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Plant and fungal biodiversity from metal mine wastes under remediation at Zimapan, Hidalgo, Mexico

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Rhizospheric fungi and organic matter encourage plant vegetation of tailings by pioneers and colonizing species.

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ABSTRACT

Plant establishment, presence of arbuscular mycorrhizal fungi (AMF) and other rhizospheric fungi were studied in mine wastes from Zimapan, Hidalgo state, Mexico, using a holistic approach. Two long-term afforested and three non-afforested mine tailings were included in this research. Fifty-six plant species belonging to 29 families were successfully established on the afforested sites, while unmanaged tailings had only a few native plant species colonizing the surrounding soils. Almost all plant roots collected were associated to AMF in these sites. The genus *Glomus* was the most abundant AMF species found in their rhizosphere; however, the *Acaulospora* genus was also observed. Other rhizospheric fungi were identified by 18S rDNA sequencing analysis. Their role in these substrates, i.e. biocontrol, pollutant- and organic matter-degradation, and aides that increase plant metal tolerance is discussed. Our results advance the understanding of fungal diversity in sites polluted with metals and present alternative plants for remediation use.

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1. Introduction

Mine tailings or mine wastes are the leftovers after the extraction of the valuable fraction of an ore. Physicochemical properties of mine wastes fluctuate greatly depending on the source of the ore, but in general all contain high concentrations of trace elements such as Pb, Ni, Cd, Cu, Mn, and Zn, and low concentrations of essential nutrients such as phosphorous and nitrogen, and organic matter (Bradshaw et al., 1978; Alloway, 1995; Walder and Chávez, 1995; Boulet and Larocque, 1998).

Mine tailings disposal sites from active or inactive mines are prevalent in Mexico. Pollutants contained in exposed tailings are easily spread by wind and water, impacting large areas, which can be estimated in dozens of hectares around (Munshower, 1994; Morris et al., 2003; Carrillo-González and González-Chávez, 2006). Zimapan, located in the Mexican state of Hidalgo, is a mining town with many tailing disposal sites. Mine activities started in 1632 and waste deposit sites have been built up ever since. Tailing heaps remained with no vegetation for many years.

Afforestation of mine wastes by the establishment of permanent plant cover is an alternative practice for remediation (Kramer et al., 2000; Mains et al., 2006), rather than abandon the sites or give physicochemical treatment to the residues. The main difficulty to implement this approach is the selection of plants that can establish and survive under mine wastes conditions. Remediation and afforestation of contaminated sites might be facilitated by selection of tolerant plant species. Latin America, including Mexico is one of the least explored areas on this regard, and there are few reports describing occurring tolerant vegetation in natural or polluted sites with mine residues (Reeves et al., 1996; Ginocchio and Baker, 2004). Reforestation studies of waste-mine soil sites have described the survival of *Pluchea sympithifolia* under such adverse conditions (Bailleres, 2003). Other plant species have been reported growing on As-contaminated soils (Flores-Tavison et al., 2003), or in other

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contaminated areas from different parts of Mexico (Díaz-Garduño et al., 2005; Carrillo-González and González-Chávez, 2006; González-Chávez et al., 2009). The need of research concerning phytostabilization and remediation of mine tailing sites in arid and semi-arid sites, such as Zimapan, has been reviewed and recognized (Mendez and Maier, 2007).

Arbuscular mycorrhizal fungi (AMF) are among the most important soil microorganisms in the soil system, and play relevant roles for the establishment and survival of plant species in stressed environments. There are some reports showing that AMF accelerate revegetation in areas affected by industrial activities such as mines, or in trace elements contaminated soils (Khan et al., 2000; Gaur and Adholeya, 2004; Gohre and Paszkowski, 2006; Hildebrandt et al., 2007). Recently, it was showed that plant and soil organisms, such as AMF and mesofaune, might play an important role in building up soil from mine residues containing high Cd concentrations (González-Chávez et al., 2009). Our group is focused in studying the transport mechanisms of heavy metals between AMF and plant roots, and the potential use of these soil microorganisms in conjunction with plant species for remediation on waste-mine substrates.

The goal of the present study was to gain information about plants and fungi, including AMF, adapted to grow on waste-mine sites at Zimapan, which contain high levels of trace elements. The study considered five mine tailing sites. Two of them have been afforested following man-induced remediation without any specific remediation protocol, whereas the other three mine tailings have followed natural plant succession.

2. Materials and methods

2.1. Study site

The region of Zimapan is located in Central Mexico in the state of Hidalgo, 20°44′ NL and 99°23′ WL in an altitude of 1780 mosl. Climatic regime is semi-arid with restricted periods of rain season at summer. The predominant vegetation is formed mainly by shrubs with cacti and mesquite trees (*Prosopis laevigata* L.).

Man-induced remediation by afforestation was initiated in some tailings called San Antonio 1 and 2 mine tailings. The afforested tailing piles were covered with sand and gravel obtained from exploitation sites of calcite, phosphorite and other non-metallic mines (Table 1). San Antonio site 1 is the only afforested tailing that was covered with soil and irrigated with local wastewater (Fig. S-1a and b). San Antonio 2 site has two tailing piles; San Antonio 2a and San Antonio 2b (Fig. S-1c and e, respectively). Both tailings were initially afforested exclusively with *Casuarina equisetifolia* L. Subsequent plant establishment occurred in both tailings, however, no records exist on the species that were intentionally introduced or naturally established. At the present time, in addition to *C. equisetifolia* trees, San Antonio 2a tailing is predominantly covered with *Opuntia* sp. and *Arundo* sp. (Fig. S-1b and d), whereas San Antonio 2b remained mostly covered with *C. equisetifolia* trees (Fig. S-1f), although several other plant species were also identified in both tailings. On the other hand, San Miguel, Pal (Fig. S-1g) and San Francisco (Fig. S-1h) mine tailing sites followed natural plant colonization, and no intentional afforestation efforts had been done to date.

2.2. Physical and chemical analysis of mine wastes

Two types of samples were collected: mine residues and rhizospheric samples. Samples were collected from January through June 2004. Mine residues were taken from the surface of mine tailings that were exposed, but depending on the site they might contain soil or even plant residues. Rhizosphere samples were taken on sites where plants grew and contained a mix of plant material, soil and spoils. Composed samples were conformed by at least six sub-samples taken from the same pile site. Each composed sample was analyzed by triplicate.

Composed samples were air dried and sieved through 2 mm plastic sieves before analysis. Soil pH was measured in water and 10 mM $CaCl_2$ (1:2.5 ratio) suspensions (Rowell, 1994). Organic matter was determined by wet digestion (Nelson and Sommers, 1982). Texture was analyzed following the Day procedure (1965). Total Cd, Ni, Pb and As, concentrations were determined after microwave digestion (300 PSI-230 °C, 20 min) with HNO_3 and quantified by ICP (Agilent 7500 ce).

2.3. Plant sampling

Plant specimens were collected from afforested mine tailing sites, as well as surrounding sites where plants were found from non-afforested tailings. Plant samples were kept alive with their rhizosphere substrate in pots until mycorrhizal analysis was performed in a period no longer of one and a half weeks after collection. For taxonomic identification and herbarium accessions, samples were processed following the protocol reported by Díaz-Garduño et al. (2005). Foliage samples with flowers or fruits were taken from the field, processed and deposited for public consultation at the Jorge Espinosa Salas Herbarium, Preparatoria Agrícola from the Universidad Autónoma de Chapingo, State of Mexico.

2.4. Arbuscular mycorrhizal fungi spore quantification and root colonization percentage

Rhizospheric soils (0–30 cm) were sampled from plants collected from afforested tailing piles, as well as from shrubs and herbs growing on the surroundings of non-managed wastes. One composed soil sample by plant was analyzed for spore abundance. Spores of AMF were isolated from 100 g of substrate by wet sieving and decanting (Gerdemann and Nicholson, 1963). Morphotypes were selected under

Table 1
Description of the studied tailing piles in Zimapan mining zone, Mexico.

Tailing name	Tailing origin	Management	Extension	Location	Altitude (mosl)
Afforested sites					
1. San Antonio 1	Tailing piles from metallic Lomo del Toro mine	Covered by sand, gravel and soil from surrounding area and reforested in 1987 mainly with *Eucalyptus camaldulensis*, *Buddleia cordata*, *Canna indica* and different Poaceae (*Bromus diandrus*, *Chloris virgata*) and irrigated with wastewater from the town during more than ten years	700 m perimeter, 0.024 km^2	20°43′52.1″ NL 99°22′43.2″ WL	1734
2. San Antonio 2	Two tailing piles (2a and 2b) afforested in 1992 with *Casuarina equisetifolia*	Tailing 2a. Covered with gravel and sand. Additionally covered by several species like *Arundo* sp. and *Opuntia* sp.	300 m perimeter, 0.005 km^2	20°43′40.8″ NL 99°22′4.8″ WL	1713
		Tailing 2b. Covered with gravel and sand. Predominates *Casuarina equisetifolia* trees but other species are also found.	423 m perimeter, 0.014 km^2	20°43′38.9″ NL 99°22′57.3″ WL	1729
Non-afforested sites					
3. San Miguel	Two tailing piles next to each other. A gray one originated from Dolores mine (El Monte) and a reddish (oxidized) one from Purísima mine (El Carrizal)	No vegetation, only some invasive shrubs and herbs in surrounding soil	215 m perimeter, 0.002 km^2 and 151 m perimeter, 0.001 km^2, respectively	20°43′38.1″ NL 99°23′51.9″WL	1673
4. Pal	Tailing pile from La Encarnación mine, extremely oxidized	No vegetation, only some invasive shrubs and herbs in surrounding soil	370 m perimeter, 0.012 km^2	20°43′32.0″ NL 99°23′11.7″ WL	1678
5. San Francisco	San Francisco basin tailings from El Carrizal and El Monte mines	No vegetation, only some pioneer herbs from the surroundings	151 m perimeter, 0.0016 km^2	20°43′58.1″ NL 99°23′51.9″ WL	1734

a dissecting microscope and preserved in polyvinyl alcohol-lacto-glycerol (PVLG) for description. Roots were cleared, stained and preserved as proposed by Phillips and Hayman (1970). Total mycorrhizal root colonization percentage was calculated analyzing two samples per plant root system. Each sample was analyzed by duplicate by the intersection method as reported by McGonigle et al. (1990). Only one sample per root system measured by duplicate was analyzed in those samples with very scarce or damaged root systems, and no standard deviation is presented for those specific samples.

2.5. Molecular identification of eukaryote organisms from mine rhizospheric substrates

In order to gain information on the kind of fungi present in the sites, total DNA was extracted from all rhizospheric substrates. The Power Soil DNA kit (MoBio, Carlsbad, CA, USA) was used following the manufacturer's instructions. Molecular identification was achieved analyzing the internal transcribed sequences (ITS) of ribosomal DNA amplified by PCR using primers ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) and ITS5 (5′ GGAAGTAAAAGTCGTAACAAGG 3′) (White et al., 1990). PCR from genomic DNA obtained from the rhizospheric substrates produced only few defined bands (1–8 PCR products per genomic DNA sample). PCR fragments were cleaned out of the gel (Geneclean Kit. Q-Bio Gen Cat. No. 1001-200) and digested with *Alu*I restriction enzyme. Same size bands showing same *Alu*I restriction pattern were grouped and considered coming from the same species. Then, only one of these PCR products was cloned into pGEM-T Easy vector (Promega, USA) and sequenced.

2.6. Molecular identification of fungi in mycorrhizal colonized roots

In an attempt to identify AMF a selection of mycorrhizal roots sections was done by visualization of external fungal hyphae after a quick staining procedure with trypan blue-lactophenol (0.05% trypan blue in lactophenol reagent) for 20 min followed by a brief wash with distilled water for 1 min.

Once mycorrhizal sections of about 5 mm were selected, genomic root DNA was extracted using DNAzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Root DNA was used for PCR to amplify the ITS rDNA variable region using primers ITS4 and ITS5. ITS4 and ITS5 primers are not specific for AMF, their selection was based on the fact that they will amplify diverse fungi present in the samples. PCR fragments were cloned into pGEM-T Easy vector according to manufacturer's recommendations, and sequenced.

PCR conditions for substrates and colonized root DNA were as follows: an initial denaturation cycle at 95 °C for 3 min, followed by 30 s at 95 °C of denaturation, 61 °C for 30 s of annealing, and extension for 45 s at 72 °C. From step 2 a total of 30 cycles were performed, followed by a final extension step of 72 °C for 5 min.

2.7. DNA sequencing and analysis

DNA sequencing was carried out by the dideoxy sequencing method with a Big Dye Terminator version 3.1 Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The products were processed by an ABI 3100 DNA sequencer (Applied Biosystems). Sequence comparisons with the non-redundant GenBank data base were performed by BLASTN searches (January, 2009) using the Megablast algorithm. The nucleotide sequences reported appear in the GenBank data base under accession numbers EU718651–EU718674

3. Results

3.1. Physical and chemical analysis of soils and mine wastes

High OM contents were found in rhizospheres collected from afforested San Antonio 1 and San Antonio 2a tailings (Table 2). In contrast San Antonio 2a, 2b and Pal mine residues had very low OM contents. San Miguel and San Francisco mine residues presented OM contents between 3 and 4%. OM content varied as the tailing was mixed with soil. pH was near the neutrality in all analyzed sites, except for the mine residue Pal, which had an acidic pH value. In relation to particle distribution, the predominant fraction was sand from 40% to 92%.

The highest Cd concentration was found in San Antonio mine residues. Ni concentration varied from 12 to 92 mg kg^{-1} in tailings, Pb concentration ranged from 910 to 3040 mg kg^{-1} and As content from 160 to 2777 mg kg^{-1} depending on the tailing. Summarizing, total content of trace elements such as Cd, Ni, Pb, and As, showed a wide range of variation among the studied mine waste sites (Table 2), and, with the exception of Ni, all other elements analyzed, surpassed environmental critical limit concentrations (Visser and Gupta, 1993; Morin and Calas, 2006).

3.2. Diversity of introduced plants

In total sixty six plant species were identified in all sites studied, which belong to 29 plant families and 63 genera (Table 3); fifty-six plant species from afforested sites and the rest from the unmanaged sites. These species included both pioneer and introduced plants. Dicotyledonous species were predominantly found on these sites. Only two monocotyledonous families were described: Poaceae represented by twelve species and Cannaceae by one. Natural revegetation has occurred mainly where soil covers have been added.

Following Poaceae family, the most represented plant family was Asteraceae with eleven species. Solanaceae was represented by five and Euphorbiaceae by four species. Many plant species were found in more than one site (Table 3). Two plant species: *Dalea* sp. and *Eragrostis* sp. were found in tailing site San Antonio 2a which contained the highest Cd concentrations detected in this study.

Eucalyptus camaldulensis, *C. equisetifolia*, *Salix* sp., *Schinus molle* are examples of some trees growing in these sites. While bushes such as *Baccharis salicifolia*, *Ricinius communis* and *Nicotiana glauca* were also present. Plants like *Arundo* sp. and some specimens from the Cactaceae family were observed growing on uncovered and exposed mine residue regions in San Antonio 1 and San Antonio 2a sites (Fig. S-1b and d).

3.3. Arbuscular mycorrhizal fungi

Four *Glomus* morphospecies were identified (Table 4): *Glomus mosseae*, *Glomus afin fasciculatum*, *Glomus afin aggregatum*, and *Glomus* type *Sclerocystis* sp. (Fig. S-2a, b) and eleven unidentifiable *Glomus* spp. *G. mosseae* spores isolated from the rhizosphere of *Opuntia* sp. at San Antonio 1 site (Fig. S-2c, d) were healthy and relatively abundant (Table 4).

Roots of almost all plants were colonized by AMF. *Cylindropuntia* sp. and plants from the Poaceae and Agavaceae family from San Antonio and San Antonio 2a tailings had high colonization percentages and morphotype diversity, as well as spore abundance. Two unidentifiable *Acaulospora* morphotypes were also found (Table 4, Fig. S-2e, f). Spores of *G. mosseae* were found in San Antonio 1, 2a and San Francisco sites.

Other ruderal species such as *R. communis*, *Tagetes micrantha* and *Chenopodium albidum* are among the highest colonized plants in San Antonio site. Some rhizospheres are associated to several spore morphotypes. This is the case for *Yucca* sp. in San Antonio 2a, *Cylindropuntia* sp., *R. communis* and *Chloris virgata* in San Antonio and the non-identified herb 1 at Pal mine tailings. In other rizospheres, AMF spores were found, however, plant roots were not colonized like *Datura stramonium* (San Antonio 1) and *Rhynchelytrum repens* (San Antonio 2a).

3.4. Molecular identification of fungi

Over one hundred PCR products were amplified from DNA isolated from different rhizospheric substrates and roots collected at three mine tailing sites from Zimapan, Hidalgo and cloned into a bacterial vector. Purified recombinant plasmids were selected for sequencing analysis. Sixteen fungal species and the rhodophite algae *Mastocarpus papillatus* were identified from these clones (Table 5). Most fungi belonged to the phylum Ascomycota, and only two to Basidiomycota (*Pisolithus tinctorius* and *Sporobolomyces lactosus*).

Table 2
Average and standard deviation of some physicochemical characteristics in studied mine wastes from Zimapan, Hidalgo.

Mine (n)	pH		Soil particles %			Organic Matter %	Concentration (mg kg^{-1})			
	H_2O	$CaCl_2$	Sand	Silt	Clay		Cd	Ni	Pb	As
1. San Antonio (rhizosphere) (3)	6.7 ± 0.30	6.3 ± 0.20	69 ± 8.5	25 ± 7.0	6 ± 1.4	9.6 ± 2.9	422 ± 168	68 ± 3	1043 ± 308	265 ± 156
2a. San Antonio (rhizosphere) (2)	6.7 ± 0.20	7.0 ± 0.15	47 ± 5.0	17 ± 5.0	35 ± 9.0	7.0 ± 9.7	9.5 ± 1	12 ± 6	285 ± 363	219 ± 227
2a. San Antonio (mine residue) (2)	7.2 ± 0.20	7.2 ± 0.14	68 ± 8.0	25 ± 7.0	6 ± 1.5	0.15 ± 0.15	814 ± 64	18 ± 3	910 ± 33	300 ± 394
2b. San Antonio (mine residue) (2)	7.0 ± 0.20	7.1 ± 0.05	36 ± 5.2	13 ± 1.0	51 ± 1.4	0.08 ± 0.02	699 ± 51	32 ± 0.5	973 ± 39	787 ± 72
3. San Miguel (mine residue) (2)	7.2 ± 0.14	6.7 ± 0.30	70 ± 1.4	18 ± 2.8	10 ± 1.4	3.14 ± 3.12	340 ± 38	92 ± 3	3040 ± 1056	2777 ± 130
4. Pal (mine residue) (2)	3.7 ± 1.8	3.7 ± 1.90	86 ± 4.4	7 ± 1.7	7 ± 1.0	0.73 ± 0.79	141 ± 54	21 ± 14	1333 ± 646	413 ± 565
5. San Francisco (mine residue) (1)	7.2 ± 0.26	7.3 ± 0.16	92 ± 1.4	4 ± 2.0	5 ± 4.0	4.29 ± 1.0	230 ± 4	44 ± 5.7	936 ± 148	160 ± 11
				Critical limits in soil (Visser and Gupta, 1993):			>5	35–75	>100	>50[a]

n = number of composed samples;
[a] Morin and Calas (2006).

4. Discussion

4.1. Physical and chemical analysis of soils and mine wastes

The OM content varied among the sites studied. The heterogeneous nature of the tailing sites comprising vegetated and exposed areas (Fig. S-1b and d) are reflected on OM results. San Antonio 1 was afforested in 1987, and it is the only site that has been artificially irrigated. It is possible that irrigation favored plant survival and subsequent OM accumulation over the years. As a comparison, San Antonio 2a and 2b mine residue samples taken on exposed areas of the tail clearly show the absence of OM.

On non-afforested sites, composed samples taken at San Miguel and Pal tails are constituted by rhizospheric and mine residue subsamples which explains such wide OM interval values. Surprisingly, the rhizospheric San Francisco composed sample contained high OM as the mine waste with less vegetation, probably because of the xanthates and hydrocarbons used during the extraction process of metals.

OM plays an important role in remediation processes by improving the residues conditions for plant colonization. Many heavy metal cations, such as Cu^{2+}, Cd^{2+}, and Zn^{2+}, form stable organic complexes with humic substances (Spark et al., 1997). Other sources of OM originated by plant residues and roots growing randomly in the tailing surroundings, can bind trace elements and change their availability (Pierzynski et al., 2002) promoting a healthier environment to initiate plant succession.

Apparently, the pH values cannot limit the plant colonization, except in Pal tailing which is very acid. The pH values were around neutrality in all analyzed sites, probably due to the presence and high contents of calcite and gypsum (Méndez and Armienta, 2003), except in Pal site, which presented an acidic pH (Table 2). Pal mine tailing has not been vegetated and is the most exposed and oxidized tailing. Probably, low pH values have stopped natural plant colonization on the waste piles where no vegetation is found (Fig. S-1g). Exposed residues, such as Pal site, are more vulnerable to run-off and wind erosion causing underground water contamination (Méndez and Armienta, 2003).

In mine residues from afforested San Antonio 2a site the predominant type of particles is sand, since the ore minerals are ground during extraction. After cover application, as shown on the rhizospheric samples, clay and silt contents were increased improving conditions for plant growth (Table 2). In general, non-afforested sites either rhizospheric or residual contained low levels of clay particles. Percentage of silt particles on the San Miguel rhizosphere/mine residue composed sample was comparable to afforested sites and this correlates with samples showing high OM content.

Unlike Ni, all the other analyzed elements were found in critical high concentrations. In some of the mine waste sites, Cd concentration exceeded by hundreds of times the critical limits proposed by Visser and Gupta (1993), as well as the maximum concentration reported for this type of spoils (390 mg kg^{-1}) (Vogel-Mikus et al., 2005). Even when Cd was one of the metals extracted from the Zimapan mines, its extraction from sphalerite is not complete, which leaves high concentrations of this element in the tailings. In the sites where the tailing have been covered with soil or gravel mixed with soil the Cd and arsenic concentration decreased. In contrast, total Pb concentrations are lower than those from other mine regions over the world (67940 and 4892 mg kg^{-1}) (Vogel-Mikus et al., 2005 and Zarei et al., 2008, respectively) or in Mexico (972–16881 mg kg^{-1}) (Gutiérrez-Ruiz et al., 2007), since Pb recovery from galena is highly efficient.

Arsenic concentrations were high in the studied sites (up to 2869 mg kg^{-1}) compared to concentrations reported for other mine regions from Mexico (21–36 mg kg^{-1}) (Mendoza-Amézquita et al., 2006) and North America (56–6000 mg kg^{-1}) (Moldovan et al., 2003). In contrast, As concentrations found in this study were low compared to the concentrations (2550–14600 mg kg^{-1}) reported by Méndez and Armienta (2003) for the same area. This discrepancy can be explained by the fact that in such study, mine residues were taken below the cover or deeper; digging until less altered material was found. The samples taken in this work were collected from the top layer (0–30 cm) and either rhizospheric or mine residues, they contained a mix of spoils and soil.

4.2. Diversity of introduced plants

An important number of plants were growing in the area. Plant species from Poaceae, Asteraceae and Solanaceae were the most representative plant families in the studied sites. Several species of these three families have been reported as the most common ones able to grow on waste-mine soil sites in South Africa, Brazil, Cuba, Italy, Canada, Mexico, New Caledonia and China (Shu et al., 2005; Prasad et al., 2006; Carrillo-González and González-Chávez, 2006; Leung et al., 2007; González-Chávez et al., 2009).

Table 3
Plant species from waste-mine sites at Zimapan, Hidalgo, Mexico.

Family	Scientific name	Tailing name
Acanthaceae	*Dicliptera peduncularis* Nees	San Antonio 1
Agavaceae	*Yuca* sp.	San Antonio 1 and 2a
Amaranthaceae	*Amaranthus hybridus* L.	San Antonio 2a
	Gomphrena decumbens Jacq.	San Antonio 2a
Anacardiaceae	*Schinus molle* L.	San Antonio 1
Asteraceae	*Baccharis salicifolia* (Ruíz & Pavón) Pers.	San Miguel
	Sanvitalia angustifolia Engelm. Ex A. Gray	San Miguel
	Simsia sp.	San Miguel
	Verbesina encelioides (Cav.) Benth. & Hook. Ex A. Gray	San Miguel
	Xanthium strumarium L.	San Miguel
	Sanvitalia procumbens Lam.	San Antonio 2a and San Miguel
	Sonchus oleraceus L.	San Antonio 2a
	Flaveria trinervia (Spreng.) C. Mohr.	San Antonio 2b
	Sclerocarpus uniserialis (Hook) B. & H. Ex Hemsl.	San Antonio 1
	Tagetes micrantha Cav.	San Antonio 1
	Zinnia peruviana (L.) L.	San Antonio 1 and 2a
Brassicaceae	*Lepidium virginicum* L.	San Antonio 1
Cactaceae	*Opuntia* sp.	San Antonio 1 and 2a
	Cilyndropuntia sp.	San Antonio 1 and 2a
Cannaceae	*Canna indica* L.	San Antonio 1 and 2a
Casuarinaceae	*Casuarina equisetifolia* L.	San Antonio 2a and 2b
Chenopodiaceae	*Teloxys ambrosioides* L.	San Antonio 1
	Salsola kali var. tenuifolia Tausch	San Antonio 1
	Chenopodium album L.	San Antonio 1
Comelinaceae	*Comelina* sp.	San Antonio 2a
Convolvulaceae	*Ipomea purpurea* (L.) Roth	San Antonio 1
Cucurbitaceae	*Sycios laciniatus* L.	San Antonio 2a
	Sicydium tamnifolium (H. G.K.) Cogn Kin D. C.	San Miguel
Euphorbiaceae	*Acalypha monostachya* Cav.	San Antonio 2a and 2b
	Euphorbia sp.	San Miguel
	Jatropha sp.	San Miguel
	Ricinus communis L.	San Antonio 1, San Francisco
Fabaceae	*Dalea* sp.	San Antonio 2a, San Miguel
	Dalea zimapanica L.	San Antonio 1
Loasaceae	*Mentzelia hispida* Willd.	San Antonio 1and 2a
Loganiaceae	*Buddleia cordata* H.B.K.	San Antonio 1
Lythraceae	*Heimia salicifolia* (H.B.K.) Link	San Miguel
Malvaceae	*Malvastrum coromandelianum* (L.) Garcke	San Antonio 2b
	Malva parviflora L.	San Antonio 1
Mimosaceae	*Prosopis laevigata* (Willd.) M.C. Johnst.	San Antonio 1, 2a, 2b and San Miguel
	Acacia farnesiana L.	San Miguel
Myrtaceae	*Eucalyptus camaldulensis* Dehnhardt	San Antonio 1 and 2b
Nolinaceae	*Dassylirium* sp.	San Antonio 1
Nyctaginaceae	*Mirabilis jalapa* L.	San Miguel
	Allionia incarnata var. glabra choisy in D.C.	San Antonio 2a
Papaveraceae	*Argemone mexicana* L.	San Antonio 1 and San Francisco
Poaceae	*Aristida adscensionis* L.	San Antonio 2a and San Miguel
	Arundo sp.	San Antonio 2a and 2b
	Bouteloua curtipendula (Michx.) Torr.	San Antonio 2a
	Cynodon dactylon (L.) Pers.	San Antonio 1 and 2a
	Eragrostis sp.	San Antonio 2a
	Leptochloa dubia (H.B.K) Nees	San Antonio 2a
	Rhynchelytrum repens (Willd.) Hubb	San Antonio 2a
	Setaria macrostachya H.B.K.	San Antonio 2a
	Bromus willdenowii Kunth	San Antonio 1
	Chloris virgata Sw.	San Antonio 1 and 2a
	Chloris verticillata Nott	San Antonio 2a, 2b, Pal
	Echinochloa crus-pavonis (H.B.K) Schult.	San Antonio 1
Rhamnaceae	*Zizyphus* sp.	San Antonio 1

Table 3 (*continued*)

Family	Scientific name	Tailing name
Rubiaceae	*Bouvardia ternifolia* (Cav.) Schlecht.	San Antonio 1
Salicaceae	*Salix* sp.	San Antonio 1
Solanaceae	*Datura stramonium* L.	San Antonio 1
	Nicotiana glauca Gram.	San Antonio 1, 2a, 2b and San Miguel
	Solanum elaeagnifolium Cav.	San Antonio 2b and San Miguel
	Solanun rostratum Dunal	San Antonio 2a
	Physalis sp.	San Antonio 2a

Some plants found in this research have also been reported in other studies. Díaz-Garduño et al. (2005); Carrillo-González and González-Chávez (2006) reported plants like *C. virgata*, *Cynodon dactylon*, *Euphorbia* sp., *Salsola kali*, *S. molle*, *Solanum elaeagnifolium* and *Xanthium strumarium* as pioneers or colonizer species on mine wastes in Zacatecas, Mexico. *Dalea* sp. and *Eragrostis* sp. were observed in the sites most contaminated with Cd. Díaz-Garduño et al. (2005) also reported these two plant species growing on a Cd contaminated site in Temascaltepec, Mexico.

In all San Antonio sites, two introduced tree species, *E. camaldulensis* and *C. equisetifolia*, were planted after setting sand and gravel (San Antonio 2a and 2b), or sand, gravel and soil (site 1) on the top of the waste piles and seemed to be adapted to these sites. However, after several years of growing, roots of *E. camaldulensis* and *C. equisetifolia* at sites 2a and 1 respectively, showed anchorage limitations. On these sites, root systems grew mainly on the top layer avoiding penetrating the waste-mine tailing directly. In consequence, as trees grew up, some fell down by wind action after some years; probably because root systems were not able to support shoot weight.

Trees, such as *Salix* sp. and *S. molle* are species of interest because their foliage can incorporate organic matter, explore and retain a large area of wastes and improve the landscape overview. Some bush species growing on tailing heaps such as *B. salicifolia* in San Miguel site, and *R. communis* and *N. glauca* in San Antonio and San Miguel sites showed similar superficially developed root systems.

Very little has been documented about the presence of the Cactaceae family in mine polluted wastes and it could represent a promising alternative in arid environments preserving resources with natural vegetation. The ectodermis of *Opuntia* sp. is an effective biosorbent of trace elements in wastewater (Barrera et al., 2006) and may become an important species for remediation in the future.

Even though afforestation of San Antonio tailings was done following no strategic planting protocol, the resulting heterogeneous plant community made out of grasses, leguminous and tree or bush species, has favored plant succession and establishment. The mechanism through which this has been achieved may have been mediated by the accumulation of organic matter from previous established plants leading to a less polluted substrate more suitable for the succession. Prasad et al. (2006) has suggested the use of phytoremediation schemes using plant species as diverse as possible.

Some advantages of the above mentioned scheme are: fast mechanical stabilization of wastes, landscape visual improvement, and a decrease of contaminant dispersion, leaching, and risks to human health. The phytoremediation process based on the empiric establishment of plant species in these contaminated sites in Zimapan, Hidalgo seems to be successful. Some of these plants should be used to revegetate Pal and San Francisco mine tailings, which presented only few plants. In addition, many of the plants reported in this study may be used in other systems to establish vegetation on contaminated sites under similar conditions.

4.3. Arbuscular mycorrhizal fungi

Glomus spp. was the most abundant genus found in the polluted sites studied, which is in accordance to other studies followed in mine wastes (Alves da Silva et al., 2005; Vogel-Mikus and Regvar, 2006; Zarei et al., 2008). The dominance of *G. mosseae* in disturbed habitats is well documented (Chen et al., 2007), and it has been suggested that high densities of *Glomus* spores are related to high organic matter contents in polluted soils, which leads AMF propagules to establish mycotrophic successional stages (Johnson, 1998; Khan et al., 1998).

In the present work, both percentages of root colonization as well as spore abundance showed high variability among different plants from the same site. This has been attributed to the heterogeneity prevailing in mine spoil piles (Vogel-Mikus and Regvar, 2006) which correlates to our studies.

R. communis and *T. micrantha* have been reported as spread plants that grow in metal rich soils and are also colonized by AMF (Khan et al., 1998; González-Chávez et al., 2009). *Ricinus* and *Chenopodium* are two genera recently studied in their abilities to extract Ni (Malarkodi et al., 2008) and other heavy metals (Bhargava et al., 2008) from polluted sites. Members of the Chenopodiaceae and Brassicaceae families are commonly reported as non-mycorrhizal in natural conditions, but have been found forming mycorrhizae in polluted sites (Orłowska et al., 2002; Regvar et al., 2006).

In the rhizosphere of *D. stramonium* and *R. repens* were found spores, but not mycorrhizal colonization. It is possible that the observed spores came from other plants or that the number of roots analyzed was not large enough to find colonization under these conditions of propagule scarceness. Even though San Antonio 1 tailing is the oldest afforested site and had the largest diversity of plants (Table 3), similar root colonization, spore abundance and AMF diversity were found when compared to the San Antonio 2a (Table 4). In contrast, the few plants found growing naturally in the surroundings of Pal and San Francisco tailings were all well colonized by AMF and showed identifiable AM morphotypes (Table 4).

In this study almost all analyzed plants were colonized by AMF, which contrasts with the reports by Regvar et al. (2006) and Pawlowska et al. (2000), in which pioneer plants in mine tailings were predominantly low or not colonized. However, Khan et al. (1998) and Leung et al. (2007) found that AMF colonization was present in all studied plants and mainly in grass species in heavy metal polluted sites, which is in concordance with the present work. Leung et al. (2007) also found high root colonization values, up to 85%, in plants in which As root concentrations were 57 times higher than in shoots. These authors concluded that plants develop different strategies for survival in contaminated sites with the help of indigenous AMF. In the present study trace metal content in plant tissues was not analyzed, but it is possible that AMF increase metal soil stabilization and have similar beneficial functions as observed by other authors in mine polluted sites (Khan et al., 2000; Hildebrandt et al., 2007; González-Chávez et al., 2009).

Table 4
AMF morphotypes, root colonization percentage (mean ± S.E) and spore abundance (spores/100 g soil) of some rhizospheric samples of plants growing in mine tailing piles at Zimapan, Hidalgo.

Tailing name	Plant	Root colonization (%)[a]	Spore abundance	Spore morphotypes
San Antonio 1	*Argemone mexicana*	0	0	–
	Nicotiana glauca	0	0	–
	Canna indica	0	1	*Glomus* sp. 6
	Datura stramonium	0	5	*Glomus* sp. 3
	Salsola kali var. tenuifolia	n.d.*	1	n.d.
	Eucalyptus camadulensis	**	0	–
	Opuntia sp.	6 ± 8	13	*Glomus mosseae*
	Bromus diandrus	6 ± 9	0	–
	Chloris virgata	30 ± 8	10	*Glomus* sp. 1, *Glomus* sp. 5 (*Sclerocystis* type)
	Dalea zimapanica	18 ± 25	11	*Glomus* sp. 1 and 7
	Lepidium virginicum	20 ± 20	5	*Glomus mosseae*
	Cylindropuntia sp.	33 ± 17	13	*Glomus* sp. 1, 4; *G. mosseae* and *Glomus afin aggregatum*
	Ricinus communis	34 ± 39	10	*Glomus* sp. 1, *Acaulospora* sp. 1., *Glomus afin aggregatum*
	Tagetes micrantha	35	7	*Glomus* sp. 1
	Chenopodium sp.	38 ± 6	0	–
San Antonio 2a	Non-identified legume	0	4	*Glomus* sp. 8
	Rhynchelytrum repens	0	13	*Glomus* sp. 1
	Arundo sp.	1	1	n.d.
	Opuntia sp.	6 ± 9 ($n = 2$)	2	n.d.
	Leptochloa dubia	8 ± 14	7	*Glomus* sp. 1 and *G. aggregatum*
	Canna indica	14 ± 14	0	–
	Chloris virgata	15	0	–
	Yuca sp.	29 ± 13	10	*Glomus* sp. 3, *Acaulospora* sp. 2, 8, 10 and *G. fasciculatum*
	Casuarina equisetifolia	34	8	*Glomus* sp. 4
	Cylindropuntia sp.	40 ± 34	12	*Glomus* sp. 1, 2, 3 and *G. mosseae*
Pal (rhizosphere from plants growing in the surroundings)	Non-identified Asteraceae	0	0	–
	Non-identified shrub 3	10 ± 12	2	n.d.
	Non-identified shrub 2	20 ± 24 ($n = 3$)	7	*Glomus* sp. 2 and 3
	Chloris verticillata	38 ± 6	4	*Glomus* sp. 4
	Non-identified herb	76 ± 21	1	n.d.
San Francisco (rhizosphere from plants growing in the surroundings)	Non-identified herb 3	22 ± 19	1	n.d.
	Non-identified herb 1	33 ± 7 ($n = 2$)	8	*Glomus* sp. 9, 11 and *G. mosseae*
	Non-identified shrub	44 ± 39	2	n.d.
	Ricinus communis	50 ± 35	2	*Glomus afin aggregatum*
	Non-identified herb 2	69 ± 19	3	n.d.

*Very few roots with vesicles; **Some hyphae penetrating the roots, n.d. = not determined.
Numbers after *Glomus* sp. indicates the specific morphotype identified.
[a] Percentage of root colonization where not specified s.e. indicates that root systems of that plant were analyzed taking one sample and analyzed by duplicate; $n = 2$ or $n = 3$ indicates that 2 or 3 plants, respectively, were analyzed for that particular species.

Like in afforested areas (San Antonio 1 and San Antonio 2a), vegetation of the Pal and San Francisco tailings should be followed by using seedling inoculated with native AMF in the nursery, and transplantation in order to increase the AMF fungal propagules in the area, which may favor revegetation. Plants found in the afforested areas represent options to select different vegetal species and use them in these unmanaged sites. Moreover, organic matter additions may increase natural remediation of these sites (accelerated natural attenuation).

AM morphological identification might be a good tool to describe AM communities in the soil but it might present limitations due to the differential sporulation by certain AM species in response to specific AMF-plant interactions or certain environmental factors. This might affect the presence of AMF spores in the rhizospheric substrates studied. Molecular studies suggest the presence of AMF colonizing root tissue which spores where not found in the surrounding plant soil (reviewed in Sanders, 2004). In order to rule out this possibility, molecular identification was attempted using genomic DNA obtained from rhizospheric substrates and AMF colonized root tissues. A small rDNA survey was conducted using ITS4/ITS5 primers. Although ITS4/ITS5 primers were chosen in this work as an attempt to amplify preferentially AMF ITS rDNA sequences, we did not succeed in obtaining any amplified product with similarity to these fungi even though morphological analysis showed the presence of AMF spores and root colonization on these sites. This is probably due to the low density sequencing approach used in this work which failed to detect AMF sequences. In the near future, more specific sets of primers and nested PCR techniques and more extensive sequencing techniques such as large scale parallel 454 sequencing (Opik et al., 2009) will be used to identify the different taxonomic groups colonizing these rhizospheric substrates and roots grown under these conditions.

4.4. Molecular identification of fungi

The redundancy observed in the analyzed samples may possibly reflect the diversity contained in the soil. This result is in disagreement to that reported by O'Brien et al. (2005), in which mass sequencing of ITS rDNA from soils showed a similar composition on asco- and basidiomycetes for different soil samples. The low diversity of sequenced fungi, and the bias observed towards the presence of members of Ascomycota in this research, may be explained by the negative effect of the pollutants over the populations of organisms that were brought for remediation in these areas and were not able to establish. It is also not possible to rule out the possibility that DNA extraction could be biased in mine waste substrates. Sequencing was not exhaustive and the possibility of undersampling can only be discarded applying massive sequencing methodologies.

Table 5
Fungi identified from the rhizosphere of mine tailing sites from Zimapan, Hidalgo, Mexico.

Sample	Plant rhizosphere	PCR insert (bp)[a]	Highest homology to species	Phylum	Percentage of homology[b]	Accession number
San Antonio 1 mine site						
M-21 037	*Cylindropuntia* sp.[d]	592	*Fusarium solani* isolate XSD-77 (EU326189.1)	Ascomycota	99% (590/592)	EU718651
M-25 037	*Cylindropuntia* sp.[d]	591	*Fusarium solani* isolate XSD-77 (EU326189.1)	Ascomycota	99% (590/591)	EU718652
M-30 037	*Cylindropuntia* sp.[d]	592	*Fusarium solani* isolate XSD-77 (EU326189.1)	Ascomycota	99% (590/592)	EU718653
M-26 037	*Cylindropuntia* sp.[d]	225	*Fusarium oxysporum* isolate FO-07 (AY928414.1)	Ascomycota	95% (218/228)	EU718654
ITS-152 IR	*Eucalyptus camaldulensis*[d]	630	*Microdiplodia* sp. XSD-38 (EU273518.1)	Ascomycota	99% (626/630)	EU718655
ITS-5 AS	Unidentified grass	568	*Fusarium oxysporum isolate* XSD-78 (EU326216.1)	Ascomycota	99% (567/568)	EU718656
ITS-11 AR	Unidentified grass[d]	564	*Phoma glomerata* isolate XSD-41 (EU273521.1)	Ascomycota	98% (558/564)	EU718657
ITS-136 IS	*Eucalyptus camaldulensis*	636	*Pisolithus tinctorius* (AY739178.1)	Basidiomycota	99% (614/615)	EU718658
San Antonio 2a–2b mine sites						
Alpha25	*Canna indica*	547	Fungal endophyte OTm-1 (AY433809.1)	Ascomycota	97% (506/521)	EU718659
Alpha 21	*Canna indica*	597	*Ophiognomonia* sp. ICMP 16022 (EU482284.1)	Ascomycota	86% (347/401)	EU718660
Alpha 22	*Canna indica*	669	Uncultured soil fungus clone 115-76 (DQ420868)	Ascomycota	99% (668/669)	EU718661
Alpha 11	Cactaceae	460	*Exophiala* sp. JS-1171 (AM182187)	Ascomycota	99% (436/441)	EU718662
Alpha13	Cactaceae	637	Herpotrichiellaceae sp. LM83 (EF060458.1)	Ascomycota	96% (616/638)	EU718663
Alpha 15	Cactaceae	595	Uncultured Xylariales (AJ879684.1)	Ascomycota	99% (570/575)	EU718664
ITS-118 HR	*Chloris virgata* Sw.[d]	575	*Cladosporium tenuissimum* (AJ300331.1)	Ascomycota	100% (575/575)	EU718665
ITS-60 FR	*Rhynchelytrum repens*[d]	568	*Fusarium oxysporum isolate* XSD-78 (EU326216.1)	Ascomycota	100% (568/568)	EU718666
ITS-35 DR	*Ricinus communis*[d]	618	*Aspergillus ustus* strain NRRL 1974 (AY373879.1)	Ascomycota	95% (558/587)	EU718667
ITS-22 BR	*Tagetes micrantha*[d]	571	*Fusarium oxysporum isolate* XSD-78 (EU326216.1)	Ascomycota	99% (568/571)	EU718668
ITS-167 JR	*Casuarina equisetifolia*	586	*Sporobolomyces lactosus* (EU551181.1)	Basidiomycota	97% (533/544)	EU718669
M-6042	*Opuntia* sp.[d]	684	*Pisolithus tinctorius* (AY739178.1)	Basidiomycota	99% (640/645)	EU718670
ITS-44 ES	*Canna indica*	437	*Mastocarpus papillatus* s.l. clade 1 voucher UBC A85238 (DQ872469.1)[c]	Rhodophyta	89% (101/113)	EU718671
Pal mine site						
P-23 AR	Unidentified grass	568	Uncultured mycorrhizal ascomycete isolate 1 (AY833044.1)	Ascomycota	99% (564/566)	EU718672
Alpha 19	Unidentified legume	631	*Phoma* sp. 2 (AF218789.1)	Ascomycota	90% (577/637)	EU718673
Alpha 20	Unidentified shrub	593	Uncultured soil fungus clone 68a21 (DQ420973.1)	Ascomycota	98% (588/595)	EU718674

[a] Number of base pairs of the sequence analyzed.
[b] In parenthesis the number of nucleotides matching the corresponding sequence/total number of nucleotides from the region showing identity. BLASTN program with the Megablast algorithm was used to obtain the highest homology to species on May 9, 2008 (http://www.ncbi.nlm.nih.gov/blast/).
[c] Indicates that the match to homologous sequence does not include homology to ITS regions.
[d] Indicates samples taken from roots.

Possible roles of identified organisms are discussed below according to the literature. An uncultured xylarial fungus and *Phoma* sp. are reported to be saprobes (Bradner et al., 1999; Liers et al., 2006), also, they are ubiquitous and indicative of organic matter mineralization.

Several of the sequences obtained in this work share homology with fungi that are described as possible phytopathogens such as *Cladosporium tenuissimum* (Fajola, 1979), *Fusarium oxysporum* (Berrocal-Lobo and Molina, 2008), *Fusarium solani* (Nelson et al., 1981), *Microdiplodia* sp. (Crous et al., 2006), and *Phoma glomerata* (Lahoz et al., 2007). The source of these fungal pathogens might be the plants or substrate used for remediation, or the water used for irrigation. A suggestive idea of the role that these fungi might be playing in these polluted sites comes from the observation that potentially pathogenic fungi have been found in association to stressed plants, while no causing disease-associated symptoms, but allowing the plant to respond and adapt to stress conditions such as heat (Márquez et al., 2007).

Symbiosis might be important for successful establishment of plants in phytoremediation processes. Besides the endomycorrhizal Glomeromycetes species described in this work, the ectomycorrhizal species *P. tinctorius* was identified. Other fungi capable of interacting with mycorrhizae were found such as an uncultured mycorrhizal Ascomycete isolated from ectomycorrhizal root tips of the mixotrophic orchid *Cephalantera damasonium*. This fungus might be part of the accompanying microflora of ectomycorrhizae (Julou et al., 2005). *Exophiala* sp. is also responsive to the presence of arbuscular mycorrhizae by decreasing its abundance when AMF are present (Tiunov and Scheu, 2005).

Other identified fungi included the fungal endophyte OTm-1, which confers abiotic stress tolerance (Rodríguez and Redman, 2008). *S. lactosus* grew on sites with high levels of wastes from oil industries (Grabińska-Loniewska et al., 1993) or petrochemical wastes (Sláviková and Grabińska-Loniewska, 1992). *Phoma* sp. isolates were reported as Cd hyperaccumulators (Yuan et al., 2007), and isolates from *P. glomerata* were able to degrade contaminants such as pentachlorophenol (Seigle-Murandi et al., 1991). *F. oxysporum* not only is important for being a wide-range phytopathogen but in the context of these mine sites it also has properties that are important for assisting plants to tolerate heavy metal contamination. This fungus was able to form carbonate crystals of Pb and Cd (Sanyal et al., 2005), and strontium (Rautaray et al., 2004) in heavy metal-contaminated water. It had the ability to remove iron from asbestos fibers (Daghino et al., 2005), and it was a degrader of the metal–cyano complex tetracyanonickelate (II) (Yanase et al., 2000). It is noticeable that from the species identified in the present study, approximately a third (5 out of 17 species) has been reported with ability to assist plants on abiotic stress, including trace element stress (Seigle-Murandi et al., 1991; Grabińska-Loniewska et al., 1993; Rodríguez and Redman, 2008; Sanyal et al., 2005; Yuan et al., 2007). This suggests that organisms developing in these sites contaminated with high levels of As, Cd, and Pb might be selected for survival and tolerance to high concentration of trace elements in polluted substrates, and probably for an increased ability to assist plant hosts to establish for remediation of mine tailing sites.

Long-term amendment of tailings, the input of wastewater, and the establishment of perennial species, modifies their biotic properties providing organic substrates and consequently, increasing

microorganism populations including different fungi. A functional microbial community allows the successful plant community to recover and with this the restoration of metal-contaminated environments (Moynahan et al., 2002). It will also be important to isolate culturable microorganisms from these sites since they are important for bioremediation purposes, possible biotechnological applications, as well as they might be of human health importance.

5. Conclusions

This field study, conducted at Zimapan, Hidalgo state in Mexico, shows information related with plants establishment and presence of rhizospheric fungi in mine wastes with high concentrations of trace elements.

A broad range of plant families and species was found on mine tailing sites which may be used for phytoremediation of other similar contaminated sites. The arbuscular mycorrhizal association was found consistently in all mine sites studied and they probably interact with their plant counterparts assisting them for establishment in these extreme conditions. Analysis of the possible roles of the identified rhizospheric fungi on these mine tailing sites suggest that they are an important component on the stabilization of areas under remediation. Field experiments involving different conditions offer the possibility of providing relevant information on the remediation process itself, even when they could be empirically man-made. This work suggests that in order to establish an efficient low-cost remediation alternative, a combination of resources might be used such as pioneers and colonizing plants and microorganisms, including beneficial associations between them.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.envpol.2009.10.034.

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