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A Preliminary Study into the Use of Tree-Ring and Foliar Geochemistry as Bio-Indicators for Vehicular NO_x Pollution in Malta

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Word Count: 5387 words (excl. references, figures, tables, and captions)

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A Preliminary Study into the Use of Tree-Ring and Foliar Geochemistry as Bio-Indicators for Vehicular NO_x Pollution in Malta

Emissions from traffic over the past few decades have become a significant source of air pollution. Among the pollutants emitted are nitrogen oxides (NO_x), exposure to which can be detrimental to public health. Recent studies have shown that nitrogen (N) stable isotope ratios in tree-rings and foliage express a fingerprint of their major N source, making them appropriate for bio-monitoring purposes. In this study, we have applied this proxy to Aleppo pines (*Pinus halepensis*) at three distances from one of the busiest roads in Malta, a country known to suffer from intense traffic pollution. Our results showed that N and organic carbon (C) stable isotope ratios in tree-rings do not vary over the period 1980-2018 at any of the investigated sites, however statistically significant spatial trends were apparent in both tree-rings and foliage. The roadside and transitional sites exhibited more positive $\delta^{15}N$ and more negative $\delta^{13}C$ values compared to those at a rural control site. This is likely due to the incorporation of ^{15}N-enriched NO_x and ^{13}C-depleted CO_2 from traffic pollution. Sampled top-soil also exhibited the $\delta^{15}N$ trend. Our results constitute the first known application of dendrogeochemistry to atmospheric pollution monitoring in Malta.

Keywords: NO_x; motor vehicles; traffic; $\delta^{15}N$; dendrogeochemistry; tree-rings; foliage; Malta

Introduction

Motor vehicles are known to be a major source of atmospheric pollutants such as NO and NO_2 (collectively termed NO_x). In Europe, motor vehicles account for just under one-third of all NO_x emissions, with the remainder largely coming from shipping and power plants [1]. Mounting concern regarding NO_x pollution from automobiles has led to the implementation of legislation aimed at limiting these emissions and the development of catalytic converters [2]. Since the 1990s, the European Union (EU) has outlined the maximum tolerable limits for pollutants emitted by diesel- and petrol-fuelled vehicles, including NO_x. This has been achieved through a series of increasingly stringent directives known as the ‘Euro Emissions Standards’, the most recent versions of which are the Euro 6 Standards for passenger and light-duty vehicles and the Euro VI Standards for heavy-duty vehicles. Despite the introduction of this legislation, however, recent studies have shown that diesel-fuelled vehicles actually emit NO_x at rates of at least 4.5 times the maximum permitted by the Euro 6 specifications, with the most significant emissions being recorded within inner-city environments [3-5]. These findings therefore highlight NO_x pollution from traffic as an important and contemporary public health issue.

This is particularly true in the case of the Mediterranean island nation of Malta, located about 90 km south of Sicily (Fig. 1), which was recently reported to have the highest percentage population exposure to pollution of any country in Europe [6]. Here, the large number of motor vehicles (mean national density: 1,150 vehicles km^{-2} [7]) is believed to be the only major source of NO_x emissions [8]. Practically the entire automobile stock is fuelled by diesel (~40%) or petrol (~60%), and a significant proportion of these vehicles are also >15 years old, meaning that they were built to comply with far less rigorous emissions standards than those defined by the Euro 6 and Euro VI directives [7]. These statistics, coupled with the fact that Malta is the smallest (area: 316 km^2) and most densely populated (1,500 people km^{-2}) EU member state, make traffic pollution a serious and contemporary public health concern. This is likely exacerbated by the development of what has been termed a ‘car culture’, in which private automobiles have become the *de facto* mode of transportation due to local perceptions of an inefficient public transport system and poor provisions for walkers and cyclists [9].

In light of these issues, further efforts at ambient air quality monitoring across Malta have been made and a network of over 90 passive air diffusion samplers now exists [10,11]. Although these samplers are easy to use and cost-effective, they cannot provide any data relating to pollutant levels prior to the date of their installation, and thus records are limited and, at best, only go back to 2004 (the installation date of the first samplers). A more thorough understanding of the state of air quality in Malta and public exposure to pollution requires knowledge of the influence of past concentrations of pollutants (such as NO_x) on health and the environment. This would be of significant value to researchers in assessing regional ambient air quality over timescales greater than those for which records are available. It would also be useful to policy makers in evaluating the effects of increased development and urbanisation.

The study of stable isotope ratios in tree-rings has gained increasing traction in understanding past atmospheric and environmental conditions [12-14]. Tree-ring nitrogen (N) stable isotope geochemistry, for instance, gives a good indication of historical N deposition, as the $^{15}N/^{14}N$ ratio in compounds produced by anthropogenic activity is known to differ greatly from that of natural compounds in soils and plant tissues [15-21]. Experimental evidence has suggested, for example, that tree-ring $^{15}N/^{14}N$ ratios are influenced by NO_x emissions from traffic. Saurer et al. [15] showed that relative ^{15}N abundances in the tree-rings of Norway spruces (*Picea abies*) increased with proximity to a busy motorway. Furthermore, elevated $^{15}N/^{14}N$ ratios were only detected in tree-rings laid down after construction of the motorway. These observations were thus explained as being the result of increased uptake of ^{15}N-enriched NO_x from traffic. Savard et al. [18] and Doucet et al. [20] identified a strong association between the increasing number of motor vehicles in the province of Quebec and decreasing trends of tree-ring $^{15}N/^{14}N$ ratios in red spruces (*Picea rubens*), white pines (*Pinus strobus*) and American beeches (*Fagus grandifolia*) growing in Quebec City and Montreal. A lack of recorded changes in local climate and land-use conditions over the time period under investigation thus made absorption of ^{15}N-depleted NO_x from traffic the most likely driver of the observed trends.

Whether an increase or decrease in tree-ring $^{15}N/^{14}N$ ratios is recorded in trees exposed to vehicular NO_x emissions depends upon the N isotopic composition of the emissions themselves. This has been the focus of a number of studies which have shown variable results, often depending on several factors such as car age, make and model, speed of travel and engine temperature [22,23]. It appears, however, that the most influential factor is the presence and function of a catalytic converter, as cars fitted with such a device emit NO_x enriched in ^{15}N [24,25], while those not fitted with one emit NO_x which is ^{15}N-depleted [26]. In either case, however, it is clear that the uptake of NO_x from traffic causes an isotopic shift from unpolluted background values.

NO_x pollution from traffic has also been shown to influence the $^{15}N/^{14}N$ ratios of foliage. Kenkel et al. [27] noted that the relative abundance of ^{15}N in needles sampled from Piñon pines (*Pinus edulis*) at roadside positions in the Grand Canyon National Park was 50% higher than that for needles sampled 15 m and 30 m away from the road. Similar results were reported by Laffray et al. [28], who showed that increased uptake of NO_x from traffic caused an elevation in the $^{15}N/^{14}N$ ratio measured in roadside purple moor grass (*Molinia caerulea*) leaves in the French Alps.

Radial tree growth has also been used as a proxy for elucidating the extent of past atmospheric pollution. Studies have shown that prolonged exposure to most pollutants results in a deleterious effect on growth which manifests as narrower annual tree-rings [29-31]. However, the effect of increased NO_x pollution on radial tree growth is not as straightforward; previous studies have found that increased deposition of NO_x can result in radial growth reduction and narrower rings [32], can induce a fertilisation effect and thus contribute to tree-ring widening [17], or may have no influence on tree-ring widths whatsoever [15]. As such, the growth response of a tree to increased loads of NO_x is complex and depends on a number of factors including tree species, soil chemistry, nutrient status, volume of pollutant emitted, and the influence of competing pollutant species such as SO_2 or O_3 [13]. Boggs et al. [33], for instance, found that the level of N saturation and tree species played a significant role in determining whether increased N deposition caused either a growth decline or a fertilisation effect in the southern Appalachian region of the United States.

The aim of this study was to determine whether tree-ring and foliar N isotope ratios are influenced by vehicular NO_x emissions in Malta where, as detailed above, traffic pollution is known to be particularly intense. We have also investigated whether these emissions have any effect on tree-ring widths. To the best of our knowledge, such a dendrogeochemical experiment has not been previously performed in Malta. Thus, if NO_x emissions from traffic are shown to influence these parameters, as has been the case in previous studies conducted elsewhere [15-21,27-32], then tree-ring and foliar isotope geochemistry and radial growth variability would represent novel and hitherto unused proxies for ambient air quality monitoring in Malta.

Materials and Methods

Study Site Description and Sample Collection Strategy

The Mdina Road is a major thoroughfare in central Malta which carries around 55,000 vehicles per day [2019 personal communication; Transport Malta; unreferenced]. Part of this road runs past the towns of Attard and Balzan, where it comes within very close proximity (~25 m) of a residential zone (Fig. 1). Given the known effects on human health of increased exposure to NO_x pollution, this section of the road was selected as the polluted site of interest (S1). Two other sites located 250 m (S2) and 3,500 m (S3) away from the main trunk of the road to the south-west were also selected for sampling. These sampling sites represent a gradient of urbanisation, with S1 being directly beside the

Mdina Road (5 m away), S2 being a transitional peri-urban site, and S3 being a rural control site. A similar sampling transect approach was employed by Saurer et al. [15].

At all selected sampling sites, it was ensured that there were no nearby agricultural activities which could have increased tree tissue N concentrations or influenced isotope ratios [34]. Furthermore, as the prevailing winds in Malta are north-westerly and westerly, there was no risk of NO_x contamination from the road along the sampling transect (Fig. 1). Annual mean temperature and precipitation at the sampling sites are about 20 °C and 600 mm, respectively. All sampling sites are also located at similar elevation. Site geology is consistent throughout, with limestone being the most dominant rock type.

Sampling was carried out in December 2018 and January 2019. At each site, five Aleppo pines (*Pinus halepensis*) were chosen and two cores per tree were sampled at breast height (~1.4 m) using a 5 mm diameter increment borer (Haglöf, Sweden). Trees selected for sampling were ensured to have no visible signs of cutting, fire damage, insect damage or disease. Current year pine needles were also hand-picked from the outer crown regions of all sampled trees. All needles were taken from the side of the tree facing the road at a height of ~1.7 m. The preference for current year needles as opposed to older ones was due to the known variation of N mass in pine needles with age [35]; the greater mass of N in younger needles would facilitate easier isotope analysis.

Soil samples were also collected from the three sites for isotope analysis. About 10 g of top-soil was gathered with a clean plastic box from a depth of 5 cm at the base of each sampled tree on the side that faced the road. The five soil samples collected at each site were then pooled into a single container and mixed with a clean spoon to generate a sample which was representative of the whole site. Our choice in only sampling the top 5 cm of soil is justified by the fact that we are only interested in whether NO_x deposition from nearby traffic has any influence on the isotopic signal of the upper soil layer. Recent results by Xu et al. [36] have shown that top-soils near busy roads are more enriched in ^{15}N than those further away primarily due to the deposition of ^{15}N-enriched NO_x and particulate dust from vehicle exhausts. Furthermore, top-soil N isotope geochemistry has been reported to be less influenced by microbial and ecological processes which are known to cause fractionations in deeper soil layers [37-41], meaning it may be more appropriate for recording the N isotope signal of deposited vehicular NO_x. Collected needle and soil samples were stored at –5 °C until they could be transported to the laboratory, thus preventing continued microbial action which may also have had an impact on isotope ratios. The samples were transported in clean capped plastic boxes to the laboratory where they were stored under vacuum (0.5 mbar) at –50 °C in a freeze-drier for nine days to remove moisture.

Sample Preparation and Dendrochronological Analysis

For each tree, a single core radius was sanded, mounted, measured (0.001 mm precision) and cross-dated using standard dendrochronological methods [42]. The second core was retained for isotope analysis. Dendrochronological analysis was conducted at the Tree-Ring Laboratory of the School of Earth and Environmental Sciences, University of St Andrews. Although it is generally accepted that for robust ring-width chronologies often 20 to 30 tree cores should be sampled [43], we chose to follow the sampling strategy used by previous dendrogeochemical studies which have successfully established reliable isotopic trends using less replicated chronologies (<10 tree cores). For example, the studies of Saurer et al. [15], Guerrieri et al. [17] and Battipaglia et al. [19] respectively sampled four, six and seven trees per site. For each of the five trees sampled per site, the raw ring-width data were aligned by pith date, allowing for comparison of mean growth as a function of cambial age [44].

Sample Preparation and Dendrochronological Analysis

The dried soil and needle samples were ground to a powder with a pestle and mortar. Carbonate was removed from the soil samples by treatment with HCl (2 mol dm^{-3}; reagent grade) in Pyrex centrifuge tubes. The acid was left to react under constant stirring with a glass rod until the reaction had visibly subsided and did not resume upon further addition of acid. The acid was then decanted after centrifugation and residual acid was washed out with three successive treatments of de-ionised water (18.2 MΩ cm^{-1}). Tree-ring cores retained for dendrogeochemical analysis were chemically treated to remove any extractable N compounds via Soxhlett extraction; first for five hours in a 1:1 v/v mixture of absolute ethanol and water, then for five hours in absolute ethanol, and lastly for one and a half hours in de-ionised water. This technique is similar to the one suggested by Sheppard and Thompson [45] and has been used in previous studies [15,19,46].

For isotope analysis, dated tree-rings identified to represent the period 1980-2018 were separated into five-year groups (1980-84, 1985-89, …, 2010-14, 2015-18) using an ultra-thin kerf razor saw. Individual rings were not analysed in case of dilution of the N isotope signal due to lateral translocation of N compounds which may be accompanied by fractionation at the ring boundaries [15]. Ring samples from the same location and time period were combined into a clean glass vial [21,47] and the pooled ring segments were then powdered using an MM-200 ball mill (Retsch, Germany).

Powdered tree-ring, pine needle and decarbonated soil samples were subsequently analysed for their ^{15}N/^{14}N ratios via combustion in an IsoLink elemental analyser connected in continuous flow mode to a Finnigan MAT-253 isotope ratio mass spectrometer (Thermo Fisher Scientific, USA). For each sample analysed, carbon (C) stable isotope ratios were also recorded as such values could potentially provide more information when interpreting the N isotope results. Isotopic compositions are reported in the standard δ-notation:

$$\delta\ (‰) = [(R_{\text{sample}} / R_{\text{standard}}) - 1] \times 1{,}000$$

where R_{sample} is the $^{15}N/^{14}N$ ratio or the $^{13}C/^{12}C$ ratio for the analysed sample and R_{standard} is either of these ratios for a selected standard (atmospheric N_2 for N and the Vienna Pee Dee Belemnite for C). Typical masses used for analysis were 14-17 mg for tree-rings, 0.3-0.5 mg for pine needles and 2-3 mg for soils with the aim of optimising signal intensity. Elemental abundances were determined from calibrated peak areas. The calibration standards used were USGS-40 and USGS-41 (both glutamic acids). USGS-62 (caffeine) was used as a quality control standard and it yielded precisions of 0.2 ‰ (1SD) for both $\delta^{15}N$ and $\delta^{13}C$. Chemical extraction work and isotope analysis were conducted at the St Andrews Isotope Geochemistry (STAiG) laboratories at the School of Earth and Environmental Sciences, University of St Andrews.

Results

All measured data and statistical test calculations can be found as part of the provided Supplementary Material.

Radial Tree Growth Analysis

The dated tree-ring cores reveal variations in the site mean ages: S1 = 41 years, S2 = 52 years, S3 = 73 years. Consequently, the common period studied was limited to the last 39 years of growth. The plotted mean cambial age-aligned ring-width series (Fig. 2) exhibit a spatial growth trend, where growth at S1 and S2 were found to be statistically similar, but also statistically higher than that at S3 via the use of a repeated measures ANOVA test ($p << 0.001$; Fig. 2).

Nitrogen and Carbon Isotope Analysis

The N isotope signals for the sampled pine needles and soils reveal a clear spatial trend, with $\delta^{15}N$ values decreasing (becoming more negative) with increasing distance to the road (Table 1). Spearman rank correlations between the measured δ^{15}N values and distance to the road are –0.80 (ρ_{needles}) and –0.83 (ρ_{soils}). Both results are statistically significant ($p << 0.01$; $n = 12$), thus confirming a decreasing relationship between N isotopic composition and increasing distance to the road in both pine needles and soils. No obvious temporal trends are apparent in tree-ring δ^{15}N values over the time period 1980-2018 (Fig. 3). However, δ^{15}N values at S1 and S2, which ranged between +3.6 ‰ and +6.8 ‰, were consistently more positive than those at S3 which, aside from a recent decline, remained fairly constant between +1.7 ‰ and +3.0 ‰. A repeated measures ANOVA test was conducted on the tree-ring δ^{15}N series measured for S1, S2 and S3, which indicated that the values for S1 and S2 are statistically indistinguishable, while that for S3 is demonstrably more negative ($p << 0.001$; Fig. 3).

With regards to the δ^{13}C values of sampled pine needles, a Spearman rank test detected a non-significant relationship between those values and distance to the road ($\rho = 0.47$; $p < 0.15$). The likely reason for this is a lack of monotonicity in our pine needle δ^{13}C data (Table 1), which most likely could be overcome through further sampling efforts. However, we argue that the spatial trend observed in the δ^{15}N proxies, in which S1 and S2 appear to be affected by traffic pollution while S3 is not, is still apparent in foliar δ^{13}C values, especially since a Pearson test revealed a good correlation with distance to the road which was statistically significant ($R = 0.90$; $p << 0.001$). No spatial trends were detected with regards to soil δ^{13}C values. In fact, there is very little variation in these values across sites (Table 1), with site mean values all being similar: S1 = –27.1 ± 0.2 ‰; S2 = –27.3 ± 0.1 ‰; S3 = –26.9 ± 0.2 ‰.

Once again, no obvious temporal trends are observed in the measured tree-ring δ^{13}C values (Fig. 4). However, with the exception of the rings corresponding to 1980-84, the δ^{13}C values for S3 are consistently more positive than those for S1 and S2. This observation was confirmed by a repeated measures ANOVA test which showed that the tree-ring δ^{13}C series for S1 and S2 are statistically indistinguishable from one another while that for S3 is significantly more positive ($p << 0.01$; Fig. 4).

Discussion

Effect of NO_x Pollution on Tree-Ring, Foliar and Soil Isotope Geochemistry

Although no apparent temporal trends in the tree-ring δ^{15}N series can be discerned, results show that there is a statistically significant spatial trend, with δ^{15}N values being consistently more positive at S1 and S2 than at S3 throughout the entire time period of interest (Fig. 3). These results are also reflected in the N isotopic signatures of sampled foliage and soils, each of which displayed statistically significant negative Spearman rank correlations with distance to the road. Thus, even though our analysis is based on a relatively small number of trees at each site, the stark contrast in δ^{15}N values between S1 and S2 on the one hand and S3 on the other for each pooled time bin as well as for different substrates is strong evidence for a robust spatial gradient.

With respect to the tree tissues (rings and foliage), the N isotopic differences between trees at S1 and S2 versus S3 most likely indicate that the major N source to trees at those sites was isotopically different (Fig. 3; Table 1). The most parsimonious explanation for these isotopic trends is the deposition and uptake of ^{15}N-enriched NO_x from traffic. This vehicular source was apparently stronger at S1 and S2, causing the observed N isotope ratios, but was significantly weaker at the rural control site S3.

This interpretation is consistent with that of previous studies which have demonstrated that the uptake of NO_x from traffic may influence the N isotopic composition of plant tissues [15-20,24,25,27,28,36]. Indeed, our results are similar to those obtained by Saurer et al. [15] and Ammann et al. [24], who analysed the effect of

NO_x from traffic on the $\delta^{15}N$ values of Norway spruce (*Picea abies*) tree-rings and needles growing at three distances away from a motorway in Switzerland. Importantly, our results are also in agreement with those studies that showed that significant isotopic trends can be identified from cores taken from a smaller number of trees at each site [15,17,19]. We note here that analysis of N concentrations in the sampled tree-rings (data not shown) did not vary significantly either through time or between the sites. This agrees with other studies which have shown that N concentrations in tree tissues are largely dependent upon physiological factors rather than environmental ones, and so tend to be tightly regulated [34,48,49].

In the case of the top-soils sampled at the three sites, there is also an evident spatial trend (Table 1), with soils nearer to the road being more enriched in ^{15}N. In their recent study, Xu et al. [36] reported similar trends in top-soils analysed at different distances from a road in China. It is well known that soil N isotope ratios are heavily influenced by both microbial and ecological processes such as nitrification, denitrification, nitrogen fixation, ammonification and nitrate leaching [37-41]. As such, there have been some questions as to the validity of using soil N isotope geochemistry as a proxy for vehicular NO_x pollution [27]. However, although such processes are known to occur in top-soils, their effect on N isotope ratios is known to be enhanced at deeper layers [36-41]. Therefore, if differing microbial pathways were the reason for the observed spatial gradients in N isotopes, we would not expect any covariance between top-soils, tree-rings and foliage. However, in all cases, top-soils are a few permille heavier than the tree-rings, which are in turn slightly heavier than the recent foliage, meaning that isotopic fractionation between different N reservoirs at each site is conserved, but the starting compositions were likely distinct [50]. Given the similarity in climate and bedrock geology, it is expected that processes contributing to isotopic shifts in the top-soils sampled at S1 and S2 are also occurring at S3 [51], and that the only major factor which differs is the proximal presence of NO_x pollution at the former two sites. Hence, we suggest that the top-soil δ^{15}N trends reported in this study can be explained by the deposition of traffic-related NO_x and particulates which are ^{15}N-enriched, similarly to the results observed and interpreted by Xu et al. [36].

The interpretation of tree-ring δ^{13}C values is more complex as this parameter is known to be influenced by a number of factors such as air pollution [52-54], climate (e.g. precipitation, temperature and drought) [55,56] and tree age [57]. Nevertheless, analysis of tree-ring C isotope ratios may yield some further insight into the effects caused by prolonged exposure to vehicular pollution. In our study, tree-ring δ^{13}C values did not possess any obvious temporal trends. When considering the dated ring segments over the 1985-2018 period, however, a significant spatial trend becomes apparent (Fig. 4). Here, tree-ring δ^{13}C values at S1 and S2 are both statistically indistinguishable from one another as well as being more negative than those at S3.

Although the trees sampled at S3 are on average older (73 years) than those at S2 (52 years) and S1 (41 years), we discount the possibility that this is the reason for the observed tree-ring C isotope trends, as such age differences (<35 years) are much smaller than those reported to cause ^{13}C enrichment in older trees (>200 years) [57]. Given that climate, site geology and elevation do not vary between sites, we argue that the spatial trends observed in tree tissue C isotopes is reflective of the effect of vehicular pollution at S1 and S2. CO_2 from fossil-fuel combustion is known to be depleted in ^{13}C [58-61], and studies have shown that CO_2 from vehicular sources causes a suppression of δ^{13}C in nearby plant tissues to more negative values [61-63]. As such, we suggest that our results reflect the greater concentrations ^{13}C-depleted CO_2 from traffic at S1 and S2 which caused more negative tree-ring δ^{13}C values at these sites. With regards to foliar δ^{13}C values, a non-significant relationship (Spearman correlation) with distance to the road was identified. However, we argue that a spatial gradient, in which S1 and S2 foliar δ^{13}C values are much more negative than those at S3, is still evident particularly in light of the strong positive correlation detected when a Pearson correlation test was applied. Once again, since there are no differences in local climate, elevation, site geology and anthropogenic activity (aside from the road itself) across the three sites, we attribute the observed foliar δ^{13}C trends to be the result of increased uptake of ^{13}C-depleted CO_2 from traffic at sites closer to the road.

Thus, our results indicate that ^{15}N-enriched and ^{13}C-depleted pollution from heavy traffic along the Mdina Road influences the N and C stable isotope geochemistry of Aleppo pine (*Pinus halepensis*) tree-rings and foliage at least 250 m away from the main trunk of the road. Furthermore, this pollution also influences the N isotope geochemistry of the top-soil, with soils at least 250 m away from the road registering enrichments in ^{15}N. Our results also raise a new question; given the fact that sections of the Mdina Road come within close proximity (~25 m) of residential zones, should there be any cause for concern with regards to public exposure to pollution from traffic and the associated deleterious health effects? Although this question goes beyond the scope of our study, we believe that our results justify further investigations into the public health of communities living within close range of main and arterial roads in Malta.

We also note that, although spatial trends reported in this study are clear and statistically significant, we were unable to detect any temporal trends from the tree-ring δ^{15}N and δ^{13}C series at the polluted sites S1 and S2. The reason for this is not known, however such a result may possibly indicate that N translocation across annual tree-rings in Aleppo pines (*Pinus halepensis*) is not associated with isotope fractionation, and thus the δ^{15}N tree-ring record for a given year is highly influenced by the isotopic composition of N translocated from tree-rings representing previous and future years. Such N isotope translocation dynamics are known to be species-dependent, as was demonstrated by Mizota et al. [64] who observed a similar N isotope translocation mechanism in red pines (*Pinus densiflora*) but not in black pines (*Pinus thunbergii*).

Potential Growth Response of Trees to Increased Vehicular Pollution

Our results have further revealed a statistically significant difference in growth rates at sites S1 and S2 compared to S3 (Fig. 2). In our experiment, conclusions regarding growth trends are difficult to reach. The reason for this is that the individual growth variability of trees is high due to climatological, ecological and physiological differences [65]. This would necessitate the sampling of at least 20 to 30 individual trees per site for robust growth trends to be estimated using traditional dendrochronological methods [42,66,67]. Nevertheless, we comment cautiously about our data. If the observed growth rates are indeed representative of the sites as a whole then it is unlikely that the observed differences are caused by climatic or geological factors due to the consistency of bedrock, regional climate and elevation across all sampling sites. Furthermore, our use of a cambial age-aligned mean ring-width series minimises any influence that tree age (i.e. higher juvenile growth) could have had on such values [44]. Thus, there would have to be some other factor driving increased growth at S1 and S2 compared to S3.

It is possible that the increased exposure to NO_x at S1 and S2 has resulted in a N fertilisation effect, as has been reported in previous studies [17,33]. Alternatively, it is possible that the reduced radial growth at S3 is a consequence of higher competition for growth resources between trees at this site [68-70], which would have been absent at the more urbanised S1 and S2 due to the presence of fewer trees. These suggestions are presently only speculative, and although we have ensured to standardise the multiple factors (e.g. substrate, climate, etc.) impacting tree growth rates [71], the inherent noisy nature of ring-width data can only be minimised through further sampling.

Conclusions

We have studied the N and C isotope geochemistry of Aleppo pine (*Pinus halepensis*) tree-rings and foliage, as well as soils, at three distances from one of the busiest roads in Malta, a country known to suffer from intense traffic pollution. Our results indicate enhanced δ^{15}N values in tree-rings, foliage and soils 5 m and 250 m away from the road compared to a rural control site 3,500 m away. Furthermore, we have also observed more negative tree tissue δ^{13}C values 5 m and 250 m away from the road compared to the rural site. It appears that these spatial isotopic differences are the result of increased emission of ^{15}N-enriched NO_x and ^{13}C-depleted CO_2 from traffic, which is then absorbed and incorporated into tree tissues. Although the use of soil δ^{15}N values as an indicator for regional NO_x pollution has been debated, we argue here that the observed N isotope trends in this study most likely reflect NO_x emission from motor vehicle traffic.

The main section of the road under investigation in this study, the Mdina Road, comes within close proximity (<30 m) of residential zones in densely populated towns and villages. Given that our results have demonstrated that pollution from traffic influences the stable N and C isotope geochemistry of trees growing at least 250 m away from the road, we suggest that there may be substantial scope for future studies to assess the extent and effects of public exposure to pollution in communities living in close proximity to main and arterial roads in Malta. We have also examined tree-ring widths at each of the investigated sites. Although our tree replication is too low to draw any definitive conclusions, future studies in this regard are recommended, as it is likely that tree-ring width variations with distance from vehicular pollution may provide an additional spatial bio-proxy for pollution monitoring studies.

Acknowledgements, Duncan V. Mifsud is the grateful recipient of an Endeavour Scholarship (Republic of Malta). The Endeavour Scholarship Scheme is part-financed by the European Union – European Social Fund (ESF): Operational Programme II – Cohesion Policy 2014-20. The authors would like to thank Vincent M. Mifsud for his assistance in sample collection. Furthermore, we would like to thank Ian Washbourne and James Weir at the University of Stirling for the loan of their Retsch MM-200 ball mill.

Declaration of Interests Statement, The authors hereby declare that they have no known conflicting interests.

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Table 1. N and C isotopic compositions of tree-rings, foliage and soils at S1 (5 m away from the road), S2 (250 m) and S3 (3,500 m).

	S1		**S2**		**S3**	
	Mean (‰)	SD (‰)	Mean (‰)	SD (‰)	Mean (‰)	SD (‰)
Tree-Rings	Site *n* = 8					
δ^{15}N	4.51	0.94	5.31	0.92	2.02	0.97
δ^{13}C	-25.80	1.72	-26.14	0.84	-24.42	0.57
Foliage	Site *n* = 4					
δ^{15}N	4.39	0.86	3.85	0.38	-1.06	0.60
δ^{13}C	-29.23	0.44	-30.66	0.33	-26.58	0.26
Soils	Site *n* = 4					
δ^{15}N	7.95	1.33	6.18	0.46	5.43	0.29
δ^{13}C	-27.13	0.17	-27.30	0.11	-26.94	0.19

Figure 1. Location of the sampling sites S1, S2 and S3. In the top right panel, the names of the constituent islands of the Maltese archipelago are given in bold italics, while in the bottom panel towns proximal to the Mdina Road (thick black line) are given in italics.

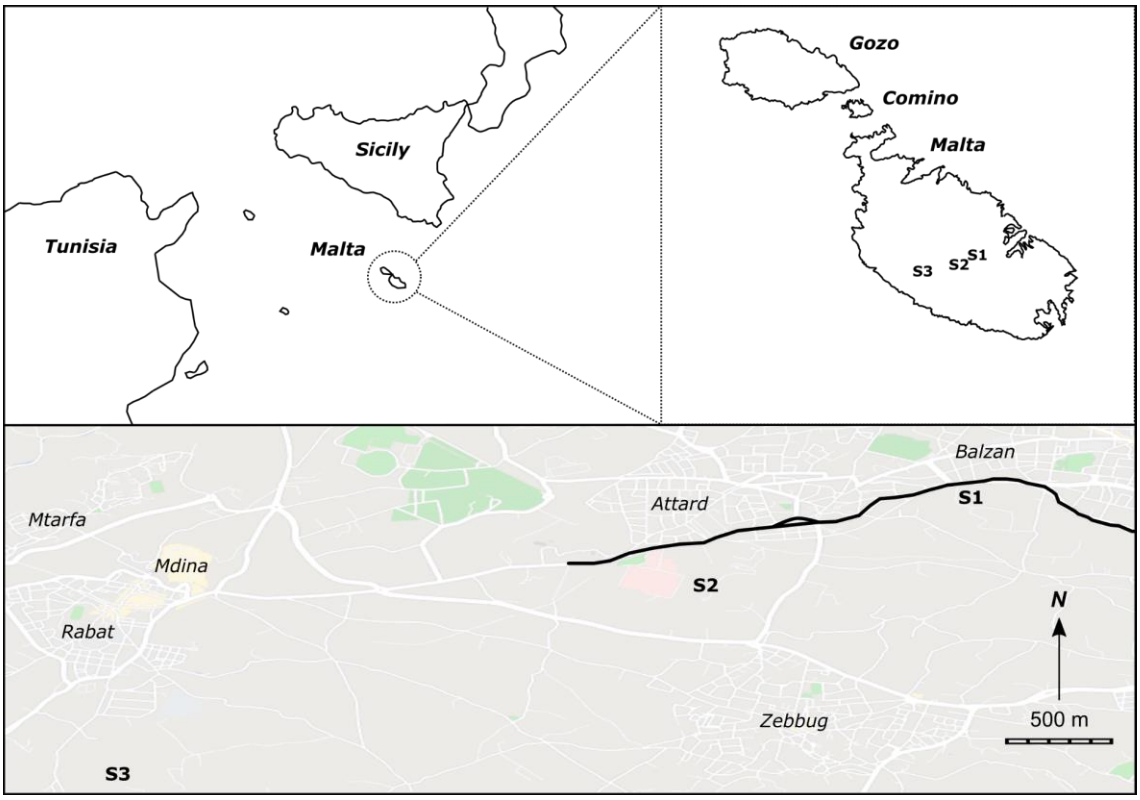

Figure 2. Pith-aligned tree-ring width series over the time period 1980-2018. On the right, repeated measures ANOVA testing showing that site mean tree-ring widths at S1 and S2 are statistically indistinguishable, and greater than those at S3.

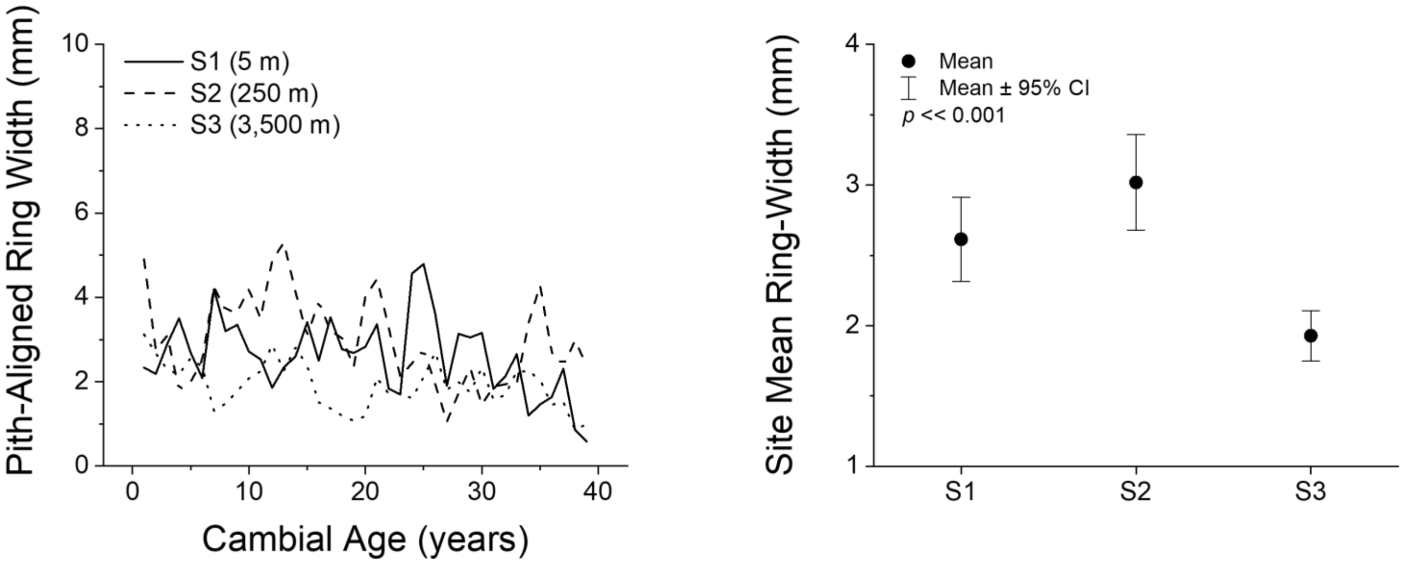

Figure 3. Tree-ring δ^{15}N series over the time period 1980-2018. On the right, repeated measures ANOVA testing showing that site mean δ^{15}N values at S1 and S2 are statistically indistinguishable, and greater than those at S3.

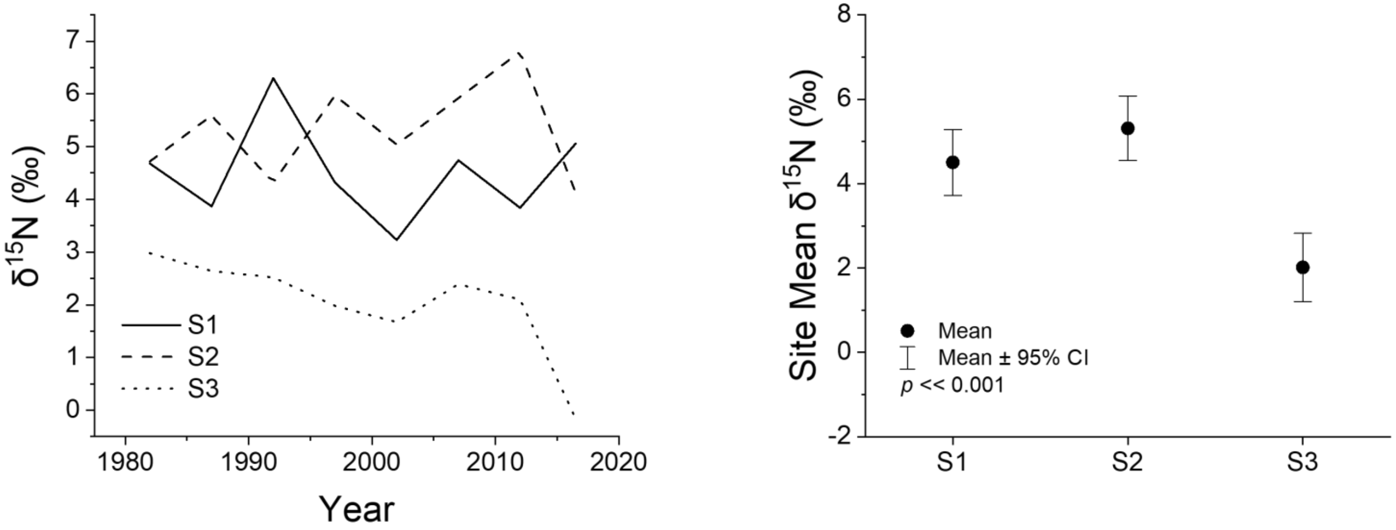

Figure 4. Tree-ring δ^{13}C series over the time period 1980-2018. On the right, repeated measures ANOVA testing showing that site mean δ^{13}C values over the time period 1985-2018 at S1 and S2 are statistically indistinguishable, and smaller than those at S3.