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# Effects of long-term heavy metal contamination on soil fungi in the Mediterranean area

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**Abstract** – The lead (Pb) and zinc (Zn) content in soil can qualitatively and quantitatively affect the microbial community and, therefore, the normal functioning of the soil ecosystem. The aim of this study was to assess the effect of long-term Pb and Zn contamination on soil fungi isolated by the dilution plate method, also in relation to vegetal topsoil. We analysed soil samples collected near dominant plant species (*Pistacia lentiscus* L., *Quercus ilex* L., *Arbutus unedo* L., and *Phillyrea angustifolia* L.) in soils suffering from heavy metal pollution as well as in unpolluted sites. The study area is located in the mining district of Ingurtosu (South-West Sardinia), where the polluted sites were severely contaminated by lead extraction activities that probably began before the Roman era. The obtained data were subjected to multivariate analysis, using Canonical Correspondence Analysis, to determine the relationship between the soil biota and the environmental variables. High concentrations of lead and zinc, with some variation with respect to the vegetation, were found in the topsoil of all polluted sites. Soil pollution places stress on sensitive micro-organisms, which causes a change in the diversity of soil mycota. A significant decline in micro-fungal abundance was observed in all polluted soils tested in this study. In addition, some interesting fungal species are discussed.

**lead / Mediterranean maquis / *Penicillium* / zinc**

**Résumé** – La présence de plomb et de zinc dans le sol peut affecter la biocénose microbienne du sol aussi bien quantitativement que qualitativement, et peut avoir une incidence sur l'activité de l'écosystème du sol. Cette étude a pour but d'estimer les effets de la contamination à long terme par le plomb et le zinc sur les microchampignons du sol, également par rapport aux espèces végétales dominantes des sites. Nous avons analysé des échantillons de sol extraits sous les végétaux dominants dans les lieux contaminés et non contaminés. L'aire d'étude est située dans la region minière d'Ingurtosu (sud-ouest de la Sardègne), les lieux contaminés ont été fortement pollués par les activités minières d'exrtraction du plomb, qui auraient débutées avant l'époque romaine. Les donnèes obtenues ont été soumises à une analyse multivariée au moyen d'une analyse canonique des correspondences afin de découvrir les relations existant entre la biocénose des

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microchampignons et les variables du milieu ambiant. Nous avons constaté de plus fortes concentrations de plomb et de zinc dans les sols contaminés, avec un degré de variation par rapport à la couverture végétale du sol. La pollution du sol provoque une pression sur les micro-organismes sensibles et ont amené, dans tous les sols contaminés un déclin significatif de l'abondance des microchampignons. Quelques espéces intéressantes de champignons sont discutées.

**maquis méditerranéen / *Penicillium* / plomb / zinc**

## INTRODUCTION

The interactions between toxic metals and fungi have long been of scientific interest. In fact, metal toxicity has been the basis of many fungicidal preparations (Ross, 1975). Several studies have shown that fungal species can resist and adapt to toxic metals (Gadd, 1993; Moore, 1998). Fungi digest their substrates externally by the excretion of appropriate enzymes. Together with these enzymes, fungi produce many extra-cellular products that can complex with or precipitate a number of heavy metals (Sayer *et al*., 1995; Morley *et al*., 1996). Fungi can also absorb and accumulate heavy metals at different cellular levels: cell wall (bio-sorption, metabolism-independent), vacuolar compartments and cytoplasmic compartments (Ross, 1975; Mullen *et al*., 1992; Morley & Gadd, 1995). The major interactions between fungi and toxic metals were reviewed by Gadd (1993).

Some metal-tolerant fungal species have been identified in several polluted areas that had potentially toxic heavy metal concentrations (Gadd, 1986; Baldi *et al*., 1990; Turnau, 1991; Abdel-Azeem *et al*., 2007). However, the effect of toxic metals on microbial abundance in natural habitats varies with the nature of the metal, type of organisms and environmental factors (Gadd & Griffiths, 1978; Duxbury, 1985; Simonicova & Frankova, 2001; Bååth *et al*., 2005).

Many researchers have investigated the effects of heavy metal contamination on the soil mycoflora in different ecosystems (Strojan, 1978; Coughtrey *et al*., 1979; Bååth, 1989; Levinskaite, 1999; Marfenina & Lukina, 1989; Abdel-Azeem *et al*., 2007). In general, heavy metal contamination is believed to reduce the abundance and diversity of fungal populations and induce selection for resistant or tolerant populations (Tatsuyama *et al*., 1975; Duxbury, 1985; Asharaf & Ali, 2007). A reduction in fungal abundance has been observed in soils polluted with Cu, Pb, Cb, As and Zn (Babich & Stotzky, 1985; Zucconi *et al*., 1996). This reduction could result in a decrease in the decomposition rate, which could lead to an immobilisation of nutrients and a reduction of soil fertility. Therefore, the evaluation of saprotrophic mycoflora in polluted areas deserves considerable attention.

The saprotrophic fungal community is strictly correlated to the organic components of soil, which largely depend on vegetal topsoil. Plant species influence the quality and quantity of organic matter in soil and the soil content of some pollutants. The pollutants can be absorbed by plant roots and translocated to the foliage (Nicklow *et al*., 1983; Preer *et al*., 1980; Leita *et al*., 1989). Nevertheless, the heavy metal content of different plant species collected in the

same sampling area shows a large degree of variation. The extent of accumulation depends on the plant and pollutant under observation (Csintalan & Tuba, 1992; Jones, 1991).

The objectives of the present work are to study the effects of long-term heavy metal contamination on the following variables: (1) the heavy metal content of the soil with respect to the vegetal cover; (2) the structure and diversity of the micro-fungal community, isolated by the dilution plate method; (3) the soil mycobiota; and (4) the vegetal topsoil with respect to a selection of specific fungal assemblages.

## MATERIALS AND METHODS

### Study area

The investigated area, Ingurtosu, is located in the south-western part of Sardinia (Italy) and belongs to the Iglesiente district, where extensive mining has been conducted during the last few decades. At present, waste materials from the de-activated mining operations are found over large areas of the Iglesiente region. These consist of gravel, sand and clayey mud. The gravel is mainly made up of fragments of quartz, schist and, to a lower extent, sulphates. Gravel plays a negligible role in surface water run-off pollution. Sand is mainly made up of sulphates, with minerals of gangue and with other materials such as sphalerite, galena, baryte, siderite, quartz, anglesite and cerussite. Both Fe and Zn sulphates from sand are solubilised by water (Caboi *et al*., 1993). The clayey mud mainly consists of mineral silicates and also contains significant amounts of Fe oxides and Zn, Ca and Mg sulphates. White clays, rich in Zn minerals, are washed away from piles of detritus by streams. The white clays contain concretions of chalk and magnesium sulphate, with small crystals of Pb, Ba and Cd sulphates derived from saturated water precipitation (Caboi *et al*., 1993).

Vegetal cover is mainly composed of broad-leaves from evergreen forests and the shrubs of evergreen sclerophylls. The most abundant species are *Quercus ilex* L., *Pistacia lentiscus* L., *Phillyrea angustifolia* L. and *Arbutus unedo* L.

### Sampling and fungal community characterisation

Soil samples were collected at two sites: a polluted sampling site and a control sampling site, located near the contaminated one, that was not affected by heavy metal transported by water run-off from the mine's detritus. Both sites are characterised by similar vegetation. According to Caboi *et al*. (1993), the pH of the area increases from pH 6.2 at the uncontaminated area to pH 6.6 at the contaminated site. At both sites, five samples of superficial soil (maximum depth 3-4 cm) were collected under each of the following plant species: *P. lentiscus* (PL), *Q. ilex* (QI), *A. unedo* (AU) and *P. angustifolia* (PA). The five soil samples were bulked to form a composite sample according to standardised methods (Davet & Rouxel, 2000). Soil mycobiota were isolated using the dilution plate method (Jhonson *et al.*, 1960). For each soil sample, ten plates were used for isolation.

Potato Agar, without added saccharose, supplemented with rose bengal (1/15'000) and chloramphenicol (50 ppm), to suppress bacterial growth, was used for primary isolation. A poor medium, as Potato Agar, was chosen to stimulate the development of slow growing fungi. After the plates were incubated for 10 days at 25°C, the developing fungi were counted. To maintain cultures, and for proper identification, pure cultures of isolated fungi were grown on standard media such as Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Czapek Yeast Agar (CYA).

Taxonomic identification, by morphology, of fungal isolates was mainly based on the following identification keys: Pitt 1979 for *Penicillium*; Raper & Fennell (1965) and Klick (2002) for *Aspergillus*; Ellis (1971; 1976) and Domsch *et al*. (2007) for the other fungi. The systematic arrangement follows the classification system reported in the $10^{th}$ edition of the Dictionary of the Fungi (Kirk *et al*., 2008).

To characterise the fungal population, parameters such as occurrence, richness, diversity and similarity were used. Abundance of fungi was calculated as a relative frequency ($Rf = n/N \times 100$, where "n" is the number of fungal colonies of one species in a sample and "N" is the total number of fungal colonies of all species in the same sample). Species richness was calculated as a number of species, while the species diversity was calculated as a Simpson's diversity index (Lande, 1996). The similarity among fungal assemblages was analysed by a Pearson's coefficient of association and a Bonferroni's probability test was performed to assess statistical significance ($P < 0.01$).

To determine the relationship between environmental factors and fungal assemblages under investigation, ordinations in the form of Canonical Correspondence Analysis (CCA; Podani, 1994) and Principal Component Analysis (PCA; Wilkinson *et al*., 1992), using SYSTAT 8.0 (Systat Inc., Evanston, IL, USA) were applied.

### Chemical analysis

The amounts of Pb and Zn were determined in each soil sample by the Inductive Coupled Plasma method (ICP; Method n° 13 – Ministry for Agriculture, Food And Forest Resources, 1994).

## RESULTS

The Zn and Pb contents of the analysed soils are shown in figure 1. The heavy metal levels in the polluted soil collected under *P. lentiscus* (PLP), *Q. ilex* (QIP), *A. unedo* (AUP) and *P. angustifolia* (PAP) sites were higher than those found in the control sites (PL, QI, AU, PA), with some variations with respect to the vegetal topsoil. In soil collected under PLP, PAP and QIP, Zn levels were twice as high as Pb levels, whereas the Pb content was greater than the Zn content in soil taken from under AUP (Fig. 1).

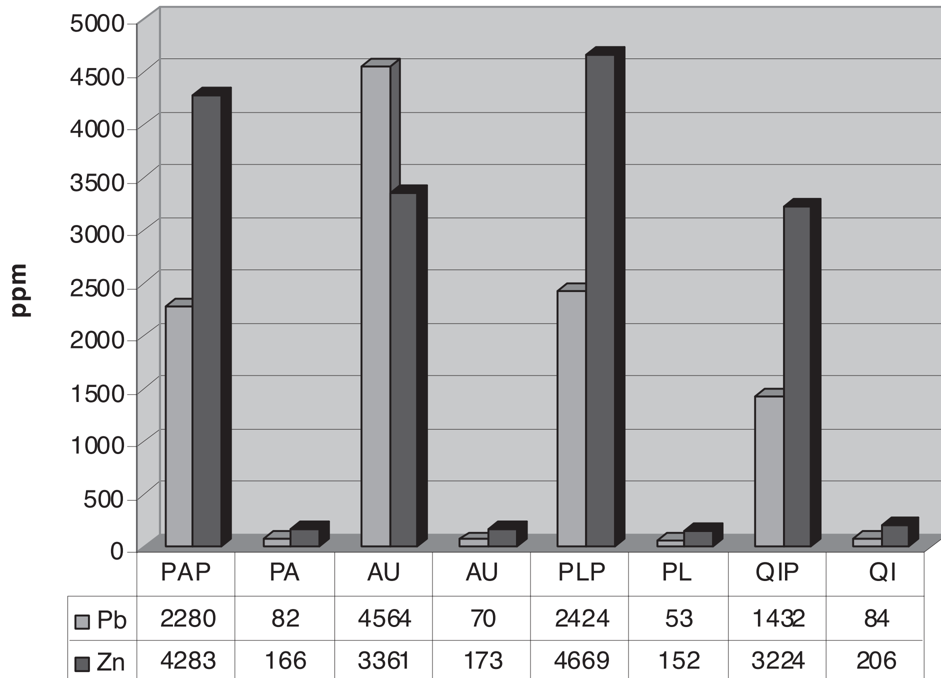


Fig. 1. Lead (Pb) and Zinc (Zn) values (ppm) in polluted (P) and control soil samples collected under *Phillyrea angustifolia* (PAP-PA), *Arbutus unedo* (AUP-AU), *Pistacia lentiscus* (PLP-PL) and *Quercus ilex* (QIP-QI).

**Fungal diversity**

As shown in table 1, it was possible to count as many as 71 species (2'008'000 UFC/g dry weight) from the eight polluted and unpolluted soil samples collected from the Iglesiente open mine district. A list of the species found and their relative frequencies (Rf), number of colonies and Simpson's species diversity indexes for each soil sample are given in table 1. The results show that Zygomycota are represented by four species (2.98% of the total isolated number), anamorphic Ascomycota by 63 species (90.36%) and mitosporic fungi by 3 species (3.19%, micelia sterilia 3.46%). The species that were isolated belong to 27 genera, the predominant genera being *Penicillium* (35 species, 60.92% of the total isolated number), *Aspergillus* (6 species, 1.74%), and *Trichoderma* (3 species, 2.10%). The remaining genera were represented by either one or two species. The most abundant species were the following: *Penicillium waksmanii* K.M. Zalessky (20.71% of the total isolate number), *Paecilomyces lilacinus* (Thom) Samson (9.58%), and *Penicillium roseopurpureum* Dierckx (7.58%). Taxonomically, the isolated species were assigned to 15 families, 11 orders, 9 subclasses, 7 classes and 2 phyla (Tab. 2).

Family Trichocomaceae had the highest contribution to the mycobiota population (45 species out of 71) followed by Hypocreaceae (3 species) and Mucoraceae (3 species). The remaining families were represented only by one or two species each.

Table 1. Relative frequencies (Rfs) of soil fungi in each sample (PL: *P. lentiscus*, control area; QI: *Q. ilex*, control area; AU: *A. unedo*, control area; PA: *P. angustifolia*, control area; PLP: *P. lentiscus*, polluted area; QIP: *Q. ilex*, polluted area; AUP: *A. unedo*, polluted area; PAP: *P. angustifolia*, polluted area). For each sample, the Richness of species, Simpson Index and number of colonies (UFC/g dry wet) is also shown

| | *PL* | *QI* | *AU* | *PA* | *PLP* | *QIP* | *AUP* | *PAP* |
|---|---|---|---|---|---|---|---|---|
| **Zygomycota** | | | | | | | | |
| *Absidia spinosa* Lendn.var. *spinosa* | 0.0 | 0.2 | 0.2 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 |
| *Mortierella alpina* Peyronel | 0.2 | 0.0 | 0.0 | 0.0 | 2.8 | 0.0 | 0.0 | 0.0 |
| *Mucor* sp.1 | 2.8 | 10.9 | 0.2 | 2.5 | 0.0 | 0.0 | 0.0 | 0.0 |
| *Rhizopus arrhizus* A. Fisch. var. *arrhizus* | 0.0 | 0.0 | 1.4 | 0.0 | 2.1 | 0.0 | 0.0 | 0.0 |
| **Ascomycota (anamorphic)** | | | | | | | | |
| *Alternaria alternata* (Fr.) Keissl. | 0.0 | 0.0 | 0.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 |
| *A. fumigatus* Fresen. | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 5.9 |
| *A. niger* Tiegh. var. *niger* | 0.0 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *A. ustus* (Bainier) Thom & Church | 0.0 | 0.2 | 0.0 | 4.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *A. versicolor* (Vuill.) Tirab. | 0.0 | 0.0 | 1.20 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *A. wentii* Wehmer | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *Aspergillus* sp.1 | 1.3 | 0.0 | 0.0 | 0.0 | 0.7 | 0.0 | 0.0 | 0.0 |
| *Beauveria bassiana* (Bals.-Criv.) Vuill. | 1.4 | 0.4 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *Cladosporium cladosporioides* (Fresen.) G.A. de Vries | 0.6 | 0.0 | 0.0 | 10.4 | 0.0 | 2.3 | 0.0 | 11.8 |
| *C. herbarum* (Pers.) Link | 0.0 | 0.2 | 0.0 | 0.0 | 2.1 | 2.3 | 0.0 | 3.9 |
| *Clonostachys rosea* (Link) Schroers, Samuels, Seifert & W. Gams f. *rosea* | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *Eladia saccula* (E. Dale) G. Sm. | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *Emericella nidulans* (Eidam) Vuill. | 0.0 | 0.0 | 0.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *Epithyrium* sp.1 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *Fusarium* sp.1 | 0.4 | 0.6 | 1.4 | 2.5 | 4.20 | 4.9 | 11.8 | 11.8 |
| *Geotrichum candidum* Link | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *Humicola fuscoatra* Traaen | 0.4 | 0.6 | 0.0 | 0.0 | 2.1 | 0.0 | 2.6 | 2.0 |
| *Leptodontidium* sp.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 17.1 | 0.0 |
| *Metarhizium anisopliae* (Metschn.) Sorokīn | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *Paecilomyces lilacinus* (Thom) Samson | 4.1 | 1.4 | 2.6 | 0.5 | 2.1 | 14.1 | 26.3 | 25.5 |
| *P. variotii* Bainier | 0.0 | 0.0 | 0.0 | 0.0 | 0.7 | 0.0 | 0.0 | 0.0 |
| *Penicillium anatolicum* Stolk | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *P. aurantiogriseum* Dierckx | 0.0 | 0.6 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *P. brevicompactum* Dierckx | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.0 | 0.0 | 0.0 |
| *P. canescens* Sopp | 0.0 | 0.0 | 0.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *P. chrysogenum* Thom var. *chrysogenum* | 0.0 | 0.0 | 1.4 | 0.0 | 0.0 | 0.0 | 10.5 | 0.0 |
| *P. citreonigrum* Dierckx | 0.0 | 0.4 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *P. citrinum* Thom | 10.3 | 11.9 | 0.2 | 0.0 | 35.0 | 1.3 | 0.0 | 0.0 |
| *P. corylophilum* Dierckx | 0.2 | 0.0 | 0.2 | 0.0 | 0.0 | 2.7 | 0.0 | 3.9 |
| *P. solitum* var. *crustosum* (Thom) Bridge, D. Hawksw., Kozak., Onions, R.R.M. Paterson & Sackin | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.3 | 0.0 | 0.0 |
| *P. donkii* Stolk | 0.2 | 0.0 | 0.0 | 0.0 | 0.7 | 0.0 | 0.0 | 0.0 |
| *P. glabrum* (Wehmer) Westling | 9.6 | 4.4 | 3.8 | 2.0 | 0.0 | 1.3 | 0.0 | 0.0 |

Table 1. Relative frequencies (Rfs) of soil fungi in each sample (PL: *P. lentiscus*, control area; QI: *Q. ilex*, control area; AU: *A. unedo*, control area; PA: *P. angustifolia*, control area; PLP: *P. lentiscus*, polluted area; QIP: *Q. ilex*, polluted area; AUP: *A. unedo*, polluted area; PAP: *P. angustifolia*, polluted area). For each sample, the Richness of species, Simpson Index and number of colonies (UFC/g dry wet) is also shown *(continued)*

| | *PL* | *QI* | *AU* | *PA* | *PLP* | *QIP* | *AUP* | *PAP* |
|---|---|---|---|---|---|---|---|---|
| *P. granulatum* Bainier | 0.0 | 1.20 | 0.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 |
| *P. implicatum* Biourge | 0.2 | 1.6 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *P. islandicum* Sopp | 0.2 | 0.0 | 0.0 | 0.0 | 0.7 | 0.0 | 0.0 | 0.0 |
| *P. madriti* G. Sm. | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.3 | 0.0 | 0.0 |
| *P. megasporum* Orpurt & Fennell | 0.0 | 0.0 | 0.0 | 3.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *P. melinii* Thom | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 11.4 | 0.0 | 0.0 |
| *P. miczynskii* K.M. Zalessky | 5.8 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *P. mirabile* Beliakova & Milko | 0.0 | 0.0 | 12.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *P. montanense* M. Chr. & Backus | 0.0 | 0.0 | 6.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *P. ochrochloron* Biourge | 1.3 | 0.0 | 1.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *P. paxilli* Bainier | 3.6 | 0.2 | 9.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *P. phoeniceum* J.F.H. Beyma | 0.0 | 0.0 | 0.0 | 1.5 | 0.0 | 0.0 | 0.0 | 0.0 |
| *P. purpurogenum* Stoll | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *P. restrictum* J.C. Gilman & E.V. Abbott | 0.4 | 1.8 | 0.2 | 9.4 | 0.0 | 0.0 | 1.3 | 0.0 |
| *P. roseopurpureum* Dierckx | 0.0 | 24.9 | 0.5 | 0.0 | 0.7 | 17.0 | 19.7 | 0.0 |
| *P. sclerotiorum* J.F.H. Beyma | 0.6 | 4.20 | 2.6 | 2.0 | 0.0 | 0.0 | 2.6 | 0.0 |
| *P. senticosum* D.B. Scott | 0.0 | 0.2 | 0.0 | 3.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *P. spinulosum* Thom | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *P. vinaceum* J.C. Gilman & E.V. Abbott | 0.0 | 0.0 | 1.20 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *P. aurantiogriseum* var. *viridicatum* (Westling) Frisvad & Filt. | 0.0 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *P. waksmanii* K.M. Zalessky | 16.1 | 23.9 | 43.6 | 39.1 | 32.20 | 8.7 | 0.0 | 2.0 |
| *Penicillium* sp.1 | 31.0 | 0.0 | 1.4 | 0.0 | 0.0 | 6.0 | 0.0 | 0.0 |
| *Penicillium* sp.2 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *Penicillium* sp.3 | 2.1 | 3.8 | 1.9 | 4.4 | 5.6 | 3.3 | 0.0 | 0.0 |
| *Pestalotia* sp.1 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *Phoma* sp.1 | 0.0 | 0.0 | 0.0 | 1.5 | 0.0 | 0.0 | 0.0 | 0.0 |
| *Sporothrix* sp.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.6 | 0.0 |
| *Trichoderma koningii* Oudem. | 0.8 | 2.6 | 0.2 | 2.0 | 1.4 | 3.3 | 0.0 | 3.9 |
| *T. piluliferum* J. Webster & Rifai | 0.0 | 1.20 | 1.20 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *Trichoderma* sp.1 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *Zasmidium cellare* (Pers.) Fr. | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 21.6 |
| **Mitosporic fungi** | | | | | | | | |
| *Acrodontium hydnicola* (Peck) de Hoog | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 17.0 | 0.0 | 0.0 |
| *Chuppia sarcinifera* Deighton | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.6 | 0.0 |
| *Scolecobasidium constrictum* E.V. Abbott | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 5.9 |
| Micelia Sterilia | 3.4 | 1.4 | 1.4 | 9.9 | 7.0 | 0.0 | 2.6 | 2.0 |
| **Richness of species** | 31 | 29 | 32 | 20 | 16 | 17 | 11 | 12 |
| **Simpson Index** | 0.850 | 0.848 | 0.776 | 0.809 | 0.761 | 0,.891 | 0.834 | 0.848 |
| **UFC/g dry wet** | 467000 | 502000 | 424000 | 202000 | 146000 | 140000 | 76000 | 51000 |

Table 2. Taxonomic assignment of the isolated taxa according to Kirk *et al.* (2008).

| *Phylum* | *Class* | *Subclass* | *Order* | *Family* |
|---|---|---|---|---|
| Zygomycota | Zygomycetes | Incertae sedis | Mortierellales | Mortierellaceae |
| | | | Mucorales | Mucoraceae |
| | | Pleosporomycetidae | Pleosporales | Pleosporaceae |
| | | | | Incertae sedis |
| | Dothideomycetes | Dothideomycetidae | Capnodiales | Davidiellaceae |
| | | | | Mycosphaerellaceae |
| | Eurotiomycetes | Eurotiomycetidae | Eurotiales | Trichocomaceae |
| | Saccharomycetes | Saccharomycetidae | Saccharomycetales | Dipodascaceae |
| Ascomycota | | | | Bionectriaceae |
| | | | | Clavicipitaceae |
| | | Hypocreomycetidae | Hypocreales | Cordycipitaceae |
| | Sordariomycetes | | | Hypocreaceae |
| | | | | Nectriaceae |
| | | Sordariomycetidae | Ophiostomatales | Ophiostomataceae |
| | | | Sordariales | Chaetomiaceae |
| | | Xylariomycetidae | Xylariales | Amphisphaeriaceae |
| | Lecanoromycetes | Ostropomycetidae | Incertae sedis | Incertae sedis |
| | Leotiomycetes | Leotiomycetidae | Helotiales | Incertae sedis |
| Mitosporic Fungi | Incertae sedis | Incertae sedis | Incertae sedis | Incertae sedis |

## Effects of pollutants on soil mycobiota

In all of the soil samples collected in the polluted area, a marked reduction in the number of fungal colonies was observed. This reduction ranged from 69% in the soil collected under PLP to 82% in the soil collected under AUP (Tab. 1). With respect to fungal species, unpolluted sites showed a higher species richness (59 species) than polluted sites (34). Nevertheless, the diversity of soil mycobiota, detected by the Simpson's species diversity index, is higher in the contaminated area (0.92) rather than the control area (0.89). With respect to the relative frequency score, 79% of the species found in the unpolluted soil samples showed a relative frequency lower than 5% and could be considered "occasional" components (Mulas *et al*., 1995); in polluted soils, this percentage dropped to 52%. Excluding the occasional species (Rf < 5), the species richness values of polluted and unpolluted soil samples are very similar (Fig. 2). The distribution pattern of mycobiota, based on the relative frequency of major species identified in the polluted and control areas is shown in figure 3. The species identified in this study could be classified into three groups. Group 1 consists of species occurring in both areas, but showing some preference for occurring in one of them (16 species), e.g., *Paecilomyces lilacinus*, *Penicillium citrinum* Thom in the polluted area and *P. waksmanii* in the control sites. Group 2 comprises of species that were restricted to the polluted areas. These species are *Acrodontium hydnicola* (Peck) de Hoog, *Aspergillus fumigatus* Fresen., *Leptodontidium* sp.1, *Penicillium melinii* Thom, *Zasmidium cellare* (Pers.) Fr. and *Scolecobasidium constrictum* E.V. Abbott. Group 3 contains species that were restricted to the uncontaminated sites. These species were *Mucor* sp.1, *Penicillium miczynskii* K.M. Zalessky, *P. mirabile* Beliakova & Milko, *P. montanense* M. Chr. & Backus and *P. paxilli* Bainier.

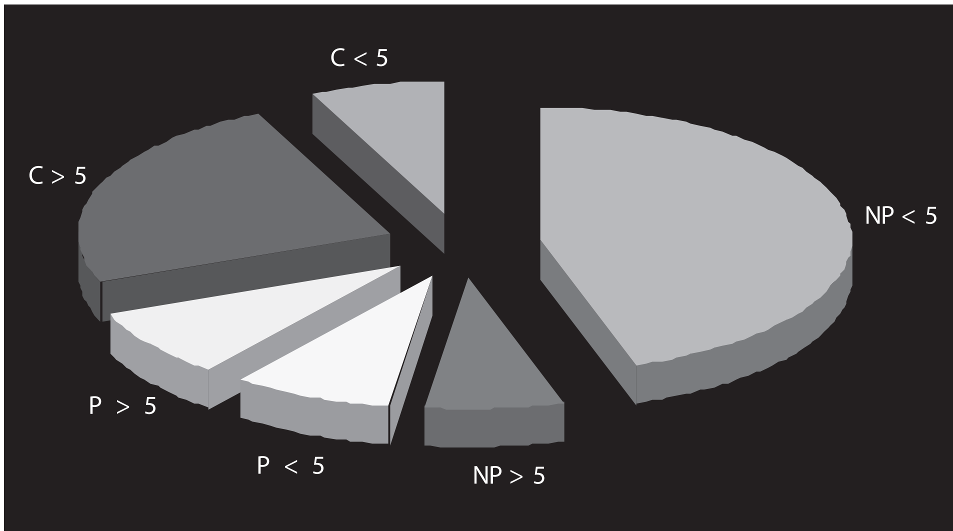


Fig. 2. Species richness of major (Rf > 5) and occasional (Rf < 5) fungal species detected in the following soil samples: observed in both polluted and control soil samples (C), exclusively observed in polluted soils (P) and exclusively observed in non-polluted soils (NP).

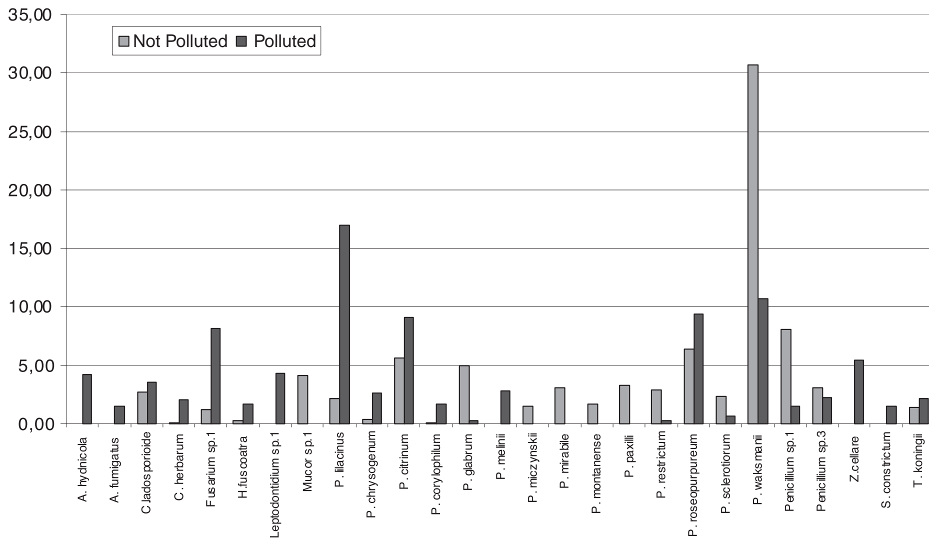


Fig. 3. The relative frequencies (Rfs) of major colonisers detected in polluted (P) and non-polluted (NP) samples.

## Effects of vegetal topsoil on the selection of specific fungal assemblages

The PCA biplot (Fig. 4) reflects the correlations among the micro-fungal assemblages identified in each soil sample taken from under the shrubs of *P. lentiscus*, *A. unedo*, *P. angustifolia* and *Q. ilex* in the contaminated and control areas. The ordination analysis showed a distribution into two groups: group 1

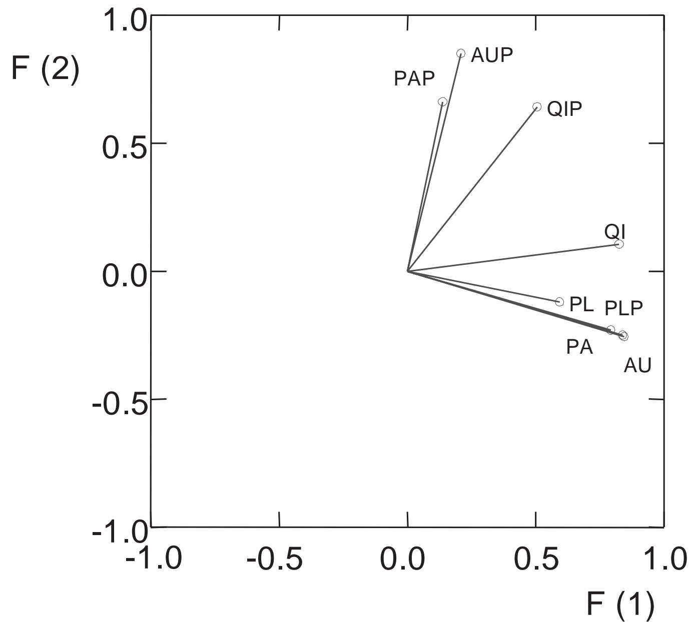


Fig. 4. PCA ordination biplot of the fungal assemblages found in each soil sample. The percentage of total variance explained by the model is 64.66% (F1 = 42.423; F2 = 22.246).

includes micro-fungal assemblages detected on PAP, AUP and QIP, while group 2 consists of fungal assemblages revealed in unpolluted (PA, AU, QI, PL) soil samples and PLP. In group 1, the fungal assemblage of AUP showed significant values of correlation with QIP (Pearson = 0.518) and PAP (Pearson = 0.496), while the correlation between QIP and PAP (Bonferroni's probability test) was not significant.

In group 2, the fungal biota of AU and PA had the highest association index (Pearson = 0.834). Moreover, the fungal assemblage of *P. lentiscus* in polluted soil (plotted near AU and PA) had elevated and significant values of association index with PA and AU (Pearson > 0.6; Tab. 3). Finally, the PL and QI fungal biota, were separated on the plot from PA, AU and PLP, along the first (F1 = 42%) and second (F2 = 23%) plot component respectively. PL and QI fungal biota also had significant values of association index with the other fungal assemblages of the group (Tab. 3).

The CCA biplot (Fig. 5) reflects the inter-relationships between the vegetal topsoil and the non-occasional species of fungal biota (Rf > 5). A group of species was separated on the right side of the plot near the unpolluted soil samples (PL, AU, PA and QI) and PLP. Some of these species showed a high Rf on a soil sample, i.e., *P. miczynskii*, *P. glabrum* (Wehmer) Westling and *Penicillium* sp.1 on PL, *P. mirabile* and *P. montanense* on AU, *P. citrinum* and *Penicillium* sp.3 on PLP, *P. restrictum* J.C. Gilman & E.V. Abbott on PA and *Mucor* sp.1 on QI. Other species with a high Rf were present in more soil samples, i.e., *P. paxilli* on PL and AU and *P. waksmanii* on PL, QI, AU, PA and PLP. Furthermore, species characterising the polluted soil were separated on the left side: *P. melinii* and *A. hydnicola* were strongly related to QIP, *Leptodontidium* sp.1 and

Table 3. Pearson's coefficient of association with significant values (P < 0.01, Bonferroni's probability test; PL: *P. lentiscus*, control area; QI: *Q. ilex*, control area; AU: *A. unedo*, control area; PA: *P. angustifolia*, control area; PLP: *P. lentiscus*, polluted area; QIP: *Q. ilex*, polluted area; AUP: *A. unedo*, polluted area; PAP: *P. angustifolia*, polluted area).

| | *PL* | *QI* | *AU* | *PA* | *PLP* | *QIP* | *AUP* | *PAP* |
|---|---|---|---|---|---|---|---|---|
| **PL** | **1** | | | | | | | |
| **QI** | ns | **1** | | | | | | |
| **AU** | **0.414** | **0.566** | **1** | | | | | |
| **PA** | **0.368** | **0.572** | **0.834** | **1** | | | | |
| **PLP** | **0.448** | **0.626** | **0.61** | **0.615** | **1** | | | |
| **QIP** | ns | **0.484** | ns | ns | ns | **1** | | |
| **AUP** | ns | ns | ns | ns | ns | **0.518** | **1** | |
| **PAP** | ns | ns | ns | ns | ns | ns | **0.469** | **1** |

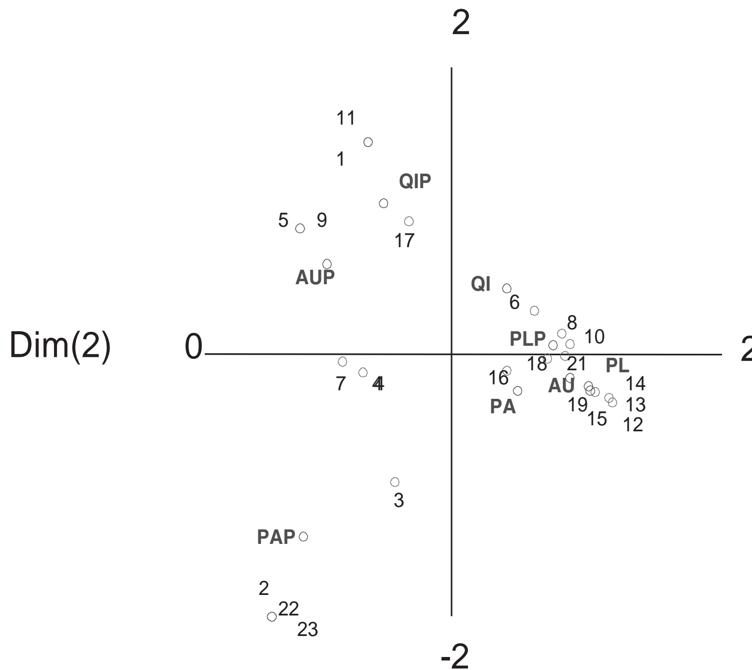


Fig. 5. CCA ordination biplot showing the relationship between major colonisers (Rf > 5) of soil and vegetal topsoil. The colonisers are as follows: 1. *Acrodontium hydnicola*; 2. *Aspergillus fumigatus*; 3. *Cladosporium cladosporioides*; 4. *Fusarium* sp.1, 5. *Leptodontidium* sp.1; 6. *Mucor* sp.1; 7. *Paecilomyces lilacinus*; 8. *Penicillium citrinum*; 9. *P. chrysogenum* var. *chrysogenum*; 10. *P. glabrum*; 11. *P. melinii*; 12. *P. miczynskii*; 13. *P. mirabile*; 14. *P. montanense*; 15. *P. paxilli*; 16. *P. restrictum*; 17. *P. roseopurpureum*; 18. *P. waksmanii*; 19. *Penicillium* sp.1; 20. *Penicillium* sp.2; 21. *Penicillium* sp.3; 22. *Zasmidium cellare*; 23. *Scolecobasidium constrictum*; PL: *P. lentiscus*, control area; QI: *Q. ilex*, control area; AU: *A. unedo*, control area; PA: *P. angustifolia*, control area; PLP: *P. lentiscus*, polluted area; QIP: *Q. ilex*, polluted area; AUP: *A. unedo*, polluted area; PAP: *P. angustifolia*, polluted area.

*P. chrysogenum* Thom var. *chrysogenum* to AUP, while *A. fumigatus*, *S. constrictum* and *Z. cellare* were related to PAP. The remaining species with high Rf values, present in more samples, were plotted in an intermediate position: *Paecilomyces lilacinus* and *Fusarium* sp.1 between AUP and PAP, *P. roseopurpureum* among AUP, QIP and QI, while *Cladosporium cladosporioides* (Fresen.) G.A. de Vries was found between PA and PAP.

## CONCLUSION

The Iglesiente open-mine district is characterised by abandoned and worked-out mines scattered over an area of approximately 100 $Km^2$. The heavy metal contamination is localised and can be attributed to the surface water run-off pollution from the slag and mine detritus of abandoned Pb and Zn mines that cover some sites. The levels of lead and zinc in soil samples showed some variation with respect to the vegetal topsoil (Fig. 1). As reported by Leita *et al.* (1989), the heavy metal content of plants collected in Iglesiente displays a wide variation. Several authors suggest that the Pb can be absorbed by plant roots and translocated to foliage, not only in grass, but also in shrubs (Leita *et al.*, 1989; Nicklow *et al.*, 1983; Preer *et al.*, 1980). Different mycorrhizal symbionts could play a significant role in the increased uptake of Pb and Zn (Gaur & Adholeya, 2004; Wong *et al.*, 2007). The variation in pollutant concentrations observed in the soils investigated in this study could be attributed to a difference in the amounts of Pb and Zn absorbed by different fungus-host plant combinations.

The data obtained in this study, using the dilution plate method, can be considered valid for the comparison of the effects of long term heavy metals contaminations on the soil fungi. However, it characterises only a portion of the total soil biota, mainly because of the selective technique used. These methods are biased in favour of rapidly growing and sporulating organisms (e.g., spore-forming fungi such as *Penicillium* and *Aspergillus*), which overwhelm the non-sporulating fungi, especially the basidiomycetes. Thus, non-sporulating fungi often get overlooked. In all soil samples collected in the polluted area, a marked reduction in the number of fungal colonies was observed (Tab. 1). The decrease in micro-fungal density caused by heavy metal contamination seen in this study is consistent with the studies of Abdel-Azeem *et al.* (2007), Bååth *et al.* (2005), Babick & Stotzky (1985), and Zucconi *et al.* (1996). Species diversity (Simpson's index) increased in the contaminated soil samples (except for PLP soils; Tab. 1). As observed by Abdel-Azeem *et al.* (2007), this increase in mycobiota diversity could be due to a fungal stress response. Consequently, fungal species better adapted to unpolluted soil and affected at intermediate rates of contamination allow other fungi, probably less competitive in non-stressed soils but better adapted to heavy metals, to colonise the organic components of soil.

Soil pollution places selective pressure on sensitive micro-organisms, thereby causing a change in the diversity of soil biota. In polluted soil samples collected under *A. unedo* and *P. angustifolia*, an overall change in the fungal assemblages was observed in comparison with the control samples (PA-PAP and AU-AUP). The *A. unedo* and *P. angustifolia* soil mycobiota, strongly correlated in the control area, respond in a similar way to the presence of contaminants, leading to a selection of new species. Among these new species, *Fusarium* sp.1 and

*P. lilacinus*, with high Rf values, were detected in both PAP and AUP soil samples. In the AUP soil with double the Pb concentration as the other contaminated soils (Fig. 1), *Leptodontdium* sp.1 and *P. chrysogenum* var. *chrysogenum* had high Rfs. These two species were detected in AUP soil only, were virtually absent in control soil (*P. chrysogenum* var. *chrysogenum* Rf = 1.4 in AU) and, as such, seemed to show a high ability to compete in the AUP soil and to tolerate the high Pb concentration.

Conversely, the fungal assemblages identified on *P. lentiscus* and *Q. ilex* from soils in polluted and control sites (PL-PLP and QI-QIP, respectively) showed significant association indices. In the PLP soil sample, the contaminants selectively favoured species such as *P. citrinum* and *P. waksmanii*, also present in the control site (PL), while in the QIP mycobiota, some new species were selected.

Vegetal topsoil appears to play a role in the selection of fungal soil. In polluted and unpolluted samples taken from under *P. lentiscus*, *Q. ilex*, *A. unedo* and *P. angustifolia*, fungal assemblages with a certain degree of correlation, but characterised by different species, were detected (Tab. 1). This could be induced mainly by the organic substances released by the plants into the soil as root exudates, litter degradation products, etc. (Mulas *et al.*, 1995; Pasqualetti *et al.*, 1999; Tempesta *et al.*, 2003).

Out of the 24 detected species with a Rf > 5 in at least one sample (Tab. 1), only six were observed in more than one soil fungal assemblage with frequencies greater than to 5. Specifically, *P. waksmanii* was present in all control samples and showed a strong reduction in polluted AUP, PAP and QIP soil samples. This species appears to present at a low specificity with respect to vegetal topsoil and was negatively influenced by high concentrations of pollutants, with the exception of soil samples collected from under *P. lentiscus*. Interestingly, *P. lilacinus* was present in AU, AUP, PA, PAP, QI and QIP, with frequencies higher in contaminated samples than in the control areas. This species is probably more tolerant to pollutants, as reported by Tatsuyama *et al.* (1975), and benefits from the reduction in competition caused by high levels of Pb and Zn. In addition, *Fusarium* sp.1, with high a Rf in AUP and PAP soils, appeared to be positively selected by the Pb and Zn pollutants. *C. cladosporioides* was commonly found in the soils collected under *P. angustifolia*, being present in both polluted and unpolluted sites with similar Rfs. Finally, *P. roseopurpureum*, with a high Rf, detected on QI, QIP and AUP and *P. citrinum* detected on PL, QI, PLP and QIP showed different behavioural trends with respect to contamination; the trend varied with the function of the topsoil.

In conclusion, the investigation of soil mycobiota has shown that long-term exposure of soils to Pb and Zn leads to a specialised micro-fungal assemblage, strongly influenced by vegetal topsoil, with a significant reduction in fungal abundance.

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