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Foliar heavy metals and stable isotope (δ^{13}C, δ^{15}N) profiles as reliable urban pollution biomonitoring tools

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ABSTRACT

Anthropogenic heavy metal pollution is an important health issue in urban areas, and therefore rapid and inexpensive monitoring in time and space is desirable. This study aimed (i) to assess the suitability of *Tilia cordata* leaves as a valuable heavy metal bioindicator, including seasonal changes in concentrations and (ii) to evaluate the use of leaf carbon and nitrogen isotope composition (δ^{13}C and δ^{15}N) as novel indicators of urban heavy metal pollution. Leaves were collected from three different pollution intensity locations (Bilbao, Vitoria, and Muskiz) in the Basque Country (northern Spain). Analysis of leaf heavy metals related to traffic emissions and δ^{13}C and δ^{15}N determinations were carried out during July-October 2018. Leaf samples from Bilbao, the most populated and traffic-intense location, showed the highest concentration of heavy metals (mainly from polluted air). Additionally, the two urban areas, Bilbao and Vitoria, showed stronger correlation between these heavy metals, indicating a traffic-related source of emissions. The source of contamination (soil or air) in relation to elements and optimal sampling time is discussed herein. On the other hand, Pearson correlation analysis revealed significant trends between leaf δ^{13}C and δ^{15}N and the studied heavy metals, especially Pb, Cr and Cd, supporting the hypothesis of δ^{13}C and δ^{15}N as tools to distinguish locations according to their heavy metal pollution levels. To our knowledge, this is the first time that δ^{13}C and δ^{15}N have been used as monitoring tools in heavy metal pollution and consequently more research is still needed to calibrate this tool through extensive vegetation screening.

1. Introduction

Rapid urbanization and industrialization during the last century have created substantial environmental pressures, with one of the most important being air pollution (Grigoratos et al., 2014). In these urban areas, in addition to gaseous pollutants, urban transportation and industrial activities release particulate matter containing heavy metals that can be a significant threat to humans and the environment (Kampa and Castanas, 2008; Adamiec et al., 2016). Between traffic heavy metals, emission of Ba, Cd, Co, Cr, Cu, Hg, Mn, Mo, Ni, Pb and Zn has been described (Bretzel and Calderisi, 2006) from brake pads, tyres, clutch plates and exhaust gas combustion (Falahi-Ardakani, 1984; Hjortenkrans et al., 2007; Adamiec et al., 2016). Exposure to chemical contaminants and particles from polluted air is associated with higher mortality and morbidity (García de Jalón et al., 2019). The World Health Organization (WHO) reported that in 2012, about 7 million people died because of fine-particulate air pollution exposure. Due to the impact on human health, heavy metal concentrations should be monitored in urban areas (Jiang et al., 2017).

Currently, the environmental quality of cities is characterized by information provided from monitoring stations that measure air quality. However, monitoring station assessment has been frequently conditioned by the high cost of instrumental monitoring methods, and difficulties in carrying out extensive sampling in time and space (Anicic et al., 2011). Therefore, there has been an increasing interest in using indirect monitoring methods such as bioaccumulation of pollutants in

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tree leaves. Comparatively, they present some advantage such as lower cost, residence time after deposition of metals in leaves and, if distributed widely enough, better spatial recording of the pollution assessment (Smodiš et al., 2004; Tomašević et al., 2004).

The use of tree leaves as bioindicators and biomonitors has been shown previously (Doganlar et al., 2012) to be efficient for environmental characterization because of the capacity of leaves to retain/absorb heavy metal pollution from the air (and the soil). Through leaves, urban trees can remove pollutants from the air via their stomata and by surface particulate matter deposition (Nowak et al., 2006). Additionally, heavy metals can be taken up by roots and further translocated and accumulated in leaves. The deposition/uptake of heavy metals in plant leaves depends on various factors, such as distance from the pollution source, differences between plants in anatomy and physiology (e.g. evergreen/deciduous, woody leaves, phytosiderophores etc.), the physical properties and the emission intensity of the heavy metals, or edaphic-climatic factors (e.g. wind and precipitation, pH, redox state) (Doganlar et al., 2012; Alahabadi et al., 2017). Due to their ubiquitous presence in most streets and parks, as well as suitable morphology, canopy structure and leaf epicuticular waxes, species in the Eurasian native genus *Tilia* have been used previously as biomonitors of heavy metal pollution (Piczak et al., 2003; Tomašević et al., 2004, Aničić2011; Serbula et al., 2013; Kalinovic et al., 2017) because of the capacity of leaves to accumulate heavy metals when growing and developing in polluted locations.

During the last decades, the stable carbon and nitrogen isotopic compositions ($\delta^{13}C$ and $\delta^{15}N$) in mosses and tree leaves have been used to identify the sources of atmospheric pollution (Xiao et al., 2011; Wang and Pataki, 2012). Due to the relatively depleted $\delta^{13}C$ in fossil fuels, their combustion results in a more ^{13}C-depleted CO_2 in urban areas than in rural locations (Widory and Javoy, 2003). On the other hand, leaf $\delta^{15}N$ has been previously used to distinguish between the two main N pollution sources, NO_x and NH_x (Pardo et al., 2007; Redling et al., 2013). Generally, higher $\delta^{15}N$ values have been reported for the main urban N-deposition form (NO_x) compared to the main rural and agricultural N-deposition form (NH_x) (Xiao et al., 2011).

However, despite this use of C and N isotopes in previous pollution works, to our knowledge, no previous studies have reported the use of both foliar $\delta^{13}C$ and $\delta^{15}N$ as indicators of heavy metal pollution, which could help to rapid asses of this type of urban pollution and the development of remediation strategies. Additionally, the combination of these two analytic tools could help to a more precise distinguish between anthropogenic contaminations sources (traffic emissions or industrial production) and differentiate from natural sources. The objectives of this study were (i) to assess air quality classification using data obtained from monitoring stations and elemental analysis carried out in tree leaves; (ii) to determine seasonal concentrations of elements in leaves of *Tilia cordata* Mill. and to assess their suitability as a heavy metal bioindicator and (iii) to assess the feasibility of leaf $\delta^{13}C$ and/or $\delta^{15}N$ as indicators of urban heavy metal pollution.

2. Materials and methods

2.1. Study area

The study was conducted in three different street locations in the Basque Country (northern Spain): Sabino Arana Street, Bilbao; Pedro de Asua Street, Vitoria; and Somorrostro Street, Muskiz (Fig. 1). The sampling sites included representative urban zones comprising (i) a high population (342,810 people) and heavy traffic with Bilbao's city centre being representative; (ii) a medium population (243,815 people) and moderate traffic intensity with Vitoria's city centre as representative and (iii) a town with a low population (7517) and low traffic intensity but some industrial activity (oil refinery), with Muskiz being representative. Population data from 2018 were obtained from the Basque Statistics Institute (Eustat, 2018).

2.2. Air pollution and weather data

For air pollution concentrations and weather variables, this study compiled data on a daily basis from the monitoring stations of the General Administration of the Autonomous Community of the Basque Country (Gobierno Vasco, 2018). The air pollution data included daily atmospheric concentration levels of NO_2, NO, NO_x, SO_2 and PM_{10}. Integrative air pollution data from July 1st (15 days before the first sampling) until the moment of the last sampling day (October 8th) were collected from the online portal of the Basque Country (https://opendata.euskadi.eus/catalogo/-/calidad-aire-en-euskadi-2018/). Furthermore, daily weather data including precipitation, humidity, and wind intensity, as well as maximum, minimum and average temperatures, were also collected during the sampling period (July-October; Fig. S1), from the online portal of the Basque Country (https://opendata.euskadi.eus/…/-/estaciones-meteorologicas-lecturas-recogidas-en-2018/). The meteorological station selected for Bilbao and Muskiz was the same because these locations are really close.

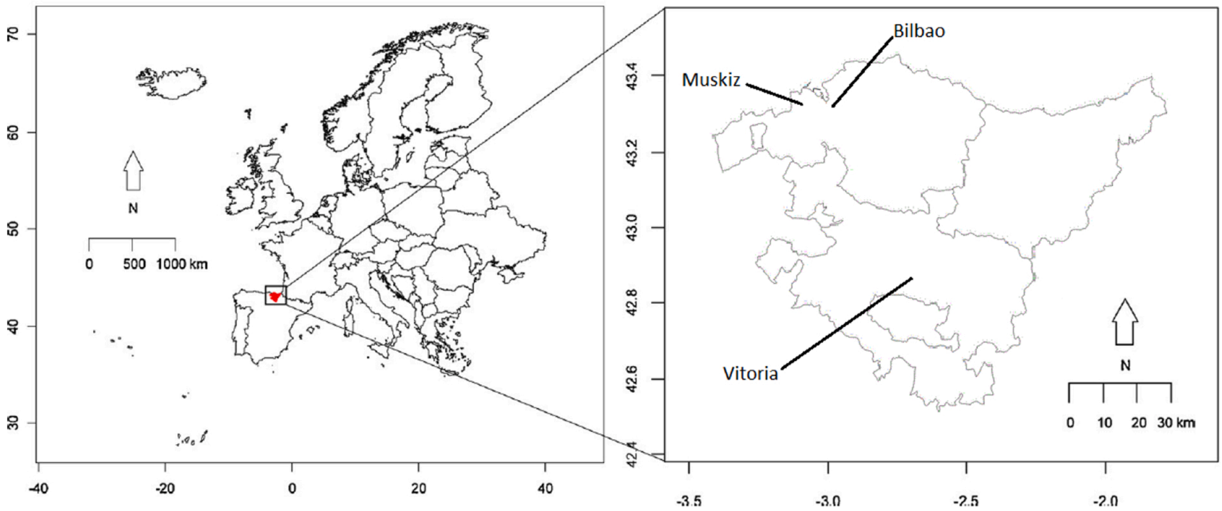

Fig. 1. Geographical location of the sampling sites in the Basque Country (Bilbao, Vitoria and Muskiz). The left panel shows the map of Europe; Basque Country province areas are enlarged in the right panel.

2.3. Sampling

In the three locations, leaves were sampled from deciduous small-leaved lime (*T. cordata*) during the sampling period of July-October (Fig. S1) in 2018. Collection of the samples was carried out according to the standardized protocol as follows. In each location six replicates were taken for each sampling time, mixing five healthy fully developed leaves randomly detached from a height of 1.5–2.0 m above the ground (usually two thirds of the canopy of each tree) while wearing polyethylene gloves to avoid contaminating the samples. The sampled material was stored in polyethylene containers and transported to the laboratory in a portable icebox (temperature below 5 °C). Once in the laboratory, samples without washing were dried at 65 °C during 48 h and stored under a low-humidity atmosphere until grinding and further analysis to estimate absorbed and deposited pollutants.

2.4. Element analyses

With the aim of a comprehensive view of leaf anthropogenic contamination, the presence and concentrations of a range of heavy metals (i.e., Al, As, Be, Bi, Cd, Co, Cr, Cu, Fe, La, Li, Mn, Mo, Ni, Pb, Rb, Sb, Se, Sr, Ti, V, and Zn) were analysed in leaf samples that previously had been ground to a fine powder. For each sample, 100 mg of milled material was weighed and stored in a 1.5 mL Eppendorf microtube. The analysis was carried out at the ionomic service of the Centre for Edaphology and Applied Biology (Murcia, Spain) using ICP/OES (inductively coupled plasma/optical emission spectrometry, iCAP 6500 Duo, Thermo Fisher Scientific, Waltham, USA).

Although considered in the original design, the following elements (As, Be, Bi, La, Mo, Sb and Se) were under the detection limit of the equipment (<0.01 mg kg-1) and were subsequently omitted from this study.

2.5. Carbon and nitrogen content and isotopic composition analyses

Leaf C and N content and isotopic composition were determined at the Serveis Cientifico-Técnics of the University of Barcelona (Barcelona, Spain) based on sample dynamic combustion, using an elemental analyser (FlashEA1112, ThermoFinnigan, Waltham, MA, USA) equipped with a MAS200R autosampler. Dried leaf samples were ground to a fine powder, and 1 mg was weighed and stored in tin capsules for elemental analyses (MX5 microbalance, Mettler-Toledo, Columbus, OH, USA), which were carried out according to Soba et al. (2019a).

Leaf C and N isotopic composition were determined using an elemental analyser (EA1108;Carlo Erba Instrumentazione, Milan, Italy) equipped with a MAS200R autosampler and coupled to an isotope ratio mass spectrometer (Delta C; Finnigan, Mat., Bremen, Germany) operating in continuous flow mode. The ^{15}N/^{14}N ratio in *T. cordata* leaf material was expressed in δ notation (δ^{15}N) following the equation as described in Soba et al. (2019b). The overall abundance of ^{13}C relative to ^{12}C (δ^{13}C) in leaf dry matter was calculated according to Farquhar et al. (1989).

2.6. Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics for Windows, Version 20.0. (IBM Corp. Armonk, NY, USA). Differences among the three locations and the five sampling dates were evaluated by two-way Analyses of Variance (ANOVA), with the location and sampling date as fixed factors. All data were tested for normality (Kolmogorov–Smirnof test) and homogeneity of variances (Levene's test). Tukey post hoc tests were used to determine statistical differences between locations. To study correlations between the variables, Pearson correlation analyses were calculated. For ANOVA and Pearson correlation analysis, the results were considered to be significant when $p < 0.05$. Heatmap and principal component analysis (PCA) for the three locations along the sampling period were conducting using XLSTAT 2008 (Addinsoft, Paris, France) software.

3. Results

3.1. Air pollutants and meteorological data

The seasonal evolution of the five air pollutants (NO, NO_2, NO_x, SO_2 and PM_{10}) measured in the three monitoring stations near to our sampling locations is depicted in Fig. 2. All pollutants follow the same pattern and could be arranged in order as Bilbao > Vitoria > Muskiz, except for $SO_{2,}$ where the lowest values throughout the sampling period were found in Vitoria.

On the other hand, daily meteorological data showed higher precipitation and lower average temperature during the sampling period in Vitoria compared to Bilbao or Muskiz (Fig. S1).

3.2. Element concentrations

The mean concentrations of metals as determined by ICP/OES of *T. cordata* leaves from three different locations in the Basque Country are shown in Table S1. One-way ANOVA indicated significant differences between locations for all the elements studied (p < 0.05). In order to reduce the complexity of the multivariate data and identify patterns between samples, principal component analysis (PCA) was performed (Fig. 3a). The PCA analysis revealed that the first two principal components explained 74.3 % of total variation and a clear separation between locations could be seen. In plot segregate the three locations and

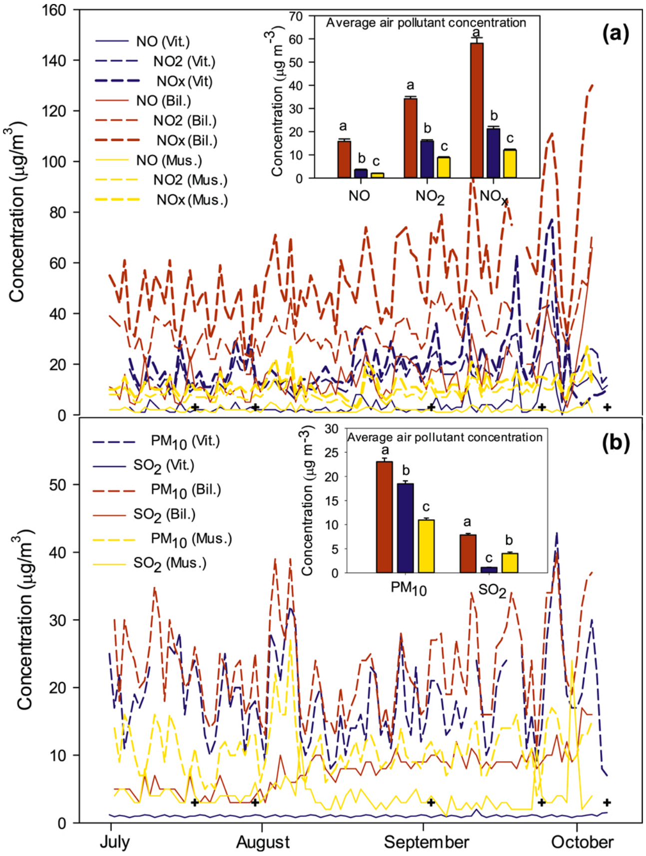

Fig. 2. Daily measures of a) NO, NO_2, NO_x and b) SO_2, PM_{10} (µg/m3) from July to October at the Bilbao (red), Vitoria (blue) and Muskiz (yellow) monitoring stations. The average values for each parameter are showed in a separate graph. Data correspond to the mean ± SE of n = 100 measures. Letters indicate significant differences between locations (Tukey post hoc test P < 0.05).

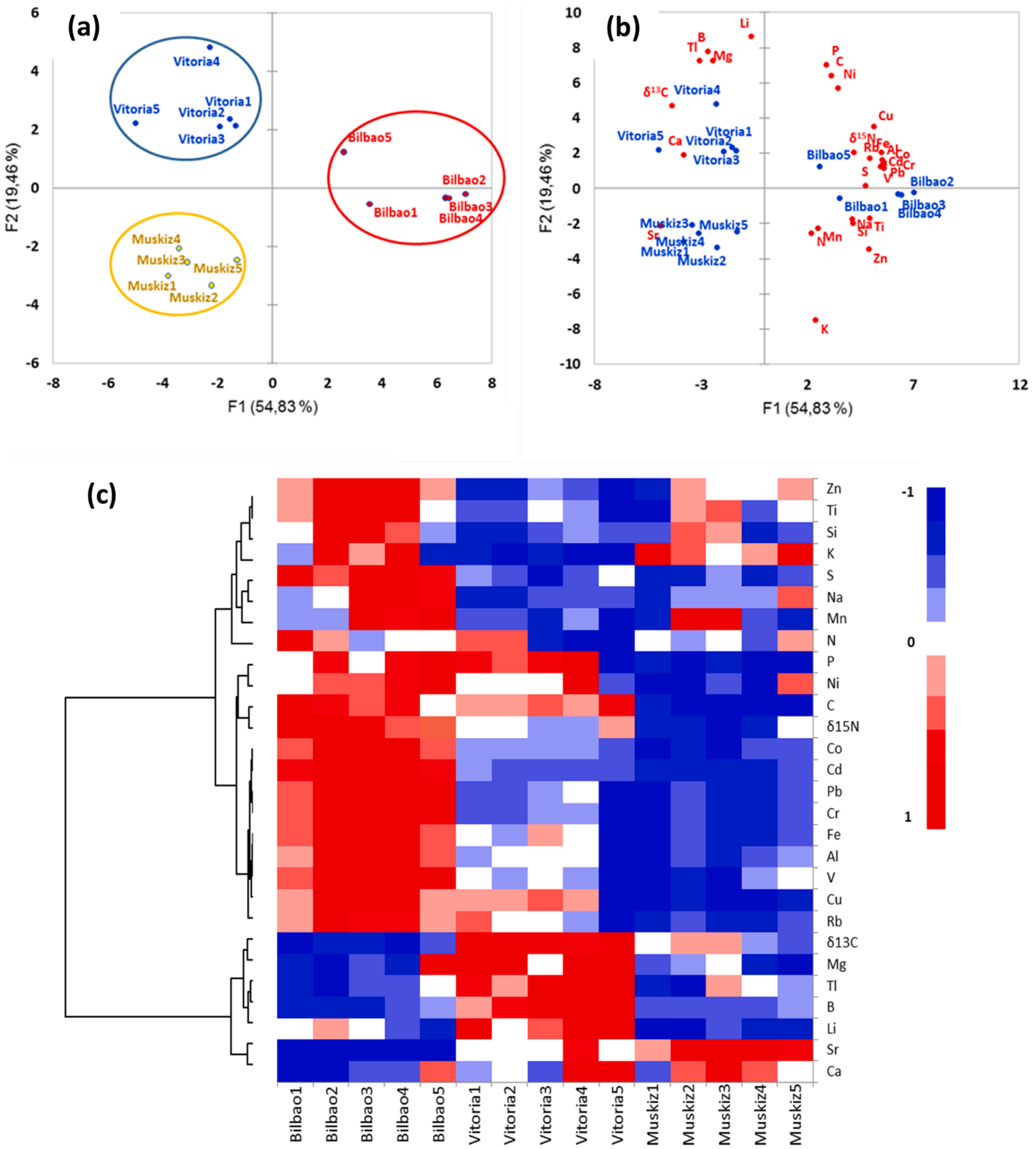

Fig. 3. Graphic representation of a) principal component analysis (PCA); b) scatter plot distribution of the different elements and c) Heatmap of analysed elements from three different locations (Bilbao, Vitoria and Muskiz) in the Basque Country, Spain and five sampling points (July-October 2018). Six replicates were examined per location and sampling point.

revealed that PC1 (54.8 % of the total variance) was mostly determined by the contributions of heavy metals related to traffic emissions (Pb, Cd, Cr, Fe, Al…), and was mainly associated with the most contaminated location (Bilbao; Fig. 3b).

With the aim of a better understanding of this response, heatmap representation and hierarchical clustering analysis were undertaken (Fig. 3c). The hierarchical clustering allowed the grouping of variables into two major clusters. Cluster 1 was mostly comprised of elements that were higher concentrations in Bilbao than at the other two locations. In contrast, cluster 2 was formed of elements that were in lower concentrations in Bilbao (Mg, Tl, B, Li, Sr and Ca). Cluster 1 could be subdivided in 2 sub-clusters: one with eight elements that were at a lower concentration in Vitoria (Zn, Ti, Si, K, S, Na, Mn and N) and the other mainly composed of heavy metals related to traffic emissions (Ni, Cd, Pb, Cr, Cu…) that had lower concentrations in Muskiz.

Pearson correlation analyses were conducted separately for the three different locations to illustrate the relationships between metals (Tables 1–3). The results revealed that metals related to traffic and industrial emissions (Al, Cd, Cr, Cu, Fe, Ni, Pb, Ti, V and Zn) were highly significant and positively correlated in the most populated and traffic-intense locations of Bilbao and Vitoria. However, in the low populated but industrial location, the correlations between these metals were not significant or, if significant, were weaker compared to Bilbao and Vitoria. All this suggests a similar pollution source, which in this case implies traffic emissions.

Finally, these ten elements that were highly correlated in the most populated locations were analysed separately for each of the three sites to determine their temporal evolution throughout the vegetative season, and the differences in concentrations between locations. The mean foliar heavy metal concentrations could be arranged in the following order: Fe > Al > Zn > Cu > Ti > Cr > Pb > Ni > V > Cd. The graphs for the selected metals are shown in Fig. 4. Differences between locations were

Table 1
Pearson correlation coefficients between elements measured by ICP/OES in leaves of *Tilia cordata* from Bilbao. The coefficients shown in bold are significant.

}	**Al**}	B}	Ca}	**Cd**}	**Co**}	**Cr**}	**Cu**}	**Fe**}	K}	Li}	Mg}	Mn}	Na}	**Ni**}	**Pb**}	P}	Rb}	Si}	S}	Sr}	**Ti**}	Tl}	**V**}	**Zn**}
Al																								
B	.104																							
Ca	.154	**,618****																						
Cd	**,593****	−.014	−.039																					
Co	**,754****	.038	.065	**,592****																				
Cr	**,845****	**,430****	**,335****	**,583****	**,632****																			
Cu	**,888****	.020	−.034	**,559****	**,660****	**,735****																		
Fe	**,940****	.148	.162	**,568****	**,655****	**,903****	**,884****																	
K	,263*	−.192	**-,414****	.121	.176	.071	**,338****	,250*																
Li	**,500****	−.109	−.152	**,421****	**,611****	**,395****	**,500****	**,459****	.076															
Mg	−.161	**,648****	**,779****	−.184	−.095	.048	**-,345****	−.209	**-,424****	−.177														
Mn	**,448****	**,754****	**,790****	.203	,274*	**,680****	,313*	**,489****	−.214	−.060	**,585****													
Na	.070	.126	.092	.002	−.043	.114	.187	.195	.021	.079	−.038	.044												
Ni	**,760****	**,410****	,313*	**,635****	**,684****	**,897****	**,596****	**,772****	−.019	,454**	.127	**,637****	.068											
Pb	**,879****	,268*	.166	**,662****	**,676****	**,873****	**,832****	**,882****	.069	**,529****	−.111	**,510****	.112	**,846****										
P	**-,385****	.131	−.162	-,262*	-,311*	-,298*	-,283*	**-,344****	,314*	-,261*	.108	−.207	.186	**-,380****	**-,438****									
Rb	.071	**-,339****	**-,507****	,249*	.199	−.156	.161	−.057	**,680****	.200	**-,358****	**-,450****	−.164	−.132	.010	**,319****								
Si	.142	−.132	−.164	.137	−.020	−.006	.160	.120	.100	.078	**-,323****	−.073	.023	.127	.228	−.201	.002							
S	,282*	,260*	.147	.002	.159	,288*	**,369****	**,347****	.239	.200	.078	,296*	.145	.211	.242	−.222	−.143	−.031						
Sr	**,330****	**,524****	**,846****	.198	,284*	**,408****	.178	,275*	**-,393****	.172	**,586****	**,690****	.031	**,450****	**,410****	**-,377****	**-,335****	.055	.157					
Ti	**,952****	−.045	.004	**,643****	**,724****	**,783****	**,890****	**,903****	,286*	**,545****	-,306*	,312*	−.001	**,713****	**,878****	**-,433****	.162	,297*	.222	,243*				
Tl	−.001	,310*	**,571****	-,277*	.051	.061	−.111	.039	−.095	−.147	**,462****	**,457****	−.016	.076	−.120	.054	**-,332****	−.159	.148	**,338****	−.116			
V	**,894****	.180	.132	**,676****	**,801****	**,857****	**,813****	**,855****	.154	**,600****	−.062	**,443****	.019	**,796****	**,879****	**-,370****	.123	−.102	,254*	**,324****	**,852****	−.112		
Zn	**,902****	.110	.127	**,583****	**,695****	**,779****	**,933****	**,879****	,246*	,523**	−.151	**,462****	.107	**,714****	**,890****	**-,454****	.085	.177	**,438****	**,363****	**,905****	−.046	**,863****	

Asterisks indicate significant differences: * $p < 0.05$, **$p < 0.01$.

Table 2
Pearson correlation coefficients between elements measured by ICP/OES in leaves of *Tilia cordata* from Vitoria. The coefficients shown in bold are significant.

	Al	B	Ca	Cd	Co	Cr	Cu	Fe	K	Li	Mg	Mn	Na	Ni	Pb	P	Rb	Si	S	Sr	Ti	Tl	V
Al																							
B	−.006																						
Ca	−.141	**,827****																					
Cd	**,645****	−.181	−.077																				
Co	.341	−.187	−.252	,473*																			
Cr	**,915****	.258	.041	,514*	.376																		
Cu	**,977****	−.035	−.165	**,634****	.370	**,911****																	
Fe	**,959****	−.012	−.145	**,620****	,445*	**,941****	**,977****																
K	**,645****	-,411*	−.345	**,685****	.404	,424*	**,669****	**,601****															
Li	−.046	**,784****	**,848****	.002	−.083	.189	−.042	.018	−.320														
Mg	.164	**,524****	**,764****	.160	-,429*	.180	.142	.091	−.083	**,573****													
Mn	.350	**,611****	**,750****	.153	−.208	,427*	.341	.335	−.086	**,714****	**,874****												
Na	−.160	**,676****	**,683****	−.292	−.187	.004	−.177	−.124	−.395	**,742****	,427*	**,630****											
Ni	**,585****	,451*	.127	,449*	.275	**,723****	**,577****	**,605****	.184	.324	.143	.248	.004										
Pb	**,861****	.395	.149	,462*	.373	**,950****	**,857****	**,881****	.391	.319	.194	,462*	.115	**,748****									
P	**,777****	.285	.138	**,555****	.326	**,773****	**,808****	**,773****	**,517****	.332	.265	,493*	.161	**,632****	**,821****								
Rb	**,724****	**-,549****	-,494*	**,641****	.394	,482*	**,748****	**,700****	**,835****	−.326	−.124	−.002	−.385	.167	,407*	**,573****							
Si	−.181	**,827****	**,694****	−.373	−.275	.088	−.171	−.118	**-,522****	**,749****	,447*	**,578****	**,881****	.233	.188	.128	**-,563****						
S	.342	−.147	.123	**,671****	.123	.178	.363	.297	**,692****	.019	.378	.146	−.266	.220	.168	.318	,426*	−.305					
Sr	.005	**,849****	**,930****	−.113	−.305	.196	−.021	.002	−.391	**,885****	**,782****	**,872****	**,786****	.204	.290	.288	−.396	**,796****	−.055				
Ti	**,968****	−.038	−.207	**,623****	.385	**,927****	**,970****	**,977****	**,617****	−.035	.078	.303	−.120	**,602****	**,857****	**,754****	**,715****	−.113	.282	−.038			
Tl	−.069	**,638****	**,629****	.008	−.170	.061	−.088	−.113	.001	**,550****	,428*	.329	,451*	.166	.197	.140	−.356	**,571****	.259	**,552****	−.083		
V	**,944****	−.252	−.356	**,694****	.404	**,830****	**,958****	**,945****	**,738****	−.217	−.016	.152	−.336	,513*	**,741****	**,690****	**,840****	−.355	.381	−.234	**,957****	−.232	
Zn	**,906****	.124	−.019	**,534****	.284	**,887****	**,937****	**,924****	**,560****	.099	.232	,465*	.096	**,553****	**,858****	**,792****	**,615****	.094	.279	.145	**,928****	.050	**,866****

Asterisks indicate significant differences: * $p < 0.05$, **$p < 0.01$.

Table 3
Pearson correlation coefficients between elements measured by ICP/OES in leaves of *Tilia cordata* from Muskiz. The coefficients shown in bold are significant.

	Al	B	Ca	Cd	Co	Cr	Cu	Fe	K	Li	Mg	Mn	Na	Ni	Pb	P	Rb	Si	S	Sr	Ti	Tl	V	Zn
Al																								
B	.035																							
Ca	.277	−.282																						
Cd	**,545****	**,518****	−.171																					
Co	.126	,406*	.163	.286																				
Cr	**,880****	.019	.103	**,611****	.083																			
Cu	,545**	.182	−.220	.266	−.009	**,476****																		
Fe	**,962****	−.074	,376*	,457*	.106	**,869****	**,488****																	
K	.160	.151	**-,668****	.268	−.152	.169	**,593****	.015																
Li	.064	.106	**,470****	.046	.012	.068	−.291	.106	**-,489****															
Mg	.119	**-,697****	**,571****	−.231	−.316	.058	−.196	.200	−.243	.170														
Mn	.308	**-,687****	**,771****	−.235	−.067	.194	−.173	,434*	**-,510****	.224	**,824****													
Na	**,684****	.164	−.200	**,632****	−.064	**,734****	**,536****	**,606****	**,545****	−.092	−.166	−.138												
Ni	,412*	.317	.073	.348	.077	**,492****	.295	,390*	.076	.246	−.187	−.027	,403*											
Pb	**,728****	.116	.062	**,514****	.059	**,716****	**,548****	**,765****	.153	−.203	−.063	.171	**,620****	,409*										
P	−.322	.235	−.090	−.148	.026	-,429*	−.105	-,385*	.029	.014	.055	−.088	-,437*	−.077	−.325									
Rb	,389*	−.103	-,383*	.207	−.225	.336	**,585****	.284	**,804****	-,388*	.079	−.033	**,582****	.229	.351	−.005								
Si	.260	**-,549****	**,632****	-,377*	.037	.100	.048	.337	−.339	.271	**,621****	**,821****	−.158	.055	.003	.015	.139							
S	**,543****	−.076	,451*	.333	−.050	,374*	.336	**,482****	.166	.044	**,505****	,373*	,390*	.206	.282	−.009	.319	.251						
Sr	,448*	−.324	**,851****	.115	.084	.328	−.128	**,516****	-,442*	.327	**,711****	**,769****	.070	.052	.210	−.180	−.174	**,493****	**,660****					
Ti	**,654****	−.348	**,606****	.081	−.118	**,585****	.273	**,708****	−.197	,400*	**,598****	**,723****	.343	,371*	,378*	−.214	.237	**,735****	,493**	**,649****				
Tl	−.122	.306	.272	.042	.051	−.142	−.166	−.083	-,362*	−.009	−.182	−.128	−.078	.062	.019	−.115	**-,555****	−.350	.091	.162	−.152			
V	**,687****	,430*	−.135	**,729****	.077	**,715****	**,497****	**,644****	.360	−.077	−.281	−.226	**,727****	,450*	**,790****	−.281	.304	-,404*	.282	.091	.170	.083		
Zn	**,784****	−.239	.132	.356	−.239	**,744****	**,528****	**,775****	.338	.075	,367*	,361*	**,730****	,375*	,594**	-,385*	**,594****	.288	**,526****	,384*	**,748****	−.245	**,565****	

Asterisks indicate significant differences: * $p < 0.05$, **$p < 0.01$.

highly significant ($P < 0.001$) in all cases. As we observed previously in the air pollutant data from the three monitoring stations (Fig. 2), Bilbao had also the highest concentration throughout the season of the ten metals analysed (Fig. 4). However, seasonal differences between sampling day or the interaction between location and date were not significant for the studied metals (only Cu and Ti showed differences between days; Fig. 4).

3.3. *Leaf carbon and nitrogen isotope composition ($\delta^{13}C$ and $\delta^{15}N$)*

Leaf δ^{13}C and δ^{15}N analysis revealed significant effects due to locations, but were also related to sampling dates and their interaction (Fig. 5). δ^{13}C values ranged from -32.5‰ to -27‰, and the most negative values were found in the Bilbao samples. On the other hand, δ^{15}N ranged from 0 to 7‰ with the highest values in Bilbao and the lowest in Muskiz, mainly at the beginning of the sampling period (early summer). A Pearson correlation analysis was carried out between δ^{13}C and δ^{15}N and the analysed elements (Table 4). In the case of δ^{13}C, significant negative correlations were found between all the metals studied (except for Li). However, for δ^{15}N significant positive correlations were found (except again for Li).

4. Discussion

4.1. *Air quality assessed using monitoring stations and metal concentration in Tilia cordata leaves*

In this study, three urban locations (the centres of Bilbao, Vitoria and Muskiz) with different populations and traffic density were analysed. The presence of heavy metals was determined in leaves of *T. cordata* (a very common urban tree species) in order to test the suitability of this species as a bioindicator for pollution through different analytical approaches.

Monitoring stations revealed that, compared with Vitoria and Muskiz, Bilbao had the highest levels of air pollutants (NO_x, SO_2 and PM_{10}; Fig. 2). Muskiz showed the lowest concentration of air pollutants; only the atmospheric SO_2 concentration was higher in Muskiz compared to Vitoria. The higher levels of SO_2 detected in Muskiz could be related to the presence of the oil refinery (Petronor) in this location, as has been observed in other studies (Pereira et al., 2007). However, as described in more detail below, these high SO_2 values did not correlate with an increase in heavy metals in this location. This lack of correlation between these air pollutants and oil refineries was also observed in a study performed in Austria (Simon et al., 2011).

In the current study, we initially analysed thirty-three elements in leaves, but seven (As, Be, Bi, La, Mo, Sb, Se) were below the detection limit (<0.01 mg kg^{-1}). In the PCA, the presence of the other twenty six elements explained most of the variation in the main principal component of the PCA (54.8 % of the total variance) and segregated the three locations, with Bilbao being associated with that elements (Fig. 3b). Foliar concentrations of metals found in our study (Table S1) were in accordance with other work that has been undertaken on deciduous trees in Europe (Reimann et al., 2001; Sawidis et al., 2011; Simon et al., 2011), including *Tilia* spp. (Piczak et al., 2003; Tomašević et al., 2004, 2011; Aničić et al., 2011; Serbula et al., 2013). Only the concentration of Cr in the leaves from Bilbao (7.65 ± 0.43 mg kg^{-1}) and the Sr concentration in Muskiz and Vitoria were higher than the concentrations reported in the literature (Tomašević et al., 2004, 2011; Aničić et al., 2011; Sawidis et al., 2011; Hu et al., 2014). The foliar concentration of Sr deserves special attention, reaching a concentration as high as 433 mg kg^{-1} in Muskiz and 316 mg kg^{-1} in Vitoria (Table S1). This is more than six to eight times the content in reference plants established by Pais and Jones (2000) (50 mg kg^{-1} for plants). Sr is not essential for plants and it is mainly absorbed from the soil via the Ca uptake pathway (Donganlar et al., 2012). The correlation between Ca and Sr was strongly positive in the three locations ($r>0.85$; $P<0.001$). The high Ca-Sr correlation,

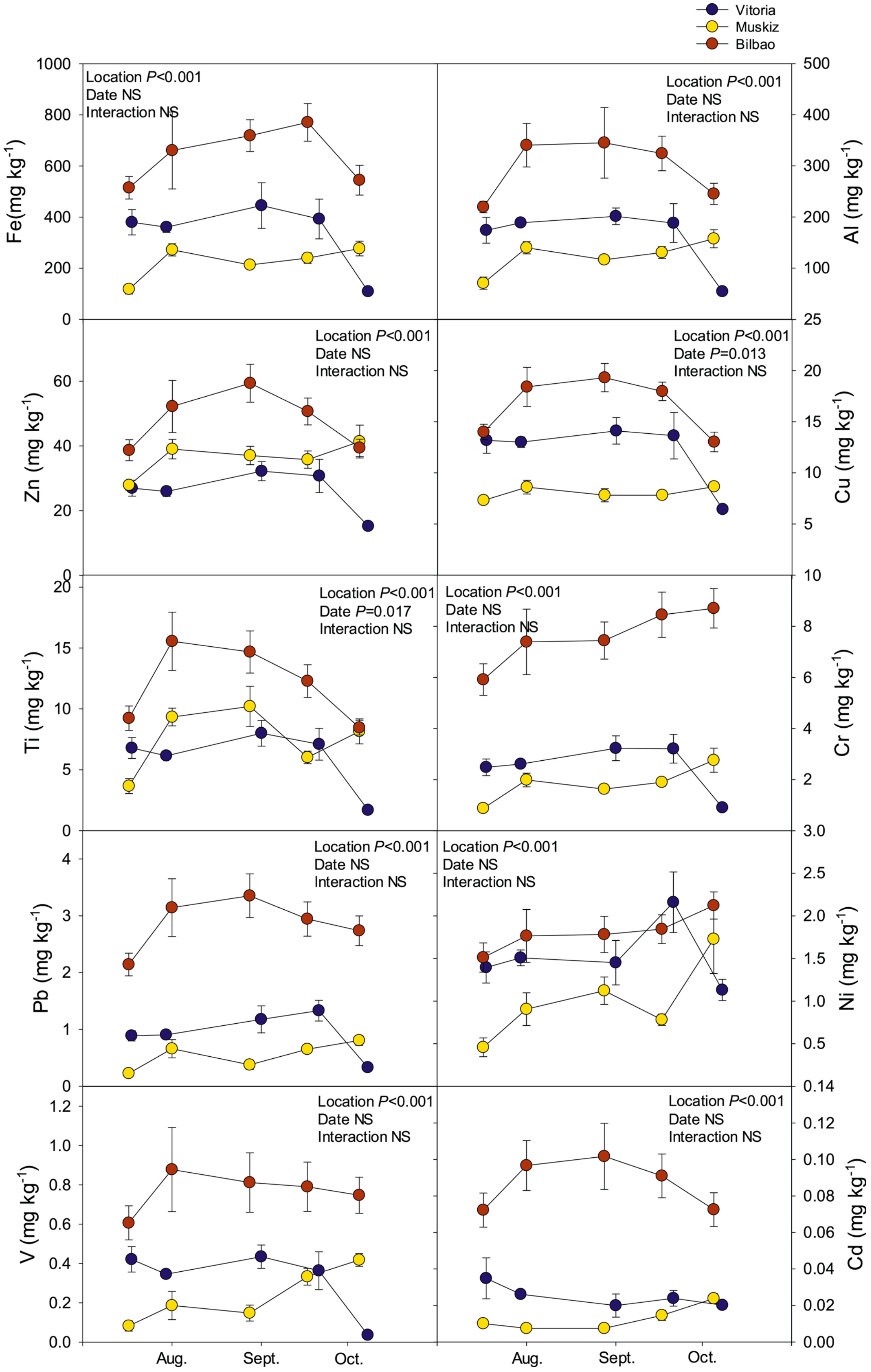

Fig. 4. Seasonal differential effects (from July to October) of location on heavy metals related to traffic and industrial emissions. Data correspond to the mean of six replicates ± SE. Results of two-way ANOVA, with the location and sampling day “date” as fixed factors, are shown in the upper part of each panel ($P < 0.05$). NS, not significant.

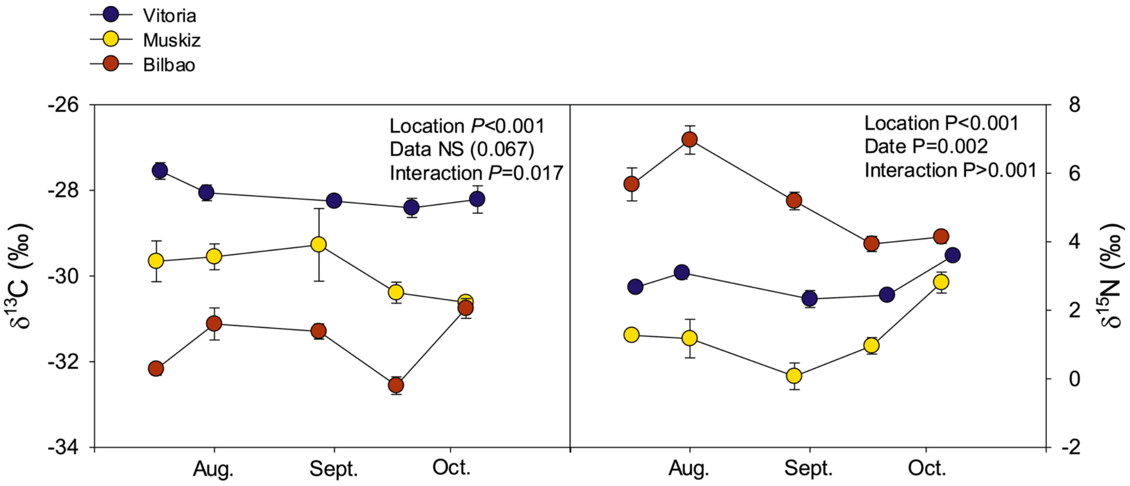

Fig. 5. Seasonal differential effects (from July to October) of location on leaf carbon and nitrogen isotope composition (δ^{13}C and δ^{15}N). Data correspond to the mean of six replicates ± SE. Results of two-way ANOVA, with the location and sampling day “date” as fixed factors, are shown in the upper part of each panel ($P < 0.05$). NS, not significant.

together with the high concentration levels detected in Muskiz-Vitoria and the absence of correlation between those elements and the others (Table 2–3) suggest that the leaf Sr found in *T. cordata* has an edaphic origin. This finding would highlight that the soils at Muskiz and Vitoria in which the sampled trees were growing might be Sr-contaminated. These very high levels of Sr are in the range known for plants from mining areas (Sasmaz and Sasmaz, 2017) and therefore deserve further attention in future studies to determine whether soil concentrations and/or the physiological properties of the plant led to accumulation, and what implication this has for *T. cordata* as an indicator to discriminate pollution sources (air, soil).

By using Pearson correlation analysis, the traffic-related origin of these metals was validated. Strongly significant correlations (r>0.6) between specific heavy metals (Al, Cd, Cr, Cu, Fe, Ni, Pb, Ti, V and Zn) were found in the two most populated and traffic-intense locations (Tables 1–2). These ten highly correlated heavy metals are strongly related to traffic emissions, brake linings, tyres, road pavement and exhaust fumes (Schauer et al., 2006; Adaemic et al., 2016). In comparison, the less populated location of Muskiz showed weaker correlations between these metals (Table 3). Additionally, most of these traffic-related metals were in the same cluster in the heatmap and were also grouped in the PCA (Fig. 3). All these facts indicate that these heavy metals appeared simultaneously and might come from the same pollution source, in agreement with the air pollution data from the monitoring stations (Fig. 2). Therefore, these ten highly correlated heavy metals (Al, Cd, Cr, Cu, Fe, Ni, Pb, Ti, V and Zn) detected from Bilbao and Vitoria were selected for further individual analysis (Fig. 4). All ten metals showed a highly significant effect of location ($P < 0.001$) and Bilbao had the highest concentrations for all heavy metals followed by Vitoria and Muskiz. Higher leaf concentrations of heavy metals in urban areas compared to industrial or rural areas has been reported before (Sawidis et al., 2011; Simon et al., 2011; Hu et al., 2014; Zhang et al., 2017) and such observations indicate the importance of traffic emissions in highly populated areas. Additionally, the difference between the concentrations found in Bilbao and the other two locations were especially consistent for three heavy metals: Cr, Cd and Pb. These three metals have been associated with brake pads, tyres and combustion of exhaust gases (Falahi-Ardakani, 1984). Therefore, the high foliar levels of heavy metals in Bilbao compared with the other urban location could be due to the higher population and are indicative of greater traffic intensity and as a result, higher levels of heavy metal pollution. These data confirm the suitability of leaves of *T. cordata* as an urban heavy metal bioindicator.

On the other hand, foliar element concentrations are known to fluctuate with time, with specific patterns for each element (Kovacheva et al., 2000). For the ten heavy metals studied here the metal concentration increased with leaf maturity, but for some elements the level decreased in autumn (Fig. 4). As noted by Liu et al. (2013), whether the metal accumulation occurs during leaf maturity/senescence or decreases at the end of the leaf cycle depends on the metal and the plant species, and this is because some plant species can remobilize and translocate elements between leaves and woody tissues. Interestingly the samples analysed during the middle of the leaf cycle (August) in the three locations had higher values and had the lowest variations in metal concentrations, so this period could be used to monitor urban contamination in comparative studies

4.2. Use of C and N isotopes (δ^{13}C and δ^{15}N) as bioindicators of environmental quality

The use of leaf carbon and nitrogen isotope composition (δ^{13}C and δ^{15}N) was analysed as a complementary tool for urban heavy metal pollution monitoring. Previous studies have indicated that δ^{13}C has potential as a bioindicator of environmental contamination (Sakata and Suzuki, 2000; Aranjuelo et al., 2014). As shown by Aranjuelo et al. (2014) in plants subjected to uranium exposure, leaf transpiration and stomatal closure were promoted as a strategy by the plants to reduce the transport of uranium toward leaves. Further, these authors also showed that uranium-exposed plants had higher δ^{13}C values.

In the current study, all the elements showed a significant negative correlation with δ^{13}C (except for Li; Table 4). The leaves with the highest heavy metal concentration had the most negative δ^{13}C. The Pearson’s correlation was especially negative in the case of Pb, Cr and Cd (r>0.55). Such a negative correlation between heavy metal content and δ^{13}C could have been caused by the high traffic intensity of the urban areas, where the air δ^{13}C is more negative than in less polluted or non-polluted areas. Indeed, lower δ^{13}C values have been associated with fossil fuel combustion, which depletes air δ^{13}C (Widory and Javoy, 2003) and, therefore the lower ambient δ^{13}C values in the atmosphere would explain the lower δ^{13}C detected in plants. In agreement with this fact, and as shown in previous studies (Balasooriya et al., 2009; Kiyosu and Kidoguchi, 2000; Lichtfouse et al., 2003; Wang and Pataki, 2012), the most negative values for δ^{13}C were found in the most traffic-intense urban area, Bilbao (Fig. 5).

Regarding δ^{15}N, the correlation was also significant for the ten metals studied in detail (except Li) although unlike δ^{13}C, the correlation was positive (Table 4). Again high correlations were detected between δ^{15}N and Pb, Cr and Cd. The high correlation between Rb and Sr and

Table 4
Pearson correlation coefficients between leaf carbon ($\delta^{13}C$) and nitrogen isotope composition ($\delta^{15}N$) and elements measured by ICP/OES in leaves of *Tilia cordata.* Data from three locations and five sampling days were pooled together. The coefficients shown in bold are significant.

}	Al}	B}	Ca}	Cd}	Co}	Cr}	Cu}	Fe}	K}	Li}	Mg}	Mn}	Na}	Ni}	Pb}	P}	Rb}	Si}	S}	Sr}	Ti}	Tl}	V}	Zn}
$\delta^{13}C$ (‰)	**-,425****	**,555****	**,417****	**-,567****	**-,313****	**-,592****	**-,445****	**-,514****	**-,308****	0.089	**,572****	**-,288****	**-,587****	**-,267****	**-,586****	−0.056	**-,298****	**-,314****	**-,594****	**,550****	**-,440****	**,429****	**-,414****	**-,481****
$\delta^{15}N$ (‰)	**,389****	**-,299****	**-,607****	**,574****	**,374****	**,499****	**,534****	**,429****	**,233***	0.154	**-,383****	−0.129	0.13	**,266****	**,543****	**,344****	**,693****	**,280****	**,334****	**-,759****	**,329****	**-,313****	**,435****	**,258****

Asterisks indicate significant differences: * $p < 0.05$, ** $p < 0.01$.

$\delta^{15}N$ (r = 0.693 and 0.759, respectively; Table 4) deserves particular attention. In the case of Rb, unlike Sr, the values found in our study (2–10 mg/kg) were lower than a previous study by Reimann et al. (2001), which showed values of 25–30 mg/kg in other urban deciduous trees like birch and willow. However, K and Rb have a relationship that is analogous to Ca/Sr, with a number of chemical similarities between the two elements and comparable behaviours in Earth superficial processes (Drobner and Tyler, 1998). In fact, Pearson correlation analysis showed that Rb had the strongest correlation with K in the three studied locations (Tables 1–3). On the other hand, phytoextraction abilities have been noted in *T. cordata* due to its ability to take up Rb from contaminated soils (Drzewiecka et al., 2019). Consequently, as observed with Sr, we consider that the levels of Rb in the leaves were of edaphic origin rather than being atmospheric contamination, and that like $\delta^{13}C$, $\delta^{15}N$ could be a novel tool to detect this edaphic contamination, alongside the traffic-related heavy metal air pollution.

While greater leaf $\delta^{15}N$ values were found in Bilbao, Muskiz showed the lowest values. In our study, a positive correlation between $\delta^{15}N$ and NO_x was found from the monitoring stations, and has been reported previously in a number of studies (Pearson et al., 2000; Pardo et al., 2007; Xiao et al., 2011; Redling et al., 2013; Kenkel et al., 2016; Cobley and Pataki, 2019). However, the use of plants for biomonitoring in these studies was focussed on the analysis of C and N isotopes to i) distinguish between the atmospheric pollution levels at different locations or ii) determine the relative contributions of different C and N pollutant sources. Data from the current study confirmed this biomonitoring function and posed the additional hypothesis that $\delta^{13}C$ and $\delta^{15}N$ might serve as reliable biomonitoring tools for heavy metal pollution in urban areas and have potential applications in a) distinguishing between locations as a function of their heavy metal pollution and b) determining the origin of this heavy metal pollution (soil or traffic air pollution). To our knowledge this is the first time that leaf $\delta^{13}C$ and $\delta^{15}N$ have been used as monitoring tools to assess heavy metal pollution and, consequently, more work is needed to confirm their efficacy.

5. Conclusions and perspectives

The current study examines the central influence of traffic on air quality. This is a major issue because different studies carried out during the last decade have shown that exposure to air pollution negatively correlates with life expectancy. Foliar heavy metal accumulation was analysed using leaves of *T. cordata*, a deciduous tree that is widely distributed. Three contrasting locations from the Basque Country, Spain, were selected for their different population levels and traffic intensity. The detailed analysis of heavy metals related to traffic emissions revealed that leaves from Bilbao, the most populated and traffic-intense location, had the highest concentrations of heavy metals, in accordance with air pollution data from monitoring stations. Additionally, Pearson correlation analysis and PCA showed that these heavy metals were likely to have an urban origin (traffic-related) instead of an industrial origin, even though an oil refinery was located near to one of the sampling points. Sampling of mature leaves in August (i.e. before senescence) ensures the highest (and thus optimal) concentration of metals in leaves for monitoring purposes. Examination of leaf carbon and nitrogen isotope composition ($\delta^{13}C$ and $\delta^{15}N$) showed significant correlations with most of the studied metals, allowing locations to be distinguished as a function of their heavy metal pollution and also identifying potential markers of edaphic contamination (Sr and Rb) that might provide a novel complementary heavy metal biomonitoring tool. Further work will be needed to confirm the origins of Sr and Rb in the test environments and to develop these heavy metals as markers of biocontamination.

Author statements

I.A., RE, CGM, JMB and D.S. fixed the experimental design. D.S.

wrote the manuscript with support from I.A. All authors discussed the results and contributed to the final manuscript. D.S, I.A, RE, AG, JA, DS, NU and LRL collected the samples and were in charge of sample analyses.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ufug.2020.126918.

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