



## Assessment of physiological state of *Betula pendula* and *Carpinus betulus* through leaf reflectance measurements



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### abstract

In this study, the evidence of a relationship between the physiological state and leaf optical properties led us to investigate the behaviour of two species – *Betula pendula* Roth and *Carpinus betulus* L. – in order to determine if the vegetation is affected by the heavy metal load of the soil from a former mining area, Rosia Montana, Romania (46°18′47″N, 23°10′15″E). Two non-destructive techniques (leaf transmittance and reflectance) were compared to assess the physiological state of the plants, and 27 vegetation indices for estimating foliar pigment content were tested. For chlorophyll assessment, the reflectance ratios  $R_{NIR}/R_{550}$  and  $R_{NIR}/R_{700}$  were the most reliable for an accurate estimation of the pigment content of the leaves. The light use efficiency (LUE) indices were within normal values, except the Red Green Ratio, indicating that the overall reactions of photosynthesis are not impaired by the specific conditions of the habitat. Differences in leaf optical properties are minimal between the two studied species and the values of the vegetation indices are showing similar trends. Both techniques performed consistently well and yielded accurate estimations of pigment content, supporting their interoperability.

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### 1. Introduction

In vegetation studies, UV–VIS–NIR spectroradiometric techniques have been demonstrated to be valuable alternatives to time-consuming and costly chemical analysis for the estimation of plant properties. Reflectance spectroscopy has important benefits compared to the traditional methods (Richardson et al., 2002). Spectral bioindicators provide new approaches to monitor the physiological state of plants considering that the stress responses affect the optical properties of plants. These non-destructive optical methods based on reflectance, absorbance and/or on transmittance of light by leaf pigments are able to assess the physiological state of a plant, faster than biochemical methods, and directly in situ. Hyperspectral data may be used to estimate pigment and water content (Gitelson and Merzlyak, 1994; Gitelson et al., 1996; Datt, 1998), plant stress (Garriga et al., 2014) or phenological changes. Analytical models were successfully developed to

optimize remotely sensed vegetation indices (VIs) for estimating leaf chemical constituents (Féret et al., 2011), to investigate changes at leaf scale (Brantley et al., 2011) or at canopy level (Zarco-Tejada et al., 2001; Clevers and Kooistra, 2012).

Broadband or narrowband greenness vegetation indices (VIs) are designed to provide information about the quality of photosynthetic pigments in vegetation. These VIs using reflectance measurements in the red and near-infrared regions are estimating the total amount of pigments, being sensitive even to small changes in vegetation status produced by stress factors.

Chlorophylls a, b, and carotenes are the most important pigments involved in the process of photosynthesis. Chlorophyll loss in plants may be associated to environmental stress; therefore, the variation of chlorophyll concentration according to the plant habitat can be used as a good indicator of stress in plants (Peñuelas and Filella, 1998).

The red-edge region has often been used for estimating chlorophyll content and for detecting plant stress (Horler et al., 1983; Wu et al., 2008). The red-edge chlorophyll index ( $CI_{red-edge} = R_{800}/R_{710} - 1$ ) and green chlorophyll index ( $CI_{green} = R_{800}/R_{550} - 1$ ) have been proposed by Gitelson et al. (2003, 2006). Due to lower absorption by chlorophyll in the red-edge region, the use of such bands reduces the saturation effect, the reflectance still remaining sensitive to chlorophyll absorption at moderate-to-high values (Schlemmer et al., 2013).

Abbreviations: Chl, chlorophyll; CCI, chlorophyll content index; LUE, light use efficiency; NDVI, Normalized Difference Vegetation Index; SR, simple ratio indices; VI, vegetation index.

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Recently, some new vegetation indices strongly sensitive to Chla, Chlb and total chlorophyll (Chlt) have been developed. The in-chlorophyll centre waveband (ICCW) (640–680 nm) or off-chlorophyll absorption centre wavebands (OCCW) are using the reflectance in these bands as optimal indices for non-destructive determination of plant Chl content (Main et al., 2011; Zhang et al., 2014).

The light use efficiency indices are quantifying vegetation's ability to use photosynthetic active radiation (PAR) for sustainable growth and for biomass production.

The stress-related pigments – carotenoids and anthocyanins – expressed by the pigment vegetation indices, tend to be present in higher concentrations when vegetation is in a weakened state. Carotenoids are protecting chlorophyll from the harmful effects of high intensity light. Anthocyanins are abundant in newly-formed leaves and in leaves undergoing senescence.

The quality of the results of such investigations is influenced not only by the indices which best assess the vegetation status but also by the correlation to the environmental on-site conditions. An optimal selection of spectral bands and an appropriate processing approach for pigment estimation among the vast array of those available are required.

In most cases, the techniques for pigment estimation have been tested for a single species or a few related species and thus, it is not clear whether they can be applied across species with varying leaf structural characteristics. In other cases, one technique (e.g. VI) was applied for different species (Sims and Gamon, 2002), with no comparison with other techniques. This study was carried out on two species of the Betulaceae family, *Betula pendula* Roth and *Carpinus betulus* L., in order to determine if the vegetation is affected by the heavy metal load of the soil from a former mining area. Correlations between chlorophyll meter readings and spectroradiometer reflectance readings were performed in order to establish if the two techniques are interoperable, and a selection of indices with optimal spectral bands which best correlate for the two species was made.

## 2. Materials and methods

### 2.1. Study area

The experiment was conducted in the former gold and silver mining area, Rosia Montana, Romania (46° 18' 47" N, 23° 10' 15" E). In the studied area, the species diversity is low being mostly attributable to the contaminated environment. The most common species are *B. pendula* Roth (European white birch) and *C. betulus* L. (European hornbeam). A systematic experimental design was made to investigate the influence of contaminated soils on optical properties of the plants. The strategy of sampling methodology was made in order to cover the entire area including polluted and non-polluted zones (for soil and vegetation).

### 2.2. Plant material

The trees from two locations – in the proximity of the deposit considered as polluted area and at 2 km distance from the mining exploitation zone considered as control – were selected randomly. In both locations, trees with comparable sizes located at distance below 10 m between them (to avoid variability of the soil) have been selected. Leaf samples from 66 birch trees (18 in control and 48 in polluted zone) and 24 hornbeam trees (9 in control and 15 in polluted zone) have been collected for analysis. The sampling points were marked by GPS readings. Measurements were made between June and July 2012 on individual leaves (minimum 3 leaves per tree), directly in the field. The leaves have been selected to be

healthy, green uniform coloured, fully developed, from the middle outer part of the trees. The leaves have been removed from the trees and measurements have been taken both, with the portable spectroradiometer and with the portable chlorophyll meter.

### 2.3. Leaf reflectance

Hyperspectral data were collected using a plant probe attached to a full range PSR-3500 portable spectroradiometer (Spectral Evolution, Lawrence, USA) with a spectral range of 350–2500 nm. The sampling interval across the 350–1000 nm is 1.5 nm with a spectral resolution of 3.5 nm (full-width-at-half-maximum), the sampling interval for 1000–1500 nm is 3.8 nm with a spectral resolution of 9.5 nm, and the sampling interval and the spectral resolution are approximately 2.5 nm and 6.5 nm, respectively, for the 1500–2500 nm range. The portable spectroradiometer is provided with a GETAC PS236 PDA (personal digital assistant) which is connected to the spectroradiometer via Bluetooth and can collect spectra with GPS readings. Data are interpolated by the DAR-Win SP data acquisition software to produce values at nanometer interval (Spectral Evolution). Leaves were scanned with a leaf clip with built-in reflectance standard (white reference). It includes also a small spot and low power illumination to keep excess heat from burning the leaf during the measurement (Lawrence, 2013). As such, the internal light source allows spectral data collection regardless of weather and time of day. Each scan represented the average of several readings on each leaf.

### 2.4. Relative chlorophyll content

Leaf relative chlorophyll content was estimated using a portable chlorophyll meter (CCM-200, Opti Science, Hudson, USA) that calculates the chlorophyll content index (CCI) as the ratio of radiation transmitted through the leaf at two wavelengths (940 nm and 660 nm).

Considering the best consistency and accuracy, from one to four separate measurements were made, usually on healthy, well developed, most fully exposed leaves from the middle part of trees. Plants or leaves damaged by insects were avoided.

### 2.5. Chlorophyll fluorescence

OS1-FL modulated fluorometer (Opti-Sciences INC, OS1-FL, Hudson, USA) was used to measure parameters of the chlorophyll fluorescence. Active fluorescence parameters ( $F_0$ ,  $F_m$ ,  $F_v$  and  $F_v/F_m$ —maximum quantum efficiency indicating photochemical efficiency of PSII) have been collected and interpreted for baseline data, as a tool for comparison with light use efficiency (LUE) indices. For the purposes of evaluation, the multiple fluorescence values were averaged.

### 2.6. Vegetation indices

Based on spectral reflectance measurements, various normalized difference vegetation (NDVI) and simple ratio (SR) indices from the literature have been calculated (Table 1). These vegetation indices were correlated with the chlorophyll content index (CCI) obtained from the transmittance measurements.

Indices selected to assess the physiological state of the plants were, Red Edge Normalized Difference Vegetation Index (NDVI705), Modified Red Edge Simple Ratio Index (mSR705), Photochemical Reflectance Index (PRI), Structure Insensitive Pigment Index (SIPID), Red Green Ratio Index (RGR Ratio), Plant Senescence Reflectance Index (PSRI), Carotenoid Reflectance Index 1 and 2 (CRI1, CRI2), Anthocyanin Reflectance Index 1 (ARI1).



Table 1  
Vegetation indices evaluated.

No	Equation	Index name	Detection	References
1	$R_{750}/R_{680}$	SR <sup>a</sup>	Chl	Richardson et al. (2002)
2	$R_{750}/R_{705}$	SR <sup>a</sup>	Chl	Gitelson and Merzlyak (1994)
3	$R_{750}/R_{700}$	SR <sup>a</sup>	Chl, stress	Gitelson and Merzlyak (1996)
4	$R_{750}/R_{550}$	SR <sup>a</sup>	Chl	Gitelson and Merzlyak (1996)
5	$R_{750}/R_{695}$	Carter index	Chl	Carter (1994)
6	$R_{750}/R_{556}$	SR <sup>a</sup>	Chl	Maccioni et al. (2001)
7	$R_{750}/R_{710}$	Zarco and Miller	Chl	Zarco-Tejada et al. (2001)
9	$R_{760}/R_{695}$	SR <sup>a</sup>	Chl	Carter (1994)
10	$R_{850}/R_{710}$	Datt derivative	Chl	Datt (1998)
11	$R_{800}/R_{680}$	SR <sup>a</sup>	Chla	Blackburn (1998)
12	$R_{800}/R_{550}$	SR <sup>a</sup>	Chl	Buschman and Nagel (1993)
13	$R_{800}/R_{635}$	SR <sup>a</sup>	Chlb	Blackburn (1998)
14	$R_{800}/R_{675}$	PSSRa	Chla	Blackburn (1998)
15	$R_{800}/R_{650}$	PSSRb	Chlb	Blackburn (1998)
16	$R_{800}/R_{500}$	PSSRc	Car	Blackburn (1998)
17	$(R_{750} - R_{680})/(R_{750} + R_{680})$	NDVI <sup>b</sup>	Chl	Richardson et al. (2002)
18	$(R_{750} - R_{705})/(R_{750} + R_{705})$	NDVI <sup>b</sup>	Chl	Gitelson and Merzlyak (1994)
19	$(R_{800} - R_{445})/(R_{800} - R_{680})$	SIP <sup>d</sup>	Car, Chla	Penuelas et al. (1995)
20	$(R_{800} - R_{700})/(R_{800} + R_{700})$	NDVI <sup>b</sup>	Chl	Gitelson and Merzlyak (1994)
21	$(R_{800} - R_{680})/(R_{800} + R_{680})$	PSND <sup>e</sup>	Chla	Blackburn (1998)
22	$(R_{800} - R_{635})/(R_{800} + R_{635})$	PSND <sup>e</sup>	Chlb	Blackburn (1998)
23	$(R_{850} - R_{710})/(R_{850} - R_{680})$	m SR <sup>f</sup>	Chl	Datt (1998)
24	$R_{red\ edge}, (R_{670} + R_{780})/2$ $700 + 40 \times (R_{red\ edge} - R_{700})/(R_{740} - R_{700})$	REIP <sup>c</sup>	Chl	Horler et al. (1983)

<sup>a</sup> Simple ratio.

<sup>b</sup> Normalized Difference Vegetation Index.

<sup>c</sup> Red edge inflection point.

<sup>d</sup> Structure Insensitive Pigment Index.

<sup>e</sup> Pigment specific normalised difference.

<sup>f</sup> Modified simple ratio of reflectance.

## 2.7. Heavy metal assessment in soil and plant tissue

The soil samples have been collected at the bottom of the trees, within 50 cm diameter, from the surface layer (0–15 cm depth), using a clean stainless steel shovel. The samples were packed in plastic bags, labeled and transported to the laboratory for further analysis. In the laboratory, the samples were dried at 105 °C for 24 h and sieved according to US-EPA 3050B. The digestion procedure for soil samples was done according to ISO 11.466 protocol, using a mixture of nitric acid and hydrochloric acid. The concentrations for Ni, Pb, Cd, Cr, Cu and Zn were determined on a ZEE nit 700 atomic absorption spectrometer using an acetylene–air (C<sub>2</sub>H<sub>2</sub>–air) flame and the adequate cathode lamps. For each heavy metal the results were averaged for each of the two distinct study sites. Leaf samples were dried in an oven at 105 °C for 24 h, weighed (0.5–5 g) and digested in nitric acid. Ni, Pb, Cd, Cr, Cu and Zn concentrations were analyzed by using the inductively coupled plasma mass spectrometry technique (SCIEX PerkinElmer Elan DRC II instrument).

## 2.8. Data analysis

All statistical analyses were conducted using the STATISTICA statistical software (Version 8, StatSoft Inc. Tulsa, USA, 2007) and Excel. To test and verify the relationship between relative chlorophyll content (from CCM-200) and hyperspectral vegetation indices (VIs), regression analyses were used and coefficients of determination (R<sup>2</sup>) were calculated.

Pearson's correlation coefficient was calculated across sampling dates in order to evaluate the sensitivity of vegetation indices to relative leaf Chl. This analysis involved determining associations between selected reflectance ratios measured in narrow wavebands and the relative concentration of Chl in leaves on a plot mean basis, each plot consisting of the trees and the soil within 10 m<sup>2</sup>.

In order to determine new wavelengths for estimating chlorophyll content, correlations of CCI values with reflectance at all

wavelengths were also determined and scatter plots were constructed to verify the type of relations (linear or non-linear). Thus, the wavelengths with maximum and minimum sensitivities to relative pigment content were identified.

In order to see if the differences between mean heavy metal concentrations in birch and hornbeam leaves from control and polluted zones are significant, the unpaired (independent) t test was used in case of both species. First, the F test was used to test equality of variance and then the proper t test formula was calculated for each metal.

## 3. Results and discussion

According to the standard reference values of ORDIN 756/1997 concerning heavy metals in soil, and compared to the normal, excessive and phytotoxic levels given in Kabata-Pendias and Pendias (1992), and Pais and Jones (1997) in plant tissues, most of the heavy metal concentrations found in soil samples and leaves samples were in the range of medium values with few exceptions (see Electronic Supplemental material). Concentrations of Cu, Pb, and Cd in soil samples from the polluted zone were slightly increased (but still below the alert or intervention threshold) compared to the control zone located at about 2 km distance. Few samples exceeded the intervention threshold of 200 mg/kg in case of Cu. In plant tissues, slightly higher concentrations compared to mean values of Cu were found in case of both species but especially in case of hornbeam (15.78 mg/kg). Most of the Zn concentrations in soil samples are in the range of normal values, but in case of both species, increased concentrations of Zn were obtained in plant tissues compared to correspondent soil samples. In case of B. pendula, in almost half of the samples, Zn concentrations were higher than 100 mg/kg, with a maximum value of 570.9 mg/kg in leaves. Only one leaf sample exceeded the threshold of 100 mg/kg in case of C. betulus. In case of both species, slightly increased concentrations of Cr and some excessive values of Ni were measured in few samples



Table 2

Pearson's correlation coefficients between vegetation indices and CCI for *Betula pendula* and *Carpinus betulus*.

No	Algorithm	B. pendula	C. betulus
1	$R_{750}/R_{710}$	0.722	0.907
2	$R_{850}/R_{710}$	0.711	0.900
3	$R_{750}/R_{705}$	0.710	0.900
4	$R_{750}/R_{556}$	0.710	0.878
5	$R_{750}/R_{550}$	0.708	0.878
6	$R_{800}/R_{550}$	0.703	0.877
7	$R_{750}/R_{700}$	0.687	0.876
8	$R_{760}/R_{695}$	0.626	0.800
9	$R_{800}/R_{635}$	0.624	0.771
10	$R_{800}/R_{650}$	0.543	0.695
11	$R_{800}/R_{680}$	0.303	0.437
12	$R_{750}/R_{680}$	0.293	0.423
13	$(R_{750} - R_{705})/(R_{750} + R_{705})$	0.706	0.907
14	$(R_{850} - R_{710})/(R_{850} + R_{710})$	0.698	0.910
15	$(R_{800} - R_{700})/(R_{800} + R_{700})$	0.675	0.883
16	□ Red edge	0.636	0.884
17	$(R_{800} - R_{635})/(R_{800} + R_{635})$	0.630	0.796
18	$(R_{800} - R_{680})/(R_{800} + R_{680})$	0.304	0.400
19	$(R_{750} - R_{680})/(R_{750} + R_{680})$	0.293	0.382
20	$(R_{800} - R_{445})/(R_{800} + R_{445})$	0.191	0.107

compared to mean values. A maximum value for Ni in case of *B. pendula* is 15 mg/kg and in case of *C. betulus* is 27.5 mg/kg (Lazăr et al., 2014).

The wavelengths sensitive to the chlorophyll content index values were selected by calculating correlation coefficients between the CCI values and each wavelength.

In case of birch trees, the wavelengths that best correlated with CCI values are within the range of 522.5–600.6 nm (Green Band), 602–614.8 nm; 697.5–700.1 nm (Red Band) and 701.5–714.8 nm (NIR). Wavelengths between 738 and 900 nm show the lowest correlations with CCI values. Reflectance in the blue and red bands shows weaker correlations with CCI values at wavelengths 400.4–500.3 nm, and 667.6–689.4 nm, respectively. The strongest correlation coefficients obtained in case of *B. pendula* ranges between –0.60 and –0.65.

In case of *C. betulus*, wavelengths which correlated best with CCI values are within the range of 525.5–600.6 nm, 602–617.6 nm and 696.1–700.1 nm and between 701.5 and 717.5 nm. Reflectance in the NIR domain, for intervals between 739.8 and 900 nm is insensitive to CCI values. Wavelengths between 400.4 and 500.3 nm and between 667.6 and 688 nm show weak correlations with CCI values. The strongest correlation coefficients obtained in case of hornbeam range between –0.75 and –0.83.

Results for the regression analysis showed that all of the vegetation indices of this study positively correlated with the CCI index. The coefficients of determination ( $R^2$ ) calculated for relationships between this index (independent variable) and the normalized and simple ratio vegetation indices (dependent variables) for all samples ( $n=90$ ) of *B. pendula* and *C. betulus*, showed that SR index was slightly superior to NDVI in its correlation with the CCI index in case of *B. pendula* and was slightly inferior to NDVI in case of *C. betulus*. Generally, our findings have shown that the simple ratio and the normalized vegetation indices correlated well with chlorophyll content, but the coefficient of determination is higher for *C. betulus* than for *B. pendula* (Fig. 1).

Our results concerning Pearson's correlation coefficients displayed in Table 2 are in accordance with previous works (Gitelson and Merzlyak, 1996; Curran et al., 1991) showing that the reflectance in spectral band near red-edge region is strongly related to chlorophyll content. The 'off-centre' chlorophyll bands support previous findings that chlorophyll absorption wavelengths (660–680 nm) are poor indicators of chlorophyll content, due to ready saturation even at low chlorophyll content (Wu et al., 2008;

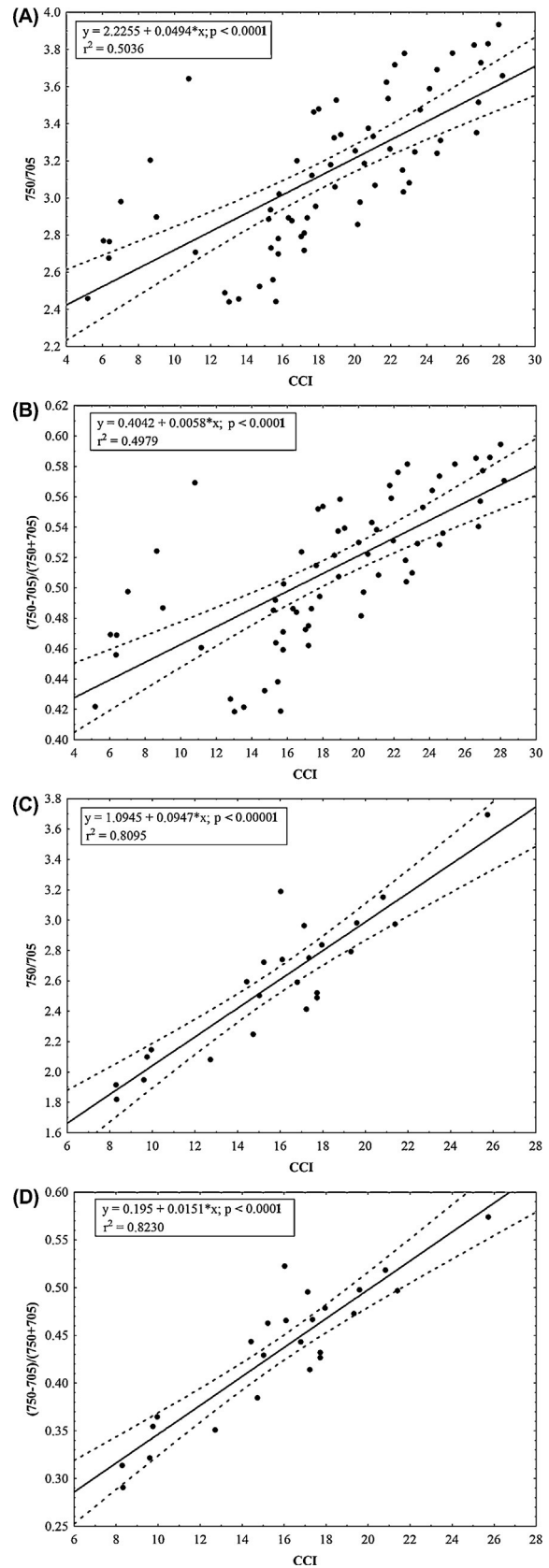


Fig. 1. The SR index ( $R_{750}/R_{705}$ ) and normalized difference index  $(R_{750} - R_{705})/(R_{750} + R_{705})$  as a function of the leaf chlorophyll content in *Betula pendula* (A, B) and *Carpinus betulus* (C, D).





Daughtry et al., 2000). Indices containing 'off-centre' wavelengths (690–730 nm) have greater sensitivity to more subtle changes in chlorophyll content (Main et al., 2011). Reflectance around 550 nm is sensitive to both chlorophyll and anthocyanin content, and near 500–520 nm is sensitive to both carotenoid and chlorophyll content.

As can be seen from Fig. 2A and B for both species, the reflectance of leaves from polluted zones is higher in VIS and in NIR parts of the spectrum compared to control zone, the form of the curves being quite similar. At wavelengths between 1500 and 1700 nm, the reflectance of samples from polluted zones are slightly lower than the samples from control zones for the birch leaves (Fig. 2A) and significantly lower for hornbeam leaves (Fig. 2B). Reflectance in this spectrum range is influenced by vegetation water content, internal structure and dry matter (Ceccato et al., 2001).

In Fig. 2C and D, concerning the reflectance in VIS, it can be seen that between 400 and 680 nm, the curves of reflectance are well separated for the samples from polluted zones and control zone. The increment of reflectance from the polluted zone compared with control spectral signature may be due to the loss of Chl in leaves (Garriga et al., 2014), to drought stress (Sun et al., 2008) or to nitrogen deficiency (Zhao et al., 2005).

The red edge (Fig. 2E and F) shows a blue shift for the polluted zone which may be associated with stress or senescence (Main et al., 2011), a situation in which water content and chlorophyll levels are decreased. According to our data, the chlorophyll content is not affected, but most probably the water status of the plants under heavy metal stress is lower than in control plants. Exposure to heavy metals may impair water uptake and water content of plants (Disante et al., 2011; Santala and Ryser, 2009). Plants exposed to heavy metals may suffer from drought stress and reduced growth even when water supply is sufficient (de Silva et al., 2012). In this perspective chlorophyll meter readings may offer a complementary tool for screening between two additive stresses.

Chlorophyll fluorescence can be used as a sensitive measurement for detection of ion/salt sensitivity and other environmental stress factors (Misra et al., 2006). Fluorescence originates in the antenna complexes of PSII as emission at 685 nm (Govindjee, 2004) and in the far-red region at 720–740 nm emitted by PS I. The ratio  $F_v/F_m$  used extensively as the maximum quantum yield of primary PSII photochemistry is ranging from 0.78–0.84 in healthy plants (Bjorkman and Demmig, 1987).  $F_v/F_m$  is often used to estimate the extent of photoinhibition of photosynthesis. Under severe stress, a decrease in the chlorophyll fluorescence ratio  $F_v/F_m$  indicates an inhibition of PSII. In our study, only two of the 90 determinations were statistically significantly lower than normal, indicating that the PSII activity was not affected by the heavy metals from the soil.

The vegetation indices selected to assess the physiological state of the plants exposed to the stressed environment are displayed in Table 3.

Red Edge Normalized Difference Vegetation Index ( $NDVI_{705}$ ) used in stress detection indicates the sensitivity of the vegetation red edge to small changes in canopy foliage content. Modified Red Edge Simple Ratio Index ( $mSR_{705}$ ) uses bands in the red edge and incorporates a correction for leaf specular reflection.

To assess the vegetation's ability to use photosynthetically active radiation (PAR), the following light use efficiency (LUE) indices have been used: Photochemical Reflectance Index (PRI), Structure Insensitive Pigment Index (SIPI), and Red Green Ratio (RGR). PRI is defined as an index of the xanthophyll cycle activity on a diurnal time scale. Xanthophyll cycle pigments adjust the energy distribution at the photosynthetic reaction centre, indicating the PSII photochemical efficiency. They also indicate the vegetation production and the stress level (Gamon et al., 1992). Various factors affect the PRI–LUE relationship, such as soil background,

temperature, or pigment content. Generally, in stressed environment both LUE and PRI decrease proportionally.

SIPI is used in cases in which there is a high variability of the canopy structure. RGR measures the relative expression of leaf redness caused by anthocyanin to that of chlorophyll. Anthocyanin Reflectance Index 1 (ARI1) is sensitive to anthocyanin in plant foliage. Increases in ARI1 indicate changes in foliage via new growth or death of leaves.

Our results are within the normal values, except the RGR which is lower, indicating a higher reflection in Green Band and a low content of anthocyanins, facts also sustained by Anthocyanin Reflectance Index 1 (ARI1) values.

Anthocyanins are considered metal stress indicators playing a major role in the protection against metal stress. Inductive synthesis of anthocyanins could be regarded as a biochemical detoxification strategy. Many studies have shown that anthocyanins are able to form strong ligand complexes with metal ions (Dai et al., 2012; Takeda, 2006). Vacuolar sequestration of excess metals in the epidermal cells suggests that the synergistic induction of anthocyanins may provide a detoxifying sink for metals, when the cell wall and extracellular exudates, the first barrier against metal stress, are surpassed (Dai et al., 2012). The role of anthocyanins may be especially critical in highly stressed conditions; when the increasing concentration of metals provides more severe stresses, anthocyanins participate more actively in plant protection. Thus, depending on the metal type, plants may choose different defense systems. In experimental studies (Baek et al., 2012), it was shown that Pb induced the smallest quantity of anthocyanins but it was the strongest inducer of carotenoids. It was also suggested that in different plants, Cu stress may induce anthocyanins as main antioxidants while the Pb stress induces carotenoids as main protectants.

Plant Senescence Reflectance Index (PSRI) incorporates wavebands from both, the visible and NIR regions. When increased, this indicates stress at canopy level or later stages of senescence. Our values are within the normal values recorded in similar studies.

The overlapping reflectance exhibited by chlorophyll and carotenoids in the visible region makes it difficult to retrieve the two bands independently. Carotenoid Reflectance Indices 1 and 2 (CRI1, CRI2) are using the difference between reciprocal reflectance at 510 and 550 nm, and between 510 and 700 nm, respectively, in order to remove the effect of chlorophyll. Higher CRIs values mean greater concentration of carotenoid relative to chlorophyll.

As can be seen in Table 3, the carotenoid content relative to chlorophyll is lower than normal. Carotenoids are known to be involved in photoprotective action by quenching the excited state of chlorophyll and in non-photochemical quenching, considered to be a second protective mechanism. Under heavy metal stress, reactive oxygen species are produced which are affecting membranes and nucleic acids (Hou et al., 2007). Previous studies evidenced that a decrease in carotenoid content is a common response to metal toxicity (Rout et al., 2001), whereas the increase is due to the involvement of the pigments in detoxifying ROS (Tewari et al., 2002; Chandra et al., 2009). In addition, metals can enhance or reduce carotenoid production depending on metal types (Fargasová, 2001; Sinha et al., 2003), metal concentration and stage of development. Arabidopsis thaliana showed gradual increases and subsequent decreases of carotenoid content, depending on increasing concentrations of metals in Cu and Hg-treated seedlings (Baek et al., 2012).

The mean heavy metal concentrations in birch and hornbeam leaves from control and polluted zones are shown in Table 4. Mean concentrations of Ni ( $p=0.1$  for birch and  $p=0.61$  for hornbeam) and Cr ( $p=0.3$  for birch and  $p=0.08$  for hornbeam) in leaves are not significantly higher in control zones compared to polluted zone. Generally, their accumulation at leaf level is not related to soil pol-



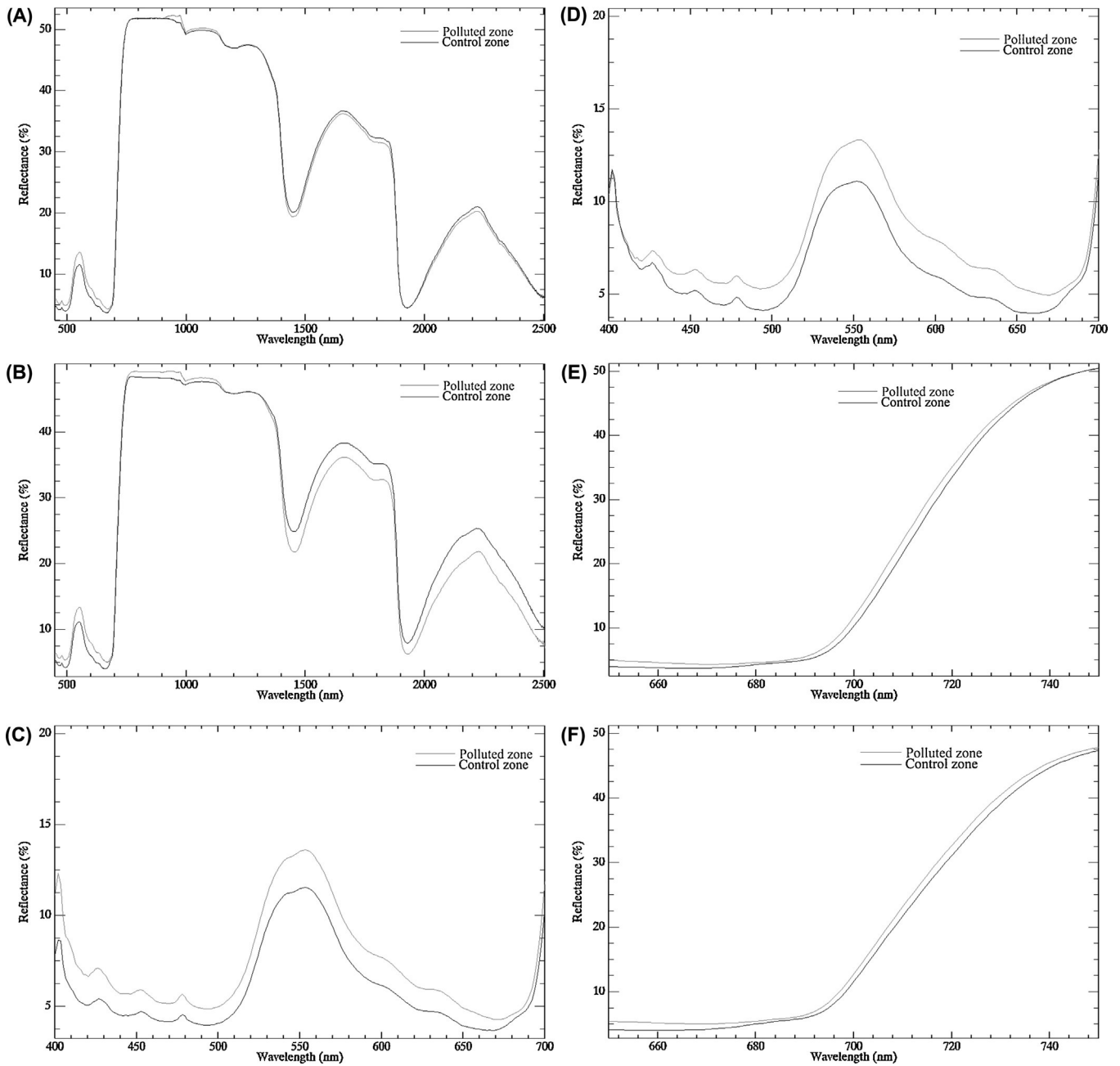


Fig. 2. Response to heavy metal exposure compared to control zone in terms of spectral ranges: 480–2500 nm for *Betula pendula* (A) and *Carpinus betulus* (B); 400–700 nm for *Betula pendula* (C) and *Carpinus betulus* (D); 680–730 nm for *Betula pendula* (E) and *Carpinus betulus* (F).

Table 3  
Vegetation Indices assessing the physiological state of *Betula pendula* and *Carpinus betulus*.

No.	Indices	Algorithm	Normal values	Recorded values —B. pendula	Recorded values —C. betulus
1	NDVI <sub>705</sub>	$(R_{750} - R_{705}) / (R_{750} + R_{705})$	0.2–0.9	0.51	0.447
2	mSR <sub>705</sub>	$(R_{750} - R_{445}) / (R_{705} - R_{445})$	2–8	4.66	3.12
3	PRI	$(R_{531} - R_{570}) / (R_{531} + R_{570})$	-0.2–0.2	0.009	0.014
4	SIP1	$(R_{800} - R_{445}) / (R_{800} - R_{680})$	0.8–1.8	0.978	0.993
5	RGR <sup>a</sup>	mean(red)/mean (green)	0.7–3.0	0.519	0.540
6	ARI1	$(1/R_{550}) - (1/R_{700})$	0.001–0.1	0.005	0
7	PSRI	$(R_{680} - R_{500}) / R_{750}$	-0.1–0.2	0.005	0.008
8	CRI1	$(1/R_{510}) - (1/R_{550})$	1–12	0.103	0.099
9	CRI2	$(1/R_{510}) - (1/R_{700})$	1–11	0.098	0.099

<sup>a</sup> Where mean (red) represents all bands with wavelengths in the red range and mean (green) represents all bands with wavelengths in the green range of the electromagnetic spectrum.



Table 4  
Heavy metal concentrations ( $\text{mg kg}^{-1}$  DW) and vegetation indices for *Betula pendula* and *Carpinus betulus* from control and polluted zones.

Samples	Item	Ni	Pb	Cd	Cr	Cu	Zn	CRII	RGR	□ Red edge
Control <i>B. pendula</i> (n = 18)	Mean	4.92	0.28	0.43	1.35	5.76	80.81	0.120	0.518	718.705
	SD	2.63	0.11	0.12	0.56	1.08	23.87	–	–	–
	C.V. (%)	53.4	39.2	27.9	41.4	18.7	29.5	–	–	–
Polluted zone <i>B. pendula</i> (n = 48)	Mean	3.70	0.34	0.31*	1.19	5.59	122.04*	0.107*	0.520	718.279
	SD	2.74	0.18	0.23	0.68	1.75	96.09	–	–	–
	C.V. (%)	74.0	52.9	74.0	57.1	31.3	78.7	–	–	–
Control <i>C. betulus</i> (n = 9)	Mean	6.11	0.42	0.05	1.50	5.82	15.35	0.114	0.547	717.647
	SD	2.64	0.13	0.03	0.21	0.66	2.08	–	–	–
	C.V. (%)	43.2	30.8	60.0	14.0	11.3	13.5	–	–	–
Polluted zone <i>C. betulus</i> (n = 15)	Mean	5.12	0.43	0.11	1.12	7.15	34.00*	0.096*	0.554	716.134
	SD	6.54	0.28	0.10	0.74	2.98	33.94	–	–	–
	C.V. (%)	127.7	65.1	90.9	66.0	41.6	99.8	–	–	–

SD: Standard deviation.

C.V.: Coefficient of variation.

\* p two tail. Marked comparisons are significant at  $p < 0.05$ .

Table 5  
Pearson's correlation coefficients between heavy metal concentrations in leaves of *Betula pendula* and *Carpinus betulus* and vegetation indices.

Sampling areas	Control area ( <i>B. pendula</i> ) n = 18		Polluted area ( <i>B. pendula</i> ) n = 48		Control area ( <i>C. betulus</i> ) n = 9		Polluted area ( <i>C. betulus</i> ) n = 15	
	Red edge	CRI 1	Red edge	CRI 1	Red edge	CRI 1	Red edge	CRI 1
Ni	–0.006	–0.342	–0.088	–0.031	–0.521	–0.402	–0.377	–0.251
Pb	–0.2	0.288	0.02	–0.027	0.098	–0.133	0.185	0.202
Cd	0.211	0.383	0.035	–0.290*	0.077	0.042	0.132	–0.275
Cr	–0.022	0.118	–0.094	0.162	–0.274	0.052	0.444	–0.044
Cu	–0.098	0.351	0.198	0.145	0.473	0.759*	0.419	0.306
Zn	0.21	0.065	0.253	–0.2	0.602	0.702*	0.287	–0.031

\*  $p < 0.05$ .

lution caused by the mining activity. Previous results obtained on soil studies from this zone suggest that Ni and Cr are not related to the mineralization of mining deposits but rather to the Cretaceous sediments occurring in the western and southern part of the investigated area (Lazăr et al., 2014).

The coefficient of variation indicating the extent of variability in relation to the mean of the population is constantly higher for polluted zones compared to control. For both species, the highest variability is for Ni and the lowest for Cu.

For birch, there is a significant statistical difference for Cd and Zn ( $p < 0.05$ ) concentrations between the control and polluted area. The Cd concentration of leaves is significantly lower and leaf Zn concentration is significantly higher at the polluted compared to the control area. In case of hornbeam, Zn concentrations are significantly higher ( $p < 0.05$ ) at the polluted compared to the control zone. The CRII is significantly higher at the control compared to the polluted zone both for birch ( $p = 0.01$ ) and hornbeam ( $p = 0.04$ ).

A Pearson's correlation was run to determine the relationship between heavy metal concentrations in leaves and vegetation indices (red edge and CRII) values. For all the samples, there is a negative correlation between Ni and the vegetation indices (Table 5). Considering the r values, most of the correlations established are weak to moderate. There was a significant negative correlation between Cd and CRII in polluted area and a strong, positive correlation between Cu and CRII and Zn and CRII for hornbeam in the control area.

Our results sustain the idea confirmed by the level of heavy metals in soil and plant tissue that in the study area the influence of heavy metals is moderate to low and that the individuals of the two species are adapted to this environment. Resistant plants adapted to the noxious conditions of contaminated soils have constitutive and adaptive mechanisms for coping with elevated soil metal concentrations. In this case, the content of total chlorophyll and the PSII activity are not affected, but values of anthocyanins and carotenoids are lower than normal, and not affected by heavy metal pollution as mirrored in the lower CRII in the polluted area for both species.

One of the objectives of this study concerning the relationships between leaf optical properties and stress level was to find indices that hold across species, mainly across plants adapted to a stressed environment. The ability to use the same indices across a wide range of vegetation types appears to be severely impaired due to the differences in leaf morphology and structure (Gamon and Surfus, 1999). Our results are indicating that the differences between leaf optical properties are minimal between the two species belonging to the same family, and the values of the vegetation indices are showing similar trends.

#### 4. Conclusions

This experiment indicates that both species, *B. pendula* and *C. betulus*, are adapted to their environment showing minimum signs of stress expressed by their optical properties, in conditions in which the level of heavy metals in soil, due to former mining activity, was medium to low.

Usually, high concentrations of metals are inducing a decrease in chlorophylls, an increase in anthocyanins and a fluctuation in carotenoid content depending on the metal types (Baek et al., 2012). In our case, the content of total chlorophyll was not affected, but anthocyanins and carotenoids values as evidenced by the reflectance indices were lower than normal. Considering that carotenoids are mainly involved in the protection against mild metal stress conditions whereas the role of anthocyanins may be especially critical in highly stressed conditions (Baek et al., 2012), one may assume that the level of stress affecting vegetation on this site is rather low.

For chlorophyll assessment, the reflectance ratios  $R_{\text{NIR}}/R_{550}$  and  $R_{\text{NIR}}/R_{700}$  are the most reliable and can be employed for an accurate estimation of the pigment content of the leaves. We found that the equations proposed by Richardson et al. (2002) for  $R_{750}/R_{680}$  and Blackburn (1998) for  $R_{800}/R_{680}$  and  $R_{800}/R_{675}$  produced poor chlorophyll content estimates when applied to our data.



Our results are showing lower than normal values of Red Green Ratio (RGR), indicating a higher reflection in Green Band and a low content of anthocyanins, facts also sustained by Anthocyanin Reflectance Index 1 (ARI1) values. Also, the carotenoid content relative to chlorophyll expressed by Carotenoid Reflectance Indices 1 and 2 (CRI1, CRI2) is lower than normal.

The other light use efficiency (LUE) indices are within normal values, indicating that the overall reactions of photosynthesis are not impaired by the specific conditions of the habitat.

Differences in leaf optical properties are minimal between the two studied species and the values of the vegetation indices are showing similar trends. Both techniques performed consistently well and yielded accurate estimations of pigment content, supporting their interoperability. In order to assess the stress level of the vegetation the portable spectroradiometer offers more complete information.

This kind of study demonstrates the potential to assess physiological status of different plant species using optical properties which may provide direct insights into structural and functional patterns and may estimate different indicators of species adapted to a certain habitat, yielding similar responses to the environment. Together with some additional information concerning soil nutrients and their interactions with heavy metals, heat stress, heavy metal concentration in plant tissue and plant stage of development, the structural and functional traits are potentially useful for the monitoring of functional shifts in ecosystems.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.flora.2015.08.003>.

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