Acta Brasiliensis 6(2): 26-34, 2022
http://revistas.ufcg.edu.br/ActaBra
Original Article
http://dx.doi.org/10.22571/2526-4338583

The effects of tropospheric ozone on plant species: New perspectives

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Received: 11 January 2022 / Accepted: 19 May 2022 / Published online: 31 May 2022

Abstract

The effect of tropospheric ozone on the physiology of plants has been well established using physicochemical analysis and visual assessment. One of the main metabolic processes, in plants that is affected by ozone, is photosynthesis. This in turn affects a number of secondary processes required for the survival of plants. This study focused on two main aspects; the qualitative determination of damage through visual assessment and the quantification of damage through the determination of the content of chlorophyll and other quality parameters using spectrophotometric techniques in a number of plant species. Three distinct setups were considered, mainly rural, urban and semiurban, representing the topography of the islands of Malta and Gozo. It was observed that chlorosis was not the sole factor contributing to the yellowing of the leaves. Another important finding was the correlation between ozone levels (50.18-69.35 ppb) and the anthocyanin content (2.57-28.99 mg/kg) of leaves. From the three plant species that were extensively studied (*Nerium oleander*, *Pinus halepensis* and *Schinus terebinthifolius*), the *N. oleander* exhibited promising results as a bioindicator for ozone-induced damage. Due to the presence of this ornamental plant in rural and urban areas, it can be used by researchers and authorities as a tool for assessment of tropospheric ozone levels.

Keywords: Anthocyanin, bioindicator, chlorophyll, chlorosis, *Nerium oleander*

Os efeitos do ozônio troposférico nas espécies vegetais: Novas perspectivas

Resumo

O efeito do ozônio troposférico sobre a fisiologia das plantas foi bem estabelecido utilizando análise físico-química e avaliação visual. Um dos principais processos metabólicos, nas plantas afectadas pelo ozônio, é a fotossíntese. Isto, por sua vez, afecta uma série de processos secundários necessários para a sobrevivência das plantas. Este estudo centrou-se em dois aspectos principais; a determinação qualitativa dos danos através da avaliação visual e a quantificação dos danos através da determinação do conteúdo de clorofila e outros parâmetros de qualidade utilizando técnicas espectrofotométricas em várias espécies de plantas. Foram consideradas três configurações distintas, principalmente rurais, urbanas e semiurbanas, representando a topografia das ilhas de Malta e Gozo. Observou-se que a clorose não foi o único factor que contribuiu para o amarelecimento das folhas. Outra descoberta importante foi a correlação entre os níveis de ozônio (50,18-69,35 ppb) e o teor de antocianina (2,57-28,99 mg/kg) das folhas. Das três espécies vegetais que foram amplamente estudadas (*Nerium oleander*, *Pinus halepensis* e *Schinus terebinthifolius*), a *N. oleander* apresentou resultados promissores como bioindicador de danos induzidos pelo ozônio. Devido à presença desta planta ornamental em zonas rurais e urbanas, ela pode ser utilizada por investigadores e autoridades como um instrumento de avaliação dos níveis de ozono troposférico.

Palavras-chave: Antocianina, bioindicador, clorofila, clorose, *Nerium oleander*

Introduction

The physiological and metabolic functions in plants are affected by a plethora of factors, from abiotic to biotic factors. In turn, changes in the physiological and metabolic activities lead to changes in a number of vital processes. Physiological and biochemical processes are quite interrelated, and these can adjust and change according to the natural ambient conditions, such as seasonality, through foliage. This leads to the acclimatization of plants to more favourable conditions for survival (Hopkins & Hüner, 2008). However, this disturbance in homeostasis can be described as biological stress (Hopkins & Hüner, 2008).

Atmospheric pollutants are an example of external stressors. Exposure to pollutants such as ground-level ozone can cause a reduction in their chlorophyll content and other photosynthetic pigments, which in turn affect a number of processes such as the normal growth of the plant, normal flowering and seed development (Giri, Shrivastava, Deshmukh, & Dubey, 2013), and the unnecessary production of secondary metabolites (Tonelli *et al.*, 2015) at the expense of energy in plants. The exposure time and the ground-level ozone concentrations can affect certain ecosystem algorithms and therefore can influence ecosystem services and in turn affect crop yields in agriculture (EEA, 2019).

Atmospheric ozone (O_3) is categorised into stratospheric and tropospheric (ground-level) ozone. The main concern is with ground level ozone, which can negatively affect biota (Agathokleous *et al.*, 2020). In this respect, the US Environment Protection Agency states O_3 exposure levels which are greater than 70 ppb for more than 8 hours (EPA, 2015). Ground level ozone is also considered as a secondary pollutant, generated through a series of reactions of other atmospheric pollutants that are emitted by industrial processes, usually from fossil fuel combustion (Sharma, Jain, Khirwadkar, & Kulkarni, 2013).

The nature of the site is a huge determinant of the O_3 concentrations, due to the fact that the pollutant can be found more prominently in rural areas rather than urban areas, despite it being formed from the presence of other atmospheric pollutants (Paoletti, De Marco, Beddows, Harrison, & Manning, 2014). Additionally, as stated by the European Union (EU), ozone is a transboundary pollutant (2001/81/EC), which in turn shows the ability of ozone to move within a region and between regions. Further legislative measures within the EU (2008/50/EC) have led to better enforcement on air pollution, resulting in rural areas in the Mediterranean, more prevalently, showing a decrease in ozone pollution (Sicard *et al.*, 2013).

The assessment of vegetal ozone damage has been carried out via various approaches and methodologies considering macro and microclimatic conditions (Shimizu, Lu, Aono, & Omasa, 2019; Mills *et al.*, 2018; Masri *et al.*, 2019; Burkey, Booker, Ainsworth, & Nelson, 2012; Gottardini, Cristofolini, Cristofori, & Ferretti, 2014). Several reports and research have been published in relation to the assessment of ozone damage to horticultural crops (Holland *et al.*, 2006; Van Zelm *et al.*, 2008). A widely accepted method for the assessment of foliar ozone damage is described by Schaub and coworkers (2016). Ozone damage is mainly identified as chlorotic mottling of leaves.

The aim of this study is to investigate the possible correlation between ground-level ozone concentrations and its impacts on plant injury, via the determination of chlorophyll content and other quality parameters, as well as from a visual-based qualitative analysis on ozone-induced symptoms.

Materials and Methods

Selection of Sites

Site selection is an important process that is highly dependent on the availability and the accessibility of the areas considered for data collection. The localities, which were studied, were chosen according to the location of ambient air monitoring stations set by the Local Environment and Resources Authority (ERA) around the island (Figure 1). The study was conducted on three sites namely, Għarb in Gozo, and Attard and Żejtun in Malta, representing three scenarios. Żejtun is a city in the south-eastern region of Malta, with a resident population of around 11,218, recorded in 2016 (https://nso.gov.mt). It is characterised by urban agglomerations. However, this is adjacent to a green area and hence considered as semi-urban. Attard is another monitoring and collection site chosen for this study. Attard is a town located in the central region of Malta, with the population reaching 10,998 in 2016 (https://nso.gov.mt), this area is considered to be urbanized, having different land-use types, both of residential and industrial nature. Consequently, this site is considered as urban. The other site selected is in Għarb, in the sister island of Malta, Gozo. The area from which the data was collected is also known as the 'San Dimitri' area of Għarb. Contrasting with the previous municipalities mentioned, Għarb is a small village, located on the western-most point of the whole archipelago, with a meagre population of 1,257 residents recorded in 2016 (https://nso.gov.mt) and hence considered as rural.

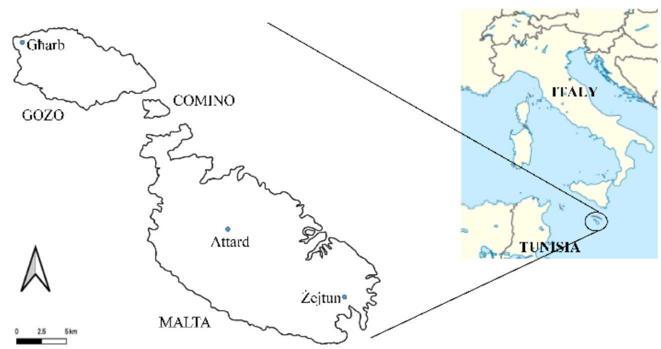

Figure 1. The three monitoring and sampling sites.

Sampling

Ozone data was obtained from the respective sites. A formal request was then sent to ERA in charge of the monitoring stations in order to acquire the localised ozone concentration data for each of the stations. The data was then organised into different periods in accordance with the sample collection.

Plant samples were physically collected from broadleaved and coniferous species, within a radius of 500 metres from the ERA air quality monitoring stations, as indicated in the ICP forest manual (Schaub *et al.*, 2016). Two sets of samples were obtained per plant; one from the upper part of the tree and another from the lower part of the tree. The plant samples were gathered from the three locations mentioned on three separate instances, between November 2019 and February 2020. Plant collection depended on the presence of the plants during the sampling season. Plants were identified through local plant identification resources (http://www.maltawildplants.com/). These included *Acacia saligna*, *Eucalyptus gomophocephala*, *Nerium oleander*, *Pinus halepensis*, *Schinus terebinthifolius* and *Tamarix africana*.

Sample Leaf Images

Leaf samples were subjected to imaging and digitizing procedures. A total of five leaves from each of the samples, that are classified as broad-leaf species, were taken for each scan. Both sides of the leaves were scanned. The specimens were then checked for any stippling and discolouration that occurs on the leaves individually.

Determination of Chlorophyll and Quality Parameters

For this procedure, approximately, 1 g of the specimen was weighed and 10 ml of ethanol were added to the specimen tubes. The tubes were refrigerated at 5°C for 4-5 days in the dark. Aliquots of 100 µl of the supernatant were diluted with ethanol (1:9) and transferred to a UV-Vis spectrophotometer (Lightwave II-WPA). A wavescan was run between 200 and 800 nm. The following parameters were then calculated as follows (Glories, 1984; Feng, Chen, Xiong, Liu, & Yang, 2017):

$$chlorophyll\ a = (13.95 \times A_{665}) - (6.88 \times A_{649})$$

$$chlorophyll\ b = (24.96 \times A_{649}) - (7.32 \times A_{665})$$

$$Total\ chlorophyll = chlorophyll\ a + chlorophyll\ b$$

$$Chlorophyll\ ratio = \frac{chlorophyll\ a}{chlorophyll\ b}$$

$$Colour\ intensity = (A_{420}.DF) + (A_{520}.DF) + (A_{620}.DF)$$

$$Tonality\ Ratio = \frac{A_{420}}{A_{520}}$$

$$Anthocyanin\ content(\mathrm{mg/kg}) = \frac{1000.V_S.DF.A_{520}}{\epsilon}$$

VS = volume of extracted sample per gramme of sample
DF = dilution factor
ε = extinction coefficient [58.3 ml(mg.cm)]
A_{420}, A_{520}, A_{620}, A_{649}, A_{665} = absorbance values at 420, 520, 620, 649 and 665 nm

Statistical analysis

Data was compiled and then statistically analysed using GraphPad Prism ver. 5.0 for Windows (GraphPad Software, San Diego, CA, USA). The Prism software was used for the determination of the level of significance between the means of samples with time and between localities, using one–way ANOVA with a Bonferroni post-hoc. Principal Component Analysis (PCA) was also used for the chlorophyll (chl) content data recorded from the spectrophotometric analysis. Along with this, the chlorophyll ratios were compared to the plant scans of the same samples. All parameters were compared to the different ozone concentrations, by considering the overall plant injury, chlorophyll content and quality parameters, as well as the determination of a possible biological indicator. The significance level was measured at $p<0.05$.

Results and Discussion

Ozone concentrations

Table 1 represents seven-day ozone concentration averages for the three sites. The guideline values (ETC/ATNI, 2020) recommend that the threshold for ozone, to safeguard the natural and semi-natural vegetation from changes in growth and seed production, must not exceed 3ppm/hour (3000 ppb/hr). For the protection of forest plant species from long-term effects and growth reductions, the AOT40 must not exceed 10 ppm/hour. These guideline values exclude the values from protecting sensitive species from short-term acute repercussions (https://wedocs.unep.org). For this current study, in all of the three areas, the concentrations were found to be below the thresholds, both when recorded on the day of the collection and in the averaged total of seven days; before, during and after the date of collection. Overall, statistical analysis revealed that the highest concentrations were found at rural area for all three periods when compared to the levels at the other two sites ($p<0.05$).

Table 1. The mean ozone levels in ppb from October 2019 till February 2020 at the urban, rural and semiurban sampling sites.

Region	Concentration		
	Sampling period 1	Sampling period 2	Sampling period 3
Urban	64.1±3.85	65.3±4.27	36.0±1.76
Rural	88.2±1.08***	76.5±2.33	43.4±1.27**
Semiurban	57.8±4.15	61.6±4.74*	31.2±1.48

Data of sampling periods: 1 (30/10/19 – 07/11/19); 2 (29/11/19 – 06/12/19). 3 (27/01/20 – 03/02/20). *$p<0.05$; **$p<0.01$; ***$p<0.001$ (within the same time period).

These recorded concentrations reveal that the ozone levels are higher in a rural setting indicating to the phenomenon that ozone levels are higher in rural rather than urban areas. This may be due to the possibility of ozone produced within an urban setting undergoing reverse reactions with nitric oxide (Guicherit, 1988), which is associated with combustion engines (Lavoie, Heywood, & Keck, 1970). Therefore, having less of this activity can lead to higher concentrations of ozone in this area. This high level of ozone in rural areas, over the accumulated dose of ozone over a threshold of 40 ppb has been reported for most Mediterranean and Central European Union countries in 2018 (ETC/ATNI, 2020). Higher levels of ozone concentrations have occurred in areas of high radiation and temperatures, and low wind speeds, which provided the necessary levels of emissions and energy for ozone formation. Studies have interpreted this change in ozone levels in terms of changes in barometric measurements (Schreiber, 1996). Schreiber (1996) also established that low pressure systems are associated with supressed surface level ozone. In this present study, it was observed that ozone levels at the three sites correlated negatively with atmospheric pressure ($r<-0.785$) but a positive correlation with temperature ($r>0.701$) indicating that temperature is a potential contributor to ozone levels.

Visual Assessment and Chlorophyll Content: Plant Injury

Samples, considered for visual checks, were taken from plants growing in a natural setting. Generally, the effects of ozone on plant species are determined in an experimental setup, where plants are exposed to different ozone levels (Macdowell, 1965).

The visual assessment was conducted in accordance with the flowchart for the diagnosis of ozone symptoms on broad-leaf species as listed in Part VII of the ICP forest manual (Schaub *et al.*, 2016). As the flowchart, within this section, is restricted to broad-leaf species, the symptoms for the *Pinus halapensis* and the *Tamarix africana* were referred to the symptoms mentioned in Part XII (Rautio, Fürst, & Stefan, Raitio, & Bartels, 2016). Younger and older leaves were collected from the species so as to determine whether there was a correlation between any possible ozone damage by visual injury found between the two leaf samples.

According to Part VIII of the ICP forest manual, the symptoms are significantly experienced by the older leaves. Alongside accelerated senescence, older leaves are the first ones that tend to develop ozone induced symptoms (Schaub *et al.*, 2016). When examining symptomatic leaves, it should be considered that the heterogeneity of the specific plant composition as well as the variability of the ecological conditions can make the visual assessment incomparable between the sites. Moreover, the absence of visible foliar injury symptoms does not necessarily suggest the absence of ground level O_3 pollution and its risks for the surrounding vegetation. It has been well established that the chlorophyll ratio, is considered to be higher in older leaves rather than that of younger leaves (Smith & Nobel, 1978). This means that if the ratio is found to be smaller in older samples, it could mean that there is possible O_3 induced damage in the plant. The foliar damage on the *Schinus terebinthifolius* leaves, particularly prominent in the younger leaves, does not indicate potential O_3 damage according to the symptom diagnosis flowchart. Although in some cases, there were symptoms of acute stippling and general discolouration in older leaves, these have not been related to ozone damage. There can be variability between the symptoms, as many foliar symptoms are known to have low specificity (Bussotti *et al.*, 2006).

There were some interveinal symptoms observed in older leaves and which are indicative of ozone damage (Schaub *et al.*, 2016), but the evidence is not very clear. Consequently, other factors may play a more significant role than ozone. These include early leaf senescence, lack of nutrients and high solar radiation, amongst others. Also, one must note that plants of the same species can react differently in the same levels of exposure, according to their ecological conditions, ontogenetic factors and genotypes (Marzuoli *et al.*, 2019). The chlorophyll ratio in this species for both dates of collection is shown as being larger in the older sample, therefore indicating that there is no ozone impact. There are two possible explanations for the reduction of chlorophyll ratios. Chlorophyll a is possibly more susceptible to degradation by O_3, and ozone could have adverse effects on the synthesis of chlorophyll, therefore synthesis of chlorophyll b is either increasing or chlorophyll a is decreasing, as compared to uninjured leaves (Knudson, Tibbits, & Edwards, 1977).

Nerium oleander is one of those species that can be found all around the islands, planted both on the kerbside and in public gardens. Although general discolouration was observed in both the older and the younger leaves, this was more prominent in the older ones. However, these visual observations were challenged by chlorophyll analysis. Given that, in most cases, the chlorophyll ratio was larger in the older leaves rather than the younger ones, this could possibly indicate that the damage found on the specimens cannot be attributed to ozone. However, in the second period for the semiurban area and in the third period for the urban and semiurban areas, the chlorophyll ratio was smaller in the older leaves rather than the younger leaves. This could possibly indicate that the damage found on the specimens can be attributed to ozone. In a study by Meletiou-Christou, Banilas, Bardis and Rhizopoulou (2011), oleander shrubs were investigated for their changes in chlorophyll and storage substances. The *Nerium oleander* shrubs used, were considered as adapted to the ambient pollution, due to the fact that they did not show any visible injury symptoms. The authors argue that the high ozone concentrations reported regionally in the Mediterranean constitute a limited threat to native plant species in general, and this can be attributed to the high concentration of ozone defence compounds in the foliage (Meletiou-Christou *et al.*, 2011).

Another plant species considered in this study was *Acacia salignia.* Some signs of tissue death was observed in the leaves of this plant. This occured in both old and young leaves indicating that this dark stippling may not be induced by O_3 damage. However the discoloration in older leaves is indicative of O_3 damage. Samples taken during the third period showed signs of ozone damage, manifested by discoloration. The extensive visible foliar damage found can possibly be due to the high ozone concentrations found in the rural area over periods two and three. The chlorophyll ratio in the *Acacia salignia* samples gathered, further strengthens the relationship between the foliar injury recorded and the O_3 concentrations found on site. This was observed by the ratio of chlorophyll for the samples, being smaller in the older rather than in the younger leaves, therefore this means that the damage is possibly induced by O_3.

Some *Eucalyptus* species in general are considered to be highly tolerant to surface ozone pollution, but the levels of tolerance are dependent on the sub-species. A study by Monk & Murray (1995) was set up to measure the growth reduction and evaluate foliar injury caused by ozone in eight species of *Eucalyptus*. In this study, the *Eucalyptus* saplings were grown under diurnally varied conditions of O_3. Significant differences were found between the different species. This study reveals differences in leaf injury and weight reduction for *E. microcorys* (90% and 30%, respectively), *E. gomophocephala* (0% and 30%, respectively) and *E. globulus* (none for both). In the present study, the *E. gomephocephala* collected from the rural area, exhibited the most visually striking with prominent damage observed on leaves (Figure 2). The older leaves had evident interveinal discolouration, which can be seen more extensively than in the younger leaves indicative of injury caused is induced by O_3. The chlorophyll ratio recorded can support this observation, with the ratio being smaller in older rather than younger leaves. There is a clear indication that at rural sites, ozone levels are higher than at urban sites, hence there is a relationship between these parameters. Following this, the *E. gomophocephala* collected on this date shows multiple signs of injury induced by O_3.

Figure 2. *Eucalyptus gomphocephala*, collected from an 'older branch' in the rural area for further analysis.

Pinus halapensis was found in both the urban and the rural locations of this study. Locally, it is a very popular ornamental tree, very common in both forested areas and is extensively found planted in public gardens all around Malta. This species used to naturally grow in the Maltese Islands, until its complete eradication in the beginning of the 19th Century (Le Houérou, 1981). Visual foliar observation for this species, is very challenging due to the nature of the foliage structure being 'needle'-like. In this present study, foliar damage was observed as yellowish spots present on both the old and the younger leaf specimens. In spite of visual difficulties, the chlorophyll ratio on the other hand, provides a better picture. Samples collected from the rural area showed a chlorophyll ratio indicative of ozone damage. The samples collected from the semi-urban area did not show any visual signs of ozone damage, but the chlorophyll ratio clearly indicates ozone damage.

Tamarix africana is an indigenous species found in different sites on the islands, and is specifically popular in coastal areas, due to their halophytic nature. To our knowledge, this species was not investigated previously in terms of ozone damage, so reference to literature was not possible. When comparing the samples collected from the different sites, one distinct difference was that the samples collected from rural area were leaner than those found at the urban area, assuming that this foliage is affected by some kind of external factor. For this species, the chlorophyll ratio was more indicative as to whether the foliar damage was due to ozone, than the visual observations. Additionally this species seemed to be more susceptible to ozone damage in an urban setup rather than a rural setup.

It has been previously reported that dry weights correlated with the total chlorophyll content (Knudson *et al.*, 1977). In this present study, to gain a better understanding as to whether leaf weight correlates with the content of chlorophyll, the Pearson correlation statistical tool was used. Fresh and dry weights were tested for potential correlated to the total chlorophyll content, chlorophyll a, chlorophyll b and the chlorophyll ratio (Table 2). There was no correlation between the fresh leaf weights and all chlorophyll parameters ($r<0.078$). The same was observed with the dry leaf weights and the chlorophyll parameters ($r<0.310$). As a result weight parameters were excluded from any further correlations.

Table 2. The correlation matrix for fresh and dry weights, total chlorophyll content, chlorophyll a, chlorophyll b and the chlorophyll ratio for leaves collected from several species between October 2019 and February 2020.

Variable	FrWt	DryWt	Chl A	Chl B	Total Chl
DryWt	-0.035				
Chl A	0.078	0.293			
Chl B	0.071	0.310	0.988		
Total Chl	0.076	0.298	0.999	0.993	
Chlorophyll ratio	0.000	-0.118	0.021	-0.108	-0.014

Values in bold are different from 0 with a significance level alpha=0.05.

Spectrophotometry for the determination of quality parameters

In this present study, leaf quality parameters were considered so as to determine whether these are affected by potential ozone pollution within the area. Although the pigmentation in leaves is predominantly due to chlorophyll, the leaf contains also other metabolites whose metabolism may be affected by external environmental conditions. Typical metabolites include flavonoids and terpenoids (Harborne, 2012). The absorbance of wavelengths by moieties particularly on flavonoids occurring within the UV and visible range of the spectrum, may be interpreted in terms of colour density, tint, percentage red, blue and yellow colours, and anthocyanin content.

Colour density is the total concentration value of the pigments present in the samples. This gives an indication of colour depth (Owusu, Abano, & Engmann, 2012). The general trend for the colour density measured for the samples, ranged between 2.59-36.69 A. Colour density may be typically diverse depending on species, for example high values in older leaves of *Schinus terebinthifolius* (36.69 A) and low values in older leaves of *Pinus halepensis* (2.72 A). In general there were no significant differences between the ranges obtained for old and younger leaves. However, within species there were variations within the ranges; for *Acacia saligna* the range was 7.46-25.09 A, whereas for *Pinus halapensis*, this was 2.72-5.74 A. Tint relates to the mixture of colour with white (Owusu *et al.*, 2012), which affects the overall reflectance (Sharafudeen, 2010). The general trend for the tint ranged from 8.1463-24.09. The highest tint was recorded in the younger leaves, and the lowest in the older leaves. The range for the older samples is between 8.15-22.89, whilst for the younger it is 8.71-24.09, both deviating slightly from the general trend. When compared to other locations, the rural area was found to have a larger range (8.15-24.09). As for the species, the widest range was found in the *Tamarisk africana*, therefore slightly deviating from the general trend. Meanwhile, the lowest range within the species was found in *Acacia saligna*.

The percentages of the red, yellow and blue colour, determine the types and amount of pigments present in the samples. Apart from the presence of chlorophyll (Milne, Toker, Rubio, & Brøndsted Nielsen, 2015) these colours represent different flavonoids classes such as the anthocyanin and proanthocyanidins. Any changes in the colour may reflect biosynthesis or degradation of particular flavonoid subclasses. For instance, a change in the red colour may be attributed to the increase in anthocyanin synthesis (Li, Yang, Jingmin, & Jun, 2019). This can therefore prove an explanation for the different degrees of damage that is observed visually. The general trend for the %red ranged from 3.49-9.43%, for the %yellow this was 76.78-89.31% and %blue ranged between 4.82-15.51%. The general trend for anthocyanin ranged from 2.57-28.99 mg/kg. The species with the highest content of anthocyanin was *Schinus terebinthiflius* and the lowest content was *Pinus halapensis*. These individual values on their own may not contribute directly to estimation of foliar damage as was observed with the chlorophyll parameters. However, comparison between these colour parameters and chlorophyll content may prove the use of such parameters as indicators of damage. The elaboration of quality parameters into old/young ratios will be discussed later.

Chlorophyll content and quality parameters

It is well known that chlorophyll content may be affected by ozone and other atmospheric pollutants. Apart from chlorophyll and hence the process of photosynthesis, the leaf produces and stores a large number of primary and secondary metabolites (Ibrahim, Jaafar, Karimi, & Ghasemzadeh, 2014). Therefore, the scope of this study extended beyond the relationship of chlorophyll to ozone damage. Prior to any correlations with ozone levels and ozone damage, this study considered the correlation of the chlorophyll content and quality parameters using Principal Component Analysis.

Through PCA, conclusions may be postulated as to what extent the values correlate with each other for the individual samples particularly with potential distinctions between old and young leaf samples. Pearson correlation statistics reveal that the colour density correlates positively with tint, anthocyanin content, chlorophyll a and b (r>0.567), while correlates negatively with %red (r=-0.524), because the colour density is mainly based on the %yellow that shows a very poor correlation to it (r=0.146). Anthocyanin content correlates to the chlorophyll a and chlorophyll b contents (r>0.837). Interestingly, %yellow does not show any significant negative correlation with chlorophyll a and chlorophyll b contents (r=<-0.055). Therefore, it is possible that the leaf chlorosis is not the sole contributor of the yellow coloration exhibited by the leaves. Ougham, Morris, and Thomas (2005) argue that the yellow coloration could also be induced by seasonal environmental changes. This can be attributed to the shorter, cooler days experienced in autumn which trigger an end to auxin production, and the supply of nutrients, sugar and water is cut off, breaking down the chlorophyll (Chu, Zang, & Tian, 2012).

It was observed from the scree plot (data not shown) that the first two components accounted for 70.23% of the total variance. In fact, the majority of the parameters studied fall within these two principal components. Figure 3 exhibits the variables and observations plots that define the correlation between the variables, and the correlation between the samples, respectively.

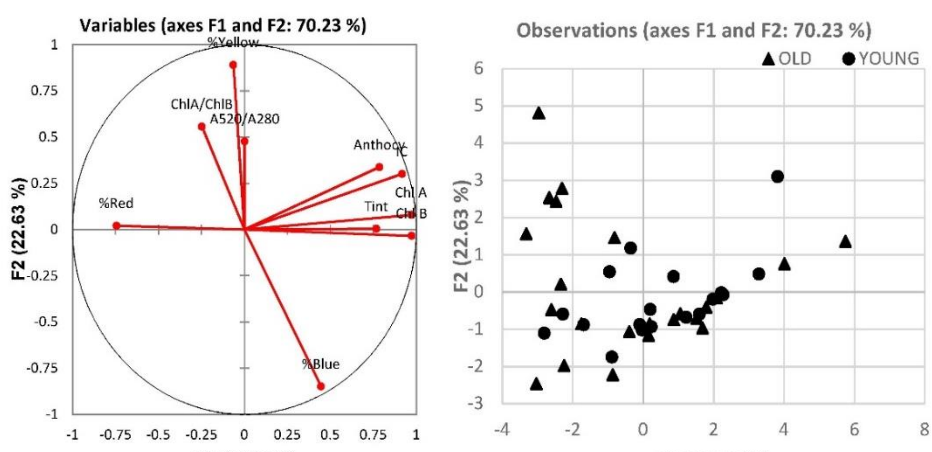

Figure 3: The variables (left) and observations (right) plots for chlorophyll content and quality parameters.

The first factor is loaded heavily on colour density, tint, %red, anthocyanin content, chlorophyll a and chlorophyll b, which represent a mix of chemical and physical parameters. The second factor is heavily loaded on %yellow, %blue, A520/A280 and chlorophyll ratio. The F1-F2 values were rearranged to represent distinctively the old and young leaves samples separately as illustrated in Figure 3. It can be concluded that both chlorophyll and quality parameters were relatively coherent in young leaves, as compared to older leaves. It may be postulated at this point that the wide variability of the parameters in older leaves, showing scattering over the whole plot, is a sign that the values for young and old leaves taken individually might not reveal any concrete data that can be compared to ozone levels and ozone damage.

Ozone damage, chlorophyll ratios and quality parameters ratios within the three localities

In order to determine whether chlorophyll and quality parameters correlate to ozone damage, ratios for every parameter in old and young leaves were considered. Ozone damage was primarily considered on the ratio of chlorophyll a to chlorophyll b in young and old leaves in accordance with other studies (Knudson *et al.*, 1977; Schaub *et al.*, 2016). Through this ratio, it is hypothesised that ozone damage is considered if the chlorophyll ratio in older leaves is smaller than that of younger leaves. That is: $(ChlA/ChlB)_{old}$ / $(ChlA/ChlB)_{young}$ < 1.

Comparing the response of plant species between the three localities it was observed ozone damage was observed 3 out of six leaf (old/young) sample combinations in the urban area, 5 out of six sample combinations in the rural area and 4 out of five sample combinations in the semiurban. In spite of the apparent low ozone damage exhibited in the urban area (56.15 ppb), the mean ozone level ranked second compared to the other two localities (rural and semiurban; 69.35 and 50.18 ppb, respectively). This variation may be in part attributed to the choice of plant species for the study. Looking in turn at the response of plants to ozone damage with respect to the chlorophyll ratio, the plants that apparently exhibited ozone damage, in descending order, are: *Nerium oleander* > *Pinus halapensis* > *Schinus terebinthifolius*.

With regards to the other species, few samples were collected and therefore a conclusion cannot be reached on these.

Table 3. Ratios for quality parameters and chlorohyll for the species under study.

	Plant	IC	Tint	%R	%Y	%B	Anth	ChlA	ChlB	ChlR	O_3D
Urban	Ta	1.223	0.700	1.371	0.959	1.091	1.702	1.174	1.425	0.824	YES
	Ph	0.683	1.529	0.702	1.073	0.781	0.463	0.651	0.524	1.241	NO
	No	1.501	1.138	0.883	1.005	1.007	1.326	1.561	1.372	1.138	NO
	St	1.002	0.953	0.992	0.945	1.605	1.050	1.634	1.590	1.028	NO
	No	1.002	0.859	1.149	0.987	1.041	1.158	1.029	1.032	0.998	YES
	ST	1.054	1.035	0.971	1.005	0.982	1.020	1.052	1.016	1.035	NO
Rural	Ph	0.822	0.493	1.871	0.921	1.186	1.577	0.736	1.065	0.691	YES
	Ta	0.321	0.779	1.280	0.996	0.937	0.407	0.308	0.192	1.605	NO
	As	0.551	0.856	1.146	0.981	1.090	0.640	0.596	0.653	0.913	YES
	Eg	0.608	0.705	1.398	0.986	0.933	0.844	0.503	0.765	0.657	YES
	As	0.870	0.943	1.059	0.998	0.995	0.921	0.852	0.896	0.951	YES
	Eg	0.948	0.913	1.100	1.005	0.881	1.031	0.858	0.881	0.973	YES
Semi urban	Ph	2.216	0.580	1.593	0.924	1.333	3.667	2.258	3.126	0.722	YES
	No	1.386	1.085	0.920	0.998	1.034	1.282	1.399	1.482	0.944	YES
	St	0.771	1.114	0.890	0.991	1.091	0.695	0.803	0.887	0.905	YES
	No	1.012	0.871	1.125	0.980	1.094	1.154	1.037	1.260	0.823	YES
	St	1.904	1.183	0.861	1.019	0.926	1.622	1.765	1.682	1.050	NO

As=*Acacia saligna*, Eg=*Eucalyptus gomophocephala*, No=*Nerium oleander*, Ph=*Pinus halepensis*, St=*Schinus terebinthifolius* and Ta=*Tamarix africana*. CI=colour density (A); Tint=Tint ratio; %R=%red; %Y=%yellow; %B=%blue; Anth=anthocyanin content (mg/kg); ChlA= chlorophyll a; ChlB= chlorophyll b; ChlR=chlorophyll ratio; O_3D=ozone damage.

This study attempted to follow the same procedure for all the quality parameter ratios as discussed previously for the chlorophyll ratios taking into account the old and young values. In general, high values for colour density indicate ozone damage. Conversely, ozone damage results in low tint values. Considering the three colours, ozone damage was exhibited as high %red and %blue but low %yellow values. High anthocyanin values were also indicative of ozone damage. However this is species specific. These ratios were also correlated with the ozone levels at the three localities using Principal Component Analysis (Figure 3) and Pearson correlation (Table 4).

PCA reveals that the rural cluster was separate from the urban cluster showing that the quality parameters responded differently to ozone levels. The rural-urban cluster intermingled with the other two clusters, exhibiting the effects on ozone levels in between the localities.

Due to insufficient data, *Tamarix africana*, *Acacia saligna* and *Eucalyptus gomophocephala*, were excluded from the correlation study. This does not mean that these species are not potential indicators of ozone damage.

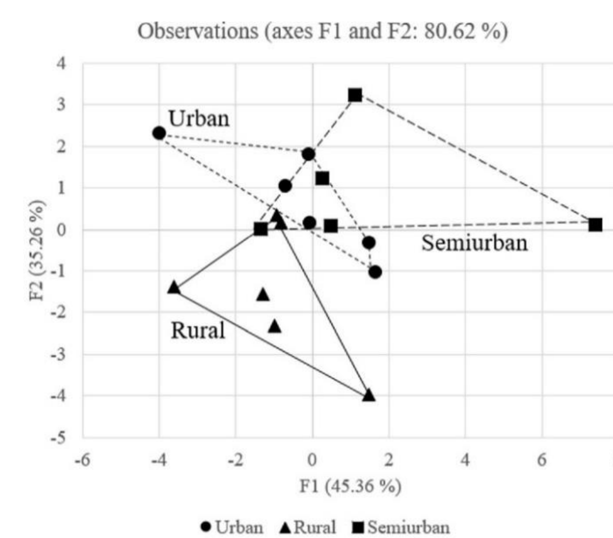

Figure 4. Observations plot for the three locations.

For instance, in a study it was argued that *Eucalyptus* species can manifest reduced weight and signs of injury in the presence of O_3 (Monk & Murray, 1995). For *Pinus halapensis*, ozone levels correlated positively with %Red (r=0.528) but negatively with IC (r=-0.596). Although this species was found at the three sites, the quality parameters do not show significant response to ozone damage. For *Schinus terebinthifolius*, ozone levels correlated positively with %Blue (r=0.791) but negatively with IC, Tint, %Yellow, and Anthocyanins (r<-0.606). Although this species was found at two sites, the quality parameters do not show significant response to ozone damage. In the case of *Nerium oleander*, ozone damage correlated positively with IC, Tint, %Yellow and anthocyanins (r>0.972), but negatively with %Red and %Blue (r<-0.872). From these quality parameters, it can be stated that the higher the ozone level, the higher is the %yellow (potential indicator of chlorosis) and the higher is the anthocyanin (stress metabolites). Previously in this study it was partially concluded that the yellow coloration is not solely attributed to potential breakdown of chlorophyll (Moser, Ulrice, & Müllera, 2008), but it may be due to the presence of particularly yellow flavonoids that absorb at a wavelength of 280nm.

Table 4. Pearson correlation for the quality parameter and chlorophyll ratios with ozone levels.

Species	IC	Tint	%R	%Y	%B	Antho	Chl A	Chl B	ChlR
Ph	-0.596	-0.392	0.528	-0.335	0.067	-0.362	-0.623	-0.499	-0.368
No	0.974	0.972	-0.964	0.986	-0.872	0.978	0.966	0.707	0.725
St	-0.791	-0.606	0.421	-0.859	0.791	-0.747	-0.288	-0.223	-0.590

Ph=*Pinus halepensis*; No=*Nerium oleander*; St= *Schinus terebinthifolius*. CI=colour density; Tint=Tint ratio; %R=%red; %Y=%yellow; %B=%blue; Anth=anthocyanin content; Chl A= chlorophyll a; Chl B= chlorophyll b; ChlR=chlorophyll ratio.

Flavonoids, in leaves, are known to increase in concentrations due to stress. The synthesis of these flavonoids is a mechanism that protects the plant from a number of hostile environmental conditions, including UV radiation, ozone, extreme weather conditions and heavy metals (Mierziak *et al.*, 2014).

Conclusion

This study was based on the determination of ozone damage on number of plant species at three sites in Malta and Gozo. These three sites represented three different scenarios that were expected to exhibit different levels of ozone levels. The plant species collected from the three sites were selected on their possible commonalities between these sites. However, this was not possible for all species. Following the extraction of chlorophyll and other plant metabolites, the samples were subjected to UV-visible analysis. Following analysis, it was concluded that three species qualified further analysis which were *Nerium oleander*, *Pinus halapensis* and *Schinus terebinthifolius*. The species that showed promising results was *Nerium oleander*. This was not only revealed by the conventional ozone damage index, derived from the ratio of chlorophyll a/chlorophyll b of old and young leaves, but also when quality parameters were challenged with ozone levels.

It was revealed that the yellowing of leaves was not solely attributed to the chlorosis due to the catabolism of chlorophyll but also due to the presence of yellow flavonoids that were prominent in UV-visible scans. On the other hand, another group of flavonoids, the anthocyanins, also correlated positive with ozone levels, and this can be explained by the fact that the production of flavonoids, considered as potential antioxidants, increases with stress. It can be concluded that *Nerium oleander* can be used as a bioindicator of ozone damage. It is worth noting, that there were other plant species that may have exhibited this potential but due to limited sampling, a conclusion cannot be drawn on these species. Furthermore, quality parameters such as %yellow and anthocyanin content can be measured alongside chlorophyll a and chlorophyll b content for the determination of ozone damage.

References

Agathokleous, E., Saitanis, C. J., Feng, Z., De Marco, A., Araminiene, V., Domingos, M., Sicard, P., & Paoletti, E. (2020). Ozone biomonitoring: A versatile tool for science, education and regulation. *Current Opinion in Environmental Science & Health, 18,* 7-13. doi: 10.1016/j.coesh.2020.04.005

Burkey, K. O., Booker, F. L., Ainsworth, E. A., & Nelson, R.L. (2012). Field assessment of a snap bean ozone bioindicator system under elevated ozone and carbon dioxide in a free air system. *Environmental Pollution, 166*, 167-171. doi: 10.1016/j.envpol.2012.03.020

Chu, A., Zhang, Y., & Tian, Y. (2012). Physiological changes of leaves of several fall color trees during color changing period in autumn and winter. *Journal of Northeast Forestry University*, *40*(11), 40-43.

Directive 2001/81/EC of the european parliament and of the council of 23 October 2001 on national emission ceilings for certain atmospheric pollutants, *Official Journal of the European Communities*, *L 309*, 22-30.

Directive 2008/50/EC of the european parliament and of the council of 21 May 2008 on ambient air quality and cleaner air for Europe, *Official Journal of the European Union*, *L 152*, 1-44.

European Environment Agency (EEA). (2019). Air Quality in Europe. Luxembourg: European Environment Agency. doi: 10.2800/822355

Environmental Protection Agency (EPA). 2015. National Ambient Air Quality Standards (NAAQS) for Ozone: Final rule, *Federal Register*, *80*(206), 65292-65468

European Topic Centres on Air Pollution, Transport, Noise and Industrial Pollution (ETC/ATNI). (2020). European air quality maps for 2018 — PM10, PM2.5, ozone, NO2 and NOX spatial estimates and their uncertainties. Eionet Report ETC/ATNI 10/2020, European Topic Centre on Air Pollution, Transport, Noise and Industrial Pollution, Norway Retrieved January 11, 2022, from https://www.eionet.europa.eu/etcs/etc-atni/products/etc-atni-reports/etc-atni-report-10-2020-european-air-quality-maps-for-2018-pm10-pm2-5-ozone-no2-and-nox-spatial-estimates-and-their-uncertainties-1

Feng, H., Chen, G., Xiong, L., Liu, Q., & Yang, W. (2017). Accurate digitization of the chlorophyll distribution of individual rice leaves using hyperspectral imaging and an integrated image analysis pipeline. *Frontiers in Plant Science*, *8*, 1238. doi: 10.3389/fpls.2017.01238

Giri, S., Shrivastava, D., Deshmukh, K., & Dubey, P. (2013). Effects of Air Pollution on Chlorophyll Content of Leaves. *Current Agriculture Research Journal*, *1*(2), 93-98. doi: 10.12944/CARJ.1.2.04

Glories, Y. (1984). La couleur des vins rouges: 2e. Partie: mesure, origine et interpretation. *Connaissance de la Vigne et du Vin*, *18*(4), 253–271. doi: 10.20870/oeno-one.1984.18.4.1744

Gottardini, E., Cristofolini, F., Cristofori, A., & Ferretti, M. (2014). Ozone risk and foliar injury on *Viburnum lantana* L.: a meso-scale epidemiological study. *Science of the Total Environment*, *493*, 954-960. doi: 10.1016/j.scitotenv.2014.06.041

Guicherit, R. (1988). Ozone on an Urban and Regional Scale. In: I. S. A. Isaksen (Ed) *Tropospheric Ozone*. NATO ASI Series (Series C: Mathematical and Physical Sciences), Vol. 227. Springer, Dordrecht. doi: 10.1007/978-94-009-2913-5_3

Harborne, A. J. (2012). Phytochemical methods: A guide to modern techniques of plant analysis. London: Chapman & Hall. doi: 10.1007/978-94-009-5921-7

Holland, M., Kinghorn, S., Emberson, L., Cinderby, S., Ashmore, M., Mills, G., & Harmens, H. (2006). Development of a framework for probabilistic assessment of the economic losses caused by ozone damage to crops in Europe. Bangor: NERC/Centre for Ecology and Hydrology Publisher.

Hopkins, W. G., & Hüner, N. P. (2008). Introduction to Plant Physiology. The University of Western Ontario: John Wiley & Sons, Inc.

Ibrahim, M., Jaafar, H., Karimi, E., & Ghasemzadeh, A. (2014). Allocation of Secondary Metabolites, Photosynthetic Capacity, and Antioxidant Activity of Kacip Fatimah (*Labisia pumila* Benth) in Response to CO_2 and Light Intensity. *The Scientific World Journal*. doi: 10.1155/2014/360290

Knudson, L. L., Tibbits, W. T., & Edwards, G. E. (1977). Measurement of Ozone Injury by Determination of Leaf Chlorophyll concentration. *Plant Physiology*, *60*, 606-608. doi: 10.1104/pp.60.4.606.

Lavoie, G. A., Heywood, J. B., & Keck, J. C. (1970). Experimental and theoretical study of nitric oxide formation in internal combustion engines. *Combustion Science and Technology*, *1*(4), 313-326. doi: 10.1080/00102206908952211

Le Houérou, H. N. (1981). Impact of man and his animals on Mediterranean vegetation. In: di Castri, F., Goodall, D. W., & Specht R. L., eds. Mediterranean-Type Shrublands. Ecosystems of the World. Amsterdam: Elsevier

Li, Y., Yang, S., Jingmin, J., & Jun, L. (2019). Spectroscopic determination of leaf chlorophyll content and color for genetic selection on *Sassafras tzumu*. *Plant Methods*, *15*(1) 1-11. doi: 10.1186/s13007-019-0458-0

Macdowell, F. (1965). Stages of Ozone Damage to Respiration of Tobacco Leaves. *Canadian Journal of Botany*, *43*(4), 420-427.

Masri, S., Hou, H., Dang, A., Yao, T., Zhang, L., Wang, T., Qin, Z., Wu, S., Han, B., Chen, J., Chen, Y., Wu, J. (2019). Development of spatiotemporal models to predict ambient ozone and NOx concentrations in Tianjin, China. *Atmospheric Environment*, *213*, 37-46. doi : 0.1016/j.atmosenv.2019.05.060

Meletiou-Christou, M. S., Banilas, G. P., Bardis, C., & Rhizopoulou, S. (2011). Plant biomonitoring: impact of urban environment on seasonal dynamics of storage substances and chlorophylls of oleander. *Global NEST Journal*, *13*(4), 395-404.

Mills, G., Pleijel, H., Malley, C. S., Sinha, B., Cooper, O. R., Schultz, M. G., Neufeld, H. S., Simpson, D., Sharps, K., Feng, Z., Gerosa, G., Harmens, H., Kobayashi, K., Saxena, P., Paoletti, E., Sinha, V., Xu, X. (2018). Tropospheric Ozone Assessment Report: present-day tropospheric ozone distribution and trends relevant to vegetation. *Elementa: Science of the Anthropocene*, *6*(1). doi: 10.1525/elementa.302

Milne, B., Toker, Y., Rubio, A., & Brøndsted Nielsen, S. (2015). Unraveling the Intrinsic Color of Chlorophyll. *Angewandte Chemie International Edition*, *54*(7), 2170-2173. doi: 10.1002/anie.201410899

Monk, R., & Murray, F. (1995). The Relative Tolerance of Some Eucalyptus Species to Ozone Exposure. *Water, Air, & Soil Pollution*, *85*, 1405-1411. doi : 10.1007/BF00477178

Moser, S., Ulrice, M., & Müllera, T. (2008). A yellow chlorophyll catabolite is a pigment of the fall colours. *Photochemical and Photobiological Sciences*, *8*, 1577-1581. doi: 10.1039/b813558d

Ougham, H. J., Morris, P., & Thomas, H. (2005). The Colors of Autumn Leaves as Symptoms of Cellular Recycling and Defenses Against Environmental Stresses. *Current Topics in Developmental Biology*, *66*(1), 135-160. doi: 10.1016/S0070-2153(05)66004-8

Owusu, J., Ma, H., Abano, E. E., & Engmann, F. N. (2012). Influence of two inocula levels of *Saccharomyces bayanus*, BV 818 on fermentation and physico-chemical properties of fermented tomato (*Lycopersicon esculentum* Mill.) juice. *African Journal of Biotechnology*, *11*(33), 8241-8249. doi: 10.5897/AJB11.4300

Paoletti, E., De Marco, A., Beddows, D. C., Harrison, R. M., & Manning, W. J. (2014). Ozone levels in European and USA cities are increasing more than at rural sites, while peak values are decreasing. *Environmental Pollution*, *192*, 295-299. doi: 10.1016/j.envpol.2014.04.040

Rautio, P., Fürst, A., Stefan, K., Raitio, H., & Bartels, U. (2016). Part XII: Sampling and Analysis of Needles and Leaves. In: UNECE ICP Forests Programme Co-ordinating Centre (ed.): Manual on methods and. Thünen Institute of Forest Ecosystems, Eberswalde, Germany: United Nations Economic Commission for Europe (UNECE).

Schaub, M., Calatayud , V., Ferretti , M., Brunialti, G., Lövblad, G., Krause, G., & Sanz, M. (2016). Part VIII: Monitoring of Ozone Injury. In: UNECE ICP Forests Programme Co-ordinating Centre (ed.): Manual. Thünen Institute of Forest Ecosystems, Eberswalde: United Nations Economic Commission for Europe (UNECE).

Schreiber, V. (1996). A synoptic climatological evaluation of surface ozone concentrations in Lancaster Country, Pennsylvania. Millersville University of Pennsylvania.

Sharafudeen, R. (2010). A spectroscopic method for quick evaluation of tint strength and tint tone of titania (rutile) pigment and factors affecting them. *Color Research & Application*, *44*(1). doi: 10.1002/col.22271

Sharma, S. B., Jain, S., Khirwadkar, P., & Kulkarni, S. (2013). The effects of air pollution on the environment and human health. *Indian Journal of Research in Pharmacy and Biotechnology*, *1*(3), 391-396.

Shimizu, Y., Lu, Y., Aono, M., & Omasa, K. (2019). A novel remote sensing-based method of ozone damage assessment effect on Net Primary Productivity of various vegetation types. *Atmospheric Environment*, *217*, 116947. doi: 10.1016/j.atmosenv.2019.116947

Sicard, P., De Marco, A., Troussier, F., Renou, C., Vas, N., & Paoletti, E. (2013). Decrease in surface ozone concentrations at Mediterranean remote sites and increase in the cities. *Atmospheric Environment*, *79*, 705–715. doi:10.1016/j.atmosenv.2013.07.042

Tonelli, M., Pellegrini, E., D'Angiolillo, F., Petersen, M., Nali, C., Pistelli, L., & Lorenzini, G. (2015). Ozone-elicited secondary metabolites in shoot cultures of *Melissa officinalis* L. *Plant Cell. Tissue and Organ Culture (PCTOC)*, *120*(2), 617-629. doi: 10.1007/s11240-014-0628-8

van Zelm, R., Huijbregts, M. A., den Hollander, H. A., Van Jaarsveld, H. A., Sauter, F. J., Struijs, J., Harm J. van Wijnen & van de Meent, D. (2008). European characterization factors for human health damage of PM10 and ozone in life cycle impact assessment. *Atmospheric Environment*, *42*(3), 441-453. doi: 10.1016/j.atmosenv.2007.09.072