



Isotope signature and ecoenzymatic stoichiometry as key indicators of urban soil functionality

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Abstract

Purpose This study aimed to assess the feasibility of the ecoenzymatic stoichiometry and isotope signature approaches as indicators of urban soil functionality, related to carbon and nutrient cycles.

Methods In Pisa and Livorno (Italy), study sites with three degrees of urbanization (natural, peri-urban and central urban sites) were selected, where holm oak (*Quercus ilex* L.) was the most common evergreen species. The urban and peri-urban sites differed in terms of NO₂ emissions. At each site, topsoil and plant litter were sampled, pH, EC, TOC, and TN were measured in soil and $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in soil and plant litter. The β -glucosidase, acid phosphatase and N-acetyl- β -D-glucosaminidase enzyme activities were also determined in soil and the ratios were calculated.

Results The $\delta^{15}\text{N}$ in plant litter increased from peri-urban to urban sites, along with the NO₂ emissions, emerging as a sensitive indicator of atmospheric N deposition. The $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ increased in soil, indicating more rapid N cycles and organic matter degradation in peri-urban and urban areas than in natural areas. The ecoenzymatic stoichiometry revealed C and P microbial limitations for all the sites studied. However, the microbial needs of C and P increased and decreased, respectively, along the urbanization gradient. Isotope abundance and microbial nutrient limitations were found to correlate with soil properties. Specifically, soil $\delta^{15}\text{N}$ was closely correlated with microbial C limitations.

Conclusion The isotope signature and enzymatic stoichiometry used as indicators revealed that the soil characteristics affected the soil carbon and nutrient cycles as well as microbial energy and nutrient needs.

Keywords Atmospheric N deposition · Carbon and nutrient cycles · Soil enzymes · Microbial limitation · Stable isotopes · Urbanization

1 Introduction

Urbanization is a major driving force in changing the environment at local, regional, and global scales. In this way, biogeochemical cycles are greatly influenced by human activities (e.g. soil disturbance and atmospheric deposition) in urban areas. Urban soils are often affected by the presence of anthropogenic materials, compaction, and mixing, which

contribute to altering directly the soil structure and indirectly the soil fertility. These physical alterations reduce the soil porosity as well as the water and air circulations, limiting the soil biota activity and organic matter degradation, with consequences for nutrient cycles. In addition, the release of air pollutants (e.g., CO₂ and NO₂) in urban areas, due to the combustion of fossil fuels, increases the nitrogen and carbon depositions on urban soils (Pereira et al. 2022; Xia et al. 2023). This can affect the C and N cycles, thus altering the functioning of the ecosystem (Lorenz and Lal 2009; Pereira et al. 2022; Xia et al. 2023). In this context, the appropriate selection of soil quality indicators is needed for the optimization of soil management, especially in designing and implementing a green infrastructure, targeted at enhancing soil functionality (Ungaro et al. 2022). The ecological indicators should be easy to measure and be sensitive to environmental changes (Karaca et al. 2011), allowing evaluation

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of the effect of green infrastructures on soil quality, especially from the perspective of increased urbanization. This approach meets the European policy of reducing net land take by 2050, remodulating the concept of urban development with a special focus on soil protection (Decoville and Feltgen 2023). From this point of view, the study of soil extracellular enzymes, and of carbon and nitrogen isotopes, acquires particular importance.

Soil microorganisms produce pools of extracellular enzymes for the acquisition of energy and nutrients, depending on microbial nutrient and energy requirements as well as resource availability (Burns et al. 2013). The enzymes most widely studied and easily measured by fluorometric methods are β -glucosidase (BG), acid phosphatase (AP) and N-acetyl- β -D-glucosaminidase (NAG), as they are proxies of soil C, P and N cycles, respectively. The BG catalyses the hydrolysis of cellobiose residues in plant debris, producing glucose, which is a C energy source for microbial growth and activities. AP activities are related to the hydrolysis of esters and anhydrides of phosphoric acid, while NAG is a N-acquiring enzyme from chitin and peptidoglycan (Moorhead et al. 2016; Adetunji et al. 2017). Urban soil properties (e.g. alkaline pH, low C/N) can influence microbial activity (Lorenz and Lal 2009), e.g. enhancing nitrification and denitrification processes (Pulikova and Gorovtsov 2022). Atmospheric N deposition can also affect the enzyme activities, as seen in N fertilization experiments. For example, Chen et al. (2018) observed an increase in NAG, as a consequence of soil acidification, derived from N addition. The N inputs in terrestrial ecosystems over time can increase the microbial C needs due to the limited degradation of recalcitrant organic carbon (Chen et al. 2018). However, N fertilization experiments have had inconsistent results due to differences in application methods, doses, and times, thus not reflecting the real effect of N on ecosystems (Wang et al. 2013).

According to the ecoenzymatic stoichiometry theory (EST), microorganisms, based on their nutrient demand, allocate their resources to the production of C, N and P-acquiring enzymes in response to soil nutrient availability, highlighting a negative correlation between enzyme production and nutrient availability. Through the ratios amongst the most representative soil enzyme activities (BG, AP, NAG) of C and nutrient cycles, EST identifies enzyme activity imbalances and therefore soil nutrient shortfalls for microbial communities (Sinsabaugh et al. 2009 and 2012; Moorhead et al. 2023; Kunito et al. 2024). In nutrient and energy-limited conditions, an imbalance among enzyme activities occurs, thus explaining the deviation from the expected enzyme ratio (BG:NAG:AP = 1:1:1). This imbalance leads to a higher production of enzymes for a specific nutrient and/or energy source, with consequences for carbon and nutrient cycles in the ecosystem (Hill et al. 2012; Sinsabaugh et al. 2009 and 2012). In fact, in limited nutrient conditions (high

C:N or C:P in litter and soil organic matter), microorganisms mainly invest their resources in the production of N or P-acquiring enzymes, reducing the N or P mineralization. Instead, the high carbon availability in litter and soil organic matter increases the microbial respiration, reducing the microbial carbon sequestration potential. Conversely, in carbon limited conditions (low C:N or C:P), the nutrient mineralization can increase and the production of enzymes for C acquisition tends to be correlated with low respiration and high microbial C sequestration potential (Zechmeister-Boltenstern et al. 2015). Cui et al. (2023) claims that carbon-limited conditions can make soil vulnerable to environmental changes given that factors such as increased atmospheric CO₂, atmospheric N deposition and warming can enhance plant growth, thus increasing the plant C input or reducing the C:N ratio in soil. These factors can indirectly increase microbial activities and respiration, leading to a loss of carbon. Moorhead et al. (2016) proposed a quantitative method (vector analysis), based on the calculation of vector lengths and angles of plotted proportional enzyme activities (C:N vs C:P acquisition). The method quantifies the relative investments in C vs N and P acquisitions (vector lengths) or P vs N acquisition (vector angles) (Moorhead et al. 2016; Cui et al. 2020). The EST approach is successfully used as an indicator of microbial nutrient limitations in sediments (Macci et al. 2021), natural soil (Giannini et al. 2020; Wu et al. 2021; Pan et al. 2023) and agricultural soil (He et al. 2021; Cui et al. 2020; Shen et al. 2023). However, little is known regarding the impact of urbanization on microbial activities (Nugent and Allison 2022; Gómez-Brandón et al. 2022) and the EST, coupled with vector analysis, can be a proxy of soil functionality (i.e. carbon and nutrient cycles) in urban areas.

Stable C and N isotopes have been widely used as indicators of biogeochemical cycles in terrestrial ecosystems, linking plant and soil processes to the atmosphere (Bahn et al. 2012). For this reason, stable isotopes have been proposed as a valuable tool in urban ecology studies (Pataki et al. 2005; Trammell et al. 2020) and in tracking pollution sources (Gong et al. 2021). The enhanced nitrification process in urban soil can lead to an enrichment of ¹⁵N in soil and in plant leaves (Pulikova and Gorovtsov 2022; Scartazza et al. 2023). Indeed, the main soil microbial processes, such as decomposition, mineralization, nitrification, and denitrification, discriminate against the heavier isotope ¹⁵N (Craine et al. 2015). The soil ¹³C can also increase as a consequence of enhanced microbial respiration and soil organic matter decomposition (Scartazza et al. 2023). In addition, the increase in CO₂ and NO₂ emissions in urban environments contributes to affecting the isotope abundance of plant and soil components. The C derived from fossil fuel burning is poor in the heavier stable C isotope, leading to a dilution of ¹³C in the atmospheric CO₂ entering the ecosystem through photosynthesis. However, due to land use change

and CO₂ emissions on a large scale, the ¹³C/¹²C ratio in the soil–plant system may not differ along an urbanization gradient (Pereira et al. 2022). The NO₂ is formed through the rapid oxidation of NO emissions by ozone and free radicals, and it can be rapidly removed from the atmosphere through dry and wet depositions (Bednova and Kuznetsov 2019). The NO₂ atmospheric depositions add ¹⁵N-enriched nitrogen forms to the soil, leading to an increase in ¹⁵N in the urban topsoil (Bednova and Kuznetsov 2019; Xia et al. 2023) and plant leaves (Scartazza et al. 2023). It has been widely demonstrated that leaves of urban trees can directly adsorb atmospheric N deposition, especially in the forms of NO₂ and NH₃, and that ¹⁵N represents a valuable tool for assessing leaf uptake of N pollution (Vallano and Sparks 2007; Gong et al. 2021). *Quercus ilex* L. is considered one of the tree species most capable of capturing large amounts of N deposition in urban environments (Alfani et al. 2000; Fusaro et al. 2016), leading to an enrichment of ¹⁵N in the leaves (Scartazza et al. 2023). Therefore, the litter of plants of this species living in an urban context can contribute to supplying the soil with organic material enriched in ¹⁵N. On the other hand, it has been suggested that N deposition in urban ecosystems may also enhance soil N cycling rates and associated ¹⁴N losses over time, leading to higher ¹⁵N values of organic matter in the topsoil (0–10 and 10–30 cm depth) due to a combination of ¹⁵N enriched N sources and greater soil N cycling rates in urban soil (Trammell et al. 2020). In support of this, a decrease in ¹⁵N was observed in topsoil and leaves following the center-periphery gradient of NO_x in subtropical urban forests (Pereira et al. 2022). The studies on C and N stable isotopes mainly focus on urban and metropolitan areas (Pereira et al. 2022; Scartazza et al. 2023). However, the comparison of peri-urban and urban sites with natural sites enforces the suitability of these isotopes as soil functional indicators.

The aims of this study were to assess the EST, coupled with vector analysis, as well as the isotope signatures as suitable soil indicators of soil functionality, along an urbanization gradient. For this purpose, in the areas of Pisa and Livorno (Italy), three sites with different degrees of urbanization (natural, peri-urban, and urban sites), dominated by *Quercus ilex* L., were selected. The soils of the three sites in each area were characterized in terms of chemical and physical properties, and the soil ecoenzymatic stoichiometry, along with the soil and litter isotope signatures, were investigated to identify their relationships with different levels of urbanization.

2 Materials and Methods

2.1 Site description

Pisa and Livorno are two medium-sized towns, approximately 20 km from each other. They are located in the

coastal strip of the Tuscany region and are influenced by the same Mediterranean climate. In the areas of Livorno and Pisa, three study sites with natural, peri-urban, and urban characteristics were selected (Fig. 1). The natural sites are in the Park of Migliarino, San Rossore-Massaciuccoli (PI-Natural) and in the Park of Monti Livornesi (LI-Natural). These are wooded areas with no presence of anthropic activities. The peri-urban sites (PI-Periurban and LI-Periurban) are characterized by residential buildings, public and private green areas, and little-used streets. The urban sites (PI-Urban and LI-Urban) have dense residential and service buildings, few green areas, and roads with intense traffic. In all the sites studied, we selected green areas where holm oak (*Quercus ilex* L.) was the most abundant evergreen tree species. In order to detect a gradient for atmospheric NO₂, we used the air quality data made available online by the environmental protection agency of Tuscany (<https://www.arpat.toscana.it>). The air data were collected by stationary stations, located in each town (Periurban and urban sites), using automatic analysers that measure atmospheric NO₂ at regular intervals. The monthly averages of the atmospheric NO₂ (µg/m³) were 14 ± 8.8 and 27 ± 10.2 for PI-Periurban and PI-Urban; 16 ± 5.7 and 33 ± 6.5 for LI-Periurban and LI-Urban.

2.2 Litter and soil sampling and preparation

Leaf litter and topsoil were sampled at about 1 m from the trunk base of holm oak trees in each site, to better evaluate the effect of *Q. ilex* litter deposition on soil properties. Three sampling points were selected for each site. In each sampling point, three litter samples were collected and mixed to obtain a composite sample. In the same points, once cleaned from sampled litter, soil samples were collected at 0–20 cm depth with a hand spade and mixed to obtain a composite sample. Soil and litter samples were air dried, and soil was sieved at 2 mm.

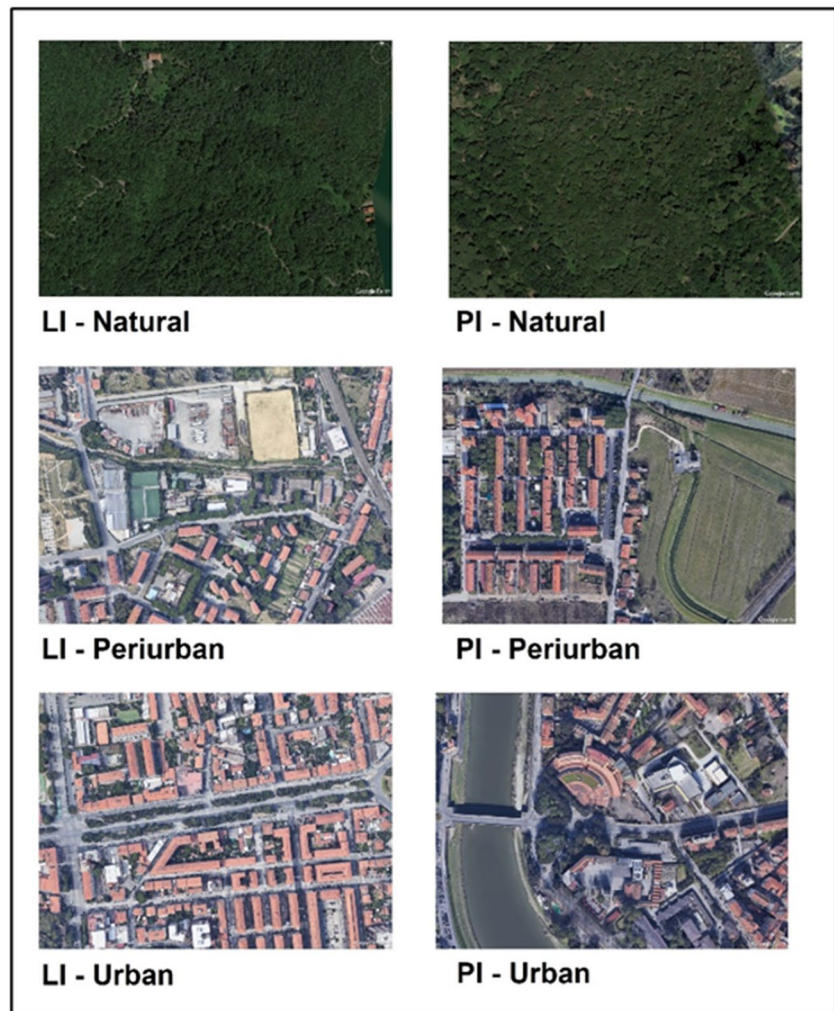
2.3 Soil physical and chemical analyses

Soil texture was determined by means of a laser granulometer Mastersizer 2000 (Malvern Panalytical Ltd., UK) equipped with a wet sample dispersion unit. Electrical conductivity (EC) and pH were determined on water extract, 1:5 and 1:2.5 (w:v), respectively, using specific electrodes (ASA-SSSA 1996).

2.4 Isotope signature

Carbon and nitrogen isotope compositions (δ¹³C, δ¹⁵N) were determined in leaf litter and soil as well as total organic carbon (TOC) and total nitrogen (TN) in soil. For the analysis of TOC and δ¹³C, soil samples were previously treated with hydrochloric acid to remove carbonates. All samples

Fig. 1 Aerial views of the study areas (PI=Pisa, LI=Livorno)



were dried and subsequently ground to a fine powder with a mortar. An aliquot of dry powder (0.5–2 mg) was then used to determine the TOC, TN and isotope composition of C and N using an IRMS (Isoprime, Cheadle, UK) coupled to an elemental analyser (NA1500, Carlo Erba, Milan, Italy). The isotope ratios $R = {}^{13}\text{C}/{}^{12}\text{C}$ and ${}^{15}\text{N}/{}^{14}\text{N}$ were determined to calculate $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, referring to the Vienna Pee Dee Belemnite (VPDB) and atmospheric N_2 standards, respectively, by applying the equation $\delta = R_{\text{sample}}/R_{\text{standard}} - 1$. The δ values were anchored using VPDB and atmospheric N_2 standards by means of IAEA standards. The precision of the stable isotope measurements was better than 0.1‰ for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$.

2.5 Ecoenzyme stoichiometry

The methods proposed by Marx et al. (2001) and Vepsäläinen et al. (2001) were used for the determination of hydrolytic enzyme activities with fluorogenic methylumbelliferyl (MUF)-substrates. The enzymes analysed were β -glucosidase (BG; EC 3.2.1.21), acid phosphatase (AP; EC

3.1.3.2), N-acetyl- β -D-glucosaminidase (NAG, EC 3.2.1.14). Re-wetted soil samples were treated with 25 ml Na-acetate buffer pH 5.5. Suspensions were obtained by treating the samples with UltraTurrax (IKA) homogeniser for 1 min at 9600 g. Aliquots of 100 μL were withdrawn and dispensed into a 96-well microplate. Finally, 100 μL of 1 mM substrate solution was added, giving a final substrate concentration of 500 μM . Fluorescence (excitation 360 nm; emission 450 nm) was measured after 0, 30, 60, 120, and 180 min of incubation with an automated fluorometric plate-reader (Infinite F200 pro TECAN) and enzyme activities were calculated as $\mu\text{mol MUB g}^{-1} \text{ C h}^{-1}$. The eco-enzyme stoichiometry approach was applied to investigate microbial resource limitation in soil of the three sites (natural, periurban, and urban). The enzyme activities (BG, AP and NAG) were normalized to TOC and the ratios $\ln(\text{NAG})/\ln(\text{BG})$ and $\ln(\text{NAG})/\ln(\text{AP})$ were calculated by the log-transformed ratio, according to method proposed by Sinsabaugh et al. (2009) and (2012). The results were reported in a scatterplot of ecoenzymatic stoichiometry, according to the method of Hill et al. (2012). The enzyme vector analysis was

also applied using the method reported by Moorhead et al. (2016), for assessing the magnitude of microbial resource limitation. The vector length (VL) and angle (VA) were calculated as follows: $VL = \sqrt{(\ln BG / \ln BG + \ln NAG)^2 + (\ln BG / \ln BG + \ln AP)^2}$; $VA = \text{Degrees}(\text{ATAN2}(\ln BG / \ln BG + \ln AP), (\ln BG / \ln BG + \ln NAG))$. Greater values of VL indicates greater C-limitation; the $VA < 45^\circ$ refers to N-limitation, while $> 45^\circ$ a P limitation.

2.6 Statistical analysis

Soil and litter analyses were carried out in three replicates and the statistical analysis was conducted using open-source R software (version 4.1.1). Data were subjected to one-way ANOVA and the HSD Tukey's test ($P < 0.05$). Pearson correlation was used to determine the relationship between soil and litter parameters, while linear regression was performed between isotope abundance and enzyme stoichiometry. Prior to one-way ANOVA and Pearson correlation, the homogeneity of variance was verified with Bartlett's test and the normality with the Shapiro–Wilk normality test. In the case normality and homogeneity was not confirmed, log-transformation was performed. Wilcoxon test was applied for non-normal data to highlight possible significant difference amongst natural and urban sites.

3 Results

3.1 Soil characteristics

The main soil properties along the urbanization gradients in Pisa and Livorno are reported in Table 1. In Pisa, pH was acidic in the natural site, while slightly alkaline in the peri-urban and urban sites. In Livorno, the soil pH ranged from neutral to slightly alkaline. The lowest EC and TP were recorded in both natural sites, while TOC was lower in PI-Urban. TN did not significantly vary along the gradient in

either of the two areas. The TOC/TN decreased from natural to urban sites in both areas.

3.2 Plant litter and soil isotope signature

In both metropolitan areas, $\delta^{15}\text{N}$ significantly increased in plant litter and in soil along the urbanization gradient (Fig. 2). The $\delta^{13}\text{C}$ did not differ in plant litter between the sites in Livorno and Pisa, while it increased in peri-urban and urban soils compared to the natural site in Livorno. In Pisa, the soil $\delta^{13}\text{C}$ was lower in urban sites, while the peri-urban site showed values similar to the natural site (Fig. 2). The $\delta^{15}\text{N}$ in litter positively correlated with $\delta^{15}\text{N}$ in soil. Both $\delta^{15}\text{N}$ in litter and soil negatively correlated with TOC, TOC/TN, and vector degree, while it correlated positively with TP, soil $\delta^{13}\text{C}$ and vector length. No significant correlations were detected between $\delta^{13}\text{C}$ in litter and soil parameters. The soil $\delta^{13}\text{C}$ showed positive correlations with TP, $\delta^{15}\text{N}$ in soil, vector length and $\delta^{15}\text{N}$ in litter. In contrast, $\delta^{13}\text{C}$ negatively correlated with vector degree, TOC and TOC/TN (Table 2).

3.3 Ecoenzymatic stoichiometry

Enzyme activities showed significant differences between natural and peri-urban/urban sites in Pisa and Livorno. Specifically, the activities of BG and AP were higher and lower, respectively, in natural than in the peri-urban and urban sites. The NAG did not differ among the sites studied in both cities (Table 3)(Fig. 3).

In Pisa, $\ln(BG)/\ln(NAG)$ was higher ($P=0.0254$) in urban and peri-urban sites than in the natural site, having the following values: 1.04 ± 0.038 ; 1.15 ± 0.051 and 1.14 ± 0.038 in natural, peri-urban and urban sites, respectively. The peri-urban and urban sites also differed for $\ln(NAG)/\ln(AP)$ ($P < 0.001$), with higher values (0.93 ± 0.035 and 0.92 ± 0.020 , respectively) than the natural site (0.79 ± 0.027). In Livorno, $\ln(BG)/\ln(NAG)$ had higher values ($P < 0.001$) in the urban (1.18 ± 0.018) and peri-urban (1.17 ± 0.011) sites

Table 1 Soil properties detected along urbanization gradient in the areas of Pisa and Livorno. EC=electrical conductivity; TOC=total organic carbon; TN=total nitrogen; TP= total phosphorus. Different letters represent statistically different values amongst sites for $P < 0.05$

Site	Texture	pH	EC dS/m	TOC %	TN %	TOC/TN	TP mg kg ⁻¹
PI-Natural	Sandy	4.7 ± 0.25a	0.46 ± 0.046a	9.4 ± 1.31b	0.27 ± 0.042a	35 ± 0.8b	224 ± 16a
PI-Peri urban	sandy-loam	7.7 ± 0.16b	1.16 ± 0.257b	6.5 ± 2.81b	0.55 ± 0.212a	13 ± 1.0a	1116 ± 450b
PI-Urban	sandy-loam	7.7 ± 0.16b	0.89 ± 0.142ab	2.7 ± 1.98a	0.22 ± 0.086a	13 ± 1.4a	666 ± 84ab
LI-Natural	loam	7.1 ± 0.04a	0.84 ± 0.02a	4.5 ± 0.43a	0.29 ± 0.042a	8.9 ± 1.60a	375 ± 51a
LI-Peri urban	sandy-loam	7.6 ± 0.02b	1.75 ± 0.07c	2.5 ± 0.03a	0.33 ± 0.018a	7.5 ± 0.48a	1478 ± 120b
LI-Urban	loam	7.6 ± 0.05b	1.48 ± 0.03b	1.8 ± 0.21a	0.28 ± 0.019a	6.6 ± 0.4a	1277 ± 13b

Fig. 2 The isotope signature in soil and plant litter detected along the urbanization gradient in the areas of Pisa (A, C) and Livorno (B, D). Different uppercase and lowercase letters represent the significant differences ($P < 0.05$) amongst sites for litter and soil, respectively. Different letters and asterisks represent statistically different values amongst sites for $P < 0.05$

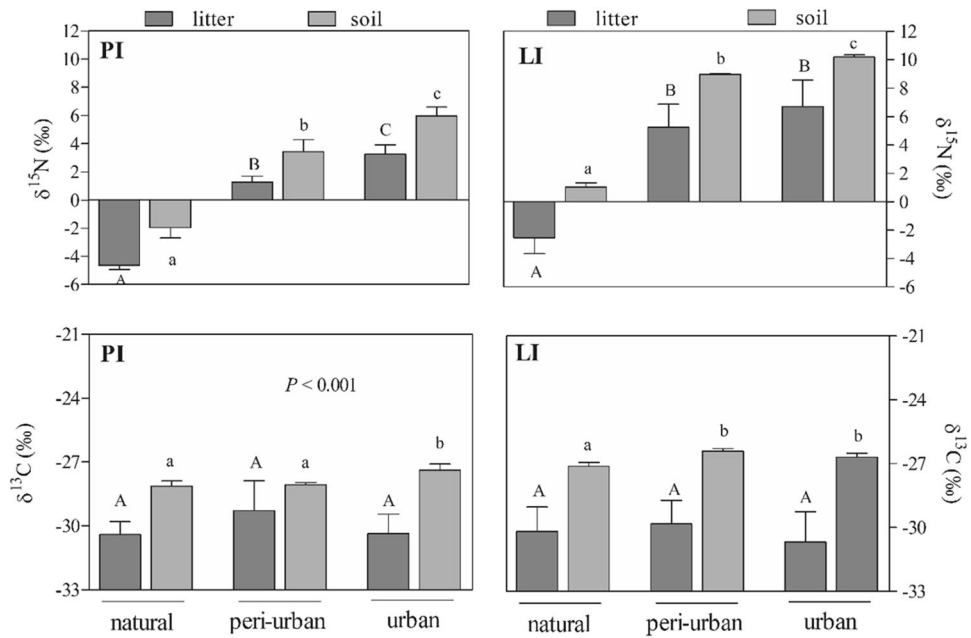


Table 2 Pearson correlations amongst litter and soil parameters. TOC=total organic carbon; TN=total nitrogen; VL=vector length; VD=vector degree. Bold values represent a significant correlation for $P < 0.05$

	TN	$\delta^{15}\text{N}$ soil	TOC	$\delta^{13}\text{C}$ soil	TOC/TN	VL	VD	$\delta^{13}\text{C}$ litter	$\delta^{15}\text{N}$ litter
$\delta^{15}\text{N}$ soil	-0.02								
TOC	0.43	-0.70							
$\delta^{13}\text{C}$ soil	-0.32	0.76	-0.87						
TOC/TN	-0.11	-0.75	0.84	-0.74					
VL	0.13	0.90	-0.69	0.62	-0.82				
VD	-0.19	-0.72	0.66	-0.55	0.87	-0.81			
$\delta^{13}\text{C}$ litter	0.30	-0.13	0.06	-0.01	-0.12	0.37	-0.13		
$\delta^{15}\text{N}$ litter	0.02	0.98	-0.65	0.69	-0.73	0.92	-0.72	0.27	
TP	0.47	0.83	-0.33	0.53	-0.63	0.81	-0.64	0.37	0.83

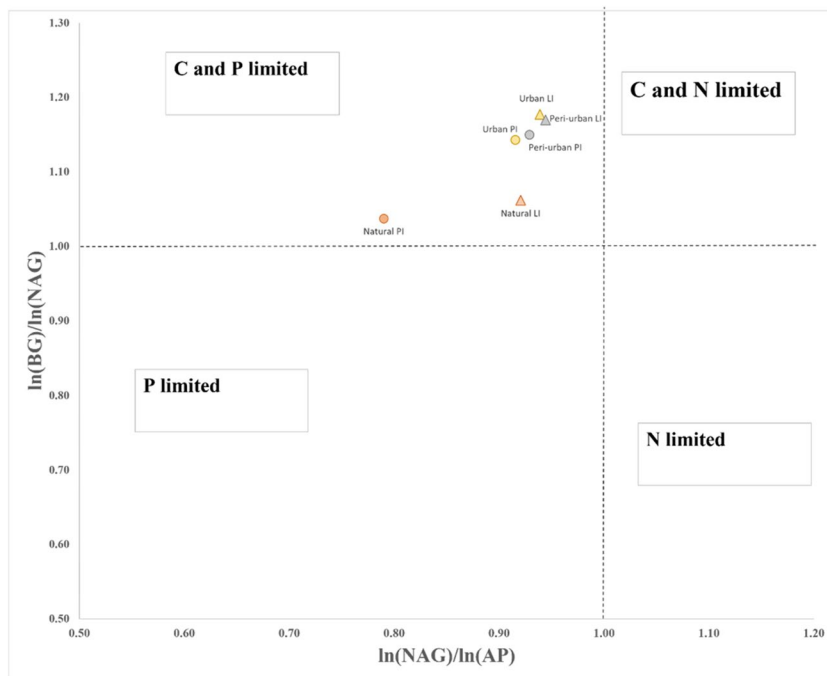
than in the natural site (1.06 ± 0.0004). In contrast, no differences were observed for $\ln(\text{NAG})/\ln(\text{AP})$ in the Livorno sites, with the following values: 0.92 ± 0.003 in natural;

Table 3 Log-transformed enzymatic activities in Pisa and Livorno areas, along urbanization gradient. BG= β -glucosidase; AP=acid phosphatase; NAG=N-acetyl- β -D-glucosaminidase. Different letters represent statistically different values amongst sites for $P < 0.05$

City	sites	$\ln\text{BG}$	$\ln\text{AP}$	$\ln\text{NAG}$
Pisa	Natural	$15.2 \pm 0.26\text{a}$	$17.0 \pm 0.27\text{b}$	$14.9 \pm 0.19\text{a}$
	Peri-urban	$16.6 \pm 0.52\text{b}$	$16.0 \pm 0.28\text{a}$	$15.4 \pm 0.17\text{a}$
	Urban	$17.0 \pm 0.49\text{b}$	$16.2 \pm 0.38\text{a}$	$15.7 \pm 0.53\text{a}$
Livorno	Natural	$16.9 \pm 0.04\text{a}$	$17.2 \pm 0.01\text{b}$	$16.4 \pm 0.01\text{a}$
	Peri-urban	$17.7 \pm 0.07\text{b}$	$16.7 \pm 0.02\text{a}$	$16.2 \pm 0.14\text{a}$
	Urban	$17.7 \pm 0.06\text{b}$	$16.7 \pm 0.08\text{a}$	$16.1 \pm 0.17\text{a}$

0.94 ± 0.02 in peri-urban; 0.94 ± 0.018 in urban sites. The vector length revealed significant differences in carbon limitation between natural and urban and peri-urban/urban sites in Pisa and between natural and urban sites in Livorno. The vector degree was higher than 45° in all the sites studied. However, a different magnitude of phosphorus limitation was observed in Pisa: the urban and peri-urban sites had lower values than the natural site. No difference in vector degree was detected along the urbanization gradient in Livorno (Fig. 4). Vector length correlated positively with $\delta^{15}\text{N}$ in litter, TP, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in soil and correlated negatively with TOC and TOC/TN. The vector degree showed positive correlation with TOC and TOC/TN, while it was negatively correlated with TP, $\delta^{13}\text{C}$ in soil and also with $\delta^{15}\text{N}$ in soil and litter (Table 2). Significant linear regression between vector length and $\delta^{15}\text{N}$ in soil was also detected (Fig. 5).

Fig. 3 Scatterplot of eco-enzyme stoichiometry in Pisa (PI) and Livorno (LI) soils, along the urbanization gradient. BG = β -glucosidase; NAG = N-acetylglutamate; AP = acid phosphatase



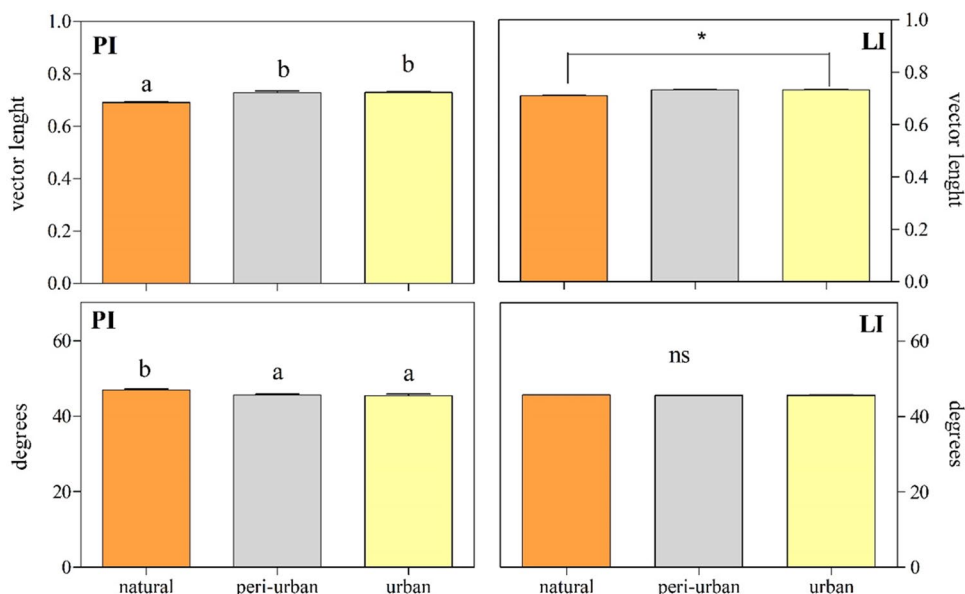
4 Discussion

Stable isotopes and enzyme activities were suitable indicators of soil functionality (i.e. carbon and nutrient cycles), along an urbanization gradient. Specifically, the $\delta^{15}\text{N}$ in plant litter was a suitable indicator for N deposition, while soil $\delta^{15}\text{N}$ was a suitable proxy of the mineralization processes. The coenzymatic stoichiometry and vector analysis detected an imbalance in microbial carbon and nutrient acquisitions. Specifically, an imbalance in enzyme activities toward C vs N and P acquisition was observed as well as an increase in phosphorous availability for the microbial

community in peri-urban and urban sites. The differences in microbial responses and soil $\delta^{15}\text{N}$ detected among the study sites were related to the soil properties, revealing an increase of the microbial carbon requirements and N mineralization along the urbanization gradient.

Natural, peri-urban and urban areas differed in terms of their soil properties. The pH in peri-urban and urban sites resulted slightly alkaline, as generally observed in urban soil, due to the presence of anthropogenic materials, such as calcareous building residues, which also affected the EC (Foldal et al. 2022). The low TOC and TOC/TN in urban and peri-urban sites could be mainly a consequence of several

Fig. 4 Vector length (unitless) and degrees evaluated for the areas of Pisa (PI) and Livorno (LI), along urbanization gradient. Different letters represent the significant differences for $P < 0.05$. ns = not significant



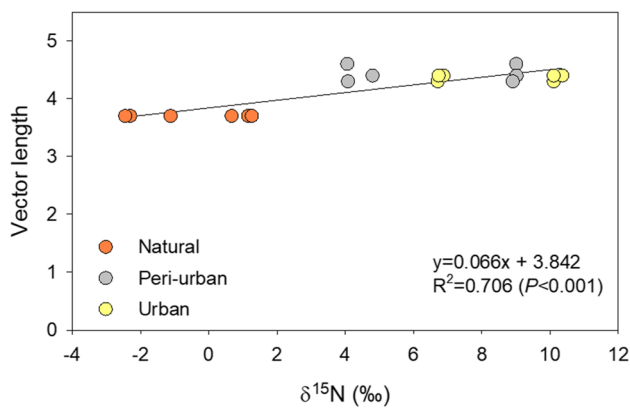


Fig. 5 Linear regression between vector length and soil $\delta^{15}\text{N}$ in all studied sites

urban factors affecting the presence of organic matter in soil. These might include soil physical disturbance and erosion, limited organic matter input and soil compaction (Yoon 2022). Soil disturbance also influences the formation process of urban soil that results in the early stage of pedogenesis (Charzyński et al. 2017), leading to serious alterations such as N deficiency, as seen for “young” soil (Lambers et al. 2008). In N-limited terrestrial ecosystems, N deposition can lead to “N saturation”, with consequences for N availability and dynamics. In particular, high levels of atmospheric N deposition can increase the TN, leading to a more rapid N cycling in urban forests than in natural woodland and its impact depends on the degree of N limitation and retention capacity of the ecosystem (Kopáček et al. 2013; Du et al. 2022). In our study, atmospheric NO_2 increased from peri-urban to urban areas in both cities, suggesting that the increase in N deposition occurred along the urbanization gradient. The enhanced N deposition increased the leaf $\delta^{15}\text{N}$ in plants growing in the urbanized sites, thanks also to the capability of *Q. ilex* leaves to capture atmospheric N deposition (Scartazza et al. 2023). Accordingly, leaf litter of the urbanized sites was ^{15}N -enriched compared to the natural sites. It is worth noting how ^{15}N enrichment of litter affected soil $\delta^{15}\text{N}$, but not the TN in soil, differently from what has been seen in forest ecosystems (Cheng et al. 2019). This is due also to the fact that N deposition could spread outside the city (Pulikova and Gorovtsov 2022). The measurement of $\delta^{15}\text{N}$ in plant litter was a sensitive indicator of atmospheric pollution in city areas with a widespread atmospheric N deposition. The consumption of available N by enhanced plant growth and/or the promoting effect of N deposition on nitrification and denitrification processes could justify the similar TN in all sites, as seen in other studies (e.g., Guo et al. 2023). The soil $\delta^{15}\text{N}$ enrichment was mainly correlated to the urban soil condition (i.e. pH, TOC, TOC/TN). In fact, the alkaline pH and the low TOC/TN can raise nitrification

and the following denitrification process, thus increasing N leaching and emission of ^{15}N -diluted nitrogen forms (Zhang et al. 2010; Hui-Juan et al. 2018; Pulikova and Gorovtsov 2022). Along the urbanization gradient, variations in isotope signature were also observed for $\delta^{13}\text{C}$ in soil, while no differences occurred in plant litter. The uniformity of $\delta^{13}\text{C}$ in plant litter suggests that plants did not differ in carbon isotope discrimination and rely on a similar isotopic CO_2 source during photosynthetic assimilation, probably due to a mixing of urban air, free troposphere and the air outside the boundary of the city area (Xu et al. 2017). The increase in the soil $\delta^{13}\text{C}$ along the urbanization gradient suggested an enhancement of microbial respiration and soil organic matter decomposition. This is mainly correlated to urban soil conditions that affected the mineralization process, confirming $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ as soil indicators of mineralization processes in urban areas (Scartazza et al. 2023).

The coenzymatic stoichiometry, coupled with vector analysis, revealed an imbalance in enzyme activities in C vs N/P acquisition and a reduction in microbial P limitation along the urbanization gradient (Hill et al. 2012; Sinsabaugh et al. 2009 and 2012; Moorhead et al. 2016). The increase in vector length (microbial C limitation) as well as the rise in BG activity suggests that microorganisms increased their C demand relative to N and P, leading to an increase in organic carbon degradation in urban and peri-urban sites (Adetunji et al. 2017; Chen et al. 2018). The significant reduction in vector degree (microbial P/N limitation), especially in Pisa, revealed a reduction in microbial P needs relative to N in peri-urban and urban sites compared to the natural site, as also confirmed by reduction in AP. On the contrary, NAG did not change along the gradient, suggesting that atmospheric N deposition did not affect the enzyme activity, as differently seen in N fertilization experiments (Chen et al. 2018). The reduction in P-acquiring enzymes was related to microbial strategy in reducing a specific nutrient-related enzyme when the nutrient is more bioavailable (Allison et al. 2010). In fact, a P enrichment in urban soil was observed, probably due to anthropogenic waste deposition (e.g. construction residues) (Hu et al. 2011), alleviating the microbial P limitation due to TP being negatively correlated with vector degree. The reduction of P limitation could support the microbial activities in organic matter mineralization in peri-urban and urban soils (Chen et al. 2014) and enhance the nitrification and denitrification processes. This is because P limitation negatively correlates with gene expressions involved in these processes (Cui et al. 2020). However, alleviating the microbial P limitation can lead to an increase in microbial metabolism, thus soil CO_2 release, contributing to the degradation of native organic matter in soil with limited C resources, as seen by Cui et al. (2022). Differences in microbial resource acquisition strategies, along the urbanization gradient, were related to soil properties, confirming the important role of

enzyme activity as an indicator of changes in soil condition (Karaca et al. 2011). The vector length correlated negatively with TOC and TOC/TN, indicating that C limitation had been accentuated by the low TOC and TOC/TN in urban and peri-urban areas. In fact, soil organic matter content is one of the main factors in supporting biological activities in urban soils (Gómez-Brandón et al. 2022). However, the N inputs in a terrestrial ecosystem over time can increase the microbial C requirements due to the reduction of recalcitrant organic carbon degradation, of which *Q. ilex* litter is rich (De Marco et al. 2010; Chen et al. 2018). The microbial C limitation was strictly correlated to $\delta^{15}\text{N}$ in soil, as confirmed by linear regression analysis, suggesting a close relationship between microbial C requirements and the mineralization process. The $\delta^{15}\text{N}$ in soil could be a proxy of microbial investments in C vs N and P acquisitions.

5 Conclusions

Isotope signature (C and N stable isotopes) and ecoenzymatic stoichiometry (vector analysis) were suitable soil indicators of soil functionality (i.e. carbon and nutrient cycles), as they are sensitive to environmental changes along the urbanization gradient. Specifically, the measurement of stable isotope in plant litter and soil gave (valuable?) insights into soil biogeochemical cycle processes, revealing an enhanced organic matter degradation as well as nitrification and denitrification in urban and peri-urban sites as opposed to natural sites. The increase in organic matter degradation was confirmed by ecoenzymatic stoichiometry and vector analysis. Along the urbanization gradient, the microbial community increased its production of enzymes for C acquisition. The reduction in microbial P limitation could support the nitrification and denitrification processes in urban soil with limited carbon sources. These indicators revealed the need to improve the carbon pool in urban soil (e.g. by increasing the plant litter input) to reduce the microbial C demand and N mineralization, thus improving the balance between microbial nutrient and energy requirements and resource availability. Although the isotope and enzyme activities are easy to measure, future studies are needed to focus on the definition of thresholds for the data interpretation so as to help policy making in the design and management of urban green infrastructures.

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Declarations

Competing interests The authors declare no competing interests.

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