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Non-destructive methodologies applied to track the occurrence of natural micropollutants in watering: *Glycine max* as a biomonitor

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ABSTRACT

Groundwater is habitually used for watering purposes in rural areas where the rainfall is not enough to adequately cover the crop requirements. However, groundwater sources could be naturally contaminated with trace micropollutants like As and associated elements (B, V and F) adversely affecting the plant health. In this work, non-destructive methodologies based on reflectance and chlorophyll emission processes were applied to assess the presence of micropollutants in watering by using a widespread crop (soybean plant). One of the most substantial results is that the co-occurrence of As, V, B and F in the watering solution clearly produced a synergistic effect in the plants. In fact, both reflectance and fluorescence techniques were proved in this work to be effective in detecting non-destructively stress by multielement treatment. Particularly, for reflectance measurements the most sensitive parameters were the derivative peak area between 480 and 560 nm and the chlorophyll content. Furthermore, it was demonstrated that it is possible to successfully use a portable hyperspectral spectroradiometer instead of a conventional spectrophotometer as the determinations performed with both instruments were positively correlated. Concerning fluorescence, variable emission of chlorophyll-a was more sensitive to stress than steady-state emission. The parameter F_v/F_0 was a valuable indicator of stress but the quantum yields of PSII and NPQ stood out as the most sensitive indices with variations of around 60 and 100% respectively.

1. Introduction

The evaluation of the optical and spectroscopic properties of plants represents a valuable tool that has been used in the last decades with diverse purposes as evaluation of plant health, harvesting conditions of fruits and stress assessment due to both biotic and abiotic factors (Iriel et al., 2014, 2015; Mendes Novo et al., 2012). These non-destructive determinations are typically based on measurements of reflectance and chlorophyll fluorescence of intact plant tissues (Gitelson et al., 2001; Lagorio, 2011). Particularly, several indices may be derived from reflectance measurements as the photochemical reflectance index (PRI), which is related to the reversible xanthophyll pigment changes during stress (Gamon et al., 1990; Peñuelas et al., 1994), the normalized difference vegetation index (NDVI), connected with the green plant biomass (Gamon et al., 1995), the normalized ratio index (NRI), related to the nitrogen content (Ferwerda et al., 2005) and the structure insensitive pigment index (SIPI) (Peñuelas et al., 1995a), associated to the ratio between carotenoids and chlorophyll content. Moreover, from both steady and non-steady state chlorophyll fluorescence, emission parameters that are strongly related to the functioning of the photosynthetic apparatus can be obtained (Goltsev et al., 2016; Iriel et al., 2014). Chlorophyll fluorescence has been thoroughly described in literature (Goltsev et al., 2016; Govindjee, 1971, 1995; Govindjee and Jursinic, 1979; Schansker et al., 2014; Strasser and Strasser, 1995, 1998; Strasser et al., 2000, 2004) and it has been used to quantify abiotic stress responses (Stirbet et al., 2018). Chlorophyll fluorescence has been shown to be an excellent method of inferring physiological mechanisms of the expansion of tor grass and was connected with the decrease of the number of genotypes as a consequence of environmental stress (Baba et al., 2016). It was also used to analyze the phytotoxic effects of heavy metals in plants at PSII levels (both on reaction centers and light harvesting complex) (Mathur et al., 2016). The activity of PSII

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was studied by means of the analysis of chlorophyll fluorescence to assess the tree tolerance to urban environments (Swoczyna et al., 2010) and the freezing tolerance of crops under variable winter conditions (Rapacz et al., 2015). Comprehensive discussions about instrument methods and applications connected with chlorophyll-a fluorescence have been reviewed by Kalaji et al. (Kalaji et al., 2014, 2016 and 2017). Among the advantages of the use of these non-destructive methodologies their low cost, quick determinations and the possibility to track big areas (for instance, a field), can be mentioned.

In the last decades, agricultural countries have been increasing the areas destined to soybean crops due to world food demand. Particularly, in Latin America it has continually increased since 1990s (FAO, 2017) and nowadays, 63% of the harvested Argentine land is planted with this commodity (Sly, 2017a,b). Because of that, soybean crop represents an excellent alternative as a biomonitor due to the wide geographical distribution of the crops in our region. Moreover, the agricultural border has been constantly expanded due to the technological advances that allowed adapting crop growth to several edaphoclimatic conditions. In rural areas the irrigation water comes from groundwater sources that could contain dissolved arsenic and others associated elements (Blanes et al., 2011; Espósito et al., 2011). In Latin America, the presence of these elements in natural environments has a geogenic origin connected with the volcanic activity of the Andes Mountains (Nicolli et al., 1989; Smedley et al., 2005). Although As is the most explored element it is present in co-occurrence with F, V and B which in aquatic environments are mostly present as oxyanions, excepting fluoride (Espósito et al., 2011). In La Pampa province, concentrations around 4 mgL^{-1} As(V) were found (Smeedley et al., 2005). In Cordoba province a maximum concentration of 6.2 mg L^{-1} V(V) was reported by Pérez Carrera et al. (2014). Moreover, several studies reported correlations between As and F concentrations in surface and ground water (Blanes et al., 2011; Rosso et al., 2011). Furthermore, it should be noticed that the effects of the isolated elements on the environment may differ from the action of these when they are concurrent (as in a real situation). In this context, our starting hypothesis consisted in proposing that the presence of trace elements of natural origin in irrigation water produce deleterious effects on plant health that are reflected in the optical properties and photochemical behaviour of soybean plants. Thus, the aim of this study was to determine how the optical and photosynthetic parameters of soybean plants were affected by irrigation with solutions of As (V), V (V), B (III) and fluoride and evaluate if a synergistic effect is produced due to the co-occurrence of them. In addition, we evaluate which non-destructive techniques and which parameters were more suitable to assess the presence of micro-contaminants through the use of a widespread biomonitor like soybean plants.

2. Materials and methods

2.1. Reagents

Irrigation solutions used in this work were prepared by the dissolution of $Na_3AsO_4.7H_2O$ (Ciccarelli), NH_4VO_3 (Sigma-Aldrich), $Na_2B_4O_7.10H_2O$ (Cicarelli) and NaF (Merck) in tap water. In this sense, separated solutions of 5 mg L^{-1} of As, B, F or V were used to assess the effect of each element on the soybean plant and a combined solution of 5 mg L^{-1} of As, B, F and V was employed to evaluate the co-occurrence effect of these trace elements. All the solutions were prepared with tap water (provided by the distribution company Agua y Saneamientos Argentinos S.A.).

2.2. Plants and growing conditions

Plants of *Glycine max* were grown from seeds (cultivar NA 5009 RG, Nidera Argentina) in plastic pots of 30 cm of diameter (5 L of capacity) containing a commercial soil substrate (Terrafertil®). Physicochemical properties of the soil were previously reported in (Cordon et al., 2018). A total of 30 pots with 3 seeds in each one was divided in groups and watered at field capacity. Watering solutions were: tap water (C), As(V) at 5 mg L^{-1}, F at 5 mg L^{-1}, B(III) at 5 mg L^{-1}, V(V) at 5 mg L^{-1} and a multielement solution (M) containing 5 mg L^{-1} of each element (As(V), B(III), V(V) and F). The experiment was conducted under natural field conditions during five months in Buenos Aires University land (34°35′41″S, 58°28′41″W). This implies from thirteen to 10 h of natural light from the beginning to the end of the experiment. Temperature and relative humidity values were recorded daily with a sensor HMP35C (Campbell Scientific, Inc.) attached to a 21X (L) Campbell data logger (Campbell Scientific Inc.). Minimum, maximum and averages values by month of both variables are shown in Table S1 of the Supplementary Material. The average wind velocity during the months in which the test was developed was maintained in a range between 14 and 18 km/h.

2.3. Leaf biospectroscopy

Recording of reflectance and fluorescence spectra were obtained from fully developed leaves in triplicate at room temperature. Spectra were averaged and various photophysical and photochemical parameters were calculated from them. Particularly, fluorescence determinations were performed after 15 min of leaf adaptation to darkness in all the cases.

2.3.1. Reflectance

Reflectance spectra of intact leaves were recorded by employing two alternative approaches for comparison. Firstly, a portable hyperspectral spectroradiometer ASD FieldSpec Pro FR (ASD, USA) equipped with the plant probe and the leaf-clip accessories allowed obtaining the reflectance spectrum *in situ* for individual leaves (non-detached leaves). In this case, the use of the leaf-clip limited the field of view (Field-Of-View, FOV) to 23 mm and it prevented the entrance of ambient light. Each spectrum resulted from the average of 10 measurements of reflectance ranging from 350 to 800 nm. Secondly, the reflectance spectra as a function of wavelength of a group of intact leaves (detached leaves) were obtained by using a spectrophotometer (Shimadzu, 3101) equipped with an integrated sphere that is able to collect the total of the reflected light. Spectra of intact leaves were recorded from 400 to 800 nm with $BaSO_4$ to adjust the 100% of reflectance level. From both reflectance measurements (individual and grouped leaves), the first derived spectra were determined as $\Delta R/\Delta\lambda$ as a function of wavelength and the area of their principal peaks (bands centred at 510 and 710 nm) were estimated. Additionally, several spectral indices such as PRI, NDVI, NRI and SIPI were calculated according to equations (1)–(4):

$$PRI = (R_{531} - R_{570})/(R_{531} + R_{570}) \quad (1)$$

$$NDVI = (R_{774} - R_{677})/(R_{774} + R_{677}) \quad (2)$$

$$NRI = (R_{570} - R_{670})/(R_{570} + R_{670}) \quad (3)$$

$$SIPI = (R_{800} - R_{445})/(R_{800} + R_{680}) \quad (4)$$

where R_λ ($0 < R < 1$) is the reflectance of the leaves at the wavelength λ.

Moreover, absorption spectra expressed as the remission function was also calculated from reflectance values using the Kubelka-Munk theory (Wendlandt and Hecht, 1966). For that, the condition of non-transmitted light (optically dense thick samples) must be satisfied in the whole range of wavelengths. Remission function spectra of leaves were calculated according to equation (5):

$$F(R)_\lambda = \frac{(1 - R_\lambda)^2}{2R_\lambda} \quad (5)$$

where $F(R)_\lambda$ is the remission function and R is the diffuse reflectance at the wavelength λ.

Additionally, remission function values calculated at 700 nm were

used to estimate the total chlorophyll (chlorophyll a + chlorophyll b) expressed in mmol cm^{-2} according to reference (Cordon et al., 2010). Precisely, the regression employed is shown in equation (6):

$$\text{Total chlorophyll} = 2.6\ 10^{-6} + 1.5\ 10^{-5} F(R)_{700} \quad (6)$$

2.3.2. Fluorescence

Non-variable (or steady-state) fluorescence spectra of intact leaves were recorded using a front face arrangement on a PTI model QM-1 spectrofluorometer under low photon flux conditions (lower than 20 μmol cm^{-2} s^{-1}) to avoid Kautsky kinetics induction. Excitation wavelength was set up at 460 nm and the emitted photons were collected between 600 and 800 nm. Correction of experimental fluorescence spectra for wavelength dependence of the excitation intensity and for the variation of the detector sensitivity with wavelengths was performed as usual (with a function provided by the manufacturer). They were additionally corrected to take into account light re-absorption effects into the leaves (by using a physicochemical model presented by Ramos and Lagorio (2004)). Therefore, true emission spectra were obtained by dividing the experimental spectra by the photon escape probability that can be calculated according to equation (7):

$$\gamma_{\lambda,\lambda_0} = \frac{1}{1 + \sqrt{\frac{F(R)_\lambda}{F(R)_\lambda + 2}}} \times \frac{1}{1 + \sqrt{\frac{F(R)_\lambda [F(R)_\lambda + 2]}{F(R)_{\lambda_0} [F(R)_{\lambda_0} + 2]}}} \quad (7)$$

where F(R) is the remission function calculated by equation (5). The parameters λ and λ_0 stand for the emission and excitation wavelengths, respectively. Function γ can be interpreted as the fraction of the luminescence emitted at wavelength λ that escapes from the leaf excited at wavelength λ_0.

The reabsorption model has been successfully applied in both biological and inert systems such as: leaves (Cordon and Lagorio, 2006; Iriel et al., 2014), fruits (Mendes Novo et al., 2012; Ospina Calvo et al., 2017; Ramos and Lagorio, 2006) and dye adsorbed onto microparticles (Iriel et al., 2002; Lagorio et al., 1998, 2001).

Variable fluorescence in intact leaves was collected with a portable amplitude pulse modulated (PAM) fluorometer (Hansatech FMS1). Operational conditions were correctly described in a previous paper (Iriel et al., 2014). Briefly, dark adapted leaves were irradiated with a modulated low intensity beam inducing a non-variable fluorescence (F_0). After that, a saturating pulse was applied and a maximum fluorescence was emitted from leaves obtaining F_m. From these measurements, an alternative expression to the maximum quantum efficiency of photosystem II photochemistry, F_v/F_0, where $F_v = F_m - F_0$ was calculated (more sensitive than the traditional expression F_v/F_m). Later, the leaves were illuminated with an actinic light that induced photosynthesis process and photochemical and non-photochemical quenching took place. When a stationary state (F_s) was attained, another saturating pulse was applied and a new maximum value of fluorescence (F_m') was obtained. From this light-adapted state, the effective quantum efficiency of photosystem II was calculated as $\Phi_{PSII} = (F_m' - F_s)/F_m'$.

The excitation energy absorbed by plants adapted to actinic light can undergo three deactivation pathways: it can be used to drive photosynthesis, it can be dissipated in photophysical processes (fluorescence, internal conversion and intersystem crossing) or it can be dissipated as heat (non-photochemical quenching, activated as a photoprotective pathway). The efficiencies of each process are called Φ_{PSII}, Φ_C and Φ_{NPQ}, respectively, and their values show how the absorbed energy is partitioned among them. These three mechanisms are competitive, thus any variation in the quantum efficiency of one may typically cause changes in the yields of the others (Maxwell and Johnson, 2000). According to Hendrickson et al. (2004), the quantum yields of non-photochemical quenching and chlorophyll photophysical decay of light-adapted leaves were calculated as: $\Phi_{NPQ} = (F_m - F_{m'}/F_m)$ x $(F_s/F_{m'})$ and $\Phi_C = F_s/F_m$, respectively (Cordon et al., 2018; Hendrickson et al., 2004). In addition, the parameters of photochemical quenching (qP) and non-photochemical quenching, qNP and NPQ, were calculated according to equations (8)–(10).

$$qP = (F'_m - F_s)/(F'_m - F_0) \quad (8)$$

$$qNP = (F_m - F'_m)/(F_m - F_0) \quad (9)$$

$$NPQ = (F_m - F'_m)/F'_m \quad (10)$$

2.4. Statistical analysis

Analysis of variance (ANOVA) and Pearson correlation analysis were performed by utilizing InfoStat software and means comparisons were performing by Fisher's LSD (Least Significant Difference) test at $\alpha = 0.05$ (Di Rienzo et al., 2010). All data sets were checked to satisfy ANOVA assumptions. Plots were performed with Sigma Plot 10.0 (Systat Software Inc., San José, CA).

3. Results and discussion

3.1. Reflectance

Analysis of reflected radiation could provide information about structure and chemical composition of plant tissues. Particularly, reflectance spectrum from leaves was analysed in order to estimate chlorophyll content and ratios related to the principal pigments in leaves that are associated to plant health. Additionally, some parameters were derived from reflectance measurements such as the first derivative spectrum as shown below.

3.1.1. First derivative of reflectance spectra

First derivative reflectance spectra of soybean plant leaves were calculated from reflectance values (data not shown) obtained from both a single leaf (ASD) and a group of them (integrating sphere) as was mentioned in the previous section. Typical spectra of leaves presented two main minimal peaks located at 525 nm (green region) related to both chlorophyll and carotenoids absorptions and 720 nm (red edge) due to chlorophyll absorption (Gitelson and Merzlyak, 1994). The spectral distributions of the derivative of reflected light for the samples treated with the solutions of isolated elements were quite similar to the controls (see Supplementary material section). However, differences appeared in plants irrigated with the M solution where a blue shift of around 5 and 10 nm was observed for bands centred at 520 and 700 nm, respectively, as it is shown in Fig. 1.

As a general rule, observed shifts in the first derivative reflectance spectra are connected with the concentration of chlorophyll in leaves (Filella and Peñuelas, 1994; Horler et al., 1983). Specifically, an increase in the chlorophyll concentration is associated with a red shift in the green band accordingly with a broader absorption reflectance spectrum. This fact was previously documented by Kochubey and Kazantsev (2007) who described a red shift as the chlorophyll concentration increases in winter wheat, maize, sugar beet and vine. A blue shift in the derivative spectra was previously observed for chicory plants irrigated with As(V) solutions (at the same concentration) where a diminution of chlorophyll concentration was also observed (Cordon et al., 2018). In the present work, the plants watered with As, F, V and B solutions (separately) presented no difference regarding the control one. In this sense, our result is similar to that found by Milton et al. who have studied the effect of As and Se solutions in soybean crops (Milton et al., 1989). In our study, only plants treated with M solution have shown a blue shift, in agreement with changes in chlorophyll content as it is presented below. These results combined with those found in literature would suggest that the effect of trace elements on the spectral data is strongly dependent on the plant species, trace element nature, exposure concentration, growing conditions, etc. From these first derivative spectra, area peaks were compared in order to determine the

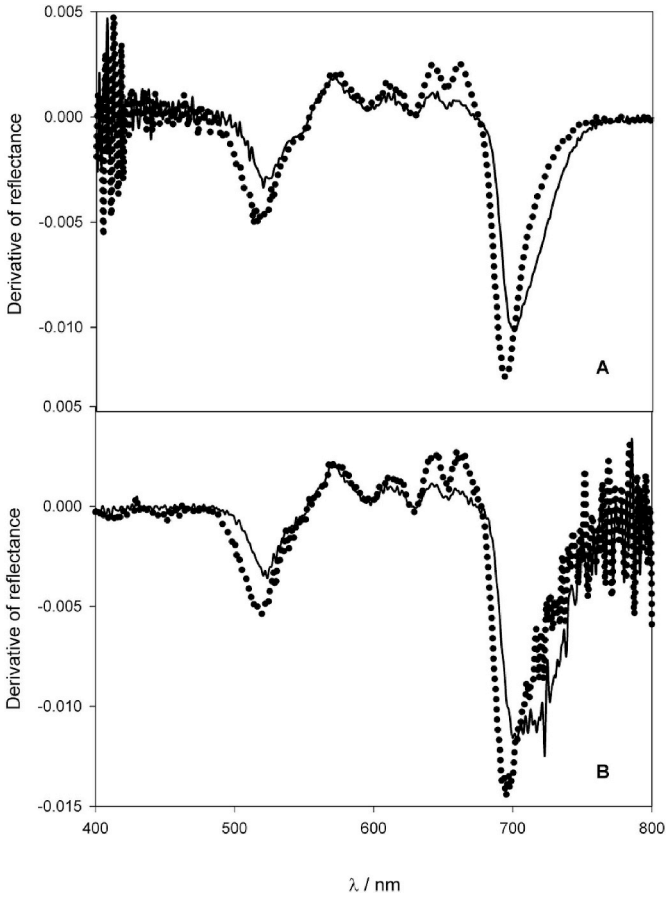

Fig. 1. Derivative reflectance spectra obtained for soybean leaves watered with: multielement solution (dotted line) and tap water (solid line). (A) Measurements performed with a spectroradiometer (ASD) and (B) measurements performed with the spectrophotometer (integrating sphere).

significances from the differences among the applied treatments. Results are presented in Fig. 2:

The area of the green band for plants irrigated with M solution showed significant differences regarding to the control. Similar results were previously observed in chicory plants (Cordon et al., 2018) and aquatic plants (Iriel et al., 2015) treated with As(V) solutions where the green band resulted more sensitivity to stress conditions. Moreover, peak areas corresponding to the green zone ($D_{480\text{-}560}$) obtained from reflectance first derivate spectra recorded with the spectroradiometer ASD and the integrating sphere (spectrophotometer) were significantly correlated (Pearson correlation coefficient = 0.952, $p < 0.001$). This is an important result considering that in both cases the leaves were measured differently. With the integrating sphere, the spectra were measured for a thick layer of leaves (zero transmittance) while with the ASD spectroradiometer spectra of single leaves were recorded. In the case of the peak area situated in the far-red zone ($D_{670\text{-}780}$), although the correlation was significant (Pearson correlation coefficient = 0.768, $p < 0.001$), it was somewhat lower than for $D_{480\text{-}560}$. This result could be due to differences between the light dispersed (%) in the NIR region that is higher for the group of leaves (Woolley, 1971). Correlation graphs are presented in the Supplementary material section.

3.1.2. Visible spectral indices

Spectral indices relate the reflectance of the plant leaves (or a canopy) at specific wavelengths with biophysical variables (Hatfield et al., 2008). In this work, the most commonly used indices based in reflectance measurements of the visible region were calculated. Particularly, NDVI is the most widely used index as estimator of the presence and good condition of vegetation (Grigera et al., 2007); PRI which is related to the carotenoids/chlorophyll ratio in green leaves; NRI which is an estimator of nitrogen content of plants, and therefore is associated with the chlorophyll a content of leaves (Bausch and Duke, 1996) and SIPI which estimates the proportion of carotenoids with respect to chlorophyll a, like PRI (Peñuelas et al., 1995b). The values for the spectral indices calculated from reflectance recorded with the ASD spectroradiometer and with the spectrophotometer were also statistically correlated. Pearson correlation coefficients were 0.939 ($p < 0.0001$) for NDVIs, 0.535 ($p = 0.0032$) for PRI, 0.836 ($p < 0.0001$) for NRI and 0.852 ($p < 0.0001$) for SIPI. Therefore, for clarity, only values from the spectroradiometer are presented here for discussion in Table 1.

It should be noted that NDVI and SIPI indices were the most sensitive parameters to the occurrence of multielements for the studied soybean plants. However, the reduction (%) in their values, although significant was not remarkably large: 11.4% for NDVI and 5.3% for SIPI. By contrast, both PRI and NRI index values exhibited no differences between the different treatments and controls.

3.1.3. Chlorophyll content

Chlorophyll content was estimated from reflectance values according to eqs. (5) and (6) for the treated and control plants. Results are presented in Fig. 3.

The chlorophyll concentration was significantly lower only for the plants treated with the multielement solution displaying a reduction in the order of 50%. Controversially, no variation was detected for plants watering with the isolated solutions although several authors reported a diminution in the chlorophyll content in presence of As. For instance, Stoeva and Bineva (2003) reported a decrease in the chlorophyll content in oat plants growing in a soil containing 160 mgAs Kg^{-1}). Furthermore, the immersion of been roots in As solutions (0, 2 and 5 mg L^{-1}) fifteen days after emergence, led to a diminution on chlorophyll concentration in leaves (Stoeva et al., 2005). Azizur Rahman et al. (2007) reported that chlorophyll content in leaves of five rice varieties decreased with the concentration of As in soils as the As content increases from 10, 20 and 30 mg of As kg^{-1} soil. However, from those results, it should also be concluded that the effect on chlorophyll content in plants seems to be dependent on the As concentration and very low concentrations could be not detected properly (Stoeva and Bineva, 2003). Moreover, we studied the effect of As in watering during the growing of chicory plants (Cordon et al., 2018). Interestingly, it was observed that chlorophyll content was reduced during the first weeks of treatment but for a longer time the plants were capable recovering displaying chlorophyll contents similar to the controls. Therefore, the time of evaluation have a secondary but very important role in the estimation of the trace elements effects.

Nevertheless, it was stated in literature that the presence of trace elements could reduce the chlorophyll content either by an inhibition of the chlorophyll biosynthesis or by destruction of chloroplasts (Baunthiyal and Ranghar, 2014; Fargašová, 1998; Mishra et al., 2016). Additionally, the decrease in chlorophyll concentration observed for the M treatment is strongly connected to the blue shift displayed by the first derivative spectrum which was discussed above.

3.2. Fluorescence

3.2.1. Chlorophyll emission spectra

Steady-state fluorescence spectra were corrected by the detector response to different wavelengths and by light re-absorption processes. Chlorophyll fluorescence spectra showed two typical bands located at 685 and 735 nm, approximately (Fig. 4A). As is well describe in literature, the band centred at 685 corresponds only to photosystem II (PSII) emission while; at 735 nm, PSI also contributes to the observed emission (Pfundel, 1998). From the corrected emission spectra fluorescence ratios ($F_{red}/F_{far\text{-}red}$) can be calculated as a quotient between the

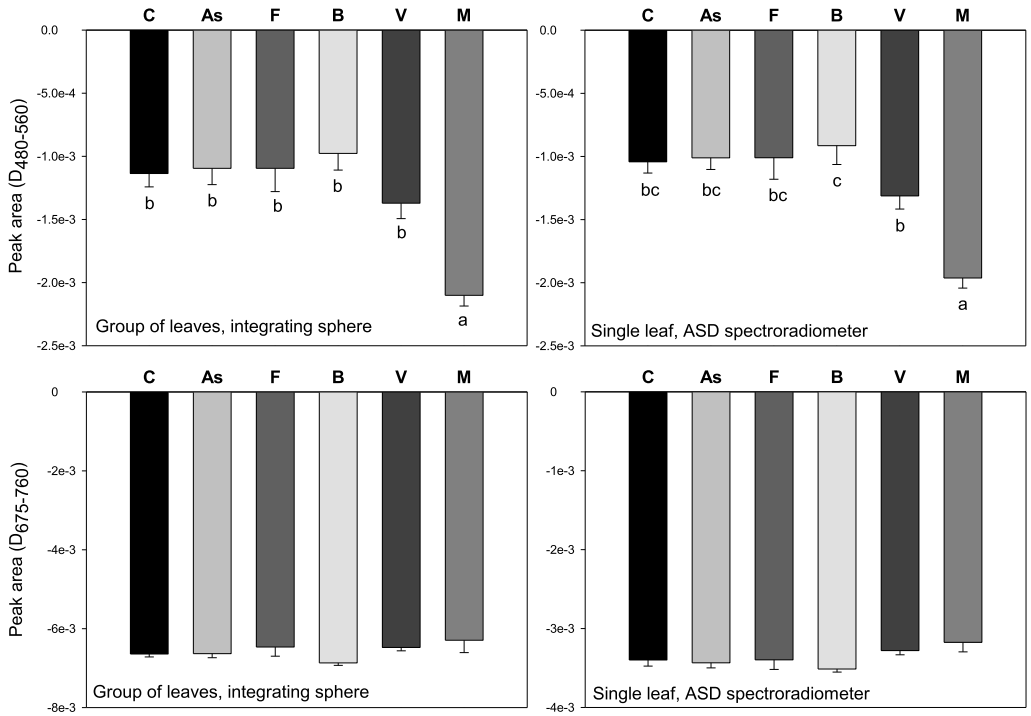

Fig. 2. Integrated area of the first derivative reflectance spectra for soybean leaves watered with solutions of As(V) 5 mg L^{-1}, F 5 mg L^{-1}; B(III) 5 mg L^{-1}; V(V) 5 mg L^{-1}; multielement solution (M) and tap water (C). Top: band located between 480 and 560 nm and bottom: band located between 675 and 760 nm. For $D_{480\text{-}560}$, different letters correspond to significant differences at $p < 0.0001$. No significant differences were observed for $D_{675\text{-}760}$. In all the cases, the error bars correspond to the standard error (n = 3).

Table 1
Reflectance indices obtained from reflectance spectra measured with the spectroradiometer ASD.

	PRI^{NS}	$NDVI^{**}$	NRI^{NS}	$SIPI^{**}$
C	-0.03 ± 0.02^{a}	0.79 ± 0.02^{b}	0.43 ± 0.03^{a}	0.75 ± 0.01^{bc}
As	-0.03 ± 0.02^{a}	0.81 ± 0.02^{b}	0.44 ± 0.03^{a}	0.76 ± 0.01^{c}
F	-0.01 ± 0.02^{a}	0.79 ± 0.02^{b}	0.40 ± 0.03^{a}	0.76 ± 0.01^{bc}
V	-0.05 ± 0.02^{a}	0.77 ± 0.02^{b}	0.46 ± 0.03^{a}	0.73 ± 0.01^{ab}
B	-0.01 ± 0.02^{a}	0.81 ± 0.02^{b}	0.41 ± 0.03^{a}	0.77 ± 0.01^{c}
M	-0.03 ± 0.02^{a}	0.70 ± 0.02^{a}	0.48 ± 0.03^{a}	0.71 ± 0.01^{a}

ANOVA results: Non-significant (NS), $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Fisher test comparison: means with a common letter are not significantly different ($p > 0.05$).

fluorescence intensities of the principal bands (Cordon et al., 2018). These ratios are presented in Table 2.

The only detected difference is a significant increase (18.4%) in the fluorescence ratio observed for the plants treated with M solution. In a normal condition (control plants) the peak ratio calculated as $F_{red}/F_{far\text{-}red}$ is connected with the stoichiometry of both photosystems in the plant (Cordon and Lagorio, 2007; Ospina Calvo et al., 2017). However, if there is any external factor affecting the electron transport along the chain, this ratio could be changed (Iriel et al., 2014). In our case, different behaviours were observed. First, the presence of As in watering conducted to a diminution in the peak ratio ($F_{red}/F_{far\text{-}red}$) from 1.93 (control plants) to 1.64 (As-treated plants). This behaviour was previously documented by our group for aquatic plants exposed to As solutions (Iriel et al., 2015a and b) where a preferential damage of PSII due to the potential presence of reactive oxygen species was suggested. There, an increase in the intensity of the fluorescence band located at 735 nm was observed and consequently, the ratio $F_{red}/F_{far\text{-}red}$ diminished displaying a negative correlation between these parameters (Iriel et al., 2015b). Otherwise, in this work soybean plants watered with the multielemental solution presented an increase in the values of $F_{red}/F_{far\text{-}red}$ regarding to control plants. This result was previously reported for *Spathiphyllum wallisi* plants in contact with atrazine solutions where a positive correlation between atrazine concentration and the peak ratio was reported. In that work, the suggested mechanism was either the blockage of the electron transport from PSII to PSI in photosynthesis or the selective destruction of PSI relative to PSII (Iriel et al., 2014).

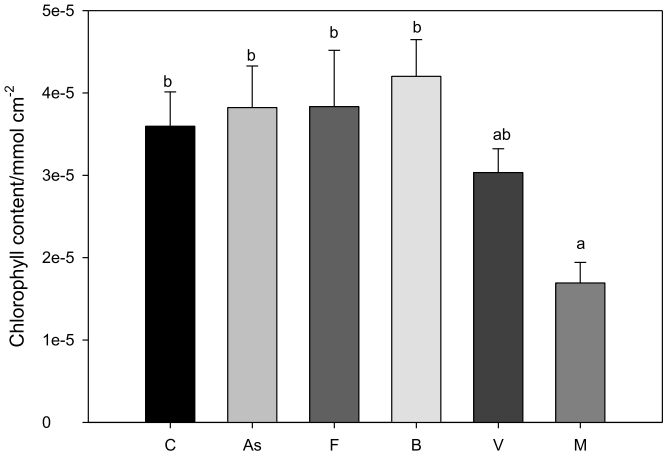

Fig. 3. Chlorophyll content calculated from reflectance values at 700 nm. Fisher test comparison: means with a common letter are not significantly different ($p > 0.05$) (ANOVA, $p = 0.0235$) (n = 3).

3.2.2. Photosynthetic parameters from variable chlorophyll fluorescence

The alternative expression to the maximum quantum efficiency of

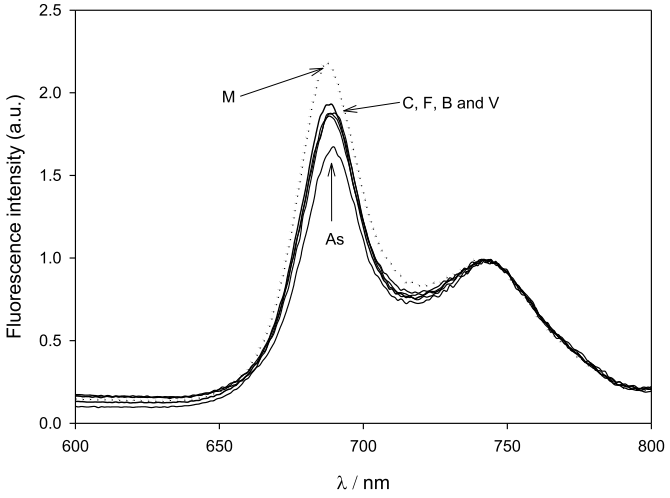

Fig. 4. Chlorophyll fluorescence spectra corrected by the detector sensitivity and by light re-absorption processes for soybean plant leaves treated with watering solutions of 5 mg As L^{-1}, 5 mg F L^{-1}; 5 mg V L^{-1}, 5 mg B L^{-1} and multielement solution (M). Spectrum of plants irrigated with tap water is denoted as C (control). Spectra were normalized in the far-red peak.

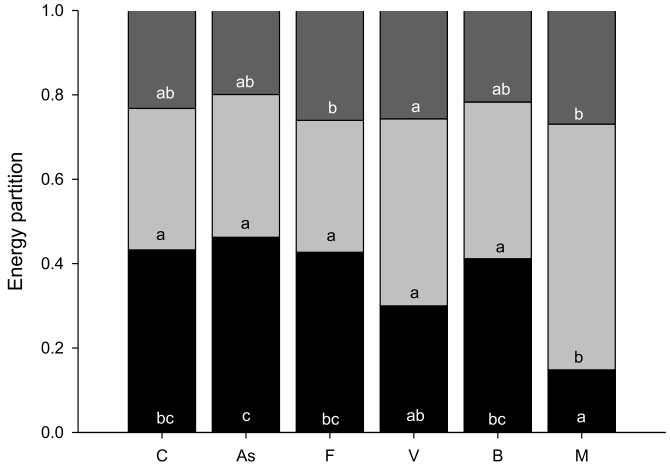

Fig. 5. Energy partition among photosynthesis (Φ_{PSII}), non-photochemical quenching (Φ_{NPQ}) and photophysical decay (Φ_C) for control and treated soybean plants.

photosystem II photochemistry (F_v/F_0) and the photochemical (qP) and non-photochemical quenching parameters (NPQ and qNP), obtained from Kautsky kinetics are shown in Table 2. The photochemical quenching was insensitive to the presence of pollutants while the other three parameters displayed significant differences between the control and plants treated with the multielement solution. In fact, for the stressed plants (M) F_v/F_0 decreased 35.5% compared to controls and the non photochemical quenching expressed as qNP and NPQ increased about 39 and 50% respectively.

The F_v/F_0 ratio is a sensitive indicator of the photosynthetic activity and it is usually preferred to F_v/F_m because the first one amplifies the variations detected by the latter ratio (Maxwell and Johnson, 2000; Ozfidan et al., 2013; Rybczyński et al., 2007). Its reduction clearly showed a deterioration of the photosynthetic capacity in the presence of the multielement solution. The lack of effects in the plants treated with the micropollutants separately (solutions that exclusively contain F, B, V or As) is not unusual since the effect of stressors not only depends on their concentration but also on the evaluated plant species. A short time ago, we found that the effect of the same concentration of As (5 mg L^{-1}) in chicory plants presented disparate results to those obtained in this new manuscript (Cordon et al., 2018). Wang et al. (2016) also evaluated the effect of As stress on chlorophyll fluorescence for plants of *Ficus tikoua*. In this case, they found a significant decrease in F_v/F_0 compared to the control plants from 320 μmol L^{-1} (approximately 34 mg L^{-1}) a value much higher than the one used in that work. Otherwise, they did not find effects on both qNP and NPQ parameters (in the range between 8 and 480 μmol L^{-1}) and they did find effect on qP from 320 mg L^{-1}. A similar antecedent was found for the case of B where Landi et al. (2013) evaluated the effects of this element on *Cucurbita pepo* and *Cucumis sativus*. They found a significant decrease of F_v/F_m and Φ_{PSII} in both species of cucurbits cultivated at the highest B concentration, i.e. 20 mg L^{-1}. However, at 10 mg L^{-1} a decrease in the F_v/F_m ratio was observed for *Cucurbita pepo* while no changes were observed for *Cucumis sativus*. They also reported an increase in qNP and a decrease in qP coefficients for plants exposed to the two tested B concentrations relative to the control plants.

Also, the repartition of the absorbed energy by plants into photochemical processes, heat dissipation and chlorophyll photophysical decay can be quantified by the estimation of the quantum yields: Φ_{PSII}, Φ_{NPQ} and Φ_C (Cordon et al., 2018; Guadagno et al., 2010; Hendrickson et al., 2004). As these three mechanisms: photosynthesis, non photochemical quenching and photophysical decay are competitive, their sum is equal to unity ($\Phi_{PSII} + \Phi_{NPQ} + \Phi_C = 1$). Fig. 5 shows the results for the energy partition among the processes.

The analysis of the energy partition presented a behaviour in line with expectations. Values for Φ_C were around 0.2 for all control and treated plants while Φ_{PSII} values were lower and Φ_{NPQ} values higher for the plants irrigated with M solution (compared to control plants); a typically response of plants under stress (Guadagno et al., 2010; Hendrickson et al., 2004; Kornyeyev and Hendrickson, 2007).

Again, only significant differences were found for the plants treated with the multielement solution. The results show that this stress causes a significant reduction in the fraction of absorbed energy destined to photosynthesis (about 65%) and a considerable increase in the fraction dissipated as heat (about 75%). These results together with the decrease in F_v/F_0 suggest a clear damage of the photosynthetic apparatus in treated plants. The fraction of absorbed light dissipated by photophysical processes remained almost constant. Hence, it is clear non-photochemical quenching (heat dissipation mechanism activated by

Table 2
Chlorophyll fluorescence parameters of leaves of soybean plants watered with 5 mg As L^{-1}, 5 mg F L^{-1}; 5 mg V L^{-1}, 5 mg B L^{-1}, multielement solution (M) and tap water (C).

	$F_{red}/F_{far\text{-}red}$ **	F_V/F_0 **	qP NS	NPQ *	qNP **
C	1.93 ± 0.06^{b}	4.37 ± 0.18^{a}	0.80 ± 0.02^{ab}	1.47 ± 0.26^{b}	0.70 ± 0.06^{b}
As	1.64 ± 0.07^{a}	3.78 ± 0.31^{a}	0.93 ± 0.09^{a}	1.47 ± 0.15^{b}	0.74 ± 0.04^{b}
F	1.86 ± 0.05^{ab}	4.44 ± 0.26^{a}	0.71 ± 0.08^{ab}	1.19 ± 0.17^{b}	0.66 ± 0.05^{b}
B	1.87 ± 0.12^{b}	3.96 ± 0.10^{a}	0.66 ± 0.05^{ab}	1.73 ± 0.10^{ab}	0.79 ± 0.02^{b}
V	1.86 ± 0.04^{b}	4.52 ± 0.18^{a}	0.82 ± 0.06^{ab}	1.73 ± 0.21^{ab}	0.77 ± 0.04^{b}
M	2.17 ± 0.09^{c}	2.82 ± 0.53^{b}	0.51 ± 0.31^{b}	2.20 ± 0.16^{a}	0.97 ± 0.06^{a}

ANOVA results: Non-significant (NS), $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Fisher test comparison: means with a common letter are not significantly different ($p > 0.05$).

actinic light) have resulted the main actuator regulating the partition of energy during the stress conditions.

3.3. Statistic global analysis

Finally, the whole set of determined parameters (for both controls and treated plants) were statistically analysed according to the Pearson Correlation Coefficients (PCC) test to methodically search for possible correlations among them. The quantum yield of photosystem II (Φ_{PSII}) displayed a significant positive correlation with the area of the reflectance derivative $D_{480\text{-}560}$ (PCC = 0.82, $p < 0.0001$) and with the chlorophyll content (PCC = 0.81, $p < 0.0001$). On the other hand, both Φ_{PSII} (PCC = −0.88, $p < 0.0001$; PCC = −0.770, $p < 0.0001$) and the chlorophyll content (PCC = −0.77, $p < 0.0001$; PCC = −0.62, $p = 0.0004$) presented a negative correlation with non-photochemical quenching (either qNP or NPQ) as expected from the results from Fig. 5. The important positive correlation between qNP and NPQ is obvious as they describe the same phenomenon (PCC = 0.85, $p < 0.0001$). The area $D_{680\text{-}780}$ was positively correlated with those parameters connected to the pigments content, obviously a very strong relationship was found with the chlorophyll content (PCC = 0.89, $p < 0.0001$), and to a lesser extent with the NDVI (PCC = 0.71, $p < 0.0001$) and SIPI (PCC = 0.71, < 0.0001) indices, which were positively and strongly correlated between them (PCC = 0.88, $p < 0.0001$).

4. Conclusions

One of the principal achievements of the work is to clearly demonstrate the synergistic effect produced by the simultaneous presence of several micropollutants. In fact, the joint presence of the various elements produced a deleterious consequence on the plants with respect to the effect exerted by each one separately (which, in turn, did not reveal differences with respect to the control). Regarding the results obtained from the reflectance measurements, it was found that whether a conventional spectrophotometer or a hyperspectral spectroradiometer is used, the determinations are positively correlated. This is undoubtedly a significant point for field measurements where the spectroradiometer is usually utilized. Both reflectance and fluorescence techniques were proved in this work to be effective in detecting non-destructively stress by multielement treatment. Regarding reflectance-based parameters, the most sensitive ones resulted $D_{480\text{-}560}$ and the chlorophyll content (which was inferred from reflectance measurements of a group of intact leaves at 700 nm). Concerning fluorescence, variable emission of chlorophyll-a was more sensitive to stress than steady-state emission. The parameter F_v/F_0 was a valuable indicator of stress but the quantum yields of PSII and NPQ stood out as the most sensitive indicators with variations of around 60 and 100% respectively.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.109368.

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