Universidade de Aveiro Departamento de Biologia
2004

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Avaliação da Ecotoxicidade de Solos: Estudo do Caso da Mina de Jales

Ecotoxicity Assessment of Soils: a Case Study from Mina de Jales

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Ecotoxicity Assessment of Soils: a Case Study from Mina de Jales

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Professor Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro, e co-orientação científica do Professor Doutor António José Arsénia Nogueira, Professor Associado, do Departamento de Biologia da Universidade de Aveiro.

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agradecimentos

A realização deste trabalho não teria sido possível sem a colaboração e o encorajamento de algumas pessoas, às quais gostaria de agradecer.

Ao Professor Doutor Amadeu Soares, como meu orientador neste estudo, por ter aceite essa responsabilidade e pelas sugestões, críticas e revisões desta dissertação. Pelos seus conselhos, e principalmente, o apoio em alturas cruciais, o meu muito obrigada.

Ao Professor Doutor António Nogueira, por ter aceite ser meu co-orientador, pelas sugestões e revisões desta dissertação e pelo apoio nas saídas de campo.

Aos meus amigos, mais que colegas, Abel, Raquelita, João, Lísia e Inêsia, por todos estes bons momentos laboratoriais, gastronómicos e festivos ao longo destes poucos, mas bons, anos.

Ao Abel e à Lísia, um especial agradecimento pela boa disposição e disponibilidade nas saídas de campo à Mina de Jales. Obrigada pela vossa amizade.

Ao meu colega e amigo Carlos, que me levou em Coimbra para as lides ecológicas e ecotoxicológicas. Obrigada pela amizade que nos une, desde essa altura... já lá vão 10 anos!

Aos meus colegas, Filipa, Quim, Henrique, Marco, Mónica, Ana Ré e Paula, pelos momentos em laboratório e não só...

Às colegas do lado, Carla, Marta, Salomé e Guida, pelos bons momentos de encontro nos corredores e no laboratório do Departamento.

Aos colegas do fundo do corredor, Catarina, Joana, Nelson, Sara e Bruno, pela boa disposição e conversas do dia-a-dia.

Ao Professor Doutor Fernando Morgado, pelo apoio e amizade.

Aos meus sempre amigos, Mafalda, Fernando, João Pinto, os manos Luís e Nuno Reis, Bruno e Rafael, pela amizade de tantos anos.

A novas amizades, que prevejo longas. Obrigada Vanda pelo apoio e amizade.

À Ana Leão, pela amizade de tantos anos. Obrigada por teres sempre um sorriso para mim.

À Rita, que me tem ajudado, mesmo tão longe, há tanto tempo... Sei que, onde quer que estejas, vais sempre olhar por nós!

Ao Miguel e à Lena, com toda a vossa amizade e apoio. Embora não sejam "oficialmente da família", já vos sinto como tal!

A toda a minha família, pelo orgulho demonstrado e apoio que me têm dado ao longo destes anos.

Ao Sérgio, por tudo! Pelo companheirismo, pela dedicação, carinho e amor destes 5 anos. Pelos conselhos que me tens dado e por me mostrares, com mais clareza, que alguns acontecimentos são meros pormenores que passam pela nossa vida. E que outros, sim, valem a pena!

Aos meus pais... por me terem dado as oportunidades que me permitiram chegar até aqui, por nunca duvidarem, por sempre acreditarem. Pelo apoio, amizade e amor demonstrados ao longo de toda a minha vida. Sei que, sem o vosso apoio, nunca conseguiria chegar onde cheguei.

À Fundação para a Ciência e a Tecnologia, pelo financiamento deste estudo através da atribuição de uma bolsa de Doutoramento (SFRH / BD / 1311 /2000).

resumo

Actualmente, a poluição dos solos é considerada um problema ambiental, podendo ser o pilar inicial da transferência de compostos químicos numa cadeia alimentar, através da assimilação de contaminantes pelas plantas, ou podendo também levar à contaminação de águas subterrâneas.

A análise química de um solo não é considerada suficiente na avaliação da toxicidade de solos, porque a biodisponibilidade de diversos xenobióticos depende de diversos factores, nomeadamente das características físico-químicas do solo (e.g. pH, matéria orgânica, presença de catiões). Por estas razões, os bioensaios são provavelmente as ferramentas indicadas para avaliar a toxicidade da fracção biodisponível de compostos químicos dos solos. Neste estudo foram desenvolvidos diversos ensaios ecotoxicológicos, utilizando organismos-teste edáficos (plantas e invertebrados) e aquáticos (cladóceros e bactérias), na avaliação do potencial tóxico de dois solos de uma mina abandonada, Mina de Jales, localizada a nordeste de Portugal. Estes solos apresentavam níveis distintos de metais pesados: o solo JNC apresentava um baixo teor em metais pesados, enquanto o solo JC apresenta uma elevada quantidade de metais pesados.

Para além destes testes foram utilizados ensaios microbianos, através da medição da actividade enzimática dos solos. Estes ensaios foram utilizados em vários tempos de amostragem, coincidindo com diferentes estágios do processo de reabilitação da mina, que foi iniciado nos finais de 2002. Com os dados obtidos dos ensaios enzimáticos, foi derivado um Índice de Qualidade dos Solos (IQS). Este índice revelou que o solo JNC tem uma baixa qualidade, quando comparado com o solo JC, cujo índice chegou a níveis próximos dos apresentados por solos nativos.

Utilizando organismos edáficos, foi obtido um padrão de toxicidade semelhante. No ensaio de emergência e crescimento com a espécie de nabo *Brassica rapa* (RCBr), observou-se uma diminuição no crescimento e peso das plantas expostas ao solo JNC, coincidente com uma resposta bioquímica de stress oxidativo. Nos ensaios de resposta de evitamento, o isópode terrestre *Porcellionides pruinosus* evitou o solo JNC, enquanto na exposição ao solo JC este organismo não demonstrou qualquer tipo de preferência. Por outro lado, o ensaio de emergência e crescimento com a aveia (*Avena sativa*), o ensaio de reprodução com o colêmbolo *Folsomia candida* e o ensaio de evitamento com a minhoca *Eisenia andrei* não demonstraram qualquer efeito na exposição de ambos os solos.

Utilizando os ensaios com organismos aquáticos, foram obtidos resultados semelhantes, na exposição ao solo JNC, embora as Unidades Tóxicas derivadas destes ensaios apresentassem valores superiores quando comparados com os outros ensaios. O Teste de Fase Sólida utilizando o Microtox® demonstrou ser o ensaio com maior sensibilidade dentro da bateria de testes utilizados. O ensaio com *D. magna* foi o único que apresentou toxicidade relativamente ao solo JNC.

Conclui-se que possivelmente a fracção biodisponível de metais pesados presente no solo JC é menor do que no solo JNC, visto que os organismos vivos são influenciados apenas pela fracção disponível dos compostos químicos e não pela sua fracção total. Outra possibilidade para a explicação das diferentes toxicidades entre os dois solos pode ser o desconhecimento da existência de algum composto químico, que não constava na análise química e que foi, por isso, negligenciado.

abstract

Soil pollution has become nowadays a key problem in ecosystems because it can act as a starting line in food web contamination by plant uptake and/or can lead to groundwater contamination.

Measuring the total amount of chemical compounds in soil is not satisfactory to evaluate its toxicity because chemical bioavailability depends on several factors, like soil physicochemical characteristics (e.g. pH, organic matter, cations). Therefore, bioassays are probably the better tools to assess the toxicity of the bioavailable fraction of contaminants in soil. In this study we carried out several ecotoxicological bioassays, using different test-organisms (e.g. plants, microorganisms, soil dwelling invertebrates, bacteria and aquatic invertebrates), to evaluate the toxicity of two soils from the surrounding area of an abandoned mine in the northeast of Portugal- Mina de Jales, in 2002. These soils showed different heavy metal contents: JNC is a soil with low heavy metal content and JC is a soil with high heavy metal content.

Soil enzymatic bioassays were performed in different time sampling to assess not only the toxicity of these two soils, but also the effect of the mine rehabilitation process (started in late 2002) on microbial communities. A Soil Quality Index (SQI) was derived from the enzymatic data. This index showed that the JNC soil presented a lower quality index when compared to JC soil, which even showed a SQI similar to indexes obtained for native or climax soils.

A similar trend was achieved using edaphic test-organisms. In the emergence and growth bioassay with *Brassica rapa*, plants showed slower growth and biomass production when exposed to JNC soil. The isopod *Porcellionides pruinosus* also avoided this soil as concluded from the avoidance behaviour response test.

The growth bioassay with *Avena sativa*, the reproduction bioassay with the collembolan *Folsomia candida*, and the avoidance bioassay with *Eisenia andrei* showed no sensitivity to compounds present in both soils. Similar results were achieved using aquatic ecotoxicological tests, although these test organisms showed higher Toxic Units for JNC soils /soil elutriates exposure. The Solid-phase Test performed with Microtox® was the most sensitive test enclosed in the test-battery. The *D. magna* bioassay was the only assay that showed toxicity responses for the JC soil exposure, by elutriates exposure.

We conclude that the bioavailable fraction of heavy metals of JC soil is lower than that of the JNC soil, since most organisms are influenced by the bioavailable fraction of metals instead of the total amount present in the soil. In addition, the soil with lower heavy metal content, and that could be uncontaminated, presented high toxicity levels. Another possible explanation is the unknown presence of other contaminants that were not regarded on the chemical analysis and were therefore neglected.

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GENERAL INTRODUCTION

SOIL - A DYNAMIC SYSTEM

Soil is a dynamic system and is considered the key component of terrestrial ecosystems, being essential for plant's growth and degradation and/or recycling of nutrients. It exhibits short-term fluctuations, such as moisture content, pH values, and oxidation reactions, being these natural variations usually the result of environmental factors or management issues.

Soil is a complex heterogeneous medium comprised of mineral and organic fractions, air and water components. These water and air components are enclosed in the fractional soil pore space, also known as soil porosity [1], which is of great importance to microorganisms and also as plants' suppliers.

Soil components are one of the major responsible for soil quality and therefore fertility, and differences in composition of soils are mainly due to their original parent material, soil formation, and the possible addition of fertilizers, pesticides, manures, or irrigation water [1].

Soil texture and structure express the importance of these soil components in the soil function. Texture refers to the relative proportion of clay, silt, and sand in soil. Different percentages of these three components will make a soil reacting in a different way when environmental and management changes occur. For example, the ability of a soil to adsorb cations (like heavy metals ions) depends on its clay fraction and also on the organic matter content. Also the arrangement of these soil particles is important to soil structure and behaviour [1]. This is one of the reasons why soil texture is important as an indicator of soil quality, but it can not be used on is own because it will act combined with other soil properties (e.g. pH, cation exchange capacity). Based on its texture, soil is usually described by the name of the dominant fraction present in soil; when no fraction is dominant the soil will be described as loam. A triangle diagram is usually used to help to classify a soil, although scientists have not reach yet a consensus on a common diagram to be used by all when describing soil texture. One of the diagram triangles that shows the composition of the textural classes in soils is used by the United States Soil Survey (Fig. 1) [1, 2].

Soil structure describes the arrangement of these soil particles, and when a soil is unattached to one another, it is called structureless or a single grained structure, as it happens with sand dunes. On the other hand, when mineral particles are packed tightly, they form a massive structure (aggregates) that have shape characteristics from the soil, and when formed naturally they are called peds [1].

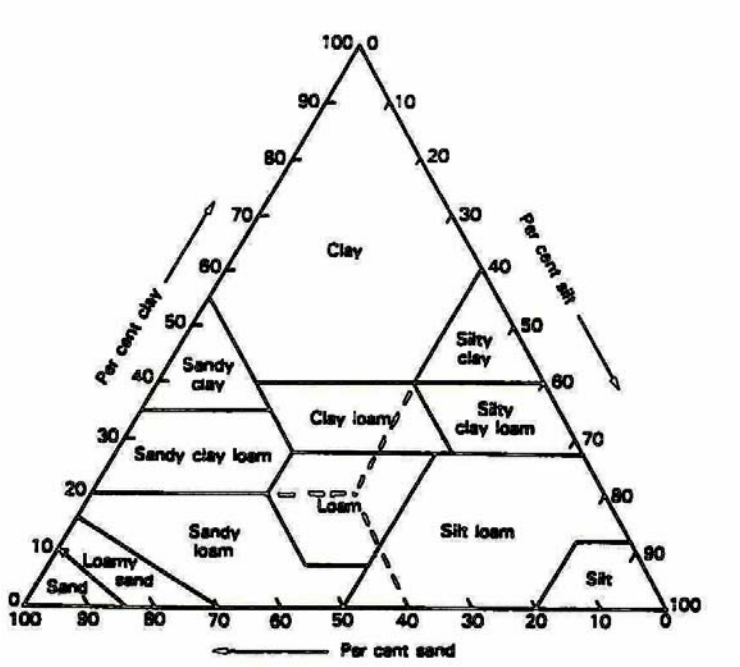


Fig. 1- Composition of the textural classes of soils used by the United States Soil Survey (sand = 2-0.05 mm; silt= 0.05-0.002 mm; clay<0.002 mm). The broken line (----) describes the loam soil (40% sand, 40% silt and 20% clay). (from [1])

The liquid phase in a soil also plays an important role in the survival of the biota. The soil water content is of major importance because it contains solutes and dissolved gases, which provide moisture for animals and transfer nutrients from soil to plants and also to groundwater or to the atmosphere. The water solutions are constituted by solutes and dissolved gases, which will play an important role in soil composition and quality. Some ions present in this soil solution are Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}, NO_3^{-}, Cl^{-}, SO_4^{2-} and HCO_3^{-}. Under anaerobic

conditions Mn^{2+} and Fe^{2+} can also be found. Some of the ions in solution can also form complexes between them and with organic acids. These complexes are soluble and they improve the supply of nutrient metals (e.g. iron) to plants by increasing their concentration in solution [1, 2].

Soil pH is another soil property that is of major importance for edaphic organisms. It is a function of H^+ concentration in soil pore water, which is in dynamic equilibrium with the negatively charged surfaces of soil particles [3]. Even though, the pH concept in soils is not as precise as in liquid solutions due to the heterogeneity of soils and to the small amount of water present in soil pores.

Clay minerals (minerals with diameters less than 2 μm) have large surfaces and act like a permanent negative charge in soils; these minerals are a result of rock weathering and have important effects on physical and chemical properties of soils. If there is a high concentration of H^+ the clay minerals will capture them, but if other cations, like Ca^{2+}, Mg^{2+} and K^+, are present they will replace H^+. The importance of clay minerals to soil fertility can be explained by the ability of retention of cations (nutrients), which will decrease their squander through water and therefore soil quality can be maintained [4].

Soil organic matter (SOM) also plays an important role in supplying nutrients to edaphic organisms, contributing to cation exchange capacity and improving soil structure. Organic matter from most soils consists mainly on plant biopolymer residues, materials derived from decomposition processes, microbial tissues and humic substances [5]. Equilibrium in sorption and desorption processes in solid-liquid interfaces are common in soils, and SOM takes part on these processes. Ions can be absorbed by these organic matter particles that act as a sorbent, as by clay minerals. SOM is composed by a so called “light” fraction (the visible fraction) and the humus fraction. The “light” fraction is constituted by carbon components originated by plant roots, stems, leaves, animal residues, sewage sludge, etc. Humus, a more stable compound, is constituted by fulvic acid, humic acid and humin [1, 2]. Decomposition products of SOM are essentially CO_2, which will be returned to the atmosphere. The plant essential nutrients like nitrogen, phosphorous and sulphur are also enclosed in organic compounds, which can be

mineralized by microorganisms and released as inorganic ions, which can finally be taken up by plants.

Although humic and fulvic fractions of humus have acid groups, their carboxyl and OH groups dissociate at 4.5<pH<7 and higher pH values, respectively. Therefore humus is negatively charged and it gains the ability to react with metal cations to form soluble and insoluble complexes, showing more stability at pH 5 with Cu and Pb and less stability with Zn and magnesium (Mg). Trivalent metals, like Fe^{3+} and Al^{3+}, are held strongly [1].

As dynamic systems, soils are subjected to significant variations by reactions of oxidation and reduction (redox), that are controlled by the aqueous free electron activity, and affect mostly elements like carbon, nitrogen, oxygen, iron and manganese [3]. These reactions are usually slow and are catalysed by soil microorganisms that live in a full range of pH and pE conditions (values that express redox activity). Microorganisms and other edaphic organisms are involved in respiration processes, consuming oxygen. As an example, when the amount of oxygen in soil becomes exhausted, anaerobic microorganisms will be predominant and some elements like Mn, Cr, Fe, Cu or Mo are gradually reduced. These reactions can be important in altering the mobility and toxicity of inorganic and also organic contaminants. As examples chromium (Cr) can exist in the soil as Cr(III) which is stable and innocuous and Cr(VI) which is mobile and harmful. Mn(III/IV) naturally oxidizes Cr(III) to the toxic Cr(VI). On the other hand, Ferrous iron (Fe(II)), FeS and soil organic matter have the capability to reduce Cr(VI) to the more stable Cr(III).

As described before, the negative charges of humus and clay minerals varies with pH. The ability of soil to hold cations that can be exchanged between soil fractions will also be pH dependent.

Living organisms can perform transformations in the environment, having a deep effect on the ability of soils to provide food and fiber for the world population. Organisms play a crucial role on soil, water and air quality, being a dominant part in the cycling of carbon (C), nitrogen (N), phosphorous (P), sulphur (S) and some micronutrient cations, like copper (Cu), iron (Fe), manganese (Mn), boron (B), molybdenum (Mo) or zinc (Zn). The interaction of these cycles is essential for soil

quality and its understanding can be a useful tool for the management of land, providing information for a rational use of fertilizers, waste disposal in soil and for the prevention of soil derived pollution of air and water [6].

The degradation of dead materials in soil is an important biological process because C is renewaled to the atmosphere as carbon dioxide (CO_2), N is turned into the available form of ammonium (NH_4^+) and nitrate (NO_3^-) and other associated elements like P, S and micronutrients appear as inorganic forms that are required by higher plants [7].

Nutrient cycling includes the conversion of organic forms to mineral and vice-versa. As plants and microorganisms uptake mineral nutrients that will be incorporated as organic molecules, edaphic organisms decompose organic detritus that are converted, in a final step, in mineral forms. The turnover of mineral to organic forms, performed by plants and microorganisms, is called immobilization, and the opposite process, i.e. the transformation of organic forms to mineral forms like ammonium, sulphate and phosphate ions, is called mineralization (oxidation reaction). This equilibrium can be expressed by the C:N ratio, and it will give information on the cycling process. When decomposition processes are taking place in soil, the C:N ration will decrease with time because carbon is lost as CO_2, while N is reused by microorganisms [7]. The decrease in the N content will reach a point when the microbial activity will also decrease due to organic carbon loss. In good quality soils this loss will take short periods to recover, depending only on the time that the organic material needs to be degraded.

As previously stated, carbon (C) is an important nutrient for plants and for live organisms and organic carbon can be found in soils, ranging from less than 1% in sandy soils to 20 % in soil of wetlands and bogs or swamps [8]. Carbon can be considered the building block of plant life and a major constituent of SOM that is also the food source for edaphic arthropods and other invertebrates. The C cycle is processed in a multicompartimental system with terrestrial, aquatic (marine and freshwaters), and atmospheric compartments (Fig. 2). The original source of C was the fundamental rocks, where intense periods of volcanic activity induced the CO_2 production, and a secondary source was probably the primitive atmosphere

with high amounts of methane gas (CH_4). In the environment there are several C reservoirs, including SOM, which constitute, at the earth's surface, the compartment that engages the higher amount of C (30-50 of C x 10^{14} Kg), and also sediments, to depths of several kms, that can have till 200 000 of C x 10^{14} Kg [6]. The annual input of C in soils is about 15% of the atmospheric CO_2 (1.10 x 10^{14} kg/year), and on the other hand the equivalent amount of C is returned to the atmosphere by decay processes [6]. The C cycle begins with the absorption of CO_2 from the atmosphere by plants, and then it is transported trough their photosynthesis process, forming organic compounds. Following this cycle, as plants are energetic sources, animals will ingest them, and the organic carbon will return to the soil compartment with animal's excretion products or after animals death.

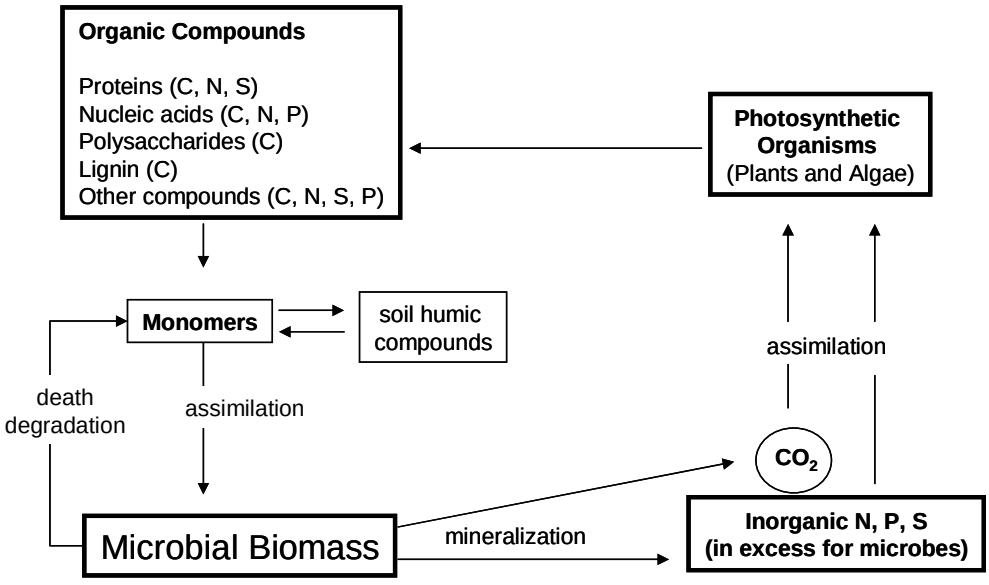


Fig. 2- The C cycle and the interchanges between compartments (adapted from [6])

Another crucial nutrient cycle for soil is the nitrogen cycle that turns nitrogen available for plants. The majority of nitrogen exists as N_2 gas in the atmosphere and this molecule is very stable due to its triple bond. However, it is very difficult for a plant to obtain nitrogen from atmosphere because this gas is non-reactive, so

they obtain nitrogen from soil as either nitrate (NO_3^-), from soil solution, or ammonium (NH_4^+), by ion exchange. This nitrogen will be of major importance to form proteins that will be used for plant growth processes. Also several soil enzymes perform nitrogen transformations like fixation, mineralization, volatilization, nitrification, leaching, denitrification and immobilization. Soil N is gained by microbial fixation of molecular N_2 and by addition of ammonium (NH_4^+), nitrate (NO_3^-) or nitrite (NO_2^-) by rainwater. The losses of N in soil occur through crop renewal, leaching and volatilization. Bacteria, cyanobacteria and plant-bacteria associations can fixate N_2 and transform it to NH_3 (ammonia) and organic N that are converted also to NH_3 and NO_3^- by mineralization processes. Organic N can also be transformed in NH_3 by ammonification and therefore this last product can suffer an oxidation process and be transformed in NO_3^- (nitrification). These two compounds can be finally assimilated by plants and immobilized by soil organisms. Ultimately to complete the N cycle, NO_3^- or NO_2^- are returned to the atmosphere as the molecular form N_2 by a biological denitrification [6].

Another important nutrient is sulphur (S), which is also a constituent of living organisms, being part of proteins and some polysaccharides, vitamins and hormones, and it is present in soils as in atmosphere and aquatic ecosystems. Sulphur dioxide can precipitate onto surfaces and be oxidised to sulphate in soil, where in high amounts can be toxic to plants. Then it can be reduced to sulphide in the atmosphere, or oxidised to sulphate in the atmosphere as sulphuric acid, a principal component of acid rain. The chemical forms of sulphur that can be found in soil have a net valence of minus 2, except for elemental sulphur which has a valence of zero. The existence of oxides or reducing mineral forms of sulphur and also nitrogen depends on the redox stage of soil. Sulphate, sulphide and sulphur are the major and most important forms of S in soils due to their ability of forming complexes with other nutrients that are essential for plants. Several chemical reactions can occur in soils catalysed by enzymes, like mineralization of organic sulphur, sulphur immobilization (reduction reaction) and sulphur oxidation. Particularly in the case of abandoned mines, S can be a serious environmental hazard where microbial and chemical reactions convert pyrite (FeS_2) to sulphuric acid (H_2SO_4) that will decrease the soil and water pH to levels that can not support

life forms. During weathering, the S in primary minerals is converted to SO_4^{2-} which is used by plants and microorganisms and turned into organic forms (e.g. cysteine, methionine of proteins, sulphates of polycchacarides). When residues return to the soil and microbial processes are carried out (decay), part of the organic S appears as H_2SO_4 and another part is incorporated as biomass and humus. Sulphur can be added to soil in fertilizers, pesticides, in irrigation waters, through precipitation or even through absorption of S gases from the atmosphere [7].

Phosphorous (P), another crucial nutrient for plant's growth, involves in its cycle interactions between plants and microorganisms. P in soil solution is in equilibrium with an amount of labile inorganic P and, as P is taken up by plants or immobilized by microorganisms, additional inorganic P is solubilized. The major reserves of P in the environment are marine sediments, terrestrial soils, dissolved inorganic phosphate in the ocean, crustal rocks as apatite and the biota or biomass, in which the amount of P in the terrestrial biota exceed 21 times the marine biota. Unlike the C, N, and S cycles, the P cycle does not have a gaseous component, and the amount of P that circulates from and to the atmosphere is of minor importance. Furthermore, lixiviation is not one route for P loss in soils, like happens with other nutrients. The movement of phosphorous in soil between available and non-available pools is affected by factors such as pH or soil temperature [7]. In diluted solutions, phosphoric acid dissociates from H_3PO_4 to $H_2PO_4^-$ or the other way depending on the addition or subtract of H+. The same can be stated between $H_2PO_4^-$ and HPO_4^{2-} and PO_4^{3-}[6]. The P content and loss in soils varies considerably and are dependent of the parent material of soil, degree of weathering and leaching. Soil P content can range from 100 mg/kg in sandstones to over 2000 mg/kg in high-phosphate limestones [6].

All these previously described components and reactions are important for the soil's function in the terrestrial environment. Soil plays a crucial role in the retention of nutrients and exceeding chemical compounds (stressors), avoiding their flow through the water path, and also as habitat for plants, animals, microbes and soil biocenoses [9].

SOIL CONTAMINATION NOWADAYS (Europe and Portugal)

Since Roman times, soils and the terrestrial environment have been issues of concern due to the beginning of agricultural practices. Some centuries ago the maintenance of soil fertility was another goal that people were focused on, mainly on erosion conservation, to ensure that crops could be grown, because soil was seen as a source on which their livelihood depended on.

With the increasing of human population, soil productivity has become a problem as some soils have became unsuitable for agriculture needs and therefore fertilizers were applied in high quantities. Additionally, another problem that has risen from urban and industrialised countries was water, air and soil pollution.

Introducing harmful substances in the environment will cause adverse effects on human health, agricultural productivity and on natural ecosystems. This is why pollution had become one of the most important environmental issues when compared to other environmental problems like soil erosion and species extinction.

The main difference between air, water and soil pollution is that in air and water this pollution tends to be diluted while in soil the tendency is to become accumulated. Soil acts like a sink for pollutants due its ability for the sorption of chemical compounds, referred before, restraining the leaching of pollutants till they reache the groundwater. But this will not inhibit totally chemical compounds to reach groundwater, particularly when the buffer ability of soil is collapsed.

Nowadays the major soil problems can be summarized as follows [10]:

- Sealing- rates of soil loss due to urbanization and transport infrastructure are increasing so that there is little space available for further expansion. In Germany, for instance, the average daily loss in soil was more than 120 ha in 1997 [10]. In the Mediterranean countries urbanization has been very quickly in coastal zones, a process also linked to tourism development. Soil sealing is also expected to increase in countries like Portugal, Finland and Ireland where urbanization has started to increase. On the other hand, some areas of the new independent states in the Eastern Europe are so heavily transformed by mining and heavy industry that are usually considered as “industrial

desertification". Loss of natural and agricultural areas due to urbanization has been noticed in Europe; in Portugal, mainly in Setúbal, artificial areas have doubled, and in Porto this increase reached 78% [10].

- Erosion- erosion by water and wind is an important problem in soils in Europe where impacts are intensified by contamination from industrial action. This problem is also increasing in agricultural areas due to more intensive practices, to the combination of severe climate, sharp slopes and poor vegetation cover. Soil erosion leads to losses in SOM, mineral nutrients and soil functions and also to problems of contamination and loss of the soil ability to sequester carbon from atmosphere. This can also lead to the disappearing of several edaphic species that are crucial for maintaining soil functions and nutrient cycles. In Europe soil loss is around 17 Mg ha^{-1} $year^{-1}$, while the formation rate of new soil is 1 Mg ha^{-1} $year^{-1}$ [7].

- Slope stability- soil plays a crucial role on the preservation of soil slope, preventing some natural disasters like flooding that sometimes occur in lowlands and also mass movements of soil. This phenomenon can cause erosion, pollution, and loss of soil resources in a complex inter-relationship of causes and effects.

- Contamination- in Europe soil contamination is not widespread (excepted for acidification) but it is considered high in restricted areas or hotspots, due to diffuse or localised sources. Although it has been observed a decrease in emissions and use of hazardous substances mainly due to policy measures, this is still balanced by an increase in economic activities. Local contamination is, above all, caused by industrial activities, former waste sites, atmospheric depositions, and intensity of agriculture practices or by former military installations [10, 11]. The seriousness of the contamination problem is linked to its consequences on ecosystems and human health and its irreversibility, because it is practically or economically impossible to restore a degraded or polluted soil in its all compartments or functions. Some remediation processes that have been developed in Europe are only focused on some of these functions and do not cover all soil compartments. Major pollutants include heavy metals, artificial radio-nuclides and organic contaminants, like PAHs, chlorinated hydrocarbons, PCBs and dioxins pesticides.

- Acidification- Soil acidification occurs as a result of the emission of acidifying pollutants from transports, industry and bio-geochemical cycles. Soils under severe acidification are difficult or impossible to be recovered if the pollutant source did not stop. This acidification problem is occurring mainly in north-western and central Europe but it is not expected to increase more due to several policies that have been undertaken for 30 years [10].

In the particular case of Portugal, the problem of contaminated soils is related to industrial and mining activities. In 1998, the National Laboratory of Civil Engineering made a report enumerating contaminated soil areas in Portugal [11]. There is no environmental legislation related to maximum permissible levels for contaminants in soils in Portugal, which creates difficulties for the implementation of the European Directives, because sometimes these directives are not adjusted to the socio-economic and structural situation of Portugal. Governments usually use benchmark values limited by other countries (e. g. Canada, USA and Germany) as values for a maximum permissible content in soils (Table I). In the Portuguese case, there is a small law on the permissible heavy metal content in soils for the culture of food for animals (Portaria n.º 732-A/96 from 11-12-1996) and a general law for environmental issues (Lei de Bases do Ambiente- Law on Environmental Bases). Additionally, the Portuguese Institute of Waste (a governmental institution) advises the use of Canadian standards (Guideline for use at contaminated sites in Ontario) as an evaluation tool for soil contamination criteria (Table I).

Every year a report on the Portuguese environmental status is being published by the Ministry of Towns, Territorial Planning and Environment (the former Ministry of Environment), which states the water, atmosphere and soil qualities, stressing the main problems in the country. In 1999, the main problem stated for soil contamination was the fact that aquifers, the water supplier for populations, depends on the soil quality and status.

Metals	Portugal (mg/Kg) (1)			EPA- ESV (mg/Kg) (2)	Canada (remediation) (3)			ESV-Protocol (mg/Kg) (4)		Canadian Guidelines (mg/Kg) (5)			German Guidelines (6) (trigger values)		Quality Standards UK "trigger Concentrations" (mg/Kg) (7)	
	pH≤5.5	5.5<pH≤7.0	pH>7.0	recommemded	agriculture	residence	industry	MPC	ESV	agriculture	residence	industry	agriculture	human (lower value)	domestic garden/ food growing area	park, paying field, open space
Alumminium				50												
Arsenic				10	20	20	20	34	10	12	12	12	0.4	25	10	40
Cadmium	1	3	4	1.6	3	12	12	1.6	1.6	1.4	10	22		10	3	15
Chromium	50	200	300	0.4	750	750	750	100	32	64	64	87		200	25	
Copper	50	100	200	40	150	225	225	40	40	63	63	91	1			
Lead	50	300	450	50	200	200	1000	140	50	70	140	600	0.1	200	500	2000
Mercury	1	1.5	2		10	10	10	2.2	0.3	6.6	6.6	50	5	10	1	20
Zinc	150	300	450	50	600	600	600	160	50	200	200	360	2			
Iron				200					200							
Manganese				100					100							
Nickel	30	75	110	30	150	150	150	38	30	50	50	50	1.5	70		
Selenium				0.81	2	10	10	0.81	0.81	1	1	3.9			3	6
Silver				2	20	20	40		2	20	20	40				

(1) Portuguese Legislation only for soils used for crop production for animal consumption; Portaria Nº 176/96 (2ª Série) from 03-10-1996.

(2) EPA-ECV source- USEPA. 2001. Supplemental Guidance to RAGS: Region 4 Bulletins, Ecological Risk Assessment. Originally published November 1995. Website version last updated November 30, 2001: http://www.epa.gov/region4/waste/ots/ecolbul.

(3) Soil Remediation Criteria for Inorganics in this Table apply only where Surface Soil pH is 5.0 to 9.0 and for Full Depth Use, the Subsurface Soil pH is

(4) Environmental Restoration, Division Manual: ERD-AG-003, Ecological Screening Values (ESVs) P.7.1, Date: 04/06/99, Page 1 of 33. Savannah River Site, http://www.srs.gov/general/enviro/erd/ffa/rdh/p75.pdf

(5) Canadian Environmental Quality Guidelines; 2002; http://www.ccme.ca/assets/pdf/e1_06.pdf

(6) German Federal Environmental Agency; Federal Soil Protection and Contaminated Sites Ordinance (BBodSchV)- http://www.umweltbundesamt.de/altlast/web1/berichte/pdf/bbodschv-engl.pdf

(7) Tentative UK "Trigger Concentrations" for Selected Inorganic Contaminants; http://www.mineralresourcesforum.org/workshops/Berlin/docs/append_8.pdf

MPC- Maximum Permissible Concentration; CCME- Canadian Water Quality Guidelines; ESV- Ecological Screening Values.

Although soil contamination is one of the major environmental problems all over the world, it seems that the Portuguese govern is devoting less importance to this issue nowadays than it gave some years ago. As far as it is known, in 1999 and 2000 the governmental reports on the environmental status, there was always a chapter concerning soil quality which stated what would be the implication of the situation [12, 13]. Unexpectedly the soil section disappeared from the 2001 report, only giving some focus on soil NO_x compounds that can be used as a measurement of air quality [14].

The increase of salt and erosion of soils in Portugal is nowadays intensified by extreme climacteric conditions, soil desertification and also by fires that have become more intense during these last years. However, the real effects of these factors are not clearly known.

Industry in Portugal is one of the main causes of soil, water and atmospheric pollution, by releasing several chemical compounds like acidic or basic compounds, solutions with heavy metals or persistent organic chemical compounds. The chemical compound emission is made by three ways: gaseous emissions, aqueous effluents and solid residues, and can be accumulated in soils. In the 1980 decade, 75% of the residues were eliminated into the soil or subsoil [11]. During a long time, residues were also deposited in dump sites nearby industries, in clean areas or stone-pits (quarries), without any impermeable device that could protect lixiviates to infiltrate into the soils. This could lead to groundwater contamination. An extremely important example was the case of an industry that produced pesticides and poisons and that dumped the end product residues in two stone-pits deactivated, nearby Sintra. Another critical case was the deposition of 32 000 tons of aluminium spoils in an open area near Setúbal [11].

Fertilizers and pesticide use and manufacture are another problem for soils. Portuguese soils used for agricultural purposes do not have sufficient nutrients for plant growth, and fertilizers are used to provide higher quality for crop production. As an example, Portuguese soils under cultivation show only 11% of the organic matter content needed and 57% of phosphorous needed for a good crop production [12]. Nevertheless, in Europe and also in few places in Portugal, there are soils with so high concentrations of P (sometimes due to cattle intensification)

that this nutrient became a risk factor to these areas. In these cases, plants do not respond to fertilizers. Another environmental risk related to P is that when water is abundant in soil, mainly due to rain, and soil can not retain more water, the exceeding part is grained as runoff or accumulated as flood. These phenomenon can cause desorption of P from the soil matrix or from the pore water phase, reaching aquatic systems like lakes, lagoons or other water compartments; this runoff can be responsible for the euthrophication phenomena [7].

Qualitatively, the fertilizers that provide nitrogen are the most used, followed by the ones with phosphorous, while potassium fertilizers are not very relevant. The use of fertilizers in Portugal increased till 1988, but after that and till 1993 a slight decrease was observed, an in all over Europe. After 1993 the use of fertilizers started to increase again, and in 1996 the total amount of fertilizers used in Portugal was 69 kg/ha, although it was one of the countries in Europe with lower usage of fertilizers [12].

Pesticides are another problem for soil quality due to its exhaustive application in agriculture but also because the residues from their production are very often dumped in to clean soil areas. From 1991 till 1998, the use of pesticides in Portugal has increased, with a particular increase of 11% in the trade of fungicides, herbicides and insecticides from 1997 to 1998. In 1998 approximately 14 382 tons of active substances from fitopharmaceutical products were sold; fungicides are the pesticides with higher commercialization (10 476 tons) [12]. Areas where contamination by pesticides is more relevant, due to its high agriculture practices, are the Algarve and Ribatejo regions, Aveiro and the low Mondego region (nearby Coimbra).

Mining was one of the most important activities in the last century in Portugal. Mine extraction has contributed to the deposition of spoils on the soil surface, treatment waters as other residues obtained through the mineral separation. Although several mining activities have cessed, the mine local area can sometimes be calamitous. These spots are commonly contaminated with heavy metals, resulting from the extraction method, and metal complexes with S, Fe Mn and Al oxides, are very often found. This complexation can induce different bioavailability and toxicity of metals in soil to humans and soil organisms.

Urban areas, stations for the treatment of waste waters, places where fuel compounds were stored, military areas and travel lines (railway and motorways) are also crucial spots that need to be evaluated and studied because of their high contamination probability. It was estimated that, in 1999, 8 millions of tons were produced as urban, industrial or hospitals residues [12].

The study and recovery actions in rehabilitation for contaminated sites started only in 1994, for the first time in Portugal, with the Chemical Industrial Complexes in Estarreja and the place where the exhibition EXPO 98, in Lisbon, was held.

HEAVY METAL CONTAMINATION IN SOILS

Criteria for metal contamination in soils are important in hazard and risk assessment, for the protection of groundwater, plants, soil-dwelling organisms, food chains, and finally human and animals that can directly ingest soil [15]. Standard values have been used as limits for the total amount of a metal that is believed to be reasonable in soil, and can be used for contamination assessment and remediation processes evaluation. The European Union does not have yet any legislation based on benchmark values and there is no consensus between countries about these triggers values; some countries have chosen some of the existing values for their own use (Table I).

These criteria are based on the lowest concentration of a metal that will produce a harmful effect and will not consider the bioavailable fraction of the heavy metal compound.

One factor that affects bioavailability of compounds in soil is their strength to bind to the soil particles. This binding will enclose a partitioning process between soil and soil water solution that will depend on pH values, dissolved organic matter (DOM), calcium (as a competitive cation, that can be adsorbed to solids turning other metals bioavailable), the solid-phase metal oxide, such as inorganic ligands, and SOM content [15-17].

Soil solid phase characteristics, like clay minerals, pH values, OM, cation exchange capacity and oxyhydroxides of Fe, Al and Mn, are important in reactive sorption reactions [18]. For this reason, several soil characteristics will be of extreme importance for heavy metal speciation processes. As an example, DOM contributes to the increase of cation exchange capability (CEC), which will also induce an increase in metal adsorption. As a result, solution metal concentration will decrease as its toxicity. Also the drying of soil can induce differences in the speciation in soil and induce higher or lower bioavailability and therefore toxicity [15].

Metals can bind to soil particles by three processes: precipitation, ion exchange and adsorption [19]. Precipitates are formed when the solubility product for the reaction between the ion metal and the ligand is exceeded. Some of these ligands consist of carbonates, hydroxides, silicates, phosphates and, in anoxic environments, sulphides. Ion exchange is related to the process where a positively charged ion is exchanged for another at a constant-charged surface. Clay minerals are one of the materials that play an important role in this process, as mentioned before. Metal adsorption is a highly pH dependent process, being associated with reactions of protons with oxide and hydroxide minerals and with humic substances [19].

The total metal in soil is distributed between the fraction bound to soil solids and the fraction dissolved in soil solution [16]. Within this, metal toxicity to different soil organisms is also related to different soil fractions [15]:

- plants- free metal ion and heavy metal content in soil pore water;
- microorganisms- free metal activity in soil pore water;
- invertebrates- organic matter and soil pore water.

Some theories, previously described for aquatic or sediment environments, have been adapted to the soil compartment to try to explain some chemical reactions and bioavailability in contaminated soils. One is the Equilibrium Partitioning Theory that assumes that chemical concentration in environmental compartments is at equilibrium [20, 21] and that, in the case of metals, the partitioning among compartments can be predicted based on partitioning

coefficients (distribution of heavy metals between the solid-phase and the pore water) [18, 22, 23]. Another model used in aquatic ecosystems is the Free Ion Activity Model (FIAM) that states that the concentration or activity of free ions is the best prediction to evaluate metal bioavailability. It is believed by several authors that models based on the free-ion activity will give generally an improved prediction on this issue because free metal ions are the only metal forms taken up by roots from the soil solution [24]. Although this model has been applied in terrestrial ecosystems with higher plants, it is still not sufficiently understood to allow the prediction of metal uptake or toxicity [15]. The Biological Ligand Model (BLM) is a theorem that derives from FIAM and it has also been applied in plant testing. In this model, the interaction between an organism and the metal ions in soil results from the competition for that metal by all soil components, and the interaction of compounds like calcium and H^+ (pH values) [24].

EVALUATION OF SOIL CONTAMINATION AND TOXICITY

Soil contamination is a crucial problem in ecosystems and in human health because chemical compounds in soils can enter food chains at its primer level – plants-, being transferred to primer consumers untill humans. The fact that chemicals can pass through the food chain can lead to a process called biomagnification where chemicals, step by step inside the food chain, will increase their magnitude in concentration.

Chemical analysis are not, by their own, sufficient to evaluate soil toxicity because analytically detectable concentrations of chemical compounds do not enable an accurate prediction of a harmful effect on organisms or on the environment as an all. They evaluate the total amount of contaminants in soil, but also for that it is necessary to know exactly what to look for. In the case of an area that is supposed to be contaminated by heavy metals, one needs to know which heavy metal compound is supposed to be analysed. If one key compound is

missing in the soil analysis, it can lead to a mistake in the conclusions for the evaluation on the contamination analysis.

Furthermore, the total amount of a chemical compound do not inform us on its toxicity because only the bioavailable fraction of that chemical will induce stress and physiological changes, since the biological availability can vary considerably depending on the chemical species, soil characteristics and environmental conditions. Another area under discussion is that when a certain fraction of a chemical compound is considered toxic for one organism, it can be less or more toxic to other organisms. Hence, the bioavailable fraction differs within organisms.

Another important point that has to be stressed is that chemical compounds do not appear lonely in the environment, so the possibility of positive or negative interactions between chemical mixtures or combined potential interactions between chemicals and different soil types has to be regarded.

Even tough, benchmark values that are usually used to evaluate toxicity can potentially be used only to distinguish sites that are almost toxic for certain, and for those that can be considered as non-toxic. Between these two extreme evaluations there is a large “black box” that can cause some problems within this process. For soil toxicity evaluation, only the direct measurement of toxicity in soil, using bioassays, can reduce these uncertainties.

Bioassays are then considered essential tools for toxicity evaluation because they are extremely influenced by physicochemical characteristics of the soil matrix and by the bioavailable fraction of the chemical compounds present in the soil. The selection of a bioassay for the assessment of soil toxicity or quality depends on the purpose that the test soil is going to be used for, and which are the target soil functions that should be evaluated.

Individual laboratory tests are often conducted to evaluate the effects of chemical compounds on specific dynamic soil processes, which can mislead on the assessment of the stressor’s effect. In some cases, there can be a soil function or structure that was not regarded in a individual test, and it could be the major target for the pollutant. Several tools have been developed to solve these problems and the use of test-batteries with the evaluation of different trophic groups or soil processes will help to fulfil this gap. The validation of laboratory

results using field toxicity tests will also contribute to Environmental Risk Assessment processes [25], because environmental factors like temperature or humidity can interact with contaminants present in soils, enhancing or reducing their toxicity.

Moreover, a suitable tool for the investigation of the potential toxicity or effects of chemical compounds on terrestrial compartments was recently developed. This tool is often called Terrestrial Model Ecosystem (TME) and it is considered a controlled and reproducible system that aims to mimic the interaction and processes of several components in a portion of the terrestrial environment [26]. TMEs were also developed to bring together laboratory and field data to structural and functional levels [27, 28], avoiding the use of only single-species bioassays.

Several bioassays have been recommended to evaluate soil quality and monitor changes in soil functions or to determine the effect of added substances, by international organisations, such as The International Organization for Standardization (ISO) or the Organization for Economic Cooperation and Development (OECD). The ISO standard 15799 include a list of advisable bioassays that can be used for soil evaluation before or even after remediation processes. These bioassays can be chosen according to the intended use of the soil and possible adverse effects on aquatic and edaphic organisms, reflecting on the habitat maintenance and retention function [29].

Included in these bioassays are those that use:

- soil fauna and flora (terrestrial test methodologies)- collembolan (*Folsomia candida*) [30], earthworms (*Eisenia andrei* or *Eisenia fetida*) [31, 32], enchytraeids (*Enchytraeus* sp.) [33], monocotyledonous and dicotyledonous plants [34, 35], these last under revision; in these tests, parameters like survival, reproduction or growth are revised.

- soil microorganisms- bioassays with microorganisms can evaluate processes like mineralization and nitrification [36, 37], the determination of the soil enzyme dehydrogenase, using two methods that are still under development [38], the determination of soil microbial biomass [39, 40], or determine the microflora abundance and activity using respiration curves [41].

- aquatic organisms (aquatic test methodologies)- using the cladoceran *Daphnia magna* [42], fresh water fishes [43], algae (which is under revision) [44] or the luminescence bioassay with the marine bacteria *Vibrio fischeri* (e.g. using MICROTOX®). With these tests, parameters like animal immobility, death, growth, and light inhibition production are evaluated.

These bioassays evaluate the habitat function of soils, as a suitable medium for organisms to live in, and the retention function of soils or their leaching potential, by studying the main role of transportation of soluble, colloid or particle fractions via water.

There are also other tests that have been developed in the laboratory using edaphic organisms, which have not been standardized yet, and that can bring good advances in the evaluation of soil toxicity or quality. One of these tests, the Soil Avoidance Response Behaviour Test with earthworms, is under standardization by the International Organization for Standardization (ISO) [45]. This test aims to obtain quick answers at lower costs in the implementation of solutions for contamination problems, as a first screening tool, and it assumes that organisms have the ability to choose or avoid a soil. Avoidance behaviour response tests can increase sensitivity and quickly assess an ecological endpoint that is not measured by any other test using the soil matrix [46]. Even though, Avoidance Behaviour Tests are not aimed to replace other ecotoxicological tests, but to be used as a complementary initial or screening test in soil contamination assessment. Although earthworms have been chosen as test-organisms in this ecotoxicological test, other edaphic organisms have shown similar ability to choose quality habitats, like isopods and enchytraeids [47, 48].

Other tests with edaphic organisms, like isopods, enchytraeids, nematodes, oribatid mites,millipedes, have been proposed [49] and can be applied in environmental assessment processes in the future.

Although microorganisms are also used as test-organisms in standardized tests, there are several bioassays that can give some information on the soil status which are based on the soil nutrient cycling. These bioassays evaluate the activity of soil enzymes related with different essential nutrients and that can be influenced by chemical compounds. For instance, heavy metals are able to reduce enzyme

activity by interacting with the enzyme-substrate complex, denaturing the protein by the interaction with its active sites or by simply affecting the microbial cell that produce the enzymes [50-52]. Not all metal species can induce these interactions, so metal speciation in the solid or liquid phases, and conclusively their bioavailability, can be assessed by measuring their effects with soil enzymes. To integrate the simultaneous measurement of several enzyme activities and to measure the microbial functional diversity of soil as a value, several quality indexes using soil enzymatic activities have been proposed [53]. Some of these indexes have been used for evaluation of the nutritional status of climax native soils [54, 55].

An important issue that has to be regarded is the choice of an appropriate control soil. When performing these bioassays several types of controls are advised, namely when testing the soil matrix [17, 29, 56]:

- An uncontaminated soil with similar physicochemical properties to the test-soil that is being evaluated, and possibly collected in the surrounding areas of the test-area;
- An inert material, like quartz sand;
- A certificated natural soil, like the Lufa 2.2 soil (Lufa Speir);
- A standardized artificial soil (see [30-32, 57]).

The choice of the control soil will depend on the goals of the ecotoxicological assessment, the future use of the test-soil and therefore the type of bioassays that will be carried out.

The use of artificial soils as controls [57] has the advantage of being already used broadly in ecotoxicological tests, it is easy to prepare, becoming a homogeneous and concise soil across laboratories or time. Even though the use of this soil type has some disadvantages: (1) this artificial soil is not suitable for microbe testing, (2) one of its constituents is kaolinitic clay which is not usually found in soils from temperate regions and (3) it lacks Al, Fe and Mn oxides that are extremely important in controlling heavy metal bioavailability, creating difficulties in the extrapolation of bioassay results to real contamination scenarios [17, 56].

As a certificated soil, Lufa 2.2 soil is advantageous because it is a natural soil but it will not embrace all soil types. Hence several soils from Europe (EUROSOILS) are being studied to be used in the future as certified reference material or control soils in the evaluation of contaminated soils, according to their pedological characteristics [58]. They were collected in France, Italy, Greece, U.K. and Germany and they hold differences in their pH, clay and organic carbon content. The use of these natural soils will represent the majority of soils in Europe and would be more suitable for all test-organisms. It will probably give more realistic information on chemical transformation, interaction, not only as control soils but also as soils for laboratory contamination testing [17].

THE CASE STUDY OF MINA DE JALES

The study that will be presented here is the evaluation of the potential toxicity of soils in the surroundings of an abandoned gold mine, in the northeast of Portugal. This mine is located in Campo de Jales (Vila Pouca de Aguiar) and got the name of the village- Jales mine (N 41º 27’ 47.2”; W 7º 35’ 11.7”) (Fig.3). It was chosen as a case study for contamination due to the known high levels of heavy metals in water and soil compartments.

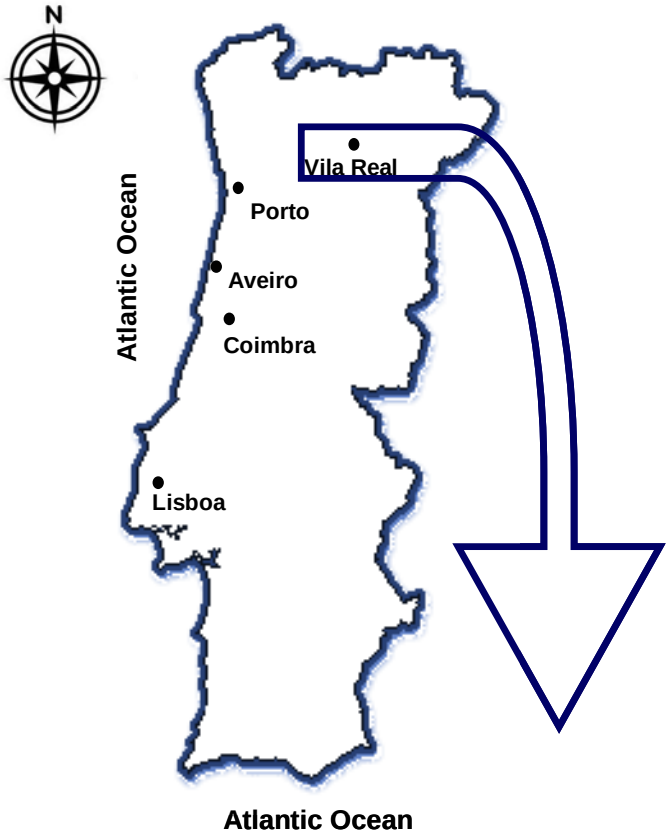
The surrounding area of the Jales mine is known to have been exploited since the Romans and, more recently, during the last century decades, with no control and with no efforts to minimize environmental damages. Since 1933, the mine has produced approximately 25 tons of gold and 100 tons of silver, having reached a depth of 620 m at the time of closing in 1992/1993 [59]

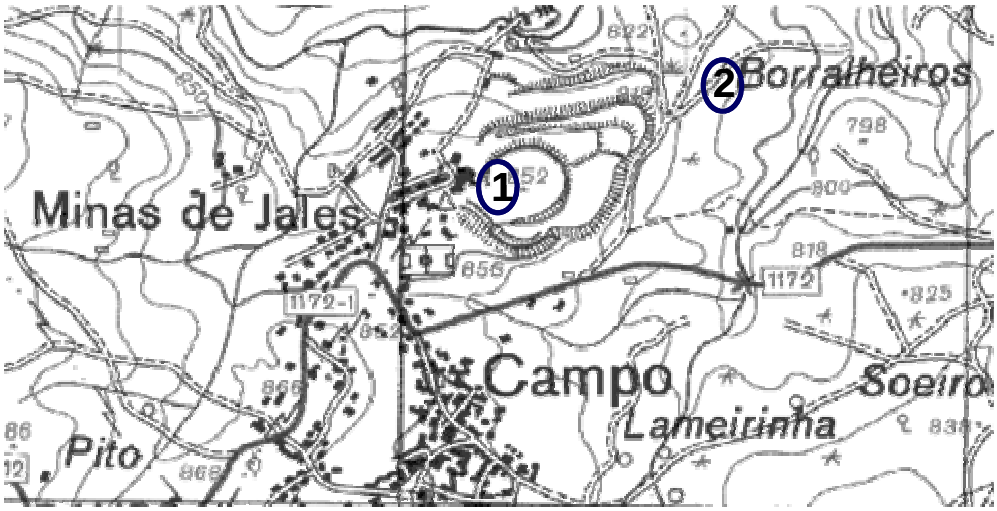
Hydrothermal gold-bearing quartz veins occur in Jales in the two directions of the subvertical fractures embebbed in Hercynian granites and schists, greywackes and quartzites from the Schist-Greywacke Complex, mainly originated during the Silurian age. Gold usually occurred associated with quartz and sulphides, as arsenopyrite, pyrite, chalcopyrite, galena, among others [59].

The mine exploitation and the mineral treatment comprised the grinding action till a certain grain size and then a concentration of free gold and sulphates. Gold, silver and lead were recovered in a final phase, by calcinations and other metallurgical procedures developed in other locations in Portugal. The materials that were produced in the final and intermediate phases and those of no need were accumulated in a spoil, reaching 5 millions of tons of material constituted, in their majority, by fine particles [59].

The contamination produced by the mine exploitation is observed in superficial as in subterranean waters, due to the mobility of heavy metals through soils, and also by wind transportation of the small particles from the mine spoil. The influence of mining is evidenced in the main watercourses by the elevated quantities of sulphate ions (SO_4^{2-}). This species is relatively innocuous, exhibiting a conservative chemical behaviour by not participating in secondary chemical reactions and its diminished concentration in aqueous systems occurs by dilution. The ions in higher abundance are iron, manganese, zinc, arsenic, lead, selenium and copper, producing appreciable quantities of sulphides.

The mine spoil is located in Cidadelha de Jales and is adjacent to a primary school; its surrounding has also been used as pasture areas for milk production cattle and also as an improvised “playground” for the village children. This will carry out serious health and environmental problems, leading to an urgent process of Terrestrial Risk Assessment: Environmental and Human. Also some problems with the mine drainage, caused by rain, induced the rupture of three platforms that were holding the sludge or mud from the mine spoil. This sludge and some other materials from the mine represent nowadays a strong environmental problem in Tinhela River that passes in the surroundings of Jales mine, and are deposited in agricultural and pasture fields.





(Instituto Geográfico do Exército (1998). Carta Militar de Portugal- Telões (Vila Pouca de Aguiar). M888. Instituto Geológico do Exército. Lisboa: 1:25 000.)

Fig. 3- Map of the study site located in Mina de Jales (Vila Pouca de Aguiar), Portugal. (1)- Area close to the Jales Mine N 41º 27' 53.9'' W 07º 34' 50.6'' (JC soil); (2)- Area 30 Km from Jales Mine N 41º 28' 36.1'' W 07º 34' 14.0'' (JNC soil).

Soils near the mine spoil were analysed for their heavy metal content by the former Portuguese Mining and Geologic Institute (nowadays the INETI Institute) in 1995, as part of a research project, and the major target compounds that showed higher concentration were mainly arsenic (As), manganese (Mn), cadmium (Cd), zinc (Zn), and lead (Pb) [60]. Within the same project it was stated that there are important geo-chemical bounds that point to the simultaneous occurrence of several contaminants, especially those of oxides and hydroxides of Fe and Mn, patterns particularly visible to Cu, Pb and Zn [60, 61].

Table II- Heavy metal content (mg/Kg) and pH values of 5 soils in the area of Jales mine (Vila Pouca de Aguiar), Portugal.

Heavy metal (mg/Kg)	**JNC soil** N 41º 28' 36.1'' W 07º 34' 14.0''	**JC soil** N 41º 27' 53.9'' W 07º 34' 50.6''	**J Epeq1** N 41º 27' 47.2'' W 07º 35' 11.7''	**JE1** N 41º 28' 13.1'' W 07º 34' 43.5''	**JE2** N 41º 28' 26.8'' W 07º 34' 44.04''
As	71	251	1249	29	20
Cd	1.9	8.2	42.8	<1.7	<1.7
Cr	6	15	4	4	2
Cu	8	24	15	7	3
Fe	7 370	17 800	5440	7870	5190
Hg	<0.05	<0.05	<0.05	0.11	<0.05
Mn	99	255	210	75	80
Ni	5	9	<4	5	<4
Pb	33	209	219	64	19
Zn	33	97	66	38	19
pH	4.14	4.47	3.74	3.79	4.00

In 2002 several chemical analysis were also made in different soil spots near the mine and within some distance from the mine, enclosed in this study (Table II).

It was observed that even 3 km away from the mine spoil (area of JNC soil), it was found high Fe and As contents in the cambisoils from this area.

In late 2002 a rehabilitation process by Exmin (a concessionary for the environmental rehabilitation of abandoned mine areas) was initiated, and the mine spoil was turned completely impermeable and covered using geosynthetic layers. Finally, it was covered by soil from the surrounding areas (10km from the mine spoil), and it is now expected that the recovered mine spoil will be naturally filled by spontaneous vegetation. Nowadays the concessionary Exmin consider the process completed and the majority of environmental problems solved.

THE RESEARCH OBJECTIVES

The global aim of this thesis was to carry out the evaluation of the potential toxicity of soils in Portugal, using Jales mine as a case study. After chemical analysis and the observation of the area, two soils was chosen, one with high heavy metal content and one with lower heavy metal content. These two soils were named JNC soil (lower heavy metal content) and JC soil (high heavy metal) also due to their location (distance from the mine), the structure of the soil and the soil use. The physico-chemical characteristics and all heavy metal analysis of JNC and JC soils and their water elutriates are reported in Table III.

To attain the global aim, the following main steps were followed:

- Application of a battery of bioassays in soils with different heavy metal contents, enclosing ecotoxicological standardized and non- standardized tests;
- Evaluation of different soil functions, like the water path (retention function), the habitat and biocenoses function, using edaphic organisms like plants, invertebrates and microorganisms.
- Evaluation of the sensitivity of the bioassays and species used in this battery.

Table III- Physico-chemical, microbial characteristics, and heavy metal content (mg/Kg) of JNC and JC soils from Jales mine (Vila Pouca de Aguiar), Portugal, and the control soil Lufa 2.2, collected in February 2002.

Parameters	units	Lufa 2.2 soil	JNC soil	JC soil
pH	-	5.03	4.14	4.47
Dry matter	%	93.8	70.25	66.19
Soil Organic Matter	%	1.28	5.07	2.88
Cation Exchange Capacity	cmol/Kg	11	4115	5492
Max. Water Holding Capacity	%	51.0	36.5	35.1
Redox Potencial	mV	30	59	47
Sand	%	77.10	20.96	23.08
Clay	%	8.0	16.8	13.6
Silt	%	14.90	27.51	25.02
Total Carbon	%	2.21	8.55	4.61
Total Nitrogen	%	0.2	0.4	0.4
Sulfur	%	0.02	0.02	0.03
Calcium	mg/Kg	1540	441	1840
Potassium	mg/Kg	292	1290	2670
Microbian Biomass N	mg/Kg	45.2	40.0	84.4
Microbian Biomass C	mg/Kg	299.6	96.9	792.2

Heavy metal (mg/Kg)	Lufa 2.2 soil	JNC soil	JC soil
As	-	71	251
Ag	-	<0.2	1.5
Al	-	14 000	10 000
Be	-	2.0	3.1
Cd	<0.2	1.9	8.2
Co	-	n.d.	n.d
Cr	9.6	6.0	15.0
Cu	1.5	8.0	24.0
Fe	-	7 370	17 800
Hg	0.07	<0.05	<0.05
Mn	-	99	255
Ni	2.7	5.0	9.0
Pb	16.8	33.0	209.0
Sb	-	0.37	2.29
Se	-	0.47	0.38
Zn	19	33	97

To address the above issued the thesis is organised in four chapters, focusing in the following issues:

Chapter I. The importance of a first screening tool in soil ecotoxicology leaded to the development of Soil Avoidance Behaviour Response Tests with earthworms (*Eisenia andrei* or *Eisenia fetida*). These tests are not yet well defined and a similar test with the isopod *Porcellionides pruinosus* was developed so that two distinct taxonomic and life strategy groups are used. Several chemical compounds were previously tested with *P. pruinosus* and *E. andrei* and the screening of the soils from the mine was made using both organisms.

Chapter II. The soil retention function was the second approach used in this study. Two bioindicator species were used, the cladoceran *Daphnia magna* and the marine bacteria *Vibrio fischeri*, because of their importance and broadly use in aquatic ecotoxicology. Consequently, they are usually advisable for the evaluation of the retention function of soil, and have proved to have high sensitivity in soil toxicity evaluation processes. Regarding the habitat function, it was also carried out a reproduction bioassay with the collembolan *Folsomia candida* to evaluate the potential toxicity of soil. Both approaches were compared in terms of sensitivity.

Chapter III. The lack of scientific information on the microbial community processes in natural contaminated soils was one of the reasons why the soil enzymatic bioassay battery was chosen in this thesis. A Soil Quality Index, previously applied in the quality evaluation of native soils, was also introduced. The recovery of soil enzymes after the rehabilitation process and the differences in their activities in the two soils were achieved.

Chapter IV. The use of plant bioassays to evaluate soil quality is considered of major importance because soil is the main source for food and fibber for humans. In this study the toxicity of the soils from Jales mine was evaluated using two plant species, *Brassica rapa* L.(Rapid-cycling Brassica) and *Avena sativa* L..

Growth, reproductive and physiological parameters were achieved for this evaluation.

In a final section, a general discussion and conclusions of this study are presented.

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CHAPTER I

Terrestrial Avoidance Behaviour Tests as Screening Tools to Assess Soil Contamination

Terrestrial Avoidance Behaviour Tests as Screening Tools To Assess Soil Contamination

(Submitted to the journal Environmental Pollution)

ABSTRACT

To assess soil quality and risk assessment, bioassays can be useful tools to gauge the potential toxicity of contaminants focusing on their bioavailable fraction. A rapid and sublethal avoidance behaviour test was developed to replace time-consuming tests used as screening tools and detect low chemical concentrations. In this study earthworms (*Eisenia andrei*) and isopods (*Porcellionides pruinosus*) were exposed during 48h to several chemicals. Isopods were exposed to lindane, dimethoate and copper sulphate and earthworms to carbendazim, benomyl, dimethoate and copper sulphate. Both species were also exposed to soils from an abandoned mine.

Isopods and earthworms were able to perceive the presence of toxic compounds and escaping from contaminated to clean soil. Furthermore the behaviour parameter was equally or more sensitive then other sublethal parameters (e.g. reproduction or growth).

Here the authors expressed the advantages of Avoidance Behaviour Tests as screening tools in ERA and present a statistical approach to derive EC_{50} values from Avoidance Behaviour Tests.

INTRODUCTION

Soil is a dynamic and complex system functioning as habitat for microorganisms, flora, animals and humans [1]. Nowadays contaminated soils have become a primordial problem since they will probably lead to, for example, groundwater contamination and biomagnification of chemical compounds through food webs, and sometimes will affect human health. Contaminants in soil have some distinct fractions depending on the contamination and soil type. Some of these chemical fractions are bioavailable and thus can be absorbed by organisms which are dependent of the soil physicochemical conditions (e.g. pH, clay content, cation exchange capacity, amount of organic matter) and on the chemical form of the element. Therefore the determination of the total chemical contents is not sufficient to evaluate the ecological risk that is inherent to a contaminated soil. To assess soil quality, bioassays can be useful tools to gauge the potential toxicity of contaminants focusing on their bioavailable fraction.

In soil ecotoxicology acute and chronic standardized tests have been developed using soil dwelling invertebrates, like earthworms [2, 3], potworms [4] and collembolans [5]. Edaphic invertebrates play a crucial role in maintaining the structure and fertility of soils, recycling nutrients, increasing aeration and drainage, and can constitute an important component of the diet of birds, reptiles or small mammals [6]. Nevertheless, the use of edaphic invertebrates in acute and chronic ecotoxicological tests have shown some disadvantages: acute tests are not ecologically relevant when compared to chronic ones, because they do not provide insight into effects on the population dynamics, while chronic tests last too long and are very labour intensive, sometimes ranging from 4 to 7 weeks. To obtain quick answers at lower costs in the implementation of solutions for contamination problems, a first screening tool is required and a rapid and sublethal avoidance behaviour test, where organisms have the ability to choose or avoid a soil, has been under development and standardization using earthworms [7-9]. The results of avoidance behaviour response tests can increase sensitivity in this evaluation, quickly assessing an ecological endpoint that is not measured by any other test using the soil matrix [10]. Even though, Avoidance Behaviour Tests are not aimed

to replace other ecotoxicological tests used nowadays, being just a complementary initial or screening test in soil contamination assessment. Earthworms have been chosen as test-organisms in ecotoxicological tests because they are common in a wide range of soils, representing 60-80% of the total soil animal/invertebrate biomass. Furthermore, by having chemoreceptors in the prostomium and sensory tubercles on their body surface, they can provide a high sensitivity to chemicals in soil [11].

Like earthworms, isopods are widely distributed and are a key species that play an important role in soil dynamics, mainly in leaf litter decomposition. They also have chemoreceptors located in an apical organ in the second antennae called aesthetascs, that can perceive chemicals and test stimuli. The antennae move continuously and a fluid excreted through channels mediates chemoreception. There are also some evidences of the existence of tricorn sensillae that are contact chemoreceptors in the tegument of isopods [12, 13]. Isopods have shown sensitivity to several chemicals present in soils because they can intake water from soil through uropods by capillary action, ingest soil or even absorb water through the cuticle [14]. The uptake of the chemical compound can influence physiological processes in isopods because these edaphic organisms are known to have low excretion rates. Zinc and Copper are two examples of toxicants that are not excreted by isopods and are deposited in granules in their internal organs [15]. Another characteristic of isopods is their ability to avoid environment limiting factors like extreme humidity, light and others [16]. For all these reasons they have been used in soil bioassays.

The objectives of this study were to test if avoidance behaviour responses by edaphic organisms can be used as a first screening tool for soil Environmental Risk Assessment and to compare the performance of two test organisms (earthworms and isopods) in choosing between two different substrates. Avoidance behaviour response tests were carried out using the earthworm *Eisenia andrei* and the isopod *Porcellionides pruinosus* exposed to organic and non-organic toxicants and also tested with natural soils from an abandoned mine to assess their performance in a real scenario.

To our knowledge, studies where earthworms avoidance behaviour tests are performed do not estimate EC_x values, like it is asked in the standardized protocol, which is currently under development from The International Organization for Standardization [8, 9, 17-19]. Hence the comparison between avoidance behaviour tests and the classical acute/chronic bioassays is difficult. In Avoidance Behaviour Response Tests, where 50% presence in one of the soils is considered as a non preferencial behaviour (endpoint), the definition of EC_{50} has to be adapted to the avoidance results. Here we present an adaptation of a standard methodology to the calculation of an EC_{50} for Avoidance Behaviour Test data.

MATERIALS AND METHODS

Test Organisms and Test Chemicals

The isopods *Porcellionides pruinosus* were obtained from a two-year laboratory culture, maintained at 25ºC with a 16:8 (light: dark) photoperiod. Only adult animals (15-20 mg wet weight) with antenna were selected for the tests and sexes were not distinguished.

The earthworms *Eisenia andrei* were obtained from a culture for compost use in the Alentejo region, in the South of Portugal. Only adult animals with a developed clitellium were selected for the test (50-60 mm length).

In these experiments organic chemicals were chosen based on their use in agriculture and on the existence of data on acute and/or chronic tests with earthworms and isopods, as well as with other edaphic invertebrates. For the isopod experiments lindane (Merck, 95% pure) and dimethoate (Sigma-Aldrich, 99.9% pure) were used, while in the earthworm's experiments the test chemicals were carbendazim (100% pure) (AgrEvo), benomyl (100% pure) (DuPont) and dimethoate. The inorganic chemical used for both test organisms was copper (II) sulphate pentahydrated (Merck), a constituent of some fungicides used in

vineyards. All chemical compounds were incorporated in Lufa 2.2 soil and several concentrations were tested (Table I.I).

TABLE I.I- Nominal and real concentrations of the organic chemical compounds used in the Avoidance Behaviour Tests (--- data not determined).

Test Organism	Control	Test Chemical in Lufa 2.2 soil Test Soil	Nominal Concentration (mg/Kg)	Real Concentration (mg/Kg)
Eisenia andrei	Lufa 2.2	Carbendazim	1 10 100	3.90 9.23 82.73
Eisenia andrei	Lufa 2.2	Benomyl	1 10 100	--- --- ---
Porcellionides pruinosus _____ *Eisenia andrei*	Lufa 2.2	Dimethoate	2.5 5 10 20	2.20 5.70 10.67 32.50
Porcellionides pruinosus	Lufa 2.2	Lindane	2 10 20 53 113 200	2.70 7.60 22.00 59.10 108.20 190.40

Soil contamination was controlled by chemical analysis by Gas Chromatography-tandem Mass Spectrometry (GC-MS/MS) for lindane and dimethoate and Liquid Chromatography-mass Spectrometry (LC-MS) for carbendazim, using two soil samples per each contaminant. Benomyl was not determined because its degradation is very fast. The recovery of the extraction method was of 95% (±14% std. dev.) for lindane, 89% (±14% std. dev.) for dimethoate and 81% (±11% std. dev.) for carbendazim. Chemical analyses were only carried out at the beginning of the experiment. No extra analysis was carried out at the end of the experiment due to the short test period of 48 hours.

TABLE I.II- Physico-chemical characteristics and main contamination of soils used: Lufa 2.2, JNC and JC from Mina de Jales (Portugal) (--- data not determined).

Parameters	units	Lufa 2.2 soil	JNC soil	JC soil
pH	-	5.03	4.14	4.47
Soil Organic Matter	%	1.28	5.07	2.88
Cation Exchange Capacity	cmol/Kg	11	4115	5492
Sand	%	77.10	20.96	23.08
Clay	%	8.0	16.8	13.6
Silt	%	14.90	27.51	25.02
As	mg/Kg	-	71	251
Al	mg/Kg	-	14 000	10 000
Cd	mg/Kg	<0.2	1.9	8.2
Cr	mg/Kg	9.6	6.0	15.0
Cu	mg/Kg	1.5	8.0	24.0
Fe	mg/Kg	-	7 370	17 800
Hg	mg/Kg	0.07	<0.05	<0.05
Mn	mg/Kg	-	99	255
Ni	mg/Kg	2.7	5.0	9.0
Pb	mg/Kg	16.8	33.0	209.0
Zn	mg/Kg	19	33	97

To evaluate the performance of these tests in real scenarios, two soils from the abandoned mine Mina de Jales, located in the northeast of Portugal (N 41.47403; W 7.57876), were chosen, due to their different heavy metal contents. JC soil is a soil located 150 m from the mine spoil and is contaminated with a high amount of heavy metals; JNC soil is located 3 Km from the spoil with a low amount of heavy metals (Table I.II). Heavy metal analysis was performed by ICP-OES. A prior soil digestion was made, with an oxidation in HCl 0.1 N.

Experimental Set up

In this study, two section chambers were used as tests containers and two behaviour avoidance tests were carried out (Fig. I.1). Earthworms and isopods were exposed to chemicals in group tests where 10 animals were placed in each test box. Additionally, an isopod individual test was performed with just one individual per box, due to the ability of isopods to produce the aggregation pheromone and consequently forming groups (aggregation behaviour).

Rectangular plastic containers were used (210 x 123 mm) in the group test. They were divided in two compartments by a removable plastic split. On the outside of the container a line representing the split place was drawn (Fig. I.1). The standardized Lufa 2.2 soil (±200g dw), used as control, was placed in one of the compartments, and the test-soil (±200g dw) was placed in the opposite compartment. The test-soil was obtained by mixing the contaminants to Lufa 2.2 soil in a water solution form (copper sulphate and dimethoate) or by mixing the chemical powder directly to the soil (lindane, carbendazim and benomyl) (Table I.I). An extra test was also performed where isopods were confronted with clean Lufa 2.2 soil in both sides of the containers. The soil humidity was adjusted to 60% of the Water Holding Capacity (WHC) in the earthworm test and to 40% WHC in the isopod tests.

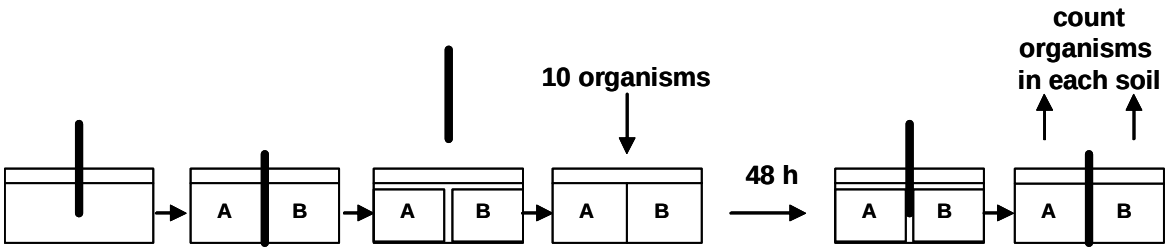

Fig. I.1- Scheme of the Avoidance Behaviour Response test set up.

The same methodology was adapted and applied to screen the quality of the field soils from Mina de Jales, by opposing Lufa 2.2 soil to these two soils. Four additional treatments for each soil were also used. The treatments were obtained by diluting the mine soils with the control soil Lufa 2.2, obtaining treatments of 12.5% (12.5% of test-soil+87.5% Lufa 2.2), 25% (25% of test-soil+75% Lufa 2.2), 50% (50% of test-soil+50% Lufa 2.2), 75% (75% of test-soil+25% Lufa 2.2) and 100% (only the test-soil). The pH values were measured in a KCl (1M) solution [20] in all dilutions, ranging from 4.61 to 4.14 in JNC soil (12.5% to 100%) and from 5.02 to 4.47 in JC soil (12.5% to 100%).

In all group tests, for each treatment or concentration, 5 replicates were used with 10 animals per replicate. In the isopod individual tests 10 replicates were used and only one animal was placed in a cylindrical box (8cm diameter and 4.5cm high). All other procedures were similar to the group tests. Earthworms were kept at 20ºC ± 2ºC, with a photoperiod of 16:8 (light: dark); isopods were exposed to chemicals at 25ºC ± 2ºC, with the same photoperiod.

After the 48 hours test period, the split was reintroduced in the marked position and the individuals were counted in each compartment containing the control and the test soil. If any animal was not found it was assumed that its death was caused by the test soil. Animals that were cut by the split were considered as being in the soil to which the animal's head was directed.

Statistical analysis

Statistical approaches in Avoidance Behaviour Tests are usually restricted to Mann-Witney U-test, when data is not normally distributed [17], or comparing the number of test-organisms in the control soil or in the test-soil in different concentrations, using Analysis of Variance or a one tailed Student's *t*-test [9]. Another approach also used is based only on the mean values and standard deviations of the number of organisms in the two sections of the test-box [18 , 19, 21, 22]. More recently, the Fischer Exact Test has been also used to analyse results from avoidance tests with collembolan and earthworms, comparing the obtained animal distribution with an expected distribution of animals which showed no avoidance [8].

In this kind of tests, the calculation of an EC_x value is asked, but till now no methodology has been presented to obtain these values. So, in Avoidance Behaviour Tests and for the evaluation of soil toxicity, the use of the "habitat function" of soil "limited" definition [9] has been proposed by several authors. In this case, the habitat function of soils is considered to be limited if less than 20% of the test organisms (on average) are found in the test soil, which indicates an impact on organisms behaviour. This is also related to the advisable measurement of the EC_{50} value presented in the currently under development standardized protocol [9], because when the mean avoidance is 50%, it means that 75% of the animals are found (on average) in the control soil. This statement assumes that when 50% of the animals are in the test-soil it is considered that the animals show no preference for both soils. Considering a total of 12 animals, it would mean that 6 animals are in the contaminated and 6 in the control soil. If at the end of the bioassay there are 3 animals in the contaminated soil and 9 in the control soil, that will mean that a total of 50% (3/6) avoided the contaminated soil, hence a 50% of avoidance. Also, if at the end of the experiment only 2 animals are found in the contaminated soil and 10 in the control soil, it will mean that 66% (2/6) avoided the contaminated soil. This last example represents the "habitat function" of soil "limited" definition, where 80% of the total animals are found in the control soil and 20% in the contaminated soil.

Two statistical approaches were adopted to treat the results obtained in this study.

First, and using the “habitat function” definition, if the percentage of live animals positioned in the contaminated/test soil was lower than 20%, the contaminated/test soil was considered to have an impact on the behaviour of the test organisms and therefore the soil was considered to be toxic or with less quality [22]. A One-Way ANOVA was also performed to assess the differences between the percentages of live animals in different treatments. Data for this analysis was transformed using equation (1), because avoidance tests are based on the ability of an organism to move away from contaminated sites, and the proportion of individuals responding (avoiding the test-soil) can be calculated as ***A***,

$$A = \frac{N - 2*T}{N} \qquad (1)$$

where **N** is the number of individuals per trial and **T** is the number of individuals observed in the test soil. Negative responses were treated as no avoidance.

Second, the EC_{50} value was calculated with the probit method, using the statistical package Minitab® [23]. Data was also previously transformed using equation (1).

RESULTS

For all group Avoidance Behaviour Response Tests EC_{50} values and 95% confidence limits were calculated (Table I.III). The EC_{50} values for the JC soil exposure for *P. pruinosus* and for JNC and JC soil for *E. andrei* were higher than 100 (i.e. the soil without dilution) due to the non avoidance behaviour response towards these exposures. Additionally the EC_{50} value for earthworms' exposure to carbendazim and benomyl was not calculated because it presented values lower than the lowest concentration used.

Isopod Avoidance Behaviour Response Tests

All tests performed with the isopod *Porcellionides pruinosus* presented less than 20% of mortality, except for the 113 and 200 mg of lindane per Kg of soil exposure, where the former reached 37.5% of mortality and the latter 28%.

When confronting the two portions of Lufa 2.2 soil, isopods showed a random distribution between the two sides of the test-box (χ^2=5.518, df=4, p>0.05).

Isopods exposed to copper sulphate showed avoidance behaviour by choosing the control soil Lufa 2.2, at the concentration 1500 mg/Kg of soil, in both the individual and group tests (Fig. I.2). When exposed to the organophosphorus insecticide dimethoate, isopods in the group test avoided the test soil at 20 and 40 mg/Kg of soil, showing a significant difference in the number of isopods present in 2.5 mg/Kg and 20 mg/Kg (One-Way ANOVA $F_{4, 19}$=12.096, p<0.05; Tukey test, p<0.05); in the individual tests, avoidance behaviour patterns were not statistically perceived (Fig. I.2), although an EC_{50} of 39.43 mg/Kg could be calculated.

TABLE I.III- EC_{50} values obtained in all Avoidance Behaviour Tests and Bioassays with *Eisenia andrei* and *Porcellionides pruinosus* (--- data not determined).

Test Organism	Test Soil	Test Type	EC_{50} (95% CL)	Concentrations with <20% ind.
Eisenia andrei	Carbendazim	Collective	< 1	10 and 100
	Benomyl	Collective	< 1	10 and 100
	Dimethoate	Collective	50.07 (37.98-66.53)	40
	Copper	Collective	181.10 (168.54-195.39)	320
	JNC	Collective	> 100	---
	JC	Collective	>100	75
Porcellionides pruinosus	Lindane	Individual	48.32 (41.03-56.06)	10, 53, 113 and 200
		Collective	48.62 (26.66-68.40)	113 and 200
	Dimethoate	Individual	39.43 (35.63-44.63)	---
		Collective	28.67 (25.57-32.60)	20 and 40
	Copper	Individual	1059.85 (983.51-1147.29)	1500
		Collective	802.26 (640.50-966.81)	1500
	JNC	Individual	64.30 (56.06-74.06)	75 and 100
		Collective	57.08 (50.82-63.66)	75 and 100
	JC	Individual	> 100	25, 50 and 75
		Collective	> 100	---

For lindane exposure, isopods avoided the contaminated soil in the group tests at 113 and 200 mg lindane/Kg of soil, where less than 20% of live animals were in the contaminated portion, while in individual tests the avoidance was observed at 10 mg/Kg and above 53 mg/Kg of soil (Fig. I.2). A high variability in the data was observed, mainly in the two lowest concentrations. Even though, there was a significant difference between 53 and 200 mg/Kg of lindane exposures (One-Way ANOVA $F_{5, 22}$=4.127, $p<0.05$; Tukey test, $p<0.05$).

To fulfil one of the objectives of this study, avoidance behaviour tests were used in natural contaminated soils. The evaluation of the two soils was made separately. After dilutions with Lufa 2.2, isopods avoided JNC soil at 75% of the field soil and 100% (soil without dilution), when exposed individually and in group (Fig. I.2). Even tough, there was no significant difference in the avoidance of isopods between all treatments (One-Way ANOVA, $F_{4, 15}$=2.188, $p>0.05$).

Isopods exposed to JC soil treatments in group bioassays did not show any preference by one of the soils. However, when exposed in individual bioassays isopods showed an avoidance performance in the 25%, 50% and 75% treatments (Fig. I.2). The average number of individuals in the test soil was similar in all treatments (One-Way ANOVA, $F_{4, 19}$=1.401, $p>0.05$), and no EC_{50} could be calculated.

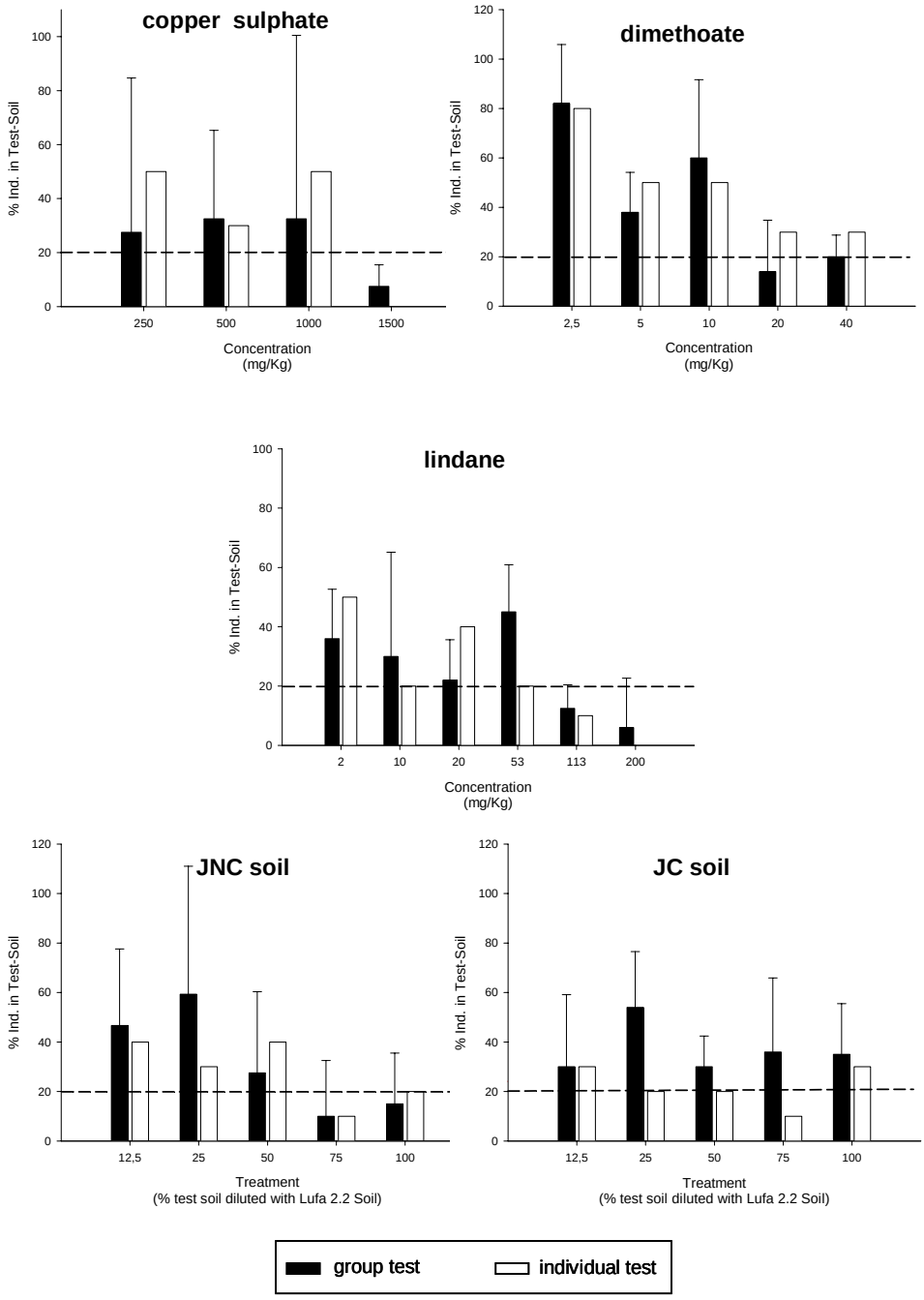

Fig. I.2- Percentage of the test-organism *Porcellionides pruinosus* in the test-soil (mean ± 95% Confidence Limits) exposed to copper sulphate, lindane, dimethoate and the two soil from Jales mine (JNC and JC soil). The dash line states the 20% "habitat function limit". The absence of the open column in the highest copper concentration in individual exposure of copper sulphate means 100% of avoidance in this concentration (i.e., zero organisms in the contaminated soil).

Earthworm Avoidance Behaviour Response Tests

Although in the Avoidance Behaviour Tests with *Eisenia andrei* some organisms were not found after the test period, and were considered dead, mortality never reached 20% in any of the concentrations used.

When exposed to copper sulphate the earthworm *Eisenia andrei* showed an avoidance behaviour at 320 mg/Kg of soil (Fig. I.3). The avoidance in copper exposure showed significant differences between 320 mg/Kg and all the other copper sulphate concentrations (One-Way ANOVA, $F_{3, 16}$=15.412, $p<0.05$; Tukey test, $p<0.05$), and also between 40 and 160 mg/Kg (Tukey test, $p<0.05$).

When exposed to the pesticide dimethoate, earthworms showed avoidance behaviour at 40mg/Kg of soil, where 20% of the animals (average) were found in the contaminated test soil (Fig. I.3). Nevertheless, there were no significant differences between avoidance values in all concentrations (One-Way ANOVA, $F_{4, 18}$=1.127, $p>0.05$).

Earthworms exposed to carbendazim and benomyl showed the same pattern, avoiding the soil at concentrations equal or higher than 10 mg/Kg of soil (Fig. I.3), what is in agreement with Hund-Rink and Wiechering [18]. Although exposure to benomyl showed a significant difference between the lowest concentration and 10 and 100 mg/Kg (Kruskal-Wallis One-Way ANOVA, H=10.274, df=2, $p<0.05$), carbendazim exposure did not present a significant difference between the concentrations used (One-Way ANOVA, $F_{2, 12}$=1.867, $p>0.05$).

When soils from the abandoned mine were tested, organisms showed no avoidance in all JNC soil treatments, including the 100% treatment (soil with no dilution). For JC soil (considered a contaminated soil), earthworms only showed some preference for Lufa 2.2 soil when they were also exposed to the treatment “75% JC soil + 25% Lufa 2.2 soil”: more than 80% were present in the Lufa 2.2 soil, hence less than 20% of the earthworms were found in the test soil “75% JC soil + 25% Lufa 2.2 soil” (Fig. I.3).

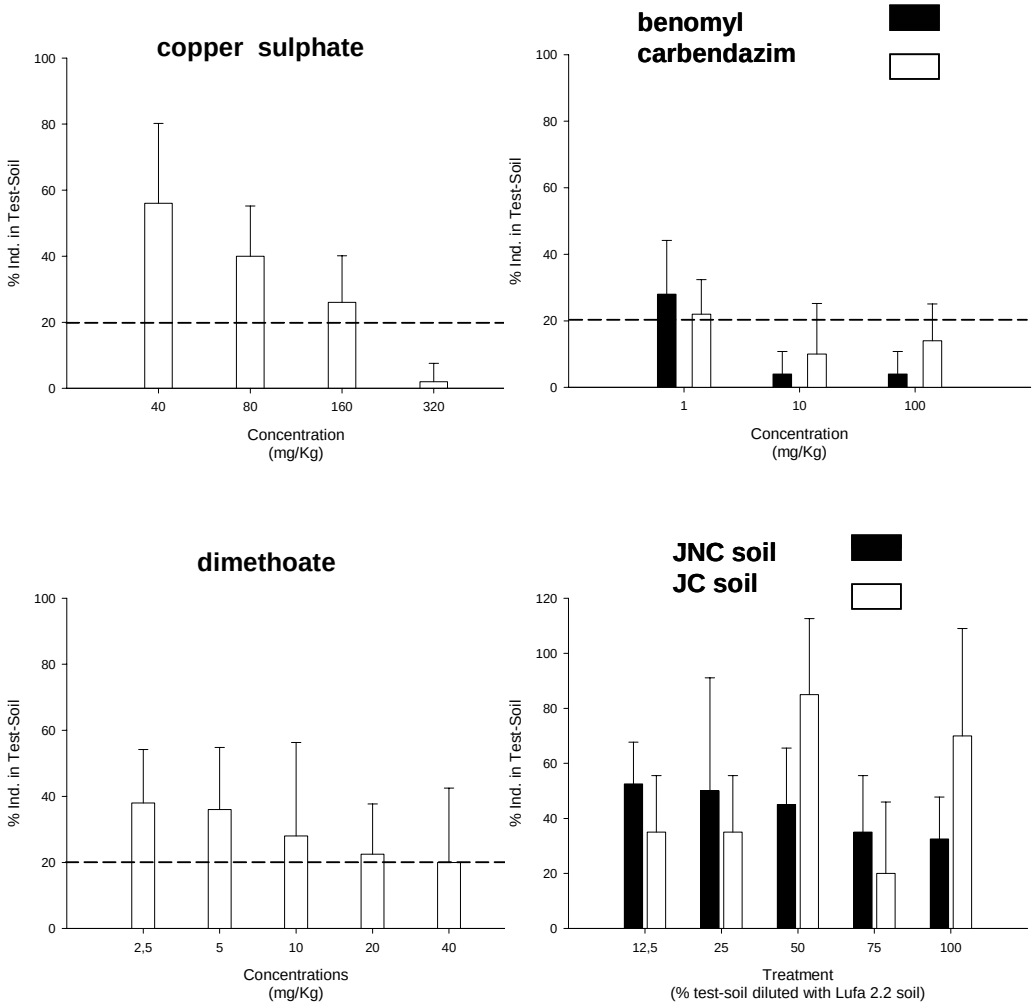

Fig. I.3- Percentage of the test-organism *Eisenia fetida* in the test-soil (mean ± 95% Confidence Limits) exposed to copper sulphate, dimethoate, carbendazim, benomyl and the two soil from Jales mine (JNC and JC soil). The dash line states the 20% "habitat function limit".

DISCUSSION

Isopods' mortality reached values higher than 20% in some chemical concentrations (113 and 200 mg/Kg lindane) which may indicate that these can be considered lethal to isopods, even if they have tried to avoid them. Despite Avoidance Behaviour Tests being considered chronic tests (evaluating a sublethal parameter), mortality can also be considered an important evaluation endpoint in these tests. This is supported by the fact that for some chemical doses isopods were found dead in the control portion. The fact that some of these chemical compounds affect the nervous system and therefore will disorientate the isopods can explain their inability to escape. Lindane is considered a persistent chemical with bioaccumulation potential and dimethoate is a ditiophosphorous insecticide that has a broader spectrum effect on arthropods by inhibiting their cholinesterase activity. Dimethoate is considered a volatile chemical compound and it might enter into organisms' body via air (e.g. respiration), in addition to pore water and soil particles ingestion. By acting as an inhibitor of acetylcholinesterase, dimethoate can also influence isopods and earthworms behaviour [24, 25]. Fábián and Petersen [26] observed that *Folsomia fimetaria* exposed to dimethoate exhibited motionless and/or uncoordinated motion. The isopod *Oniscus asellus* when exposed to lindane showed some changes in a stress protein level, which would comprise the adjustment of the nervous system [27]. Hence, when present in very high concentrations chemical compounds with these characteristics can affect orientation and consequently animals survivorship.

Isopods Avoidance Behaviour Tests

From the results from the group and the individual isopods' tests, it is possible to hypothesize that the production of an aggregation pheromone can be also important for the recognition and evasion from chemical compounds. However, this remains to be tested. In some situations isopods can escape from contaminated places only by accompanying others that have perceived the presence of chemicals. The active principle responsible for this aggregation

phenomenon on isopods was observed on their faeces and it is thought that they communicate by a response to chemical stimuli through their antennae [16]. The aggregation pheromone is secreted from the epithelial tissues on the mid or hind gut into the lumen where it will impregnate the faeces. For this reason animals with anosmia (animals with no antenna) were not used. Individual tests were run to give a more conclusive answer because aggregation was observed in some trials, producing high variability in the results. A possible example of this phenomenon was observed by the high confidence limits obtained in the exposure to copper sulphate, demonstrating the variability between replicates. Furthermore when exposed to dimethoate isopods avoided the soil with higher contamination only in group exposure, while individually they did not show any clear preference.

Some studies with edaphic species exposed to lindane have been performed, mainly considering bioaccumulation of this persistent compound [28-31] and its effects on reproduction [32 , 33]. In this study the exposure of isopods to lindane showed the same trend as former studies observing avoidance at 20 mg/Kg and with EC_{50} values of 48.32 and 48.62 mg/Kg in the individual and group tests, respectively. This was also supported by the bioaccumulation study with *P. pruinosus* [28] where the authors found an LC_{50} value of 76.3 mg/Kg after 2 days and 23.5 mg/Kg after one week. The sensitivity of terrestrial isopods to lindane was also confirmed by the influence of this chemical compound on the activity of the stressor protein *hsp 70*, showing an initial peak of the *hsp 70* activity during the first three days of exposure to lindane [27].

When exposed to dimethoate in this study, *P. pruinosus* showed the same trend as the isopod *Porcellio scaber* exposed to dimethoate in Lufa 2.2, where an EC_{50} value on growth of 17.5 mg/Kg was calculated [34].

Copper is known to be essential for the production of hemocyanine in isopods. Its high accumulation and storage in the mid gut lobs can also be explained as a detoxification process [35]. Due to this, ecotoxicological studies with isopods exposed to copper have reported very high EC_x values [36, 37]. The exposure of *P. scaber* to Lufa 2.2 soil contaminated with copper chloride resulted in an EC_{50} value of 1858 mg/Kg for juveniles growth and a LC_{50} value of 3755 mg/Kg [37]. This study showed that *P. pruinosus* is more sensitive than other

isopod species. Moreover, avoidance behaviour of this species is more sensitive than other ecotoxicological parameters. The EC_{50} value of 802 mg/Kg (for group tests) and the avoidance of soil with a concentration of copper higher than 1500 mg/Kg are lower than the results that have been reported for *P. scaber* [37].

Earthworm Avoidance Behaviour Tests

Earthworms exposed to benomyl exhibited the same behaviour pattern as in a study by Hund-Rink and Wiechring that also used an Avoidance Behaviour test [18]. Earthworms avoided the soil test when contamination was 10 mg/Kg or higher. For carbendazim, earthworms also showed the same pattern. A range of concentrations of benomyl (8.3, 56, 112 mg/Kg) used in a spermatogenesis study caused abnormalities in the ultrastructure of sexual organs of *E. fetida* [38]. In other studies, using a soil microcosm, the exposure of earthworms to carbendazim showed an EC_{50} value for the biomass loss of 1.9 mg/Kg and a LC_{50} of 6.2 mg/Kg [39]. The latter values were in agreement with the results obtained for this study with carbendazim exposure, where less than 20% of the animals were found in the 10 and 100 mg/Kg concentrations, although it was not possible to calculate the EC_{50} value.

The Avoidance Behaviour Tests with dimethoate showed similar results when compared with other studies. Martikainen observed IC_{50} values for biomass change in the range of 14.4 to 42.9 mg/Kg of soil for the earthworm *Aporrectodea caliginosa tuberculata* which are in the same range of the EC_{50} value found in this study [25].

Although the EC_{50} value found for the earthworm avoidance tests with copper is higher than 100 mg/Kg, it is in agreement with the study of earthworms' population (*Lumbricus rubellus* and *Dendrodilus rubidus*) where concentrations above 100 mg /Kg of soil were found to be toxic even if earthworms can still be

found in places where the copper concentration is above this value [40]. Burrows and Edwards also showed that earthworms biomass was reduced at 200 mg/Kg of copper and that an exposure of 100 mg/Kg revealed no effects in this parameter [39]. As expected, in this study earthworms were more sensitive than isopods when exposed to this chemical compound.

Avoidance Behaviour Bioassays

The performance of Avoidance Behaviour Bioassays in the evaluation of the quality/toxicity of soils from one real scenario, an abandoned mine, was also tested. When isopods and earthworms were exposed to the JNC soil their behaviour was quite different. Although individuals of *E. andrei* did not show any avoidance behaviour, the isopods *P. pruinosus* presented a clear avoidance on the 75% and 100% treatments, in individual and group bioassays, being therefore considered more sensitive. This fact was not expected because JNC soil is a silt loam soil with low amount of heavy metals, almost all below the Ecological Screening Values [41] or the Canadian Environmental Quality Guidelines (in http://www.ccme.ca/assets/pdf/e1_06.pdf) with high organic matter content, and could be considered clean by the Portuguese legislation (Portaria nº 176/96 (II Série), 3/10/1996).

On the other hand, JC soil was expected to be toxic because of its chemical characteristics. Nevertheless it was found that when test-organisms were in contact with this soil they did not avoid it and the majority seemed in fact to prefer JC soil when confronted with Lufa 2.2 soil. This might be explained by a low bioavailable fraction of heavy metals present in this soil or by an interaction between chemical compounds that would be traduced in a lower toxicity. Additionally chemical information not provided by the chemical analysis (i.e. the existence of a non-expected compound in soil) may also explain the results.

Anyway, this reinforces the fact that chemical analyses, per se, are not sufficient to evaluate the potential toxicity of soils.

Using the terminology from BioQuest International [42], the avoidance bioassay classified JNC soil as a soil with slight toxicity.

CONCLUSIONS

The results observed in this study show that Avoidance Behaviour Tests can be regarded as a valuable tool in the screening evaluation of soil contamination. The use of these tests as first approaches for contaminated sites evaluation will bring a quick information for future decisions on the evaluation procedure.

Additionally, different species should be used in this kind of tests because species react and respond differently to chemical stimulus, as shown in the soil mine bioassays.

For this reasons statistics for this kind of tests have to be improved and established, so that more sound information can be derived from the ISO standardized protocol that is currently being developed [9] using the statistical methodology for calculating EC_{50}s (derived for standard methodologies) presented here.

This will mean that Avoidance Behaviour Tests can be used, with clear advantages, both as first screening tools in Terrestrial Risk Assessment and also in Soil Quality Criteria studies, warranting quantitative assessment of the contaminant(s) bioavailability and toxicity.

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CHAPTER II

Evaluation of the Toxicity of Two Soils from Jales Mine (Portugal) Using Aquatic Bioassays

Evaluation of the Toxicity of Two Soils from Jales Mine (Portugal) Using Aquatic Bioassays

(Submitted to the journal Chemosphere)

ABSTRACT

Soil contamination can be one path for streams and ground water contamination. As a complement of chemical analysis and total contaminants determination, bioassays can provide information on the bioavailable fraction of chemical compounds, focusing on the retention and on the habitat function of soils. In this study the evaluation of toxicity of two soils from the abandoned Jales mine (Portugal) regarded both functions. The retention or buffer capacity of soils was tested with bioassays carried out using as test-organisms the cladoceran *Daphnia magna* and the marine bacteria *Vibrio fischeri*. The habitat function of soils was evaluated with the reproduction bioassay with the collembolan *Folsomia candida*. The Microtox solid-phase test was performed with *V. fischeri* using soil as test medium, and soil elutriates were extracted to perform the Microtox basic test, and an immobilization and reproduction bioassay with *D. magna*. The marine bacteria showed high sensitivity to JNC soil and to JNC soil elutriates, while JC soil or soil elutriates exposure did not cause any toxic effect. In the bioassays with *D. magna*, organisms showed sensitivity to JNC and also to JC soil elutriates. Both mobilization and reproduction features were inhibited. The bioassay performed with *F. candida* did not reflect any influence of the contaminants on their reproduction. Although JNC soil presented lower heavy metal contents, elutriates showed different patterns of contamination when compared to JC soil and elutriates, which indicates different retention and buffer capacities between soils. Results obtained in this study pointed the sensitivity and importance of soil elutriates bioassays with aquatic organisms in the evaluation strategy in soil ERA processes.

INTRODUCTION

Soil contamination has become an environmental problem, not only regarding soil as a source of fibber and food but also regarding the possibility of groundwater contamination. Analytical chemistry is usually the traditionally detection method for soil or water contamination. The spectrum of substances analysed is based on the area's history and not all contaminants may be evaluated, such as some metabolites that can be formed during degradation. These methods can not differentiate between chemical compounds that are available to biological systems from those which are inert, complexed or in unavailable forms, like happens with heavy metals [1].

To solve this gap, bioassays are considered useful tools to assess the bioavailable fraction of contaminants in soils. From the Equilibrium Partitioning Theory (EqPT) edaphic organisms are only sensitive to the bioavailable fraction of chemical compounds. Contaminants that can be uptaken are found in the interstitial water (aqueous phase) and in soil particles and dissolved organic matter (soil solid phase) [2-4].

To cover several functional soil levels, a stepwise approach using bioassays should be established. A first level should comprise the soil retention function, studying the potential of chemicals to be mobilized via the water path, followed by a second and third levels that would evaluate the biological function of soil and the suitability for plant survival/growth, respectively [5, 6].

The main effect of the retention function is to immobilize substances due to the adsorption function of the soil matrix. The increase of water in soil and therefore the decrease in immobilization caused by high solubility can lead to the transport of contaminants and their entrance in the groundwater system.

Ecotoxicological tests in aqueous soil extracts or elutriates are based on the assumption that edaphic organisms are affected by chemical compounds through the soil pore water (aqueous phase), although they are considered less ecologically relevant than tests that use the soil matrix as an all. There are some tests, already standardized by the OECD, ISO and the European Union, to evaluate the toxicity in waters that can be used to test soil elutriates [7]. These

tests comprise several trophic levels like microorganisms, primary consumers, secondary consumers or producers.

Aquatic bioassays with elutriates have been specially used to estimate the hazard of landfills and groundwater resulting from disposal of solid wastes in landfills, as part of a test battery where some terrestrial species are also placed in contact with the waste extracts [8-10]. Furthermore, some studies have also used this type of approach to evaluate the toxicity of contaminated natural soils or sediments [6, 11-16].

The aim of this study was to evaluate the potential toxicity of two soils from an abandoned mine in Portugal (Mina de Jales) to groundwater systems using aquatic bioassays, as a first function level evaluation of soil toxicity. The bioassays chosen were the *Daphnia magna* immobilization and reproduction tests, and the Microtox test using marine luminescent bacterium *Vibrio fischeri* that has been used as a standard government ecotoxicological bioassay in several countries, like Canada, The Netherlands, France, Germany, Sweden and Spain [17, 18]. Additionally a second level was also evaluated, now in terms of habitat function, using the reproduction test with the collembolan *Folsomia candida*. Finally the results from the aquatic and terrestrial bioassays were compared.

MATERIALS AND METHODOLOGY

Two soils were collected in an abandoned mine in the northeast of Portugal, Mina de Jales (Vila Pouca de Aguiar) (N 41º 47’ 40.3’’; W 07º 57’ 87.6’’) and studied, using both soil matrix and elutriates as test media. One of the soils shows a high heavy metal content (JC soil) and was collected 150 m from the mine spoil (N 41º 27’ 53.9’’; W 07º 34’ 50.6’’); the other soil has a low heavy metals content (JNC soil) and was collected 3 Km from the mine spoil (N 41º 28’ 36.1’’; W 07º 34’ 14.0’’).

Controls were selected according to the objective and bioassay procedure. The soil Lufa 2.2, obtained from Speir, Germany, was used as control when bulk soil was used as test medium while its elutriates were used in the luminescent

bacteria Basic test as control. This soil was chosen in detriment of an artificial soil [19] because it has often been used as a control in soil ecotoxicological testing and it is a natural soil. The control usually used for aquatic ecotoxicological tests, the ASTM solution [20], was used as control in the aquatic bioassays and also as the diluents medium.

Soil pH was determined following the methodology of ISO 10390 [21].

Elutriates extraction

Soil elutriates were obtained by a combination of processes in order to make available in water chemical compounds present in the test soils [5, 12, 22, 23]. As heavy metals were the main contamination and therefore the main objective of this study, the extraction procedure with water (used only for hydrophilic compounds) was the main extraction used. The exception was one of the bioassays performed for the MICROTOX Basic tests where elutriates were obtained using the Microtox extraction solution (Azur Environmental, Carlsbad, CA, USA).

Firstly, distil water was added to the soil (wet basis) in 2:1 proportion and it was mixed in the dark for 24h in a benchtop orbital shaker. These mixtures were then centrifuged for 20 min., at 10000 g, and finally filtrated using a vacuum filtration apparatus through glass microfibre filters (Whatman GFC ∅ 47 mm, 1.2 μm porosity). Elutriates were stored at 4ºC up to the testing time, but never exceeding one week. The same procedure was followed when extraction was made with the Microtox extracting solution in a 1:4 ratio (soil:solution).

Elutriates were analysed for heavy metal content by ICP-AES (Inductively Coupled Plasma- Atomic Emission Spectroscopy) and pH values, oxygen content and electrical conductivity were also measured. The bulk soil heavy metal content was analysed by ICP-OES.

Bioassays with Daphnia magna

Two bioassays were carried out with the cladoceran *Daphnia magna*: an immobilization and a reproduction test, according to OECD guidelines [24], using individuals from clone F [25] and initiating each test with third- to fifth- brood neonates (< 24h). For each treatment and control 10 animals were used; daphnids were transferred to new test medium every two days. In the reproduction test animals were fed daily with *Pseudokirchneriella subcapitata* (also known as *Selenastrum capricornutum*) and an organic additive was added [26].

The ASTM solution was used has the negative control for the immobilization bioassay (acute test), and the Lufa 2.2 soil elutriate solution was used has positive control. The two elutriate solutions obtained from JNC and JC soils were diluted with the ASTM solution and several treatments were obtained containing 6, 12.5, 25, 50, 75, 100% of the elutriate of the test soil. After 24 and 48h daphnids were observed to check their mobility/death.

In the reproduction bioassay (chronic test), the dilution of soil elutriates were based on the results from the immobilization test. For the JNC soil, elutriates were diluted with ASTM making treatments of 0.25, 0.5, 1, 2 and 4% of the JNC elutriate; for the high heavy metal content soil, treatments of 1, 2, 4, 8 and 16 % of the JC elutriate were used. In the chronic test the number of juveniles produced during 21 days and the length of daphnids (body length excluding the anal spine and antennas) were also measured at the end of the tests.

Physico-chemical parameters like pH values, oxygen content and electrical conductivity were measured in the diluted elutriates used in all bioassays. No adjustment was made in the pH values nor in the O_2 content of elutriates used in the bioassays, because changes could cause differences in the heavy metal bioavailability.

A reference test with potassium dichromate ($K_2Cr_2O_7$) was also performed as a positive control. The 24h LC_{50} value was in the range of the values (0.6-1.7 mg/L) stated in the OECD protocol [24].

MICROTOX® bioassay

MICROTOX® was used to evaluate the inhibition of the luminescence in the marine bacteria *Vibrio fischeri* in two different tests: the Solid Phase Test (SPT) and the Basic Test, using the soil matrix and soil elutriates as test media. In these bioassays the toxicity of both soils and elutriates from JNC, JC and Lufa 2.2 soils were evaluated, following the protocols from the MICROTOX® [27].The protocol for the extraction of elutriates for the light inhibition bioassay (MICROTOX®) advises the use of the Microtox extraction solution (Azur Environmental, Carlsbad, CA, USA) but, and in addition to this, water extraction elutriates (same procedure as for the *Daphnia magna* protocol) were also evaluated.

The Solid Phase or direct contact bioassay was conducted as recommended by the manufacturer [27]. Thus, 0.3 g of soil were suspended in 3 ml of solid phase test diluent (Azur Environmental, Carlsbad, CA, USA), and it was shaked for 15 sec. A series of dilutions were made in glass cuvettes and the luminescent bacteria *Vibrio fischeri* was added to each dilution and control (just the diluent). After 20 min of incubation, filter columns were inserted in the glass cuvettes and pushed downwards to settle the solids. The filtrate was then transferred to a new cuvette in the Microtox incubation block and 5 min after the bacterial luminescence was measured using a Microbics Model 500 toxicity analyser (Microbics).

The soil concentration corresponding to a 50% reduction in luminescence was computed using Microtox data Collection and Reduction software (Microbics) and reported as % dry soil.

For the Basic Test, three kinds of soil elutriates were tested for their toxicity. Water extraction elutriates were evaluated in a proportion of 1:2 and 1:4 soil: water (g:ml) and elutriates were also obtained using 4 parts of Microtox diluent to 1 part of soil sample (1:4). All elutriates were obtained by the same procedure as explained above.

Elutriates were pipetted into glass cuvettes and the salinity was adjusted with MOAS (Azur Environmental, Carlsbad, CA, USA); a series of dilutions were made as in the protocol of the manufacturer [27]. Five and 15 min after transferring the bacteria to the elutriates vials, their toxicity was evaluated and a 50% reduction in

luminescence was computed using Microtox data Collection and Reduction software (Microbics) and reported as % of elutriates.

Folsomia candida bioassay

The *Folsomia candida* reproduction bioassay was based on the standard protocol ISO 11267 [19]. In this bioassay, 10 collembolans were exposed to three soils: Lufa 2.2 (control), JNC and JC soils, and every week soil moisture was adjusted to the initial moisture value of 40-60% Water Holding Capacity of the soil. After a period of 28 days, adults' mortality and the number of juveniles produced were evaluated. The methodology used for counting juveniles consisted of the addition of water and dark ink to soil replicates and, as juveniles were located at the water surface tension, we could photograph them. The computer programme SigmaScan Pro5, an image measuring software, was used to count the number of individuals in each photograph (replicate). Additionally, a reference test was also performed using Betanal as test substance spiked in soil Lufa 2.2. The results obtained were consistent with the EC_{50} values between 100 and 200 mg/Kg that are advisable in the protocol [19].

Statistical Analysis and Parameters Calculation

The immobilization of daphnids was observed and reported and the LC_{50} value was calculated using the Probit Method [28].

One-Way ANOVA analysis were performed using the SigmaStat statistical package [29], and whenever significant differences between treatments were found, post-hoc multiple comparisons were conducted [30].

For the reproduction bioassay with *D. magna* LOEC and NOEC values were determined.

In the Microtox Bioassays EC_{50} values were calculated using the software from Microbics Corporation.

For a more convenient interpretation of the toxicity data, all toxicity values were converted into TU, i. e. the inverse of the X (where X=LC, EC_{50}, NOEC or LOEC) expressed in %: TU= [1/X] x 100.

RESULTS

The heavy metal content and pH values of soil elutriate and bulk soil can be found in Table II.I.

Daphnia magna bioassays

In the immobilization bioassays with the cladoceran *D. magna*, it was observed that the elutriates from the soil with lower heavy metal content (JNC soil) exhibit higher toxicity than those from soil JC, with high heavy metal content. JNC soil elutriates exposure originated a LC_{50} of 51.11% after 24h, while JC elutriates showed a LC_{50} of 95.26% after the same period. The LC_{50} values for JNC and JC soil elutriates after 48h were, respectively, 15.81% and 51.60%.

Using these results a reproduction test was carried out using as highest elutriates concentrations 8% of JNC elutriate and 32% of JC elutriate. Even so, when exposed to these two highest treatments, individuals died just after 48h. So, reproduction tests were performed with treatments of 0.25, 0.5, 1, 2 and 4% of JNC elutriates and 1, 2, 4, 8 and 16% JC elutriate dilutions.

Table II.I- Heavy metal content and pH values of JNC and JC soils and soil elutriates.

Parameters	Soil Elutriates (μg/l)		Bulk Soil (mg/Kg)	
	JNC Soil	JC Soil	JNC Soil	JC Soil
Zn	290	2238	33	97
Cd	2.7	44.0	1.9	8.2
Pb	< d.l.	47	33	209
Co	15	16	n. d.	n. d.
Ni	< d.l.	23	5	9
Be	5.8	4.0	2.0	3.1
Al	10710	645	14 000	10 000
Fe	6	56	7370	17800
Mn	1143	1117	99	255
Cr	< d.l.	< d.l.	6	15
Cu	< d.l.	< d.l.	8	24
As	28	< d.l.	71	251
pH	4.28	5.00	4.14	4.47

The Lufa 2.2 soil elutriates did not cause any immobilization in the daphnids during the 48h period of the bioassay.

In the chronic bioassay and when exposed to JNC soil elutriate dilutions, the number of juveniles per daphnid increased significantly (One-Way ANOVA, $F_{5, 53}=17.82$, $p<0.001$) in the four lower dilutions (0.25, 0.5, 1 and 2%) but in the highest dilution (4%) the number of juveniles per female was lower but not significantly different from the control (Fig. II.1). A similar trend was observed for the two lowest dilutions of JC soil elutriates (1 and 2%), but the 16% dilution exposure showed a significant decrease of 8% in the production of juveniles per daphnid (One-Way ANOVA, $F_{5, 50}=36.02$, $p<0.001$) (Fig. II.1).

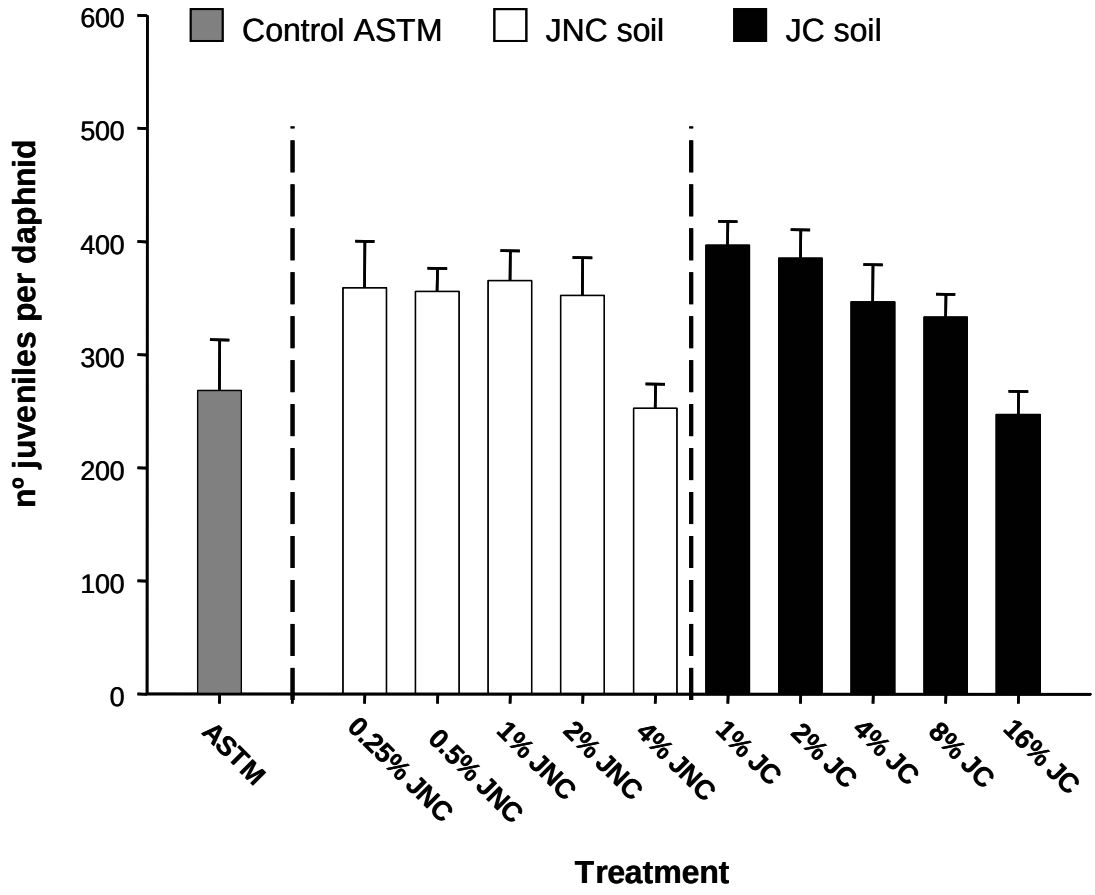

Fig. II.1- Number of juveniles produced per *D. magna* under different dilutions of JNC and JC soil elutriates, over 21 days. Data are expressed as mean number of juveniles per female ± 95% confidence limits.

After the 21 day period of the test, daphnids were measured and their length was compared. Here again the same trend was observed as in the production of juveniles. The exposure to dilutions of 1 and 2% of JNC lead to a slight increase in the females length (One-Way ANOVA, $F_{5, 53}$=12.59, p<0.001), while the highest treatment (4%) presented no effect on their growth (Fig. II.2). Also when exposed to JC elutriates dilutions there was a significant increase in the growth in dilutions of 1 and 2% of JC soil elutriates, but a significant decrease of 10% when daphnids were exposed to the 16% dilution was also observed (One-Way ANOVA, $F_{5, 52}$=39.61, p<0.001) (Fig. II.2).

Comparing soil elutriates with the control, the time to achieve the 1st brood was not statistically different in the JNC elutriate exposure, but it showed a significant decrease in the 8%JC and 16% JC elutriate exposure (Kruskal-Wallis One-Way ANOVA on Ranks, H=22.301, df=5, $p<0.01$, followed by the Dunn's Method).

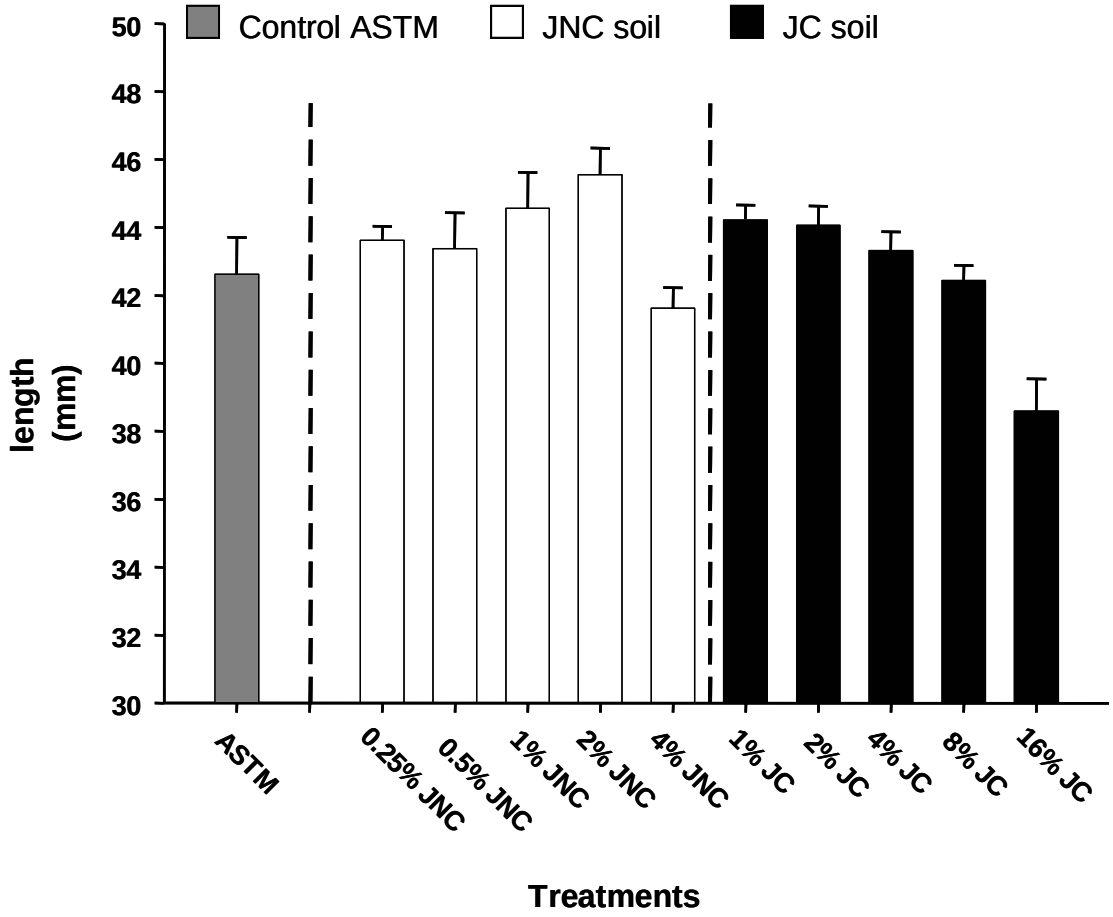

Fig. II.2- Body size of *D. magna* after 21 days of exposure to several dilutions of JNC and JC soil elutriates. Data are expressed as mean length of female ± 95% confidence limits.

Comparing the cumulative production of juveniles in the five broods that were obtained for almost every female exposed to JNC soil elutriates, there was no significantly difference between the number of juveniles in the first brood within all treatments (Kruskal-Wallis One-Way ANOVA, H=1.795; df=5; p=0.877). The same trend was observed in the 2nd brood (Kruskal-Wallis One-Way ANOVA; H=6.656, df=5; p=0.248) and 3rd brood (One-Way ANOVA; $F_{5, 53}$=1.799; p=0.129). The effects of the exposure to JNC elutriates were only observed in broods 4 (One-Way ANOVA; $F_{5, 53}$=2.769; p=0.027) and 5 (One-Way ANOVA; $F_{5, 42}$=15.723; p<0.001). When exposed to JC soil elutriates, differences within broods were observed immediately in the first brood (One-Way ANOVA; $F_{5, 51}$=6.879; p<0.001, after log transformation).

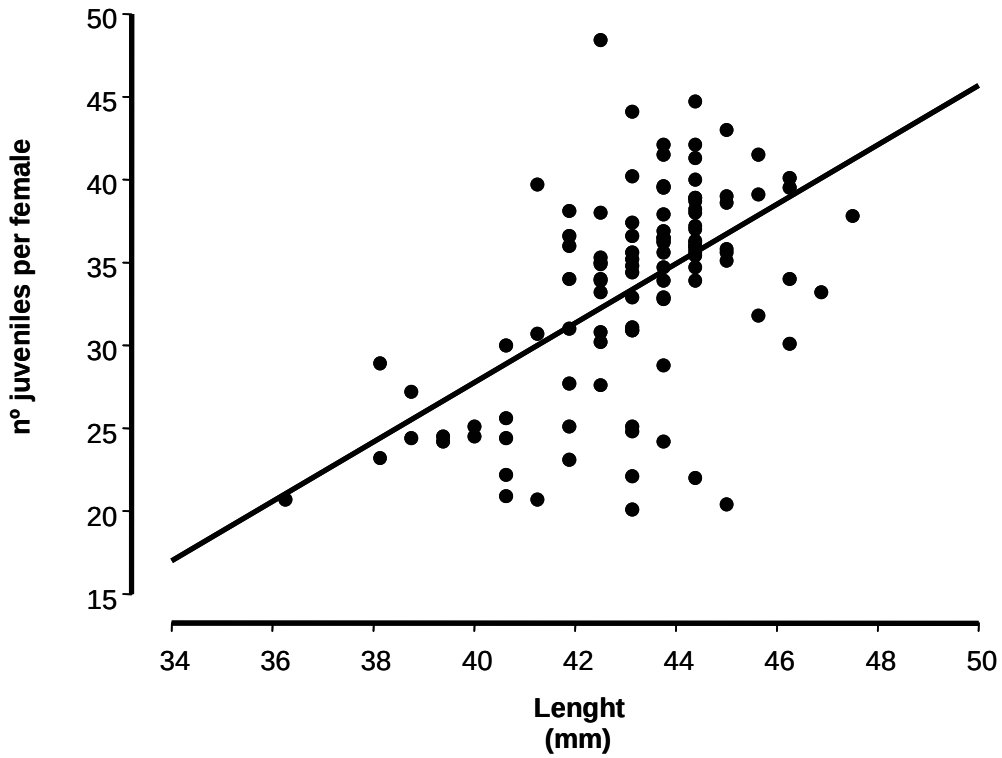

Fig. II.3- Production of juveniles as a function of female body length (mm) in *D. magna* exposed to all soil elutriate dilutions and control.

The presence of aborted eggs was observed only in dilutions with 2% and 4% of JNC soil elutriate. In the 1st brood of daphnids exposed to these JNC soil dilutions only few aborted eggs were observed, increasing in number in the 3rd, 4th and 5th broods.

A significant correlation between the mean number of juveniles per female and the length of each female was found (Pearson Correlation; r=0.553; n=106; $p<0.05$) and the regression model computed (Fig. II.3).

Vibrio fischeri bioassays

Only JNC soil caused an inhibition of the light production in the bacteria *V. fischeri* while JC and Lufa 2.2 soil showed no influence on this parameter. Although only EC_{50} values are presented in Table II.II, no EC_{10} values were obtained from the exposure of the bacteria to JC and Lufa 2.2 soils/ soil elutriates. Comparing all bioassays performed with the luminescent bacteria *V. fischeri*, the Solid Phase Test (SPT) showed the lowest EC_{50} value within all tests.

Table II.II- EC_{50} values obtained for the marine luminescent bacteria bioassays. Values are presented in % of soil elutriate (n.t.- no toxicity; SPT- Solid Phase Test).

	EC_{50} (%)						
Soil Type	**SPT**	**Basic Test**					
	soil	water extract (1:2)		water extract (1:4)		Microtox extract liquid (1:4)	
		5 min	15 min	5 min	15 min	5 min	15 min
Lufa 2.2	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
JNC	2.57	27.21	n.t.	n.t.	62.34	32.83	29.90
JC	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.

n.t.- with no toxicity response

The water extractability can be considered wicker when compared to the Microtox extract liquid performance because the TU values obtained for ratios 1:4 (soil:liquid) extractions were higher in the Microtox liquid extraction.

As expected, the water extracts in ratio 1:2 expressed the double of toxicity when compared to the 1:4 ratio water extractions.

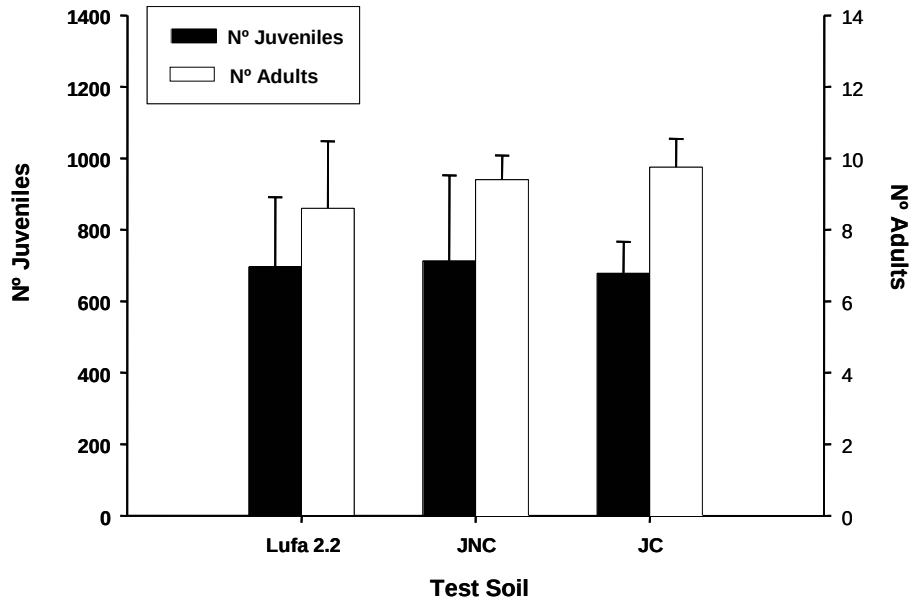

Fig. II.4- Number of juveniles produced and survival of adults of *F. candida* exposed to Lufa 2.2 (control), JNC and JC soils. Data are expressed as mean number of juveniles per replicate and number of live adults over 28 days ± 95% confidence limits.

Folsomia candida reproduction bioassay

After the exposure of the collembolan *Folsomia candida* to both soils JNC and JC from Mina de Jales during 21 days, no significant differences were observed in the production of juveniles when compared to the exposure to the

control Lufa 2.2 soil (One-Way ANOVA, $F_{2, 11}$=0.0569, p=0.945) (Fig. II.4). The mortality of adults exposed to JNC and JC soils after 21 days was 6% and 2.5%, respectively.

DISCUSSION AND CONCLUSIONS

From the bulk soil chemical analysis it was concluded that some heavy metal compounds (e.g. Be, Ni, Cd and Zn, only in JNC soil) were not considered important because their levels were below the threshold values that are advised in several countries like Canada, The Netherlands or Germany (e. g. Canadian Environmental Quality Guidelines; http://www.ccme.ca/assets/pdf/e1_06.pdf). However, elutriate chemical analysis revealed their importance because they were toxic to *Daphnia magna*, being above the advisable level for heavy metals in water, ([31], available in http://www.esd.ornl.gov/programs/ecorisk/documents/tm96r2.pdf). It was interesting to note that arsenic was not extracted to water from the JC soil, although it was detected in JNC soil elutriates. This might indicate a strong adsorption of As to another soil compound (e.g. clay, chloride ions or organic matter) in the JC soil, thus determining its availability to edaphic organisms. The different adsorption of heavy metals observed in JNC and JC soils might also be related to the existence of complexed forms or ionic forms of the compound that bound differently to soil particles. pH is clearly the dominant factor that controls the distribution of metals between the soil and the aqueous phase, although the effect of other characteristics cannot be ignored (e.g. soil organic matter, which is correlated inversely related to sorption and desorption of contaminants [32]).

Moreover, Ni, Be and Cd show low levels in the bulk soil chemical analysis, but were detected at higher levels in water extraction. This seems to indicate the presence of the major fraction of these chemical compounds in the soil pore water, resulting in a high bioavailability to edaphic organisms.

The differences in chemical adsorption to different soils and, consequently, their different availability to organisms, is an argument for the use of a test battery with organisms from different trophic levels and different environmental compartments. Chemical analysis can not give clear information or quantify the effects of chemical compound mixtures when they are present in the environment. In contrast, bioassays provide a biological response to all the compounds within a mixture and their realistic interaction (e.g. how they act or react in the presence of others), thus being a useful method for assessing the biological impact of effluents, leachates and contaminated water and soils [16].

JNC soil would probably not be categorised as contaminated using conventional analytical methods, but the elutriates caused significant effects in all aquatic organisms used in this small test battery, although in the *Folsomia candida* reproduction test no impact on survival or reproduction was observed. Furthermore, JC soil would be considered as a high contaminated soil, mainly due to its high levels of As and Pb, but the elutriates were only toxic to *Daphnia magna* (immobilization and reproduction test) and not to the other test species.

When daphnids were exposed to JNC and JC soil elutriates the number of juveniles increased in the higher elutriates dilutions. Body length also increased in the same treatments. Conductivity values in JNC soil elutriates reached a range values between 531 and 545 μS/cm, and JC soil elutriates also showed similar values (539-577 μS/cm). Although conductivity was similar between dilutions and the control, this fact might be explained by the possible increase of both micronutrients in these dilutions and of the dissolved organic matter, which might have improved the metabolism of daphnids.

In the bioassays with the JNC soil elutriates differences between treatments and the control were not observed in the 1st, 2nd and 3rd broods. This is not in agreement with the study made by Guilhermino *et al.* for the exposure of *D. magna* to different chemical compounds like sodium bromide, 3,4-dichloroaniline, cadmium and parathion [33]. Even though, exposure of daphnids to JC soil elutriates results were in accordance to this indication.

Daphnids' 1st brood appears in 8 and 16% JC earlier than in the ASTM control, which can be explained by an energetic input to achieve a premature

reproduction, trying to leave a lineage in case of females early death. As a result of this energetic input, the number of offspring decreased in these lowest dilutions of JC soil elutriates.

Some substances, even hydrophilic, can not be extracted from soil because they can adsorb to the organic carbon or clay particles that exist in the soil. So Microtox SPT is a good choice for the evaluation of the potential toxicity of soils. In this study the sensitivity of the SPT compared with the Basic test was higher, providing higher TU values and therefore a higher sensitivity to the toxicants present in the bulk soil (Table II.III). This fact was also reported by Doherty [34] when comparing results of Microtox tests conducted with pore water, aqueous elutriates, organic solvent extracts and whole sediment. Notwithstanding the limitations or difficulties in practice that SPT can bring (e.g. sometimes being difficult to make accurate dilutions of soil particles), solid phase material is more sensitive than aqueous extracts mainly due to the fact that the bacteria is in direct contact with the contaminants present in the bulk soil and not only with those which have been extracted by water or some other extracting solution.

Although, the SPT toxicity appears to be associated with sample composition, i.e. soil characteristics, there are disadvantages on its use because it has been stated that some differences in sand and clay content of clean soils give different toxicity patterns. The bacteria can be adsorbed to clay particles and the luminescence is diminished, resulting in low EC_{50} values [34]. JNC and JC soil have similar percentages of clay (16.79% and 13.60%), so the amount of bacteria adsorbed to both soil might be similar. When comparing to Lufa 2.2 soil, another approach has to be done because this is mainly a sandy soil with 77% of sand and 8% of clay. In this case the bacteria are mainly free in the soil matrix, so the soil non/toxic potential is not dependent on the “clay content” issue.

Table II.III- Toxic Units (TU) calculated for all bioassays. For the Microtox bioassays TU are obtained from EC_{50} values; for the *Daphnia magna* immobilization and reproduction bioassays, TU were obtained by LC_{50} and LOEC values, respectively.

	Toxic Units (TU EC_{50})							**TU [LC_{50} (48h)]**	**TU [LOEC]**
Soil Type	**SPT**	**Basic Test**		**Basic Test**				**Daphnia magna**	
	soil	water extract (1:2)		water extract (1:4)		Microtox extract liquid (1:4)		immobilization test	reproduction test
		5 min	15 min	5 min	15 min	5 min	15 min		
Lufa 2.2	n.t.	n.t.	n.t	n.t.	n.t.	n.t.	n.t.	n.t.	n.d.
JNC	38.94	3.67	n.t	n.t.	1.60	3.05	3.35	6.33	12.50
JC	n.t.	n.t.	n.t	n.t.	n.t.	n.t.	n.t.	1.94	6.25

SPT- Solid Phase Test; **n.d.-** not determined; **n.t. -** with no toxicity response

When comparing the three test organisms and their different test medium and test parameters that were used, the Microtox SPT was the most sensitive test regarding JNC soil, since the *Folsomia candida* bioassay did not show any toxicity level for this soil and the soil elutriates produced lower toxicity values.

For the JC soil the *Daphnia magna* reproduction test was the only test (despite the immobilization test) that showed effects on the exposure of this species to the soil elutriates, but this could not be anticipated from the chemical analysis made to the test soils. Although with higher heavy metal levels than JNC soil, JC soil showed lower potential toxicity when compared to JNC soil.

Elutriate chemical analysis and ecotoxicological testing of soil elutriates might be a good tool to shed some light over unexpected results. In this study the elutriates analysis and ecotoxicological testing showed that some heavy metals are more bioavailable than others and also that their availability is dependent from the soil type. Aluminium is probably the main toxicant causing major effects on the used test organisms when exposed to JNC elutriates. On the other hand, zinc content and bioavailability might be the cause of toxicity of JC soil elutriates to *Daphnia magna*. Additionally it might also be possible that there are other interactions, not necessarily additive, between the heavy metal species that are present in the soils and also in elutriates, since an additive effect would result in much higher toxicities, especially in the JC soil case.

Our results clearly show that it is essential the use of a multiple test battery to screen the toxicity of soil in association with chemical analysis and soil characteristics, to produce accurate recommendations regarding remediation processes or interventions.

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CHAPTER III

Microbial Activity in Two Soils from Jales Mine (Portugal): an Ecotoxicological Approach

Microbial Activity in Two Soils from Jales Mine (Portugal): an Ecotoxicological Approach

(Submitted to the journal Soil Biology and Biochemistry)

ABSTRACT

Microorganisms play a crucial role in the decomposition processes and nutrient cycling, and therefore in soil quality. Soil enzymes have shown sensitivity to contaminants, as heavy metals, due to their interaction with the specific reaction sites of enzymes, reducing the formation of the reaction products. In this study several enzymatic bioassays were applied (dehydrogenase, acid phosphatase, arylsulfatase, urease and β-glucosidase) to soils with different heavy metal content, from the abandoned Jales mine (Portugal), before, during and after a rehabilitation process (years 2002, 2003 and 2004, respectively). Additionally, the mineralization of N and the microbial biomass of C and N were also measured. This information will be derived into a Soil Quality Index (SQI), usually applied to native soils. The results obtained in this study indicated that dehydrogenase, arylsulfatase and N-mineralization showed a recovery in soil microorganisms, but no information on the influence of contaminants in soils was derived. Microbial biomass C and N also showed an increase from 2002 to 2004 and soil organic matter and pH influenced the enzymatic activities, mainly dehydrogenase, acid phosphatase and arylsulfatase. The soil with lower heavy metal content presented a low SQI and the soil considered highly contaminated showed a good SQI, similar to those obtained in native soils. Although not expected, these results are in concordance with results obtained in other bioassays, showing the influence of the bioavailable fraction on soil processes.

INTRODUCTION

Soil quality is intimately related with physicochemical soil properties as with soil biological functions, and their evaluation will help to characterize the fertility and productivity of soils. Recently some attention has been directed to soil habitat function, requiring the assessment of toxicity factors that influence microorganisms and all processes that they mediate [1].

Some authors [2-4] have suggested that biological or biochemical properties that have demonstrated to be useful for the detection of soil quality changes are related with nutrient cycles. Nitrification or respiration are usually recommended as toxicity endpoints in specific guidelines but other endpoints like soil enzymatic activities can also be considered [5], presenting as main advantages cost-effectiveness and quickness.

Soil enzymatic activity can be used as a sensitive gauge in studies addressing heavy metals contamination. These chemical compounds are able to reduce enzyme activity by interacting with the enzyme-substrate complex, denaturing the protein by the interaction with its active sites or by simply affecting the microbial cell that produce the enzymes [6-8]. Not all metal species can induce these interactions, so metal speciation in the solid or liquid phases, and conclusively their bioavailability, can be assessed by measuring their effects with soil enzymes.

The use of single enzyme bioassays has been criticized by several authors [2, 3, 9, 10], because each enzyme catalyses a specific reaction, using a specific substrate. So, enzymatic batteries are advisable in soil quality studies.

In this study, the use of soil enzymes was chosen to evaluate the contamination, toxicity and therefore the quality of two soils from an abandoned gold mine “Mina de Jales”, located in the northeast of Portugal. During 1993 the mine was abandoned and in September 2002 the rehabilitation of the area started, reaching its end in late 2003. During 3 years, four enzymatic activities (arylsulfatase, urease, acid phosphatase and β-glucosidase) were determined due to their participation and importance in different nutrient cycles in soil. N-mineralization was also determined as an additional parameter in the N-cycling

approach and the soil enzyme dehydrogenase was chosen because it is considered an endocellular enzyme, being an integral part of microorganisms and therefore an indicator of physiological active organisms, also having an apparent role in oxidation of organic matter [11-13].

The main goals of this study were to:

- Evaluate the effects of heavy metals in the two soils in different years, matching different rehabilitation stages: February 2002 (no rehabilitation), February 2003 (during rehabilitation) and February 2004 (after rehabilitation);
- Evaluate the effects of season in the selected soil enzymes (February 2003, July 2003 and November 2003) in the two soils from Jales mine surroundings;
- Use Lufa 2.2 soil as a control to the methodology used in all sampling times;
- Apply a soil quality index, previously developed to be used in native soils, to the results obtained for Lufa 2.2 soil and the two soils from the mine [3, 9, 10]. This index integrates some of the enzymes measured and will be an indication of the soil biochemical balance.

MATERIAL AND METHODS

Study area

The study was carried out in the abandoned mine “Mina de Jales” area (near Vila Pouca de Aguiar) in the northeast of Portugal (N 41º 27’ 47.2’’; W 07º 35’ 11.7’’). Two distinct areas were chosen due to their different heavy metal content. The area nearby the mine spoil is surrounded by agriculture and pasture fields with vegetation composed by *Alnus glutinosa* (L.) Gaertn., *Carpinus betulus* L., *Castanea sativa* Miller, *Frangula alnus* Miller, *Fraxinus angustifolia* Vahl, *Malus domestica* Bork, *Pinus pinaster* Aiton, *Pinus sylvestris* L., *Quercus pyrenaica* Willd., *Quercus robur* L., *Salix atrocinerea* Brot.. Although harvested during the

Summer, the sampled area has no use in terms of agriculture or pasture. The soil collected from this area (N 41º 27' 53.9''; W 07º 34' 50.6'') hereafteridentified as JC soil, has high heavy metal concentrations (Table III.I). Another chosen area was located 3 Km far from the mine spoil and is surrounded by trees like *Alnus glutinosa* (L.) Gaertn., *Carpinus betulus* L., *Castanea sativa* Miller, *Frangula alnus* Miller, *Fraxinus angustifolia* Vahl, *Pinus pinaster* Aiton, *Pinus sylvestris* L., *Quercus pyrenaica* Willd., *Quercus robur* L., *Salix atrocinerea* Brot.. This study area was mainly used for cow and horse pasture (N 41º 28' 36.1''; W 07º 34' 14.0'') and the soil collected from this area, hereafter identified as JNC soil, has lower heavy metal contents when compared to the JC soil (Table III.I).

The northeast region of Portugal is considered a humid zone with mild and rainy climate, strongly influenced of the Atlantic Ocean. Temperatures in Winter are considered mild and very high temperatures are frequent during the Summer.

Soil sampling and soil analysis

In each selected area 10 soil samples (0-10 cm from the surface soil) were collected and stored in open plastic bags and then transported to the laboratory. In the lab, the soils were sieved on a <2-mm mess and physico-chemical analysis were then conducted. Soil samples were pooled together and stored in plastic bags at 4ºC until enzymatic analysis was completed. pH values were determined in a KCl (1M) solution [14] and soil moisture was measured by the difference in weight before and after drying soil, at 105ºC, in an oven, for 11h. The organic matter content was determined by the loss-ignition method, after the determination of soil moisture content, from loss in weight after 5h at 540ºC (adapted from [15]). Microbial biomass of C and N were measured based in a standardized protocol ISO 14240-2 [16] and the total N was determined by a Kjeldahl's digestion.

The sampling procedure and enzymatic activity analysis were carried out in February 2002, February 2003 and February 2004 to compare the different stages of the rehabilitation process which started in September 2002 and ended in late 2003. Sampling was also carried out during July and November 2003. These two

late months and February 2003 were also compared to investigate seasonal differences in the enzymatic activity. February is representing the winter and cold season, July is representing the highest temperatures and one of the lowest moisture periods during the year, and November is the month after the highest input of organic matter in soil.

Table III.I- Physico-chemical and biological characteristics and main contamination of soils Lufa 2.2, JNC and JC from Mina de Jales (Portugal).

Parameters	units	Lufa 2.2 soil	JNC soil	JC soil
pH	-	5.03	4.14	4.47
Dry matter	%	93.8	70.25	66.19
Soil Organic Matter	%	1.28	5.07	2.88
Cation Exchange Capacity	cmol/Kg	11	4115	5492
Max. Water Holding Capacity	%	51.0	36.5	35.1
Redox Potencial	mV	30	59	47
Sand	%	77.10	20.96	23.08
Clay	%	8.0	16.8	13.6
Silt	%	14.90	27.51	25.02
Total Carbon	%	2.21	8.55	4.61
Total Nitrogen	%	0.2	0.4	0.4
Sulfur	%	0.02	0.02	0.03
Calcium	mg/Kg	1540	441	1840
Potassium	mg/Kg	292	1290	2670
Microbian Biomass N	mg/Kg	45.2	40.0	84.4
Microbian Biomass C	mg/Kg	299.6	96.9	792.2

Heavy metal (mg/Kg)	Lufa 2.2 soil	JNC soil	JC soil
As	-	71	251
Ag	-	<0.2	1.5
Al	-	14 000	10 000
Be	-	2.0	3.1
Cd	<0.2	1.9	8.2
Co	-	n.d.	n.d
Cr	9.6	6.0	15.0
Cu	1.5	8.0	24.0
Fe	-	7 370	17 800
Hg	0.07	<0.05	<0.05
Mn	-	99	255
Ni	2.7	5.0	9.0
Pb	16.8	33.0	209.0
Sb	-	0.37	2.29
Se	-	0.47	0.38
Zn	19	33	97

Heavy metal content were only analysed in the soil samples collected in February 2002, and N content and Microbial biomass of C and N were evaluated in February 2002 and 2004.

Additionally, enzymatic determination procedures were also applied to Lufa 2.2 soil, a soil commonly used as a control soil in ecotoxicological tests or bioassays, originated from Speir, Germany (Landwirtschaft Umweltschutz Forschung Analytik, Speir). Three different Lufa 2.2 soil patches were used: one obtained from Speir in the beginning of 2002 and used for enzymatic determination when field samples where collected in February 2002 and February 2003; a second one obtained from Speir in May 2003 and used for enzymatic determination at the same time as the field samples from July 2003 were performed and a third one obtained during August 2003 and used for the other enzymatic determinations (November 2003 and February 2004).

Enzymatic Activities

Dehydrogenase (DHA) (EC 1.1.1.49) activity was determined by the suspension of soil samples in a triphenyltetrazolim chloride (TTC) solution and incubated for 24h at 40°C. The triphenyl formazan (µg TPF / g.dm / h) produced is extracted with acetone and measured photometrically at 546nm. This methodology is similar to the ISO draft that is being developed by ISO [17] and was based on the methods of Dick *et al.* [18]. To determine Acid Phosphatase (EC 3.1.3.2) activity, soil samples were suspended in a buffered p-nitrophenyl phosphate solution and incubated for 2 h at 35 °C. The p-nitrophenol produced (µg pNP / g.dm / h) was coloured and measured photometrically at 405 nm in a microplate reader [18-20]. Arylsulfatase (EC 3.1.6.1) activity was determined by incubating soil samples in a buffered potassium-p-nitrophenylsulfate solution at 37ºC, for 1 h. The accumulation of p-nitrophenol (µg pNP / g.dm / h) was measured as in the phosphatase protocol based on Dick *at al.* [18]. The method for β-Glucosidase (EC 3.2.1.21) activity determination was adapted from methodologies of Tabatabai [20]

where soil samples were incubated in a buffered p-nitrophenyl-β-D-glucoside solution. The measurement of p-nitrophenol (µg pNP / g.dm / h) was measured as previously explained. Urease (EC 3.5.1.5) activity was determined following the protocol previously described by Shinner *et al.* [18] and Kandeler and Gerber [21], where soil samples were suspended in a borate buffer and urea solutions and incubated for 2 h at 37ºC. The release of NH_4^+ (µg N / g.dm / 2h) was measured in a microplate reader at 690nm. For the N-mineralization determination soil samples were incubated in water at 40ºC and 1 week later NH_4^+ was extracted with potassium chloride. NH_4^+ was measured as in the Urease methodology [18].

All enzymatic activity determinations were performed in 5 replicates plus 3 controls.

Soil Quality Index

To integrate the simultaneous measurement of several enzyme activities and to express the microbial functional diversity of soil as one value, several enzyme index have been proposed [2].

In this study soil biochemical balance was expressed by the equation 1, which determines the total amount of N of soils as a function of microbial biomass C, N-mineralization capacity, and three enzymatic activities (Acid Phosphatase, Urease and β-Glucosidase), as used by Cepeda *et al.* [9] for native soils under climax vegetation.

[Equation 1]

$$\text{Total N } (x10^{-3}) = 0.38 \text{ microbial biomass C} + 1.40 \text{ N-mineralization capacity} + 13.60 \text{ Acid Phosphatase} + 8.90\ \beta\text{-Glucosidase} + 1.60 \text{ Urease}$$

A ratio was calculated with the total N obtained from the biochemical properties, using equation 1 (Nc), and the total N content of soils measured by the Kjeldahl's method (Nk), and transformed in percentage. For climax native soils, the Nc/Nk ratio is usually 100% or higher [3]. In Equation 1, microbial biomass C is expressed in mg/Kg, N-mineralization in mg/Kg, Acid Phosphatase and β-Glucosidase in μmol p-NP g^{-1} h^{-1} and Urease in μmol N g^{-1} h^{-1}.

Statistical analysis

Results were analysed using different statistical methods according to the goals and questions addressed.

Soil enzyme activities were compared by one-way analysis of variance ANOVA [22], using the SigmaStat statistical package [23]. To compare the differences in their enzymatic activity between soils and sampling time, a Two-Way ANOVA was performed. When significant differences were found, following the analysis of variance, a post-hoc multiple comparison Tukey Test was used to assess differences between soils and sampling times.

To evaluate the weight of abiotic environmental parameters on the ecosystems biotic fraction, multivariate analysis has been used in ecology, but this type of analysis is not widely used in ecotoxicology [24]. In this study Redundancy Analysis (RDA) was performed with CANOCO [25], using enzymatic activities as species and organic matter content, pH and soil moisture as environmental parameters. Enzymatic data was transformed with the internal function ln (x + 1) to standardize and normalize all the data. The Monte Carlo Permutation method was used to assess marginal effects associated with environmental factors.

RESULTS

Changes in pH values and organic matter content in JNC and JC soils throughout the sampling periods are presented in Fig. III.1.

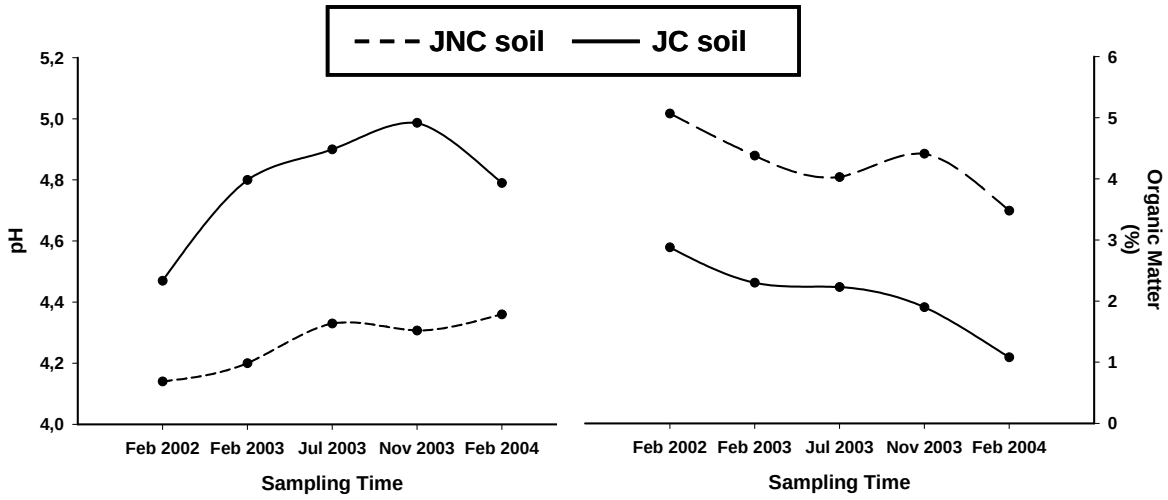

Fig. III.1- pH values and organic matter content (%) of JNC and JC soils collected from Jales Mine (Portugal) recorded during all sampling period (from February 2002 till 2004).

Enzymatic Activities in Lufa 2.2 soil

During different sampling times, all soil enzymes showed different activities in the Lufa 2.2 soil (Table III.II).

Acid phosphatase and urease showed activity values in the same range and can be considered stable during this sampling times. Nevertheless, statistical differences were found between sampling times in acid phosphatase activity (One-Way ANOVA, $F_{4,20}$=8.206, $p<0.05$) and urease activity (One-Way ANOVA, $F_{4,17}$=7.717, $p<0.05$).

On the other hand DHA and N-mineralization showed different activity ranges and non normal distributed data. DHA activity was in the same range in February 2002 and 2003, but from different ranges in the other sampling time (Table III.II).

β-glucosidase activity in Lufa 2.2 soil was also statistically different during sampling times (One-Way ANOVA, $F_{4,\ 17}$=292.98, p<0.05), but the activities measured showed high variability.

Table III.II- Enzymatic activities of Lufa 2.2 soil during all sampling periods (average ± 95% confidence limits).

Lufa 2.2 Soil Enzymatic Activity	Sampling Time				
	February 2002	February 2003	July 2003	November 2003	February 2004
Dehydrogenase (μg TPF / g.dm / h)	3.22 (3.17-3.26)	1.63 (1.62-1.64)	21.39 (21.02-21.76)	19.39 (19.06-19.73)	14.84 (14.79-14.88)
Acid Phosphatase (μg p-NP / g.dm / h)	93.84 (93.72-93.94)	84.41 (84.33-84.50)	84.25 (84.09-84.41)	95.00 (95.13-94.86)	83.11 (82.99-83.24)
Arylsulfatase (μg p-NP / g.dm / h)	199.50 (199.01-199.99)	7.22 (7.17-7.28)	161.69 (161.29-162.09)	55.02 (54.86-55.19)	232.95 (232.59-233.31)
β-glucosidase (μg p-NP / g.dm / h)	3651.45 (3650.28-3652.62)	-42.31 * (-46.92- -37.69)	198.02 (195.22-200.82)	1260.08 (1252.11-1268.05)	1237.33 (1229.50-1245.15)
Urease (μg N / g.dm / 2h)	22.04 (21.99-22.08	10.20 (10.17-1023)	16.98 (16.90-17.05)	12.01 (11.90-12.12)	11.80 (11.69-11.90)
N-mineralization (μg N / g.dm / 2h)	1.75 (1.74-1.76)	1.70 (1.68-1.72)	3.93 (3.92-3.95)	1.73 (1.72-1.74)	6.33 (6.29-6.36)

* unrealistic result

Arylsulfatase activity in Lufa 2.2 soil was significantly different in all sampling periods (One-Way ANOVA, $F_{3,\ 16}$=257.02, p<0.05), showing different ranges in its values. This pattern was also obtained for N-mineralization process (Kruskal-Wallis One-Way ANOVA, H = 13,086, df=4, p= 0,011).

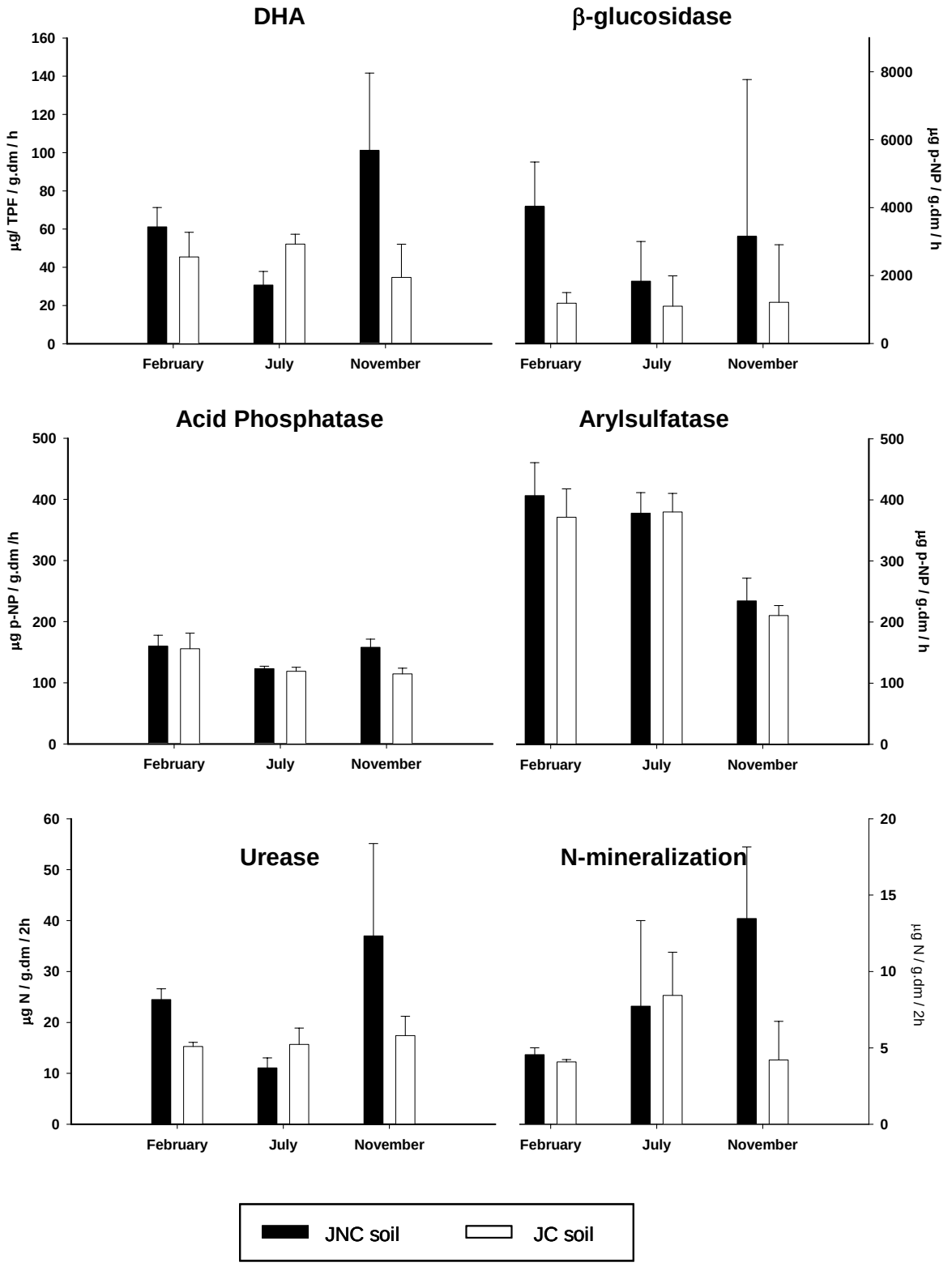

Fig. III.2- Enzymatic activities (average ± 95% confidence limits) of JNC and JC soils collected from Jales Mine (Portugal) recorded during different season periods in 2003 (February, July and November).

Enzymatic Activity during different Season sampling

During the year 2003, the activity of the soil enzyme DHA was significantly different in the three months of February, July and November in the soil JNC (Two-Way ANOVA, $F_{2,16}$=34.704, p<0.05). On the other hand, JC soil DHA activity did not show significant differences in its activity during the year (Tukey Test, p>0.05). During the three sampling months JNC and JC soil were always different (Tukey Test, p<0.05) (Fig. III.2).

However, there was only a significant difference between the two soils in November in their acid phosphatase activity (Two-Way ANOVA, $F_{2,24}$=9.252, p<0.05; Tukey Test, p<0.05). Acid phosphatase activity in JNC soil was higher in February and November, presenting a decrease in the Summer. JC soil, on the other hand, showed the lowest activities in July and November (Fig. III.2).

There were no significant differences in the arylsulfatase activity between the two soils JNC and JC (Two Way ANOVA, $F_{1,24}$=2.879, p=0.103). In both soils, this enzymatic activity was 1.5 times higher in February and July than in November (Fig. III.2).

Urease activity data did not show a normal distribution during the sampling time. In February and November, the two soils differed significantly in their urease activity (Two-Way ANOVA, $F_{2,24}$=9.385, p<0.05; Tukey Test, p<0.05), but during the Summer their urease activity was similar (Fig. III.2). During this sampling year, the urease activity of JC soil was stable but the enzyme activity of JNC soil showed differences in all three sampling period, reaching its highest value in November (Tukey Test, p<0.05).

For the β-glucosidase activity there was a significant difference between the two soils from the mine during February and November (Two-Way ANOVA, $F_{2,19}$=3.061, p=0.07; Tukey Test, p<0.05). During July a lower β-glucosidase activities was observed in both soils. Even though, this enzymatic activity did not show significant differences in the JC soil during the year.

The N-mineralization process showed significant differences between the two soils JNC and JC only during November (Two-Way ANOVA, $F_{2,14}$=16.055, p<0.05; Tukey Test, p<0.05). Also during this month JNC was 2.5 times higher than in

February. In JC soil, N-mineralization presented its higher values in July (Fig. III.2).

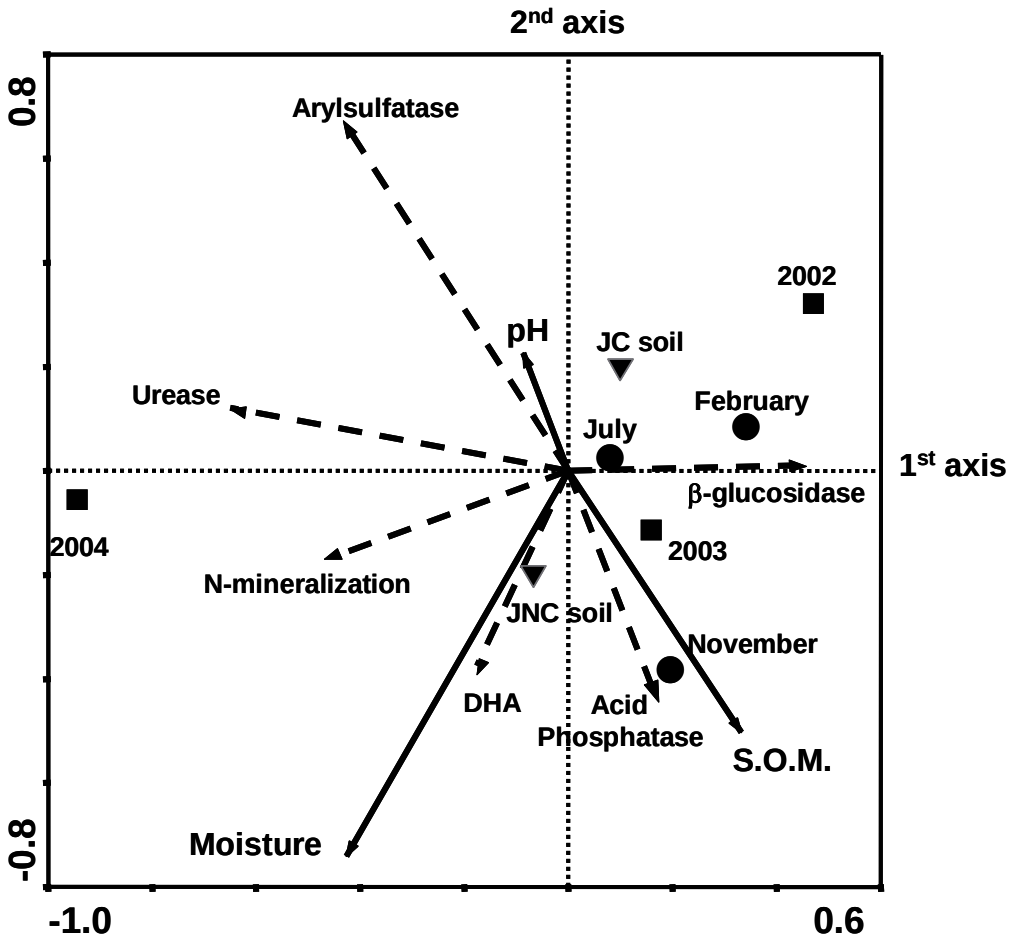

Fig. III.3- Redundancy Analysis (RDA): triplot of enzymatic activities and environmental variables (▼centroid for soil type; ●centroid for the sampling months; ■centroid for sampling year; **- - - -** enzymatic activity; —— environmental parameters).

The RDA analysis showed that the variability of our data is explained by the 1st axis in 91.6% (19.4% of the variability is associated with the enzymes and the remaining 72.2% is explained by the interaction between enzymes and environmental factors). The 2nd axis explains 6.6% of the variability (1.4% of the

variability is associated with the enzymes and the remaining 5.2% is explained by the association of enzymes and environmental parameters). The eigenvalues for the 1st and 2nd axis were 0.194 and 0.014, respectively (Fig. III.3).

Seasonal changes are clearly explained by the 2nd axis in association with the opposite gradient of pH and SOM (higher pH values and lower SOM in February and July, and lower pH values and higher SOM occur in November).

Moisture affected significantly and positively DHA activity and negatively acid phosphatase activity, which is affected positively by SOM. The pH values obtained for the soils were positively related to the values for arylsulfatase activity (Fig. III.3).

β-glucosidase activities are negatively correlated with the enzymes related with the N-cycle (urease and N-mineralization) (Fig. III.3).

Enzymatic Activities before and after rehabilitation

Soil enzymes showed different patterns considering their activity before, during and after the mine's rehabilitation (Fig. III.4).

During the three sampling years there were no statistically differences in the acid phosphatase activity between the JNC and the JC soils (Two-Way ANOVA, $F_{1,24}$=4.168, p=0.052). Both soils showed a significant decrease in their phosphatase activity in February 2004, when compared to the two previous years (Two-Way ANOVA $F_{2,24}$=31.106, p<0.05; Tukey Test, p<0.05).

The urease activity did not show significant differences between soils JNC and JC in February 2004 (Two-Way ANOVA $F_{2,18}$=9.456, p>0.05; Tukey Test, p<0.05). This enzymatic activity presented a high increase in February 2004 in the JNC soil, reaching twice the value obtained before the beginning of the mine rehabilitation. Urease activity of JC soil showed a different behaviour, presenting a decrease in this enzymatic activity in February 2003, when compared to 2002, but recovering again to similar values in February 2004.

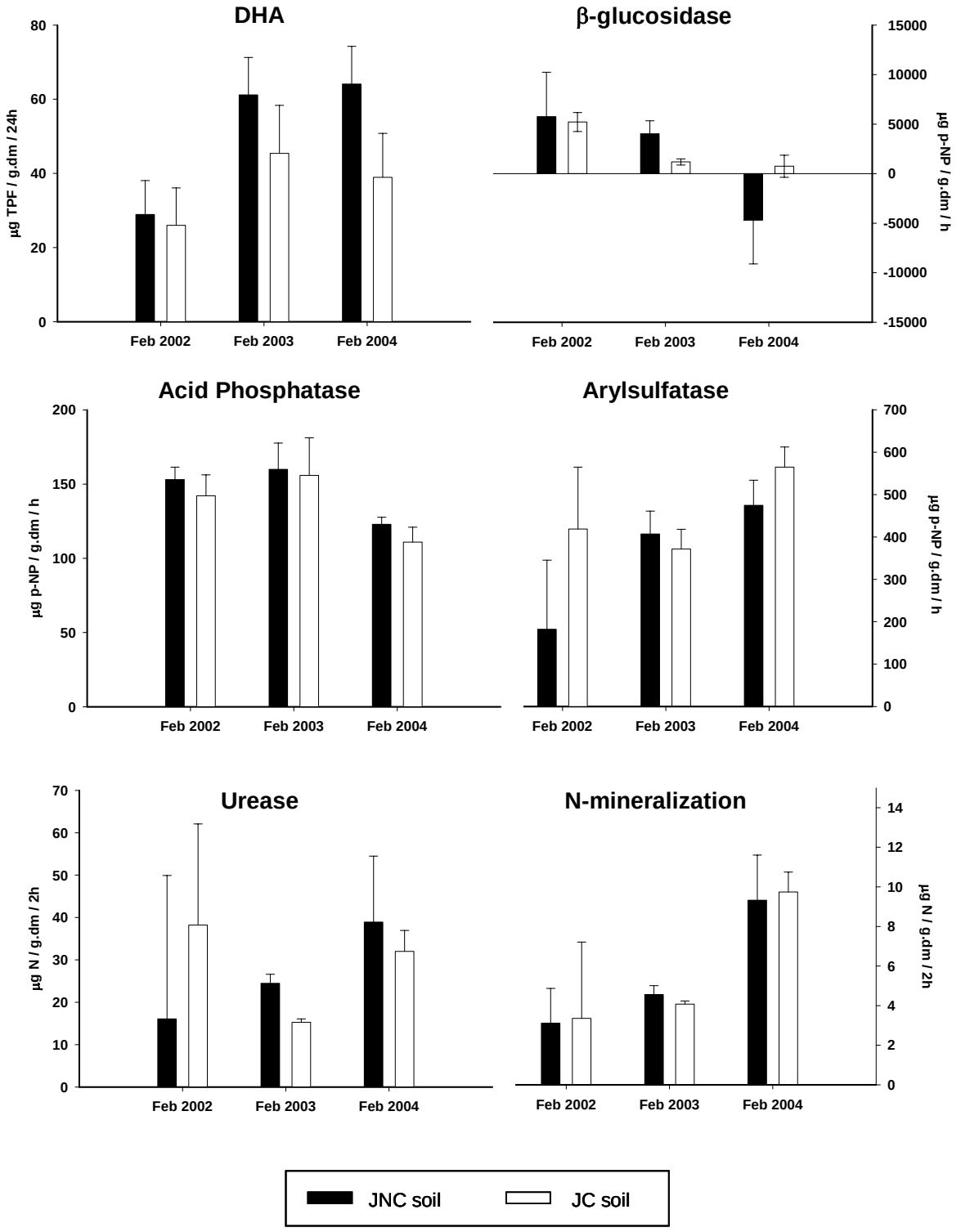

Fig. III.4- Enzymatic activities (average ± 95% confidence limits) of JNC and JC soils collected from Jales Mine (Portugal) recorded before, during and after the rehabilitation process (February 2002, February 2003 and February 2004).

JNC soil showed negative values in its β-glucosidase activity in February 2004. Before and during the rehabilitation process, JNC soil showed similar values on this enzymatic activity. With a different pattern, JC soil showed a decrease in the β-glucosidase activity during these 3 years, reaching values 7 times lower in 2004 than in 2002.

Comparing the different years of rehabilitation, the DHA activity increased twice in JNC soil and 1.5 times in JC soil in 2004, when compared to its activity in 2002. Before rehabilitation (2002), the two soils presented similar activities, but during the first year of rehabilitation (February 2003) JNC soil showed a big increase in this enzyme activity which turned to be significantly different from the activity in the JC soil (Two-Way ANOVA, $F_{2,24}$=4.120, $p<0.05$).

In the JNC soil the arylsulfatase activity increased significantly from February 2002 till 2004, reaching mean values 2.5 times higher than those from 2002 (Two-Way ANOVA, $F_{2,24}$=7.272, $p<0.05$; Tukey Test, $p<0.05$). The soil JC also showed a significant increase in its values but only in 1.3 times when comparing its activity in February 2002 and 2004 (Tukey Test, $p<0.05$).

Although the two soils from the mine showed similar mineralization of nitrogen between them and between February 2002 and 2003 (Two-Way ANOVA, $F_{2,16}$=0.206, $p=0.816$; Tukey Test, $p>0.05$), on February 2004 they showed an increase in this mineralization process in both soils. The increase observed in February 2004 reached a value three times higher when compared to the year before rehabilitation (February 2002).

From the triplot of Fig. III.3, it can be observed that the 1st axis explains the distribution of the sampling years, placing the year 2002 in an opposite part of the triplot when compared to the year 2004. This distribution occurs along a gradient defined by urease and N-mineralization (associated with 2004) and β-glucosidase (associated with 2002).

Table III.III- Soil quality index calculated using enzymatic activities, microbial biomass C and nitrogen content of Lufa 2.2, JNC and JC soils in February 2002 (before rehabilitation) and February 2003 (after rehabilitation).

YEAR	SOIL	Microbial Biomass N (mg/Kg)	Microbial Biomass C (mg/Kg)	N-mineralization (mg/Kg)	Acid Phosphatase (μmol p-NPg^{-1}h^{-1})	β-glucosidase (μmol p-NPg^{-1}h^{-1})	Urease (μmol p-NPg^{-1}h^{-1})	Nc (equation)	Nk (measured) (mg/Kg)	Soil Quality Index (%)
2002	Lufa 2.2	45.20	299.60	1.73	0.61	3.57	0.0047	0.16	0.20	78.14
	JNC	40.00	96.90	4.55	1.15	5.63	0.0037	0.11	0.40	27.23
	JC	84.40	792.20	4.08	1.12	5.09	0.0045	0.37	0.40	91.83
2004	Lufa 2.2	38.10	415.40	6.33	0.60	8.90	0.0848	0.25	0.20	127.07
	JNC	84.00	361.50	9.32	0.88	0.00	0.2797	0.16	0.55	29.62
	JC	91.40	637.03	9.74	0.80	3.10	0.2301	0.29	0.29	101.59

Soil Biochemical Balance

The soil biochemical balance was calculated through an index usually used for native soils with climax vegetation, as previously mentioned in the Material and Methods section. This index was applied to both soils JNC and JC collected only in February 2002 and February 2004, and also to Lufa 2.2 soil, using the enzymatic activities obtained in this two sampling times.

The values obtained, ranging from a minimum value of 27.23% to a maximum of 127.07%, are presented in Table III.III.

DISCUSSION

The absence of a real control soil is one of the major problems that have been raised in ecotoxicological approaches when assessing field soil quality. To overcome this problem some strategies have been recommended [26, 27]. One is the use of the Lufa 2.2 soil as a control. This soil has been used in ecotoxicological bioassays with plants and invertebrates, and its use has been compared to a commonly used artificial soil [28]. Lufa 2.2 soil spiked with contaminants has shown, in some cases, higher toxicity to organisms than chemical compounds incorporated in the artificial soil [29], and the composition of the artificial soil encloses kaolinitic clay that is not typical of temperate zones and unsuitable for endemic microorganisms [30]. Therefore the Lufa 2.2 soil has also been chosen as a control in bioassays to test field soil quality, being suitable for all organisms. Another strategy that has also been used is to choose a natural soil from the surrounding area where the test-soil is collected. This soil should have similar physico-chemical characteristics when compared to the one to be tested, and that is hard to achieve in most of the cases.

In this work we decided to choose the Lufa 2.2 soil as a control because it has shown some stability in its physico-chemical characteristics (e.g pH, cation

exchange capacity, etc) throughout the years, and even if it would not present considered ordinary enzymatic values, it might function as a control to the laboratory techniques used. Previous studies demonstrated that JNC soil presented toxicity to aquatic and edaphic organisms (Chapter I and II); hence JNC soil could not be chosen as a natural control soil for the JC soil evaluation approach, contrary to our initial expectations.

Even though, and measuring different enzymatic activities through out the year and in different years, the results obtained in the microbial activity showed that Lufa 2.2 soil is not enzymaticaly stable through different sampling time. Acid phosphatase was the only enzyme that showed some stability in its activity during all sampling time.

Some values for the enzymatic activities have been reported for different soils from several countries, and that can be considered as confidence quality limits [19].

Lufa 2.2 soil showed very low values for all enzymatic activities when compared to these confidence limits, with the exception of arylsulfatase that showed similar activity in February 2002, July 2003 and February 2004, and β-glucosidase, which activity was extremely high in February 2002, November 2003 and February 2004, and in an opposite trend negative values in February 2003.

DHA activity in soils from Europe, USA and Australia has been reported in the literature with values ranging from 9 to 1760 mg TPF Kg^{-1} $24h^{-1}$, with a mean of 337 mg TPF Kg^{-1} $24h^{-1}$ [19]. The activity values obtained in this study were always in the range of these values, with JNC and JC soils presenting mean values twice or higher than the mean value of 337 mg TPF Kg^{-1} $24h^{-1}$.

Literature data shows that the Acid Phosphatase activity values in soil from Europe, New Zealand and USA range from 23 to 2100 mg pNP Kg^{-1} h^{-1}, with a mean value of 617 mg pNP Kg^{-1} h^{-1}[19]. Lufa soil and the two soils from the mine presented lower values when compared to this mean value from literature.

Arylsulfatase activity measured in this study in JNC and JC soils was always higher than the mean value of 80 mg pNP Kg^{-1} h^{-1} (ranging from 7 to 340 mg pNP Kg^{-1} h^{-1}) obtained from literature [19]. In these two soils this enzymatic activity in some of the sampling times was higher than 340 mg pNP Kg^{-1} h^{-1}.

Although it has been reported for β-glucosidase activity in soils from the USA mean values of 148 μg pNP g^{-1} h^{-1} (38 to 720 μg pNP g^{-1} h^{-1}), our results were mostly higher than 1000 μg pNP g^{-1} h^{-1}, with the exception of Lufa 2.2 soil in July 2003 (198.02 μg pNP g^{-1} h^{-1}) and also the non expected negative values obtained in samples from Lufa 2.2 (February 2003) and JNC soil (February 2004).

Mean values of 202 mg pNP Kg^{-1} $2h^{-1}$ (22 to 422 mg pNP Kg^{-1} $2h^{-1}$) of urease activity have been reported for New Zealand and USA soils [19], which are not similar to our findings. Lufa 2.2 soil always showed lower activities, reaching its higher values (22.04 μg pNP g^{-1} h^{-1}) in February 2003. The soils from the mine showed low urease activities, always lower than the mean value reported, and never reaching values higher than 40 μg pNP g^{-1} h^{-1}.

Seasonal Effects

Seasonal effects have been reported as a major cause in variability in soil enzyme activities [31-33].

In the JC soil DHA, urease and β-glucosidase activities did not show any effects induced by season, and they might be considered stable. Once the enzymes are stable in soil they manifest resistance to moisture, temperature and other environmental changes [33]. As JC soil is not used for any agricultural or pasture purposes, and it has been harvested only once a year in June (maintaining plant roots), the soil and all microorganisms might become stable and adapted to seasonal changes as to the accumulation of heavy metals during the years. Nevertheless, acid phosphatase, arylsulfatase and N-mineralization activities were affected by season conditions. Acid phosphatase activity started decreasing during Summer and maintained lower activities in November as observed by Kang and Freeman [34] and Turner *et al.* [31] while the arylsulfatase activity showed a decrease only in November. The lower values in the activity of these two enzymes in Autumn were also observed by the same authors.

In the JNC soil, all enzymes showed lower values in their activity in July. The exception was arylsulfatase activity, because November was the sampling time where the lowest values were measured. However, JC soil did not show this trend, and this can be explained by the extreme temperature conditions that were observed in the Summer of 2003, one of the hottest Summers in the last two decades, as reported by other authors [19, 31, 33-35].

During Spring and Summer it was observed the presence of horse and cow cattle in the area where the JNC soil was collected. The input of phosphorous and nitrogen in this period could explain why N-mineralization showed an increase in its activity from February till its highest values in November. This may also explain the highest increase of urease and acid phosphatase activities in November. Grazing regimes, like those observed in the JNC soil collecting area, can be also important for the instability of soil enzymes and for changes in C content. Therefore it can influence enzymatic activities in soil in the Winter months, when grazing activity is lower [33]. Although an input of vegetable material in soils in November is expected, it was observed that the organic matter content in both soils decreased in this month, comparing to the values of February 2002.

In both JNC and JC soils arylsulfatase activity showed a different behaviour during the year when compared to DHA, urease, β-glucosidase and acid phosphatase. Arylsulfatase activity in this study was higher in February and June and decreased in November, which agrees with the findings of Kang and Freeman [34], where the decrease of moisture content increased the enzymatic activity. Arylsulfatase activity and high pH values were also closely related (Fig. III.3).

However, these differences between sampling months cannot discharge the rehabilitation process that was carried out since the second half of 2002.

Rehabilitation Procedure Effects

In both soils the rehabilitation process induced an increase in soil enzymatic activities, with the exception of β-glucosidase and acid phosphatase. JNC soil

denoted higher enzymatic activities and also an increase in the Microbial Biomass C and N in 2004, showing a recovery on the microorganisms population, during and at the end of the rehabilitation process, where the mine spoil was delimited and covered which prevented dusts and fine particles to disperse through the air flow.

Acid Phosphatase was related with changes of pH (increasing with acidic pH values) and straightly related with SOM contents, which was also reported by [36, 37]. These authors also reported that arylsulfatase presented the opposite trend with pH values, increasing with the increase of pH.

When pH values reach a range between 4 and 6, like happened with JNC and JC soils, $H_2PO_4^-$ is the dominant form of P in soil. With the rehabilitation process it was expected an increase in the acid phosphatase activity, because the content of the ionic form is stable and the other forms are not available. Consequently it would be expected that the enzyme catalysed a reaction where $H_2PO_4^-$ would originate as products H_3PO_4 and an alcohol by the donation of H^+ by water [38]. The reasons why this did not happened might be related to retention processes of P, by processes of absorption by plants or even by a stronger inhibition of the enzymatic activity due to the precipitation process of P produced by other ions (e.g. Fe or Al).

The DHA, arylsulfatase and N-mineralization were the enzymes that produced a better and positive response to the mine rehabilitation process, while urease was not concise in the results and acid phosphatase and β-glucosidase produced a decrease in its activity afterwards. DHA is strictly related to live cells and has proved to be a good indicator of soil quality [11, 12], and the increase of the mineralization process will improve the transformation of organic forms to mineral forms, which is of extreme importance to plants. Consequently their increase is a good indication for soil quality improvement. Also SO_4^{2-} is essential for plants and is also immobilized by edaphic organism; its production is promoted by arylsulfatase activity which also presented an increase in both soils from the mine area [38].

From February 2002 till 2004 it was observed an increase in the Microbial Biomass C that can be responsible for the decrease/ degradation of the organic

matter content in both soils collected from the mine. But it also induced the decrease in β-glucosidase activity that was unexpected and contradictory to what was found in several studies where this enzyme was always positively and highly related to the Microbial Biomass C content [4, 36, 39].

The increase in enzymatic activities related with the N-cycle was also related with the increase of the microbial biomass N, especially noticed in the JNC soil, where this value doubled in February 2004, when compared to 2003. Additionally, urease and N-mineralization showed an opposite trend when compared to β-glucosidase. This fact can be explained by the cycling of C and N during mineralization and immobilization processes. When decomposition processes are taking place in soil, the C:N ration will decrease with time because carbon biomass is lost as CO_2, while N is reused by microorganisms [38]. The decrease in the N biomass will reach a point when the microbial activity will also decrease due to organic carbon loss. In good quality soils this loss will take short periods to recover, depending only on the time that the organic material needs to be degraded.

The high concentration of Fe found in JC soil can act as an inhibitor of enzymes, diminishing their activities, but also can make complexes with other ions like SO_4^{2-} and with other heavy metal complexes, reducing the heavy metal toxicity [40].

Although all enzymatic activities were higher in JNC soil than in JC soil, with the exception of arylsulfatase in 2002 and 2004 and N-mineralization that showed no difference between soils, the soil quality index showed that JC soil had higher quality than JNC soil. This can be explained by the differences in the microbial biomass C and the nitrogen content of both soils, which also play an important role in the activity of soil enzymes. In JC soil, the amount of N available decreased nearly 30% and the microbial biomass C decreased 20% from 2002 to 2004. Nevertheless, its quality index was maintained near the 100 value.

This index has been only applied in native soils and was never applied in stressed soils. The results obtained with this index follow the same trend of other results obtained for these two soils using other soil bioassays (see Chapter I, II

and IV). Even though, these values have to be enclosed within results from other bioassays and also with the chemical analysis.

The index values obtained for JC and Lufa 2.2 soils are similar to values obtained for native soils [2, 9, 10], which is difficult to explain because both soils are affected by contamination (JC soil) and by management (Lufa 2.2 soil). Lufa 2.2 soil is a natural collected soil, and consequently disturbed, from a place mainly dedicated to its commercialization and JC soil has been stressed for some years with heavy metal contamination. Thus more attention has to be paid when applying this quality index to stressed soils, although the values obtained are in accordance with the results from other bioassays.

Regarding the methodologies used, β-glucosidase activity protocol might need to be changed in accordance to different organic matter content, because the negative values that were presented in this study were obtained three times, repeating the same procedure, due to the strangeness and surprise of the values. One possible change in the methodology can be the increase in the concentration of the $CaCl_2$, to avoid the presence of very small particles that produced turbidity in samples (both controls, without the enzyme substrate, and the “samples”, with the addition of the enzyme substrate), not solved with the filtration processes.

As a final remark, we think that the study of enzymatic activities in soil is a useful tool to check how nutrients are involved in the recycling of soils and how contamination or other disturbing factor is affecting soil quality. Nevertheless, this assessment should be done as part of an integrative test battery, to fulfil the microbial gaps that usually exist in the ERA, but can not probably be used by their own as “a single enzymatic battery”.

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CHAPTER IV

Toxicity Assessment of Two Soils from Jales Mine (Portugal) Using Plants: Growth and Biochemcial Parameters

Toxicity Assessment of Two Soils from Jales Mine (Portugal) Using Plants: Growth and Biochemical Parameters

(Submitted to the journal Ecotoxicology and Environmental Chemistry)

ABSTRACT

Contaminants in soils can enter food chains through initial producers. Bioavailable contaminants can induce growth, reproductive or biochemical changes in plants. To evaluate the bioavailable fraction of heavy metals in two soils from the Jales mine surroundings, bioassays with the plants *Brassica rapa* (RCBr) and *Avena sativa* were performed (14 day standardized test with both species and a Life Cycle Bioassay (LCB) with *B. rapa*). The two soils from the mine showed different heavy metal contents: JNC soil with low heavy metal concentrations, while JC soil presented high heavy metal contents. After the 14 day bioassay, biochemical parameters (protein and MDA content and catalase and peroxidase activities) were also measured. Results from these bioassays stressed the difference between species sensitivity, with *A. sativa* showing no toxicity effects when exposed to both soils. On the other hand, *B. rapa* showed a decrease in growth parameters when exposed to JNC soil and no changes when exposed to JC soil. The LCB confirmed this trend for plants exposed to JNC soil, concerning reproductive parameters, but also showed that JC soil was affecting *B. rapa* in terms of flowers and seed pods production. The biochemical assays showed that plants affected by heavy metals in the 14 day bioassay, which had demonstrated growth inhibition, also showed oxidative stress, with an increase in MDA production, reduction of protein content as reduction of catalase and peroxidase activity. All plant bioassays carried out in this study revealed that JNC soil, although with lower heavy metal contents, had a higher bioavailable fraction when compared to JC soil, consequently increasing its toxicity to plants.

INTRODUCTION

Nowadays chemical pollution in the terrestrial environment has been considered a threat for living organisms, affecting human population by changing crop and livestock quality and production. Therefore Terrestrial Risk Assessment is often divided into Human Health Risk Assessment, including the exposure of humans to the environment, and Ecological Risk Assessment, which protects ecosystems that will be also considered as a human resource [1].

The structure and function of ecosystems are considered the second and third levels to be studied in Environmental Risk Assessment, whereas the first level is strictly related with the water path system (retention function) [2, 3]. This structural and functional assessment will evaluate the capability of soil to be used for agricultural purposes, using, among others, ecotoxicological plant bioassay endpoints for the evaluation of soil contamination and/or quality. According to the Scientific Committee on Toxicology, Ecotoxicology and the Environment [1, 4, 5] the adverse effects on the terrestrial environment include the evaluation of: (1) effects on soil functions, mainly as plant substrate and as habitat for edaphic organisms (essential for the nutrient life cycle); (2) effects on plant biomass production; (3) effects on edaphic organisms, particularly above-ground and foliar invertebrates, that are preys for other organisms and act also as important pollinators, detritivores, pest controllers, etc.; (4) accumulation of toxic compounds in food items, that will lead to biomagnification through food chains.

Bioassessment can therefore be used as a tool to detect the presence of hazardous chemicals in the environment, evaluating also the effects of mixtures, with combined effects that can be expressed as synergism, additivity and antagonism, demonstrating the bioavailability of contaminants to different species [6].

Bioassays with vascular plants are considered to be versatile tools to identify the effect of pollutants present in soils as well as to verify the success of remediation processes of contaminated soils. Plant bioassays used in the evaluation of natural soils are based on test procedures for the assessment of effects of single chemicals in defined media [7, 8]. After a first screening

evaluation, this bioassay can be used preferably when agricultural or pasture fields are tested for their soil quality or contamination, focusing on early seedling survival and plant growth.

Notwithstanding, the results obtained in these bioassays are a consequence of biochemical processes after chemical exposure. The exposure to stressors can promote the production of reactive oxygen species (ROS) within the cells. In addition to this production, it has been shown that ROS are naturally formed during the regular plant metabolism, where the photosynthetic electron transport is a major source of ROS [9-11]. ROS can also directly damage proteins, amino acids and nucleic acids and cause peroxidation of membrane lipids [12]. One important ROS is H_2O_2, which is able to diffuse across cell membranes and can be formed by the transformation of superoxide ($O_2^{\cdot -}$), a reaction catalysed by superoxide dismutase (SOD) [11, 13]. The toxicity of H_2O_2 is weak compared to other ROS, but it can produce $^{\cdot}OH$ (hydroxyl radical) in the presence of transition metals, that is the most reactive active oxygen. Hence, scavenging of H_2O_2 is essential to prevent oxidative damage of plant cells. Enzymatic protection is partly carried out by catalase and peroxidase, by degrading H_2O_2, and influencing the level of lipid peroxidation, and by other enzymes acting in other ROS (e.g. SOD eliminates $O_2^{\cdot -}$ radicals) [13].

Environmental stressors and the consequent production of ROS promote the expression of genes that are associated with the synthesis or activation of enzymes that will be involved in the removal and detoxification of these toxic products [9]. Therefore a modification on the amount and activities of several enzymes involved in scavenging oxygen radicals and in the elimination of ROS may be observed.

Several experiments carried out in laboratory, where plants are grown in highly controlled conditions (e. g. hydroponic mediums) have shown that enzymatic activities are sensitive indexes for the adaptation and response of the plants to stress factors (e.g. [12-14]). As an example, catalase (EC 1.11.1.6; CAT) is one of the main enzymes in plants scavenging H_2O_2 (a ROS) and is located in peroxisomes/glyoxysomes and, in some plant species, in the mitochondria. This enzyme does not consume reducing power and has a high reaction rate, however

with poor affinity for H_2O_2. The main function of catalase is to convert hydrogen peroxide, a powerful and potentially harmful oxidizing agent, to water and molecular oxygen ($2H_2O_2 \rightarrow 2H_2O + O_2$) [15], but it also uses hydrogen peroxide to oxidize toxins including phenols, formic acid, formaldehyde and alcohols.

Peroxidase (EC 1.11.1.7; POD) is well known for its important role in root initiation set up [16] and its activity has been reported to increase with senescence advancement [17]. It is known, like catalase, to be implicated on the removal of H_2O_2. Peroxides can be metabolised directly by peroxidases, particularly in the cell wall, and also by catalase in the peroxisome. The oxidative membrane damage and the effect on fragmentation of fatty acids (lipid peroxidation) can be determined by the measurement of the malondialdehyde (MDA) content.

In this study, the knowledge on plant biochemical processes and their responses to environmental stressors will be applied to real scenario conditions, i. e. using plants grown in naturally contaminated soils. We will evaluate the quality/ toxicity of two soils from an abandoned mine located in the northeast of Portugal, Mina de Jales (Vila Pouca de Aguiar), using plant bioassays with Rapid Cycle *Brassica rapa* L. (RCBr) and *Avena sativa* [7]. Seedling germination and growth tests, regularly enclosed in test-batteries during remediation processes [18, 19], were carried out to monitor the toxicity of contaminated soils. Additionally, enzymatic bioassays, which have been previously developed for measuring biochemical adaptations, usually under controlled conditions, were adapted to this real scenario situation. Catalase and peroxidase activity, and MDA and protein contents were measured.

MATERIALS AND METHODS

Mine Location and Soil collection

The study was carried out in the abandoned mine, Jales mine, in the northeast of Portugal, near Vila Pouca de Aguiar (N 41º 27' 47.2''; W 07º 35' 11.7''), where two distinct areas were chosen due to their different heavy metal contents (Table IV.I). The first area is located nearby the mine spoil and it is surrounded by agriculture and pasture fields. Although harvested during summer, the sampled area has no use in terms of agriculture or pasture. The soil collected from this area (N 41º 27' 53.9''; W 07º 34' 50.6'') is hereafter identified as JC soil, due to its high heavy metal concentrations. The second chosen area was located 3Km far from the mine spoil and was mainly used for cow and horse pasture (N 41º 28' 36.1''; W 07º 34' 14.0''); the soil collected from this area is hereafter identified as JNC soil, due to its lower heavy metal concentrations relatively to the JC soil. The two soils are classified as silt loam soils and their soil characteristics are presented in Table IV.I.

To diminish the disturbance caused by sampling, soil collection was conducted to cause minimal changes in soil biota and its activity, according to The International Organization for Standardization protocol 10381-6 [20]. In the two sampling areas, several samples were randomly made, with a maximum of 15 cm depth, and were mixed in only one bulk sample in February 2002 [21].

After collection, samples were kept in plastic bags not completely closed, to allow gas exchange. Moreover, compaction during transportation was also avoided. The pre-treatment of soil samples in laboratory was made by two procedures. For physico-chemical properties determination (e.g. pH, nutrient status, cation exchange capacity, redox potential and heavy metal analysis) soil was sieved with a 5 mm mesh and stored at room temperature. For biological assessment (microbial biomass C and N) and plant bioassays, soil was sieved with a 2mm mesh, without getting previously dried to avoid differences in heavy metal speciation [22] or high changes on biological properties (e.g.

microorganisms). Soil was stored at 4ºC after sieving, trying to preserve its biological properties.

Table IV.I- Physico-chemical, microbial characteristics, and heavy metal content of Lufa 2.2, JNC and JC soils.

Parameters	units	Lufa 2.2 soil	JNC soil	JC soil
pH	-	5.03	4.14	4.47
Dry matter	%	93.8	70.25	66.19
Soil Organic Matter	%	1.28	5.07	2.88
Cation Exchange Capacity	cmol/Kg	11	4115	5492
Max. Water Holding Capacity	%	51.0	36.5	35.1
Redox Potencial	mV	30	59	47
Sand	%	77.10	20.96	23.08
Clay	%	8.0	16.8	13.6
Silt	%	14.90	27.51	25.02
Total Carbon	%	2.21	8.55	4.61
Total Nitrogen	%	0.2	0.4	0.4
Sulfur	%	0.02	0.02	0.03
Calcium	mg/Kg	1540	441	1840
Potassium	mg/Kg	292	1290	2670
Microbian Biomass N	mg/Kg	45.2	40.0	84.4
Microbian Biomass C	mg/Kg	299.6	96.9	792.2

Heavy metal (mg/Kg)	Lufa 2.2 soil	JNC soil	JC soil
As	-	71	251
Ag	-	<0.2	1.5
Al	-	14 000	10 000
Be	-	2.0	3.1
Cd	<0.2	1.9	8.2
Co	-	n.d.	n.d
Cr	9.6	6.0	15.0
Cu	1.5	8.0	24.0
Fe	-	7 370	17 800
Hg	0.07	<0.05	<0.05
Mn	-	99	255
Ni	2.7	5.0	9.0
Pb	16.8	33.0	209.0
Sb	-	0.37	2.29
Se	-	0.47	0.38
Zn	19	33	97

Organic matter content (%) was measured by the procedure on the Loss of Weight on Ignition (LOI) adapted from Storer [23]. After dried at 105ºC, for 11 hours, the soil was heated at 360ºC for two hours. The LOI was calculated as:

LOI (%) = (SW105 – SW360) x 100 / SW105

where SW105 is the soil weight at 105ºC and SW360 is the soil weight at 360ºC.

Plant Growth Bioassays

The methodology of bioassays used to evaluate the toxicity/ quality of two soils from Jales mine was adapted from the standard protocol from ISO 11269-2 [7, 8].

Two species, a monocotyledonous and a dicotyledonous, were selected, based on the species list presented in [7]: *Avena sativa* L. (oat) and the *Brassica rapa* L. RCBr (turnip), respectively. Oat caryopses were obtained from an agriculture store in Aveiro, Portugal, whereas turnip seeds were acquired from Crucifer Genetics Cooperative, University of Wisconsin- Madison, Madison, WI, USA. Standard field Lufa 2.2 soil was chosen as control soil, because it has been commonly used as a control soil in ecotoxicological tests or bioassays [24-26]. This soil was obtained from Landwirtschaft Umweltschutz Forschung Analytik, Speir, Germany.

The following tests were performed with both species:

(1) a 14 day bioassay to evaluate the effect of JNC and JC soil on *A. sativa* and *B. rapa* based on the protocol ISO 11269-2 [7] ;

(2) a 14 day bioassay based on the protocol ISO 11269-2 [7] was performed with the test-soil whose exposure showed inhibition on the growth parameters of the species after 14 days [after test (1) has been performed]; in these bioassay the test-soil was diluted with the control soil Lufa 2.2, making 5

treatments: 12.5% (12.5% of test-soil + 87.5% control soil), 25% (25% test-soil + 75% control soil), 50% (50% test-soil + 50% control soil), 75% (75% test-soil + 25% control soil) and 100% (only the test-soil).

(3) a 6 weeks life-cycle test to evaluate the effect of JNC and JC soil on *B. rapa* based on the protocol ISO 11269-2 [7].

Plastic pots (100 mm Ø, 90 mm height) with 400 ± 50 g of soil (control or test-soil) were used, and 10 seeds or caryopses were placed at a maximum depth of 1 cm from the soil surface. The soil moisture was maintained by a fiberglas wick (between 5-10 mm Ø) that was located at the pot's bottom, where a hole was previously made [19, 27]. The pot was then placed on the top of an upside down similar pot with the two sides open (Fig. IV.1). The two pot set was placed in a tray with water and the maintenance of soil moisture was made by capillarity through the fiberglas wick. Bioassays were carried out at 22±3ºC, with an illumination of 12000 lx, in a 16:8 (light:dark) photoperiod.

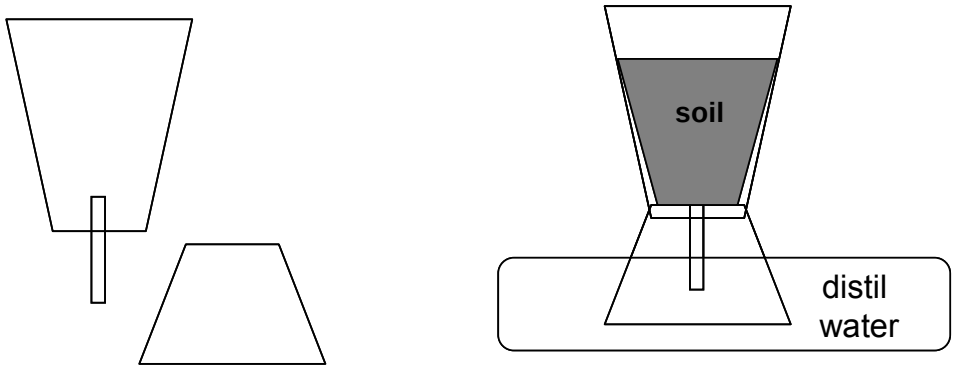

Fig. IV.1- Scheme for the setup of pots in the plant bioassays, to maintain the water content of soil.

In the first week of each bioassay, seeds and caryopses germination time was reported, and the reduction from 10 to 8 plants per vessel was made. After 14 days plants were harvested and growth (measured as shoot length, dry and fresh

weight) was recorded in all bioassays. In the life-cycle bioassay with *B. rapa*, a similar procedure was used, but only 4 plants were harvested after 14 days and the rest at the end of the test period (6 weeks). At days 24 and 25, plants went through cross- pollination, using artificial bee sticks, in order to obtain seed pods. At the end of the bioassay, growth parameters (shoot length, biomass production) and reproductive parameters (number of flowers and pods) were recorded. The Relative Growth Rate (RGR) was calculated using measurements from days 14 and 42 (6 weeks), based on Radford [28, 29]:

[Equation 1] $RGR = (\ln W_2 - \ln W_1) / (t_2 - t_1)$,

where,
RGR- Relative Growth Rate (g/day)
W_1- plant dry weight in t_1 (g)
W_2- plant dry weight in t_2 (g)
t_1- number of days in the first time measurement
t_2- number of days in the second time measurement

Changes in plant colour or other symptoms of cell illness or death were also recorded.

Physiological parameters

Physiological parameters were measured in the 14 day bioassays with: *A. sativa* exposed to Lufa 2.2, JNC and JC soils, and *B. rapa* exposed to different dilutions of JNC soil and to the control Lufa 2.2 soil. The physiological parameters for the turnip exposure to JC soil were not possible to determine.

Lipid peroxidation was determined by malondialdehyde (MDA) content according to what was described in [13], where tissue samples were homogenized

in trichloroacetic acid and centrifuged. The supernatant was mixed with two acids (trichloroacetic and thiobarbituric acids) and heated at 95ºC. Then it was quickly cooled and centrifuged again. The supernatant was read at 532 nm and 600 nm to determine MDA concentration.

Enzyme extraction was made by homogenising shoots in ice K-phosphate buffer 0.05M (pH 7.8), containing 5 mM cysteine, 0.1 mM ethylenediamine tetraacetic acid, 1% polyvinyl pyrrolidone, and 0.2% Triton X-100. The homogenate was centrifuged at 8000 g for 15 min., at 4ºC, and the supernatant was dialysed for 24h against 1 dm3 10mM K-phosphate buffer (pH 7.8). Then it was again centrifuged (2500 g for 10 min, at 4ºC) and used for enzymatic determination [13].

Peroxidase activity was determined as described by Takahama and Egashira [30]. Generally, this enzyme was determined in 1ml of reaction mixture of guaiacol, H_2O_2 and Na-Pi (pH 6). The oxidation of guaiacol was followed by the increase of *A* at 470 nm (assuming the extinction coefficient of 26.6 nM^{-1} cm^{-1}).

Catalase activity was measured using the methodology described by Aebi [31].The assay mixture contained 0.1 M potassium phosphate buffer (pH 7.8) and 10 μl of fresh extract. While the absorbance of the mixture was measured continuously at 240 nm, a 20 μl-aliquot of 1 M H_2O_2 was added. The expression of one unit of CAT activity is nmol substrate decomposed per minute (assuming the extinction coefficient of 39.4 M^{-1} cm^{-1}).

Protein content was determined using the "Total Protein Kit, Micro" (Sigma), according to Bradford [32].

Statistical Analysis and Parameters Calculation

The comparison between soil exposures was made using One Way ANOVA tests to our endpoints data [33]. If data were not normally distributed and data transformation did not correct for normality, a Kruskal-Wallis One Way Analysis of Variance on Ranks was then performed [33].

EC_{50} values were calculated, when possible, using a sigmoidal function, like the Hill function.

RESULTS

Seed Emergence and Growth Parameters in Brassica rapa bioassays

Seed emergence was reported for *B. rapa* after 2, 3 and 7 days. No significant differences on the germination rate between the control soil and JNC and JC soils from the mine (Kruskal-Wallis One-Way ANOVA, H = 4,493 df=2 P(est.)= 0,106 P(exact)= 0,131; One-Way ANOVA, $F_{2, 9}$=1.444, p>0.05; One-Way ANOVA, $F_{2, 9}$=0.632, p>0.05, respectively) were found. Plant seeds showed as mean overall values for germination 92.5% for Lufa 2.2 and JNC soils, and 87.5% for JC soil.

The exposure of *B. rapa* to JNC soil produced significant effects on biomass production (fresh weight) when compared to the same parameter in the control exposure (Kruskal Wallis One-Way ANOVA, H=33.682, df=2, p<0.001, Dunn's Method, p<0.05). The length of the plants exposed to this soil showed a decrease of 45% when compared to the control (One-Way ANOVA, $F_{2, 44}$=48.366, p<0.001). Contrarily, JC soil exposure did not affect the biomass production although it was observed a slight decrease in the length of plants, when compared to the Lufa 2.2 soil exposure (Fig. IV.2).

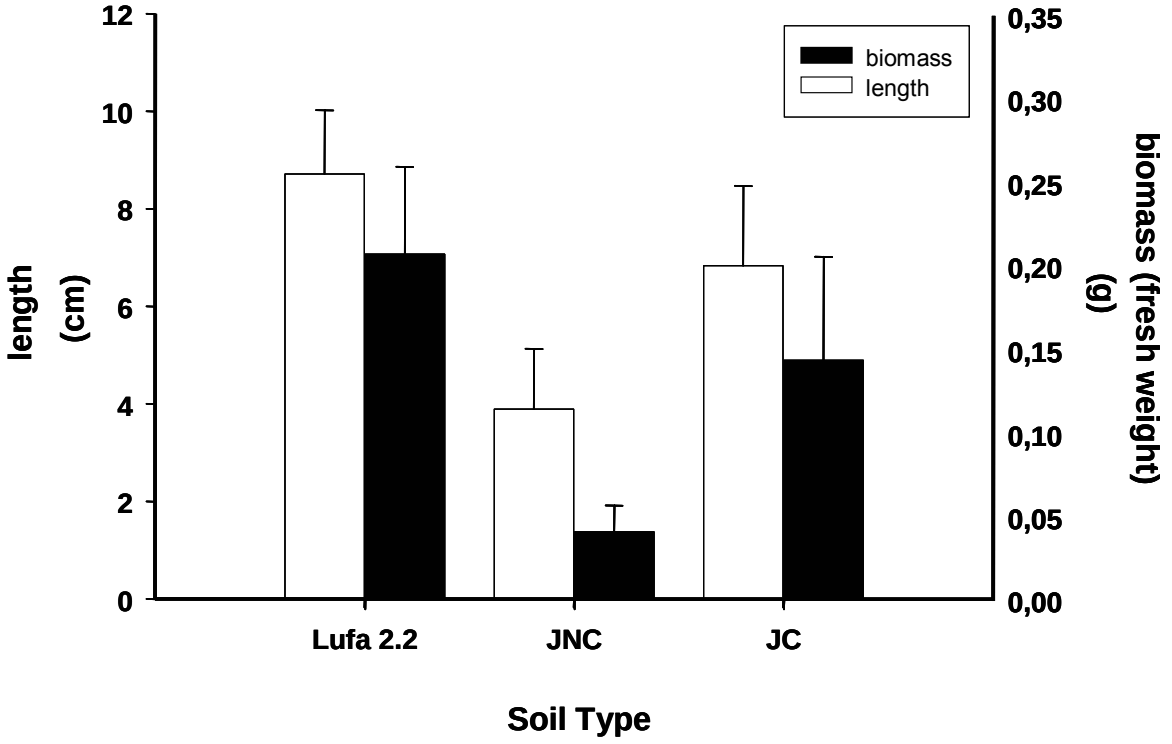

Fig. IV.2- Length and biomass production (FW) (mean ± 95% confidence limits) of *B. rapa* RCBr exposed to the control soil Lufa 2.2 and the two soils from Jales mine: JNC and JC soil.

As JC soil did not produce conclusive effects on seeds emergence nor on plant's growth, it was only performed a test with dilution treatments with the JNC soil.

Plants growth showed a significant decrease in plant's length in the 75% treatment (75% JNC soil + 25% Lufa 2.2 soil), and 100% (only JNC soil) treatments (Kruskal-Wallis One-Way ANOVA, $H=108.848$, $df=5$, $p<0.001$, Dunn's Method, $p<0.05$) (Fig. IV.3). Also between the 75% and the 100% treatments there was a significant decrease in their length (Dunn's Method, $p<0.05$). The same trend was observed for the fresh weight and dry weight biomass production (Fig. IV.3) (Kruskal-Wallis One-Way ANOVA, $H=120.020$, $df=5$, $p<0.001$ and Kruskal-Wallis One-Way ANOVA, $H=131.301$, $df=5$, $p<0.001$, respectively).

For these three parameters it was possible to see a clear dose-response relationship EC_{50} values were calculated (Table IV.II).

Table IV.II- EC_{50} values obtained from the exposure of *Brassica rapa* plants to different treatments of JNC soil (%- percentage of JNC soil present in the dilution treatment).

Parameter	EC_{50} value (%)	± 95% confidence limits (%)	r^2	p
Length	67.69	(67.29 - 68.08)	0.558	<0.05
Fresh Weight	47.83	(47.44 - 48.21)	0.559	<0.05
Dry Weight	43.08	(42.75 - 43.40)	0.652	<0.05

At the end of the test period, there was a significant difference in the number of flower botons between treatments (Kruskal-Wallis One-Way ANOVA, H=102.446, df=5, p<0.001). There was no differences between the two lower treatments and the control (Dunn's Method, p>0.05), but there was a significant decrease in the production of the flower botons in the 50%, 75% and 100% treatments (Dunn's Method, p<0.05).

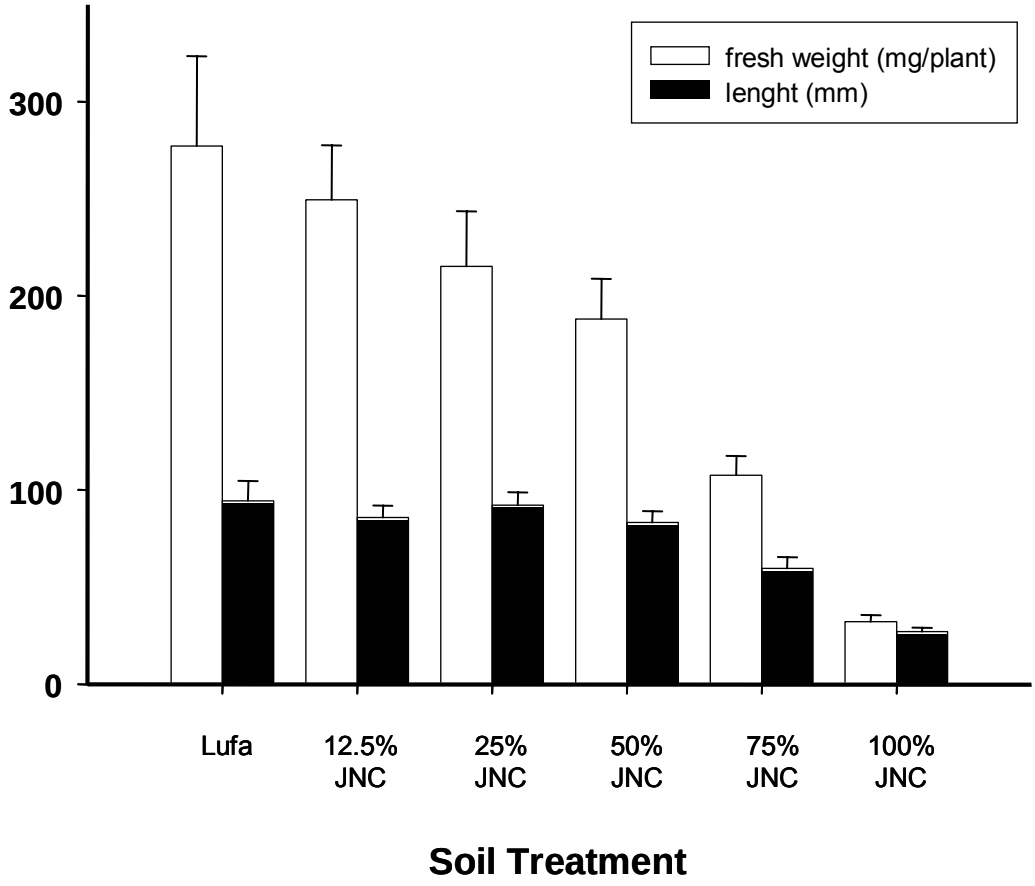

Fig. IV.3- Length and biomass production (FW) (mean ± 95% confidence limits) of *B. rapa* RCBr exposed to the control soil Lufa 2.2 and treatments of JNC soil (diluted with the control).

Life-Cycle Bioassay with Brassica rapa

For the first 14 days, results obtained in the life-cycle bioassay (LCB) were similar to the ones obtained in the 14 day's bioassay.

After 16 days of the beginning of the test, the number of flower botons was significantly different between the control and the two soils from the mine (One-Way ANOVA, $F_{2,\ 45}$=11.479, $p<0.05$). After 21 days the number of flowers originated from the botons observed at day 17, presented a significant difference between the soils (One-Way ANOVA, $F_{2,\ 9}$=9.550, $p<0.05$), while JNC soil

produced a total of 1 flower within the 16 plants in the pots, JC soil produced 18 and Lufa 2.2 soil 54 flowers. At the end of the test, the control soil produced more flowers reaching a total of 94 (per 16 plants), JC soil produced 22 (per 16 plants), and in the JNC soil exposure there were no flowers after the 6 week exposure period (Fig. IV.4). Also, the biomass production (fresh weight) was significantly different between the three soils (Kruskal-Wallis One-Way ANOVA, H=36.397, df=2, $p<0.001$), where JNC showed very small plants only with cotyledon leaves and one row of definitive leaves. The dry weight measurement produced similar results, with all soils showing significant differences (One-Way ANOVA, $F_{2, 9}$= 48.585, $p<0.001$).

When evaluating the length of the plants at the end of the 6 weeks, there was only a significant difference between Lufa 2.2 soil and JNC soil (Kruskal-Wallis One-Way ANOVA, H=31.963, df=2, $p<0.001$; Dunn's Method, $p<0.05$), whereas JC soil presented similar length values when compared to the control (Dunn's Method, $p>0.05$).

The number of seed pods produced per plant followed the same trend (Fig. IV.4) as the length parameter, with significantly differences between Lufa 2.2 soil and JNC soil (Kruskal-Wallis One-Way ANOVA, H=21.174, df=2, $p<0.001$; Dunn's Method, $p<0.05$), although the mean production of pods in JC soil was lower when compared to Lufa 2.2 exposure.

At the end of the 6 weeks' period, all the plants exposed to the JNC soil were very small, had yellow leaves (chlorosis) and some also showed some signs of necrosis (browning colour). Plants exposed to Lufa 2.2 and JC soil showed in few cases a light yellow mottling on their leaves.

RGR was calculated using the dry weight (DW) of plants in day 14 and in day 42. Lufa 2.2 presented a RGR of 0.031 g/g/day, JNC a value of -0.040 g/g/day and JC 0.007 g/g/day.

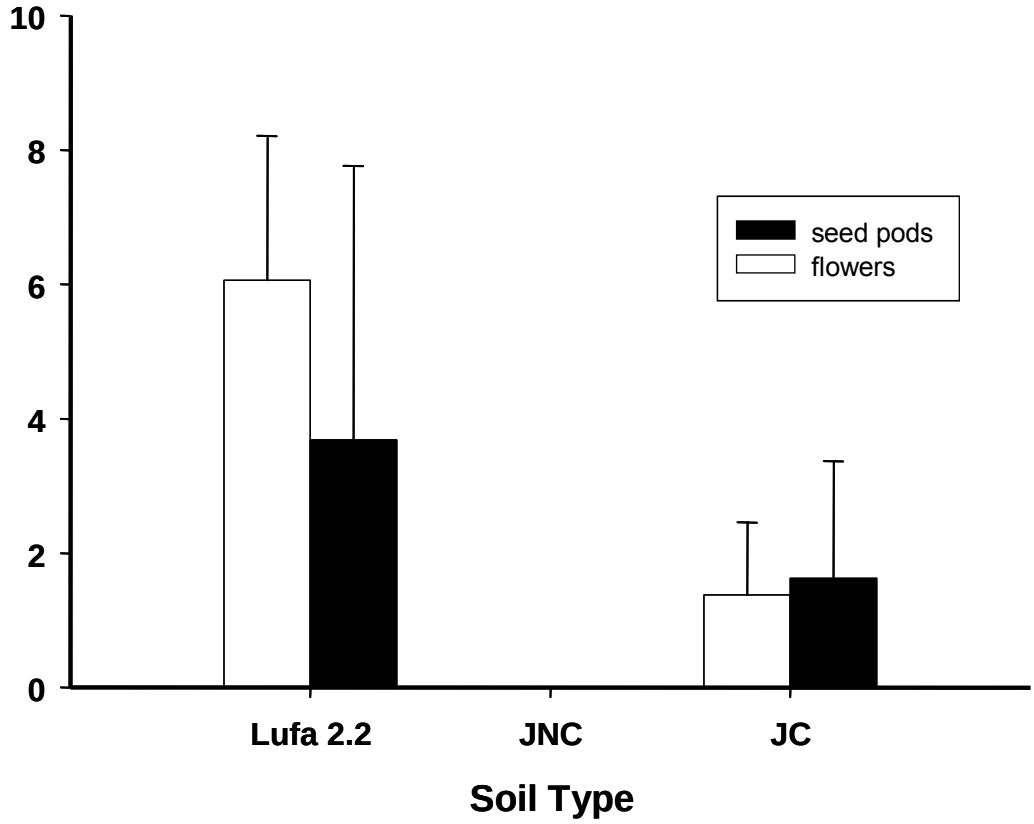

Fig. IV.4- Reproduction parameters (number of seed pods and flowers) of *B. rapa* RCBr exposed to the control soil Lufa 2.2 and the two soils from Jales mine: JNC and JC soil, after 6 weeks (Life-Cycle Bioassay). Data is expressed as mean values ± 95% confidence limits.

Caryopses Emergence and Growth Parameters in the Avena sativa bioassay

In the *A. sativa* bioassay, the overall average germination rates of the caryopses were 87% for Lufa 2.2 soil, 77% for JNC soil and 100% for JC soil.

The biomass production (dry weight) data was not distributed normally and statistical approaches reported inconclusive data. The same trend was observed for the length of oat plants. Plants in JNC soil showed a significant decrease of 36.5 % in their fresh weight when compared to the control soil exposure (One-Way

ANOVA, $F_{2, 79}$=11.285, p<0.01) (Fig. IV.5). There was no evidence of yellowing mottling on leaves.

Since no clear effects were observed when comparing the soils from the mine with the control, no dilution soil bioassays were performed.

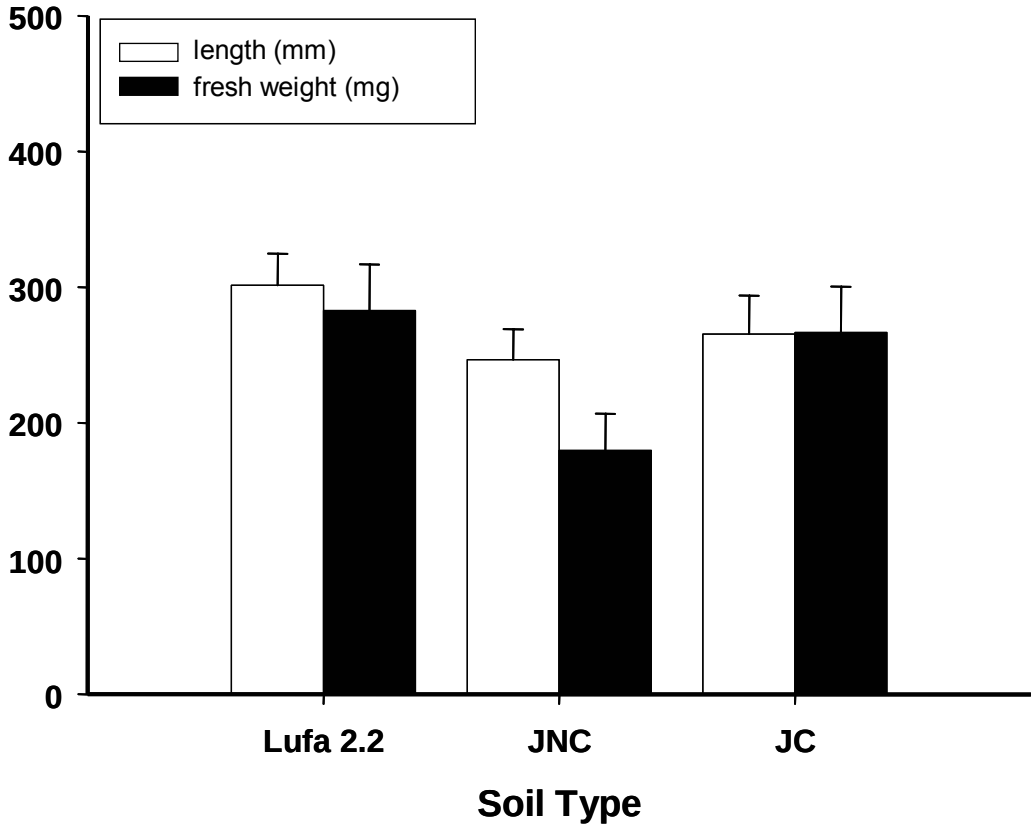

Fig. IV.5- Length and biomass production (FW) of *Avena sativa* exposed to the control soil Lufa 2.2 and the two soils from Jales mine: JNC and JC soil. Data is expressed as mean values ± 95% confidence limits.

Biochemical parameters

Physiological parameters were measured in the JNC treatments exposure for *B. rapa* and for the 14 day bioassay with *A. sativa* exposed to Lufa 2.2, JNC and JC soils.

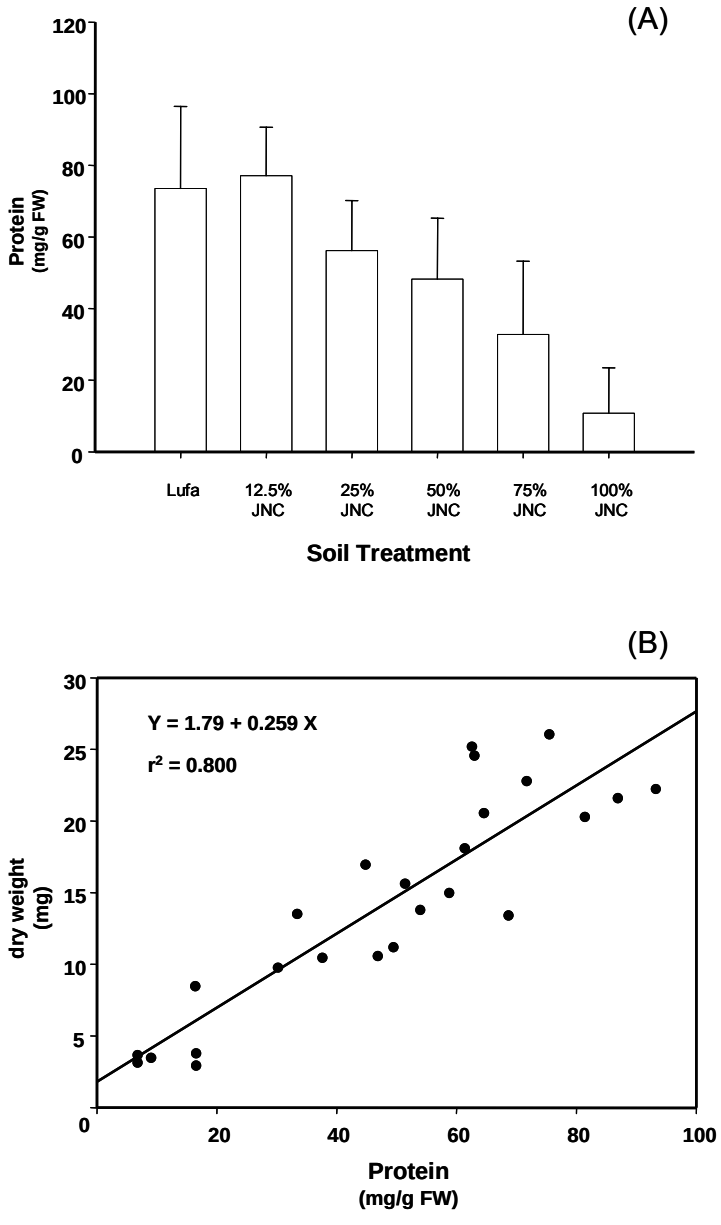

Fig. IV.6- Protein content of *B. rapa* RCBr exposed to the control soil Lufa 2.2 and treatments of JNC soil (mean ± 95% confidence limits) (A) and its correlation with the biomass production (DW basis) (B).

For the *B. rapa* bioassay, the protein content showed a significant decrease in 50%, 75% and 100% JNC treatments (One-Way ANOVA, $F_{5, 17}$=19.230, p<0.001 Dunnett's Method, p<0.05), with an EC_{50} of 60.19% ± 36.36 (average ± st. error) (r^2=0.796; p<0.05) (Fig. IV.6A). It was observed a significant correlation between the plant's dry weight and the protein content (Fig. IV.6B; Pearson Correlation, n=29, r^2=0.800, p<0.05).

The opposite trend was observed for the MDA content, where an increase at 50%, 75% and 100% treatments was observed (One-Way ANOVA, $F_{5, 27}$=42.354, p<0.001, Dunnett's Method, p<0.05) (Fig. IV.7).

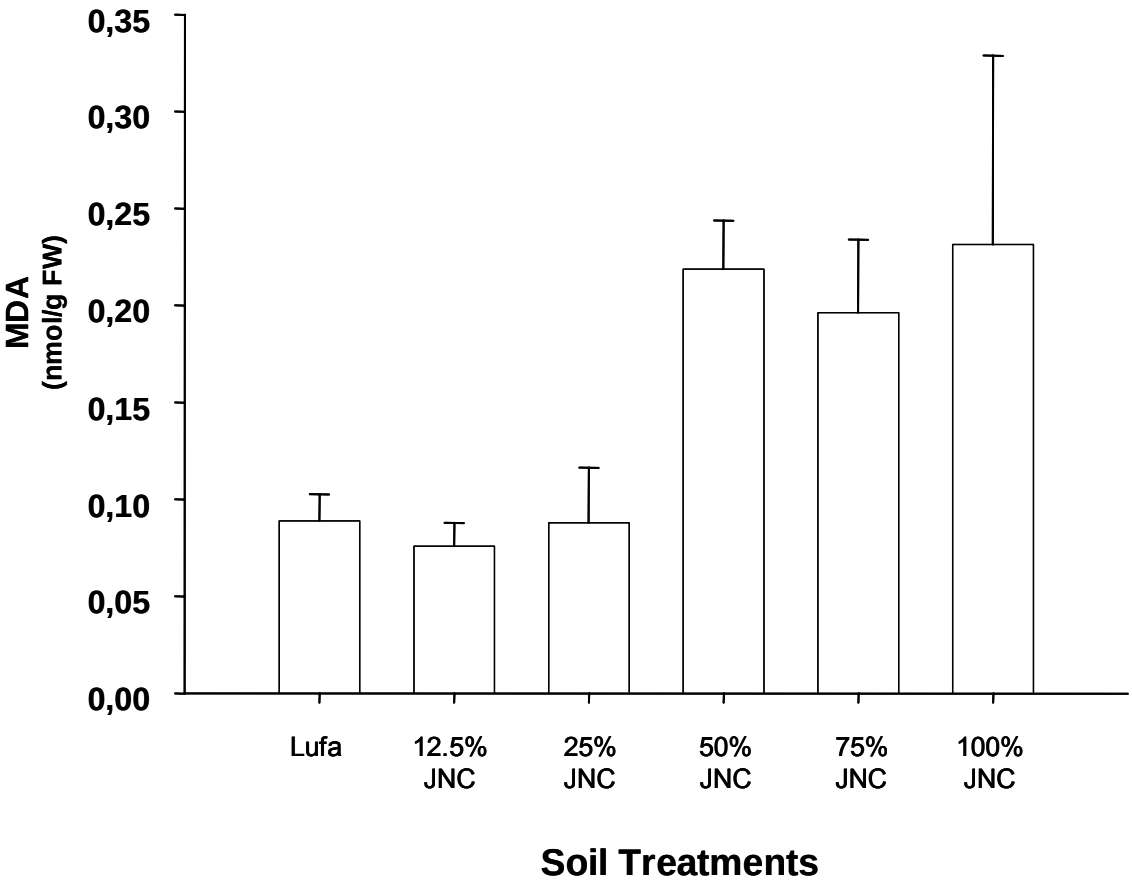

Fig. IV.7- MDA content (mean ± 95% confidence limits) in *B. rapa* RCBr exposed to the control soil Lufa 2.2 and to treatments of JNC soil (diluted with the control Lufa 2.2 soil).

Peroxidase and catalase activities showed similar response pattern between them. In the peroxidase bioassay there was an increase in its activity in the 25% treatments (Fig. IV.8), which was followed by a decrease till the 100% treatment, significantly different from the 25% treatment (Kruskal-Wallis One-Way ANOVA, H = 16.880, df=5, p = 0.005, Tukey Test, p<0.05). The catalase activity also showed an increase in its activity, starting on the 25% treatment and reaching the highest value of 3.767± 0.533 (mean ± st. error) in the 50% treatment, and again, after this hormesis it started to decrease till it had reached the 100% treatment. The 100% treatment caused significant lower values (0.510 ± 0.094) (mean ± st. error) in the catalase activity when compared with the control soil (2.309 ± 0.421) (One-Way ANOVA, $F_{5, 18}$=8.928, p<0.001; Dunnett's Method, p<0.05).

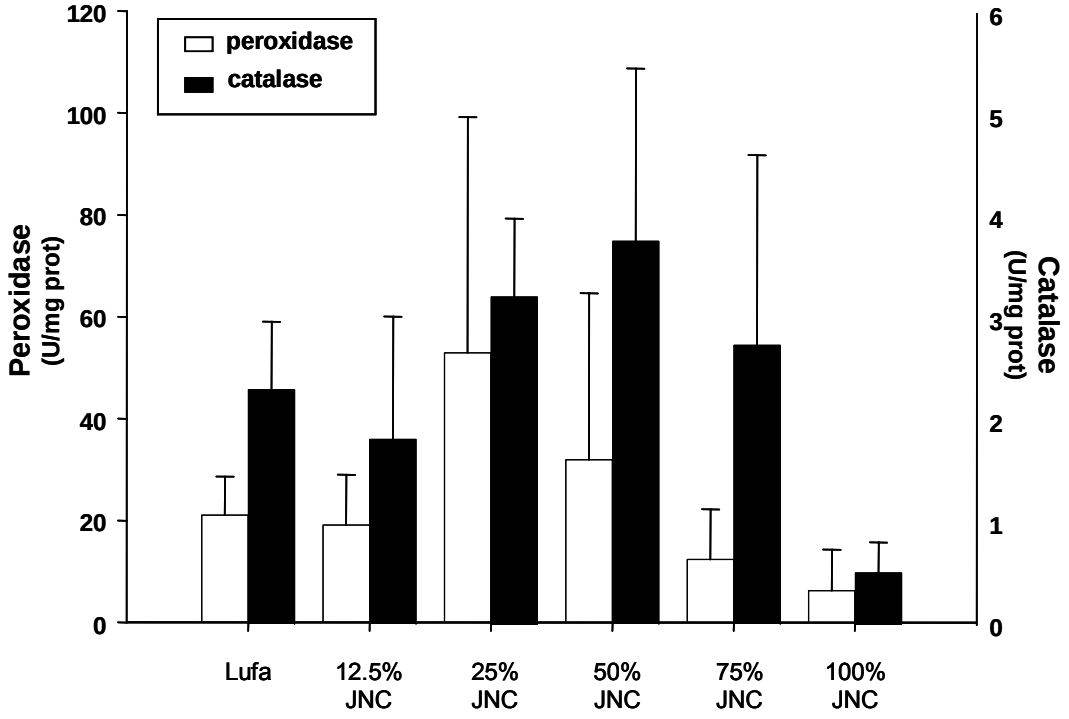

Fig. IV.8- Peroxidase and catalase activities (mean ± 95% confidence limits) measured in *B. rapa* RCBr exposed to Lufa 2.2 soil (control) and treatments of JNC soil (diluted with Lufa 2.2).

In the *A. sativa* 14d bioassay the exposure to the three soils did not cause significant differences in the biochemical parameters measured (MDA content- One-Way $F_{2, 6}$=0.01, p>0.05; protein content- One-Way ANOVA $F_{2, 7}$=0.820, p>0.05; peroxidase activity- One-Way ANOVA $F_{2, 5}$=0.846, p>0.05; catalase activity- One-Way ANOVA $F_{2, 7}$=0.134, p>0.05) (Table IV.III).

Table IV.III- Biochemical parameters measured in *A. sativa* after 14 days of exposure to Lufa 2.2, JNC and JC soils. Data is expressed as mean values ± st. error.

Soil	MDA Content (nmol/g FW)	Protein Content (mg/g FW)	Peroxidase Activity (U/mg prot)	Catalase Content (U/mg prot)
Lufa 2.2	0.006 ± 0.000	41.56 ± 3.18	43.08 ± 4.20	1.70 ± 0.24
JNC	0.005 ± 0.001	36.33 ± 2.19	50.27 ± 4.83	2.21 ± 0.70
JC	0.005 ± 0.003	38.05 ± 2.55	41.04 ± 9.42	2.14 ± 0.55

DISCUSSION

Seed emergence and early seedling growth Bioassays

Although showing high heavy metal contents, JC soil did not show toxic effects on the parameters measured for *B. rapa* and *A. sativa*. There was a high sensitivity of *B. rapa* to JNC soil whereas *A.sativa* poorly reflected the phytotoxicity

potential of this soil. This feature was also found for the same species of oat exposed to different soil types with several contamination levels [34]. Moreover, Fjälloborg and Dave [35] evaluated the effect of sludge contaminated with copper (Cu) and antimony (Sb) (incorporated in soil) on radish, lettuce and oat species, and they observed that oat did not showed any dose relationship between its growth (expressed as FW biomass) and Cu or Sb contamination. Another case study that found a lower sensitivity of oat when compared to other plant species (turnip and cress) was carried out by Gong *et al.* [19]. Here, regarding the shoots biomass production, oat was less sensitivity when was exposed to organic pollutants.

The exposure of the two soils did not cause any influence on the seed emergence ability. This feature was also observed in other studies where significant effects on plant growth were observed, and seed emergence was not so sensitive to pollutants as shoot growth parameters for different plant species [19, 35, 36].

In the *B. rapa* bioassays shoot length showed similar results after 14 days and after 6 weeks. JNC soil exposure caused a significant decrease in plants growth, as shown by their length and biomass production, while JC soil did not cause any prejudice on this endpoints. JC and Lufa 2.2 soil exposure showed similar seed pod productions, while JNC did not present any seed pod production. This was an expected feature due to the smallest or null production of flowers in plants exposed to JNC soil.

Unexpectedly, and although the number of flowers produced by the exposure to Lufa 2.2 and JC soils was significantly different, the number of seed pods were similar. One hypothetical explanation for this fact might be that plants exposed to JC soil, as a result of exposure to a certain heavy metal concentration, allocated all the reproductive energy to the production of seed pods, whereas plants exposed to Lufa 2.2 soil (clean soil) produced more flowers of which some (probably the better fitted) originated seed pods, and had reproductive success. The fact that JNC exposed plants did not bloomed, expresses that reproductive effort and consequently survival of some plant species in this soil area or re-forestation with new species might be endangered. Although out of the scope of

this work, it would be interesting to explore the influence of stressors on the mechanisms (e.g. hormones, receptors/signals interaction, and anatomical development) that regulate the reproductive process in exposed plants, and that could give some more information on this feature.

Regarding the RGR value, JNC soil was the only soil producing plants with a negative value. This might indicate that these plants are unable to balance their osmotic, nutritional or toxic adjustment and might be related also with the occurrence of a senescent phenomenon. Negative growth rates might be related with the extreme use of stored nutrients [37].

Seed production proved to be as sensitive as length in the LCB as was shown in a study where *B. rapa* was exposed to mercury and zinc in a LCB [38]. LCB have shown to be important in the soil toxicity evaluation because they reflect the effects of toxicants in both growth and reproduction. Using the rapid cycling *Brassica rapa* it is possible to follow all life stages due to its rapid and reliable development, producing mature seed when plants are still very short [38]. Although the parameters length and biomass showed similar trends in the 14d bioassay and the LBC, there were differences between soil exposures in reproductive parameters like the number of flowers produced. Although JC soil did not show any effects on the endpoints in the 14d bioassay, differences in flower production, when compared to the control, could be observed.

Additionally to the heavy metal bioavailability, the C:N ratio is an important factor that can help to understand the results obtained for the *B. rapa* exposure to JNC and JC soils. C: N ratio for JNC soil is 21.38, while JC soils presented a lower ratio of 11.53. Usually, soils with a C:N ratio near 20 indicate that there is a delay on the ability of N to plants, values of 10 being considered as an optimal ratio for C and N cycling.

Biochemical bioassays

It was expected that the biomass production would exhibit a strong correlation with the protein content of plants, because proteins are one of the intermediates for the production of growth tissues. This decrease, together with the observation of chlorosis in plant leaves exposed to JNC soil strongly suggests that the photosynthetic apparatus is affected, and rubisco (ribulose bisphosphate carboxylase oxygenase), the most abundant protein in leaves, is probably affected. This enzyme is a major player in photosynthesis, being involved in biochemical processes by which plants transform sunlight, water from the soil, and carbon dioxide from the air to the carbohydrates that they need for growth, conditioning therefore plant biomass production. Also, during leaf senescence, this protein may be a source of amino acids for other growing regions (e.g. reproductive organs) [39].

In the exposure of several JNC soil treatments, growth parameters were sensitive at the 75% treatment, in length, fresh weight cases, and in the 50% in the dry weight case. The MDA production and protein content were more sensitive to this exposure than length and fresh weight, inducing a significant increase and a decrease, respectively, in the 50% treatment.

The increase of MDA production in exposed plants was expected because, as available toxicants increase in soil solution, their absorption will increase, and the lipid peroxidation through the possible excessive generation of free radicals will be incremented. Similar responses have been reported for exposure with heavy metals [9].

The increase in the peroxidase and catalase activities at the 25% and 50% JNC treatment, respectively, indicates an attempt of mitigate and repair the damage caused by reactive oxygen species that were generated by this exposure. The increase of peroxidase activity in the 25% treatment still prevented the lipid peroxidation in cell membranes. This fact was shown by the maintenance of productivity of MDA levels in this treatment exposure, but was not strong enough to avoid lipid peroxidation in cell membranes in the 50% treatment exposure. Consequently the MDA production in the 50% treatment was significantly

increased. The peroxidase activity trend was inverted when plants were exposed to the two highest treatments (75 and 100% JNC), as observed for SOD activity of rice (*Oryza sativa* L.) exposed to microsystins [40], because their activity was affected by the contaminants bioavailability.

In the *A. sativa* biochemical bioassays, it was confirmed the trend shown in the seed emergence and growth bioassays. Plants exposed to Lufa 2.2, JNC and JC soils did not exhibited oxidative stress.

As a conclusion of this study we can say that plant bioassays are good tools to use in the evaluation of soil quality. However, some species sensitivity might be one factor of concern because *A. sativa* did not show any response in the bioassays that were carried out. Probably the use of four plant species, with two monocotyledonous and two dicotyledonous species, would improve the evaluation of the test soil.

Additionally, the prolongation of the 14d bioassay till the all life cycle of *B. rapa* RCBr is completed, could be one advantage for this evaluation process.

Also, the biochemical approach responded in lower treatments when compared to the other bioassays, showing that it can be used as a complementary tool to be carried out after the 14d bioassay or even the LCB. Even though, more studies have to be carried out to confirm oxidative responses towards contamination in soils.

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GENERAL DISCUSSION AND CONCLUSIONS

GENERAL CONCLUSIONS AND DISCUSSION

The Mina de Jales area is one of the Portuguese areas that enclose highly concerning environmental problems. Water courses, ground water, soil and air are the main compartments that should be revised to assess environmental and human risk.

In this study, soil was the main compartment evaluated, although ground water was also regarded by the evaluation of the water path and the soil buffer capacity, using aquatic bioassays.

The pedosphere is a complex and dynamic system that has the ability to react against changes and harmful substances or processes by a balanced and buffered mode. It is influenced by numerous substance flows and interactions (chemical, physical or biological), many of them still unknown, and that can bring soil as a constant environmental variable system [1].

Thus, before starting the evaluation of the potential toxicity of JNC and JC soils from the Mina de Jales area, a chemical analysis provided information on the different heavy metal contents, with JC soil presenting high heavy metal concentrations and therefore considered toxic. The JNC soil showed low heavy metal contents, although arsenic and aluminium were present in moderate and high quantities, respectively. Arsenic has been analysed and identified by several institutions (e.g. the former Portuguese Geological and Mining Institute, nowadays the INETInnovation) in soils from this mine, but there is no information available about the presence of Al in prior analytical procedures. This confirms the fact that simple determinations of total soil metal contents by chemical analysis can often neglect chemical compounds that are present and remain undetected simply because they are not analysed. Additionally, these analyses will not provide any information on the bioavailable fraction of these chemicals, which depends on the chemical form (speciation) of the metal and consequently if it is absorbed to soil particles/ constituents or if it is free in the soil solution medium.

Therefore, bioassays can help to assess the potential environmental risk that is inherent in a soil, by assessing the effects of the total bioavailable fraction of the pollutants.

In this study a test-battery was applied, using different trophic groups for the evaluation of soils potential toxicity and different results and sensitivities were achieved.

As a first screening test, the soil avoidance behaviour tests supported the first evidence about the toxicity of the two soils, evaluating the habitat function. The first test performed with edaphic organisms showed that JC soil was not avoided by neither the earthworms (*Eisenia andrei*) nor the terrestrial isopods (*Porcellionides pruinosus*) (Table I). Moreover, earthworms even showed preference by some treatment dilutions (50 and 100% JC soil), when compared to the clean soil Lufa 2.2. Comparing both test-organisms used in this screening bioassay, *P. pruinosus* showed higher sensitivity to JNC soil exposure, avoiding treatments 75% and 100%, while *E. andrei* did not avoid any of the treatments. Using the guidelines adopted from BioQuest International [2], the avoidance behaviour tests showed slight to moderate toxicity, with an EC_{50} value of 57%. At the end of this study, the results of the avoidance tests with *P. pruinosus* were confirmed.

Aquatic bioassays provided the information on the retention function and buffering capacity of the two soils and the chemical analysis in elutriates gave also the data on some of the bioavailable fraction. Water elutriates can translate the dilution of the pore water, one of the bioavailable fractions of chemical compounds in soil. Substances that are lightly sorbed to organic matter can also be released to water elutriates in the extraction process, while soil and water are shacked.

One of these aquatic bioassays was performed using Microtox® testing [3]. The bioluminescent bacteria *Vibrio fischeri* did not reflect any toxicity towards JC soil or soil elutriates. When in contact with JNC soil particles it presented the highest toxicity unit (38.94), which can be considered “extreme” by the quantitative labels used in the BioQuest International [2]. Soil elutriates (1:2 dilutions) also indicate high toxicity but 10 times lower than soil particles, with a TU of 3.675.

Table V.I- EC_{50}, LOEC and NOEC values obtained from the Terrestrial Bioassays and Soil Quality Index derivation for JNC and JC soils.

Bioassay	Test-Soil	Endpoint	Toxicity	LOEC	NOEC	LC/EC_{50}
Porcellionides pruinosus Avoidance Behaviour bioassay	JC	avoidance	No	-	-	-
	JNC		Yes	75	50	50.08
Eisenia andrei Avoidance Behaviour bioassay	JC	avoidance	No	-	-	-
	JNC		No	-	-	-
Brassica rapa 14 day bioassay	JC	all	No	-	-	-
	JNC	length	Yes	75	50	84.60
		FW	Yes	75	50	66.11
		DW	Yes	50	25	50.68
		protein content	Yes	50	25	60.19
Avena sativa 14 day bioassay	JC	all	No	-	-	-
	JNC		No	-	-	-
Soil Quality Index (%)	JNC	microbial	low quality	27.23		
	JC		high quality	91.83		

The *Daphnia magna* bioassays evaluated lethal and sublethal toxicity and both JC and JNC soil elutriates presented high toxicity levels, being JNC soil elutriates those with higher sublethal and lethal toxicity. Although in the evaluation of mortality, given by the immobilization parameter, JNC soil elutriates showed 3 times higher toxicity than JC soil elutriates, in reproduction trends the difference was accentuated in 4 times. When compared with the *Vibrio fischeri* bioassays with soil elutriates, both chronic and acute tests with *D. magna* produced higher

toxicity values. This cladoceran was the only test organisms that showed a clear response to JC soil, by reducing their reproductive and life trends (Table II).

Table V.II- EC_{50}, LOEC and NOEC values obtained from the Aquatic Bioassays in the toxicity evaluation of JNC and JC soil elutriates.

Bioassay	Test-Soil	Endpoint	Toxicity	LOEC	NOEC	LC/EC_{50}
Daphnia magna bioassays	JC	immobilization	Yes	-	-	51.60
		no juveniles	Yes	16	8	-
		female length	Yes	16	8	-
	JNC	immobilization	Yes	-	-	15.81
		no juveniles	Yes	4	2	-
		female length	Yes	4	2	-
Vibrio fischeri luminiscence bioassay (MICROTOX)	JC	all	No	-	-	-
	JNC	soil	Yes	-	-	2.57
		1:2 water elutriate	Yes	-	-	27.21
		1:4 water elutriate	Yes	-	-	62.34
		1:4 extractant elutriate	Yes	-	-	29.90

Although tests with soil elutriates have shown high sensitivity, there are some practical features that have to be mentioned. Soil elutriates were filtrated with glass fibber filters, which has been proved recently to be a strong adsorption material for several trace heavy metals [4]. As a result, heavy metal content in soil elutriates, used for chemical analysis, is probably lower than the raw elutriates before filtration, especially heavy metals like Pb and Ag, and as consequence their toxicity might be considered underestimated.

Plant bioassays were also carried out and reflected two opposite results, depending on the plant species used. In one hand, the turnip *B. rapa* (RCBr) showed toxicity effects when exposed to JNC soil, whilst JC soil did not affect their growth (Table I). On the other hand, the oat species *A. sativa* did not express any toxic effects both in terms of growth and biochemical parameters. The evaluation of biochemical parameters in *B. rapa* exposed to the JNC soil treatment also lead to an accurate measurement of toxicity and were in concordance with the results obtained for the early seedling bioassay. This enzymatic bioassays have been used to assess oxidative processes that occur in plants grown in contaminated hydroponic solution [5-9], and the extrapolation of these methodologies to plants raised in real contaminated soils could be an improvement in the assessment of ecotoxicological effects.

Regarding the C and N cycling in soil, the C:N ratio was 21.38 for JNC soil and 11.53 for JC soil. Since N was similar in both soils, this might reflect the higher loss of C in JC soil by decomposition processes (mineralization), turning available more nutrients like ammonium, sulphate or phosphate ions to plants. This may explain the higher uptake of N by plants in the JC soil, which will probably be recovered, depending only on the degradation time of organic materials [10]. As general rule, conditions that increase decomposition of organic matter will result in a narrowing of the C:N ratio. The C:N value of 10 has been considered as an optimal ration for C and N cycling, whereas 20 is a value related to the delay of the availability of N as a fertilizer for growing plants. This is also in accordance with our results from the plant bioassay with *B. rapa*.

In this study another biochemical approach (soil enzymatic activities) was used. Soil enzymes have proved to be sensitive in the evaluation of soil quality and several enzymatic bioassays, based on the nutrient cycles, have been developed (e.g. [11, 12 , 13]).

The evaluation of soil enzymes can and should be performed during different time samplings, because microorganisms activity differs in different environmental conditions [14]. As at the time of the work a rehabilitation process in the Jales mine was being implemented, it was decided to evaluate the recovery of several soil enzymes in 3 sampling years. DHA, arylsulfatase and urease activities and N-

mineralization processes responded in a positive way to the rehabilitation process, showing a recovery in their activity. The same trend was observed for the Microbial Biomass of Carbon and Nitrogen that also indicated a recovery in the microbial population. The enzymatic activity bioassays could not distinguish, in terms of toxicity, differences between JNC and JC soils, although some soil enzymes showed higher activities in the JNC soil.

Incorporating these enzymatic activities into a Soil Quality Index (SQI) has proved to be a good measure for the achievement of the quality status of native or climax soils [15-17]. Nevertheless, to our knowledge, this index was never applied to contaminated soils or to soils suspected to be nutrient poor. SQI values obtained for JNC and JC soils were compared to the same index for Lufa 2.2 soil and to the results from the other bioassays used in this battery (Table I). Unexpectedly, JC soil SQI was similar to the values obtained for native climax soils, i. e. close or higher than 100% [15]. Nevertheless, the index values are in accordance with the results obtained for the other bioassays, where JNC soil showed very low quality and JC soil proved to be non toxic for all edaphic test-organisms used. Additionally, the β-glucosidase activity bioassay must be revised for future use and incorporation in the SQI, due to problems that occurred while measuring its activity and that can be caused by high SOM contents.

All the bioassays used can be ranked, in order of increasing sensitivity, as follows:

Avena sativa ≅ ***Folsomia candida*** ≅ ***Eisenia andrei*** < ***Porcellionides pruinosus*** ≅ ***Brassica rapa*** (growth) ≅ ***Brassica rapa*** (biochem.) < Microtox Basic Test < ***Daphnia magna*** < Microtox Solid-phase

Increasing sensitivity

A similar trend of species sensitivity was observed by Carter *et al.* [2] in the evaluation of three sites in Atlantic Canada, contaminated with PCBs and heavy metals. The author stated that the Solid-phase test of Microtox® was the most sensitive bioassay, followed by the lettuce seedling and algae test, and the less sensitive test-organisms was the earthworm *E. andrei*.

In the review of bioassays for soil, freshwater and freshwater sediment in Canada, Keddy *et al.* (1995) concluded that earthworms showed less sensitivity when compared to other test-organisms like several plant species, aquatic organisms (*D. magna* and algae) and bacteria [18]. The algae test was the most sensitive.

In another study [19], soil samples from a petroleum harbour in Amsterdam were evaluated for their toxicity using a test battery. Again, different species showed different toxicity sensitivity, and the collembolan *F. candida* appeared to be less sensitive than earthworms or plants. Bioassays with soil elutriates presented more concise results when the test organisms were *D. magna* and *Vibrio fischeri* (Microtox® tests).

Another similar approach was made by Sheppard *et al.* (1993), in the evaluation of Zn and Hg toxicities in sandy, garden and clay soils [20]. The sandy soil showed higher toxicities of Zn and Hg, due to the lower content on organic matter or clay, and earthworms were again considered as the species with the lowest sensitivity to the chemicals. By the contrary, the *B. rapa* life cycle bioassay presented lower EC_{50} values followed by the aquatic tests with *D. magna* and Microtox®.

Even often considered ecologically not relevant for soil toxicity evaluation, *D. magna* and the marine bacteria *Vibrio fischeri* have shown high toxicity responses to soil elutriates in this study, as in other studies, as mentioned before. Oat (*A. sativa*) and the springtail *F. candida*, commonly used in ecotoxicological tests, showed no sensitivity to toxicants present in soils. Different species sensitivity stresses the need to include in test-batteries representative organisms from different trophic levels. Regarding edaphic animals in general, they have different uptake routes that can consist on chemical compounds uptake through the skin, in the case of soft body invertebrates, through soil ingestion (via the gut wall), via

drinking, or special organs like ventral tubulus in springtails or uropods in isopods. These differences are related to species body structure, but also to behaviour or preferences of habitat types, like soil layers or the humus layer [21, 22]. Also different plant species can react differently to chemical compounds due to different physiological processes that are taking place and that make them more or less sensitive to chemicals (hyperaccumulative plant species and high sensitive plants) [23, 24]. While hyperaccumulative plants can effectively remove cationic and anionic forms of toxic metals, sensitive plants will enter in oxidative stress.

In addition to chemical compounds threshold values in soil, there is also data available on NOEC and LC_x /EC_x values, compiled in a RIVM report [25], for arsenic, chromium, cadmium and copper to terrestrial species and soil processes, that could provide thresholds for chemical evaluation. Even though there is still no information, regarding the influence on soil characteristics and chemical mixtures on toxicity data, that could help in the evaluation processes. As an example, the NOEC value for *E. andrei* exposed to Cd is 5.3 mg/Kg, a value lower than the 8.2 mg/Kg present here in the JC soil. Anyway, when we exposed the earthworms to this soil they even showed preference for that soil. Additionally several species of earthworms (not identified) were observed in this soil, while only few specimens of earthworms were observed in the JNC soil, although not during the all year.

The increase in the microbial biomass C and N in the JNC soil showed a clear recovery of the microbial population. From the year 2002 to 2004, the microbial biomass C increased 3.7 times and the microbial biomass N doubled. In the JC soil the trend was different, i. e. no recover was observed.

We can conclude that the bioavailable fraction of heavy metals of the JC soil (higher heavy metal content) is lower than that of the JNC soil (lower heavy metal content), since most organisms are influenced by the bioavailable fraction of metals instead of the total amount present in the soil. In addition, the soil with the lower heavy metal content, that could be considered uncontaminated, presented high toxicity levels. Another likely reason for these toxicity levels is the possible unknown presence of other contaminants that were not regarded on the chemical analysis and were, therefore, neglected.

One of the major outputs of this study was the demonstration of the role of ecotoxicological testing methodologies for the evaluation of soil functions and the importance and urgent need to incorporate these methodologies within a structure of protection and evaluation by the Portuguese laws and government. Establishing and applying soil values as a unique way to evaluate soil quality might be considered pointless, because in soils a high number of chemical compounds can be found, complexed in different ways, forming several combinations due to physico-chemcial soil properties, and therefore behaving differently. Even tough, bioassays are not raised to be considered as competitive tools to chemical analysis, but rather as a mandatory supplement.

Soil ecotoxicology has evolved from aquatic ecotoxicology and, in an initial stage soil toxicity evaluation was made using methodological adaptations of pre-existing protocols for water quality assessment. The major progress in terrestrial ecotoxicology was the development of several new tests with edaphic organisms, especially chronic studies and investigations regarding sublethal endpoints. Nowadays, more tests are being developed to improve the evaluation of soil toxicity, reducing costs and time. Bioassays such as the Avoidance Behaviour Test with earthworms and isopods, tests with enchytraeids, soil enzymatic activity measurements or the BIOLOG methodology for the microbial and fungi identification are examples of recently developments in soil ecotoxicology (e. g. [26-28]). Other approaches on decomposition processes performed by invertebrates, like the bait lamina test, or by microorganisms, like the litter bags method, are used to evaluate how resident decomposers are dealing with soil contamination (e. g. [19, 29, 30]).

Despite using in this study bioassays covering several trophic levels, much more work is needed to assess the effects of heavy metals present in soil, water and air compartments, as part of the risk analysis in an ERA process in the Jales mine area. This study should be completed with other bioassays, like the earthworm reproduction, BIOLOG techniques, bioaccumulation studies, or even the tissues' analysis of plant and animals that live in the mine area.

Additionally, a Human Health Risk Assessment should be performed in Mina de Jales, because breeding cattle and top predators are also exposed to heavy

metals by grazing on the mine area. The transfer of contaminants through meat, milk and vegetables from agriculture should be regarded as a major hazard for human population. Now that the mine's rehabilitation is finished, some attention has to be paid if remediation processes are expected to be developed in soils from that area. As it was observed here, the bioavailability of heavy metals in the JC soil (closer to the mine spoil) is lower and any changes on pH values, SOM or cationic exchange capacity can be reflected in an increase of heavy metal bioavailability.

In summary, Jales mine is one case study that can be taken as an example for future evaluation of contaminated sites. In Portugal, all evaluation procedures are based on chemical analysis and no testing with organisms is carried out. Therefore, nowadays no real assessment of toxicity is accomplished in contaminated areas because there is no information on the bioavailability of chemical compounds, generally present in complex mixtures and not as isolated and single substances.

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