N deposition effects, further measurements of photosynthetic pigment compositions such as Chl *a*/*b* and carotenoids are suggested to reveal the impacts of N addition on photosynthesis-related parameters and plant growth performances (e.g., Arróniz-Crespo et al., 2008; M. Li et al., 2018; Ochoa-Hueso et al., 2014; Zhang et al., 2016). In both beech forests, Chl *a*/*b* was reduced by N fertilization in the 2016 growing season, due to a decrease of Chl *a* at Cansiglio and a relatively greater increase of Chl *b* than Chl *a* at the Collelongo site. Similarly, in *Leymus chinensis* Zhang et al. (2016) found a significant rise in Chl *b* due to N addition, while M. Li et al. (2018) revealed constant or enhanced values of both Chl *a* and Chl *b* in *Cupressus lusitanica* after different N treatments. In both beech forest sites, the leaves sampled are typical sun leaves developed in full light with a relatively elevated Chl *a*/*b* ratio (> 2.7-2.8), mainly due to a minor investment of Chl *b* in PSII (Scartazza et al., 2016). The Chl *a*/*b* ratio is a valuable indicator of N partitioning within a leaf because it is positively correlated with the ratio of PSII cores to light harvesting chlorophyll-protein complex (LHCII) (Terashima and Hikosaka, 1995). It has been predicted that the ratio of PSII to LHCII (and the Chl *a*/*b* ratio) should increase with the decreasing of N availability (Kitajima and Hogan, 2003), while it has not been demonstrated the contrary, i.e. that an increase in N availability for plants should be associated with a decrease of leaf Chl *a*/*b* ratio, as in our case. However, N plays important roles in photosynthesis and the production of light-harvesting pigments and diphosphoribulose carboxylase (Wu et al., 2008), and variations in leaf Chl *a*/*b* are usually related to morphological and physiological differences in plants and to plant response to alterations involving re-arrangements and distribution of photosynthetic pigments between the two photosystems (Maina and Wang, 2015; Y. Li et al., 2018). This could explain the constitutive differences found in leaf Chl *a* and Chl *b* concentrations found at Cansiglio and Collelongo, as well as the modification of Chl *a*/*b* and carotenoids during the 2016 and 2018 growing season. Moreover, the relative increases of carotenoids observed at the Collelongo site with both N doses during 2016 and 2018 and at Cansiglio with the High N treatment in 2018 (Fig. 2 C, D) can be due to an up-regulation of the synthesis of these pigments to protect the photosynthetic apparatus to possible photo-oxidative damages caused by the effects of N depositions, as found in similar experimentations (M. Li et al., 2018; Zhang et al., 2016).

The multivariate analysis helped identify the most representative factors influencing this complex forest system, highlighting the apparent differences between the two forest stands. The site of Cansiglio showed an overall higher response to further N inputs than the site of Collelongo in both years of sampling. It is known that the effects of N addition differ depending on abiotic factors, such as soil pH and exchangeable base cations (Mao et al., 2020). The higher soil pH and cation exchange capacity in Collelongo might represent a better-buffered system against acidification (Bowman et al., 2008) than in Cansiglio. These soil properties may explain why we found fewer imbalances in foliar nutrient stoichiometry for the central site.

In accordance with the result of the CDA, imbalances in nutrient stoichiometry due to N fertilization were greater during the 2016 growing season (Fig. 4 A), when both sites had relatively low fruit production, and Collelongo was interested by a severe late frost event (D’Andrea et al., 2019; 2021). N additions determined a detectable enrichment of N foliar concentration in the Northern site, combined with an overall depletion of all macro and meso-nutrients, except for Ca in 2018 (Fig 4 A-B). In contrast, in the Southernmost site, treatments did not significantly affect foliar nutrient concentrations in the last sampling year (Fig. 4 B). Hence, the interaction with the late spring freezing event may have emphasized the effect of N fertilization on foliar nutrient concentrations at the site, which we did not observe during the 2018 growing season. Although many studies had already demonstrated the consistent reduction of beech forest productivity during years interested by frosts events (e.g. D’Andrea et al., 2020; Gazol et al., 2019; Vitasse et al., 2019), much less is known about the impact on canopy biochemistry and morphology. Even if the Collelongo forest had to mobilize C reserves to produce a second leaf flush after the severe frost foliar damage (D’Andrea et al., 2021), the concentration of nutrients did not drop below deficiency thresholds for optimal physiological functions (Mellert and Göttlein, 2012; Stefan et al., 1997). Nevertheless, we observed a consistent reduction of P, S, Ca, and K foliar concentration in High N fertilized plots compared to control plots, which we did not find during the 2018 growing season. St. Clair et al. (2009) observed a similar reduction in foliar nutrient concentrations after a defoliation event in Aspen trees, although the study did not consider interaction with N depositions. As other authors suggested, excess of N could directly affect tree sensitivity to early frost episodes in spring (Jönsson et al., 2001). Hence, an increased frequency of climate extreme events could lead to higher depletion of foliar nutrient resources in presence of elevated N deposition, potentially undermining the health and resilience of the forest ecosystem.

Photosynthetic pigment concentrations also varied more remarkably in 2016 than in the 2018 growing season, with a reduction in the relative content of Chl *a* to Chl *b* in both sites in the first year, in addition to changes in carotenoids, which persist in 2018. This was also demonstrated by the different directions of Chl *b* in respect to the other pigment components in 2016 and of carotenoids to total Chls ratio in 2018 (Fig. 4). Photosynthetic pigments are early sensitive indicators of plant response to environmental stresses (Y. Li et al., 2018; Zhang et al., 2016), so changes in their synthesis and composition might reflect the level of plant acclimation to the increased N deposition. This was more evident at both sites in 2016, when the total chlorophyll and carotenoid amounts and the relative content of Chl *a* and *b* (Chl *a/b*) were affected.

A last consideration should regard the potential limitations of the experimental setup applied in the study, in particular the application of N fertilizer directly to the soil which bypasses the relevant natural process of canopy N uptake. Indeed, estimates suggest that tree canopies can retain a significant percentage of incoming atmospheric N to forests. Substantial amounts of N can be obtainable by the plant from leaf or branch surfaces, which could supplement nutrition from the soil (Nair et al., 2016). Canopy N fertilization is a direct addition of N to plant metabolism, and could potentially affect tree growth and physiological processes such as photosynthesis more readily than direct soil application (Sparks, 2009). As a result, the response of beech forests to natural N deposition could have been underestimated.

Conclusions

We found that chronic N addition differently affected leaf biochemical and stoichiometric composition of two monospecific beech forests, developed in contrasting climatic regions of Italy. Leaf nutrients and photosynthetic pigments have proved to be good indicators of beech acclimation to environmental and changes, pigments showing an earlier response to N deposition, while nutrients a later and more stable one possibly due to the complex relations of soil availability and plant biomass partitioning. The modifications observed in nutrient and photosynthetic pigment composition accentuated and reflected the natural differences between the Northern (Cansiglio) and Central (Collelongo) sites, being the former closer to N saturation conditions than the latter.

Despite the potential limitations of the study, which relied on the traditional spreading of fertilizer to the soil and could mis-represent the effects of N deposition onto the canopy, our results provide a useful insight into the future effects of ongoing N deposition on beech in Italy. We suggest that eutrophic beech forests, developed on nutrient-rich soils for several years, should not manifest, at least over the short to medium period, critical nutritional and physiological impairments in response to elevated N input, but only modifications indicative of a developing stress response. Seasonal extremes and high fruit production in 2016 may also have influenced results.

Further investigations over an extended period are needed to understand the dynamics and limits of the observed variations in leaf stoichiometry and pigment composition, and their interactions with climate variability and extremes at the two contrasting sites.

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Figure captions

Fig. 1. Leaf N:P and N:S ratios in beech forests at two experimental sites (Cansiglio, white bars; Collelongo, grey bars) subjected to N addition treatments (Control; Medium, 30 N kg ha^{-1} y^{-1}; High, 60 kg N ha^{-1} y^{-1}) in 2016 and 2018 (panels A and B). Mean ± standard error (*n* = 3). Horizontal lines refer to minimum (dotted) and maximum (dashed line) critical values for the species, according to the normal range for nutrient concentrations suggested by Mellert and Göttlein (2012) for N:P ratio and by Stefan et al. (1997) for N:S ratio. Results of two-way ANOVA analysis (factors: site and N treatment, N) are given in panel C. Significant ($p \leq 0.05$) effects of site, N and their interaction are in bold. The columns identified by different letters are significantly different (differences between treatments or sites, simultaneously; SNK test).

Fig. 2. Total leaf chlorophyll (Chl *a*+*b*) and carotenoid concentrations in *F. sylvatica* trees subjected to N addition treatments (Control; Medium, 30 N kg ha^{-1} y^{-1}; High, 60 kg N ha^{-1} y^{-1}) at the Cansiglio (white bars) and Collelongo (grey bars) sites for 2016 and 2018 sampling years (panels A to D). Mean ± standard error (*n* = 3). Statistics as in Fig. 1. Results of the two-way ANOVA analysis (factors: sites and N treatment, N) are given in panel E.

Fig. 3. Correlation between leaf N concentration (N) and total chlorophyll concentration (Chl *a*+*b*) in Cansiglio and Collelongo forests in 2016 and 2018. Points correspond to measurements on individual plants (*n*=3-9) from each experimental plot (Control, grey dots; Medium, dark grey squares; High, black triangle during the two sampling campaigns. The Pearson’s coefficient and significance level (p) of linear regressions are also shown.

Fig. 4. Principal component analysis showing the difference in nutritional status of *F. sylvatica* trees at the two sites (Cansiglio and Collelongo) and exposed to different N fertilization treatments (Control, Medium, High), along the first two canonical variate axes during the first (2016, A), and the second year (2018, B) of sampling.

Graphical abstract

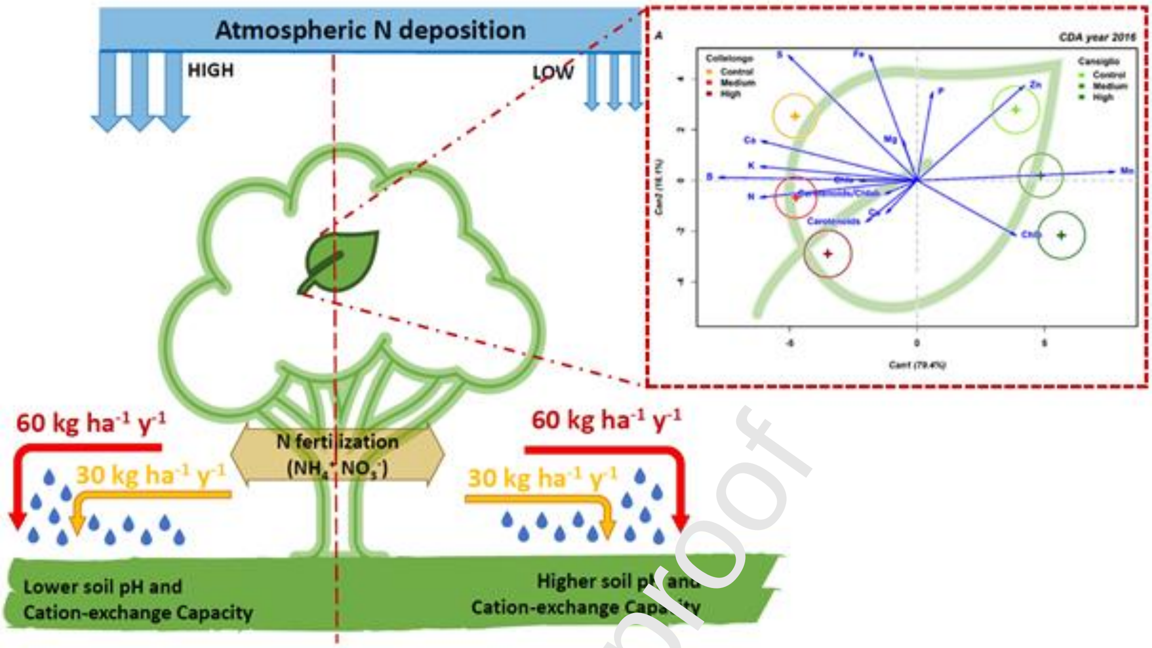
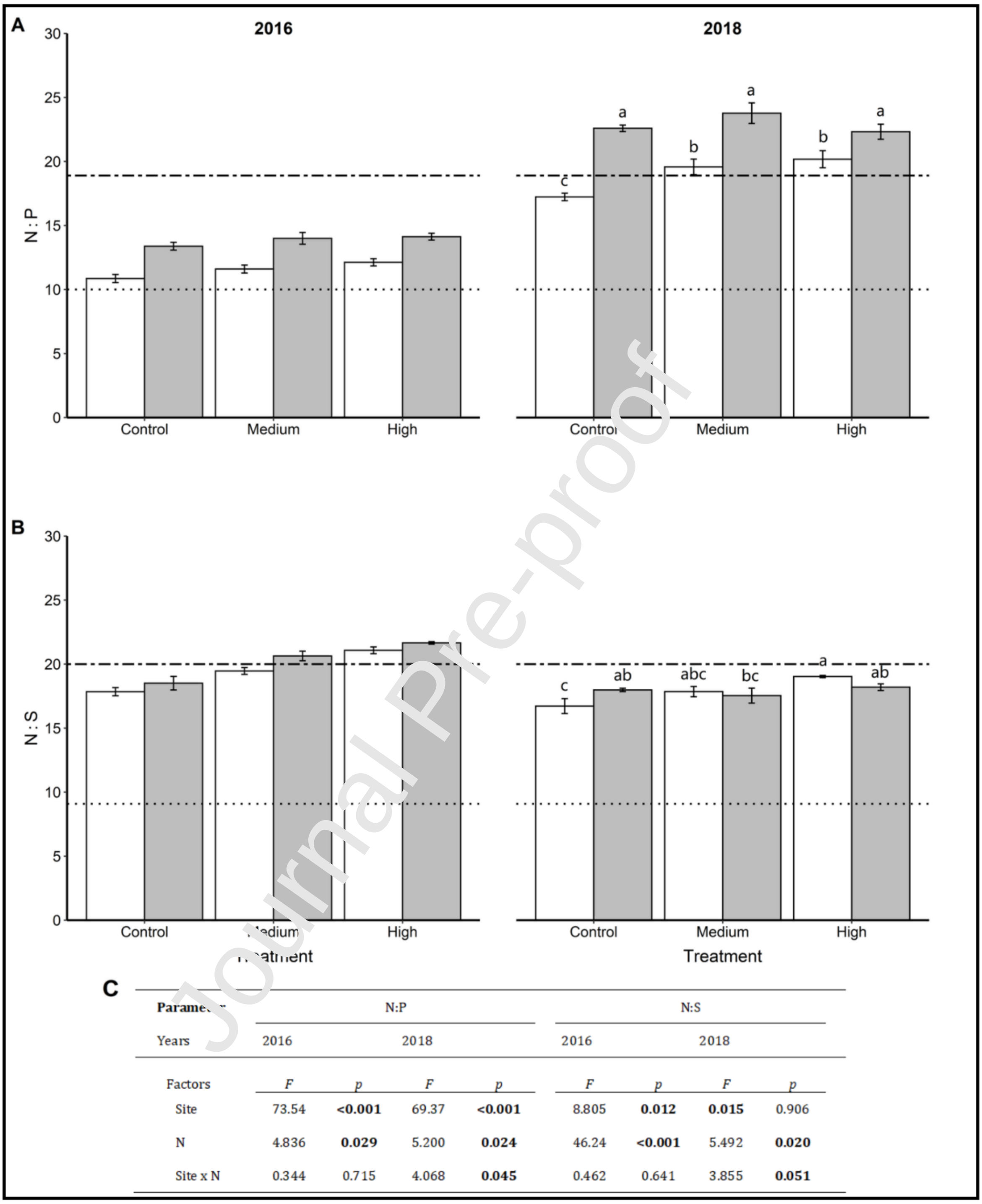
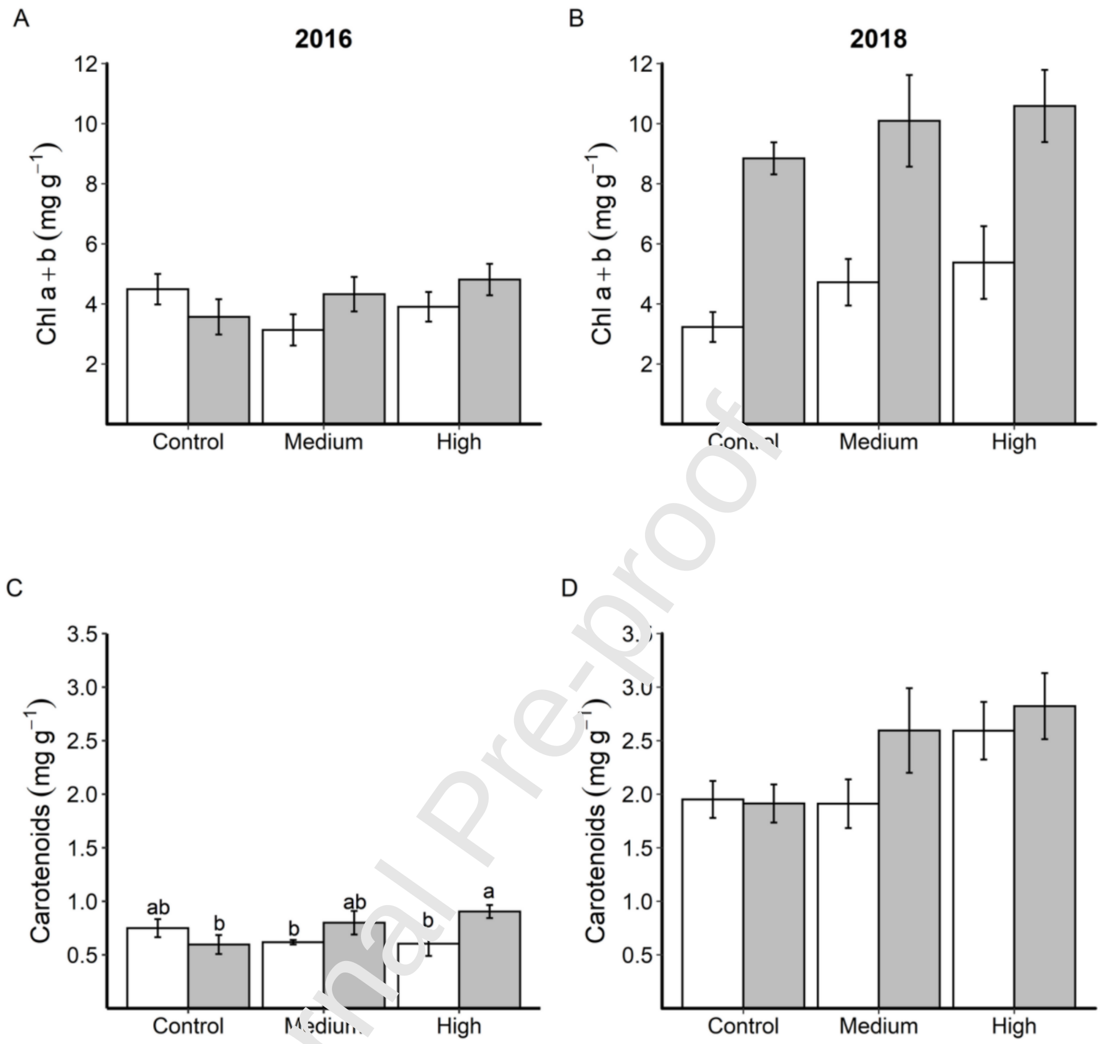


Fig. 1



Parameter	N:P				N:S			
Years	2016		2018		2016		2018	
Factors	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Site	73.54	**<0.001**	69.37	**<0.001**	8.805	**0.012**	**0.015**	0.906
N	4.836	**0.029**	5.200	**0.024**	46.24	**<0.001**	5.492	**0.020**
Site x N	0.344	0.715	4.068	**0.045**	0.462	0.641	3.855	**0.051**

Fig. 2



E

Photosynthetic pigments (mg g^{-1})	Chl (a+b)				Carotenoids			
Year	2016		2018		2016		2018	
Factors	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Site	0.688	0.415	41.92	**<0.001**	2.443	0.132	1.741	0.194
N	0.588	0.564	1.982	0.151	0.492	0.618	4.243	**0.021**
Site x N	1.982	0.162	0.020	0.98	3.99	**0.032**	0.927	0.404

Fig. 3

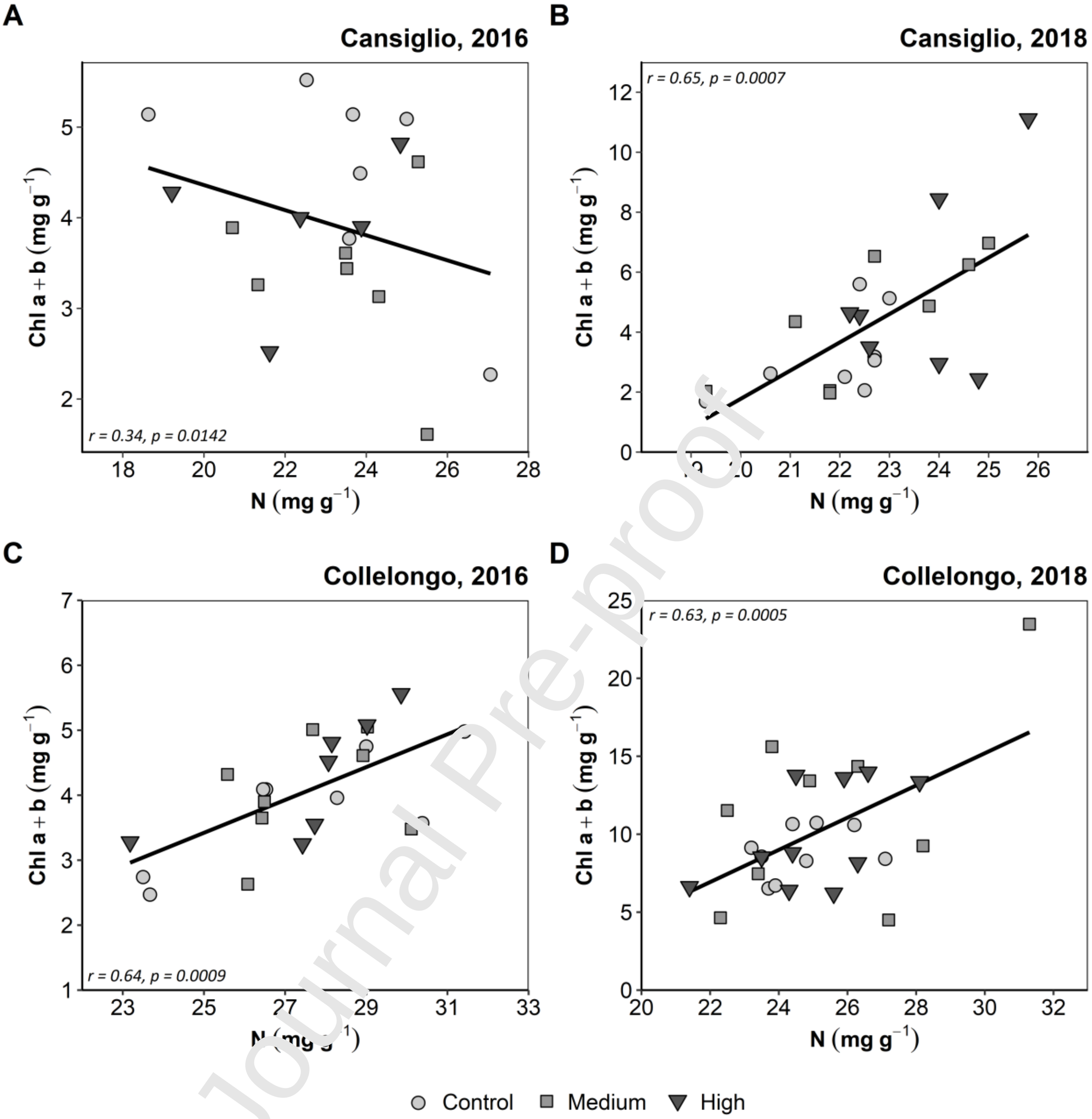

A
Cansiglio, 2016
Chl a + b (mg g^{-1})
5
4
3
2
r = 0.34, p = 0.0142
18
20
22
24
26
28
N (mg g^{-1})
B
Cansiglio, 2018
r = 0.65, p = 0.0007
12
10
8
6
4
2
0
Chl a + b (mg g^{-1})
20
21
22
23
24
25
26
N (mg g^{-1})
C
Collelongo, 2016
7
6
5
4
3
2
1
Chl a + b (mg g^{-1})
r = 0.64, p = 0.0009
23
25
27
29
31
33
N (mg g^{-1})
D
Collelongo, 2018
r = 0.63, p = 0.0005
25
20
15
10
5
0
Chl a + b (mg g^{-1})
20
22
24
26
28
30
32
N (mg g^{-1})
○ Control
■ Medium
▼ High

Fig. 4

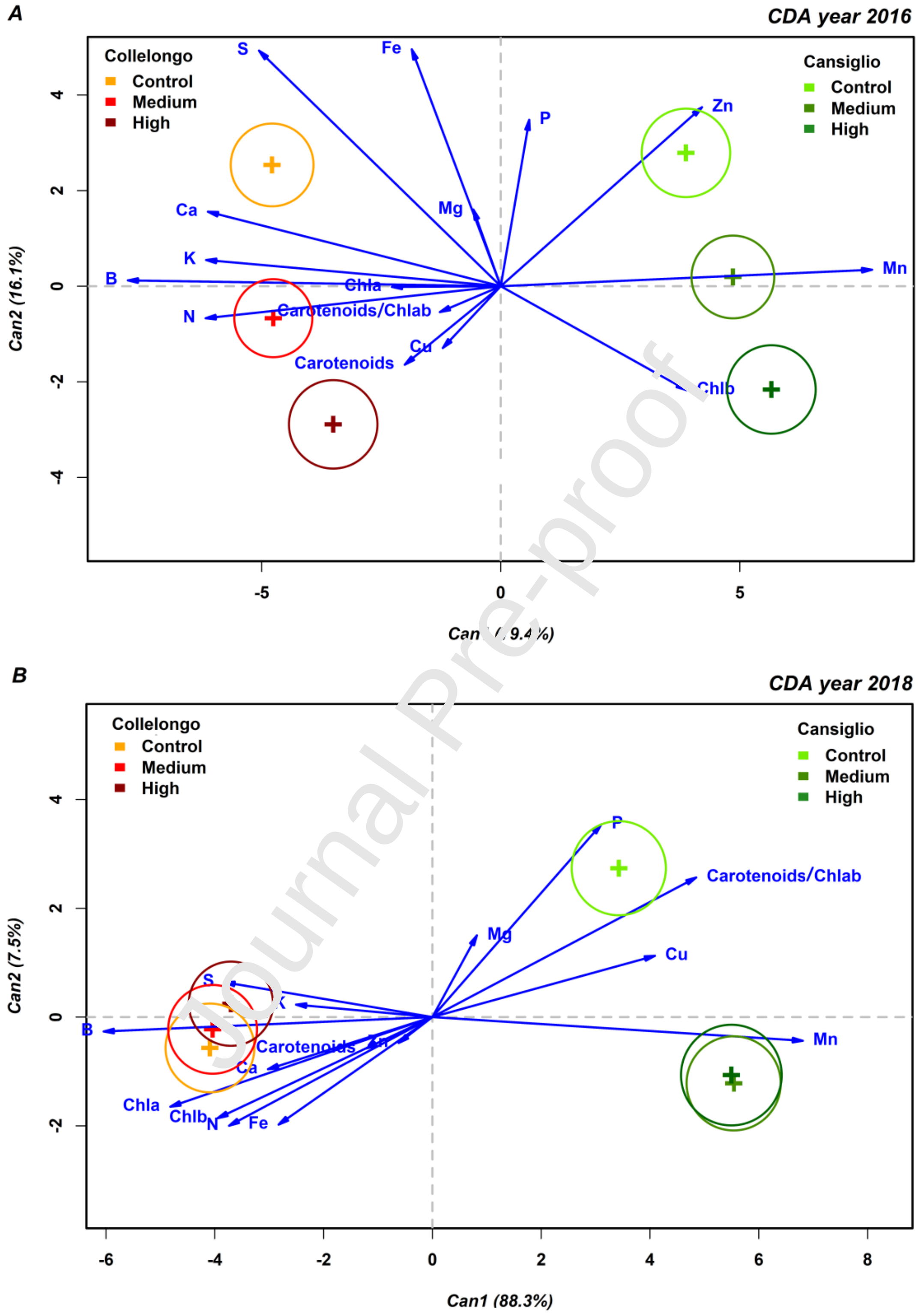

A
CDA year 2016
Collelongo
🟧 Control
🟥 Medium
🟥 High
Cansiglio
🟩 Control
🟩 Medium
🟩 High
S
Fe
P
Zn
Mg
Ca
K
B
Chla
Mn
N
Carotenoids/Chlab
Cu
Carotenoids
Chlb
4
2
0
-2
-4
-5
0
5
Can2 (16.1%)
Can1 (79.4%)
B
CDA year 2018
Collelongo
🟧 Control
🟥 Medium
🟥 High
Cansiglio
🟩 Control
🟩 Medium
🟩 High
P
Carotenoids/Chlab
Mg
Cu
S
K
B
Mn
Carotenoids
Ca
Chla
Chlb
N
Fe
4
2
0
-2
-6
-4
-2
0
2
4
6
8
Can2 (7.5%)
Can1 (88.3%)

Table 1. General description of Cansiglio and Collelongo experimental sites. Stand structural data from July 2015; N deposition data are an average for the period 1998-2017; climatic data are an average for the period 1981-2010. Total N soil con refers to mineral horizon A.

	Unit	Cansiglio	Collelongo
Latitude		46° 03’ 29’’ N	41° 50’ 58’’ N
Longitude		12° 22’ 45’’ E	13° 35’ 17’’ E
Elevation	m a.s.l.	1100	1560
Management		High forest	Coppice converted to high forest since 1950
Tree species		*Fagus sylvatica* L.	*Fagus sylvatica* L.
Stand age	years	130-140	105-125
Stand density	trees ha^{-1}	168	740
Mean tree DBH	cm	45	27
Mean tree height	m	29	21
FAO soil type		Haplic Luvisol	Humic Alisol
Annual precipitation	mm y^{-1}	1767	877
Mean annual temperature	°C	7.2	7.4
Mean annual total N deposition (open field) *	kg N ha^{-1} y^{-1}	17.7	5.99
Mean annual total N deposition (throughfall) *	kg N ha^{-1} y^{-1}	12.6	5.58
Total soil N concentration	%	0.34	0.67
Soil field capacity	% volume	21	14
Soil net N mineralization**	kg N ha^{-1} y^{-1}	95	120
Soil pH (at 20 cm depth)		4.3	5.6

* Cecchini et al., 2021. **In 2015, at the beginning of the experimental study.

Table 2. Macro- (N, P, K) and meso-(S, Mg, Ca) nutrients concentration in beech leaves collected at the two experimental sites in 2016 and 2018. Mean ± standard error (*n* = 3). For each parameter, the results of 2-way ANOVA analysis (factors: Site and N treatment) are also presented for each sampling year (2016 and 2018). Values of significant level (p) and F are reported; significant differences (p ≤ 0.05) are indicated in bold. Fertilization treatments correspond to 0 (Control), 30 (Medium) and 60 kg N ha^{-1} y^{-1} (High).

Macronutrients (mg g^{-1})	N		P		K	
Year	2016	2018	2016	2018	2016	2018
CANSIGLIO						
Control	23.5 (±0.98)	22.0 (±0.61)	2.17 (±0.06)	1.28 (±0.03)	6.05 (±0.21)	5.21 (±0.40)
Medium	23.6 (±1.04)	22.7 (±1.08)	2.04 (±0.01)b	1.16 (±0.05)	5.76 (±0.25)	5.00 (±0.30)
High	22.8 (±0.62)	23.6 (±0.35)	1.91 (±0.05)	1.17 (±0.06)	5.51 (±0.29)	4.90 (±0.26)
COLLELONGO						
Control	27.1 (±0.84)	24.6 (±0.73)	2.02 (±0.06)	1.09 (±0.02)	7.19 (±0.26)	5.53 (±0.48)
Medium	27.5 (±0.59)	25.6 (±1.39)	1.97 (±0.02)	1.08 (±0.05)	7.75 (±0.09)	5.66 (±0.40)
High	27.0 (±0.38)	25.0 (±0.58)	1.91 (±0.05)	1.12 (±0.04)	6.60 (±0.37)	5.78 (±0.51)

	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Site	37.41	**<0.001**	10.59	**0.007**	1.321	0.273	9.390	**0.010**	44.01	**<0.001**	3.598	0.082
N	0.393	0.683	0.729	0.502	4.647	**0.032**	1.145	0.351	4.070	**0.045**	0.006	0.994
Site x N	0.102	0.904	0.439	0.655	0.933	0.420	1.318	0.304	1.888	0.194	0.261	0.775

Mesonutrients (mg g^{-1})	Ca		Mg		S	
Year	2016	2018	2016	2018	2016	2018
CANSIGLIO						
Control	9.70 (±0.27)	8.12 (±0.02)	1.75 (±0.12)	1.61 (±0.03)	1.32 (±0.06)	1.32 (±0.02)
Medium	9.03 (±0.27)	7.93 (±0.42)	1.46 (±0.06)	1.39 (±0.04)	1.21 (±0.04)	1.27 (±0.03)
High	8.16 (±0.16)	8.71 (±1.00)	1.35 (0.10)	1.53 (±0.10)	1.08 (±0.02)	1.24 (±0.02)
COLLELONGO						
Control	14.25 (±0.58)	9.55 (±0.48)	1.69 (±0.18)	1.45 (±0.04)	1.46 (±0.04)	1.37 (±0.05)
Medium	13.70 (±1.93)	10.1 (±0.42)	1.35 (±0.07)	1.42 (±0.15)	1.33 (±0.03)	1.46 (±0.08)
High	12.22 (±1.00)	9.11 (±1.05)	1.53 (±0.29)	1.42 (±0.14)	1.25 (±0.02)	1.37 (±0.05)

	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Site	33.746	**<0.001**	5.658	**0.035**	0.002	0.967	1.068	0.322	19.373	**<0.001**	10.570	**0.007**
N	1.887	0.194	0.019	0.981	2.351	0.138	0.804	0.470	16.395	**<0.001**	0.777	0.482
Site x N	0.060	0.942	0.870	0.444	0.472	0.635	0.465	0.639	0.171	0.845	1.054	0.379

Table 3. Micro-nutrients (Mn, Fe, Zn, Cu and B) concentrations in beech leaves as a function of N fertilization at the two experimental sites (Collelongo and Cansiglio) in 2016 and 2018. Mean ± standard error (*n* = 3). For each parameter, the results of a 2-way ANOVA analysis (factors: Site and N treatment) are also presented for each sampling year (2016 and 2018); significant differences (p ≤ 0.05) are indicated in bold. Abbreviation as indicated in Table 2.

Micronutrients (μg g^{-1})	Mn				Fe				Zn				Cu				B			
Year	2016		2018		2016		2018		2016		2018		2016		2018		2016		2018	
CANSIGLIO																				
Control	0.85 (±0.05)		0.74 (±0.07)		109.7(±13.8)		80.7 (±3.17)		24.2 (±1.26)		24.7 (±0.53)		9.62 (±0.76)		8.71 (±0.36)		34.3 (±0.85)		11.4 (±0.36)	
Medium	1.01 (±0.09)		1.09 (±0.12)		95.4 (±3.39)		86.4 (±4.23)		23.5 (±0.40)		25.9 (±0.49)		9.93 (±0.66)		8.28 (±0.35)		30.3 (±0.39)		10.2 (±0.03)	
High	0.98 (±0.12)		0.95 (±0.08)		89.1 (±3.23)		85.7 (±2.93)		20.1 (±0.83)		24.8 (±2.37)		11.2 (±0.18)		8.54 (±0.37)		28.8 (±0.21)		9.96 (± 0.52)	
COLLELONGO																				
Control	0.85 (±0.06)		0.17 (±0.04)		118.6 (±8.5)		101.1 (±5.6)		18.7 (±0.85)		25.8 (±1.73)		10.7 (±0.35)		7.42 (±0.48)		49.8 (±0.73)		18.5 (±1.76)	
Medium	0.20 (±0.01)		0.15 (±0.01)		97.6 (±2.25)		93.2 (±7.75)		16.9 (±2.65)		25.9 (±3.73)		10.5 (±0.33)		7.13 (±0.47)		48.3 (±3.80)		19.8 (±1.38)	
High	0.33 (±0.04)		0.23 (±0.02)		82.3 (±2.93)		91.9 (±4.03)		14.0 (±1.41)		26.8 (±2.85)		10.4 (±1.46)		7.05 (±0.21)		48.7 (±1.09)		19.5 (±0.83)	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Site	139.0	**<0.001**	164.2	**<0.001**	0.06	0.808	7.67	**0.017**	27.31	**<0.001**	0.31	0.586	0.20	0.661	17.46	**0.001**	166.87	**<0.001**	111.89	**<0.001**
N	0.76	0.487	2.47	0.126	8.28	**0.005**	0.09	0.913	5.15	**0.024**	0.05	0.956	0.41	0.671	0.47	0.636	2.25	0.149	0.05	0.951
Site x N	1.47	0.269	2.32	0.141	0.63	0.549	1.32	0.303	0.07	0.930	0.09	0.915	0.87	0.442	0.10	0.905	0.87	0.445	1.02	0.390

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Declaration of competing interest

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