

Soil Arsenic toxicity impact in the growth and C-assimilation of *Eucalyptus nitens*

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Abstract

The selection of suitable plant species that can live in contaminated and abandoned mining areas is a goal environmental managers and policy makers. In this framework, the use of a fast-growing forestry species such as *Eucalyptus nitens* in the recovery of As from artificial contaminated soils was studied during a long-term experiment. Roots can accumulate levels ranging between 69.8 and 133 $\mu\text{g g}^{-1}$ for plants treated with 100 As and 200 As, respectively, while leaves between 9.48 $\mu\text{g g}^{-1}$ (200 As) and 15.9 $\mu\text{g g}^{-1}$ (100 As) without apparent morphological damage and toxicity symptoms. The performance of the photosynthetic apparatus showed a gradual impact in some gas exchange parameters such as net photosynthetic rate (P_n), stomatal conductance to H_2O (g_s) and the transpiration rate (E), particularly with the use of the highest As concentration (200 As), but without significant impacting the PSII performance. The effects of As on the uptake and translocation of Ca, Fe, K and Zn revealed two major interferences. The first one related with Zn where a moderate antagonism was detected, and the second one related with Fe, where a particular enrichment in *E. nitens* leaves was noted in both As treatments. Thus, it seems to exist a synergistic action with impact in the levels of the photosynthetic pigments in As treated plant leaves, compared with control plants. In face of the acquired data, *E. nitens* must be considered as an alternative when phytoremediation processes are put in practice in our country.

Highlights

- Root system of *E. nitens* prove to be efficient in the recovery of As from artificial contaminated soils.
- The impact of the studied As concentrations on some gas exchange parameters was noted but without compromising the PSII performance.
- The effects of As on the uptake of Fe and Zn revealed two opposite trends – a synergistic effect regarding Fe and an antagonistic effect for Zn.
- As a frost resistant species, *E. nitens* is recommended for phytoremediation, especially in areas where winter temperatures are too low.

1. Introduction

Ecosystem contamination by heavy metals and metalloids due to natural or anthropogenic activities is a major concern related with the effects on both human life and remaining biodiversity. In this context, reducing anthropogenic emissions of heavy metals is therefore a focus of international and European Union action (EEA, 2019) since natural phenomena, such as, vulcanism and natural fires cannot be confined and/or controlled.

The evaluation of the contamination degree by heavy metals of different compartments such as agricultural soils (Reboredo et al 2019), water basin sediments (Carrondo et al 1984; Reboredo and Ribeiro, 1984; Martins et al 2017) or atmosphere (Ferreira et al 2020) has been the target of different

researchers, beyond studies regarding the effects at metabolic (Loureiro et al 2006) and cellular levels (Reboredo and Henriques, 1991; Lidon et al 1993; Vassilev et al 2002).

Within industrial activities, mining is one that greatly contributes with large inputs of heavy metals to the aquifers and soils with possible transfer to the food chain. The mining activity in Portugal flourished until the first half of the 20th century when the overwhelming majority of the mines were closed, such as Urgeiriça (Pereira et al 2013), Lousal (Ferreira da Silva et al 2005; Reis et al 2007) among others. As a result of these long-term activities huge mine tailings enriched with heavy metals and metalloids were formed, which contribute to soil and aquifer contamination through rainwater percolation (Batista et al 2012), plus aerial dispersion to surrounding areas.

In a similar manner the disposal of huge quantities of extractive wastes in Europe is a major concern – 900 Mton/year of extractive waste is being generated and stored in tailing facilities or ponds corresponding to 26% of the EU's current waste output (Eurostat, 2018). The very high contamination of soils situated on, or in the vicinity of a mine dump area, was evaluated regarding the mobilization of different heavy metals to green bean, courgette, oranges and figs (Reboredo et al 2018). The authors concluded that the regular intake of these edible plants might pose a great risk to human health due to the high Cu levels detected which can induce chronic toxicity in humans. Human exposure from contaminated soils around gold mine tailings dumps, as it happens in Krugersdorp (South Africa), clearly indicates that children were more at risk than adults. Also, cancer-related risks associated with metal and metalloid exposure among children were also higher than in adults (Ngole-Jeme and Fantke, 2017).

The so-called Iberian Pyrite Belt (IPB) is a large area encompassing Southern Portugal and Spain, where polymetallic sulphides extraction occurs as it happens in Neves-Corvo mine located in Portugal which produces copper, zinc and lead concentrates. Arsenic sulphides are commonly associated with these orebodies and despite the stability and insolubility under reducing conditions, when oxidation takes place (exposition to atmospheric conditions) the dissolution of sulfides by rainwater may lead to the release of As, among other elements, to the environment.

In this context, mine tailings of abandoned or active mines may constitute a potential hazard to fauna, flora and human health. Furthermore, the long-term exposure to inorganic arsenic can induce bladder and lung cancer, as well as skin lesions in humans (EFSA, 2014) such as the so-called "*black-foot disease*" first reported in Taiwan in the 1960s (Tseng, 1977) mainly due to the consumption of water from well, with high As levels.

In plants, the deleterious effects of As range from the replacement of inorganic phosphate in biochemical reactions in the case of pentavalent arsenic, to the increment of reactive oxygen species, or the binding of trivalent arsenic to thiol reactive compounds (Finnegan and Chen, 2012). Arsenic-mediated oxidative stress causes an array of metabolic dysfunctions in plants ranging from the reduction of growth and biomass, interference with photosynthesis, imbalance in nutrient uptake, reduction in stomatal conductance, suppression in ATP synthesis, among others (Chandrakar et al 2016).

Shaibur et al (2009) observed in hydroponic cultures of barley a decrease in the chlorophyll index and Fe concentration in shoots of the As-treated plants compared with the control plants, while for *Eucalyptus camaldulensis*, root and leaf biomass (on a dry weight basis) decrease as the concentration of the As supplied to the soil increases (Ahmad et al 2018).

The effects of As on the leaves of the aquatic plants *Vallisneria gigantea*, *Azolla filiculoides* and *Lemna minor* show that fluorescence and reflectance properties for the first two species were sensitive to As. Changes in fluorescence spectra could be interpreted in terms of preferential damage in photosystem II. Also, the quantum efficiency of photosystem II was also affected, decreasing upon arsenic treatment (Iriel et al 2015).

The Directive 2006/21/EC3 on the management of waste from the extractive industries provides guidance of actions to prevent or reduce (as far as possible), the adverse effects on the environment and the resulting risks on human health (European Commission, 2021). In order to mitigate the effects several approaches can be taken such as Remediation which is focused on the removal of pollutants from a closed mining site in order to clean up the contaminated land to safe levels and Rehabilitation with the aim of returning the land to some degree of its former state (DeCarb/Interreg Europe, 2019).

The use of phytoremediation, as a low-cost process, can be implemented in order to alleviate contaminant dispersion through an efficient root system uptake, as observed in some polluted salt marsh areas (Caçador and Duarte, 2011; Anjum et al 2014) or abandoned mining sites (Durante-Yáñez et al 2022; Dradrach et al 2020), although the success of this process depends of the selected plant species and soil characteristics.

Tree species, due to longevity, large biomass accumulation and extensive rooting, might be more suitable for phytoremediation than herbaceous species (Yan et al 2020) and the genus *Eucalyptus* sp. has been used with success worldwide in both *in vivo* (Mughini et al 2013) and *in vitro* assays (Reboredo et al 2021; El Rasafi et al 2021) in soils contaminated with heavy metals. *Eucalyptus* plantations, mainly *Eucalyptus globulus* are widely spread in Portugal, managed in coppice systems for pulp production, mainly in 10–12 years rotations and occupying currently, 812,000 ha in the mainland (Reboredo and Pais, 2014).

Despite *E.globulus* is the main species used in afforestation, *Eucalyptus nitens* is under evaluation due to its high tolerance to frost (Gomes and Canhoto, 2003), which is a strong handicap to the former species. In this context, it can be used as an alternative in some regions of Portugal where winter temperatures are too low.

In natural conditions plants can be exposed to multiple abiotic stresses and the health status of the plant can be monitored, using the photosynthetic pathway as probe of the global plant functioning (Iriel et al 2015; Kalaji et al 2016; Reboredo and Lidon, 2012) in order to detect interferences leading to growth decline and yield reduction. Thus, this type of approach can also be used *in vitro* conditions.

In this framework and taking into account the prevalent levels of As in agricultural soils surrounding the Neves-Corvo mining area (Reboredo et al 2018) an experiment with *Eucalyptus nitens* growing in soil artificially contaminated with As during six months, was implemented. This assay aimed to verify the plant capability to uptake and translocate As to the different organs, the As impact on photosynthetic performance (assessed through leaf gas exchanges, chlorophyll a fluorescence parameters), as well as the As interference on the uptake of other important nutrients, in order to determine the potential of *E. nitens* for phytoremediation of soils from contaminated mining areas.

2. Materials And Methods

2.1. Plant material and experimental design

Eleven months old *Eucalyptus nitens* Deane & Maiden plants were collected from Altri Florestal S.A. and transplanted to 5 L pots, with 3 L of substrate SIRO Universal/Portugal (pH: 5.5–6.5; humidity: 50–60%, electrical conductivity: 0.6–1.2 (mS m⁻¹), N: 80–150 mg L⁻¹; P₂O₅: 80–150 mg L⁻¹ - correspond to 35 and 65 mg P; K₂O: 300–500 mg L⁻¹ - correspond to 249 and 415 mg K; organic matter: >70%). Plants were then acclimated from September to December to natural conditions in Campus da Caparica, Portugal (Gps - 38° 39' 41, 5" N, 9° 12' 24, 0" W). The mean air temperature was 16.0 °C, and the average of maximum and minimum temperature were 21.9 °C and 10.1 °C, respectively (IPMA, 2016). The mean values of total annual rainfall reached 600 mm.

A total amount of 72 plants were selected to obtain three distinct groups of 24 plants *i.e.*, a control group and two As treatments. Arsenic was added to the soil in January as NaAsO₂, soluble in 100 ml bidistilled water in two distinct concentrations, 100 (100 As) or 200 (200 As) µg As ml⁻¹, while control plants received the same volume of bidistilled water only. After soil contamination in the end of January (T0 moment), plant and soil analysis were carried every two months, *i.e.*, by the end of March (T1), May (T2) and July (T3), *ca.* 2, 4 and 6 months after soil contamination.

2.2. Growth analysis

Along the experimental period the growth parameters specific leaf area ratio (SLA), the leaf weight ratio (LWR) and the leaf area ratio (LAR) were evaluated (Lambers and Poorter, 2004). Photosynthetic pigments (total chlorophyll and total carotenoids) were extracted in 80% acetone, determined spectrophotometrically, and calculated according to the formulae of Lichtenthaler (1987). In each case four replicates were used for the determination of the above-mentioned parameters.

2.3 Leaf gas exchanges analysis

Net photosynthesis (P_n), stomatal conductance to water vapour (g_s) and transpiration rate (Tr) were evaluated under photosynthetic steady-state conditions after at least 2 h of illumination (10:00–11:00, which preliminary evaluations showed to be the daytime with higher P_n and g_s values), using a portable open-system infrared gas analyser (CIRAS 3, PP Systems, USA). Measurements were done by 1st April,

2nd June and 30th July, with an irradiance of *ca.* 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and providing an external $[\text{CO}_2]$ of *ca.* 390–400 $\mu\text{L L}^{-1}$.

2.4. Chlorophyll a fluorescence data

Chlorophyll (Chl) fluorescence parameters were evaluated on the same dates (environmental conditions) and leaves used for gas exchange measurements, using a PAM-2000 system (H. Walz, Effeltrich, Germany), as previously described (Rodrigues et al 2016; Semedo et al 2020). Calculations and meanings of the several parameters followed the formulae discussed elsewhere (Kramer et al 2004; Krause and Jahns, 2004; Klughammer and Schreiber, 2008). Measurements in dark-adapted leaves included the F_0 (minimum fluorescence from excited Chl *a* molecule from the antennae), and F_v/F_m (maximal PSII photochemical efficiency). A second set of parameters, evaluated under photosynthetic steady-state conditions (*ca.* 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of natural irradiance) and superimposed saturating flashes (*ca.* 8000 $\mu\text{mol m}^{-2} \text{s}^{-1}$), included the F_v'/F_m' (PSII photochemical efficiency of energy conversion under light exposure), q_L (photochemical quenching based on the concept of interconnected PSII antennae, representing the proportion of energy captured by open PSII centers and driven to photochemical events (Kramer et al 2004; Klughammer and Schreiber, 2008), and F_s/F_m' - predictor of the rate constant of PSII inactivation (Stirbet and Govindjee, 2011).

Additionally, estimates of photosynthetic quantum yields of non-cyclic electron transfer ($Y_{(II)}$), photoprotective regulated energy dissipation of PSII ($Y_{(NPQ)}$), and non-regulated energy dissipation of PSII as heat and fluorescence ($Y_{(NO)}$), where $[Y_{(II)} + Y_{(NPQ)} + Y_{(NO)} = 1]$, were also calculated (Kramer et al 2004; Huang et al 2011). Finally, it were evaluated the PSII photoinhibition indexes (Werner et al 2002; Martins et al 2016) that included: A) chronic photoinhibition (PI_{Chr}), representing the percent reduction in F_v/F_m at each temperature relative to the maximal F_v/F_m obtained during the entire experiment - the latter for the control treatment; B) dynamic photoinhibition (PI_{Dyn}), representing the decline in F_v/F_m that is fully reversible overnight, being measured as the percent reduction in midday F_v'/F_m' relative to F_v/F_m at each evaluation date, relative to the maximal F_v/F_m from the entire experiment - the latter for the control treatment; C) total photoinhibition (PI_{Total}).

$$PI_{\text{Chr}} = [(F_v/F_m)_{\text{max}} - (F_v/F_m)_{\text{pd}}] / (F_v/F_m)_{\text{max}} \times 100\%$$

$$PI_{\text{Dyn}} = [(F_v'/F_m')_{\text{pd}} - (F_v'/F_m')_{\text{mid}}] / (F_v'/F_m')_{\text{max}} \times 100\%$$

$$PI_{\text{Total}} = PI_{\text{Chr}} + PI_{\text{Dyn}}$$

2.5. Metal determination in soil and plant samples

For determination of As, Ca, Fe, K and Zn contents, plants were removed from the soil, washed twice with tap water and a third time with deionised water, and separated in roots, stems and leaves, which were then dried at 60 °C for 72 h until a constant weight. Thereafter, plant samples were powdered and stored. Soil drying followed a process similar to that of plant material, before analysis.

The elemental determination in soil, roots, stems and leaves, was performed in triplicate for each treatment, in a total of 36 samples per collection date, through an X-ray Analyser (Thermo Scientific, Niton model XL3t 950 He GOLDD+, USA) in accordance with Environmental Protection Agency (EPA) method 6200 (EPA, 2007). Detection limits using the optimum “mining” mode for a period of 120s under high purity helium (He) were: As = 5 $\mu\text{g g}^{-1}$; Ca = 350 $\mu\text{g g}^{-1}$; Fe = 25 $\mu\text{g g}^{-1}$; K = 500 $\mu\text{g g}^{-1}$; Zn = 6 $\mu\text{g g}^{-1}$; K = 500 $\mu\text{g g}^{-1}$. Soil reference materials - NRCan Till-1 (NRCan, 1995) and plant reference materials (Orchard Leaves - SRM 1571) were run before the beginning of analyses and after every five samples. The recovery values ranged between 91% and 96%.

2.6. Micro-Energy Dispersive X-ray Fluorescence (m-EDXRF)

In order to overcome the limit detection of the portable XRF, stems and leaves were also monitored for As determination using a μ -Energy Dispersive X-Ray Fluorescence (μ -EDXRF) Bruker M4 Tornado™ system. Dehydrated samples were previously reduced to powder in a mortar and transformed into pellets of 2 cm in diameter and 1 mm thick. This pellet was then glued onto a mylar sheet in a plastic frame and placed directly onto the X-ray beam for analysis. From each sample, three pellets were made and submitted to analysis.

Quantification of the total spectra of the obtained maps was performed with the fundamental parameters method of the built-in ESPRIT software and the recovery rate was checked against a set of standard reference materials—Orchard Leaves (NBS 1571) and Sea Lettuce (BCR-279). The achieved detection limit with this setup can be seen in Pessanha et al (2009) and Gallardo et al (2016) is around 3 $\mu\text{g/g}$. The recovery values for As were 115% for the Orchard Leaves (NBS 1571) and 99% for the Sea Lettuce (BCR-279). In both SRM, the certified value was within the measured average \pm standard deviation concentration interval.

2.7. Statistical analysis

Data were statistical analysed with comparison of means with two-way ANOVA, considering differences overtime or between As treatments followed by a Tukey’s test for multiple mean comparisons using the SPSS statistical package (Version 14.0). A 95% confidence level was adopted for all tests.

3. Results

3.1. Arsenic accumulation

Among plant organs, roots showed the greatest As accumulation along the experiment, although the pattern of accumulation was similar in both plants treated with 100 $\mu\text{g As ml}^{-1}$ (100 As) and 200 $\mu\text{g As ml}^{-1}$ (200 As). From March till May the levels in the roots increase (Table 1) decreasing after that to approximately 70 $\mu\text{g g}^{-1}$ and 106 $\mu\text{g g}^{-1}$ for plants treated with 100 As and 200 As, respectively. The accumulation of As in the leaves of plants treated with 200 As, slightly decrease from March till May (14 $\mu\text{g g}^{-1}$ vs 13 $\mu\text{g g}^{-1}$, respectively) decreasing to 9.5 till the end of July. Conversely, plants treated with

100 As do not exhibit detectable As levels in March but gradually raised until $16 \mu\text{g g}^{-1}$ the end of experiment in July (Table 1).

Table 1

– Variation of the content of As in different organs of *E. nitens* plants submitted to 100 or 200 $\mu\text{g As ml}^{-1}$ expressed as $\mu\text{g g}^{-1}$; Concentration of As in the substrata where *E. nitens* grown.

March- T1		Control	100 As	200 As
	Leaf	<i>BDL</i>	<i>BDL</i>	$14.0 \pm 0.6a$
	Stem	<i>BDL</i>	<i>BDL</i>	<i>BDL</i>
	Root	<i>BDL</i>	$72.8 \pm 14.5 a,s$	$118 \pm 32.0 a,r$
	Soil	<i>BDL</i>	$17.7 \pm 6.49br$	$25.9 \pm 4.55ar$
May - T2	Leaf	<i>BDL</i>	$10.0 \pm 0.5br$	$13.3 \pm 0.4ar$
	Stem	<i>BDL</i>	$3.24 \pm 0.25^*$	$4.30 \pm 0.22^*$
	Root	<i>BDL</i>	$90.1 \pm 26.7 a,s$	$133 \pm 18.4 a,r$
	Soil	<i>BDL</i>	$34.1 \pm 6.37ar$	$41.8 \pm 17.3ar$
July - T3	Leaf	<i>BDL</i>	$15.9 \pm 1.6ar$	$9.48 \pm 2.22as$
	Stem	<i>BDL</i>	$5.04 \pm 0.17^*$	$7.25 \pm 0.36 a$
	Root	<i>BDL</i>	$69.8 \pm 14.1 a,s$	$106 \pm 5.0 a,r$
	Soil	<i>BDL</i>	$19.8 \pm 6.08br$	$31.4 \pm 6.06ar$

Different letters after the mean values \pm standard deviation (n = 3) express significant differences over time (a, b) or between As treatments in each date (r, s); (*) Concentrations determined by μ -EDXRF; *BDL* = Below the Detection Limit

The accumulation of As in the stems was only detected in May in both treatments with levels $< 5.0 \mu\text{g g}^{-1}$ As, slightly increasing till July reaching $5.04 \mu\text{g g}^{-1}$ in plants treated with 100 As, and $7.2 \mu\text{g g}^{-1}$ in plants treated with 200 As. Regarding the substrata, the accumulation of As reach a maximum of $41.8 \mu\text{g g}^{-1}$ in May with the 200 As treatment, declining in July to $31.4 \mu\text{g g}^{-1}$. Soils contaminated with 100 As also exhibited the highest levels in May ($34.1 \mu\text{g g}^{-1}$) declining to $19.8 \mu\text{g g}^{-1}$, in July.

3.2. Plant growth and foliar traits

All studied leaf traits (Fig. 1), Specific leaf area (SLA), leaf weight ratio (LWR), and leaf area ratio (LAR) showed a declining pattern along the experiment. That was significant in SLA for control plants only by July, for control and 200 As plants in LWR in May and July, and for all plants for LAR also in the latest two evaluation dates. However, there was no significant differences between As and control plants in all

evaluation dates, thus denoting an absence of As impact. Regarding photosynthetic pigments, both chlorophylls and carotenoids concentrations appears to be stimulated by the levels of As used, since 100 and 200 As treatments had always higher levels than control ones (Fig. 2)

3.3. As impact in leaf gas exchanges

The As impact was mostly absent by the first date of evaluation in P_n , g_s and E (Fig. 3). However, with the persistence of As submission in time, significant impacts were observed in May and July for the three parameters, as compared with their respective controls. The impacts were also dose related, with the greatest reductions observed with un the 200 As plants, and with 100 As counterparts showing an intermediate decline. Therefore, maximal differences to the control values were observed in the latest evaluation date (July) when 200 As showed declines of 60%, 72% and 67%, for P_n , g_s and E , respectively.

3.4. As Impact in chlorophyll a fluorescence

Although some impact tendencies were already observed in May (T2), significant As impacts were mostly observed in the plants submitted to the highest dose treatment (200 As) and in the last date for the PSII functioning/dissipation processes as compared with the control. More in detail, F_0 was not modified by As treatments within each date, but the 200 As plants showed an increased value by the last evaluation in comparison to the first one, similarly to the impact in the maximal photochemical efficiency of PSII (F_v/F_m) – Table 2.

The photochemical use of energy by the photosynthetic machinery can be assessed by the estimate of the quantum yield of non-cyclic electron transport ($Y_{(II)}$), and the photochemical quenching (q_L). Both parameters were moderately impacted by both As doses in May, but showed significant negative impacts in 100 and 200 As plants in July, without differences between As doses. These impacts were not accompanied by significant declines in the actual PSII photochemical efficiency of energy conversion (F_v'/F_m'), regardless of As dose. Still, a tendency to a lower value in the 200 As plants was observed by the last evaluation date, in accordance with a significant increase in the PSII inactivation (F_s/F_m'), and a consistent rise (non-significantly) of the chronic photoinhibition (PI_{Chr}), and total photoinhibition (PI_{Total}) indexes in these same plants.

Table 2

– Variation of leaf chlorophyll a fluorescence data along the experiment with *E. nitens* plants submitted to 0 (Control), 100 (100 As) or 200 (200 As) $\mu\text{g As mL}^{-1}$ treatment. The parameters included the minimal fluorescence, F_0 , maximal photochemical efficiency of PSII, F_v/F_m , the estimate of the quantum yield of non-cyclic electron transport, $Y_{(II)}$, the quantum yield of regulated energy dissipation of PSII, $Y_{(NPQ)}$, the quantum yield of non-regulated energy (heat and fluorescence) dissipation of PSII, $Y_{(NO)}$, the photoprotective sustained thermal dissipation, q_N , photochemical quenching based on the concept of interconnected PSII antennae, q_L , the actual PSII photochemical efficiency of energy conversion (F_v'/F_m'), the predictor of the rate constant of PSII inactivation (F_s/F_m'), as well as the dynamic photoinhibition (PI_{Dyn}), chronic photoinhibition (PI_{Chr}), and total photoinhibition (PI_{Total}).

Treatment	March (T1)		May (T2)		July (T3)	
	F_0					
Control	0.18 ±	0.00 a,r	0.18 ±	0.01 a,r	0.22 ±	0.01 a,r
100 As	0.18 ±	0.00 a,r	0.18 ±	0.00 a,r	0.22 ±	0.02 a,r
200 As	0.20 ±	0.01 b,r	0.20 ±	0.01 b,s	0.28 ±	0.02 a,r
	F_v/F_m					
Control	0.80 ±	0.02 a,r	0.82 ±	0.01 a,r	0.77 ±	0.01 a,r
100 As	0.77 ±	0.02 a,r	0.81 ±	0.00 a,r	0.77 ±	0.02 a,r
200 As	0.80 ±	0.00 a,r	0.80 ±	0.01 a,r	0.70 ±	0.02 a,s
	$Y_{(II)}$					
Control	0.26 ±	0.05 a,r	0.28 ±	0.03 a,r	0.27 ±	0.05 a,r
100 As	0.23 ±	0.05 b,r	0.8 ±	0.03 a,s	0.16 ±	0.02 ab,s
200 As	0.21 ±	0.01 a,r	0.19 ±	0.05 a,r,s	0.14 ±	0.00 a,s
	$Y_{(NPQ)}$					
Control	0.32 ±	0.06 a,r,s	0.54 ±	0.03 a,r	0.41 ±	0.08 a,s
100 As	0.24 ±	0.06 a,s	0.55 ±	0.06 a,r	0.54 ±	0.03 a,r,s
200 As	0.37 ±	0.03 b,r	0.56 ±	0.05 b,r	0.59 ±	0.04 a,r
	$Y_{(NO)}$					

For each parameter, different letters after the mean values ± standard error (n = 8) express significant differences over time (a, b) or between As treatments within each date (r, s).

Treatment	March (T1)		May (T2)		July (T3)	
Control	0.42 ±	0.07 a,r	0.17 ±	0.01 b,r	0.32 ±	0.04 a,r
100 As	0.50 ±	0.04 a,r	0.27 ±	0.04 b,r	0.30 ±	0.02 b,r
200 As	0.42 ±	0.04 a,r	0.25 ±	0.09 b,r	0.27 ±	0.03 b,r
q_N						
Control	0.64 ±	0.07 b,r	0.84 ±	0.01 a,r	0.71 ±	0.11 ab,r
100 As	0.53 ±	0.10 b,r	0.75 ±	0.06 ab,r	0.81 ±	0.04 a,r
200 As	0.71 ±	0.03 a,r	0.76 ±	0.12 a,r	0.84 ±	0.03 a,r
q_L						
Control	0.36 ±	0.06 a,r	0.34 ±	0.02 a,r	0.45 ±	0.10 a,r
100 As	0.31 ±	0.05 a,r	0.14 ±	0.06 a,s	0.29 ±	0.02 a,s
200 As	0.35 ±	0.04 a,r	0.22 ±	0.08 a,r,s	0.29 ±	0.03 a,s
F_v'/F_m'						
Control	0.49 ±	0.04 a,r	0.54 ±	0.03 a,r	0.47 ±	0.10 a,r
100 As	0.47 ±	0.06 ab,r	0.61 ±	0.03 a,r	0.41 ±	0.03 b,r
200 As	0.43 ±	0.02 ab,r	0.57 ±	0.08 a,r	0.37 ±	0.03 b,r
F_s/F_m'						
Control	0.75 ±	0.06 a,r	0.72 ±	0.03 a,r	0.73 ±	0.05 a,s
100 As	0.77 ±	0.05 a,r	0.82 ±	0.02 a,r	0.84 ±	0.01 a,r,s
200 As	0.80 ±	0.01 a,r	0.81 ±	0.03 a,r	0.86 ±	0.00 a,r
PI_{Chr}						
Control	7.12 ±	1.57 a,r	5.54 ±	1.80 a,r	7.28 ±	0.66 a,r
100 As	5.16 ±	1.76 a,r	6.69 ±	2.47 a,r	7.33 ±	1.79 a,r
200 As	6.30 ±	1.23 a,r	7.18 ±	2.30 a,r	7.62 ±	2.31 a,r

For each parameter, different letters after the mean values ± standard error (n = 8) express significant differences over time (a, b) or between As treatments within each date (r, s).

Treatment	March (T1)		May (T2)		July (T3)	
	PI_{dyn}					
Control	39.4 ±	12.1 ^{a,r}	35.3 ±	3.7 ^{a,r}	37.9 ±	12.4 ^{a,r}
100 As	35.8 ±	6.8 ^{ab,r}	24.5 ±	9.3 ^{b,r}	45.3 ±	5.9 ^{a,r}
200 As	45.5 ±	2.3 ^{a,r}	28.9 ±	11.2 ^{a,r}	41.3 ±	3.3 ^{a,r}
	PI_{Total}					
Control	40.5 ±	5.4 ^{a,r}	35.3 ±	3.7 ^{a,r}	42.6 ±	12.7 ^{a,r}
100 As	41.6 ±	7.0 ^{ab,r}	24.5 ±	4.7 ^{b,r}	49.6 ±	4.1 ^{a,r}
200 As	46.9 ±	2.1 ^{ab,r}	30.4 ±	9.3 ^{b,r}	54.4 ±	3.1 ^{a,r}
For each parameter, different letters after the mean values ± standard error (n = 8) express significant differences over time (a, b) or between As treatments within each date (r, s).						

Nevertheless, the absence of greater impacts in the PSII functioning in 200 As plants in the same date (July) was likely associated with the increase of photoprotective processes, as reflected in the rises of the estimate of regulated energy dissipation of PSII ($Y_{(NPQ)}$), and the non-photochemical quenching (q_N) (significantly only for the first), whereas non-regulated energy dissipation processes ($Y_{(NO)}$) did not show any increase, thus denoting that deleterious photo inhibitory impacts were not present (as compared with the control plants).

For most of the above studied parameters the 100 As usually displayed an intermediate value between control and 200 As plants in the last evaluation date (either closer to control or to the 200 As treatment values), thus somewhat revealing a relationship between the applied As doses and the impacts in the performance of the photosynthetic apparatus. Finally, the dynamic photoinhibition (PI_{dyn}) index showed only minor fluctuations, both along the experiment and between treatments.

3.5. Elemental accumulation

3.5.1. Calcium

At the moment T1 (March) the uptake of Ca by the roots was reduced in the presence of As - control plants contained 3.89% while both As treated plants have 2.89% (Fig. 4). A similar pattern was noted also for the stems, but not in leaves where the concentrations of Ca were not significant different. By the end of the experiment, control plants exhibited the highest Ca concentration in all the organs although the differences in the mean values had not been significant different. However, it must be emphasized that the highest As treatment (200 As) correspond to the lowest Ca concentrations in the roots and stems while maintaining a similar Ca level in the leaves compared with plants treated with half of the As concentration (Fig. 4). The highest concentrations were generally observed in the roots regardless of the

treatments, with levels in general above 3.5%, but only in leaves it was observed an increase in Ca content from March to July, irrespective of treatments.

3.5.2. Potassium

The K concentrations were higher in leaves and stems than in roots. The levels of K in the roots of all treatments decreased from March to July although the concentrations were alike in the last sampling date. In stems and leaves the concentrations of K did not show significant differences between treatments in each date, except for stems in March between control and 200 As plants, the latter one showing a decline followed by a recovery in May and July (Fig. 4). By the end of the experiment the lowest levels in the leaves were measured in control plants with 2.40% compared with 2.76% of 100 As and 2.52% of 200 As treated plants.

3.5.3. Iron

The accumulation of Fe by the different organs presents a pattern along the experiment which is not unique. Regarding the accumulation by the leaves an increase in the Fe concentrations was noted from March to July in As treated plants, while in the same period control plant declined from $111 \mu\text{g g}^{-1}$ to $93 \mu\text{g g}^{-1}$. Furthermore, the increase of Fe in $200 \mu\text{g As ml}^{-1}$, is much more pronounced than in plants treated with half of this concentration – 200 As plants have $242 \mu\text{g g}^{-1}$ Fe in July whereas 100 As plants have $149 \mu\text{g g}^{-1}$ Fe (Fig. 5).

As for the roots an increase in Fe uptake was also observed from March to July in both control and 100 As plants, while in 200 As a strong decline was verified from May to July *i.e.*, from $1233 \mu\text{g g}^{-1}$ Fe to $492 \mu\text{g g}^{-1}$ Fe. The accumulation of Fe by the stems is strongly affected by the As treatments in March, reaching a peak in May in the case of As treatments only, declining thereafter to concentrations lower than those verified at March, except the 200 As plants which increase from 202 to $224 \mu\text{g g}^{-1}$ Fe between T1 (March) and T3 (July). Despite this variability, the roots are by far the main accumulator organ (Fig. 5).

3.5.4. Zinc

The highest levels were found in the roots, while stems and leaves exhibited similar concentrations. At T1 (March) there is a clear effect of the As concentrations used on the levels of Zn in the different organs compared with control plants. In May (T2) this trend is slightly mitigated while in July (T3) the Zn levels in control plants are higher than similar values observed in As treated plant organs. For example, leaves from controls contained $38.2 \mu\text{g g}^{-1}$ Zn while plants treated with 100 As and 200 As had 25.2 and $28.3 \mu\text{g g}^{-1}$ Zn, respectively. A similar result was observed in the roots – control plants with $61.8 \mu\text{g g}^{-1}$ and 100 As and 200 As with $52.9 \mu\text{g g}^{-1}$ and $43.7 \mu\text{g g}^{-1}$ respectively. This pattern which is extensive to the stems indicate a moderate antagonistic effect of As on the uptake of Zn and later translocation the above ground organs (Fig. 5).

4. Discussion

4.1 As accumulation and plant growth

It is well known that As might affect plant growth and development and the negative effects are generally attributed to As-induced accumulation of reactive oxygen species (ROS) and a consequent lipid peroxidation and damage to cellular membranes although other major impacts include the availability of essential nutrients, photosynthesis, carbohydrate metabolism, lipid metabolism, protein metabolism, and sulfur metabolism (Zhang et al 2021).

When studying the effect of As at concentrations 0.05 and 0.2 mg/L on tomato and cabbage cultivated in different substrata - sand, sandy silt, and silt soil, Sandil et al. (2021) observed that increasing As in irrigation water did not affect the photosynthetic machinery. Arsenic concentrations of 0.05 and 0.2 mg/L did not induce phytotoxic symptoms other than reduction in biomass in some cases.

Different doses of As under the form of sodium arsenite (NaAsO_2) - 0.5, 1.0, 2.0, 4.0 mg L^{-1} were supplied to *Eucalyptus camaldulensis* for 18 months and it was observed that As concentration increases in both roots and leaves, with increasing supplying doses (Ahmad et al 2018). For example, plants submitted to 0.5 mg L^{-1} contained 14.8 and 2.7 mg Kg^{-1} As in the roots and leaves respectively, while plants treated with 4.0 mg L^{-1} presented in the same organs 37.2 and 6.6 mg Kg^{-1} .

The variability of As accumulation by eucalyptus depend mainly of the type of experiment (soil or hydroponic cultures) and selected species, beyond the time of exposure and As compound used. If the experimental assay is conducted *in vivo* the type of the soil and its characteristics and climatic conditions were of extreme importance.

Using hydroponic culture, it was possible to measure a maximum of 315 $\mu\text{g As g}^{-1}$ in the roots and 10 $\mu\text{g g}^{-1}$ As in the leaves of *E. grandis* x *E. urophylla*, after 14 days exposure to 30 mg As L^{-1} in the form of Na_3AsO_4 (Wang et al 2019), which indicates that the availability in this medium is much more favourable than when soil cultures were used. In our case, using potted plants, As concentration in roots ranged from close to 70 $\mu\text{g As g}^{-1}$, up to a maximum of 133 $\mu\text{g g}^{-1}$, the latter in the 200 As plants (in May), when a concentration of 13 $\mu\text{g g}^{-1}$ was also detected in the leaves.

The tested *Eucalyptus* species (*E. cladocalyx*, *E. melliodora*, *E. polybractea*, *E. viridis*) growing in gold mine tailings during five years growth, accumulate up to 5.1 $\mu\text{g g}^{-1}$ As in mature leaves (King et al 2008) leading the authors to conclude that the absence and/or tolerance of As effects on trees is a useful characteristics of a plant for phytostabilisation. In the same framework, the growth of 13 *Eucalyptus* clones in agricultural fields contaminated with As, Cd, Cr, Pb, Cu, and Zn (Mughini et al 2013) lead to an average value of 2.9 $\mu\text{g g}^{-1}$ As, with a maximal value of 7.8 $\mu\text{g g}^{-1}$ As. The accumulation of As, Cu, Pb, and Zn was significantly higher in leaves than in stems and branches. These findings clearly show that

extrapolation derived from studies *in vitro* must be done with great care (Reboredo, 1991; Reboredo, 1994).

The grow of *Eucalyptus camaldulensis* in strongly contaminated sites by a mine-spill in 1998 (soils contained up to 1069 mg kg⁻¹ of As and 4086 mg kg⁻¹ of Pb), along the Guadiamar River valley (SW Spain) was studied by Madejón et al (2017), concluding that despite of its tolerance to a wide variety of soil conditions and rapid growth rate, the accumulation in *E. camaldulensis* leaves is relatively low, and below toxic levels. Other studies conducted in greenhouse and field experiments also show that *E. camaldulensis* is a good candidate for phytostabilization of Cd-contaminated soils through the amendment of substrata with cow manure, pig manure, and organic fertilizer (Meeinkuirt et al 2016) which is particularly relevant when the species exhibit a rapid growth in parallel with a great biomass increment which is also the case of *E. globulus* whose response to high concentrations of As (200 µg As ml⁻¹) do not disturb negatively plant metabolism, indicating that this species is suitable for plantation in contaminated areas (Reboredo et al 2021).

4.2 As interaction with macro and micronutrients

The whole elements studied (Ca, K, Fe, and Zn) are essential to plant nutrition with the following adequate concentrations in dry tissues: 0.5%, 1.0%, 100 mg Kg⁻¹ and 20 mg Kg⁻¹, respectively (Evert et al 2013). Their role in plant metabolism is diverse. For example, Fe is taken up as Fe²⁺ and is needed for chlorophyll synthesis and enzyme activation, while Zn is taken up zinc as Zn²⁺ and is required for protein breakdown and in enzyme activation (Mahler, 2004). Calcium has a main structural role in the cell wall and membranes, it is a counter-cation for anions in the vacuole, and the cytosolic Ca²⁺ concentration is an important intracellular messenger (Thor, 2019), while K which is abundantly present in the cytosol has an essential role in enzyme activation, protein synthesis, osmoregulation, ionic balance, photosynthesis, opening and closing of stomata and also stress resistance since plant adaptive responses to several abiotic and biotic stresses are mediated by regulation of intracellular K⁺ homeostasis (Pandey and Mahiwal, 2020).

The response of *Pteris cretica* and *Spinacia oleracea* shoots to As treatments (plants were grown in pots in As(V) spiked soil (20 and 100 mg/kg) showed that the highest treatment affected growth, and Fe and Zn content in *S. oleracea* but not in *P. cretica* where an increase in the concentrations of these elements was observed, comparatively with control data (Zemanová et al 2021) which is related with the status of *P. cretica* as an As-hyperaccumulator while *S. oleracea* is an As-root excluder.

The interaction of As and Fe was studied in hydroponic cultures of barley during two weeks (Shaibur et al 2009). The chlorophyll index and the Fe concentration decreased in shoots of the As-treated plants compared with the control plants. Also, As reduced the concentration of P, K, Ca, Mg, Mn, Zn and Cu. Conversely, Yu et al. (2016) when studying the effects of iron fertilizer on two cultivars of *Ipomoea aquatica* grown in As-contaminated soils at different As concentrations, observed that the application of

the fertilizer generally enhanced the growth of the plants, which may be related to the reduction of As toxicity since a significant reduction in the uptake of As was noted.

In our case it seems to occur a continuous enrichment of the Fe content of the leaves throughout the experiment in parallel with a decrease in the roots in the plants treated with 200 As, whereas in a previous study with *Eucalyptus globulus* (Reboredo et al 2021) a decrease in the same organ was noted as a result of As treatments. This particular Fe enrichment and taking into account the role of Fe in chlorophyll biosynthesis (Mahler, 2004) is in agreement with the increase in the levels of both total chlorophyll and carotenoids in plants treated with As, as seen throughout the experiment. Although in the overwhelming majority of the cases it was reported that As inhibits the photosynthetic pigments in diverse plants ((Shaibur et al 2009; Chandrakar et al 2016; Zemanová et al 2020; Zhang et al 2021) in some cases it was noted a neutral effect of As or even a stimulus (Miteva, 2002; Sghaier et al 2015).

In fact, when studying the plant growth and pigment content of tomato (*Lycopersicon esculentum*), cultivated on soils, polluted with arsenic (As) in sublethal doses (15, 25, 50, and 100 mg kg⁻¹), it was observed that the lower As doses (15 and 25 mg kg⁻¹) stimulated the synthesis of pigments, while the higher ones (50 and 100 mg kg⁻¹) had an antagonist effect (Miteva, 2002). In the same context, Sghaier et al (2015) verified that the halophytic shrub, *Tamarix gallica*, growing during three months in the presence of different concentrations of As (0, 200, 500 and 800 µM) is a tolerant plant regarding As and the levels of total chlorophylls and carotenoids did not vary, despite the increase of As concentration. Except for the 500 µM treatment, no morphological or visible symptoms of toxicity were detected, concluding that *T. gallica* is able to cope with high concentrations of As under 500 µM. These data indicate that the variability in the response depends on the plant species, the As concentrations and the chemical species of the element, beyond characteristics of experimental conditions.

It appears that exists an antagonistic relationship between Zn and As *i.e.*, Zn in soils was found to reduce As availability as well as its accumulation by the leafy vegetable (*Ipomoea aquatica*), particularly at an elevated application rate of 3 mg/L Zn (Sanchary and Huq, 2017), which agrees with our results since the overwhelming Zn levels in the different organs are higher in control than in As treated plants.

The fern *Pteris vittata* is a hyperaccumulator of As and thus several attempts have been made to understand how the high levels of As influence the distribution of both macro and micronutrients in the plant. In this context the plant has been submitted to increasing levels of As applied to the soil (from 0 to 500 mg As kg⁻¹) during 6 months (Tu and Ma, 2005). In what concerns the interaction between As and K it was verified that the young fronds had the highest K concentrations while the old fronds the lowest ones. Also, at higher As levels, an enrichment in K in the fronds was observed to balance excessive anions caused by As hyperaccumulation (Tu and Ma, 2005) which agrees with our results since in the end of the experiment the lowest levels in the *E. nitens* leaves were measured in control plants with 2.40% compared with 2.76% of 100 µg As mL⁻¹ and 2.52% of 200 µg As mL⁻¹ treated plants.

Interestingly when a similar experiment was performed with *E. globulus* with similar concentrations (100 $\mu\text{g As mL}^{-1}$ and 200 $\mu\text{g As mL}^{-1}$), control plant leaves had, in the end of the experiment, the lowest K levels while the highest levels were observed in plants treated with 200 $\mu\text{g As mL}^{-1}$ (Reboredo et al 2021). A similar pattern was noted in the roots while in the stems the levels of K were much more alike.

4.3. As impact in the performance of the photosynthetic apparatus

The performance of the photosynthetic apparatus showed only minor, if any, impacts in the first date of evaluation, as reflected in the absence of significant impacts in gas exchange (Fig. 2), and fluorescence (Table 2). Yet a gradual impact was observed with time. In fact, a different situation was found in the intermediate evaluation point (May) after 4 months of As exposure, P_n significantly declined only under maximal exposure (200 As plants), what was in accordance with the reduction in the use of energy through photochemistry ($Y_{(II)}$, q_L). Notably, in 200 As plants such P_n reduction seemed to be closely associated with the large stomatal closure (which also led to a E decline), but not with impacts at the PSII performance, as revealed by an absence of significant changes in the PSII photochemical efficiency (F_v/F_m , F_v'/F_m'), PSII inactivation (F_s/F_m'), photoprotective thermal dissipation ($Y_{(NPQ)}$, q_N) or in the photoinhibition indexes.

In fact, until May the F_v/F_m values were similar regardless the treatments with values around 0.8 which agrees with Björkman and Demmig (1987) which indicate that the F_v/F_m ratio is nearly constant in unstressed leaves. Nevertheless, in July a reduction of 9.1% in the F_v/F_m value was noted in plants treated with 200 As compared with control and 100 As which can be indicative of a smooth stress. Zemanová et al. (2020) observed a decrease of F_v/F_m values in both young and old fronds of the As hyperaccumulator *Pteris cretica* L., when treated with 100 mg As per kg soil and 250 mg As per kg soil - with 100 As the decrease ranges between 12.5% and 14.3%, for young and old fronds, respectively, climbing to 29% in old fronds treated with 250 As, thus indicating a faster progression of senescence in the latter case.

The same situation of 200 As plants in May seemed to be observed in the 100 As plants by the last evaluation date (in July, with 6 months of As exposure), since with a few exceptions (P_n , $Y_{(II)}$, q_L) these plants showed values of gas exchanges and fluorescence parameters without significant differences to the control plants. However, the 200 As plants showed additional impacts by the end of the experiment, similarly to reports in *E. globulus* submitted to the same As treatments (Reboredo et al 2021). In fact, the 200 As plants of *E. nitens* showed the maximal P_n reduction of the entire experiment, together with a reduction of the photochemical use of energy ($Y_{(II)}$, q_L) but also with significant impacts in the PSII, with loss of photochemical efficiency (F_v/F_m), and a greater inactivation status (F_s/F_m'). Still, As did not cause increases in the photoinhibitory indexes (PI_{DYN} , PI_{CHR} , PI_{TOTAL}), and in $Y_{(NO)}$.

This latter parameter is usually stable even under environmental stressful conditions (Busch et al 2009), but its increase reflects non-photochemical quenching attributable to photoinactivation and non-regulated energy (heat and fluorescence) dissipation in PSII (Busch et al 2009; Kramer et al 2004; Huang et al 2011). In this way, the maintenance of $Y_{(NO)}$ even in the 200 As plants after 6 months of As exposure, suggests that, despite the reduction of photochemical energy conversion ($Y_{(II)}$), the non-photochemical energy dissipation in PSII, related to the protective down-regulation of light-harvesting function ($Y_{(NPQ)}$) (Huang et al 2011), was capable to minimize the PSII impacts, in contrast with *Pisum sativum* Pb-treated plants, where photoprotective mechanisms (evaluated through NPQ) were suppressed (Kycko et al 2019). Also, q_N and NPQ were found to increase in barley seedlings exposed to Cu (80 μ M) during six days, but decreased under a Fe (1.5 mM) treatment, thus indicating different action mechanisms/impacts (Lysenko et al 2020). Therefore, our findings denoted a capability of the photosynthetic machinery of *E. nitens* to respond and cope with high levels of As to a certain extent.

Furthermore, since *E. nitens* is high tolerance to frost as recognized by Gomes and Canhoto (2003), this plant can be used as an alternative in phytoremediation processes in some mining abandoned areas of Northern Portugal where winter temperatures are too low and impeditive of an efficient uptake and/or translocation mechanism of major heavy metals or metalloids present in the substrata.

5. Conclusions

Roots are unquestionably the main accumulator organs reaching a peak in both treatments in May, with 90.1 μ g g⁻¹ and 133 μ g g⁻¹ for plants treated with 100 As and 200 As, respectively, declining thereafter. The translocation to the leaves reaches a maximum of 15.9 and 14.0 μ g g⁻¹ for plants treated with 100 As and 200 As, respectively, showing that in the current experimental conditions *E. nitens* was tolerant and efficient in the extraction of As from the matrix. The performance of the photosynthetic apparatus showed a gradual impact with time in some gas exchange parameters such as net photosynthetic rate (P_n), stomatal conductance to H₂O (g_s) and the transpiration rate (E), particularly with the use of 200 As, but without impacting with significant changes the PSII performance, *i.e.*, the PSII photochemical efficiency, the PSII inactivation or the photoprotective thermal dissipation. The highest levels of As seem to stimulate the accumulation of Fe in the leaves which is in agreement with the increase of total chlorophyll in both As treated plants, comparatively with control plants. Regarding the effects of As on Zn levels it seems to exist a moderate antagonistic effect on the uptake of Zn and later translocation to the above ground organs which is more pronounced with the highest As treatment. In general, this species is able to cope with As contaminated substrata surrounding mining areas, although the As extraction by the root system would be expected to be slower than that monitored in soil pots.

Declarations

Human Ethics

“Not applicable”

Consent for publication

All authors agreed with the content and gave explicit consent to submit the manuscript.

Availability of supporting data

All relevant data are included in the manuscript.

Competing interests

The authors have no financial or non-financial interests to disclose.

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Authors' contributions

Conceptualization, F.H.R. and F.C.L.; formal analysis, F.H.R., F.C.L., J.P., M.G., and J.C.R.; funding acquisition, F.H.R. and F.C.L.; investigation, J.C.R., F.H.R., F.C.L.; and J.P.; methodology, F.H.R., F.C.L., J.C.R., and M.G.; project administration, F.H.R. and F.C.L.; supervision, F.H.R., F.C.L., and J.C.R.; writing—original draft, F.H.R. and J.C.R.; writing—review and editing, F.H.R., F.C.L., J.C.R., M.M.A.S and M.M.S. All authors have read and agreed to the published version of the manuscript.

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Figures

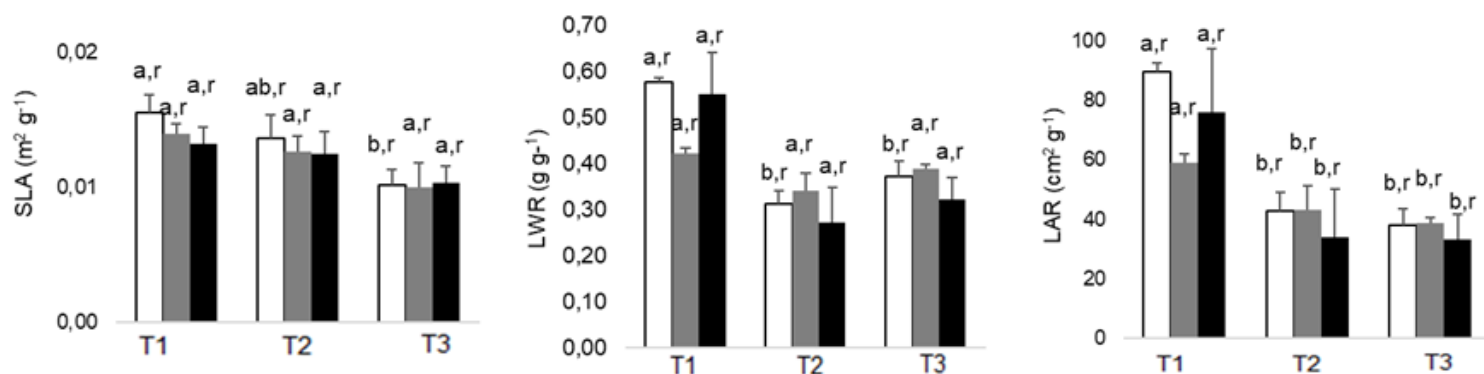


Figure 1

Variation in specific leaf area (SLA) leaf weight ratio (LWR) and leaf area ratio (LAR), in *Eucalyptus nitens*, throughout the experimental assay (T1-March; T2-May; T3-July). The mean values \pm standard error (n = 4) followed by different letters express significant differences over time (a, b) or between As treatments (r, s), for the control (white), 100 As (grey), and 200 As (noir) treatments.

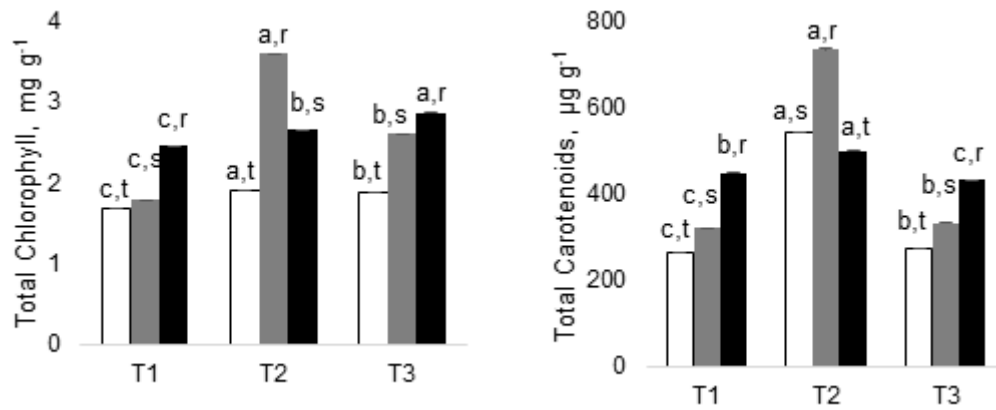


Figure 2

Variation in Total Chlorophyll and Total Carotenoids in *Eucalyptus nitens*, throughout the experimental assay (T1-March; T2-May; T3-July). The mean values \pm standard error ($n = 4$) followed by different letters express significant differences over time (a, b) or between As treatments (r, s), for the control (white), 100 As (grey), and 200 As (noir) treatments.

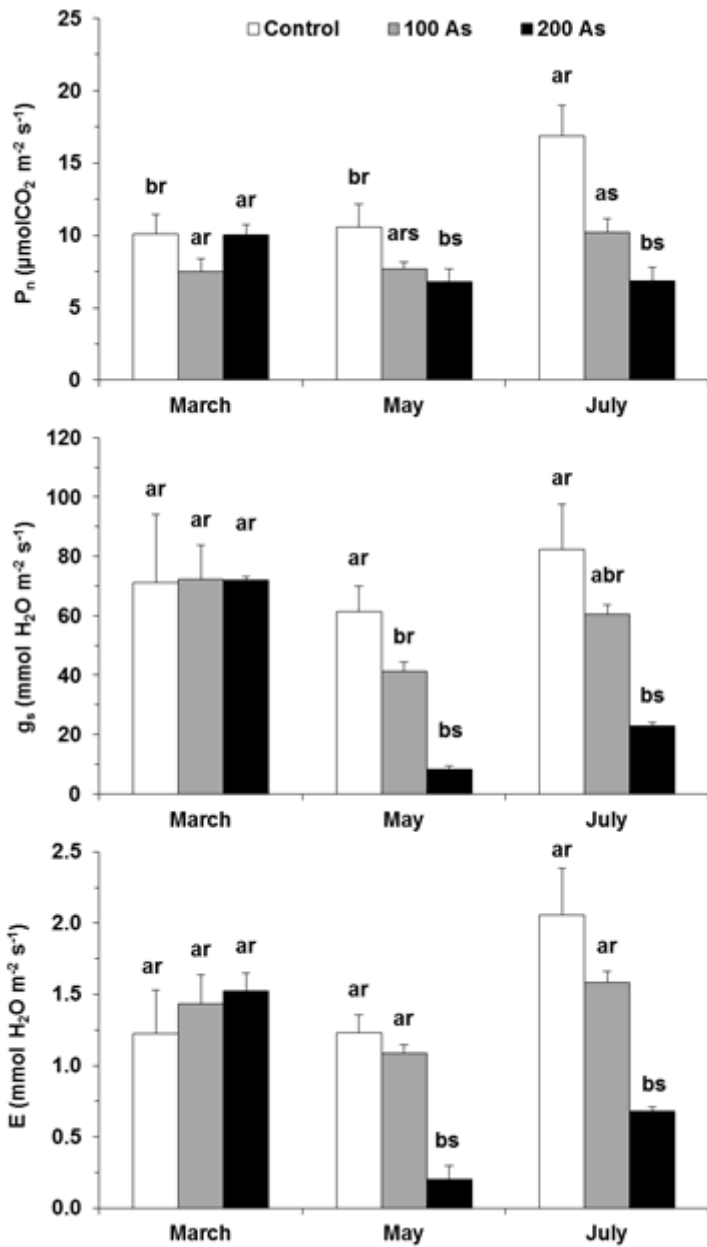


Figure 3

Variation of leaf gas exchanges parameters along the experiment in *E. nitens* plants submitted to 0 (Control - white), 100 (100 As - grey) or 200 (200 As - noir) $\mu\text{g As mL}^{-1}$ treatment. The parameters included net photosynthetic rate (P_n), stomatal conductance to H_2O (g_s) and the transpiration rate (E). For each parameter, different letters after the mean values \pm standard error ($n = 4-6$) express significant differences over time (a, b) or between As treatments within each date (r, s).

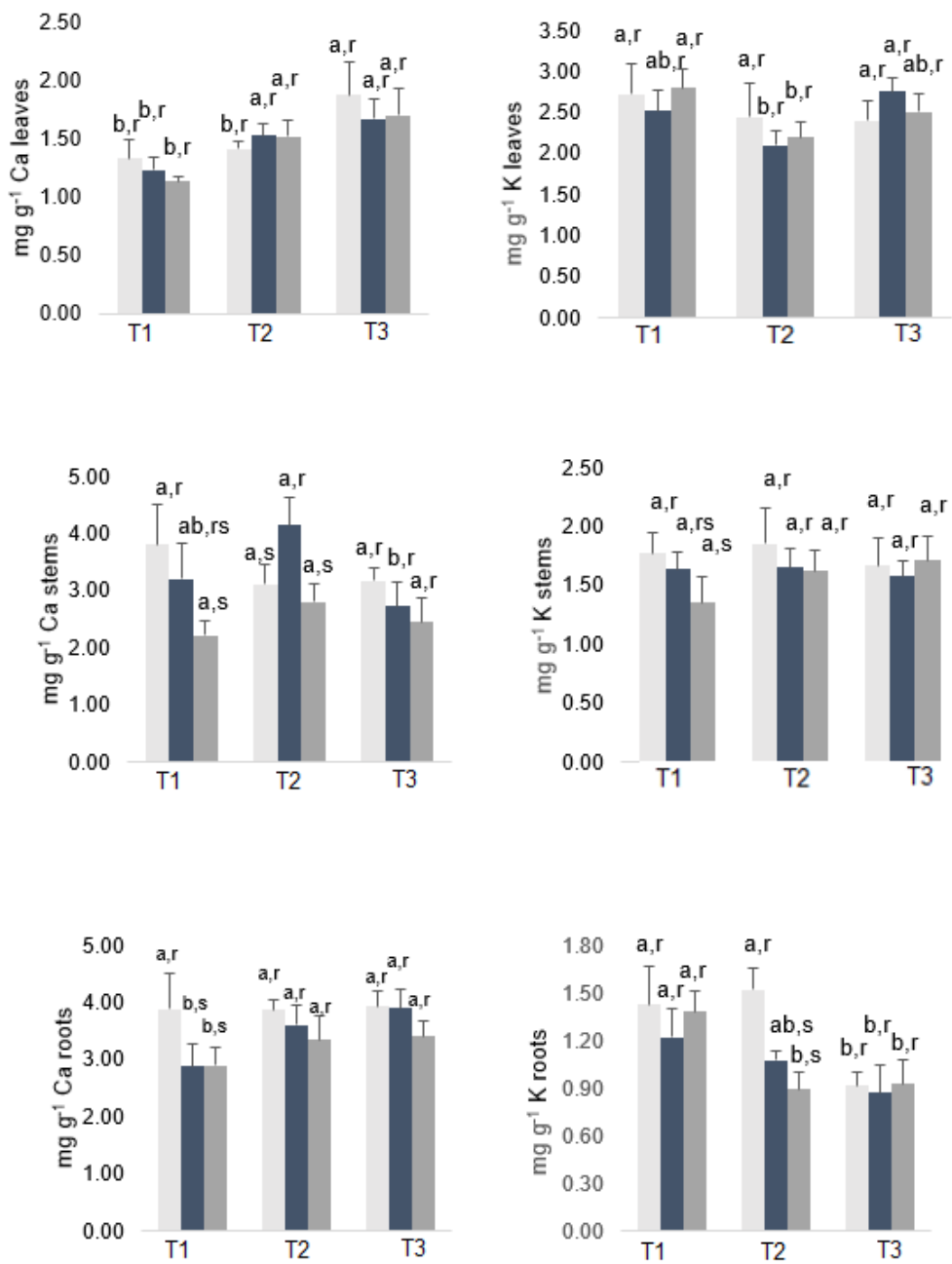


Figure 4 - Average values of Ca (left) and K (right) in the leaves, stems and roots of *E. nitens* As treated-plants, throughout the experimental assay. Mean values are expressed as mg g⁻¹ ± standard deviation. Different letters indicate significant differences at the 0.05 significance level. T1 = March; T2 = May; T3 = July: ■ 0 µg As (Control), ■ 100 µg As ml⁻¹, ■ 200 µg As ml⁻¹.

Figure 4

See image above for figure legend.

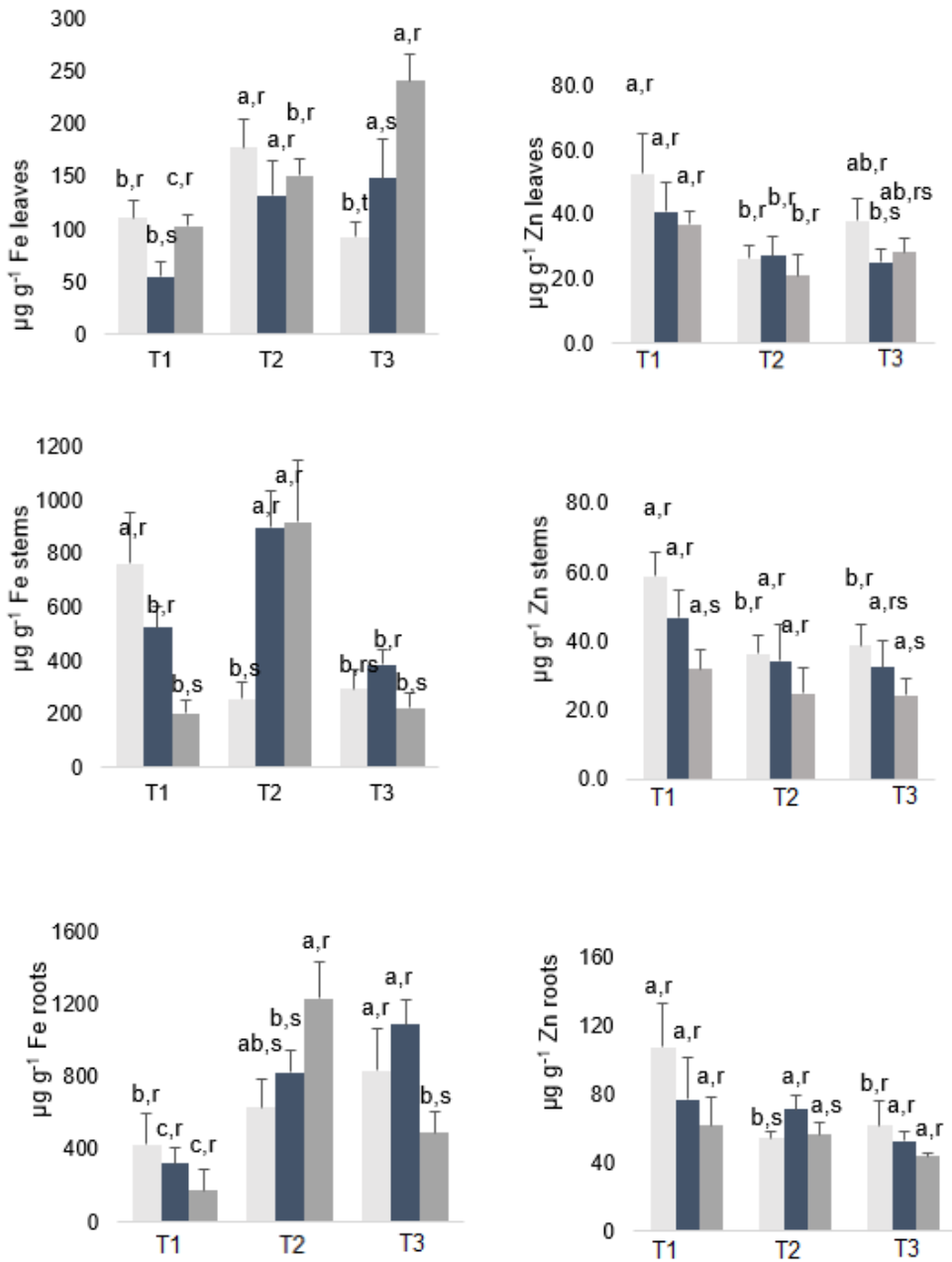


Figure 5 - Average values of Fe (left) and Zn (right) in the leaves, stems and roots of *E. nitens* As treated-plants, throughout the experimental assay. Mean values are expressed as $\mu\text{g g}^{-1} \pm$ standard deviation. Different letters indicate significant differences at the 0.05 significance level. T1 = March; T2 = May; T3 = July; \square 0 $\mu\text{g As ml}^{-1}$ (Control), \blacksquare 100 $\mu\text{g As ml}^{-1}$, \blacksquare 200 $\mu\text{g As ml}^{-1}$.

Figure 5

See image above for figure legend.