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# Effect of arsenic on reflectance spectra and chlorophyll fluorescence of aquatic plants

Analia Iriel [a], Gavin Dundas [a], Alicia Fernández Cirelli [a], Maria G. Lagorio [b,*]

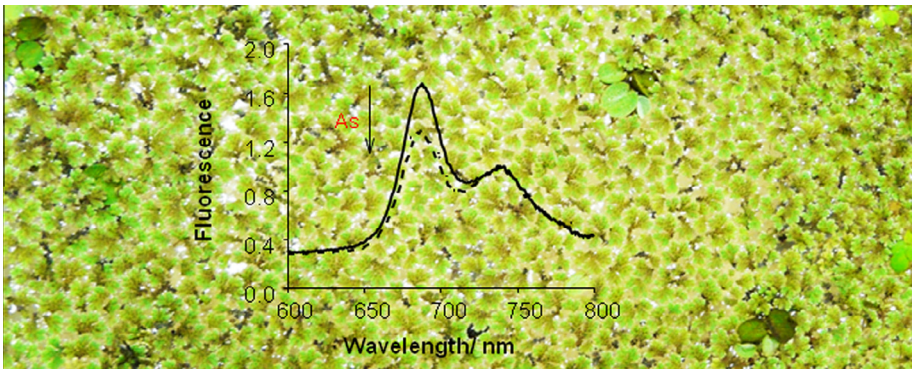
[a] Instituto de Investigaciones en Producción Animal / INPA(UBA-CONICET) / Centro de Estudios Transdisciplinarios del Agua (CETA), Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Av. Chorroarín 280, C1427CWO Buenos Aires, Argentina

[b] INQUIMAE / Dpto. de Química Inorgánica, Analítica y Química Física, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón II, 1er piso, C1428EHA Buenos Aires, Argentina

## HIGHLIGHTS

- *Vallisneria gigantea*, *Azolla filiculoides* and *Lemna minor* were grown in aquatic media with As (V).
- Reflectance in the UV–VIS–NIR range was performed.
- Initial (non-variable) and variable chlorophyll fluorescence were both recorded.
- Photosynthetic parameters were calculated.
- Photophysical data for the treated plants were compared with signals from control plants.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Arsenic pollution of groundwater is a serious problem in many regions of Latin America that causes severe risks to human health. As a consequence, non-destructive monitoring methodologies, sensitive to arsenic presence in the environment and able to perform a rapid screening of large polluted areas, are highly sought-after. Both chlorophyll – a fluorescence and reflectance of aquatic plants may be potential indicators to sense toxicity in water media.

In this work, the effects of arsenic on the optical and photophysical properties of leaves of different aquatic plants (*Vallisneria gigantea*, *Azolla filiculoides* and *Lemna minor*) were evaluated. Reflectance spectra were recorded for the plant leaves from 300 to 2400 nm. The spectral distribution of the fluorescence was also studied and corrected for light re-absorption processes. Photosynthetic parameters ($F_v/F_m$ and $\Phi_{PSII}$) were additionally calculated from the variable chlorophyll fluorescence recorded with a pulse amplitude modulated fluorometer.

Fluorescence and reflectance properties for *V. gigantea* and *A. filiculoides* were sensitive to arsenic presence in contrast to the behaviour of *L. minor*. Observed changes in fluorescence spectra could be interpreted in terms of preferential damage in photosystem II. The quantum efficiency of photosystem II for the first two species was also affected, decreasing upon arsenic treatment. As a result of this research, *V. gigantea* and *A. filiculoides* were proposed as bioindicators of arsenic occurrence in aquatic media.



* Corresponding author.
*E-mail address:* mgl@qi.fcen.uba.ar (M.G. Lagorio).

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## 1. Introduction

The presence of arsenic in groundwater is a problem for rural and periurban areas in Latin America because the continued ingestion of this element, even in low concentration, is associated with illnesses such as hyperkeratosis, gastric complications, liver fibrosis, peripheral neuropathy, and cancer (Sinha et al., 2007). In Argentine, about 4 million inhabitants are exposed to drinking water with arsenic concentrations higher than 10 $\mu g\ L^{-1}$ which is the safe limit for drinking water recommended by the World Health Organization (McClintock et al., 2012).

The origin of arsenic in Latin America is geogenic, and it is related to volcanic activity. In Chaco-Pampean plain of central Argentine the high concentration of arsenic in groundwaters comes from quaternary deposits of loess and dacitic volcanic ash (Nicolli et al., 1989; Smedley et al., 2005; Bundschuh et al., 2012). In natural waters it occurs principally as As (V) and As (III) depending on the oxygenic conditions of the environment (Smedley and Kinniburgh, 2002; Smedley et al., 2005). Organic forms can be produced by biological activity (Ferguson and Gavis, 1972), but they are quantitatively neglected in natural waters. Due to its geogenic origin it is normally accompanied by several trace elements such as V, B, U and F. These associations are probably due to preferential mobilization of these anions under high-pH conditions where silicates hydrolysis takes place (Smedley et al., 2005). From these elements, F is found in the highest concentration. Additionally, several studies have observed a relationship between arsenic and fluoride concentrations showing a linear correlation between them in both surface and groundwater (Rosso et al., 2011).

In a comprehensive study, local scientists reported several low-cost technologies as sustainable options for the removal of arsenic from drinking water in Latin America (Litter et al., 2012). Though the majority need further investigation prior to implementation, they provide promising routes for arsenic removal. Within this group of technologies macrophytes are seen as an attractive alternative for remediation of contaminated areas. This is a simple, ecofriendly, cost-effective and extremely sustainable cleanup technology for water treatment (Mikryakova, 2002; Miretzky et al., 2004; Sasmaz and Obek, 2009; Miretzky et al., 2006). For terrestrial plants, ferns have been proposed as hyperaccumulators because they are able to translocate As from roots to leaves (Ma et al., 2001; Wang et al., 2011). As a disadvantage, even in the presence of a hyperaccumulator, some authors determinated that it is not enough to remediate soils due to the lower biomass growth and a scarce roots depth. In Georgia, modified plants have been developed that are helpful to remove arsenic but, their use is conditional upon the possibility of including a new species in the environment (Dhankher et al., 2002; Zhao et al., 2009).

High arsenic concentrations in soils and water are regularly connected with negative effects in the physiological state of plants (Stoeva and Bineva, 2003). Parameters such as chlorophyll content, length of roots and leaves size are known to be affected (Stoeva et al., 2005; Rahman et al., 2007; Rahman Shaibur and Kawai, 2009; Meharg and Jardine, 2003), while the effect on chlorophyll fluorescence and reflection properties is poorly understood.

Chlorophyll fluorescence is strongly connected with plant photosynthesis and for this reason it is used as a sensitive tool to detect damage at the photosystem level. In fact, chlorophyll fluorescence from plants is a detectable signal which can be measured at some distance from the studied sample in a non-intrusive way (Schreiber et al., 1995; Cerovic et al., 2002). The light energy absorbed by Chlorophyll-a in plants can undergo three deactivation pathways: start of the electron transfer which leads to photosynthesis, energy dissipation as heat or energy emission as fluorescence. These three processes take place in competition and as a consequence the decrease in the yield of one of them leads to an increase in one of the other two. These competitive pathways explain the observed increase in chlorophyll fluorescence that occurs when a photosynthetic tissue is transferred from the dark to the light. In fact, when Chlorophyll-a in PSII is excited, it transfers electrons to the primary acceptors (quinone A, QA) in the photosynthetic chain. Once the QA has accepted an electron it is not able to accept another until it has been transferred to the next acceptor quinona B (QB). During this time (with closed reaction centres) the fluorescence emission increases from an initial value $F_0$ up to a maximum value $F_m$ (Fig. 1). This period of time is usually in the order of 1 s. Later, fluorescence starts to fall (fluorescence quenching) for several minutes to finally reach a stationary state ($F_s$). The variation in Chlorophyll fluorescence quantum yield as a function of time is called Kautsky kinetics (Maxwell and Johnson, 2000).

Chlorophyll fluorescence has being explored in literature for the design of biosensors capable of monitoring pollutants (Védrine et al., 2003; Durrieu et al., 2006; Sassolas et al., 2012) in the media even before the chemical composition of present pollutants is known.

In literature, there are different results about arsenic effect on photosynthetic electron transport. Stoeva et al. studied physiological parameters in bean plants treated with arsenic and they concluded that it has a negative effect on the growth, chlorophyll content and photosynthesis rate (Stoeva et al., 2005). In maize, these authors also have found a diminution on the maximum photosynthetic efficiency (Stoeva et al., 2003/4). However, in soybean plants, Milivojevic et al. concluded that there was no effect of As on the photosynthetic electron transport (Milivojević et al., 2006). Miteva and Merakchiyska studied the effect of As (III) in contaminated soils, and found changes in the chloroplast shape similar to changes observed in plants under the effect of other stress factors such as heavy metals, temperature and water stress (Miteva and Merakchiyska, 2002).

Spectral reflectance features of plants have been also reported as sensitive, non destructive signals of pollutants presence and they are fully used in remote sensing (Hatfield et al., 2008; Emengini et al., 2013). Several prediction models were designed with hiperaccumulators species that correlate the arsenic content in soils with the reflectance data in red edge region (Slonecker et al., 2009a,b).

The main purpose of our work was to evaluate the effects of arsenic on the optical and photophysical properties of leaves of different aquatic plants and to explore reflectance and fluorescence emission as potential indicators of arsenic toxicity. An additional

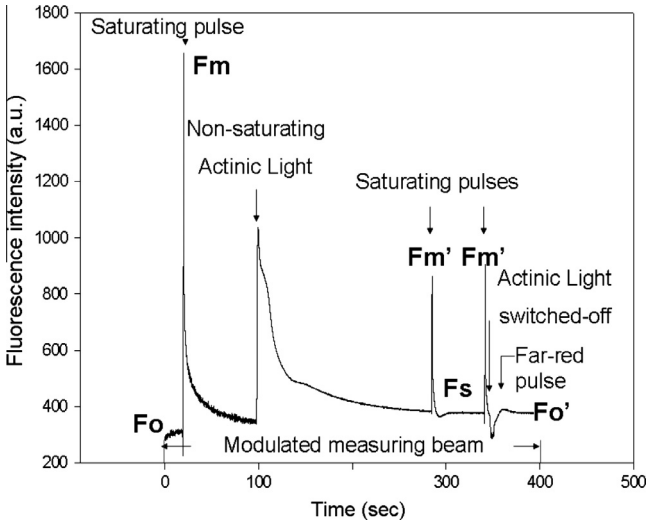


**Fig. 1.** Variable chlorophyll fluorescence recorded with a pulse-modulated fluorometer for a typical plant leaf.

aim of this work was to evaluate the possibility of detecting photosystem damage through the interpretation of Chlorophyll fluorescence spectra.

## 2. Materials and methods

### 2.1. Plants and reagents

The species used in this study were *Vallisneria gigantea*, *Azolla filiculoides* and *Lemna minor*. The plants were obtained from a local store and were cleaned with HClO (Baker) 5% to remove traces of organic matter, and rinsed with distilled water. Plants were then put in plastic containers in a ratio of 10 g of plant for each litre of liquid. Plastic containers were covered with plastic film to avoid water evaporation during the experiment and the plants were illuminated using artificial light. The plants were kept at room temperature (about 20 °C) with illuminating cycles of 16 h light and 8 h dark, in which light was emitted at a photosynthetic photon flux density of 200 μmol $m^{-2}$ $s^{-1}$. The photosynthetically active radiation (PAR) was measured with a sensor provided by Cavadevices SA. Stock solution of As (V) was prepared by dissolving $Na_3AsO_4.7H_2O$ (Biopack, Argentina) in milliQ water, and fluoride stock solution was obtained from NaF (Aldrich). All reagents used were analytical grade. Working solutions were prepared by dilution of stock solutions in water from a 70 m-depth well (geographical coordinates 34°32′8851″S and 58°6′16086″W) to reach a final concentration of 2 ppm in all cases. The well water had the following physico-chemical parameters: pH = 7.20 ± 0.05, conductivity = 1.134 ± 0.002 mS $cm^{-1}$, total dissolved solids = 0.578 ± 0.001 g $L^{-1}$, $Cl^-$ = 89 ± 1 ppm, $SO_4^{2-}$ = 74 ± 1 ppm, $NO_3^-$ = 4.4 ± 0.1, $NO_2^-$ = 0.023 ± 0.001, ppm, $F^-$ = 0.3 ± 0.1 ppm, alcalinity = 517 ± 5 ppm, total hardness as Calcium carbonate = 595 ± 9 ppm, $Na^+$ = 93 ± 1, $K$ = 3.0 ± 0.1 ppm. These parameters were determined using standard methods described in APHA (1995). Conductivity and pH were measured using a combined meter Hanna HI 255. Total dissolved solids were calculated from the weight of the residue of evaporation after drying at a temperature of 110 °C. Alcalinity and total hardness were determined by tritation as usual. Ionic compounds were analyzed by ion Chromatography using a Dionex DX 100 chromatograph. The plants were immersed in these media and water samples were obtained daily to determine quantitatively As and F concentration in solution. Plants immersed in water without additional aggregates were used as controls. Fluoride concentrations were evaluated using a selective electrode (ThermoOrion/model 0609) according to APHA (1995). Arsenic concentrations were determined by ICP-OES (Perkin Elmer, Optima DV 2000).

### 2.2. Reflectance

Thick layers of leaves were put in a holder and covered with quartz windows to fix them in place. Spectra were obtained in a Shimadzu 3100 spectrophotometer equipped with an integrating sphere. Barium sulphate was used as a standard for 100% reflectance. Diffuse reflectance was recorded as a function of wavelength from 300 to 2400 nm for groups of stacked intact leaves. From these data the remission function, $F(R)$, a quantity proportional to chromophore concentration, was calculated as: (Iriel and Lagorio, 2009)

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

### 2.3. Initial fluorescence ($F_0$)

Emission spectra of intact plants were obtained under low photon flux conditions as not to induce the photosynthetic process. In these conditions, the initial fluorescence $F_0$ was recorded as a function of wavelength. Measurements were carried out on a PTI Model QM-1 spectrofluorometer using a front-face geometry. Prior to measurements, plants were dark-adapted for 15 min. Spectra were recorded from 600 to 800 nm using an excitation wavelength of 460 nm. As usual, emission curves were corrected for changes in the detector responsivity with wavelength. The selected excitation wavelength led to the highest fluorescence signal.

Due to the overlap of the leaves' absorption and emission spectra, there is an artifact in experimental spectra that enhances the band at 735 nm compared to the band at shorter wavelength. As a consequence, emission spectra are distorted and the peak ratio is underestimated. To eliminate light reabsorption artifacts, thick layer fluorescence spectra were corrected for reabsorption according to Ramos and Lagorio (2004). True emission spectra were obtained by dividing experimental spectra by:

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

where $\lambda$ and $\lambda_0$ stand for the emission and excitation wavelengths respectively. Function $\gamma$ can be interpreted as the fraction of the luminescence emitted at wavelength $\lambda$ that escapes from the optically thick sample excited at wavelength $\lambda_0$. The method employed here was developed by our working group and has been successfully applied to correct fluorescence spectra of leaves (Ramos and Lagorio, 2004; Cordon and Lagorio, 2006) and fruits (Mendes Novo et al., 2012).

### 2.4. Variable fluorescence (Kautsky kinetics)

Variable fluorescence was investigated using a pulse-modulated chlorophyll fluorometer (Hansatech FMS1), on leaves that had been previously dark-adapted during 15 min. The instrument had a 594 nm amber modulating beam for excitation which induced a pulse fluorescence signal under conditions where ambient light was excluded. The modulating beam used had very short duration pulses (1.8 μs) with long off periods between pulses. The integrated amount of incident radiation upon the sample from the modulating beam was smaller than 0.05 μmol $m^{-2}$ $s^{-1}$, low enough to avoid significant physiological change in the sample. The saturating pulse (halogen light) was fixed at 14400 μmol $m^{-2}$ $s^{-1}$ with duration of 0.4 s. A halogen light source of about 600 μmol $m^{-2}$ $s^{-1}$ provided the actinic light. The instrument varied the sampling rate from 10 Hz to 20 kHz (low frequency for $F_0$ measurement and high frequency during application of actinic or saturating light). Fluorescence detection was performed by a PIN photodiode with a long pass filter for wavelengths longer than 700 nm (Schott RG 695, 3 mm thickness). The experiments were started recording the minimum fluorescence signal from dark-adapted samples ($F_0$ in Fig. 1) with the modulating beam. Then the saturating pulse was applied and the maximum fluorescence $F_m$ was recorded allowing calculation of $F_v/F_m$ ($F_v = F_m - F_0$). Following, the samples were exposed to the actinic light during several minutes. After this time period, a steady state fluorescence value $F_s$ was reached, and a new saturating pulse was applied to record the maximum fluorescence for light-adapted leaves ($F_m'$). Another saturating pulse was applied, the actinic light was automatically turned-off and the far-red pulse was applied for $F_0'$ determination. From these measurements, the maximum quantum efficiency of PSII photochemistry ($F_v/F_m$) and the quantum efficiency of PSII ($\Phi_{PSII} = (F_m' - F_s)/F_m'$) were calculated (Lichtenthaler et al., 2005; Mendes Novo et al., 2012).

Reflectance and fluorescence measurements were performed on leaves after seven days of treatment.

### 2.5. Statistical analysis

Analysis of variance and Pearson correlation analysis of the results were performed using the statistical software InfoStat®. Correlation significance was evaluated with Fisheŕs test.

## 3. Results and discussion

### 3.1. Arsenic uptake in plants

During the experiment arsenic and fluoride concentrations were monitored to assess the ability of plants to remove them, and to evaluate a possible synergistic effect. It was found that fluoride concentrations remained constant during the experiment in all cases (data not shown). Of the plants studied only *V. gigantea* was efficient in arsenic uptake with a 17% of remotion both in the case of presence and absence of fluoride. *Lemna* and *Azolla* exhibited very low absorption under the same conditions, around 2–4%. These results were found after seven days of treatment.

In a thorough review about arsenic phytoremediation using macrophytes (Rahman and Hasegawa, 2011), results for arsenic uptake from different aquatic plants were compiled. Several authors have reported that some species of aquatic macrophytes accumulate high amounts of arsenic from water (Robinson et al., 2005; Alvarado et al., 2008; Mishra et al., 2008; Rahman et al., 2008; Zhang et al., 2008). Working with an initial arsenic concentration of 0.15 mg $L^{-1}$, Alvarado and co-workers have found a removal efficiency of 18% for *Eichhornia crassipes* (a value close to our removal efficiency for *V. gigantea*) and 5% for *L. minor*, (value close to our result for the same species), (Alvarado et al., 2008). Other authors have reported much higher values. In fact, Mishra and co authors studied and compared the removal efficiency for arsenic in *E. crassipes*, *L. minor* and *Spirodela polyrrhiza*, finding values of 80%, 60% and 40% respectively. In that work, an initial arsenic concentration of 0.05 mg $L^{-1}$ was used. Zhang and coworkers reported that different strains of *Azolla* showed a large variation in arsenic accumulation. In particular, the lower arsenic uptake was obtained by them for *A. filiculoides*. High removal efficiency (32–65%) has also been reported for *Salvinia notans* L. (Rahman et al., 2008).

### 3.2. Absorption spectra from leaves

Variations in NIR region (above 800 nm) could not be detected (data not shown) so in this study only the visible region will be considered. From these data, the remission function spectrum for each plant was calculated using Eq. (1) and they are shown in Fig. 2.

As a general rule three regions of interest can be distinguished: from 400 to 500 nm, from 500 to 600 nm and from 600 to 800 nm in the visible spectrum.

Absorption spectra of leaves have a contribution of several pigments, such as chlorophyll, carotenoids and anthocyanins. The spectrum of chlorophyll has a minor band at 400 nm and a principal band around 680 nm (Lagorio, 2011). Carotenoids and anthocyanins absorb around 400 and 530 nm respectively (Iriel and Lagorio, 2010) and their biosynthesis is related to stress factors (Yaryura et al., 2009; Misyura et al., 2013) and senescence process (Procházková et al., 2009). Absorption spectra for *L. minor* did not show any change upon As treatment. *A. filiculoides* and *V. gigantea*, instead, both showed increased absorption in the region from 400 to 500 nm (Fig. 2). An increase around 530 nm was also present for *A. filiculoides*. The spectral behaviour observed for *Vallisneria* and *Azolla* plants could be related to an increase in the biosynthesis of carotenoids and flavonoids as a defensive response towards As

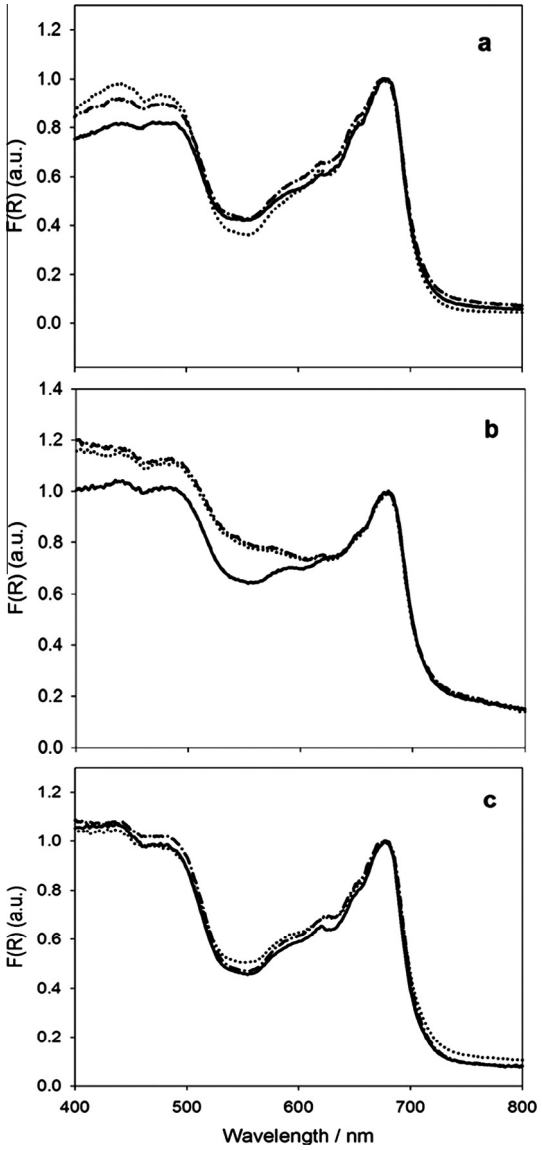


**Fig. 2.** Remission function as a function of wavelength for: (a) *Vallisneria gigantea*, (b) *Azolla filiculoides*, (c) *Lemna minor*. Spectra are normalized at the red maximum. Full line: control plant, dot line: plant with As, dashed and dot line: plant with As and F.

effect. In addition, *Azolla* tissue was observed to turn brown. This behaviour has been previously reported in literature and it was attributed to Radical Oxygen Species (ROS) generation that appeared as a stress response. It is well documented that ROSs may be generated through the conversion of arsenate to arsenite in the plant with simultaneous damage to DNA, proteins and lipids. Several studies found correlations between lipid peroxidation, Superoxide dismutase (SOD) activity and phytochelatins production and arsenate concentrations in *Holcus lanatus* L. (Hartley-Whitaker et al., 2001). In maize, antioxidative enzymes like catalase, GSHS-transferase and SOD have been shown to increase after exposure to arsenate and arsenite (Mylona et al., 1998). An increase in $H_2O_2$ concentrations was determined in *Xanthoria parietina* (L.) Th Fr. treated with As concentrations ranging from 0.01 to 10 ppm (Pisani et al., 2011), additionally cell damage was reported.

In literature, it is well documented that a high arsenic concentration cause oxidative stress due to rapid reduction from arsenate to arsenite via cytochrome/cytochrome oxidase, (Mascher et al., 2002) using oxygen as a final electron acceptor (Tamaki and Frankenberger, 1992).

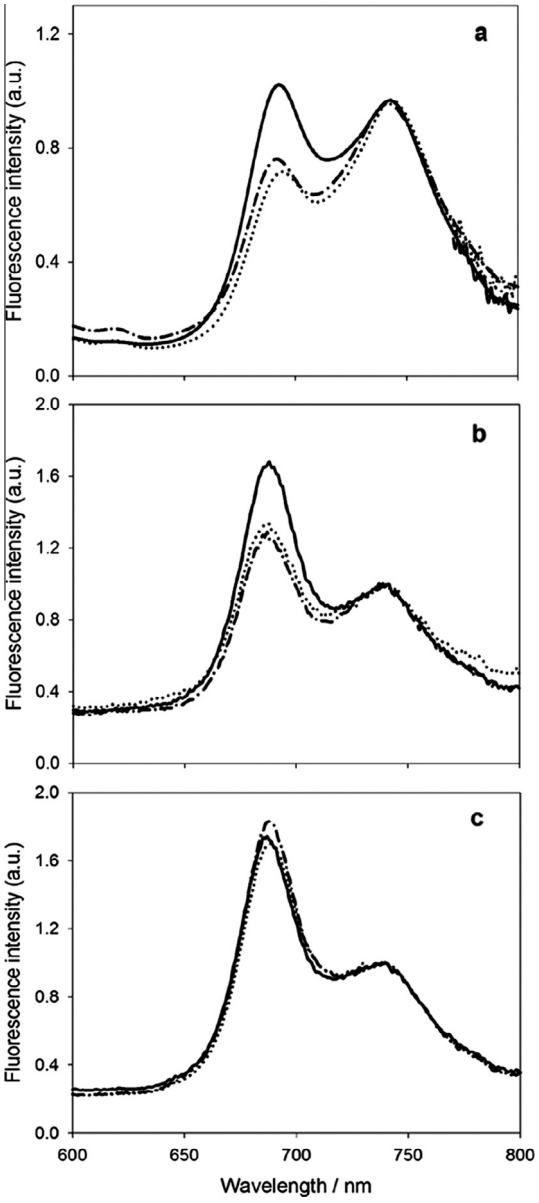


**Fig. 3.** Fluorescence spectra ($F_0$) for dark adapted leaves of *Vallisneria gigantea* (a), *Azolla filiculoides* (b) and *Lemna minor* (c) at excitation wavelength of 460 nm. Spectra are corrected for the detector response to wavelengths and for light re-absorption processes. Full line: control plant, dot line: plant with As, dashed and dot line: plant with As and F.

### 3.3. Variable and non-variable fluorescence emission from leaves

Fig. 3 shows chlorophyll fluorescence spectra for intact plants of *V. gigantea* (a), *A. filiculoides* (b) and *L. minor* (c) obtained with the standard fluorometer under low photon flux irradiation, corrected for the detector response to emission wavelengths and additionally for light re-absorption processes according to Eq. (2).

The obtained spectra showed the typical chlorophyll emission spectrum with two bands corresponding to photosystems fluorescence. Several authors agree that at room temperature both PSI and PSII are responsible for the band in the far red, around 735 nm, while at 680 nm emission is only attributed to PSII (Pfündel, 1998). The peak fluorescence ratio **F680/F735** is thus connected to the balance between them, and was found to vary in the presence of several factors related to plant health (Iriel et al., 2014).

In Table 1, results obtained for the fluorescence ratios (experimental and corrected for light re-absorption), the maximum quantum efficiency of PSII photochemistry ($F_v/F_m$) and the quantum efficiency of PSII ($\Phi_{PSII}$) are summarized.

Both experimental and corrected fluorescence ratios (F680/F735) for As-treated samples did not vary appreciably in the case of *L. minor* and decreased for *A. filiculoides* and *V. gigantea*. This fact showed a relative decrease in the fluorescence of PSII compared to PSI emission suggesting either preferential damage in PSII or inhibition of $e^-$ transfer from PSI. The joint presence of F and As decreased slightly the fluorescence ratio for *A. filiculoides* and *V. gigantea*.

It is already known that PSI and PSII reaction centres are the place with major generation of ROS due to their particular conditions (Asada, 2006). A study on induced leaf necrosis of *Arabidopsis thaliana* showed that the electron transport beyond QA and the reduction of the end acceptors at the PSI acceptor side were inhibited (Chen et al., 2012).

Parameters from measurements of Kaustky kinetics were obtained in triplicate and are shown in Table 1. For $F_v/F_m$ ratio no significative differences have been found for the whole set of plants and treatment. For $\Phi_{PSII}$ a clear diminution was observed for *Azolla* and *Vallisneria* plants, while *L. minor* showed erratic results. In general, fluoride presence did not modified As effect on photophysical parameters.

## 4. Conclusions

Absorption spectra for *L. minor* did not show any change upon As treatment. *A. filiculoides* and *V. gigantea*, instead, both increased absorption in the region from 400 to 500 nm in the presence of As. An increase around 530 nm was also present for *A. filiculoides*. This spectral behaviour could be related to an increase in the

**Table 1**
Non-variable and Variable fluorescence parameters for *Vallisneria gigantea*, *Azolla finiculoides* and *Lemna minor*. $F_{red}/F_{far\ red}$: Ratio of fluorescence intensities in the red (685 nm) and far-red (735 nm), $F_{red}/F^{e}_{far\ red}$: Ratio of fluorescence intensities in the red (685 nm) and far-red (735 nm) corrected for light re-absorption, $F_v/F_m$: maximum quantum yield of PSII for dark adapted leaves, $\Phi_{PSII}$: quantum yield of PSII. Statistical analysis was performed using ANOVA. All data represent the means ± S.E. (from at least three independent series of experiments). Within each column, for each species separately, different letters indicate significant differences ($p < 0.05$).

| Plant species | Treatment | $F_{red}/F_{far\ red}$ | $F_{red}/F^{e}_{far\ red}$ | $F_v/F_m$ | $\Phi_{PSII}$ |
|---|---|---|---|---|---|
| *Vallisneria gigantea* | Control | $0.55 \pm 0.02^a$ | $1.07 \pm 0.03^a$ | $0.83 \pm 0.01^a$ | $0.58 \pm 0.02^a$ |
| | As | $0.40 \pm 0.01^b$ | $0.79 \pm 0.02^b$ | $0.81 \pm 0.01^a$ | $0.46 \pm 0.03^b$ |
| | As–F | $0.35 \pm 0.01^b$ | $0.72 \pm 0.02^b$ | $0.80 \pm 0.01^a$ | $0.46 \pm 0.03^b$ |
| *Azolla filiculoides* | Control | $1.02 \pm 0.03^a$ | $1.70 \pm 0.05^a$ | $0.71 \pm 0.04^a$ | $0.77 \pm 0.01^a$ |
| | As | $0.86 \pm 0.03^b$ | $1.35 \pm 0.04^b$ | $0.71 \pm 0.02^a$ | $0.61 \pm 0.01^b$ |
| | As–F | $0.82 \pm 0.02^b$ | $1.28 \pm 0.04^b$ | $0.75 \pm 0.01^a$ | $0.62 \pm 0.02^b$ |
| *Lemna minor* | Control | $0.91 \pm 0.03^a$ | $1.72 \pm 0.05^a$ | $0.77 \pm 0.01^a$ | $0.64 \pm 0.03^a$ |
| | As | $0.97 \pm 0.03^a$ | $1.70 \pm 0.05^a$ | $0.73 \pm 0.01^b$ | $0.45 \pm 0.05^b$ |
| | As–F | $0.97 \pm 0.03^a$ | $1.80 \pm 0.05^a$ | $0.75 \pm 0.01^b$ | $0.61 \pm 0.03^a$ |

biosynthesis of flavonoids as a defensive response towards As action for *Azolla* and *Vallisneria*. The fluorescence ratio (red/Far-red) for As-treated samples did not vary in the case of *L. minor* and decreased for *A. filiculoides* and *V. gigantea*. This fact showed a relative decreased in the fluorescence of photosystem II compared to photosystem I emission, suggesting any kind of preferential damage in photosystem II for the two last species. Finally, the quantum efficiency of PSII photochemistry decreased around 15% for As treated leaves of *A. filiculoides* and *V. gigantea*. Similarly to what happened with other optical properties, the quantum efficiency of PSII photochemistry did not change appreciably for *L. minor*.

As a final conclusion, *V. gigantea* and *A. filiculoides* were sensitive to As presence even in the presence of fluoride and may be proposed as bioindicators of As occurrence.

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