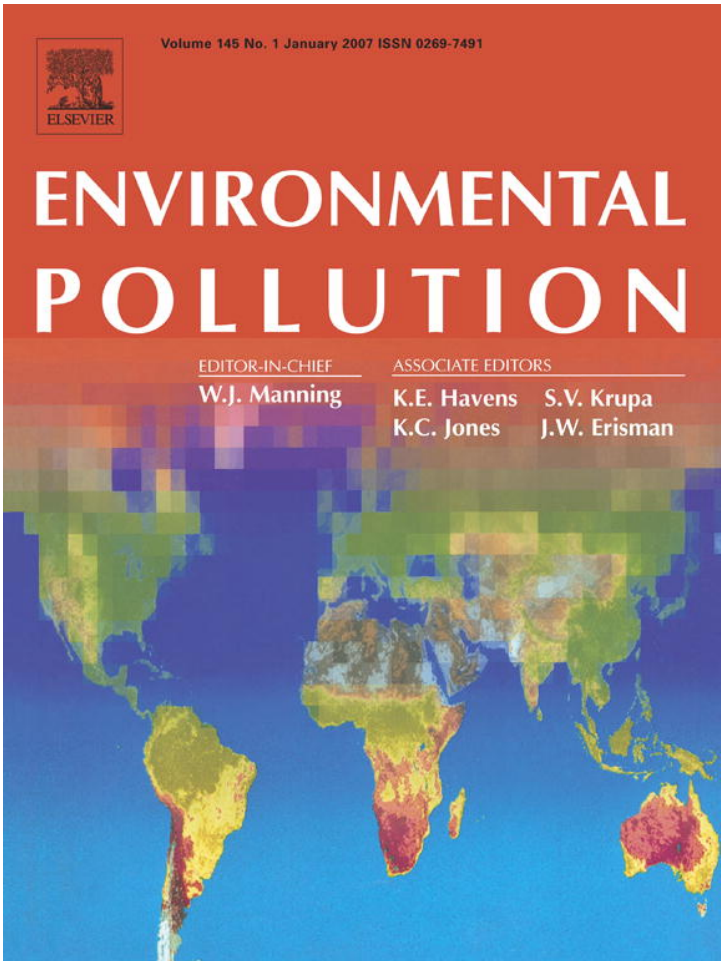
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Response of *Pinus halepensis* Mill. seedlings to biosolids enriched with Cu, Ni and Zn in three Mediterranean forest soils

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Biosolid-borne Cu, Ni and Zn did not show negative effects on Pinus halepensis seedlings performance after application on three Mediterranean forest soils.

Abstract

We investigated the response of *Pinus halepensis* seedlings to the application of biosolids enriched with Cu, Ni and Zn on three Mediterranean forest soils under semiarid conditions. One-year-old seedlings were planted in lysimeters on soils developed from marl, limestone and sandstone which were left unamended, amended with biosolids, or amended with biosolids enriched in Cu, Ni and Zn. Enriched biosolids increased plant heavy metal concentration, but always below phytotoxic levels. Seedlings receiving unenriched biosolids showed a weak reduction in Cu and Zn concentration in needles, negatively affecting physiological status during drought. This effect was alleviated by the application of enriched sludge. Sewage sludge with relatively high levels of Cu, Zn and Ni had minor effects on plant performance on our experimental conditions. Results suggest that micronutrient limitations in these soils may be alleviated by the application of biosolids with a higher Cu, Zn and Ni content than those established by current regulations.

Keywords: Pinus halepensis; Biosolids; Heavy metals; Photosynthesis

1. Introduction

Forest soils in the Mediterranean basin have been intensively exploited (Naveh, 1982), resulting in the loss of soil organic matter and nutrients (Vallejo et al., 2000). Decreases in soil fertility may constraint spontaneous vegetation recovery once human pressure is released (Vallejo and Alloza, 1998), and the same would apply to restoration actions (Serrasolses and Alloza, 2004). Thus, increases in soil fertility may be needed to foster ecosystem recovery. Biosolids represent an easily accessible source of organic matter and nutrients that can be used to restore degraded ecosystems. Sewage sludge reutilization has been recommended as the best practicable environmental option (Hall, 1999) for the management of this organic residue, in spite of the fact that environmental risks may impose restrictions on its use (European Commission, 2000). Detailed information on the effects of biosolids on ecosystems is needed to maximize the benefits and minimize the environmental risks of biosolid use in ecological restoration.

Biosolids contain variable amounts of heavy metals which could compromise plant growth. Woody species can be very sensitive to moderate concentrations of heavy metals. These elements may reduce biomass accumulation in tree seedlings

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(Kukkola et al., 2000), inhibit root growth (Arduini et al., 1995; Hartley et al., 1999), decrease the availability of essential elements (Kabata-Pendias and Pendias, 1992), and modify root morphology and architecture, compromising root capacity to explore soils (Arduini et al., 1994). The excess or deficiency of essential metals may also inhibit protein and enzyme function, and thus impair photosynthetic electron transport at the reaction centers (Cakmak, 2000; Maksymiec, 1997). Heavy metals may indirectly affect seedling performance by reducing plant ability to access and transport soil resources, particularly water (Barceló and Poschenrieder, 1990).

However, most studies on the effects of heavy metals on tree seedlings have been performed under laboratory and greenhouse conditions, and extrapolations to field conditions are difficult. For example, dryland soils are frequently carbonated (Fuller and Tucker, 1977) and heavy metal availability greatly reduced (Brallier et al., 1996; Illera et al., 2000). Periodic soil drying and low moisture availability may also affect heavy metal availability (Pascual et al., 2004). Despite the urgent need for a knowledge-based biosolid application, there is little information on the effect of heavy metal contaminated biosolids on Mediterranean woody plants.

This paper focuses on the short-term effect of biosolids enriched with Cu, Ni and Zn on the morphology and physiology of *Pinus halepensis* seedlings planted under semiarid Mediterranean conditions. *Pinus halepensis* is one of the most widespread tree species in the Mediterranean basin, and indeed one of the few species that can thrive under semiarid conditions. The experiment was performed on three contrasted soil types, representing common forest soils in the Mediterranean basin.

2. Materials and methods

2.1. Experimental design

The experiment was carried out in Alicante (E Spain), under semiarid conditions (mean annual rainfall 310 mm, mean annual temperature 17.5 °C). We selected three soil types common in the Mediterranean basin. We used a *Regosol* derived from marl with high carbonate content, loamy texture and high pH (8.4); a *Rendzic Leptosol* derived from limestone, decarbonated brown-red earth with clayey texture and pH 8.4, and a *Typic Haploxerept*, loamy soil developed from red sandstone, with slightly acidic pH (5.9) and lower OM content than the previous two soils (Table 1). The two basic soils were collected from Ayora (Valencia), and the soil developed from sandstone was collected from Sierra de Espadán (Castellón). For clarity we will refer to them according to their dominant lithology, i.e., Marl, Limestone and Sandstone soils, respectively. The top 50 cm layer of each soil was collected and homogenized by sieving through a 2 cm mesh. We used each soil type either unamended (Control), mixed with a biosolid from a domestic water treatment plant, or mixed with a heavy metal-enriched biosolid (Table 2). We artificially increased the heavy metal content of the biosolid by adding a heavy metal solution to a fresh, anaerobically stabilized biosolid rather than using a biosolid from waters contaminated in origin. In this way, we prioritized control on the heavy metal content of the biosolid. The heavy metal solution contained $ZnSO_4$, $CuSO_4$ and $NiSO_4$ to attain a concentration that was well above the level permitted in European and Spanish regulations (Council Directive 86/278/EEC, 1986) for agricultural application of biosolids (1750 mg Cu kg^{-1}, 400 mg Ni kg^{-1} and 4000 mg Zn kg^{-1}, dry weight of biosolid; Table 2). We selected Cu, Ni and Zn because they frequently limit the agricultural application of biosolids in the Region of Valencia. Unaltered and enriched biosolids were allowed to slowly dry down to 40% moisture content, and then were thoroughly mixed with the soil.

We filled fifteen 38 × 38 × 70 cm lysimeters per soil type with, from bottom to top, a 10 cm layer of limestone gravel, a 20 cm layer of unamended soil, and a 30 cm layer of either unamended soil, a homogeneous mixture of soil with dried biosolid, or a mixture of soil with dried biosolid that was previously enriched in heavy metals (a total of 45 lysimeters, 5 replications per treatment).

The biosolid application rate corresponded to 60 Mg ha^{-1} (850 g d.w per lysimeter). Previous studies have shown this to be an adequate dose for woody seedlings (Berry, 1977; Roldán et al., 1996). In February 2003 we planted one 1-year-old *Pinus halepensis* seedling per lysimeter. Seedlings came from a public nursery (Sta. Faz, Alicante; Spain), 10 km from the area were the lysimeters were located. They were grown on 350 cm^3 forest containers filled with peat and cocopeat. At the time of planting, current year needles had not yet flushed. The lysimeters were rainfed, except for two waterings applied at planting, and in late summer (25 mm each).

Table 1
Basic physical-chemical properties of the three top soils used in the experiment, and total soil concentration of Zn, Cu and Ni (extracted with aqua regia) in control soils (C), soils amended with biosolid (SS), and soils amended with biosolid enriched with heavy metals (SSM) (Toribio and Romanyà, 2005; in press)

Soil	Treatment		Marl	Limestone	Sandstone
Sand		%	40	23	43
Silt		%	40	34	40
Clay		%	20	43	17
CEC		cmol + kg^{-1}	3.8	10.0	3.9
$CaCO_3$		%	51	15	4
pH (H_2O)			8.4	8.4	5.9
TOC		%	2.8	2.4	0.4
Cu	Control	mg kg^{-1}	1.6	12.0	7.0
	SS		3.3	14.0	8.0
	SSM		24.0	44.0	31.0
Ni	Control	mg kg^{-1}	6.3	29.0	16.0
	SS		6.5	30.0	18.0
	SSM		12.0	35.0	20.0
Zn	Control	mg kg^{-1}	13.0	52.0	10.0
	SS		17.0	56.0	14.0
	SSM		66.0	122.0	64.0

Table 2
Biosolid properties before and after the application of a solution enriched with Cu, Ni and Zn

pH		7.4
Moisture (%)		63
C (%)		32
N (%)		2.0
P (%P)		1.2
K (%K)		0.5
Ca (% Ca)		15
Mg (% Mg)		0.5
Fe (mg kg^{-1})		10 668
	Unenriched	Enriched
Cd (mg kg^{-1})	<0.4	<0.4
Cu (mg kg^{-1})	101	2098
Ni (mg kg^{-1})	16	666
Pb (mg kg^{-1})	34	34
Zn (mg kg^{-1})	395	5377
Hg (mg kg^{-1})	2.6	2.6
Cr (mg kg^{-1})	53	53

2.2. Soil measurements

We monitored soil volumetric water content by Time Domain Reflectometry (TDR Tektronik 1502C Cable Tester) with one vertical set of probes (0 to 20 cm depth), and two horizontal sets of probes, at 20 cm and 35 cm depth, respectively. By the end of the experiment, soil salinity (0–30 depth) was estimated from the electrical conductivity of extracts from a saturated soil-paste (Rhoades, 1982), and soil pH (0–30 depth) was determined from a 1:2.5 (w/v) solution of soil and deionized water. For further details of soil analyzes see Toribio and Romanyà (2005; in press).

2.3. Evaluation of seedling performance

We evaluated seedling performance by measuring seedling morphology, biomass allocation, gas exchange, chlorophyll fluorescence and concentration of heavy metals in current-year stem and roots.

A Li-Cor 6400 portable closed photosynthesis system (Li-Cor Inc. Nebraska, US) was used to measure photosynthetic rate (A) and transpiration (E). Gas exchange measurements were conducted once in May and twice in August, before and after watering, in all seedlings. Sampled needles were scanned to estimate needle surface area (see below). A portable fluorometer PAM-2001 (Walz, Effeltrich, Germany) was used to measure photochemical yield (ϕ_{PSII}) on the same dates.

In October 2003 we harvested all seedlings and carefully washed the root system. We separated aboveground tissues grown during the experiment (hereafter 'new shoots') from those grown in the nursery. Roots colonizing the soil were separated from those confined in the original root plug. The needles of the new shoots and the roots colonizing the soil were digitized by scanning on an A3 flatbed scanner (HP Deskscan) fitted with a transparency adaptor at 300 dpi, using an 8-bit grayscale. We analyzed the images with specific software (WinRhizo, Regent Instruments, Québec, Canada) to obtain needle and root surface area and length.

2.4. Heavy metal concentration in plant tissues

All biomass fractions were oven dried at 65 °C for two days and the dry weight determined. Specific root length was calculated in the new roots colonizing the soil, as the ratio between root length and root biomass, and specific needle weight was calculated in the new shoots as the ratio between needle biomass and needle surface area. The current year tissues were digested in a heating block at 250 °C with a mixture of sulfuric acid and hydrogen peroxide (1:1, v:v) (Jones and Case, 1990). Digests were analyzed for Cu, Ni and Zn by ICP-OES (Perkin Elmer Optima 4300 Inductively Coupled Plasma – Optical Emission Spectrometry). *Olea europaea* leaf standard reference materials (BCR: CRM 062, Commission of the European Communities Bureaus of Reference, Brussels) were digested and analyzed for quality control.

2.5. Statistical analysis

We used analysis of variance (soil type and biosolid amendment as fixed factors, with three levels each) to evaluate treatment effects on seedling performance. When results showed a significant treatment effect, we used Tukey's HDS test to establish pairwise comparisons between levels of each factor. We used regression analysis to evaluate the degree of covariation between dry weight and heavy metal concentration in new roots. All analyses were performed by using SPSS 10.0 (SPSS Inc., 1999).

3. Results

3.1. Soil properties

After eight months, soil EC was higher in amended soils than in control soils ($F = 53.49$, $P < 0.001$). Anyway, EC was always ≤2 dS m^{-1}, hence no strong osmotic effect should be expected. Heavy metal enrichment had no effect on soil salinity. Treatments did not affect soil pH in any soil type ($F = 2.20$, $P = 0.139$) (Table 3). Soil moisture was highest on Limestone and lowest on Sandstone, according to expected water holding capacity differences related to soil texture. We found no overall effect of biosolid amendment on soil moisture content. A significant Soil × Treatment interaction reflected that biosolid application significantly increased soil moisture at the 20 cm depth in Limestone soils immediately after watering ($F = 7.99$, $P = 0.06$) and seven days later ($F = 7.99$, $P = 0.006$) (Fig. 1). One month later, soil moisture content was still higher, but differences were marginally significant ($F = 3.01$, $P = 0.09$).

3.2. Bioaccumulation of heavy metals

No effect was found of soil type on Cu and Ni concentration in new shoots, but seedlings planted on Limestone showed higher Zn concentration in this biomass fraction. New shoots concentration of Cu and Zn increased in seedlings amended with enriched biosolid (Table 4). Significant increases ranged from more than 100% (Cu in seedlings planted on Marl) to less than 20% (Zn in seedlings planted on Limestone). Interestingly, the unenriched biosolid application resulted in a trend towards lower Cu and Zn concentrations in this fraction, which was apparently compensated by the incorporation of heavy metals in biosolid. We found no significant effect of the treatments on Ni concentration in new shoots. Ni concentrations were low, particularly in control seedlings planted on Marl, where they were close to the detection limit (0.05 mg kg^{-1}).

Cu, Zn and Ni concentrations in the current year tissues were substantially higher in roots than in needles, particularly in seedlings receiving enriched biosolid. The heavy metal concentration in both the new roots of seedlings planted on control soils and the soils amended with unenriched biosolid was similar. Increases in the heavy metal concentration in

Table 3
Soil electrical conductivity in a saturated paste (EC) and pH eight months after planting on three Mediterranean forest soils with no amendment (C), amended with biosolid (SS), and amended with biosolid enriched with metals (SSM)

Soil	EC (dS m^{-1})			pH		
	C	SS	SSM	C	SS	SSM
Marl	0.79 ± 0.03a	1.79 ± 0.20b	1.94 ± 0.16b	8.1 ± 0.0a	7.9 ± 0.1a	7.9 ± 0.0a
Limestone	0.88 ± 0.06a	2.07 ± 0.19b	2.03 ± 0.19b	8.0 ± 0.0a	7.7 ± 0.2a	7.9 ± 0.0a
Sandstone	0.64 ± 0.04a	1.42 ± 0.12b	1.87 ± 1.15b	7.2 ± 0.2a	7.1 ± 0.1a	7.1 ± 0.2a

Means and standard errors of $n = 5$ replicates are shown. Different letters within a single row indicate significant differences ($P < 0.05$).

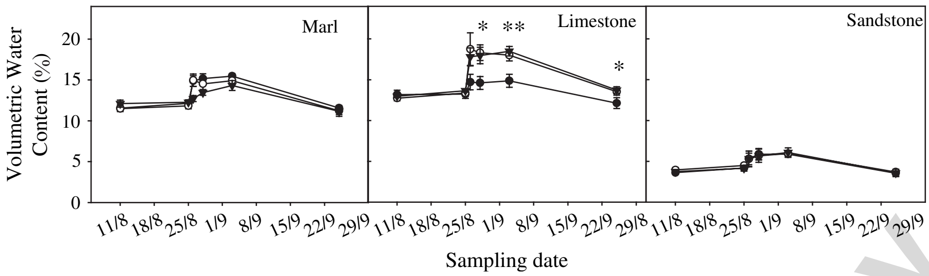

Fig. 1. Changes in volumetric water content in the three soil types: amended with biosolid (triangles), biosolid enriched with heavy metals (white circles) and unamended soil (black circles), at 20 cm depth. An experimental watering was applied on August 26th. Data represent means and standard errors of $n = 5$. Significances of ANOVA are explained in the text (*$P < 0.1$, **$P < 0.01$).

the new roots of seedlings amended with enriched biosolid ranged from less than 100% (Zn on Sandstone) to a 17-fold increase in the Cu concentration of plants growing on Marl. The increased Ni concentration in roots of seedlings amended with enriched biosolid was significant only on Marl.

3.3. Seedling physiology and morphology

Physiological status was strongly influenced by soil type. Seedlings planted on Marl performed better than those planted on Limestone or Sandstone during the peak of summer, as reflected by the higher net photosynthesis rate and photochemical yield (Fig. 2). During the period of maximum drought, unenriched biosolid had a marginal negative effect on net photosynthesis ($F = 2.87$, $P = 0.074$). This effect disappeared in plants amended with biosolid enriched in heavy metals. Similarly, quantum yield was consistently higher in seedlings amended with enriched biosolid than in those amended with unenriched biosolid and control seedlings ($F = 6.283$, $P = 0.010$).

Response to watering was highly dependent on soil type. Net photosynthesis rate and quantum yield increased in seedlings planted on Sandstone after irrigation; thus, by the second sampling date, photosynthesis rate and quantum yield were higher in these seedlings than in those planted on Marl. Seedlings planted on Limestone showed no changes in physiological status 5 days after watering.

We found no significant effect of soil type or treatment on seedling physiological status in April, when growing conditions were optimum (data not shown), or after the August watering.

We found a significant effect of soil type on total biomass accumulation, and a marginal effect of soil type on root collar diameter (Table 5). Seedlings growing on soils developed from Marl grew faster than seedlings planted on Limestone or Sandstone, and showed higher potential for soil colonization. Biomass allocation belowground was also higher in seedlings growing on Marl than in those planted on other soil types, as reflected by a higher root weight ratio (RWR). Seedlings planted on Marl showed higher specific root length.

We found no significant effect of enriched or unenriched biosolid, on aboveground total biomass and morphology. The significant Soil × Treatment interaction in root biomass accumulation reflected the opposite effect of amendments on seedlings planted on Limestone (increased biomass in amended seedlings) and Marl (decreased biomass in amended seedlings).

Table 4
Cu, Ni and Zn concentration in current year tissues of *Pinus halepensis* seedlings eight months after planting on three Mediterranean forest soils with no amendment (C), amended with biosolid (SS) or amended with biosolid enriched with heavy metals (SSM)

Soil	Treatment	Cu (mg kg^{-1})		Ni (mg kg^{-1})		Zn (mg kg^{-1})	
		Shoot	Root	Shoot	Root	Shoot	Root
Marl	C	1.0 ± 0.1	1.5 ± 0.4	0.05 ± 0.01	0.3 ± 0.1	13.3 ± 1.6	39.5 ± 7.4
	SS	1.6 ± 0.2	7.8 ± 0.8	0.05 ± 0.01	0.8 ± 0.2	14.8 ± 1.8	44.4 ± 11.3
	SSM	2.2 ± 0.2	25.8 ± 3.2	0.05 ± 0.01	5.1 ± 0.5	20.9 ± 1.2	86.0 ± 6.2
Limestone	C	1.4 ± 0.2	3.3 ± 0.6	0.66 ± 0.53	1.1 ± 0.4	20.3 ± 2.2	40.0 ± 4.6
	SS	1.3 ± 0.2	10.2 ± 4.2	0.27 ± 0.16	5.8 ± 3.7	17.1 ± 3.7	47.1 ± 8.3
	SSM	2.0 ± 0.1	30.0 ± 6.9	0.05 ± 0.01	4.4 ± 2.3	23.8 ± 1.6	73.2 ± 17.2
Sandstone	C	2.0 ± 0.3	4.6 ± 1.2	0.70 ± 0.31	2.8 ± 1.3	18.2 ± 1.5	47.3 ± 8.4
	SS	0.9 ± 0.1	3.7 ± 1.2	0.57 ± 0.24	6.9 ± 1.2	14.7 ± 0.9	32.9 ± 11.9
	SSM	2.6 ± 0.3	18.4 ± 2.1	0.75 ± 0.22	9.5 ± 0.6	23.5 ± 2.1	65.3 ± 6.2
2-way ANOVA							
	Treatment	19.31**	42.38**	0.64	5.98*	11.39**	10.57**
	Soil type	1.38	0.82	5.14	16.51**	3.36*	0.48
	T × S	4.66**	3.26*	0.64	1.72	0.62	0.99

Means, standard errors of $n = 4$–5 replicates and results of a two-way ANOVA are shown. Asterisks indicate significant differences at $P < 0.005$ (**) or $P < 0.05$ (*) (Tukey's test, $P < 0.05$).

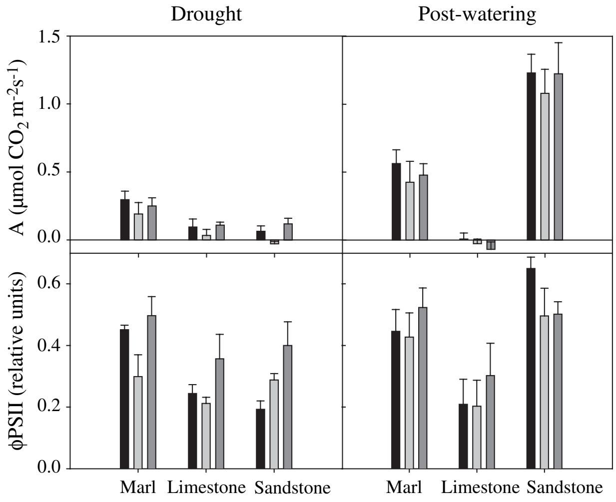

Fig. 2. Net photosynthesis (A) and photochemical yield (ϕPSII) in *Pinus halepensis* seedlings amended with biosolid (light grey), biosolid enriched with heavy metals (dark grey) or unamended (black bars). Measurements were performed on August 11th during the peak of summer drought. Data represent means and standard errors of $n = 4–5$.

Biomass of roots colonizing the soil was negatively affected by the application of biosolid enriched in heavy metals on Marl, but not on the other soil types (Fig. 3). The negative relationship found between the EC in soils developed from Marl and the amount of roots colonizing the soil ($r^2 = 0.639$, $P = 0.001$, $n = 14$) suggests that root growth was inhibited by salinity, perhaps by transient peaks in EC not detected in the regular soil measurements. On the contrary, we found a positive but weak relationship between salinity and fine root accumulation in Limestone ($r^2 = 0.323$, $P = 0.02$, $n = 15$).

We analyzed the relationship between the residuals of the regression between root biomass colonizing the soil and soil EC (when it was significant), and heavy metal concentration in the same roots, to avoid interferences with the effect of soil EC on root biomass. The relationship was negative and statistically significant for Zn, and only marginally significant for Ni, in seedlings planted on Marl (Table 6).

4. Discussion

Information on heavy metal contents in *Pinus halepensis* is scarce. To our knowledge, critical levels for Cu, Zn and Ni have not been established for seedlings of this species. Adequate levels of these elements in plants range from 3 to 6 mg Cu kg^{-1}, 27 to 150 mg Zn kg^{-1} and 0.1 to 6 mg Ni kg^{-1} (Boardman et al., 1997; Kabata-Pendias and Pendias, 1992; Rademacher, 2001). Cu and Zn concentrations in needles of unamended seedlings and seedlings amended with unenriched biosolid (except for those planted on Sandstone) can be considered deficient (Boardman et al., 1997; Kabata-Pendias and Pendias, 1992), or critical (Marschner, 1995), and they were well below the toxicity levels suggested by Kabata-Pendias and Pendias (1992) and Marschner (1995) (20–30 mg Cu kg^{-1} and 100–300 mg Zn kg^{-1}).

Ni concentrations in shoots were always very low, and did not show significant responses to the addition of enriched or unenriched biosolid. This is in agreement with the lack of change in total Ni concentration in amended soils (Table 1).

Application of enriched biosolids increased foliar concentrations of Cu and Zn, but still the values were at the lower limit of deficiency, and toxicity was unlikely. The form in which the heavy metals were applied (i.e. as soluble salts; Kiekens et al., 1984; Oudeh et al., 2002), may have contributed to these increases. Soil pH increased in Sandstone soils throughout the course of the experiment, probably due to atmospheric inputs that are relatively high in this area (Carratalá, 1993; Sanz et al., 2002). Thus, our results may be valid for slightly-to-highly carbonated soils, but not for acidic soils. Soil pH has been described as the main factor controlling heavy metal solubility in soils (Martínez and Motto, 2000; Rieuwerts et al., 1998). Cu, Ni and Zn concentration in plants

Table 5
Morphology of *Pinus halepensis* seedlings grown for eight months on three Mediterranean forest soils with no amendment (C), amended with biosolid (SS) or amended with biosolid enriched with heavy metals (SSM)

Soil	Treatment	Height (cm)	Diameter (mm)	Biomass (g d.w.)		RWR	Specific Root Length (cm mg^{-1})	Specific Needle Wt. (mg cm^{-2})
				Shoot	Root			
Marl	C	17.5 ± 0.6	5.0 ± 0.2	9.5 ± 0.7	6.6 ± 0.4	0.67 ± 0.01	18.3 ± 1.0	5.3 ± 0.1
	SS	16.3 ± 0.9	5.2 ± 0.1	8.5 ± 0.9	5.6 ± 0.4	0.64 ± 0.01	18.7 ± 1.3	5.1 ± 0.1
	SSM	15.9 ± 0.5	5.2 ± 0.2	8.1 ± 0.5	5.2 ± 0.2	0.63 ± 0.01	20.8 ± 1.2	4.8 ± 0.2
Limestone	C	16.8 ± 0.7	4.6 ± 0.3	7.1 ± 0.4	4.4 ± 0.2	0.61 ± 0.02	14.6 ± 1.6	5.4 ± 0.2
	SS	16.7 ± 1.0	4.9 ± 0.3	8.1 ± 0.8	5.3 ± 0.4	0.64 ± 0.01	15.3 ± 1.7	4.8 ± 0.2
	SSM	17.1 ± 0.9	4.8 ± 0.1	7.3 ± 0.2	5.0 ± 0.1	0.64 ± 0.01	14.1 ± 1.1	5.0 ± 0.2
Sandstone	C	16.1 ± 0.8	5.1 ± 0.2	6.8 ± 0.7	4.1 ± 0.4	0.59 ± 0.03	14.1 ± 1.1	4.8 ± 0.3
	SS	15.6 ± 0.6	5.0 ± 0.2	7.0 ± 0.8	4.3 ± 0.3	0.60 ± 0.01	14.6 ± 2.0	5.3 ± 0.2
	SSM	15.6 ± 0.8	5.1 ± 0.1	6.9 ± 0.8	4.1 ± 0.4	0.58 ± 0.02	17.7 ± 1.3	4.6 ± 0.2
2-way ANOVA (*F* values)								
Treatment		0.64	0.33	0.34	0.86	0.38	1.71	2.30
Soil type		1.65	2.96	5.49*	16.36**	11.30**	6.47**	0.88
T × S		0.35	0.19	0.63	2.66*	1.43	1.24	1.98

The root weight ratio (RWR) was calculated as the ratio between total root dry weight of the plant and total plant weight. Means, standard errors of $n = 4–5$ replicates and results of a two-way ANOVA are shown. Asterisks indicate significant differences at $P < 0.005$ (**) and $P < 0.05$ (*) (Tukey's test).

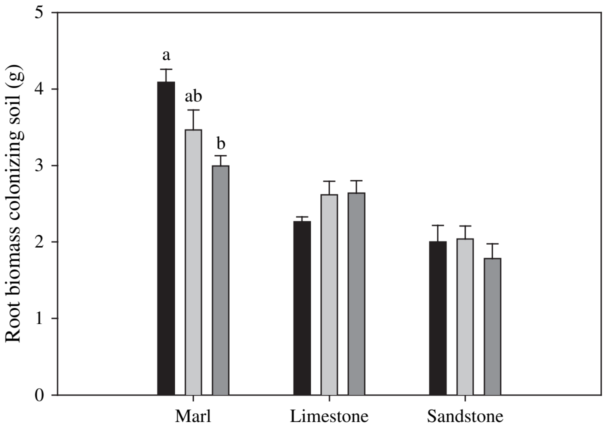

Fig. 3. Biomass of roots colonizing the three soil types as affected by the application of biosolids (light grey), biosolids enriched with heavy metals (dark grey), or unamended (black). Data represent means ± SE ($n = 4–5$). Results of a Tukey test ($P < 0.05$) are indicated by letters.

on unamended soils suggest that bioavailability was higher on Sandstone than on Limestone and Marl. Differences in pH, clay and carbonate content may be responsible for these results (García-Rizo et al., 1999; Martínez and Motto, 2000; Rodríguez-Rubio et al., 2003). But the increase in Cu and Zn concentration in roots of seedlings amended with polluted biosolids was lower in those planted on Sandstone than in those planted on more carbonated soils. Decreases in the heavy metal concentration in seedlings after organic matter application have been associated with sorption onto organic compounds and inorganic fractions (Pascual et al., 2004; Rate et al., 2004; Sánchez-Monedero et al., 2004). Results suggest that the sewage sludge matrix buffered the release of sludge-borne metals, or caused a reduction in the availability of weaker-bound indigenous heavy metals in sandstone soil (Merrington et al., 2003; Smith, 1996). The decrease in heavy metal concentration in seedlings amended with unenriched biosolids, particularly in those planted on Sandstone, provides further support to this hypothesis.

In pines, heavy metal transport to shoots is commonly low (Bargagli, 1998; Fuentes et al., 2003; Kukkola et al., 2000). In the present experiment, heavy metal concentration in roots was substantially higher than in shoots. Several mechanisms may explain this, including immobilization of metals at the cell wall-plasma membrane interface, and selective transport (Kabata-Pendias and Pendias, 1992; Reichman, 2002). As a result, heavy metal concentration in needles was relatively low even for seedlings amended with enriched biosolid.

Table 6
Significance of the relationship between heavy metal concentration in new roots and the residuals of the relationship between the biomass of roots colonizing the soil and the electrical conductivity of soil extracts (EC)

Soil		Cu	Ni	Zn
Marl	r^2	0.069	0.252	0.447
	P	0.41	0.096	0.017
Limestone	r^2	0.042	0.002	0.011
	P	0.483	0.866	0.706

Seedling ecophysiological status was slightly improved during the driest period by heavy metal application, as reflected by the significant increase in photochemical efficiency in needles of seedlings amended with enriched biosolids as compared to those receiving unenriched biosolids. Cu and Zn deficiency affects metabolic processes like photosynthesis and respiration (Adams et al., 2000; Cakmak, 2000). Cu interacts with PSII electron transport either directly, by participating in the electron transfer as a constituent of an electron carrier, or indirectly, by controlling the membrane protein composition within the PSII complex (Mysliwa-Kurdziel et al., 2004). Thus, our results suggest that biosolids enriched in heavy metals at the levels used in this study may have a weak positive effect on plant ecophysiology by releasing micronutrient limitation. This result is consistent with a general trend towards increased Cu, Ni and Zn concentrations in the needles of seedlings amended with enriched biosolids as compared to those receiving unenriched biosolids, and agree with the higher Cu, Ni and Zn leaching found by Toribio and Romanyà (2005) in the soils amended with enriched biosolids.

The low response to biosolid application on biomass accumulation and allocation and plant growth, was somewhat unexpected as *Pinus halepensis* usually increases growth when amended with biosolids at doses similar to the ones used in the present experiment (Zagas et al., 2000). However, most of the experiments with *Pinus halepensis* have been performed under dry-to-subhumid climatic conditions. Under the semi-arid conditions of the present experiment, low water availability probably increased the concentration of soluble salts, counterbalancing the positive effects resulting from organic matter and nutrient inputs. The negative relationship found between the biomass of roots colonizing the soil and soil salinity in soils developed from Marl supports this hypothesis. Heavy metal addition had no effect on biomass accumulation and allocation. This is in agreement with the relatively low heavy metal concentration found in needles, and further supports the idea that no toxicity levels were reached in any of the three soils tested (Table 4). However, at this point we cannot ascertain whether the negative effect of Zn and Ni on the biomass of fine roots colonizing the soil was a result of incipient phytotoxicity, or a shift in biomass allocation patterns (i.e., reduced biomass allocation belowground) as a consequence of the released micronutrient limitation (Chapin et al., 1987). It is worth noting that in the current experiment biosolids were thoroughly mixed with the soil. This is not feasible in regular field plantations, and may have affected the outcomes of the experiment (Fuentes et al., 2002).

5. Conclusions

Biosolids that may be rejected for agricultural uses due to excess Cu, Ni and Zn, did not show toxicity effects on the *Pinus halepensis* seedlings planted under the conditions of this study. Toxicity by Cu, Ni and Zn seems unlikely at the application rates presented in our study. Biosolid application in ecological restoration differs from that in agricultural crops and land reclamation. In the former, biosolid use may be justified as a kick-off treatment to improve early growth in planted seedlings, and it must be restricted in time (e.g., one single application before planting), and space (application not widespread, but confined in planting holes or lines) to prevent undesirable effects of excessive disturbance. Thus, biosolid application in ecological restoration may have a lower environmental impact than its use in agriculture. Furthermore, in soils where low micronutrient availability is likely (weakly-to-highly carbonated soils, high pH, semiarid climate), the use of biosolids enriched in Cu, Ni and Zn may help to attenuate micronutrient limitations, improving seedling ecophysiological status. Biosolid enriched in Cu, Ni and Zn may affect ecosystems at other levels, and thus, our results can not be directly transferred to managers. But they indicated that plant performance may not be impaired by the application of enriched biosolids within the range of conditions and application rates tested in the present experiment.

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References

Adams, M.L., Norwell, W.A., Philpot, W.D., Peverly, J.H., 2000. Micronutrient status. Spectral detection of micronutrient deficiency in 'Bragg' soybean. Agronomy Journal 92, 261–268.

Arduini, I., Godbold, D.L., Onnis, A., 1994. Cadmium and copper change root growth and morphology of *Pinus pinea* and *Pinus pinaster* seedlings. Physiologia Plantarum 92, 675–680.

Arduini, I., Godbold, D.L., Onnis, A., 1995. Influence of copper on root growth and morphology of *Pinus pinea* L. and *Pinus pinaster* Ait. seedlings. Tree Physiology 15, 411–415.

Barceló, J., Poschenrieder, Ch, 1990. Plant water relations as affected by heavy metal stress: a review. Journal of Plant Nutrition 13, 1–37.

Bargagli, R., 1998. Trace Elements in Terrestrial Plants: An Ecophysiological Approach to Biomonitoring and Biorecovery. Springer-Verlag and R.G. Landes Company.

Berry, C.R., 1977. Initial Response of Pine Seedlings and Weeds to Dried Sewage Sludge in Rehabilitation of an Eroded Forest Site. USDA Forest Service Research Note, vol. 249.

Boardman, R., Cromer, R.N., Lambert, M.J., Webb, M.J., 1997. Forest plantations. In: Reuter, D.J., Robinson, J.B. (Eds.), Plant Analysis. An Interpretation Manual. ASPAC. CSIRO Publ., Collingwood, pp. 505–566.

Brallier, S., Harrison, R.B., Henry, C.L., Xue, D., 1996. Liming effects on availability of Cd, Cu, Ni and Zn in a soil amended with sewage sludge 16 years previously. Water, Air and Soil Pollution 86, 195–206.

Cakmak, I., 2000. Possible roles of Zinc in protecting plant cells from damage by reactive oxygen species. New Phytologist, 146, 185–205.

Carratalá, A., 1993. Caracterización de la Precipitación en la Comunidad Valenciana: Distribución Espacial y Temporal. Ph. D. Thesis. University of Alicante.

Chapin III, F.S., Bloom, A.J., Field, C.B., Waring, R.H., 1987. Plant responses to multiple environmental factors. BioScience 37, 49–57.

Council Directive 86/278/EEC. Commission of the European Communities, 1986. Council Directive 12 on the Protection of the Environment, and in Particular Soil, When Sewage Sludge is Used in Agriculture. Official Journal of the European Communities L181, 6–12. 04/07/1986.

European Commission, 2000. Working Document on Sludge. European Commission., Brussels. Third Draft. (ENV.E.3/LM, 2000).

Fuentes, D., Cortina, J., Valdecantos, A., Casanova, G., Vallejo, V.R., 2002. Sensitivity of Mediterranean woody species to high inputs of biosolids. In: Faz, A., Ortíz, R., Mermut, A.R. (Eds.), Sustainable Use and Management of Soils in Arid and Semiarid Regions. Quaderna Ed., Cartagena, Spain.

Fuentes, D., Disante, K., Cortina, J., 2003. Response of Mediterranean woody species to high concentrations of heavy metals. In: Gobran, R.G., Leep, N. (Eds.), Proceedings of 7th International Conference on the Biogeochemistry of Trace Elements, 1, pp. 148–149.

Fuller, W.H., Tucker, T.C., 1977. Land utilization and disposal of organic wastes in arid regions. In: Elliot, Stevenson (Eds.), Soils for Management of Organic Wastes and Waste Waters. American society of Agronomy, Inc., pp. 505–566.

García-Rizo, C., Martínez, J., Pérez, C., 1999. Environmental transfer of zinc in calcareous soils in zones near old mining sites with semi-aridic climate. Chemosphere 39, 209–227.

Hall, J., 1999. Ecological and economical balance for sludge management options. In: Langenkamp, H., Marmo, L. (Eds.), Proceedings of Workshop on Problems Around Sludge. European Commission (EUR 19657). Joint Research Centre, Stresa (NO) Italy, pp. 155–172.

Hartley, J., Cairney, J.W., Freestone, P., Woods, P., Meharg, A.A., 1999. The effects of multiple metal contamination on ectomycorrhizal Scots pine (*Pinus sylvestris*) seedlings. Environmental Pollution 106, 413–424.

Illera, V., Walter, I., Souza, P., Cala, V., 2000. Short-term effects of biosolid and municipal solid waste applications on heavy metals distribution in a degraded soil under a semi-arid environment. The Science of the Total Environment 225, 29–44.

Jones, J.B. Jr., Case, V.W., 1990. Sampling, handling, and analyzing plant tissue samples. In: Westerman, R.L. (Ed.), Soil Testing and Plant Analysis. SSSA Book Series 3. Madison, WI, pp. 389–427.

Kabata-Pendias, A., Pendias, H., 1992. Trace Elements in Soils and Plants. CRC Press, Boca Raton, FL.

Kiekens, L., Cottenie, A., Landschoot, G., 1984. Chemical activity and biological effect of sludge-borne heavy metals and inorganic metal salts added to soils. Plant and Soil 79, 89–99.

Kukkola, E., Rautio, P., Huttunen, S., 2000. Stress indicators in copper- and nickel-exposed Scots pine seedlings. Environmental and Experimental Botany 43, 197–210.

Maksymiec, W., 1997. Effect of copper on cellular processes in higher plants. Photosynthetica 34, 321–342.

Marschner, H., 1995. Mineral Nutrition of Higher Plants. Academic Press Inc, London.

Martínez, C.E., Motto, H.L., 2000. Solubility of lead, zinc and copper added to mineral soils. Environmental Pollution 107, 153–158.

Merrington, G., Oliver, I., Smernik, J., McLaughlin, M.J., 2003. The influence of sewage sludge properties on sludge-borne metal availability. Advances in Environmental Research 8, 21–36.

Mysliwa-Kurdziel, B., Prasad, M.N.V., Strzalka, K., 2004. Photosynthesis in heavy metal stressed plants. In: Prasad, M.N.V. (Ed.), Heavy Metals Stress in Plants. From Biomolecules to Ecosystems, second ed. Springer-Verlag, Berlin, pp. 146–182.

Naveh, 1982. Mediterranean landscape evolution and degradation as multivariate biofunctions: theoretical and practical implications. Landscape Planning 9, 125–146.

Oudeh, M., Khan, M., Scullion, J., 2002. Plant accumulation of potentially toxic elements in sewage sludge as affected by soil organic matter level and mycorrhizal fungi. Environmental Pollution 116, 293–300.

Pascual, I., Antolín, M.C., García, C., Polo, A., Sánchez-Díaz, M., 2004. Plant availability of heavy metals in a soil amended with a high dose of sewage sludge under drought conditions. Biology and Fertility of Soils 40, 291–299.

Rademacher, P., 2001. Atmospheric Heavy Metals and Forest Ecosystems. International Cooperative Program on Assessment and Monitoring of Air Pollution Effects on Forests. Federal Research Center for Forestry and Forest Products (BFH), Hamburg, Germany.

Rate, A.W., Lee, K.M., French, P.A., 2004. Application of biosolids in mineral sands mine rehabilitation: use of stockpiled topsoil decreases trace element uptake by plants. Bioresource Technology 91, 223–231.

Reichman, S.M., 2002. The Responses of Plants to Metal Toxicity: a Review Focusing on Cooper, Manganese and Zinc. Australian Minerals and Energy Environment Foundation, Melbourne.

Rhoades, J.D., 1982. Soluble salts. In: Page, A.L., Miller, R.H., Keeney, D.R. (Eds.), Methods of Soil Analysis, Part 2, 2nd ed. American Society of Agronomy, Soil Science Society of America, Madison, WI, US, pp. 403–430.

Rieuwerts, J.S., Thorntorn, I., Farago, M.E., Ashmore, M.R., 1998. Factors influencing metal bioavailability in soils: preliminary investigations for the development of a critical loads approach for metals. Chemical Speciation and Bioavailability 10, 61–74.

Rodríguez-Rubio, P., Morillo, E., Madrid, T., 2003. Retention of copper by a calcareous soil and its textural fractions: influence of amendment with two agroindustrial residues. European Journal of Soil Science 54, 401.

Roldán, A., Querejeta, I., Albaladejo, J., Castillo, V., 1996. Survival and growth of *Pinus halepensis* Miller seedlings in a semi-arid environment after forest soil transfer, terracing and organic amendments. Annales Des Sciences Forestieres 53, 1099–1112.

Sánchez-Monedero, M.A., Mondini, C., de Nobili, M., Leita, L., Roig, A., 2004. Land application of biosolids. Soil response to different stabilization degree of the treated organic matter. Waste Management 24, 325–332.

Sanz, M.J., Carratalá, A., Gimeno, C., Millán, M.M., 2002. Atmospheric nitrogen deposition on the east coast of Spain: relevance of dry deposition in semi-arid Mediterranean regions. Environmental Pollution 118, 259–272.

Serrasolses, I., Alloza, J.A., 2004. Condicionantes edáficos en la restauración forestal mediterránea. In: Vallejo, V.R., Alloza, J.A. (Eds.), Avances en el Estudio de la Gestión del Monte Mediterráneo. Fundación CEAM.

Smith, S.R., 1996. Agricultural Recycling of Sewage Sludge and the Environment. CAB International, Wallingford, UK.

Toribio, M., Romanyà, J.,, 2005. Leaching of heavy metals (Cu, Ni and Zn) and organic matter after sewage sludge application to Mediterranean forest soils. Science of the Total Environment, doi:10.1016/j.scitotenv.2005.10.004.

Vallejo, V.R., Alloza, J.A., 1998. The restoration of burned lands: the case of Eastern Spain. In: Moreno, J.M. (Ed.), Large Forest Fires. Backhuys Publ., Leiden, The Netherlands, pp. 91–108.

Vallejo, V.R., Bautista, S., Cortina, J., 2000. Restoration for soil protection after disturbances. In: Trabaud, L. (Ed.), Life and Environment in the Mediterranean. WIT Press, Southampton, pp. 301–343.

Zagas, T., Ganatsas, P., Tsitsoni, T., Hatzistathis, A., 2000. Influence of sewage sludge application on survival and early growth of forest species. In: Proceedings of Thrace 2000. Protection and Restoration of the Environment 1, pp. 583–590.