

## RESEARCH ARTICLE

# Has COVID-19 Lockdown Affected C and N Level and Isotope Composition in Urban Soils and Plant Leaves?

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This study aimed to evaluate how seasonal variations in environmental conditions and atmospheric NO<sub>2</sub> impact C and N cycle in an urban environment by determining their elemental concentration and isotopic composition ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ ) at spatial scale (urban and peri-urban sites) and species level (evergreen plants). Leaves and soil across the medium-sized city of Pisa were collected over 1 year including COVID-19 lockdown, taking advantage of the unprecedented containment measures causing a substantial NO<sub>2</sub> drop. The enrichment in heavier isotopes of organic matter in urban soil was most likely due to the long-term contribution of both  $\delta^{15}\text{N}$ -enriched depositions and greater C and N cycling rates in comparison with peri-urban soil. Leaf  $\delta^{15}\text{N}$  represented a valuable proxy of the urbanization degree depending on microclimate and N inputs from atmospheric NO<sub>2</sub>. Leaf  $\delta^{13}\text{C}$  showed a seasonal trend linked to plant functional types, with significant differences between sites and species. Differently from *Nerium oleander* and *Pittosporum tobira*, *Quercus ilex* showed a positive correlation between  $\delta^{13}\text{C}$  and NO<sub>2</sub>, highlighting the positive effect of N deposition on its intrinsic water-use efficiency. Moreover, the lockdown-induced NO<sub>2</sub> reduction was reflected in a decreasing trend of leaf N concentration and change in intrinsic water-use efficiency depending on the plant species and urbanization degree. *Q. ilex* showed the best adaptability to the more NO<sub>2</sub>-polluted site, being able to uptake and immobilize high amounts of  $^{15}\text{N}$ -enriched atmospheric depositions into its leaves without toxic effects. Overall, these results must be considered in urban greening programs to improve air quality in NO<sub>2</sub>-polluted areas.

## Introduction

Various gases and solid particles in the atmosphere have originated from natural and anthropogenic emission sources, with the latter becoming globally dominant since the beginning of industrialization [1]. The most common air pollutants consist of particulate matter (PM), nitrogen dioxide (NO<sub>2</sub>), sulfur dioxide (SO<sub>2</sub>), carbon monoxide (CO), and ozone (O<sub>3</sub>) [1]. Besides industry, road transport is one of the main sources of air pollution in urban environments, where CO, SO<sub>2</sub>, and NO<sub>2</sub> are directly released during the combustion of fossil fuels. Such pollutants have rapidly increased in many cities with accelerating urbanization and industrialization, leading to a substantial impact on ecosystems and human health [1,2].

In the last decades, the application of strict legislations and the implementation of new technologies have contributed to a global decline of major anthropogenic air pollutant levels such as CO and SO<sub>2</sub>, whereas atmospheric concentration of NO<sub>2</sub> has remained constant [2]. In fact, a recent data from the World

Health Organization showed that 77% of people in 74 countries still breathe unhealthy levels of NO<sub>2</sub> without differences between high- and low-/middle-income countries [3]. Besides, increasing anthropogenic emissions of reactive N such as NO<sub>2</sub> into the environment can impact the C and N cycle, thus altering the biodiversity and functioning of ecosystems and ecological processes [4,5]. Therefore, actions such as nature-based solutions to reduce NO<sub>2</sub>-induced pollution are crucial for safeguarding ecosystem function.

In plants, C and N are essential nutrient sources for diverse cellular functions, and their acquisition and metabolism are closely related. This results in an intricate regulatory machinery that controls C and N interaction ensuring an optimal growth and development [6–8]. However, as plants are in continuous interaction with the atmosphere–soil system, any disturbance such as urbanization, anthropogenic emissions, and climatic conditions can alter the interaction between C and N. Moreover, plants' response also depends on their metabolic status, developmental stage, cell type, and species [6–8], thus highlighting

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the complex process behind the capacity of plant species to adapt and acclimate against adverse conditions. For instance, studies related to urban plant physiology demonstrated that the status of key environmental services can be affected by  $\text{NO}_2$ -polluted sites independent of the city scale [9] and dependent on the species [10,11]. Therefore, choosing the right species, adapted to a specific region, based on its  $\text{NO}_2$ -removal capacity [12,13] might maximize air quality in an urban environment.

The use of stable isotopes of C and N has considerably increased to study environmental processes and to trace pollution sources [12,14]. Moreover, leaf stable isotope composition (i.e.,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) has been used to evaluate leaf-level physiological responses of urban forest patch trees compared with trees growing at reference forest sites [15].  $\delta^{15}\text{N}$  represents a valuable indicator of road transport-produced nitric oxide levels and land use changes in an urban environment [12]. However, seasonal studies integrated over time are needed to understand the factors influencing the C and N cycle. Therefore, we assessed the alteration of C and N inputs and processes in the urban ecosystem by the analysis of C and N elemental and isotopic composition in leaves and soils across the city of Pisa over 1 year including COVID-19 lockdown. This period allowed the study to take place during a complete and strict lockdown when unprecedented containment measures on transportation, non-essential business, and citizen mobility were applied, thus resulting in a substantial drop of atmospheric  $\text{NO}_2$  [16]. The main aim was to evaluate how the lockdown-induced atmospheric  $\text{NO}_2$  reduction impacts the C and N cycle in different environments. This was possible by determining the patterns of elemental and isotopic composition at spatial scale (urban and peri-urban areas), at species level (*Quercus ilex*, *Nerium oleander*, and *Pittosporum tobira*), and over time (as a function of seasons).

## Materials and Methods

### Site and species description

Two study sites were selected across Pisa municipality (Italy) named “Borghetto” (43°42′46.8″N, 10°24′34.6″E) and “Passi” (43°43′35.7″N, 10°23′58.7″E). Detailed description and selection of the sites, their soils, and plant species are reported by Huaranca Reyes et al. [9]. Briefly, Borghetto is in the central urban area, while Passi is a peri-urban area next to the rural sites (Fig. S1). The soils of the 2 sites were similar in terms of the following properties: sandy loam texture, alkaline pH, and electrical conductivity lower than the limit for saline soils; also, the soil temperature did not show significant differences between the 2 sites in the studied period. Three adult individuals of each evergreen species were selected: holm oak (*Q. ilex* L.), oleander (*N. oleander* L.), and pittosporum [*P. tobira* (Thunb.) W.T. Aiton]. In both sites, *Q. ilex* were more than 40 years old, 7 m height, and 0.5 m diameter at breast height; *N. oleander* were more than 20 years old, 2 m height, and 2.5 m basal canopy diameter; and *P. tobira* were more than 20 years old, 1.75 m height, and 1 m wide. The study was performed from September 2019 (late summer) to August 2020 (midsummer) covering the Italian lockdown period due to COVID-19 applied at the national level (March 2020 to May 2020).

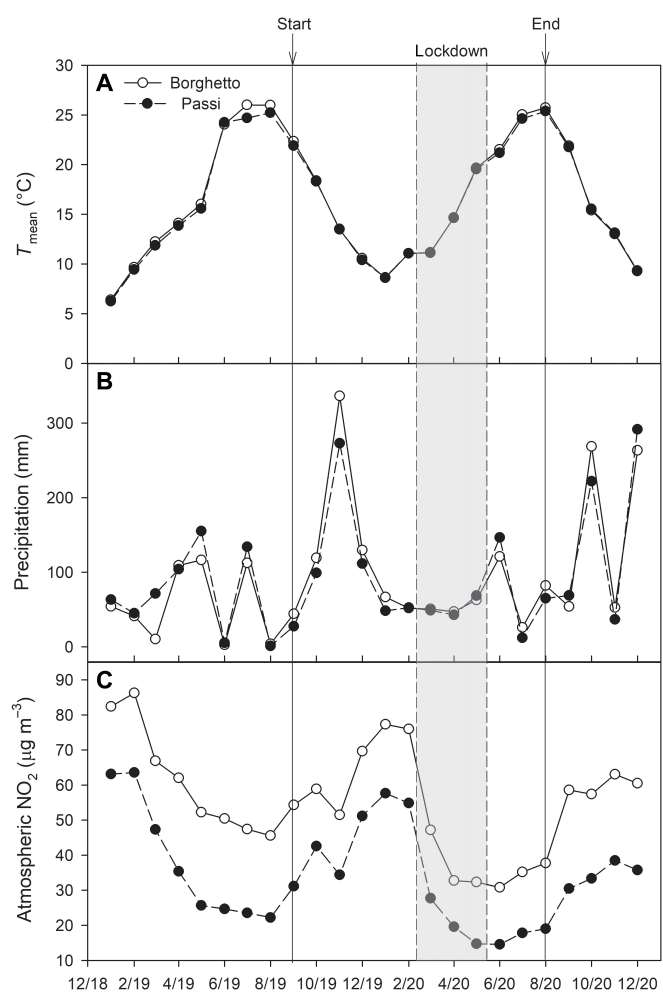
### Climate and air quality

Climate and air quality data were collected from automatic analyzers in each site. The temperature (Fig. 1A) and precipitation

(Fig. 1B) data were downloaded from “Servizio Idrologico Regionale” (<http://www.sir.toscana.it/>) and “Meteo I Passi” (<https://www.meteopassi.it/>), respectively. The data of atmospheric  $\text{NO}_2$  (Fig. 1C) were collected from “Agenzia Regionale per la Protezione dell’Ambiente della Toscana” (<http://www.arpat.toscana.it/>).

### Plant and soil sampling

In both sites, at least 10 fully expanded mature leaves from 3 individuals of each plant species were collected from the sunny-exposed canopy, pooled, and used as replicates for each sampling date. Sampling during 2019 was performed monthly from September to December, while sampling during 2020 was performed in January, April, and August. The leaves sampled faced the road (southeast oriented) and were at the same height as follows: *Q. ilex* lower canopy at 3.5 m, and *N. oleander* and *P. tobira* at 1.5 m. Soil sampling was performed in September 2019, January 2020, and April 2020. Six soil samples were collected from each site within 1 m from the sampled plants at 0 to 20 cm depth, air-dried at 20 °C, and sieved with 2-mm mesh.



**Fig. 1.** Seasonal change of the average monthly (A) mean temperature, (B) precipitation, and (C) atmospheric  $\text{NO}_2$  concentration in Borghetto and Passi. Data were collected from January 2018 to December 2020. The period of this study started in September 2019 (late summer) and ended in August 2020 (midsummer), covering the lockdown period due to COVID-19 highlighted in gray (March 2020 to May 2020).

## Carbon and nitrogen concentration and stable isotope determination

Leaf and soil samples were oven-dried at 45 °C until constant weight and ground to a fine powder with a mortar in the laboratory. Soil samples were treated with 10% hydrochloric acid to remove carbonates [17]. An aliquot of leaf and soil powder (about 1 to 2 mg) was weighed and analyzed for C and N concentration (dry mass basis, %) and isotope composition ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ ) using an elemental analyzer (Model NA 1500, Carlo Erba, Milan, Italy) coupled to an isotope ratio mass spectrometer (Isoprime Ltd., Cheadle, UK). The isotope ratios of C ( $R = {}^{13}\text{C}/{}^{12}\text{C}$ ) and N ( $R = {}^{15}\text{N}/{}^{14}\text{N}$ ) were measured to calculate  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  referring to the Vienna–Pee Dee Belemnite (VPDB) and atmospheric  $\text{N}_2$  standards, respectively, as:  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N} = R_{\text{sample}}/R_{\text{standard}} - 1$ . Anchoring was pursued on the VPDB and atmospheric  $\text{N}_2$  scales by means of IAEA standards. The precision of sample measurements was better than 0.1‰ for both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ .

## Statistical analysis

After the confirmation of homogeneity of variance (Bartlett's test), data were subjected to analysis of variance (ANOVA), with a 0.05 probability level ( $P$ ). The latter analysis was performed on the soil (2 way-ANOVA) and plant (3 way-ANOVA) conducted using STATISTICA 6.0 (StatSoft, Inc., Tulsa, OK). The ANOVA for soil samples was used to compare the effects of site ("Borghetto" and "Passi") and date (sampling time) factors and their interactions, while for leaf samples, it was used to compare the effects of site ("Borghetto" and "Passi"), species (*P. tobira*, *N. oleander*, and *Q. ilex*), and date (sampling time) factors and their interactions. The means separation of factor effects was accomplished with the Fisher's least significant difference test ( $P \leq 0.05$ ). Linear regressions to test the relationships between single variables of interest were performed by a regression analysis (determination coefficient [ $R^2$ ] and probability value [ $P$ ]).

## Results and Discussion

### Concentration and isotope composition of C and N in urban and peri-urban soils

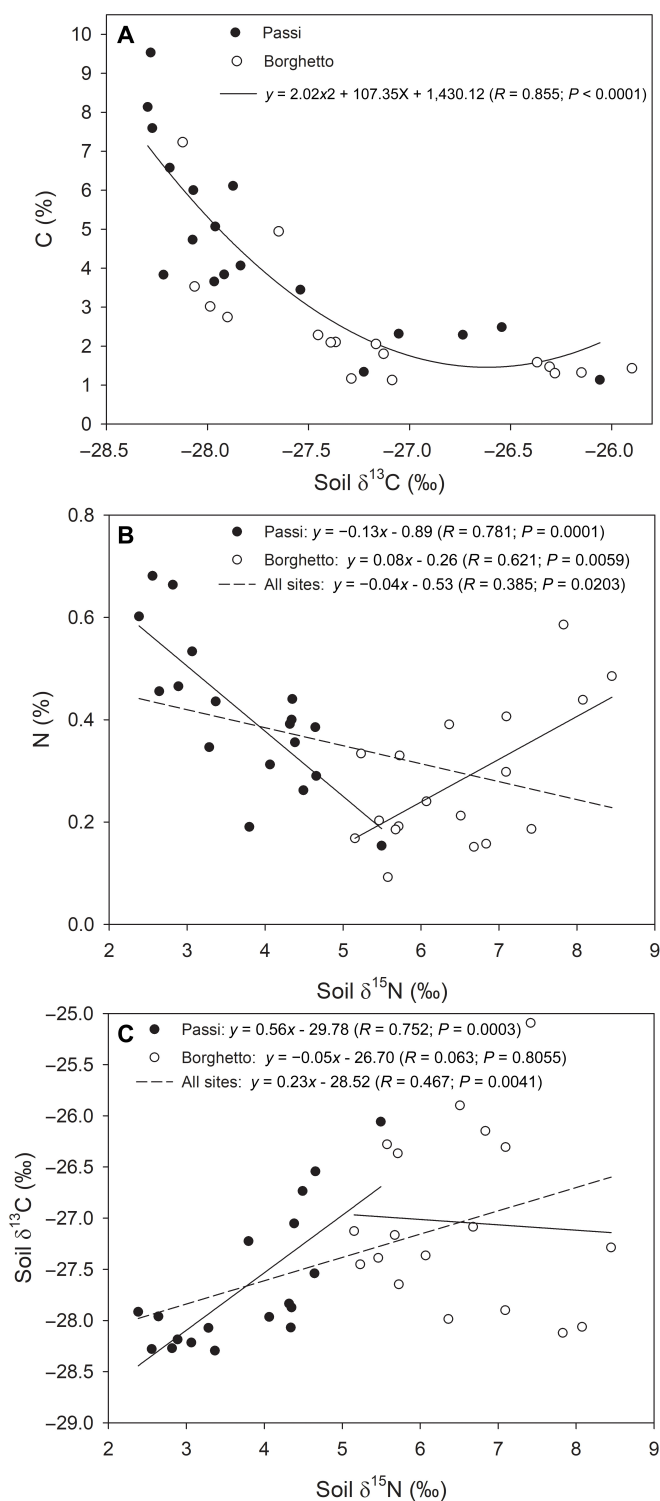
The enrichment in the heavier isotopes  $^{13}\text{C}$  and  $^{15}\text{N}$  in the urban soil of Borghetto compared to the peri-urban soil of Passi (Table) suggested a strong influence of the urbanization degree on both C and N cycling [14]. In fact, environmental conditions (e.g., temperature and soil water content, urban fragmentation, and N depositions) strongly affect C and N cycling in urban soils [4]. Urban heat island increases mean monthly soil temperature in urban soils, promoting microbial respiration and soil organic matter (SOM) decomposition [18]. On the other hand, dry N depositions tend to be isotopically enriched [19–21] and contribute to increasing SOM decomposition [22] and N cycling rates [23]. Consequently, both the heat island and N deposition may promote SOM decomposition in urban soil with the release of  $^{15}\text{N}$ -depleted forms by ammonia volatilization and nitrate leaching, leading to a progressive  $^{15}\text{N}$  enrichment of the remaining N pool. Moreover, high pet dejections in urban green areas can also enhance losses of  $^{15}\text{N}$ -depleted nitrogen, leaving the remaining N pool enriched in  $^{15}\text{N}$  [24]. Furthermore, soil decomposers fractionate against  $^{13}\text{C}$  during microbial decomposition of organic material and soil

**Table.** Isotopic carbon and nitrogen status in soil from urban and peri-urban sites. The 2-way ANOVA for the effect of site (Borghetto and Passi), date (sampling time: September 2019, January 2020, and April 2020), and their interaction are shown. Data are shown as mean  $\pm$  SE. The  $P$  values in bold indicate significant differences.

	Borghetto	
	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
September 2019	$-26.15 \pm 0.27$	$6.20 \pm 0.35$
January 2020	$-27.25 \pm 0.21$	$6.60 \pm 0.48$
April 2020	$-27.72 \pm 0.35$	$6.69 \pm 0.44$
	Passi	
	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$
September 2019	$-27.12 \pm 0.34$	$3.98 \pm 0.46$
January 2020	$-28.16 \pm 0.51$	$3.21 \pm 0.26$
April 2020	$-27.74 \pm 0.38$	$4.07 \pm 0.29$
2-way ANOVA ( $P$ )		
Site	<b>0.0018</b>	<b>&lt;0.0001</b>
Date	<b>&lt;0.0001</b>	0.7662
Site $\times$ Date	0.2320	0.4717

respiration, causing a consumption of SOM associated with a  $^{13}\text{C}$  enrichment of microbial biomass and its by-products [25]. Accordingly, in both sites, a  $^{13}\text{C}$  enrichment with decreasing soil organic C concentration was observed (Fig. 2A), and Borghetto showed higher  $\delta^{13}\text{C}$  than Passi during the autumn–winter period (Table).

A contrasting relationship between soil  $\delta^{15}\text{N}$  and N concentration was observed in the 2 sites, with Passi and Borghetto showing a dilution and an enrichment in  $^{15}\text{N}$  with increasing soil N concentration, respectively (Fig. 2B). This result seems to indicate that the spatial–temporal variability in soil  $\delta^{15}\text{N}$  of Passi was mainly dependent on the isotopic fractionations occurring during the SOM decomposition processes, thus leading to a simultaneous enrichment of both C and N heavy stable isotopes in the remaining SOM, as highlighted by the positive and high significant relationship between  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  (Fig. 2C). Conversely, the greater atmospheric  $\text{NO}_2$  concentrations in Borghetto (Fig. 1C) associated with the higher  $\delta^{15}\text{N}$  values of SOM (Table) suggested a contribution of  $^{15}\text{N}$ -enriched depositions, which could directly affect the  $\delta^{15}\text{N}$  variability of SOM without altering its  $\delta^{13}\text{C}$ . This fact could explain the lack of a significant relationship between  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ , and the positive relationship between  $\delta^{15}\text{N}$  and N concentration in the urban site (Fig. 2B and C). Hence, our quantitative and isotopic data of C and N supported the hypothesis that  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values in urban soils are most likely a combination of the direct contribution of  $^{15}\text{N}$ -enriched N sources and the greater soil C and N cycling rates associated with  $^{12}\text{C}$  and  $^{14}\text{N}$  losses over time, which are dependent on the urbanization degree, microclimatic conditions (e.g., heat islands), and  $\text{NO}_2$ -induced pollution [14]. Moreover, as Borghetto was characterized by homogeneous microclimatic conditions and soil properties [9] but with much higher atmospheric  $\text{NO}_2$  concentration compared to Passi



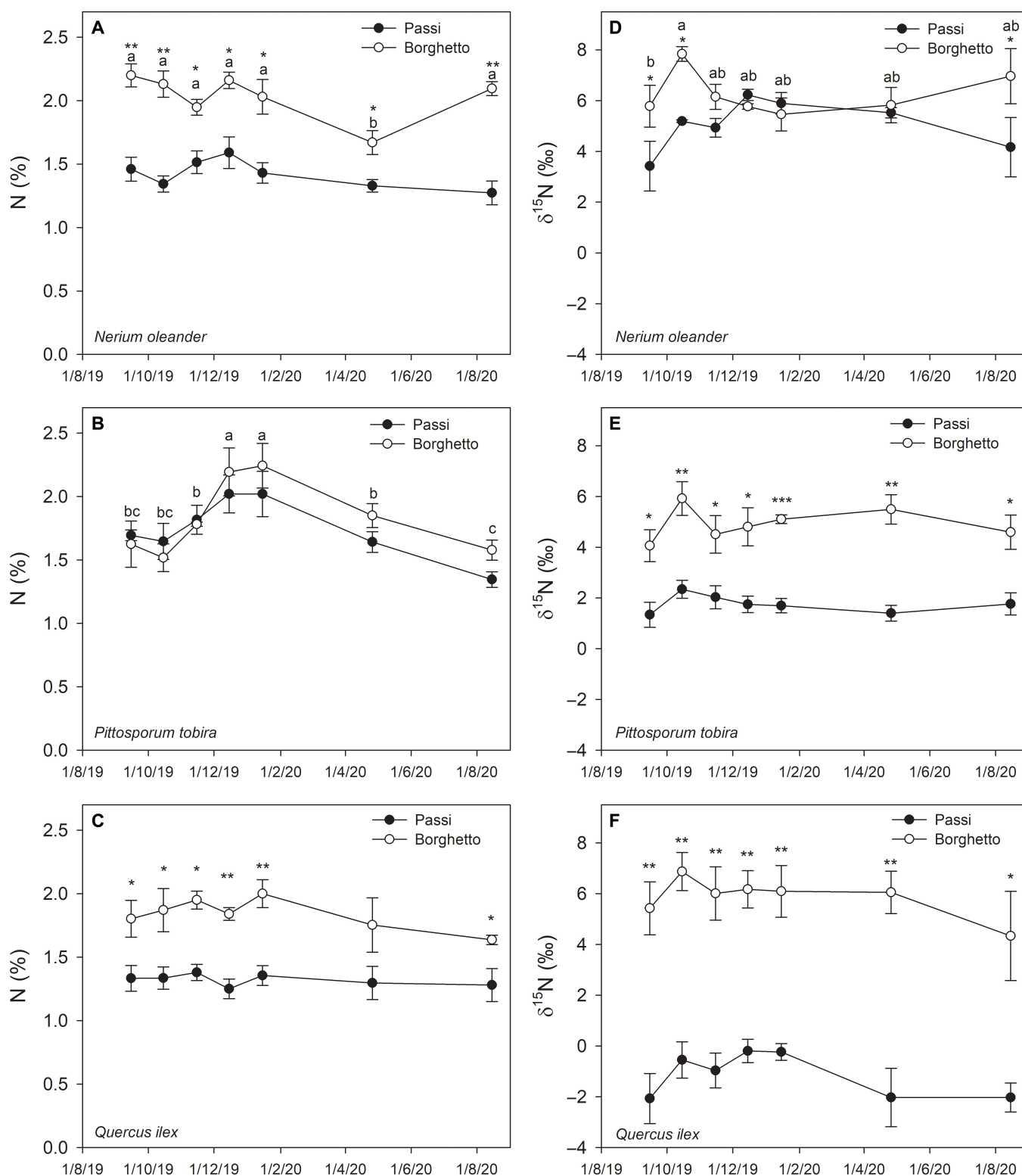
**Fig. 2.** Elemental and isotopic carbon and nitrogen composition in soil from urban and peri-urban sites. Regressions between (A) organic C and  $\delta^{13}\text{C}$ , (B) N and  $\delta^{15}\text{N}$ , and (C)  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  for soil samples collected from urban (Borghetto) (open circles) and peri-urban (Passi) (filled circles) sites. Data were collected in September 2019, January 2020, and April 2020.

(Fig. 1C), it is plausible that the isotopic differences between sites were mainly attributed to different spatial contribution of  $^{15}\text{N}$ -enriched depositions. It is worth noting that the N concentration and  $\delta^{15}\text{N}$  of SOM were not significantly affected

by the lockdown despite the transient drop of atmospheric  $\text{NO}_2$ . This could be because N of SOM is determined by the long-term balance between N inputs to the soil, through N depositions and plant residues, and N losses due to microbial SOM decomposition processes that show a relatively slow turnover rate.

### Seasonal changes of leaf N concentration and isotope composition in evergreen woody vegetation of urban and peri-urban sites

The generally higher leaf N concentration in Borghetto compared with Passi (Fig. 3A to C) further suggested an increase of N supply to plants due to the enhanced N turnover in urban soils. This indicated a different microbial activity due to differences in microclimate conditions, litter composition, and/or N depositions. A higher turnover rate of SOM could be related to the generally low leaf C/N ratio in Borghetto (Fig. S2) and its possible stimulating effect on litter decomposition [26], thus promoting the release of inorganic N forms available for plant uptake. Moreover, cities are hot spots of N deposition [27]; hence, the higher atmospheric  $\text{NO}_2$  concentration in Borghetto compared with Passi (Fig. 1C) could contribute to directly enhance leaf N concentration in all the evergreen species. Accordingly, a previous finding demonstrated that the direct incorporation of atmospheric  $\text{NO}_2$  in leaves can represent between 3% and 16% of the total plant N assimilation [28]. Moreover, urban trees are able to remove between 0.79 and  $1.11 \text{ g m}^{-2} \text{ year}^{-1}$  of atmospheric  $\text{NO}_x$  [13]. Interestingly, it was found that among different functional groups, the evergreen plants highly increase the rates of N in their leaves after  $\text{NO}_2$  treatment [11]. In this study, *Q. ilex* at Borghetto exhibited much higher leaf N concentration than that at Passi throughout the year (Fig. 3C), suggesting that this species was able to uptake high amounts of N from atmospheric depositions incorporating them into leaf N compounds [29], notably amino acids [30]. According to this hypothesis, higher N concentration has been observed in leaves of *Q. ilex* from urban areas than in leaves from remote areas [31]. Similar results were observed in trees of *Q. ilex* grown in urban soils poor in N [32]. Moreover, an increase of leaf N due to N deposition has been previously reported in *Q. ilex* trees [10]. Similarly, a higher concentration of leaf N in urban (Borghetto) than in peri-urban (Passi) sites was observed in leaves of *N. oleander* (Fig. 3A), suggesting that oleander shrubs were also able to uptake N atmospheric compounds. Accordingly, Meletioui-Christou et al. [33] found an elevated chlorophyll content in *N. oleander* growing in  $\text{NO}_2$ -polluted sites, indicating an increase in the total N organic compounds [34]. Conversely, *P. tobira* showed only slight differences in leaf N concentration between sites and a clear seasonal trend, with an increase during the cold and wet period (December 2019 to January 2020) (Fig. 3B). This could be due, in addition to a high N deposition during winter in both sites (Fig. 1C), to the seasonal changes in N allocation. In fact, Meletioui-Christou and Rhizopoulou [35] showed that N accumulated in mature leaves of *P. tobira* during the wet period could be translocated to the expanding leaves for supporting both vegetative and reproductive growth during the next spring, suggesting that the N withdrawal from mature leaves may be interpreted as a N recycling process. It is worth noting that the lockdown caused a general decreasing pattern of leaf N concentration (Fig. S3A and B) that could be partly associated



**Fig. 3.** Seasonal change of nitrogen concentration (N) and isotope composition ( $\delta^{15}\text{N}$ ) in leaves of Mediterranean evergreen woody species from urban and peri-urban sites. (A to C) N (dry mass basis, %) and (D to F)  $\delta^{15}\text{N}$  (‰) in (A, D) *N. oleander*, (B, E) *P. tobira*, and (C, F) *Q. ilex* leaf samples collected from urban (Borghetto) (open circles) and peri-urban (Passi) (filled circles) sites. Data are shown as mean  $\pm$  SE. Different lowercase letters indicate significant differences between means over time ( $P < 0.05$ ), while asterisks indicate significant differences between sites at specific dates (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

with the reduced uptake of N by atmospheric depositions due to the sharp reduction of  $\text{NO}_2$  concentration (Fig. 1C). However, some differences were observed between species (Table S1). A significant decrease in N concentration was observed in leaves

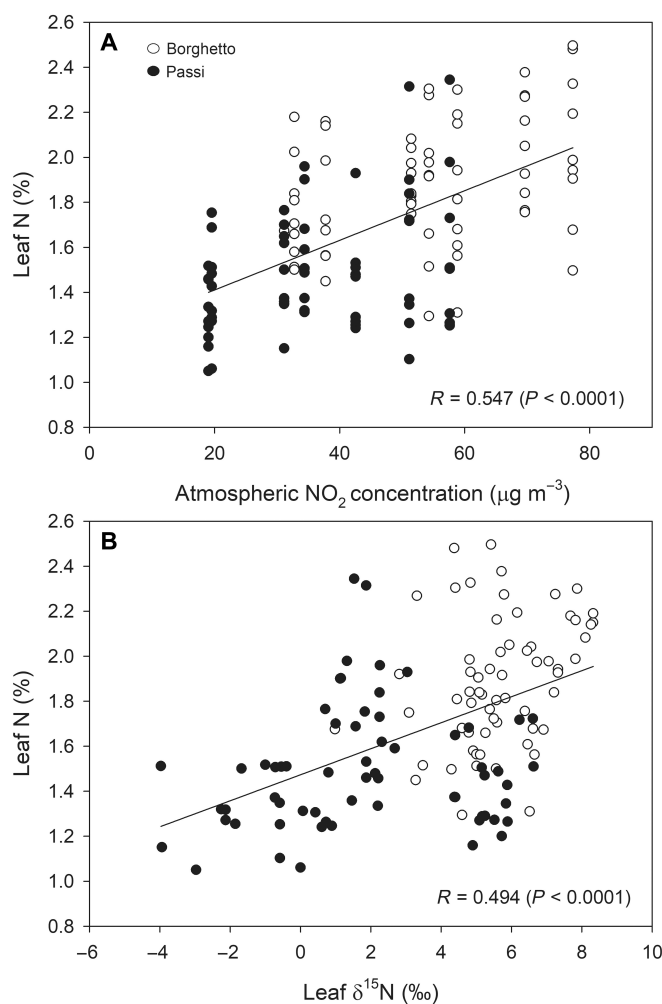
of *N. oleander* from January to April 2020 (Fig. 3A), especially in the urban site of Borghetto, suggesting a sudden effect of the lockdown-induced reduction of atmospheric  $\text{NO}_2$  on N supply in this species. Similarly, beyond January 2020, *P. tobira* showed

a significant decrease of leaf N concentration associated with an increase of C/N (Fig. 3B and Fig. S2B). Conversely, *Q. ilex* did not show significant differences in leaf N concentration between pre- and post-lockdown period (Fig. 3C), which may be related to its slow growth rate and long persistence leaves that were slightly affected by transient changes of environmental conditions. Interestingly, this species also showed a decreasing trend in leaf N concentration in the urban site of Borghetto from January to April 2020 (Fig. 3C) when the leaf N concentration was not significantly different with respect to Passi. Hence, this result suggested that the lockdown period smoothed out the differences in N availability between urban and peri-urban sites.

Leaf N concentration was often found to be positively correlated with leaf  $\delta^{15}\text{N}$  values, indicating a high N supply due to enhanced N turnover in the soil (e.g., [36]). Moreover, higher N availability in urban forest patches may lead to enriched foliar N concentration and  $\delta^{15}\text{N}$  compared with nearby rural sites [37,38]. Here, a strong positive impact of vegetation N uptake on  $^{15}\text{N}$  isotopic signals was found (Fig. 3D to F), supporting the hypothesis of enhanced N fluxes (and increased losses of  $^{15}\text{N}$ -depleted compounds) in the more urbanized site of Borghetto, reflecting a more open N cycle. Additionally, it has been reported that the pollution-derived N deposition tends to be isotopically enriched [19–21]. In fact, the  $\delta^{15}\text{N}$  in leaves represents a valuable biomarker of  $\text{NO}_x$  emitted by road transport and a reliable predictor of land use change, following the same trend as N concentration along a center–peripheral urban gradient [12]. Consequently, N stable isotopes have been proposed as useful tools for estimating  $\text{NO}_x$  removal capacity by urban trees [13] and as indicators of leaf uptake of atmospheric N pollution [28]. Thus, the positive relationships between leaf N with both  $\delta^{15}\text{N}$  and atmospheric  $\text{NO}_2$  concentration found in this study (Fig. 4) may be most likely due to a combination of greater N deposition on plant canopy and its incorporation into leaf compounds with increasing urbanization degree. However, some differences among species were observed (Table S1), with *Q. ilex* showing the highest  $\delta^{15}\text{N}$  difference between Borghetto and Passi (Fig. 3F). This suggested that *Q. ilex* may be able to uptake and immobilize high amounts of  $^{15}\text{N}$ -enriched atmospheric depositions into its leaves. The same inter-site differences, with  $^{15}\text{N}$  enriched values in leaves of Borghetto compared with Passi, were observed for *P. tobira* and *N. oleander* (Fig. 3D and E), although the latter species did not show any significant inter-site isotopic difference between November 2019 and April 2020 (Fig. 3D). This result was most probably due to a drastic pruning intervention carried out on *N. oleander* plants during late autumn in Passi, which negatively affected their physiological performance [9], exposing the remaining old and new developing leaves to the sharp increase of atmospheric  $\text{NO}_2$  concentration during winter (Fig. 1C).

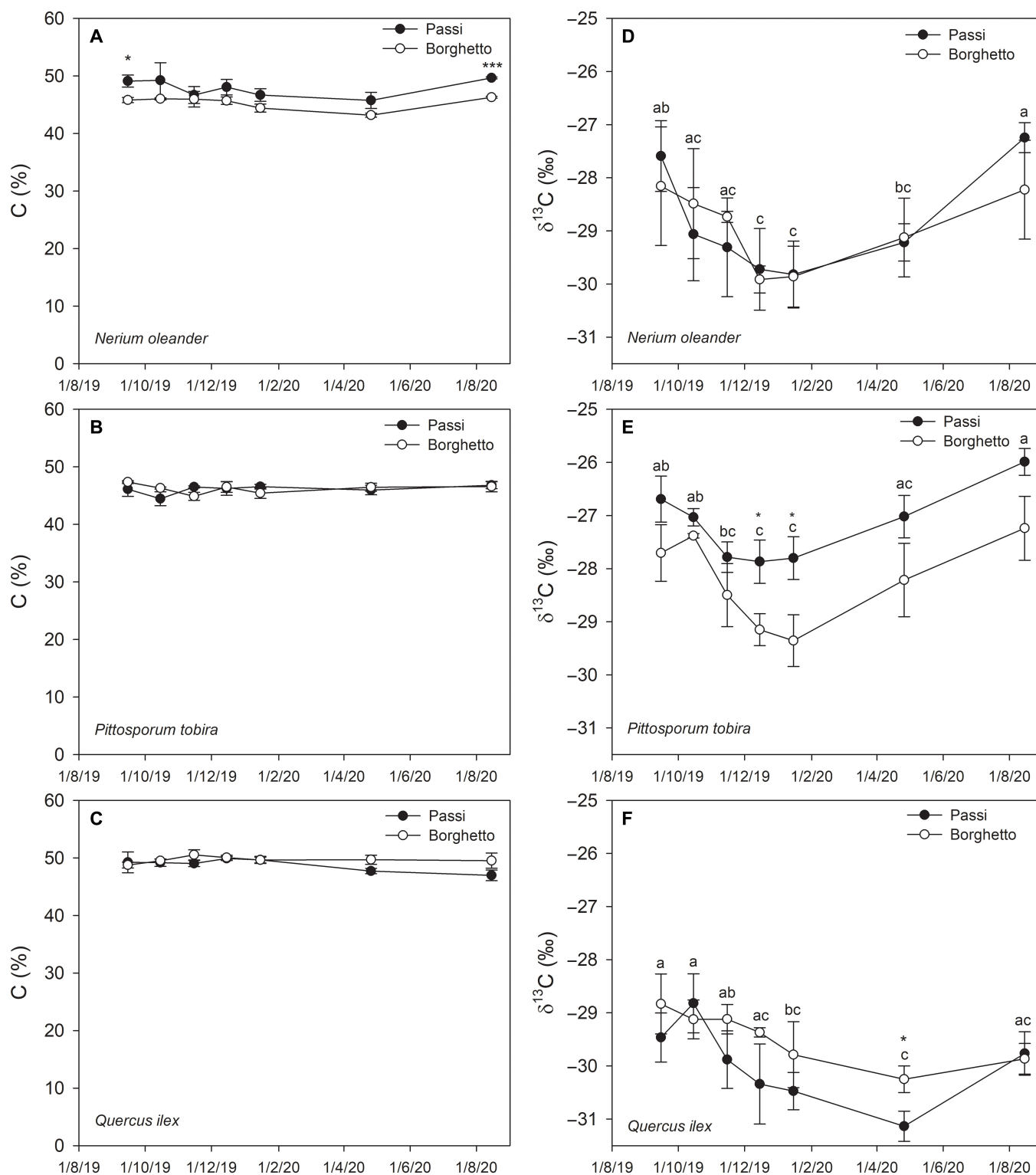
### Seasonal changes of leaf C concentration and isotope composition in evergreen woody vegetation of urban and peri-urban sites

A seasonal trend in  $\delta^{13}\text{C}$  without significant changes in C concentration was observed in leaves of all evergreen species (Fig. 5 and Table S1). Seasonal variations, such as air temperature and rainfall, affect vapor pressure deficit and soil moisture, influencing stomatal conductance. This, in turn, alters photosynthetic C isotope discrimination ( $\Delta$ ), leading to leaf  $\delta^{13}\text{C}$



**Fig. 4.** Correlations of leaf N content with (A) atmospheric  $\text{NO}_2$  concentration and (B) leaf  $\delta^{15}\text{N}$  across the study transect. Correlation coefficients ( $R$ ) are shown across the entire gradient including each Mediterranean woody species (*N. oleander*, *P. tobira*, and *Q. ilex*) grown in urban (Borghetto) (open circles) and peri-urban (Passi) (filled circles) sites. Data were collected from September 2019 to August 2020.

seasonal dynamics [39]. In fact, lower leaf  $\delta^{13}\text{C}$  indicates an increase of  $\Delta$  associated with higher intercellular to atmospheric  $\text{CO}_2$  ratio ( $C_i/C_a$ ), which is dependent on the balance between the supply of  $\text{CO}_2$  to the leaf intercellular air spaces by stomatal conductance ( $g_s$ ) and the net photosynthetic  $\text{CO}_2$  uptake ( $A$ ). Hence, leaf  $\delta^{13}\text{C}$  is considered a valuable proxy for time-integrated intrinsic water-use efficiency (iWUE), i.e., the ratio between  $A$  and  $g_s$  [40]. Pereira et al. [12] found that emissions derived from fossil fuel, biogenic respiration, microclimatic conditions, and distinct land uses (e.g., urban fragmentation) interfere with leaf  $\delta^{13}\text{C}$  at different levels in subtropical urban forests, and highlighted that new studies including seasonal analyses are needed to integrate over time the physiological responses of urban forests. Here, our data indicated wide seasonal and inter-specific differences in leaf  $\delta^{13}\text{C}$ , with *N. oleander* and *P. tobira* showing higher leaf  $\delta^{13}\text{C}$  value (i.e., enhanced iWUE) than *Q. ilex* (Fig. 5D to F). Moreover, these shrubs showed a clear seasonal isotopic trend (Fig. 5D and E), with a  $^{13}\text{C}$  dilution pattern from summer to autumn–winter (2019 data) strictly related to the decreased air temperature and increased precipitation (Fig. 1A and B). Thus, our data indicated a  $\Delta$



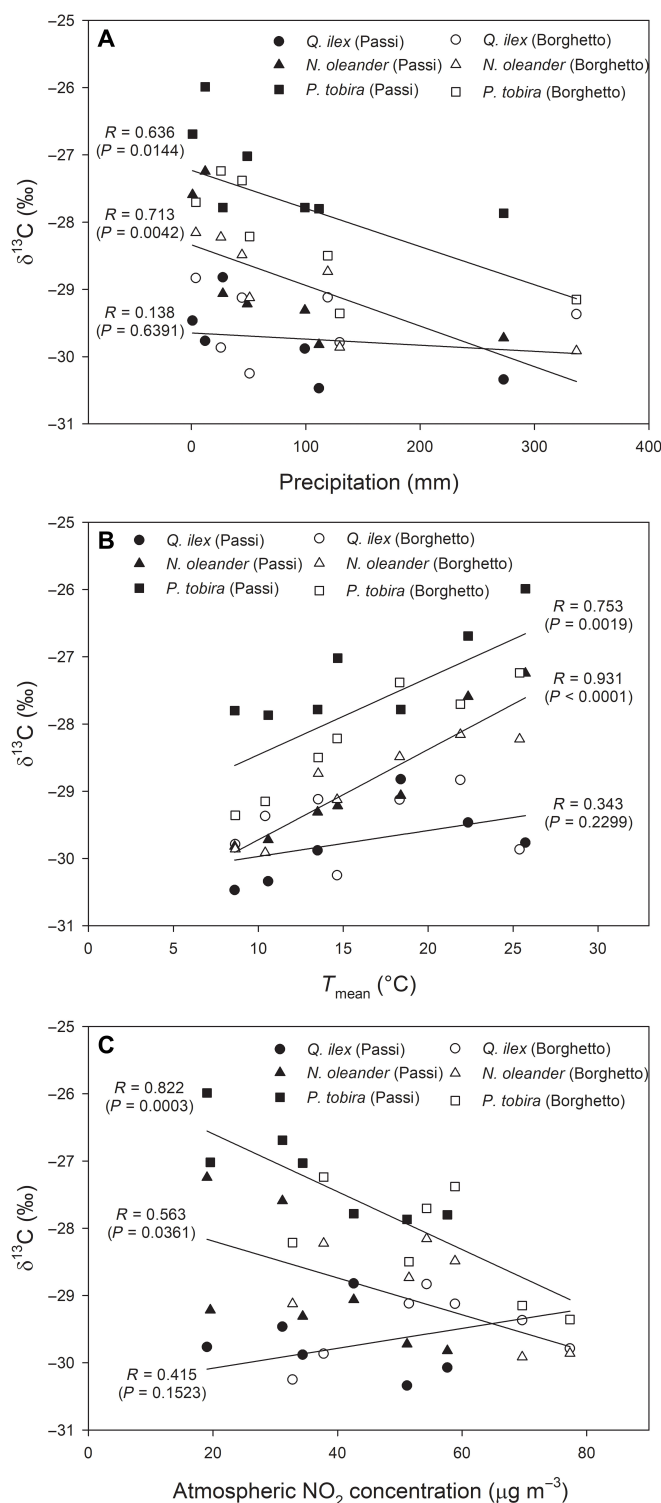
**Fig. 5.** Seasonal change of carbon concentration (C) and isotope composition ( $\delta^{13}\text{C}$ ) in leaves of Mediterranean evergreen woody species from urban and peri-urban sites. (A to C) C (dry mass basis, %) and (D to F)  $\delta^{13}\text{C}$  (‰) in (A, D) *N. oleander*, (B, E) *P. tobira*, and (C, F) *Q. ilex* leaf samples collected from urban (Borghetto) (open circles) and peri-urban (Passi) (filled circles) sites. Data are shown as mean  $\pm$  SE. Different lowercase letters indicate significant differences between means over time ( $P < 0.05$ ), while asterisks indicate significant differences between sites at specific dates (\* $P < 0.05$ , \*\*\* $P < 0.001$ ).

decrease during the driest and hottest seasonal periods in *N. oleander* and *P. tobira* species, suggesting an enhanced iWUE to reduce water losses and preserve leaf water status. However, *P. tobira* showed relatively higher  $\delta^{13}\text{C}$  values throughout the

year compared with *N. oleander* (Fig. 5D and E), indicating that the first species was able to maintain higher iWUE due to a stronger stomatal control. Accordingly, a drought-avoidance mechanism involving stomatal closure to minimize water loss

through transpiration during drought periods in *P. tobira* has been hypothesized [41,42]. Moreover, lower  $g_s$  values in *P. tobira* than in *N. oleander* were recorded during late summer in the same sites of this study [9]. Therefore, the relatively higher  $\delta^{13}\text{C}$  values in *P. tobira* during summer (Fig. 5E) may be related to its response to the reduction of soil water availability by increasing its iWUE. Conversely, the lower  $\delta^{13}\text{C}$  values in *N. oleander* (Fig. 5D) could be explained by considering its ability to maintain higher levels of stomatal gas exchange despite the increasing drought conditions [9,43,44]. In fact, the hydraulic behavior of *N. oleander* suggests an isohydric behavior of ostensibly drought-tolerant plants, which preserves gas exchange at the cost of more negative internal water potentials and risk of hydraulic failure and embolisms [43,45]. On the other hand, *Q. ilex* showed the lowest  $\delta^{13}\text{C}$  values throughout the year, reaching a minimum in April 2020 during the lockdown period (Fig. 5F). In agreement with our isotopic data, *Q. ilex* showed higher  $C_i$ , and lower  $g_s$  and transpiration rates compared with *P. tobira* and *N. oleander* [9], and its maximum seasonal values of  $g_s$  and  $A$  were reported in spring [46]. Moreover, it has been reported that the transpiration rates of *Q. ilex* is much lower than those of some perennial sclerophyllous trees due to its stomatal control and low cuticular transpiration [47]. Altogether, it suggested that the xerophytic characteristics of leaf epidermic surface and deeper root system of *Q. ilex* trees with respect to shrub species contributed to a conservative strategy, assuring relative low amounts and variability of water losses throughout the season. Consequently, this may lead to a low  $\delta^{13}\text{C}$  variability and a lack of clear relation with environmental parameters (e.g., air temperature and precipitation) in *Q. ilex* (Fig. 6A and B).

Significant differences in leaf  $\delta^{13}\text{C}$  between sites and species were observed (Table S1). In particular, *P. tobira* leaves at Borghetto showed generally lower  $\delta^{13}\text{C}$  values than that at Passi, especially during winter (Fig. 5E). Previous studies demonstrated that the photosynthesis of this species was sensitive to atmospheric pollutants [9], and its photosynthetic apparatus was impaired by high atmospheric  $\text{NO}_2$  concentrations [11]. Therefore, the high  $\text{NO}_2$  level at Borghetto may have a greater impact on the photosynthetic capacity of *P. tobira* than on its stomatal conductance, contributing to increasing  $C_i/C_a$  and, consequently,  $\Delta$  especially during winter when atmospheric  $\text{NO}_2$  concentration sharply increased (Fig. 1C). This hypothesis was supported by the significant negative correlation between  $\delta^{13}\text{C}$  and atmospheric  $\text{NO}_2$  concentration observed in *P. tobira* (Fig. 6C). This correlation may also indicate an increase of iWUE with decreasing atmospheric  $\text{NO}_2$  concentration during the lockdown. A similar correlation was also detected in *N. oleander*, although this species did not show significant differences in leaf  $\delta^{13}\text{C}$  between Passi and Borghetto (Fig. 5D). These data suggested that the possible changes in stomatal and non-stomatal constrains did not alter the  $C_i/C_a$  ratio in *N. oleander* and, hence, the isotopic signal in the studied sites. Finally, a  $^{13}\text{C}$  enrichment in *Q. ilex* leaves at Borghetto with respect to Passi was only observed in April 2020 during the lockdown period (Fig. 5F), indicating a higher iWUE in spring for the trees growing in the more urbanized and  $\text{NO}_2$ -polluted site. Moreover, the correlation between  $\delta^{13}\text{C}$  and atmospheric  $\text{NO}_2$  concentration showed an opposite pattern in *Q. ilex* when compared with the other studied species (Fig. 6C), suggesting that *Q. ilex* increased iWUE with increasing  $\text{NO}_2$  concentration. Accordingly, a positive effect of N deposition on WUE of global terrestrial ecosystems has been previously hypothesized [48],



**Fig. 6.** Correlation of leaf  $\delta^{13}\text{C}$  values with (A) precipitation, (B) mean temperature, and (C) atmospheric  $\text{NO}_2$  concentration across the study transect. Correlation coefficients ( $R$ ) are shown for each Mediterranean evergreen woody species. Leaves of *Q. ilex* (circle), *N. oleander* (triangle), and *P. tobira* (square) grown in urban (Borghetto) (open symbols) and peri-urban (Passi) (filled symbols) sites were collected from September 2019 to August 2020.

especially for those species on which  $\text{NO}_2$  has no toxic effects [49]. Thus, *Q. ilex* seems to well tolerate high  $\text{NO}_2$  concentrations, suggesting that its photosynthetic capacity was not impaired or even stimulated in the urban site of Borghetto. This



may lead to an increase of iWUE in spring, when *Q. ilex* showed the maximum seasonal level of stomatal gas exchanges [46]. This particular behavior could be due to the higher leaf N concentration (Fig. 3C and F) and better N use efficiency and partitioning [9] in *Q. ilex* growing in more urbanized and polluted sites. Accordingly, Fusaro et al. [50] found that N addition could improve the photosynthetic capacity of *Q. ilex* and stimulate an alternative antioxidant response such as the emission of volatile organic compounds, playing a key role to counteract the detrimental effect of other dangerous pollutants such as O<sub>3</sub>. Overall, our data indicated that the seasonal variation of leaf δ<sup>13</sup>C in Mediterranean urban evergreen species could be due to a combination of microclimatic conditions, urban fragmentation, and N depositions. The reduced NO<sub>2</sub> concentration during the lockdown period differently affected leaf δ<sup>13</sup>C and iWUE in the studied species, suggesting a possible effect of this air pollutant on stomatal and non-stomatal factors and affecting their photosynthetic activity. Finally, the inter-species comparison highlighted the potential of *Q. ilex* in reducing the atmospheric NO<sub>2</sub> concentration without undergoing toxic effects, thus combining the advantage of maintaining a good physiological performance and improving important ecosystem services in urban environments.

## Conclusion

The isotopic data confirmed as δ<sup>13</sup>C and δ<sup>15</sup>N in soil and plant represent valuable proxies of urbanization degree and soil-plant-atmosphere interactions in an urban environment. Here, we unraveled how seasonal changes of microenvironmental variables (i.e., precipitation and temperature), urban fragmentation (urban vs. peri-urban sites), and N inputs from atmospheric NO<sub>2</sub> enhanced the application of the isotopic approach. The <sup>15</sup>N enrichment in soil and leaf samples in urban environments was most likely due to a combination of faster N cycling rate and higher N deposition in the highly urbanized and polluted site of Borghetto compared to the peri-urban site of Passi. Seasonal changes of microclimatic condition and N deposition were the main drivers affecting leaf δ<sup>13</sup>C and iWUE in the studied species. The lockdown caused a sharp decrease of atmospheric NO<sub>2</sub> concentration that was reflected in a general reduction of leaf N concentration of evergreen species, especially in leaves of *N. oleander* and *P. tobira*, which showed a faster turnover rate than *Q. ilex*. Conversely, SOM was only slightly affected by lockdown, being subjected to a much slower turnover, and thus reflecting inter-site differences in the long-term balance between N inputs and outputs in the soil system. Although all species responded to higher atmospheric NO<sub>2</sub> level by increasing their leaf N concentration and isotopic composition, *Q. ilex* showed the highest adaptability to urban environmental conditions. In detail, our data suggested that *Q. ilex* might be able to uptake and immobilize higher amounts of <sup>15</sup>N-enriched atmospheric depositions in leaves with positive effects on its physiological performance and iWUE, while the photosynthetic performance of *P. tobira* seems to be negatively affected by higher concentrations of NO<sub>2</sub> leading to a reduced iWUE. Altogether, our findings highlighted the importance of studying the dynamics and effects of atmospheric NO<sub>2</sub> pollutant on the C and N cycling-related parameters and their alterations in an urban context. The knowledge on physiological adaptation mechanisms and inter-specific differences produced in this study could be taken in consideration for urban greening

programs to improve air quality and promote ecosystem services in NO<sub>2</sub>-polluted areas.

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## Data Availability

Data are contained within the article.

## Supplementary Materials

Table S1. Three-way ANOVA used to test the effects of species (*Q. ilex*, *P. tobira*, and *N. oleander*), site (Borghetto and Passi), date (sampling time), and their interactions for the studied leaf traits.

Fig. S1. Site map.

Fig. S2. Seasonal change of C/N ratio in leaves of Mediterranean evergreen woody species from urban and peri-urban sites.

Fig. S3. Total leaf N concentration from *Q. ilex*, *P. tobira*, and *N. oleander* across the study transect during pre- and post-lockdown period.

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