

Metal uptake of *Nerium oleander* from aerial and underground organs and its use as a biomonitoring tool for airborne metallic pollution in cities

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Abstract The analysis of the airborne particulate matter—PM—incorporated to plant leaves may be informative of the air pollution in the surroundings, allowing their use as biomonitoring tools. Regarding metals, their accumulation in leaves can be the result of both atmospheric incorporation of metallic PM on aboveground plant organs and root uptake of soluble metals. In this study, the use of *Nerium oleander* leaves as a biomonitoring tool for metallic airborne pollution has been assessed. The metal uptake in *N. oleander* was assessed as follows: (a) for radicular uptake by irrigation with airborne metals as Pb, Cd, Cr, Ni, As, Ce and Zn (alone and in mixture) and (b) for direct leaf exposure to urban PM. Plants showed a high resistance against the toxicity of metals under both single and multiple metal exposures. Except for Zn, the low values of translocation and bioaccumulation factors confirmed the excluder behaviour of *N. oleander* with respect to the metals provided by the irrigation. For metal uptake from airborne pollution, young plants grown under controlled

conditions were deployed during 42 days in locations of the city of Zaragoza (700,000 h, NE Spain), differing in their level of traffic density. Samples of PM_{2.5} particles and the leaves of *N. oleander* were simultaneously collected weekly. High correlations in Pb concentrations were found between leaves and PM_{2.5}; in a lesser extent, correlations were also found for Fe, Zn and Ti. Scanning electron microscopy showed the capture of airborne pollution particles in the large and abundant substomatal chambers of *N. oleander* leaves. Altogether, results indicate that *N. Oleander*, as a metal resistant plant by metal exclusion, is a suitable candidate as a biomonitoring tool for airborne metal pollution in urban areas.

Keywords *Nerium oleander* · Immission · Traffic pollution · Inhalable particles · PM_{2.5} · Metals · Biomonitoring

Introduction

Urban air pollution is a serious concern for human health; as example, airborne particulate matter is related to the increase of respiratory and cardiac diseases (Künzli et al. 2000; Pope et al. 1995; Seaton et al. 1995). The breathable fraction of that particulate matter presenting diameters under 2.5 μm (PM_{2.5}) is especially worrying because it is more strongly associated than inhalable fraction (PM₁₀) with acute respiratory health effects, as it has been reported in children (Schwartz and Neas 2000). Metals are one of the main components of PM_{2.5} (USEPA 1996). Moreover, it has been described that certain toxic metals such as Pb, Cd, Ni and Cu are better associated with PM_{2.5} than PM₁₀ fraction of the airborne particulate matter (Samara and Voutsas 2005).

Although we have spatial and quantitative detailed information of metal emission in different cities (Hjortenkrans and

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Bergbäck 2005; Kakareka et al. 2004), there is still a lack of immission data for these pollutants. The need of a large amount of sampling stations, whose operations are quite expensive, is an insuperable drawback for implementing the monitoring programs at high spatial resolution that prevent an adequate environmental risk assessment for metal exposure in urban environments. An alternative method that may help in tackling this obstacle could be the use of widespread organisms, as biomonitoring tools.

Large number of higher plants, such as *Ligustrum japonicum*, *Olea europea*, *Pinus brutia*, *Platanus orientalis*, *Populus alba*, *Populus nigra*, *Robinia pseudoacacia*, *Capsella bursa-pastoris*, *Tillandsia capillaries* and *Phoenix dactylifera*, have been used as biomonitoring tools in urban environments (Aksoy et al. 1999; Al-Alawi and Mandiwana 2007; Al-Shayeb et al. 1995; Çelik et al. 2005; Pignata et al. 2002; Sawidis et al. 1995). These studies showed that particulate matter adheres to leaves and may be absorbed, being thus used to assess the air pollution in the plant surroundings.

Nerium oleander is evergreen xerophyte species widespread in Zaragoza which has been proposed as a biomonitor of heavy metals in Mediterranean areas by several authors (Aksoy and Ozturk 1997; Dongarra et al. 2003; Fernández Espinosa and Oliva 2006; Mingorance and Oliva 2006; Mingorance et al. 2007; Rossini Oliva and Mingorance 2006). In this way, significant correlations between metal concentrations in surface soil and leaf samples have been obtained (Aksoy and Ozturk 1997), as well as between *N. oleander* leaves and PM10 particles (Fernández Espinosa and Oliva 2006). In addition, *N. oleander* is able to survive in extremely heavy metal polluted soils due to its strong excluder behaviour, as it has been reported (Franco et al. 2012, 2013).

In biomonitoring studies of metal pollution, it is difficult to distinguish between atmospheric and soil contribution. For this reason, both sources of metals should be considered. By means of washing *N. oleander* leaves, a maximum contribution of the airborne pollution of 56, 46, 37 and 39 % for Pb, Cd, Zn and Cu, respectively, has been reported by Aksoy and Ozturk (1997). There are only a few studies of *N. oleander* plants grown in metal polluted soils under controlled conditions. These studies showed synergistic uptake of Cu/Zn and Pb/Cd in *N. oleander*, being Pb and Cd mainly accumulated in roots (Drakatos et al. 2000; Kadukova et al. 2006; Kalavrouziotis and Drakatos 2002). Anyway, important aspects as the metal uptake and translocation ratios or the simultaneous presence of different metals in the soil have not been yet taken into account.

Thus, the aim of this study is to assess the use of *N. oleander* as a biomonitor of the immission of the airborne metal pollution related to inhalable particles (PM2.5) in the urban area of Zaragoza (700,000 h, NE Spain). To achieve this goal, two experimental approaches were designed: (a) greenhouse pot experiments in which *N. oleander* plants were

treated with metals related to urban pollution (Pb, Cd, Cr, Ni, As, Ce and Zn), with the objective of both to assess the ability of resistance and to characterize the metal uptake behaviour of *N. Oleander*; and (b) a screening experiment in which samples of PM2.5 particles and samples of *N. oleander* leaves were collected simultaneously in seven locations within the urban area of Zaragoza city in order to study their correlation. This work was complemented with low temperature-scanning electron microscopy and energy dispersive X-ray analyses (LT-SEM-EDX) of *N. oleander* leaves from both experiments, in order to study the differences in the ultrastructure of leaf tissues and in the micro-localization of metals.

Materials and methods

Greenhouse pot experiment

Eight month-aged plants of *N. Oleander* L. cv. “Rosa Bartolini” in a height range of 15–20 cm were selected for this experiment. Plant material was provided by Garden Center Aznar (Zaragoza, Spain). Plants were transferred into 20 L plastic pots filled with organic substrate (Universal Substrate Blumenerde, Capriflor®) and silica sand in a 3:1 ratio as growth substrate. Plants were irrigated with tap water (pH 7.0; 644 $\mu\text{S}/\text{cm}$ at 25 °C; trace elements were under detection limits) up to water holding capacity twice per week during 20 days as acclimatization period and kept into a greenhouse (20 \pm 2 °C; 40–60 % humidity). A randomized block design was carried out as follows: eight treatments (Pb, Cd, Cr, Ni, As, Ce, Zn and mixture) \times 3 doses (0, 10 and 100 μM) \times 3 replicates (pots) plus 1 control \times 4 replicates (each experimental unit consisted of one pot with one plant).

Metal solutions were prepared using CdCl₂.2.5H₂O; CrCl₃.6H₂O; NiCl₂.6H₂O; Ce(SO₄)₂.4H₂O; Na₂AsO₄.7H₂O; PbCl₂; and ZnSO₄.H₂O (all PA-ACS grade from PANREAC®, Barcelona, Spain) dissolved in deionized water. Separate solutions were prepared for Pb, Cd Cr, Ni, As and Zn treatments and all metals together for mixture treatment. Metal solutions for treatments and tap water for control were used to irrigate plants up to water holding capacity twice per week during 42 days. After irrigation, pH and EC were measured in the substrate of treated and control pots (pH 5.34–6.09; EC 195–409 $\mu\text{S}/\text{cm}$ at 25 °C). Heights of plants were measured every 7 days using a measuring tape. After 42 days of treatment, substrate and plant samples were collected.

Screening of airborne metals using air sampler and *N. oleander* leaves

Samples of PM2.5 particles and samples of *N. oleander* leaves were collected simultaneously in seven different sites (COR, STA, JES, NAV, TFL, MAD and DEL, details in Fig. 1).

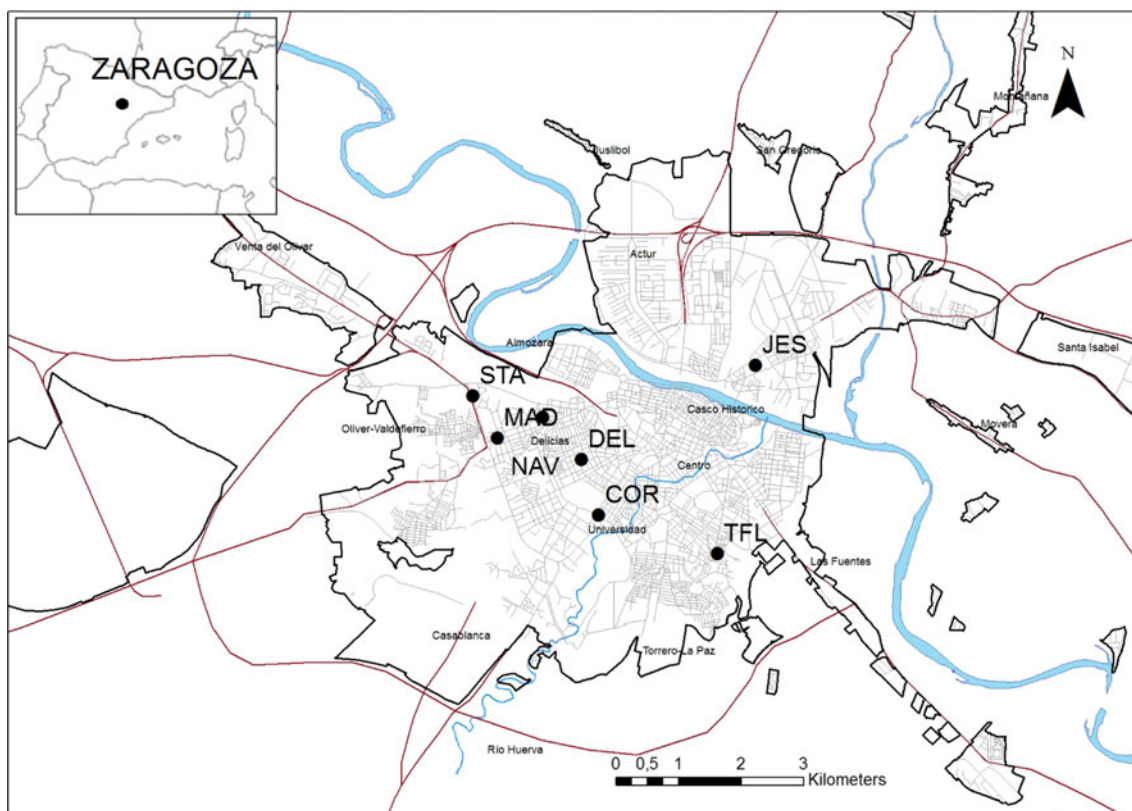


Fig. 1 Sampling sites were *N. oleander* and PM_{2.5} samplers were located. The high-capacity autoroutes surrounding the city are indicated as red lines

These locations were selected because of their differences in the road traffic intensity as follows: 7552, 13,101, 306, 8107, 20,859, 20,646 and 14,734 vehicles per day (2010 yearly data provided by the City Council of Zaragoza). The sampling period ranged from September 2011 to August 2012. At each sampling point, four plants were placed, together with an air sampler MicroVol 1100 (Ecotech ©, Knoxfield, Australia), equipped with a PM_{2.5} inlet (collecting particles under 2.5 μm Ø). The sampler worked in continuous with a flow rate of 3 l/min. Fiberquartz filters of 47-mm diameter and 0.45- μm pore size were used as collection media. Soils of pots were covered with plastic to avoid the deposition of atmospheric pollution.

Samples of airborne particles and leaves of *N. oleander* were collected every 7 days during 42–47 days, depending on the location. Accordingly, between 6 and 7 samplings were done at every location, each one consisting in one filter containing PM_{2.5} and plant leaves. Healthy leaves (2–4) from a younger and completely developed whorl (with size similar to the older ones) were selected and cut off. Samples were dried 48 h at 60 °C and then ground to a fine powder and analyzed for metals. Concentrations of metals in both leaves and filters from each sampling were normalized per day of exposure to urban pollution and per dry weight, in order to plot and statistic calculations. Therefore, metal results were expressed as nmol g^{-1} dry weight day^{-1} for leaves and as $\text{nmol g}^{-1} \text{m}^{-3}$ air day^{-1} for the PM_{2.5}.

Analytical determinations

Substrate samples were air-dried and sieved at the minor than 2 mm prior to the analyses. Quantitative paper-filtered (Filter-Lab, S A) 1:2.5 and 1:5 substrate/water extracts made by mixing air-dried soil and deionised water, shaking at 180 rpm for 1 h and waiting for 30 min prior to using a digital pH meter and a conductivity meter (WTW Multiline P4 and Hach-Lange HQ) were performed respectively (Cabezas et al. 2008). The EDTA fraction was extracted from 10 g of substrate with 100 mL of 0.1 M ethylene-diamine tetra-acetic acid (EDTA) shaken in plastic flasks at 180 rpm for 4 h at room temperature (Chojnacka et al. 2005). For the total concentration of metals (pseudo-total), 100 mg of substrate and 5 mL mixture of hydrochloric-nitric acid (4:1) were digested in an open-vessel microwave digestion system (Ultraclave Microwave Milestone) for 20 min at 220 °C (Mingorance and Oliva 2006).

Plant material was thoroughly washed with tap water and was given a final rinse with deionised water. Leaves, stems and roots were dried at 60 °C for 48 h to determine dry weight. Dried matter was ground and digested as described above (Mingorance and Oliva 2006). Whole filters were also digested by the same protocol (Mingorance and Oliva 2006). Filtered EDTA extracts and acid digests were analysed for metal concentrations by simultaneous inductively coupled plasma (ICP/OES, Mod. ICAP 6500 DUO

THERMO©). Calibration curves were performed using standard stock solution 1.0 mg/L diluted in 10 % nitric acid. Recovery for metals ranged from 80 % (Cr, Pb) to 92 % (Zn). The detection limit for all metals was established at 0.5 mg Kg⁻¹ dry weight.

Low temperature-scanning electron microscopy and microanalysis

The *N. oleander* leaf pieces were mounted on aluminium stubs with adhesive (Gurr®, optimum cutting temperature control; BDH, Poole, UK), cryo-fixed in slush nitrogen (−196 °C), cryo-transferred to a vacuum chamber at −180 °C and then fractured using a stainless steel spike. Once inside the microscope, the samples underwent superficial etching under vacuum (−90 °C, 120 s, 2 kV) and then overlaid with Au for observation and microanalysis with a Zeiss digital scanning microscope (DSM 960). Fractured samples were observed at low temperature with a digital scanning electron microscope (Zeiss©, model DSM 960, Oberkochen, Germany) using secondary and back-scattered electrons. Secondary electron images (1024 × 960 pixels) were obtained at 133 eV operating at a 35° take-off angle, an accelerating voltage of 15 kV, a working distance of 25 mm and a specimen current of 1–5 nA.

Microanalysis was carried out in specific leaf areas (approximately 15 × 15 μm) using an energy dispersive X-ray microanalysis (EDX) Pentafllet apparatus (Pentafllet©, Oxford, UK). Areas tested were adaxial epidermis, palisade parenchyma, xylem vessels, spongy parenchyma and abaxial epidermis. Whenever possible, a single cell area was targeted. Semiquantitative element analysis was obtained using standard ZAF (atomic number, absorption and fluorescence) correction procedures, using Link Isis v3.2 Software (Link Isis©, Oxford, UK). Eight points of analysis per leaf tissue and three leaves per treatment were analyzed. Due to the limitations of the technique (LT-SEM-EDX) and according to the purpose to compare airborne vs root via, one representative sample (three leaves) from NAV location was selected as airborne polluted leaves. The thickness of the different leaf layers (adaxial epidermis, palisade parenchyma, spongy parenchyma and abaxial epidermis) was measured manually with image analysis software (Photoshop CS3; Adobe, San Jose, CA, USA) using images similar to those shown in Fig. 8 (total, 18 images = 3 images × 3 leaves × 2 treatments—airborne polluted and 100 μM mixture).

Statistical analyses

Values in the tables indicate mean values ± standard error (SE). The obtained data have normal distribution; therefore, differences among treatments were analysed by one-way analysis of variance (we focused on increasing the

power of the analysis to detect differences between metals in a clearer way), followed by a post hoc multiple comparisons of means using the Duncan test ($P < 0.05$). Simple linear regressions between the concentration of each metal in leaves vs PM2.5 particles, plant organs (shoot and root) vs substrate (total and EDTA extractable) were calculated. The Pearson coefficients (R) were used to assess how well the regression line represents the data. Statistical analyses were performed using SPSS PC 10.0 package software.

Results

Impact of metals on plant growth

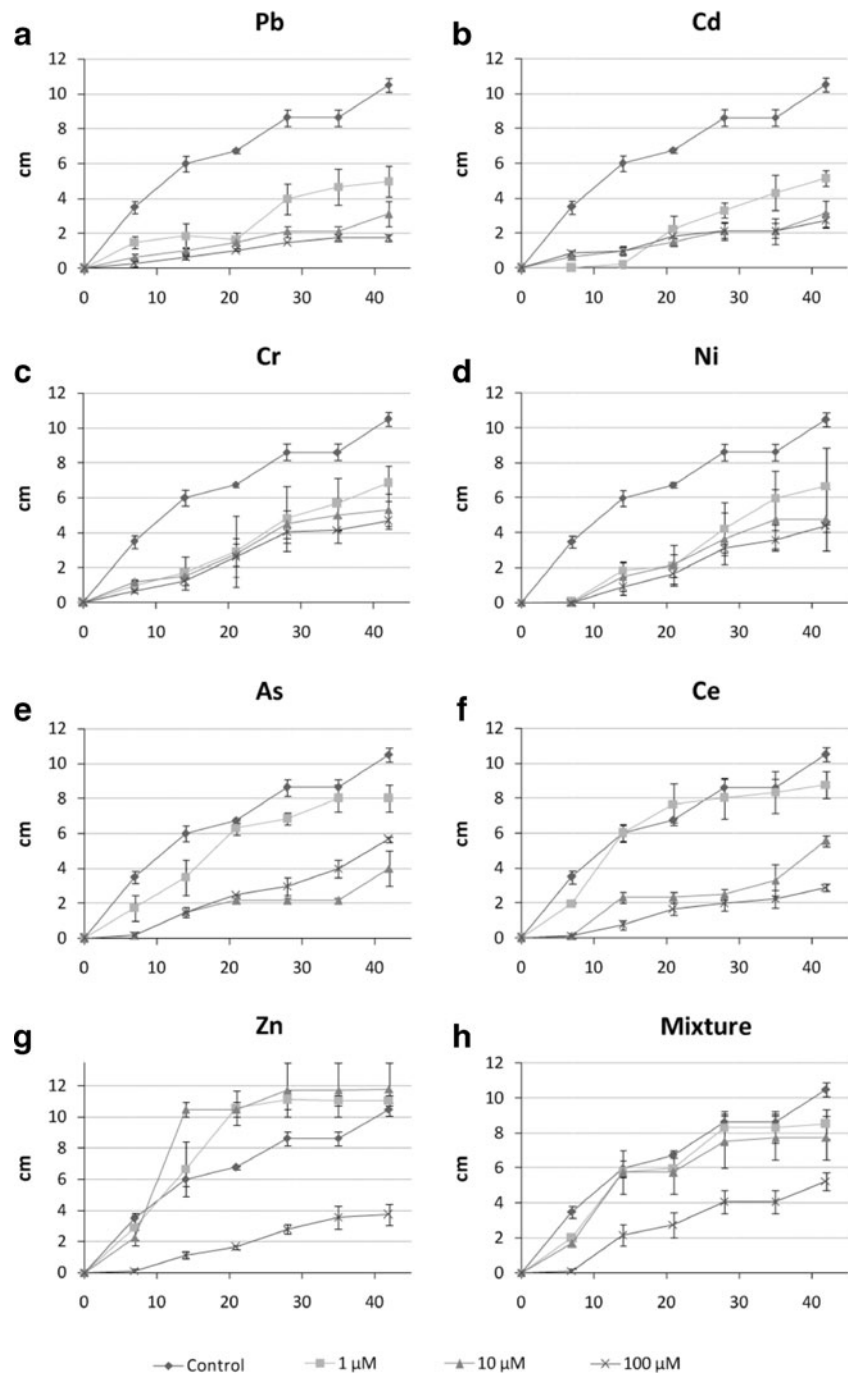
Exposure to metals inhibited plant growth impacting both heights and weights. Figure 2 showed the height of *N. oleander* shoots after 7, 14, 21, 28, 35 and 42 days of treatment. In general, higher decreases in height of treated plants in comparison to the control were observed with increasing metal dose and exposure time. Among metals, Pb, Cd, Cr and Ni showed a notable reduction of the weights even at 1 μM concentration (Fig. 2a–d). Significant decreases in heights in comparison to the control were only observed at 10 and 100 μM for As and Ce (Fig. 2e, f). Likewise, only the 100 μM concentration of Zn and mixture treatments reduced significantly the height of *N. oleander* plants (Fig. 2g, h). In the case of Zn, a higher weight in 1 and 10 μM treated plants than control was obtained (Fig. 2g).

The percentage of reduction in weight of treated plants in comparison to control at the end of the pot experiment (42 days) was shown in Fig. 3. A reduction in the biomass of treated plants was observed in all cases but 1 μM As and 1 μM Ce. A higher reduction of the biomass with increasing doses was observed though only significantly for the Cd, As, Zn and mixture treatment. For the 100 μM dose, the highest percentage of weight reduction was observed as followed: Cd (58.6 %), Pb (44.9 %), Cr (40.7 %), As (37.2 %), Zn (33 %), Ni (30.2 %), Ce (29.5 %) and mixture (28.8 %).

Metals in the growth medium

The total concentration of metals in the growth substrate was higher with increasing doses (Fig. 4). The highest metal concentrations in the substrate were observed for 100 μM treatments. Regarding the presence of other pollutants in the medium, lower substrate concentrations in the mixture treatment than those supplied alone were obtained for Pb, Cd and Zn at 10 and 100 μM (Fig. 4a, b and g) and for Cr and Ni at 10 μM (Fig. 4c, d). However, higher substrate concentrations for As and Ce in the mixture than in the alone supply at all doses were observed

Fig. 2 Height (cm) of *N. oleander* shoots at 7, 14, 21, 28, 35 and 42 days under control (diamond) and Pb (a), Cd (b), Cr (c), Ni (d), As (e), Ce (f), Zn (g) and mixture (h) treatments at 1 (square), 10 (triangle) and 100 μ M (cross) doses. Error bars indicate the standard error of three replicates



(Fig. 4e, f). Thus, the maximum levels of total substrate concentration (nmol g^{-1}) were as follows: Pb (587), Cd (499), Ni (461), Cr (392), Zn (352), As (229), and Ce (183). These values were notably lower than those established as the threshold for non-contaminated soils in the region (BOA 2008), in all cases except for As (still lower but close to the limit value) and Cd (four- to -fivefolds higher than the limit value). The average percentage of EDTA-extractable metal with respect to the

total concentration in substrate was shown in Fig. 4h. The highest percentage of EDTA-extractable metal was observed for Cd (84 %) followed by As, Ni, Pb (55–57 %), Ce (27 %), Zn (14 %) and Cr (9 %).

Uptake and translocation of metals

Metal concentrations in root and shoot of *N. oleander* were shown in Fig. 5. Plant concentrations of Ce were

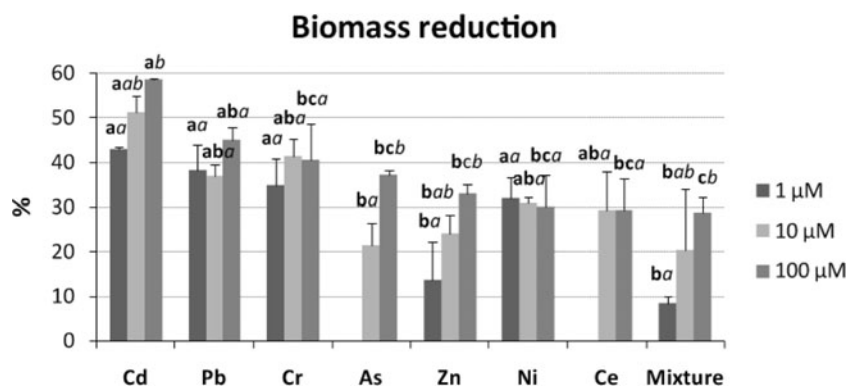


Fig. 3 Percentage of biomass reduction of *N. oleander* shoots (g f.wt.) after 42-day-treatments with Cd, Pb, Cr, As, Zn, Ni, Ce and mixture treatments vs. control at 1 (dark grey), 10 (light grey) and 100 μM (medium grey) doses. Data are expressed as mean ± SE. Different *non-italic*

letters indicate significant differences among treatments ($P < 0.05$; $n = 3$). Different *italic* letters in the same treatment indicate significant differences among doses ($P < 0.05$; $n = 3$)

under the limit of detection ($<0.5 \text{ mg Kg}^{-1}$). Root and shoot concentrations were higher with increasing doses for all pollutants but Zn. In all cases except for root Zn concentrations at 1 and 10 μM, lower plant concentrations in the mixture treatment than those in the corresponding separated ones were observed. Thus, the maximum concentration ($\text{nmol g}^{-1} \text{ d.wt.}$) of metals in plants under one-metal treatments (alone) at 100 μM doses were the following: As (1539), Zn (683), Cr-Cd-Ni (85–107), Pb (29) and Ce (n.d.) for root and Zn (683), As (163), Cr, Cd, Ni (19–20), Pb (12) and Ce (n.d.) for shoot (Fig. 5). High positive correlation coefficients between substrate and plant concentrations were obtained in all cases but Zn (Table 1). Table 2 showed the translocation factor (TF) and the bioaccumulation factors (Total-BF and EDTA-BF) for *N. oleander* plants defined as follows:

$$TF = \frac{\text{Shoot concentration}(\text{nmolg}^{-1}\text{d.wt.})}{\text{Root concentration}(\text{nmolg}^{-1}\text{d.wt.})}$$

$$\text{Total-BF} = \frac{\text{Shoot concentration}(\text{nmolg}^{-1}\text{d.wt.})}{\text{Substrate total concentration}(\text{nmolg}^{-1}\text{d.wt.})}$$

$$\text{EDTA-BF} = \frac{\text{Shoot concentration}(\text{nmolg}^{-1}\text{d.wt.})}{\text{Substrate EDTA concentration}(\text{nmolg}^{-1})}$$

The *N. oleander* plants showed low translocation factors ($TF < 1$) for all pollutants but Zn. Regarding BF_s, levels ranged between 0.01 and 0.09 for Pb, Cd and Ni in all cases, 10-fold higher for As and EDTA-Cr and between 2 and 31 in the case of Zn (Table 2). For Cr, As and Zn, higher values in EDTA-BF than in Total-BF were found.

Airborne metal pollution accumulated in leaves

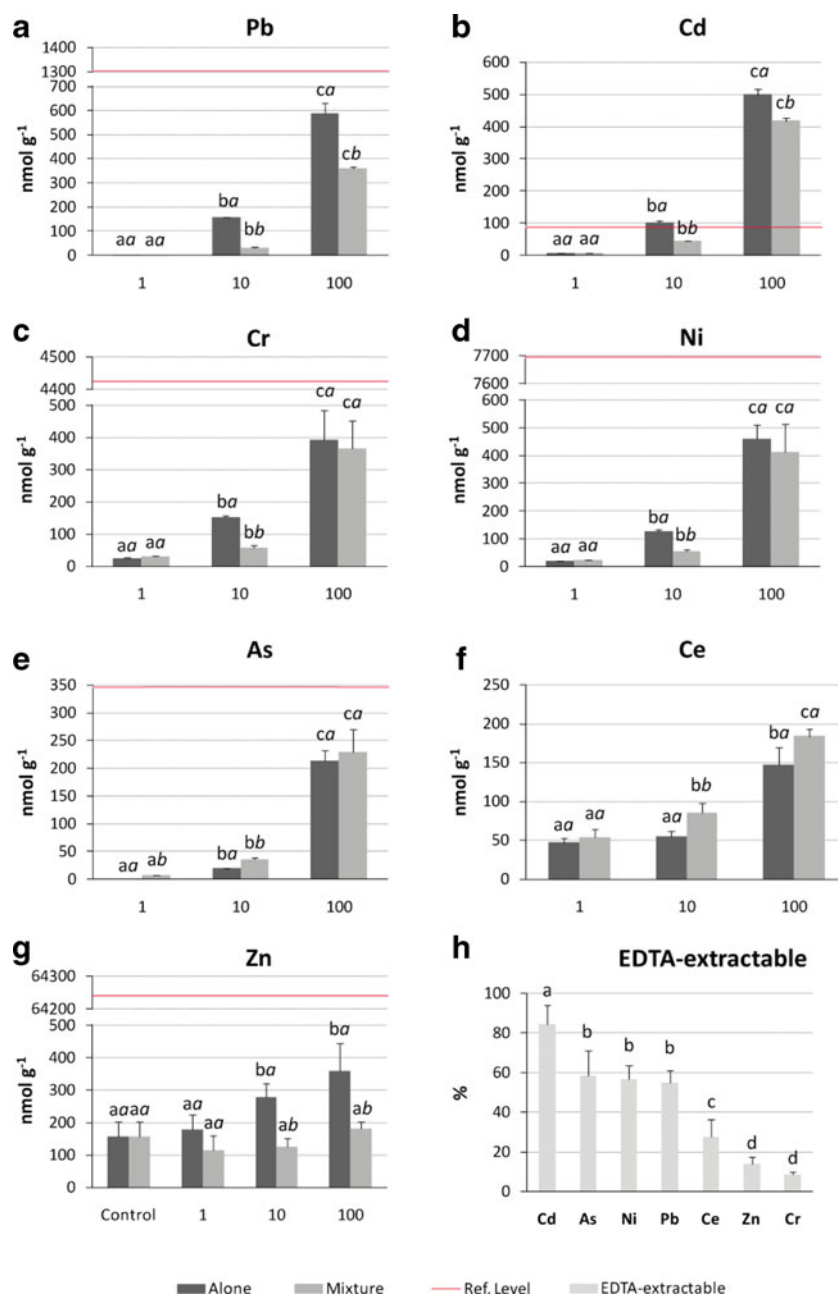
Concentrations of Cd and As in PM_{2.5} particles were below the limit of detection in all cases, as well as those of Ce and Ni for most of the collected samples (data not shown). Taking together all samples collected from the different sites, a significant linear correlation in the average Pb concentration between *N. oleander* leaves and PM_{2.5} particles was found (Fig. 6). Considering the metals separately, significant linear correlations between the concentration of metals of *N. oleander* leaves and PM_{2.5} were found for Fe and Pb (in NAV), Zn (in TFL) and Ti (in JES). As shown in Fig. 7, leaves accumulate metals as a function of the metals accumulated as PM_{2.5} in the air samplers.

Structural and chemical composition of leaves using LT-SEM-EDX

Leaf tissue structural information of cryo-fractured *N. oleander* leaves of airborne polluted (blue) and 100 μM mixture treated (red) plants was obtained using LT SEM (Fig. 8a–d). Leaves exposed to airborne pollution were thinner than 100 μM mixture treated leaves, with a 16.6 % reduction in total leaf thickness (Fig. 8a, b). The 100 μM treated plants show also a more compact mesophyll tissue than the airborne polluted ones (Fig. 8a, b). Figure 8c, d shows a notable number of particles trapped in substomatal chambers of airborne polluted leaves which were not presented in 100 μM mixture treated plants. The size and the abundance of the substomatal chambers in the abaxial surface of *N. oleander* leaves were shown in Fig 9.

EDX analysis (semiquantitative analysis) in the different layers of leaf cross, including adaxial epidermis, palisade parenchyma, xylem vessels, spongy parenchyma, and abaxial epidermis, showed no differences in the relative metal signals

Fig. 4 Concentration of metals in the substrate (nmol g^{-1}) after 42-day-treatments with Pb (a), Cd (b), Cr (c), Ni (d), As (e), Ce (f) and Zn (g), in both alone (dark grey) and mixture (medium grey) supply, at 1, 10 and 100 μM doses. Red line indicates the allowable limits in urban soils in the Aragon region. Percentage of EDTA-extractable metal with respect to the total concentration of metal in the substrate (light grey) (h). Data are expressed as mean \pm SE. Different *non-italics* letters indicate significant differences among doses ($P < 0.05$; $n = 3$). Different *italics* letters in the same treatment indicate significant differences among alone and mixture supply ($P < 0.05$; $n = 3$). Zn treatment included control concentrations



across the leaf section (details in Tab. S1 at Electronic Supplementary Information). Likewise, no differences in the EDX results between airborne polluted and 100 μM mixture treated leaves were found.

Discussion

The plants exposed to metals via root uptake were able to survive in all cases. These results are in agreement with the extremely high ability of resistance against the toxicity of metals reported by (Franco et al. 2012, 2013) for

this plant under field conditions. Height measurements indicated that doses and exposure time were the main factors involved in the growth inhibition by metals (Figs. 2 and 3). The same behaviour has been widely reported in the literature (Kabata-Pendias and Pendias 1992). The exposure to metal mixture resulted in a lower biomass reduction than those found in single treatments (alone) (Fig. 3). This fact is in accordance to the lower concentration of metals in the mixture than in the single treatments found in the substrate, except for As and Ce, (Fig. 4), and also in *N. oleander* plants in all cases (Fig. 5). The lower substrate concentrations of Pb, Cd,

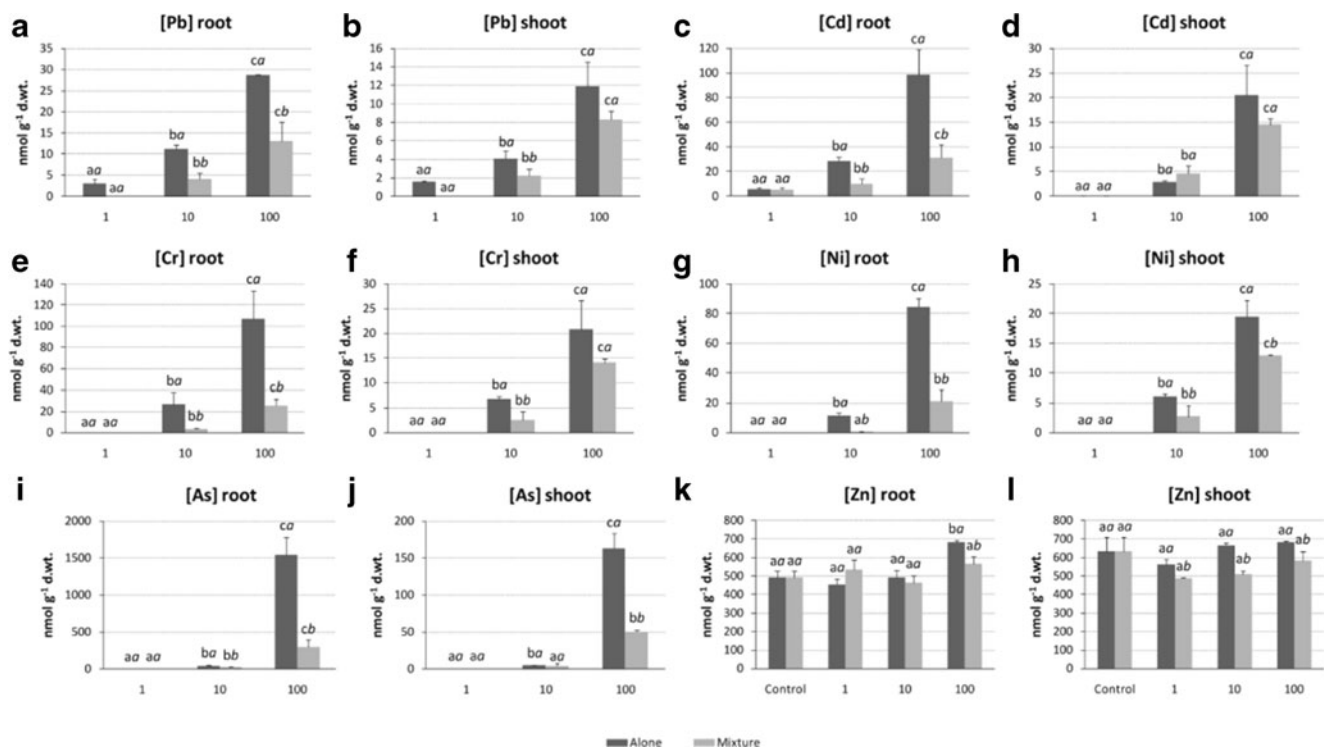


Fig. 5 Root and shoot concentration of metals (nmol g⁻¹ d.wt.) after 42-day-treatments with Pb (**a, b**), Cd (**c, d**), Cr (**e, f**), Ni (**g, h**), As (**i, j**) and Zn (**k, l**), in both alone (dark grey) and mixture (light grey) supply, at 1, 10 and 100 μM doses. Data are expressed as mean ± SE. Different non-

italics letters indicate significant differences among doses ($P < 0.05$; $n = 3$). Different italics letters in the same treatment indicate significant differences among alone and mixture supply ($P < 0.05$; $n = 3$). Zn treatment included control concentrations

Cr, Ni and Zn in the mixture than in the single treatments could be due to the competition of metals for the binding sites in the substrate.

Table 1 Pearson correlation coefficients between plant (shoots and roots) concentrations of metal, in both alone and mixture supply, and substrate concentrations of both EDTA-extractable and total metal

		Shoot		Root	
		Alone	Mixture	Alone	Mixture
Pb	Total	0.789**	0.966**	0.933**	0.984**
	EDTA	0.892**	0.963**	0.927**	0.991**
Cd	Total	0.753**	0.973**	0.869**	0.838*
	EDTA	0.829**	0.996**	0.932**	0.970**
Cr	Total	0.814*	0.968**	0.960**	0.975**
	EDTA	0.816*	0.938**	0.981**	0.813*
Ni	Total	0.970**	0.951**	0.992**	0.999*
	EDTA	0.911**	0.975**	0.953**	0.812*
As	Total	0.996**	0.961**	0.995**	0.868**
	EDTA	0.993**	0.953**	0.999**	0.933*
Zn	Total	-0.024	0.021	0.555	-0.280
	EDTA	0.276	-0.241	0.853**	0.090

**Significant at probability level $P < 0.01$; *significant at probability level $P < 0.05$

The mixture of metals supplied to the substrate could lead to a prompt saturation of the binding sites and subsequent lixiviation of the remained metals in solution (Alloway 1995). However, when only one metal was added, a higher fraction of the metal was retained by the substrate. In the case of Ce and As, tetravalent and metalloid, respectively, their different physico-chemical characteristics in comparison to the Pb, Cd, Cr, Ni and Zn, all of them are bivalents except Cr (trivalent), could involve a different behaviour with respect to the binding sites of the substrate. An opposite behaviour between As and heavy metals with respect to their interaction with the soil fractions has been reported in a previous study (Fitz and Wenzel 2002).

An indirect approach to assess the relative affinity of each metal to the binding sites of the substrate is the determination of the bioavailable fraction of the metal in the substrate. The estimated bioavailability of metals is strongly dependent on the components of the growth media, concentration of the metal, the plant species and the selected extractant solution (Vázquez et al. 2008). Taking into account the high cation-exchange capacity (CEC) of the substrate, due to its high content in organic matter, a stronger extractant as EDTA than inorganic salts was selected. The order of bioavailability was Cd > As ~ Ni ~ Pb > Ce > Zn > Cr (Fig. 4h). A low bioavailability is a characteristic trail of Pb that is widely reported in the literature (Adriano 2001). Thus, in this study, the

Table 2 Translocation factor (TF) and bioaccumulation factors (Total-BF and EDTA-BF) for *N. oleander* plants treated with Pb, Cd, Cr, Ni, As and Zn in both alone and mixture supply

	TF		Total-BF		EDTA-BF	
	Alone	Mixture	Alone	Mixture	Alone	Mixture
Pb	0.34±0.04	0.63±0.09	0.02±0.01	0.01±0.01	0.06±0.01	0.04±0.03
Cd	0.13±0.02	0.36±0.05	0.02±0.01	0.02±0.01	0.04±0.02	0.05±0.03
Cr	0.48±0.20	0.61±0.21	0.06±0.02	0.04±0.01	0.44±0.13	0.55±0.26
Ni	0.24±0.02	0.73±0.31	0.02±0.02	0.04±0.02	0.02±0.02	0.09±0.02
As	0.13±0.01	0.32±0.13	0.51±0.25	0.37±0.25	0.70±0.11	0.58±0.08
Zn	1.15±0.12	1.13±0.16	2.11±0.13	5.48±0.86	31.29±14.64	30.86±11.03

Data are expressed as mean ± SE

bioavailability of Pb (55 %) could be overestimated by the EDTA-extractable metal because of the high stability of the Pb-EDTA complex (Niinae et al. 2008). This is in agreement to the high concentration of Pb found in the substrate (Fig. 4), which indicated an important retention of Pb by the substrate. According to our data, Cd was the most bioavailable metal (84 %). Likewise, a high bioavailability of Cd has also been reported in soils (Kabata-Pendias 2004). In the case of As, its estimated EDTA bioavailability could be shift by its opposite behaviour with respect to the biding sites in comparison to the metals and its close relationship to phosphorous (Fitz and Wenzel 2002). However, it is worth to mention that the most bioavailable metals, Cd (84 %) and As (57 %), are also those whose substrate concentrations were higher and closer to the reference levels reported in soils (BOA 2008), respectively (Fig 4b and e). This fact pointed out at Cd and As as the most potential hazardous pollutants in the growth media.

The order of toxicity of the metals with respect to *N. oleander* assessed by the maximum percentage of biomass reduction was Cd>Pb~Cr>As>Zn~Ni~Ce~mixture (Fig. 3). The toxicity of these metals in *N. oleander* is the

product of several factors related to the substrate (concentration and bioavailability), mentioned above, and factors related to the plant. Regarding the latter, Zn has a clear different pattern with respect to the other metals: higher biomass under 1 and 10 µM Zn treatments (Fig. 3), high values in root and shoot concentrations of Zn and no increase of them with increasing doses (Fig. 5k, l). No significant correlations were found between substrate concentration and plant (Table 1), but higher TF and BF than for the other metals were found (Table 2). The different pattern of Zn is due to the fact that Zn is a nutrient unlike the other metals considered as pollutants. This fact involved the existence of specific uptake and translocation mechanisms of Zn as well as a higher toxicity threshold of Zn in plants (Marschner 1995).

In the case of As, high root and shoot concentrations (Fig. 5j, k) and 10-fold higher BF (Table 2) were observed. These results could be explained by the antagonism between As and P. Arsenic and phosphorous are intimately linked in plants due to the chemical similarity between them. It is thought that As to be taken up by plants via P uptake system and the subsequent toxicity in plant is due to the disturbance of many physiological phosphate functions (Fitz and Wenzel 2002; Meharg and Hartley-Whitaker 2002). Thus, a high correlation in shoot concentrations between As and P was observed (Tab. S2 Electronic Supplementary Information). Regarding the rest of the metals, the low values of TF and BF indicated an excluder behaviour of *N. oleander* (Baker 1981). The same excluder behaviour of *N. oleander* with respect to heavy metals has been reported by (Franco et al. 2012, 2013) under field conditions.

LT-SEM pictures (Fig. 8a, b) showed a more disrupted leaf tissue structure in *N. oleander* plants polluted via root uptake than those via airborne deposition. These findings confirmed the inhibition of plant growth, both in height (Fig. 2h) and in weight (Fig. 3), induced by the 100 µM mixture treatment. The higher concentration of metals in the substrate in the pot experiment (Fig. 4) than those in the leaf surface in the screening experiment (Figs. 6 and 7) would explain the different grades of alteration in the leaf anatomy. Similarly, less thickness and more disorganized tissues in *Beta vulgaris* leaves

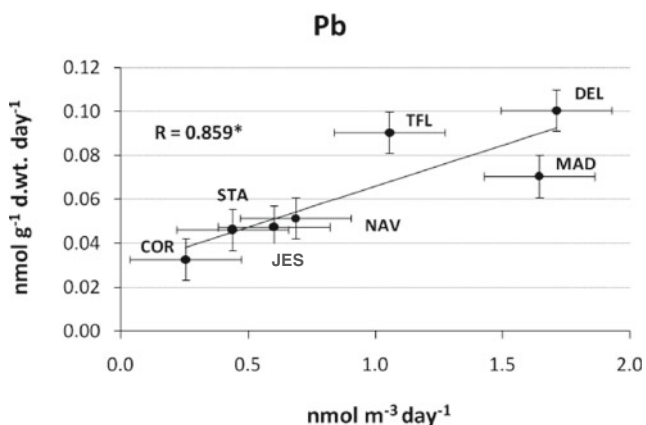


Fig. 6 Correlation in the concentration of Pb along the sampling period (42 days) between *N. oleander* leaves ($\text{nmol g}^{-1} \text{ d.wt. day}^{-1}$) and PM2.5 particles ($\text{nmol g}^{-1} \text{ m}^{-3} \text{ day}^{-1}$) collected in each sampling site (R = Pearson coefficient, *significant at probability level $P < 0.05$). Error bars are standard deviations

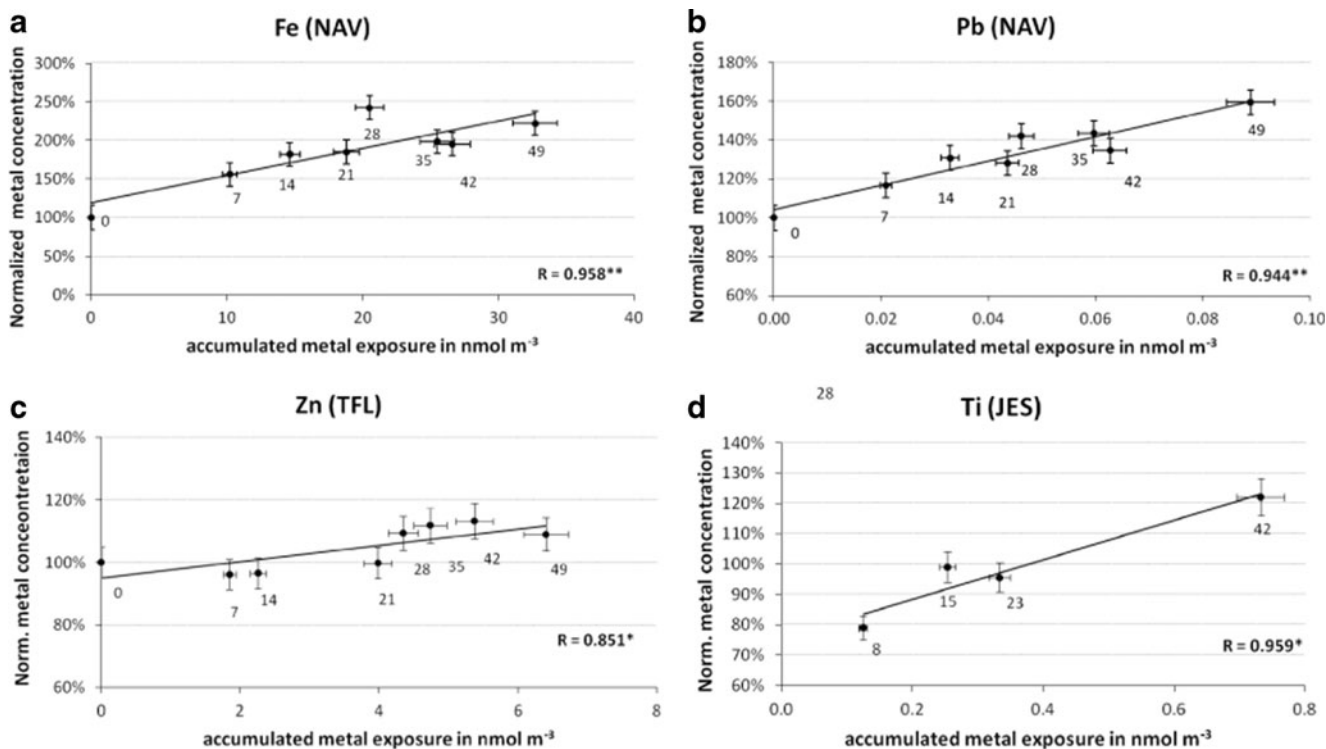


Fig. 7 The increase of metals accumulated in *N. oleander* leaves (normalized vs day 0) as a function of the accumulated exposure to the metals present in the surrounding PM2.5 (R =Pearson coefficient,

**significant at probability level $P < 0.01$ and $*P < 0.05$). The number below each point indicates the days of exposure to PM2.5

with increasing doses of Zn have been reported by Sagardoy et al. (2010). In biomonitoring studies, the nature of the leaf surface is an important factor that affects the deposition process of airborne particulate matter on the leaf. Thus, the capture of particles in the substomatal chambers from the airborne pollution observed in this study (Fig. 8c, d) could result in an important contribution to the total amount of metals in *N. oleander* leaves, especially in this species due to the large size and number of these chambers in its abaxial side (Fig. 9). Similar findings in the ability to trap airborne particulate matter based on the leaf surface characteristics between *Lamana camara* and *N. oleander* were suggested by Fernández Espinosa and Oliva (2006).

The experiments exposing plants to urban pollution showed that Pb was incorporated in leaves as a function of the Pb present in the PM during the sampling period (Fig. 6). This significant relationship was also found for certain sites as NAV (see Fig. 7b), showing a clear exposure-related increase of the Pb in leaves. Other metals with significantly high correlations between *N. oleander* leaves and PM2.5 were Fe, Zn and Ti (Fig. 7a, c, d). Similar high correlations for Fe and Zn in *N. oleander* plants expose to air pollution were found by Fernández Espinosa and Oliva (2006) and Aksoy and Ozturk (1997), respectively. The % of Pb bioaccumulated from roots during the 42 days exposure to urban pollution has

been also calculated. The value, 18 ± 6 %, strongly indicates that the rest of Pb found in leaves is the result of the exposure to urban pollution (calculations are shown at Tab. S3 Electronic Supplementary Information).

Lead has been reported as the main metal supplied by aerial deposition in urban areas in proportion to the density of traffic and distance from the roadside (Aksoy and Ozturk 1997; Dongarra et al. 2003). But in our case, it should be noticed an apparent lack of accordance between traffic intensity (JES \ll COR \ll NAV \ll STA \ll DEL \ll MAD \approx TFL, see “Screening of airborne metals using air sampler and *N. oleander* leaves” section) and Pb airborne pollution (COR $<$ STA $<$ JES \approx NAV $<$ TFL $<$ MAD \approx DEL). But this discordance is explained by the use of traffic intensity values that have been calculated in a different period, having thus only a qualitative value to characterize the potential level of air pollution at each site. Even though, as shown in Fig. 6, *N. oleander* leaves accumulate Pb as a function of the metals present as PM2.5, being thus a good indicator of the air pollution in their surroundings.

The lack of pattern and differences in the relative metal signals across the leaf section between 100 μ M mixture treated and air polluted leaves indicated a non-significant penetration of the metals through *N. oleander* leaf tissues exposed to polluted air. This result supported the importance of the airborne particle matter trapped in the substomatal

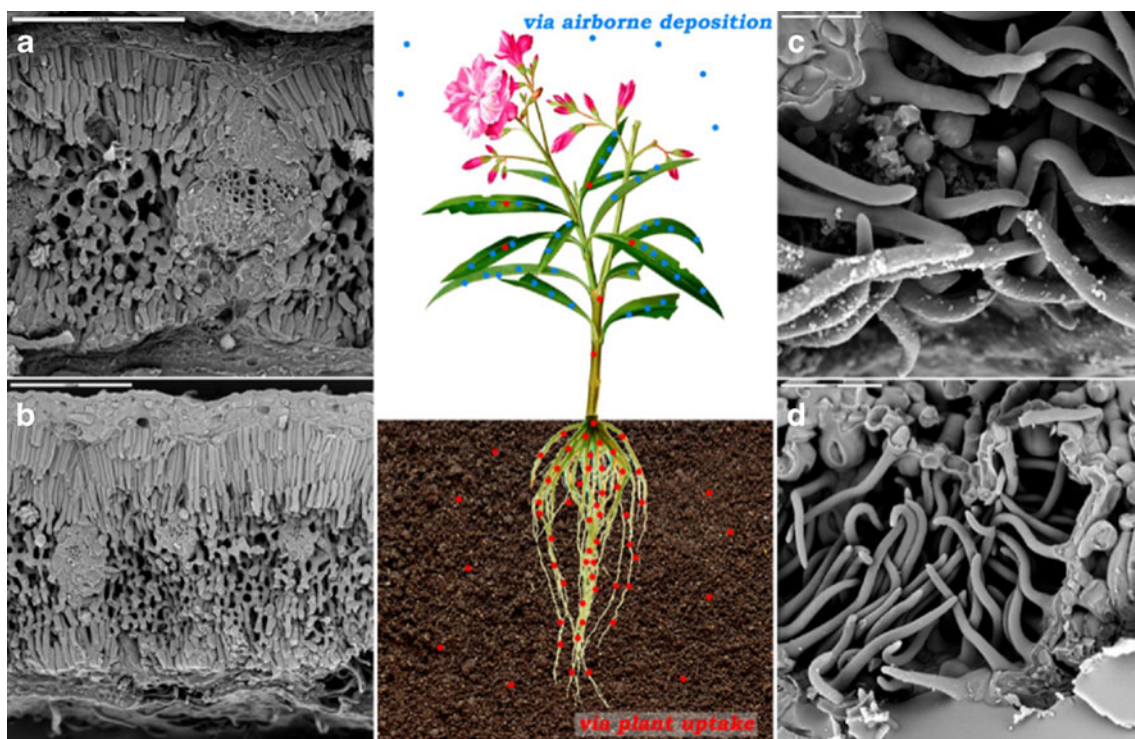


Fig. 8 Freeze–fracture low-temperature scanning electron micrographs of leaf transversal sections from airborne polluted (blue) and 100 μM mixture treated (red) *N. oleander* leaves. Leaf transversal sections on the left give three-dimensional information on the internal structure of the leaf, confirming the disorganisation caused by 100 μM mixture (b) in comparison to airborne pollution (a). Detailed zoom of the substomatal chambers showed the presence of particulate matter in to airborne

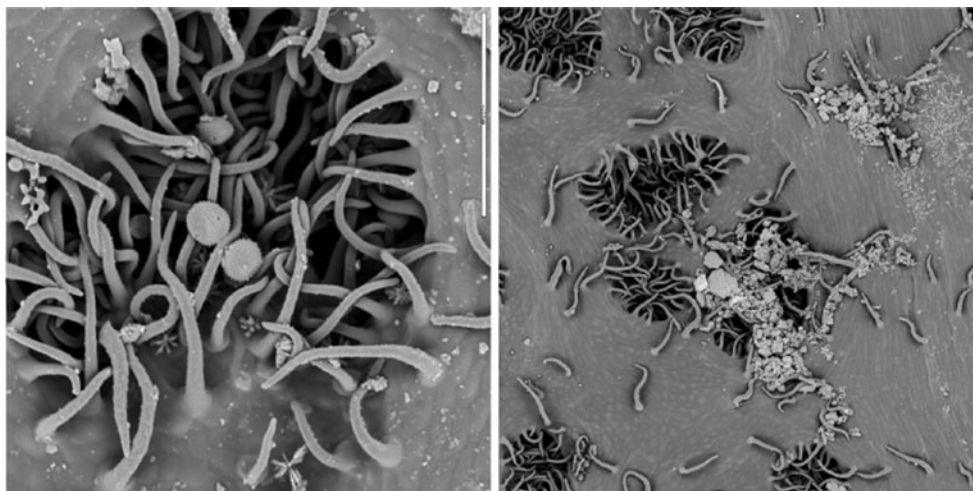
polluted (c) in comparison to 100 μM mixture treated (d) leaves. In the middle part, a scheme of the *N. oleander* plant as a biomonitor of airborne metal pollution where metals from air pollution (blue points) are deposited on its leaves, and metals from soil pollution (red points) are retained mainly in root and only a few amount of those reach shoot. Scale bars—upper left corners—represent 200, 200, 20 and 50 μm (a, b, c, d)

chambers of *N. oleander* leaves when they are used as bio-monitoring tool. Regarding the 100 μM mixture treated leaves, these EDX results would also confirm the notably excluder behaviour of *N. oleander*. In this sense, preferential accumulation of metals in leaf tissues was only reported in *N. oleander* plants grown under extremely high levels of heavy metals (Franco et al. 2012, 2013).

Conclusion

Screening data indicated high correlations in the concentration of Pb and in lesser extent of Fe, Zn and Ti between *N. oleander* leaves and PM_{2.5} particles. *N. oleander* showed a high ability of resistance against the toxicity of trace metals, particularly for Cd and As that were considered the most

Fig. 9 Low-temperature scanning electron micrographs of leaf abaxial surface from airborne polluted *N. oleander* leaves. Scale bars—upper right corners—represent 100 and 200 μm (left and right pictures, respectively)



potential hazardous metals according to their bioavailability and reference levels in soil. In comparison to single treatments, metal mixture treatments showed a lower retention of Pb, Cd, Cr, Ni and Zn in the substrate, lower plant concentrations of metals and subsequently a lower inhibition of plant growth. Except for Zn, *N. oleander* showed an excluder behaviour with respect to the metal pollutants present in the growth media.

LT-SEM analyses showed the capture of particle matter from the airborne pollution in the large and abundant substomatal chambers of *N. oleander* leaves. The leaves also showed the ability of accumulating metals (Pb and others) as a function of the metals presents as PM_{2.5} in their surroundings. All these findings indicated *N. oleander* plants as a suitable tool to assess the immission of the airborne metal pollution, especially for Pb, related to inhalable particles (PM_{2.5}) in urban areas (see Fig. 6).

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