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Nutritional Status of Mediterranean Trees Growing in a Contaminated and Remediated Area

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Abstract Soil contamination may contribute to forest decline, by altering nutrient cycling and acquisition by plants. This may hamper the establishment of a woody plant cover in contaminated areas, thus limiting the success of a restoration program. We studied the nutritional status of planted saplings of Holm oak (*Quercus ilex* subsp. *ballota* (Desf.) Samp.), white poplar (*Populus alba* L.), and wild olive tree (*Olea europaea* var. *sylvestris* Brot.) in the Guadiamar Green Corridor (SW Spain) and compared it with established adult trees. Soils in this area were affected by a mine-spill in 1998 and a subsequent restoration program. The spill resulted in soil acidification, due to pyrite oxidation, and deposited high concentrations of some trace elements. In some sites, we detected a phosphorus deficiency in the leaves of *Q. ilex* and *O. europaea* saplings, as indicated by a high N:P ratio (>16). For *O. europaea*, soil contamination explained 40% of the variability in leaf P and was negatively related to chlorophyll content. Soil pH was a significant factor predicting the variability of several nutrients, including Mg, P, and S. The uptake of Mg and S by *P. alba* was greater in acidic soils. The monitoring of soil pH is recommended since long-term effects of soil acidification may negatively affect the nutritional status of the trees.

Keywords Heavy metal · Tree nutrition · Phosphorus · *Quercus ilex* · *Olea europaea* · *Populus alba* · Soil remediation

1 Introduction

Soil contamination can affect nutrient cycling and acquisition by trees leading to nutrient disorders in forests sites. Competitive interactions between pollutants, such as trace elements, and nutrients in the soils and in the roots may alter the availability or uptake of essential elements. For example, these interactions have been described for As and P (Lambkin and Alloway 2003), Tl and K (Kwan and Smith 1991), or Cd and Ca (Perfus-Barbeoch et al. 2002). Root growth and morphology can also be altered by trace elements (Arduini et al. 1994; Reichman et al. 2001; Domínguez et al. 2009), reducing root ability to absorb essential nutrients. Elevated trace element concentrations can alter microbial activity and com-

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position in soils (Pennanen et al. 1998; Tuomela et al. 2005), affecting plant–microorganism interactions that are essential for plant nutrition such as mycorrhizae (Kieliszewska-Rokicka et al. 1997; Hartley-Whitaker et al. 2000).

Mediterranean environments are frequently deficient in N and P (Henkin et al. 1998; Rodà et al. 1999). Nutrient limitations may affect the growth of afforested woody plants in Mediterranean areas (Romanyà and Vallejo, 2004; Villar-Salvador et al. 2004). Therefore, fertilization often improves the performance of planted seedlings (Querejeta et al. 1998; Vallejo et al. 2000; Fuentes et al. 2007a). The management of contaminated sites should address possible tree nutrient deficiencies associated with the contamination event or its subsequent remediation. However, little is known about the effects of soil trace elements on the nutrition of Mediterranean trees, despite the increasing use of metal-rich amendments in the afforestation of Mediterranean sites. On one hand, soil contamination may exacerbate nutrient deficiencies in plants. On the other hand, many semi-arid areas in the Mediterranean region are characterized by a relatively high pH and carbonate content, which may result in a low availability of some micronutrients such as Cu or Zn for plants (Adriano 2001). The application of metal-enriched material such as sewage-sludge to semi-arid calcareous soils may improve the seedling performance, by releasing micronutrient limitations (Fuentes et al. 2007a). Thus, the influence of soil contamination on plant nutrition depends on the type of contaminant (essential vs. non-essential trace element), the dose, the type of soil and the soil organic matter content. The latter two factors determine the background availability of essential trace elements, the capacity for the retention of the added contaminants, and, therefore, the possible phytotoxic effects (Illera et al. 2000; Oudeh et al. 2002; Toribio and Romanyà 2006).

The Guadiamar Green Corridor (SW Spain) is one of the largest cases of soil remediation in Europe in the last decade. This area was contaminated by a mine-spill in 1998 and a large-scale restoration program was implemented, which included the addition of soil amendments and the afforestation with native Mediterranean woody plants. Soil types in the area range from sandy-loam to calcareous clay-loam. Therefore, this wide variability allows exploring the influence on soil type on the dynamics and effects of contamination. Several recent studies have shown the dynamics of trace elements in soils and plants from the Guadiamar Green Corridor following remediation (Madejón et al. 2004; Cabrera et al. 2008; Domínguez et al. 2008). However, there is a lacuna of information on the ecophysiological responses of the trees growing in this area. Given the high trace element concentrations in soils, the pollutants may detrimentally affect plant processes at the root level, and hence affect plant nutrition.

In this work, we studied the chlorophyll content and nutritional status, as plant-health indicators of trees in the Guadiamar Green Corridor. We assessed the influence of soil conditions on these plant variables. We selected three tree species (*Olea europaea* var. *sylvestris, Populus alba*, and *Quercus ilex* subsp. *ballota*), which are the most abundant in the Guadiamar Green Corridor. We aimed to determine: (1) whether there was any disorder in nutritional status or chlorophyll content of trees growing under these altered soil conditions and (2) the relative influence of soil contamination on plant nutrient status over a range of soil properties, including different pH, texture, organic matter content, and nutrient availability. As a secondary objective, we sought to elucidate relationships (synergistic, antagonistic, or neutral) that exist between essential and non-essential trace element concentrations at the leaf level, since these relationships could help to explain any nutritional changes induced by soil contamination.

2 Materials and Methods

2.1 Study Area and Studied Species

The Guadiamar River Green Corridor (SW Spain) has a semi-arid Mediterranean climate with mild rainy winters and warm dry summers. The average annual temperature is 19°C (monthly mean minimum of 9°C in January and maximum of 27°C in July). The average annual rainfall is 484 mm and potential evapotranspiration is 1,139 mm (period 1971–2004). Soils of the area are mostly neutral or slightly alkaline, with the exception of some terraces on the Northern bank, which are acidic.

In 1998, a mine-spill affected some 4,286 ha of the river basin. The sludge that covered the soils was composed by polymetalic sulfides (mainly pyrite), with average concentrations of 380 g kg^{-1} of Fe, 9.3 g kg^{-1} of Zn, 8.6 g kg^{-1} of Pb, 5.7 g kg^{-1} of As, 1.5 g kg^{-1} of Cu, 40 mg kg^{-1} of Cd, and 43 mg kg^{-1} of Tl, as main contaminants (ITGE 1999).

After the accident, an emergency cleanup removed sludge and contaminated topsoil. Organic matter and calcium-rich amendments were added with the aim of immobilizing trace elements and improving soil fertility. Sugar beet lime was the most used amendment, which had 70–80% of $CaCO_3$ (pH 9) and N, P, and K concentrations up to 9.8, 5.1, and 5.3 g kg^{-1}, respectively (Madejón et al. 2006). Application rates ranged from 3 to 50 t ha^{-1}, depending of the degree of contamination of the site (Arenas et al. 2008).

The revegetation of around 2,700 ha with native Mediterranean tree and shrub species started in 1999. One of the goals of this program was the long-term establishment of a continuous vegetation belt for wildlife to migrate along the Guadiamar River between the Doñana National Park in the South (where the river flows into) and the Sierra Morena Mountains in the North. Depending on the local conditions, the target tree and shrub species used for afforestation were those typical of riparian forests, such as *P. alba*, *Fraxinus angustifolia*, and *Salix atrocinerea* or those typical of drier upland forests, such as *Q. ilex* subsp. *ballota*, *O. europaea* var. *sylvestris*, *Ceratonia siliqua*, *Phillyrea angustifolia*, *Pistacia lentiscus*, *Rosmarinus officinalis*, and *Retama sphaerocarpa*. Seedlings were grown in a local nursery and then planted out after 1 year. The planting density ranged from 480 to 980 plants per hectare.

For this study we selected the most abundant tree species used in this restoration program: Holm oak (*Q. ilex* subsp. *ballota* (Desf.) Samp.), wild olive tree (*O. europaea* var. *sylvestris* Brot.), and white poplar (*P. alba* L.).

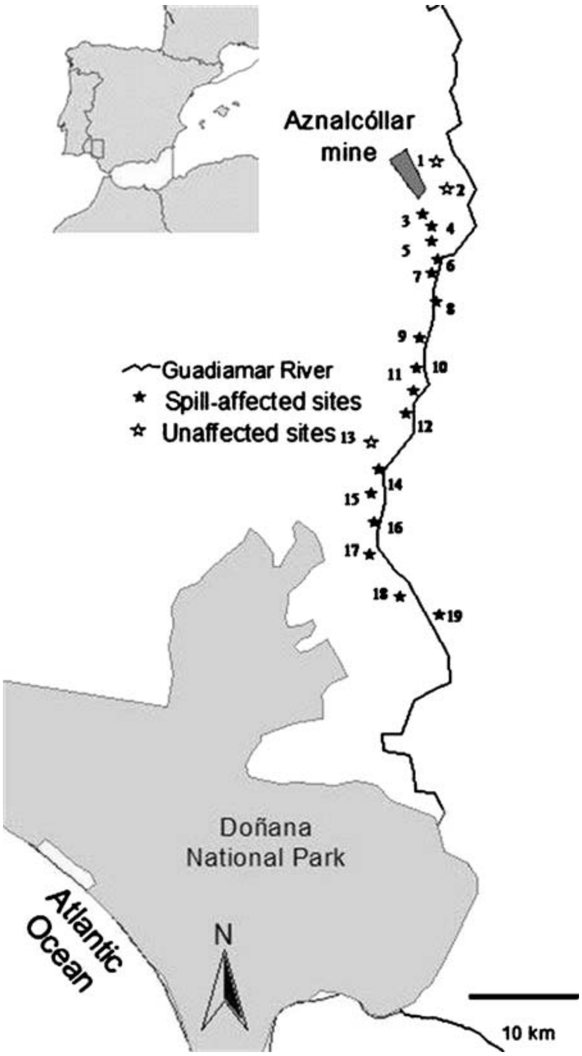


Fig. 1 Map of the Guadiamar River Valley (SW Spain) in the Iberian Peninsula (*insert*) and location of the sampling sites

2.2 Plant and Soil Sampling

Sampling was carried out during autumn of 2005, 7 years after the remediation works. Nineteen sites along the Green Corridor were selected (Fig. 1), from the unaffected areas upstream of the tailings dam (37°30′, 6°13′ W), 38 km down to the Southern limit of the Doñana saltmarshes (37°13′ N, 6°14′ W). Three of these sites were unaffected by the spill, but also were afforested and included within the Corridor; they will serve as "blank" references for this study. Several soil types are present along the sampled area. Bedrocks in the North of the basin (including the unaffected sites 1 and 2) are mostly slate and schist, and soils are mostly acidic sandy loams. In the Central part of the basin, predominant bedrocks are lime and calcarenite, and soils are neutral and basic loams (including the unaffected site 13). Soils in the South of the basin are mostly clay loams, due to the vicinity of the saltmarshes in the South of the basin.

Samples of *P. alba* and *Q. ilex* were collected from ten sites and *O. europaea* from 11 sites. Where possible, at each site we also sampled adult trees that survived the spill, for comparison with planted saplings. There were no individuals of *P. alba* nor adult trees of the other two species at the non-affected sites.

At each site, we selected three to ten individual trees of each species, depending on their abundance. Around each tree, the leaf litter was removed and soil samples were taken from the root-zone at 0–25 cm, using a spiral auger with a diameter of 2.5 cm. Two cores were taken at opposite sides of the trunks to make a composite soil sample for each tree. A composite leaf sample was taken for each selected tree, by randomly collecting four to eight leaf subsamples around the crown. We collected fully expanded leaves from the outer canopy, avoiding shaded leaves from the inner parts of the crown. For adult trees, we used a pole to reach the leaves of the medium and upper parts of the crown. Leaves were placed in a chilled, dark storage container for transportation to the laboratory. Between 15 and 25 plant samples per species and life-stage (adults and saplings) were collected. The total number of plant (and correspondent soil) samples was 116 (52 adults and 64 saplings).

2.3 Sample Preparation and Chemical Analyses

Immediately upon returning from the field, we selected ten leaves from each plant sample and carefully washed them with deionized water. In these leaves we used a SPAD-502 chlorophyll meter (Minolta Camera Co. Ltd. Osaka, Japan) to determine the Chlorophyll Content Index (CCI) of each leaf, by taking three measurements per leaf. The CCI is a non-destructive, dimensionless variable, which is linearly related to the total chlorophyll concentration per unit of area. This relationship differs for leaves of different species, so we did not compare across species. The rest of each plant sample was washed thoroughly with deionised water, dried at 70°C for at least 48 h and ground using a stainless-steel mill.

Plant material was analyzed for N using a Kjeldahl digestion. The rest of macronutrients (Ca, K, Mg, P, and S) and trace elements (As, Bi, Cd, Cu, Pb, Tl, and Zn) were extracted by wet oxidation with concentrated HNO_3 under pressure in a microwave digester. Macronutrients (except N) were analyzed by inductively coupled plasma optical emission spectrophotometry (ICP-OES; Thermo Jarrel Ash Corporation). Trace elements were analyzed by inductively coupled plasma mass spectroscopy (ICP-MS; Perkin Elmer, Sciex-Elan 5000).

To ensure that the CCI was a reliable measurement of the total leaf chlorophyll of the studied species, a calibration was conducted in the autumn 2007. Ten trees per species and life-stage were selected in four of the sampling sites; in each of them two to three leaves were collected and treated as described above. The chemical analysis of the chlorophyll concentration was performed in two disks of 5 mm diameter of each leaf; for *P. alba* and *Q. ilex* chlorophyll was extracted with N,N-dimethylformamide and determined by spectrophotometry according to Moran (1982). For *O. europaea*, the extraction with N,N-dimethylformamide was not complete and methanol was used as extractant in a subsample of leaves; chlorophyll was then determined by spectrophotometry according to Wellburn (1994). The best fits between CCI and chlorophyll concentrations were obtained by exponential regressions (Fig. 2).

All soil samples were oven-dried at 40°C until a constant weight was obtained, then sieved to <2 mm,

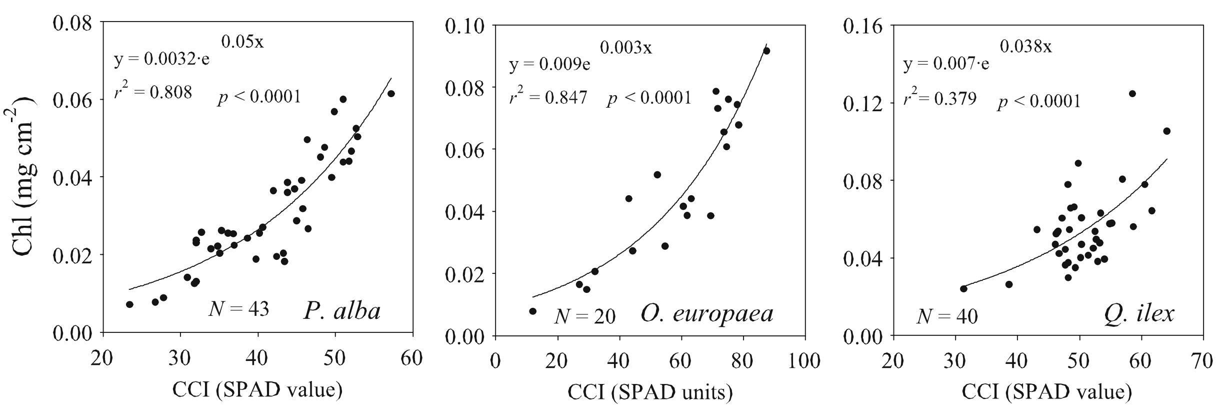


Fig. 2 Relationship between the Chlorophyll Content Index (CCI) estimated by a SPAD meter and total chlorophyll concentration (Chl) for the three studied species. Exponential function and parameters are indicated

for the analysis of general properties. A fraction of each sample was then ground in an agate mortar to <1 mm for trace element analysis.

Soil texture was determined by the hydrometer method (Gee and Bauder 1979). The pH was determined potentiometrically in a 1:2.5 soil–water suspension. Organic matter content was analyzed by dichromate oxidation and titration with ferrous ammonium sulfate (Walkley and Black 1934). Available P was determined by sodium bicarbonate extraction (Olsen et al. 1954) and available K was analyzed by extraction with ammonium acetate (Bower et al. 1952). Total organic N was analyzed by the Kjeldahl method (Kammerer et al. 1967). For total trace element concentrations (As, Bi, Cd, Cu, Pb, Tl, and Zn), the <1 mm soil fraction was digested using concentrated HNO_3 and HCL (1:3 *v*/*v*, aqua regia) and analyzed by ICP-MS.

The quality of the analyses was assessed by analyzing different reference materials: NCS DC 73350 (white poplar leaves, China National Analysis Center for Iron and Steel) for macronutrients and trace elements; BCR-62 (olive tree leaves, European Community Bureau of Reference) only for plant trace elements and CRM 141R (calcareous loam soil, European Community Bureau of Reference) for soil trace elements. Our experimental values showed recoveries from the certified values of 86% to 96% (plant macronutrients), 81% to 105% (plant trace elements), and 83% to 91% (soil trace elements).

2.4 Data Analyses

Principal component analyses (PCAs) were performed to explore the relationships between soil factors. Factor–variable correlations were considered as relevant when factor loadings were ≥0.60. Significant differences in the concentrations of plant nutrients and the chlorophyll content between saplings and adult trees of each species were analyzed using the *t*-test. Linear correlation analyses were performed within the concentrations of elements and the chlorophyll content in the leaves to investigate possible interferences of trace element accumulation on the leaf nutrient content. With the mean values of nutrient and chlorophyll concentrations in each sampling site, we performed univariate linear regressions with some of the soil properties of the corresponding sites (pH and an index of soil contamination, see below). Weighted regressions were used because the variances were highly different among sites. The inverse of the variance of the studied concentrations was used as the weight variable.

On an individual tree basis, multiple regression models were used to investigate the variation of nutrient concentrations and the chlorophyll content in leaves in relation to various soil predictors. We used the Mallow's *Cp* coefficient for the selection of the best subset of the models. Mallow's *Cp* is a special case of the Akaike Information Criterion and is less dependent than *R*-square on the number of predictors in the model. We selected models with minimum *Cp* coefficients values (Mallow 1973). The soil variables used as predictors were pH, clay content, N, P, K concentrations, and a Contamination Index (CI). The CI incorporates all the major soil contaminants, since all contaminants were mutually correlated and were therefore unsuitable individually as predictors. CI is the first component of a PCA of the total elemental concentrations in the soil, with the soil contaminants (As, Bi, Cd, Cu, Pb, Tl, and Zn) having factor loadings >0.75 (Domínguez et al. 2008). The more contaminated the soil is, the more positive the CI is. In each model, we checked that the tolerance of the selected soil predictors was ≥0.40, to avoid error inflation due to possible collinearity of the predictors.

The significance level was fixed at the 0.05. To avoid the increase of type I error derived from multiple testing, we controlled the 'false discovery rate', (FDR) at the 5% level, as suggested by García (2004). We used an adapted FDR procedure (Hochberg and Benjamini 2000) to calculate a threshold value ($p_t \leq 0.05$) for each test, to which individual *p*-values were compared. Therefore, only *p*-values not exceeding a threshold (p_t) value were considered as significant. The p_t values for each test are reported in the "Results" section.

Data that were log-normally distributed were log-transformed for all these statistical analyses. All analyses were performed with STATISTICA v. 6.0. (StatSoft Inc., Tulsa, USA).

3 Results

3.1 Soil Conditions

Soil properties were heterogeneous in the afforested Guadiamar Green Corridor (Table 1). Soils at the

Table 1 Soil properties (mean values and minimum and maximum in parentheses) in the Guadiamar Green Corridor on affected and unaffected sites

Variable	Unaffected sites			Affected sites		
	Site 1 ($N = 5$)	Site 2 ($N = 6$)	Site 13 ($N = 6$)	North (sites 3–7, $N = 25$)	Central (sites 8–12, $N = 41$)	South (sites 14–19, $N = 33$)
pH	8.4 (5.5, 8.3)	5.5 (5.1, 6.3)	7.9 (7.4, 8.1)	7.1 (3.5, 8.4)	6.0 (2.4, 8.1)	8.1 (7.0, 8.5)
Silt (%)	32.1 (31.7, 32.5)	31.2 (29.7, 32.2)	24.4 (17.6, 31.3)	25.1 (12.7, 32.7)	33.3 (19.3, 45.4)	29.9 (18.7, 41.9)
Clay (%)	54.2 (53.1, 55.5)	21.1 (17.3, 28.4)	23.3 (11.5, 34.0)	25.1 (14.2, 39.2)	23.8 (14.8, 33.6)	24.5 (17.0, 39.2)
Sand (%)	13.7 (12.7, 14.9)	47.7 (39.8, 52.9)	52.3 (34.7, 70.9)	49.8 (32.2, 73.1)	42.9 (21.3, 62.9)	45.6 (27.0, 62.5)
OM (g kg^{-1})	66.3 (63.0, 68.3)	18.1 (7.8, 25.7)	16.3 (11.6, 22.6)	22.4 (3.0, 71.2)	24.1 (12.7, 45.4)	22.6 (8.99, 71.2)
N (g kg^{-1})	1.24 (1.12, 1.31)	0.99 (0.6, 1.4)	0.89 (0.48, 1.17)	1.16 (0.39, 2.17)	1.33 (0.59, 3.1)	0.65 (0.08, 1.21)
P (mg kg^{-1})	8.4 (6.3, 10.0)	10.8 (5.7, 20.6)	12.4 (4.6, 17.6)	35.6 (14.3, 100)	15.9 (1.7, 101)	18.7 (3.7, 41.5)
K (mg kg^{-1})	463 (438, 482)	201 (125, 309)	267 (230, 295)	144 (22, 357)	172 (18, 378)	223 (95, 404)
CI	−3.37 (−3.4, −3.30)	−2.87 (−3.33, −2.27)	−4.99 (−5.23, −4.88)	0.44 (−3.59, 4.62)	1.31 (−2.8, 9.21)	−0.17 (−1.94, 2.91)

OM organic matter, *CI* Contamination Index (see Materials and Methods for further explanations)

Northern areas were mostly sandy-loam and loam; while most frequent soils at Central and Southern areas were loam and clay-loam. The pH values were highly variable within the affected sites and included extremely acid soils (pH<4). Many soils (45%) had N-concentrations <1 g kg^{-1}, a value considered low for Southern Spanish loamy soils (CAC 1992). Similarly, 50% of the sampled soils had low organic matter content of <20 g kg^{-1}. The availability of P was highly variable, especially within the affected area, ranging from 1.7 to 101 mg kg^{-1}. Nearly 40% of the soils had a comparatively low P availability (<13 mg kg^{-1}). However, 40% of soils had a high Olsen-P concentration (>20 mg kg^{-1}). Most of the soils had normal to low levels of available K (<200 mg kg^{-1}). Trace element concentrations (As, Bi, Cd, Cu, Pb, and Zn) were relatively high in the spill-affected sites (Appendix (Table 6)). Arsenic was the most important contaminant, with an average concentration of 129 mg kg^{-1} in affected sites; it is remarkable that this average value is much higher than the upper limit of the range of normal values for agricultural soils (40 mg kg^{-1}, Bowen 1979). The most contaminated sites (as indicated by the Contamination Index) were located at the Northern and Central areas of the Guadiamar Green Corridor.

The PCA of the soil data revealed three factors with eigenvalues >1, explaining 73% of the variance (Table 2). Factor 1 had high weightings for soil texture, organic matter, and available K, the latter two were negatively correlated with sand content. Factor 2

Table 2 Results of the principal component analysis (factor loadings) applied to the soil properties ($N = 116$)

Variable	Factor 1 (38.7%)	Factor 2 (22%)	Factor 3 (12.3%)
pH	−0.40	0.79	0.11
Silt	−0.68	−0.31	−0.21
Clay	−0.87	−0.16	0.03
Sand	0.92	0.20	0.09
OM	−0.66	−0.34	0.25
N	−0.23	−0.65	0.47
P	0.14	0.29	0.86
K	−0.73	0.48	0.09
CI	0.47	−0.59	0.12

The percentage of variance explained by each factor is also indicated

OM organic matter, *CI* Contamination Index

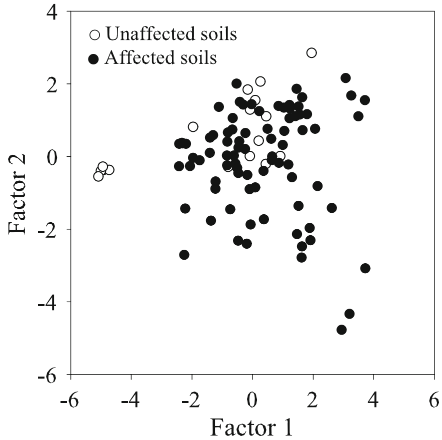


Fig. 3 Principal component analysis of the studied soil properties (N = 116). Soil-factor correlations are shown in Table 2

was defined by soil pH, negatively correlated with total N and contamination (CI). Factor 3 was associated with Olsen-P. The negative correlation between CI and pH was especially high in the Central areas ($r = -0.50$, $p = 0.001$). The distribution of the soil samples in the PCA diagram (Fig. 3) illustrates the high variability of the soil properties. Even the unaffected soils had distinct textures and organic matter contents, as indicated by their positions along the Factor 1 axis. The range of coordinates in the Factor 2 axis indicates the variability in pH values and the trend of lower pH in the contaminated soils.

3.2 Leaf Chlorophyll and Nutrients in Trees

The average Chlorophyll Content Index values (CCI, hereafter referred as "chlorophyll" for simplicity) were 67, 37, and 50 for the leaves of *O. europaea, P. alba*, and *Q. ilex*, respectively. The afforested *Q. ilex* saplings had lower leaf chlorophyll than adult trees (Table 3). For the other two species, there were no differences between life-stages. Considering the average chlorophyll content in the leaves of each site individually and for each species, there was no significant relationship with soil contamination (Fig. 4).

Leaf nitrogen levels were similar for the three species, ranging between 12 and 16 g kg^{-1}. Phosphorus concentrations varied from 0.70 g kg^{-1} (*Q. ilex* saplings) up to 1.25 g kg^{-1} (*P. alba* adults). *P. alba* had the highest concentrations for Ca, Mg, and S (Fig. 5). For each species, there were some differences between adult trees and the afforested saplings (Table 3). In most cases, these differences indicated a higher leaf concentration of nutrients in adult trees, with the exception of P concentrations for *O. europaea*, which were lower in the adult trees. The ratios between N and S, P, K, and Mg (respectively) also varied between life-stages (Table 4). In particular, there was a significant difference in the ratio N:P between *Q. ilex* saplings (mean value of 19) and adults (mean value of 15) which indicates a relative deficiency in P content of the afforested oak saplings.

Table 3 Results of the t-test for the comparison of Chlorophyll Content Index (CCI) and nutrient concentrations in adult trees and saplings for each species

	O. europaea		*P. alba*		*Q. ilex*	
	N = 15 (A), 25 (S)		N = 22(A), 18 (S)		N = 15 (A), 21 (S)	
	t-value	p	t-value	p	t-value	p
CCI	0.65	0.5201	−0.06	0.9493	*−3.86*	*0.0006*
Ca	−1.82	0.0761	*−3.00*	*0.0048*	*−3.05*	*0.0044*
K	1.42	0.1630	*−2.52*	*0.0159*	−1.78	0.0846
Mg	*−3.68*	*0.0007*	−1.34	0.1890	−0.21	0.8350
N	1.25	0.2176	−0.04	0.9706	*−2.24*	*0.0316*
P	*2.95*	*0.0054*	−0.92	0.3610	*−4.30*	*0.0001*
S	*−2.97*	*0.0051*	*−3.20*	*0.0028*	−0.56	0.5796
N:K	−0.53	0.5994	*2.62*	*0.0126*	1.36	0.1826
N:Mg	*3.53*	*0.0011*	0.81	0.4248	1.73	0.0935
N:P	−1.66	0.1049	0.91	0.3681	*3.95*	*0.0004*
N:S	*2.92*	*0.0058*	*2.61*	*0.0130*	0.83	0.4151

Significance levels after controlling the FDR at the 5% level was p_t=0.0316. Significant values are indicated in italics

A adult trees, *S* saplings

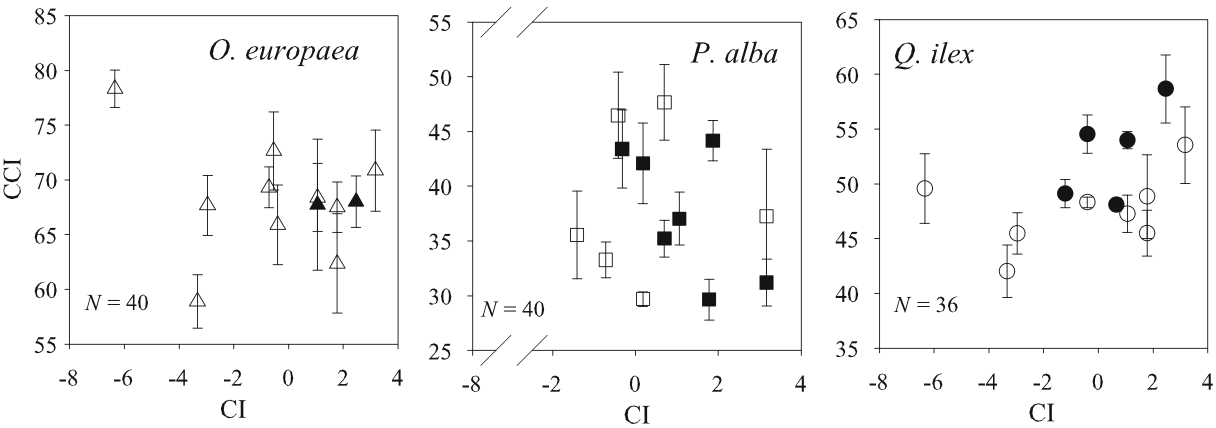


Fig. 4 Relation between Chlorophyll Content Index (CCI, mean±standard error) in the leaves of the studied species and the soil degradation level (indicated by a Contamination Index, CI). *Filled* and *open symbols* correspond to adult trees and saplings, respectively

An analysis at the site level revealed a negative relationship between soil contamination and the P concentrations in the leaves of *O. europaea* (Fig. 6). For this species, soil pH was positively correlated with P concentrations and negatively correlated with the N:P ratio (Fig. 6). Consequently, in the most contaminated sites, the P concentrations were much lower than in the unaffected sites. For example, the wild olive saplings growing on sites 4 and 10 (affected) had some 33% less P than those on unaffected sites.

For *P. alba*, there was an increase in leaf S with higher soil contamination levels (Fig. 7). Soil pH was highly correlated with the average leaf S at each site (Fig. 7) and showed marginally significant correlations ($0.05>p>0.004$) with leaf Mg (negative) and N:S (positive). For *Q. ilex*, we did not find any similar relationship between the soil contamination level and the leaf concentration of any nutrient.

3.3 Soil Factors Influencing Leaf Chlorophyll and Nutrient Concentrations

The models obtained by multiple regressions, considering each tree and its surrounding soil individually, reflect the influences of soil conditions on leaf chlorophyll and nutrients. In the case of P concentrations in the leaves of *O. europaea* adults, soil contamination was the only significant predictor in the model and explained a 40% of the variance of leaf P (Table 5). In these trees, contamination was also negatively related to the N:S ratio, although soil texture had a higher relative importance in the variance of this variable (indicated by the β coefficient of the model). In the *O. europaea* saplings, soil contamination negatively influenced the plants' chlorophyll content and the N:Mg ratio. For chlorophyll, the model was highly significant and explained a high percentage of the variance ($r^2=0.72$, $p<0.001$). For N:Mg ratio, soil pH was also a significant predictor and its influence was higher than that corresponding to contamination. In these saplings, soil pH positively influenced leaf Mg; thus, under acidic conditions the N:Mg increased, due to a probable decrease in Mg uptake. Likewise, there was a negative correlation between soil pH and the N:P ratio (Table 5).

In contrast to *O. europaea*, the uptake of some nutrients by *P. alba* responded positively to soil contamination or acidification. For example, soil contamination was a positive predictor for leaf K in adult trees; decreasing pH increased the leaf S and, marginally ($p>0.025$), the leaf Mg in the saplings. The exception was the N to P ratio: for this variable soil pH was a negative predictor, explaining a 30% of the variance; this indicates that, under acidic conditions, the trees showed a relative P deficiency.

Similarly to *P. alba* trends, contamination was positively correlated with leaf S in *Q. ilex* saplings and negatively with the N:S ratio.

3.4 Interactions Between Leaf Trace Elements and Chlorophyll and Nutrients

Some of the above-mentioned patterns were related with the interactions between non-essential elements (such as As, Pb, and Cd) and nutrients in the leaves of the trees. For example, in *O. europaea* saplings chlorophyll and

Fig. 5 Nutrient concentrations (mean and standard error) in the leaves of the studied species. *Gray* and *white bars* correspond to adult trees and saplings, respectively

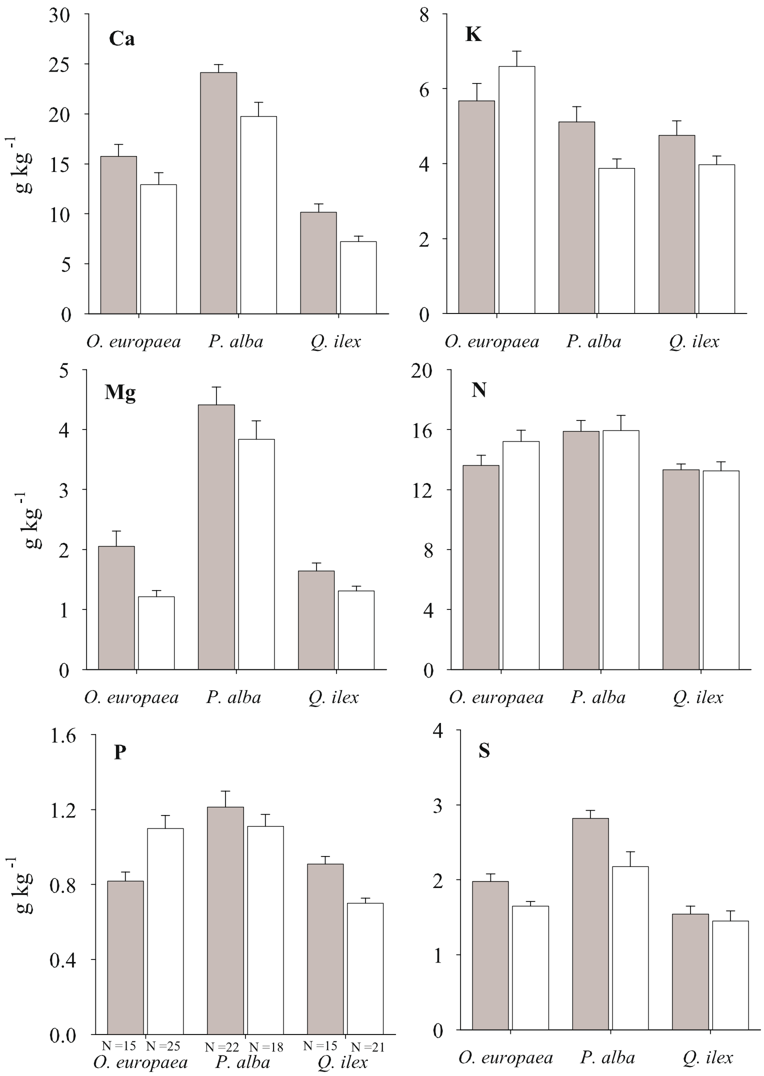


Table 4 Ratios between macronutrients (mean±standard error) in the leaves of the studied plant species

Ratio	*Olea europaea*		*Populus alba*		*Quercus ilex*	
	Adult (N = 15)	Sapling (N = 25)	Adult (N = 22)	Sapling (N = 18)	Adult (N = 15)	Sapling (N = 21)
N:K	2.70 ± 0.35	2.52 ± 0.23	3.45 ± 0.30	4.36 ± 0.24	3.02 ± 0.20	3.59 ± 0.29
N:Mg	7.85 ± 0.86	14.8 ± 1.4	4.03 ± 0.38	4.5 ± 0 .49	8.93 ± 0.79	10.9 ± 0.9
N:P	16.9 ± 0.6	15.0 ± 1.1	14.1 ± 1.0	14.9 ± 0.7	15.0 ± 0.6	19.2 ± 0.8
N:S	7.15 ± 0.50	9.34 ± 0.43	5.78 ± 0.32	7.78 ± 0.68	9.08 ± 0.56	10.2 ± 0.7

Significance levels in the comparison between adult trees and saplings are indicated in Table 3

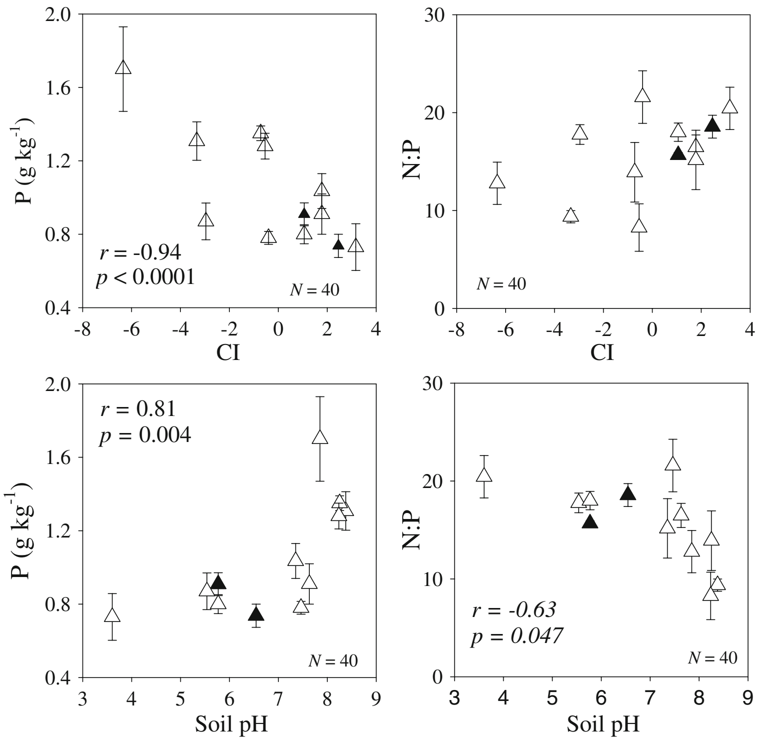


Fig. 6 Relationship between Contamination index (CI, *plots in top row*) or soil pH (*plots in bottom row*) and leaf P and N:P in the leaves of *O. europaea* (mean±standard error). *Filled* and *open symbols* correspond to adult trees and saplings, respectively. If significant (p_t=0.004), correlation coefficients and *p* values are indicated. *Values in italics* are marginally significant (0.05>*p*>0.004)

Pb showed a negative relationship (r=−0.73, p<0.001). Likewise, P was negatively correlated with As in the adult olive trees (r=−0.68, p=0.005), and, marginally, in the saplings (r=−0.436, p=0.037). A marginal influence of Bi on P levels was also observed for the saplings (r=−0.439, p=0.036). The K concentration showed a highly significant relationship with As in the leaves of the adult olive trees (r=−0.81, p<0.001).

In contrast, for the other two species there were some positive interactions between trace elements and some nutrients, especially for *P. alba*. For example, between K and Cu (r=0.62, p=0.002) and Tl and Mg (r=0.711, p=0.001) in adult trees. Again, the exception was related to leaf P, having a marginal and negative interaction between As and P in *P. alba* saplings (r=−0.57, p=0.016).

4 Discussion

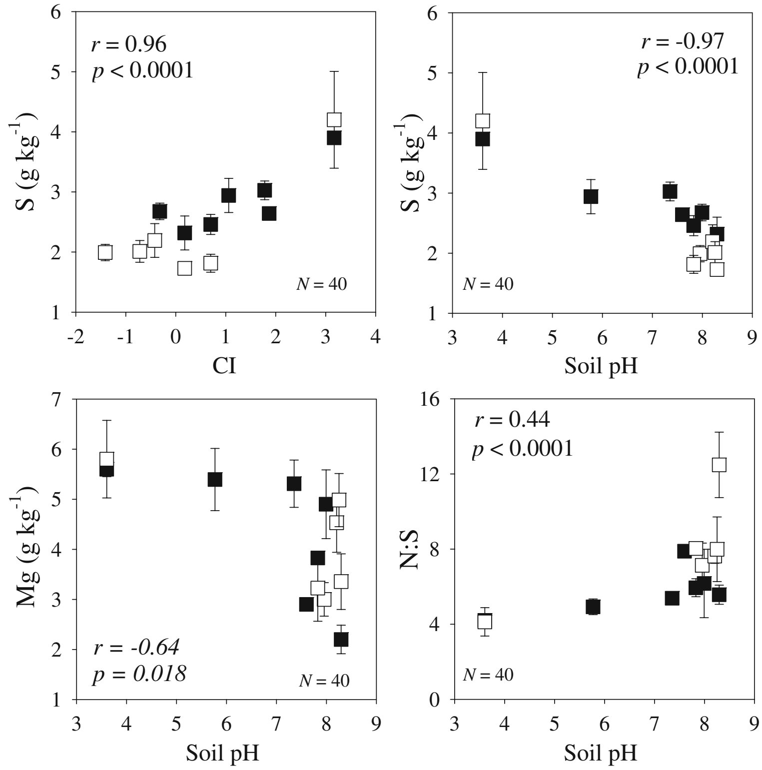
4.1 Nutritional Status of the Trees

Soil contamination frequently leads to nutrient disorders in plants. Thus, the monitoring of the nutritional status of trees should be addressed in polluted areas, since the enhanced nutrient deficiencies could limit the success of a restoration program. Tree nutritional and health status depends on many soil factors, which determine the availability of nutrients, as well as the bioavailability of potentially toxic elements. In this study, we assessed the nutritional status of three different tree species, growing in afforested sites with different degree of soil degradation (elevated trace element concentrations and acidification).

We found some evidences of nutrient deficiencies in the studied species. In general, the afforested saplings had lower nutrient concentrations than the adult trees. For adult *O. europaea*, N and K leaf concentrations were lower than the values reported for trees growing on uncontaminated sites (Madejón 2004); and were below the optimal thresholds for cultivated olive (according to Fernández-Escobar 1997). At some affected sites, the N:P ratio in both adult and sapling trees of *O. europaea* was above 16, indicating a P limitation (Koerselman and Meuleman 1996).

The concentrations of all macronutrients in *P. alba* (in both adult trees and saplings) were suboptimal (according to Kopinga and van den Burg 1995). However, the nutrient levels were similar to those

Fig. 7 Relationship between Contamination index (CI) or soil pH on each sampling site and leaf S and Mg and N:S ratio in the leaves of *P. alba* (mean± standard error). *Filled* and *open symbols* are adult trees and saplings, respectively. *Values in italics* are marginally significant (0.05>*p*>0.004)



reported for adult trees growing in a nearby uncontaminated riparian forest (Madejón et al. 2004).

The leaf concentrations of N and P in *Q. ilex* saplings were below the levels reported for seedlings of the same species, but growing under optimal conditions (Cornelissen et al. 1997). Mean N:P ratios in saplings over the whole study area were much higher than 16 (Table 3), indicating a strong P limitation (Koerselman and Meuleman 1996).

4.2 Influence of Soil Conditions on Leaf Nutrients and Chlorophyll

In the Guadiamar Green Corridor we found a high level of heterogeneity in soil properties, 7 years after the soil remediation works, which partly explained the differences in the concentrations of some nutrients in the trees growing there.

The soils in the affected area still presented high concentrations of trace elements, with a high variability even within each site, due to the irregular sludge deposition and the irregular cleanup of the soils. For example, in a same site soils beneath adult individuals were more contaminated and had lower pH than those beneath afforested saplings, due to the difficulty of removing the sludge and topsoil from forested areas (data not shown). The bioavailability of the cationic trace elements in the Guadiamar area is primarily determined by soil pH, with soil organic matter content or soil texture having little influence (Domínguez et al. 2009). Thus, in the acidic sites the interactions between soil contaminants and plant nutrition may be greater. Soil pH was one of the most important soil factors explaining nutrient variability in the leaves of trees.

A decrease in soil pH was associated with contamination, due to the leaching of acids generated by the oxidation of sulfides in the remnant of sludge in the soils (Kraus and Wiegand 2006). Sugar beet lime, which had 70–80% $CaCO_3$, was the most used amendment. The application rates during the remediation work were higher in the most contaminated sites (Arenas et al. 2008). Nevertheless, the most contaminated sites were highly acidified and carbonate content was lower than 1% (Domínguez et al. 2008). Therefore, the carbonates supplied in the amendments may have already been attenuated by acid generation from the contaminating sludge. In contrast, the

Table 5 Results of the General Multiple Regression Models, where either the Contamination Index (CI) or soil pH significantly affected the leaf Chlorophyll Content Index (CCI) or nutrient concentrations

Species	Leaf variable	Soil predictor	β	*p*	Model adjusted r^2	Model *p*
O. europaea adults	P	CI	−0.53	0.032	0.39	0.025
N = 15	N	CI	*−0.49*	*0.049*	*0.43*	*0.033*
		Clay	*0.70*	*0.010*		
	S	CI	*0.53*	*0.048*	*0.23*	*0.048*
	N:S	CI	−0.67	0.002	0.65	0.003
		Clay	0.70	0.002		
O. europaea saplings	CCI	CI	−0.47	0.007	0.72	<0.001
N = 25		Clay	−0.61	0.003		
		OM	−0.46	0.002		
	K	Clay	−0.52	0.008	0.66	<0.001
		pH	−0.89	<0.001		
		P	0.34	0.021		
		K	1.21	<0.001		
	Mg	pH	0.73	0.006	0.32	0.022
		P	−0.55	0.014		
		K	−0.64	0.012		
	N:P	pH	−0.61	<0.001	0.62	<0.001
		P	0.68	<0.001		
	N:Mg	CI	−0.39	0.047	0.50	0.003
		pH	−0.74	0.002		
		P	0.71	<0.001		
		K	0.86	0.003		
P. alba adults	K	CI	0.97	0.013	0.37	0.009
N = 22		pH	0.53	0.047		
	S	pH	−0.46	0.014	0.70	<0.001
		N	0.28	0.046		
	N:K	CI	*−0.79*	*0.012*	*0.24*	*0.047*
	N:P	pH	−0.57	0.010	0.25	0.024
P. alba saplings	Mg	pH	*−0.56*	*0.030*	*0.26*	*0.030*
N = 18	S	pH	−0.81	<0.001	0.87	<0.001
		OM	0.31	0.030		
	N:P	pH	−0.61	0.020	0.32	0.020
Q. ilex saplings	S	CI	0.73	<0.001	0.65	<0.001
N = 21	N:S	CI	−0.68	<0.001	0.43	<0.001

Significance level (after controlling the FDR at the 5% level) was p_t=0.025. Values in italics are marginally significant (0.05>*p*<0.025)

nutrient concentrations, particularly P, in the soils still reflected the application of the amendments. On average, the spill-affected sites had significantly higher Olsen-P than non-affected sites (data not shown). Some 40% of the soils had P concentrations higher than 20 mg kg^{-1}, which is significantly higher than the background values for this region. Contaminated soils in central areas had P concentrations up to 100 mg kg^{-1} (Table 1). This reflects the high P content of the sugar beet lime (up to 5.1 g kg^{-1}, Madejón et al. 2006). Under experimental conditions, the addition of sugar beet lime to soils from the

Guadiamar Green Corridor resulted in the enrichment in P to levels up to three times higher than P levels in the unamended soils, 30 months after application. The relative increase of nutrient concentrations after application was higher for P than for other nutrients (Pérez-de-Mora et al., 2007).

Some of the detected nutrient deficiencies, particularly P, in the afforested trees were significantly related to either the level of soil contamination or to soil acidification. For example, there was a strong negative interaction between the index of soil contamination and the P uptake for *O. europaea*.

There are several possible explanations for these negative interactions among trace elements and nutrients. Arsenic may interfere with plant P uptake, since arsenate and phosphate are analog ions; they compete in the soil matrix and As is transported across the plasma membrane through phosphate transport systems (Meharg and Hartley-Whitaker 2002). Arsenic was one of the most important soil contaminants in the Guadiamar Green Corridor; the mean As concentration of 130 mg kg^{-1} and maximum of 340 mg kg^{-1} (Appendix 1) were high in comparison with P concentrations (mean of 23 mg kg^{-1} and maximum of 100 mg kg^{-1}, Table 1). In fact, for *O. europaea* we found negative correlations at the leaf level between As and P; therefore, some competitive interaction may be taking place at the root level. However, this effect would be greater on basic soils, since anionic trace elements such as arsenate are more mobile at higher pH (Adriano 2001). In contrast, we found that trees growing in acidic sites showed the highest P deficiencies.

This could be explained by a decrease in P availability in acid soils, due to increases in P sorption capacity and decreases in the rate of organic phosphorus mineralization (Paré and Bernier 1989; Carreira et al. 1997). Oxides of Al, Fe, and Mn dominate P sorption in acid soils. Under acidic conditions, P sorption by Fe oxides could thus reduce the P availability. However, acidity and contamination were not related to P availability in the studied soils. Therefore, the decrease in the P concentrations in the leaves of some plants (mainly *O. europaea*) in the most degraded sites cannot be atributable to a limited availability of soil P, but to some interaction between soil degradation and P uptake by roots or P translocation to the leaves.

The toxicity at the root level may be hampering the nutrient acquisition in the most contaminated and acidic soils, since there the solubility of toxic trace elements is higher at low pH (Greger 1999). Several studies with woody species have reported root damage by trace elements, but low contaminant transport from roots to shoots (Arduini et al. 1996; Wisniewski and Dickinson 2003; Fuentes et al. 2007b). However, non-specific morphological damage at the root level may hamper the uptake of several nutrients, and we have found that P uptake was reduced, while the uptake of other nutrients was enhanced in the most acidic and contaminated sites.

That P is the most affected nutrient may be due to the precipitation of phosphates with soluble cationic trace elements in the rhizosphere (Rolfe 1973; Ruby et al. 1994), which might be enhanced in the most contaminated and acidic sites. The precipitation of cationic elements with phosphate can reduce the bioavailability of these elements in soils, ameliorating their toxicity and reducing the accumulation of trace elements in the leaves (Brown et al. 2004; Bosso et al. 2008). This mechanism of trace element-phosphate interaction could contribute to keep the low trace element accumulation in the leaves of *O. europaea*, even in highly contaminated and acidic sites.

For the other two species, *Q. ilex* and *P. alba*, the interactions between soil degradation and P uptake were less clear. Nevertheless, there were also some effects of soil contamination on the N:P ratios of the leaves. The reason why *O. europaea* is the most sensitive species, in terms of P nutrition, to soil contamination is unclear and deserves further research. Different plant species can have different mechanisms for P uptake and *O. europaea* may be less efficient in accessing P from metal–phosphate complexes than the other two studied species. In general, phosphorus is a frequent limiting nutrient in Mediterranean ecosystems and can limit the growth of Mediterranean woody plant seedlings (Sardans et al. 2004; Villar-Salvador et al. 2004). Thus, the inhibition of P uptake by soil degradation could hamper the development of a woody plant cover on this remediated and afforested area.

In contrast to the negative effects on phosphorus nutrition, for *P. alba* (and to a lesser extent also for *Q. ilex*) the altered soil conditions (with high level of trace elements and low pH) enhanced the uptake of several nutrients. This unexpected result may be a short-term result of soil acidification. In forest soils with a pH<4, Ca^{2+}, Mg^{2+}, and K^{+} are released to the soil solution

(Rehfuess 1989; Foster et al. 1989). In the short-term, this may enhance the nutrient uptake and tree growth (Likens et al. 1996; Tomlinson 2003). *P. alba* (a fast-growing deciduous tree) may respond faster to this release of cationic nutrients than *O. europaea* and *Q. ilex* (slow-growing evergreen trees). However, if cation inputs to the soil are low, a possible long-term loss of cationic bases due to soil acidification may negatively influence the uptake of cationic nutrients by the trees in the future (Likens et al. 1996; Tomlinson 2003).

In general, the chlorophyll content was not sensitive to the nutritional changes induced by soil degradation. In particular, chlorophyll content did not reflect the P deficiencies, which were the main detrimental effect of soil degradation on tree nutrition. The only significant interaction between chlorophyll and soil contamination occurred for *O. europaea*, coinciding with a decrease in leaf Mg, and therefore with an alteration in the N:Mg ratio. Leaf N and Mg are mostly contained in chlorophyll, so changes in chlorophyll content may be more related to N or Mg limitations. The use of the chlorophyll estimates (CCI) as a rapid and non-destructive measurement of tree health may become more important if some Mg deficiencies appear in the future, as a possible long-term effect of soil degradation.

5 Conclusions

There are nutrient deficiencies, particularly of phosphorus, in the trees growing in many sites of the Guadiamar Green Corridor. Soil contamination and subsequent soil acidification contribute to these P deficiencies, especially for *O. europaea*. High levels of available P were found on affected soils. Since there were no important soil nutrient limitations in these afforested lands, the benefits of soil fertilization are likely to be small. Soil pH was found to be the most important factor affecting the nutritional status of the trees. In contrast to P, the uptake of some cationic nutrients was enhanced by soil acidification, especially in *P. alba*. Soil pH monitoring in the area is advisable, since acidification may led to nutrient disorders in the trees, influencing the long-term growth and ecosystem functioning in the Guadiamar Green Corridor.

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Appendix

Concentration of trace elements in soil and leaves (adapted from Domínguez et al. 2008).

Table 6 Mean and range (minimum–maximum) of total concentrations (mg kg^{-1}) of trace elements in soils in the Guadiamar Green Corridor, from spill-affected and unaffected sites

	Surface soils (0–25 cm)		Deep soils (25–40 cm)	
	Affected (*N*=100)	Unaffected (*N*=17)	Affected (*N*=100)	Unaffected (*N*=17)
As	129 (49–339)	17 (13–20)	95 (18–438)	16 (14–17)
Bi	1.64 (0.57–5.40)	0.30 (0.15–0.40)	1.35 (0.33–4.29)	0.25 (0.18–0.35)
Cd	1.44 (0.44–3.05)	0.23 (0.07–0.37)	1.27 (0.22–3.28)	0.17 (0.10–0.25)
Cu	115 (66–198)	32 (13–43)	110 (30–238)	24 (14–31)
Pb	210 (73–607)	47 (15–65)	179 (38–519)	31 (18–38)
Tl	1.17 (0.55–4.02)	0.29 (0.16–0.43)	0.82 (0.20–3.15)	0.27 (0.23–0.33)
Zn	457 (183–768)	109 (47–149)	376 (103–954)	82 (53–99)

Table 7 Trace element concentrations (mean±standard error) in leaves of adult trees and afforested saplings from affected sites in the study area

	O. europaea		*P. alba*		*Q. ilex*	
	Adult (N = 15)	Sapling (N = 16)	Adult (N = 22)	Sapling (N = 18)	Adult (N = 15)	Sapling (N = 14)
As	0.42 ± 0.07	0.22 ± 0.03	0.63 ± 0.06	0.34 ± 0.05	0.59 ± 0.07	0.54 ± 0.14
Bi	0.016 ± 0.03	0.034 ± 0.010	0.018 ± 0.005	0.010 ± 0.03	0.036 ± 0.004	0.016 ± 0.001
Cd	0.06 ± 0.01	0.08 ± 0.02	4.90 ± 0.58	0.96 ± 0.13	0.14 ± 0.02	0.29 ± 0.09
Cu	6.55 ± 0.55	7.32 ± 0.80	8.16 ± 0.47	8.07 ± 0.54	13.1 ± 0.9	7.18 ± 0.31
Pb	1.02 ± 0.09	0.77 ± 0.07	1.28 ± 0.06	1.12 ± 0.08	3.18 ± 0.40	1.72 ± 0.21
Tl	0.014 ± 0.003	0.013 ± 0.003	0.053 ± 0.012	0.007 ± 0.002	0.029 ± 0.009	0.013 ± 0.003
Zn	57.0 ± 5.2	28.2 ± 2.5	605 ± 45	176 ± 22	83.5 ± 8.4	76.2 ± 16.4

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