<table>
<thead>
<tr>
<th>CONTENTS</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author’s declaration of originality</td>
<td>iii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>v</td>
</tr>
<tr>
<td>Symbol, acronym and abbreviation list</td>
<td>vi</td>
</tr>
<tr>
<td>List of figures and tables</td>
<td>vii</td>
</tr>
<tr>
<td>Abstract</td>
<td>ix</td>
</tr>
<tr>
<td>Resumo</td>
<td>x</td>
</tr>
</tbody>
</table>

**CHAPTER I: INTRODUCTION**

1.1. Background .................................................. 11
1.2. Coal mining and the soil contamination problem due to metals .... 13
1.3. General Objective ............................................. 15
    1.3.1. Operational objectives .................................. 15
1.4. Dissertation structure ................................. 16

**CHAPTER II: METHODOLOGY**

2.1. Tier 1: Simple Screening Stage for ERA Process in S. Pedro da Cova Abandoned Coalmine .... 17
2.2. Study area and sampling collection method .......................... 17
2.3. Chemical line of evidence and physicochemical characterisation of the soil samples ........ 18
2.4. Ecotoxicological line of evidence: ecotoxicological tests with the whole soil matrix and soil elutriates ......................... 19
    a) Soil preparation ........................................ 19
    2.4.1. Avoidance test with *Eisenia andrei* Bouché .......... 19
    2.4.2. Bioluminescence immobilization test with bacteria *Vibrio fischeri* Beijerinck (Microtox®Omni, Basic Solid-Phase test) .... 20
2.4.3. Plant germination and growth test ....................... 21
    2.4.4. Mortality or immobilisation acute test .............. 22

**CHAPTER III: RESULTS AND DISCUSSION**

3.1. Chemical line of evidence and physicochemical characterisation of the soil ................. 23
3.2. Ecotoxicological line of evidence: ecotoxicological tests with the whole soil matrix and soil elutriates ........................................... 26
    3.2.1. Avoidance test with *Eisenia andrei* Bouché .......... 26
    3.2.2. Bioluminescence immobilization test with bacteria *Vibrio fischeri* Beijerinck (Microtox®Omni, Basic Solid-Phase test) .... 26
    3.2.3. Plant germination and growth test ........................ 29
        a) Endpoint: Emergency of seeds ....................... 33
        b) Endpoint: Fresh biomass ......................... 34
        c) Endpoint: Dried biomass ......................... 34
    3.2.4. Mortality acute test with *Daphnia magna* Straus ....... 35
3.3. Risk Characterisation ...................................... 35

**CHAPTER IV: CONCLUSION AND RECOMMENDATIONS**

Conclusions and recommendations .................................... 37
References .................................................................. 38
Author’s Declaration of Originality

I, the undersigned Evidóquio Roné Alzira Maculuve, hereby certify and declare on my honour that this scientific research related to the master’s dissertation is the result of my personal research and guidance of my supervisor and co-supervisor and it’s not plagiarised. Its contents are original and all consulted sources are properly mentioned in the text, notes and in the final references.

I also declare that this research has not been submitted to any other institution in order to obtain any academic degree.

Porto, September 2014

____________________________________
(Evidóquio Roné Alzira Maculuve)
Dedication

In loving memory of my beloved grandfather and uncles:

Martins (Nhaca) de Oliveira (Grandfather)

Salomão Martins de Oliveira (Uncle)

Guilherme (Gui) Martins de Oliveira (Uncle)

Rostino Martins de Oliveira (Uncle)

Rest in Peace.

“Deus dá as batalhas mais difíceis aos seus melhores soldados”

Papa Francesco
Acknowledgements

Firstly, thanks God for the blessings and insights that has allowed in my life and in my family, and also for giving me enough health, strength and faith during this two years I spent in Porto city.

My special gratitude and appreciation goes to my supervisor Professor Dr.ª Ruth Maria Oliveira Pereira for her teachings, endurance, guidance, dedication and facilitating my research by sharing her knowledge and time to make this work more valuable. My special acknowledgements are also extensive to my co-supervisor Professor Dr. Jorge Manuel Espinha for his simplicity, availability, willing to help and for the contribution he gave during the days we have been going to study area collecting the samples and also thanks to Dr.ª Joana Ribeiro. My special thanks goes as well to Professor Dr. Eduardo Silva from the University of Aveiro for the chemical analysis of soil samples.

I must acknowledge as well to the Erasmus Mundus ACP II Programme for giving me the opportunity to have brilliant and unique moments in Europe and mainly for having academic and cultural exchange; particularly thanks to Bárbara Costa and Ana Paiva.

To my mother, Carolina Alzira Martins de Oliveira, for the endorsements. My brothers, sister, nephews, nieces, cousins, uncles, aunts and my grandmother for always being by my side.

I will always appreciate and keep into my mind forever what I’ve learned from my Professors, particularly Rubim Almeida, in the Science Faculty, Biology Department.

I am grateful for the hospitality, wisdom support, integration and advices from my faculty colleagues of the Ecology, Environment and Territory Master’s 2012. Special thanks to: João Martins, Daniela Torres, Cristiana Maia, Cláudia Oliveira, Paula Portela, Juliana Barros Carvalho, Joana Oliveira, Cristiano Freitas, Ricardo Cardoso, Cristina Santos, Manuel Samussone and Simónia Sanches.

I must express my particular gratitude to Ana Gavina for the support gave in the laboratory experiments and must also thank to the Laboratory Technicians of the Faculty of Science, Lilyana and Teresa, and to the Securities, Paulo and Marco.

A deep appreciation to Horácio Fulane, André Gonçalves (Messias), André Pereira and Maria Zita whose friendship and hospitality supported and entertained me over the two years of our friendship.
Symbol, acronym and abbreviation list

Chem – Chemical
ECotox – Ecotoxicological
EPA – Environmental Protection Agency
ERA – Environmental Risk Assessment
EU – European Union
GPS – Global Positioning System
ISO – International Organisation for Standardisation
LoE – Line of evidence
OECD – Organisation for Economic Co-operation and Development
REF – Reference
SDI – Strategic Diagnostics Inc
US – United States
USEPA – United States Environmental Protection Agency
List of figures and tables

Fig. 1: Geographical localization of S. Pedro da Cova abandoned coal mining area within the national territory and Porto metropolitan area (on the right). Representative map of the sampling transects T0.0 (in the centre of the mine heap with 1 sampling point) T1 (on the South with 4 sampling points), T2 (north-northeast with 3 sampling points) and T3 (south-southwest with 3 sampling points) and corresponding sampling plots in the study area.  

Fig. 2: Eisenia andrei Bouché http://environmentprogress.com/key-research-articles/effect-of-tio2-nanoparticles-in-the-earthworm-reproduction-test/ online available on August 6th, 2014. 

Fig. 3: Device used for measuring luminescence of Vibrio fischeri Beijerinck, model 500 analyzer. 

Fig. 4: Plant germination and growth test (Zea mays). 

Fig. 5: Daphnia magna Straus http://en.wikipedia.org/wiki/Daphnia_magna online available on August 6th, 2014. 

Tab. 1: Average and standard deviation (STDEV) values resulting on physicochemical parameters and the geographic coordinates of different sampling sites. The highest values are those highlighted. 

Tab 2: pH optimum and tolerance of soil test organisms. 

Tab. 3: Total metal concentrations (mg/kg) in the soil samples collected in S. Pedro da Cova coal mine and Hazard concentrations for 5% of species (HC5) proposed by Jänsch et al. (2007). 

Fig. 6: Avoidance assay percentage performed with E. andrei after exposed in soil samples and OECD artificial soil used as a reference soil. The error bars correspond to standard deviation and the avoidance significant differences are represented by * or ** indicating to significantly avoided and highly significantly avoided/preferentially selected by earthworms, respectively. (Fisher’s Exact Test, p ≤ 0.05). 

Tab. 4: Shows the results of the acute effects of the bioluminescence assay with V. fischeri Beijerinck obtained with the Microtox® BSPT protocol for each soil sample. The highlighted values are those very toxic soil samples. 

Fig. 7: Average values of the seeds emergency recorded for Avena sativa (oats) after exposition in soil samples and OECD artificial soil. The error bars correspond to standard deviation (Dunnett’s test, p ≤ 0.05). 

Fig. 8: Average weight values of fresh mass recorded for Avena sativa (oats) after 14 days of exposition in soil samples and OECD artificial soil. The error bars correspond to standard deviation and the significant differences in fresh mass weights are
represented by * indicating to significant differences from the control group equal to reference soil (Dunnett’s test, p ≤ 0.05).

Fig. 9: Average weight values of dry mass recorded for Avena sativa (oats) after 14 days of exposition in soil samples and OECD artificial soil. The error bars correspond to standard deviation and the significant differences in dry mass weights are represented by * indicating to significant differences from the control group equal to reference soil (Dunnett’s test, p ≤ 0.05).

Fig. 10: Average values of the seeds emergency recorded for Zea mays (maize) after exposition in soil samples and OECD artificial soil. The error bars correspond to standard deviation (Dunnett’s test, p ≤ 0.05).

Fig 11: Average weight values of fresh mass recorded for Zea mays (maize) after 14 days of exposition in soil samples and OECD artificial soil. The error bars correspond to standard deviation and the significant differences in fresh mass weights are represented by * indicating to significant differences from the control group equal to reference soil (Dunnett’s test, p ≤ 0.05).

Fig. 12: Average weight values of dry mass recorded for Zea mays (maize) after 14 days of exposition in soil samples and OECD artificial soil. The error bars correspond to standard deviation and the significant differences in dry mass weights are represented by * indicating to significant differences from the control group equal to reference soil (Dunnett’s test, p ≤ 0.05).

Fig. 13: Average values of the seeds emergency for Brassica rapa (cabbage) after exposition in soil samples and OECD artificial soil. The error bars correspond to standard deviation and the significant differences of the emergencies are represented by * indicating to significant differences from the control group equal to reference soil (Dunnett’s test, p ≤ 0.05).

Fig. 14: Average weight values of fresh mass recorded for Brassica rapa (cabbage) after 14 days of exposition in soil samples and OECD artificial soil. The error bars correspond to standard deviation and the significant differences in fresh mass weights are represented by * indicating to significant differences from the control group equal to reference soil (Dunnett’s test, p ≤ 0.05).

Fig. 15: Average weight values of dry mass recorded for Brassica rapa (cabbage) after 14 days of exposition in soil samples and OECD artificial soil. The error bars correspond to standard deviation and the significant differences in dry mass weights are represented by * indicating to significant differences from the control group equal to reference soil (Dunnett’s test, p ≤ 0.05).
Fig. 16: Average percentage of mortality of *D. magna* recorded for the non-diluted elutriates obtained for each soil sample collected in *S. Pedro da Cova* abandoned coal mining area. Error bars represent Standard deviation.

Tab. 5: Values of risks calculated for the chemical (ChemLoE) and the ecotoxicological (ECotox) lines of evidence, integrated risk and STEDV, for the different soil samples collected in *S. Pedro da Cova* abandoned coal mining area.
Abstract

Coal mining industry, in particular, is one of the many anthropogenic actions, which should be considered whether the mine is in operating or deactivation stages because of its potential hazardous in terms of modifications made in the surrounding environment from which the impacts goes since the devastation of the vegetation up to the changes into soil physical, chemical and biological composition and contamination of the surface and groundwater. Beyond that, some heap sites are also used to dump large amounts of the construction waste material increasing the possibilities of both, environmental and human health risks. With a great focus on the wastes from extractive industries, and aimed in attaining such objective the European Directive 2006/21/EC (EC, 2006) in the article 20 established that: “each member country should drawn an inventory of the abandoned waste facilities within their territories, which have the potential to cause negative impacts on human health and the environment”. Thus, according to article 21, appropriate methodologies like risk assessment procedures should be followed. This investigation was performed to apply the Dutch framework for the risk assessment of contaminated sites to S. Pedro da Cova abandoned coal mining area, performing the first tier of the evaluation process, integrating both, the chemical and the ecotoxicological lines of evidence. In order to deal with the problem in that contaminated specific site, in terms of methodology, the approach used was Tier 1 screening stage for ERA process of the ecological risk assessment from the Dutch’s framework based in a Triad approach from which were integrated simultaneously information from the two lines of evidence: chemical and ecotoxicological. For such purpose soil samples were collected in three different transects defined in the area. Physical and chemical parameters of soils (pH, % of OM, water holding capacity, conductivity and total metal contents) were assessed, followed by ecotoxicological assays performed with the whole soil matrix and with soil elutriates. The results of the risk integration confirmed that the toxic effect was most likely caused by numerous factors as physicochemical features and high metal concentrations. Risk characterization indicated the existence of risks especially located in the south and north part of the heap (T0.0, T1.4, T2.1, T2.2 and T3.2). However, a great difference was recorded between the risks calculated based on the chemical and the ecotoxicological line of evidence for soils T1.2 T1.3, T1.4, T2.2 and T2.3 once again all for transect 1 and 2. So, further investigation to Tier 2 is needed for these sampling points.

Keywords: coal mining sites, ecological risk, metals, toxic and inert residues, soil compartment and ecotoxicology.
Resumo

A indústria extrativa do carvão, em particular, é uma entre várias ações antrópicas, que deve-se ter em consideração em qualquer um dos estágios da vida duma mineradora, quer na fase de exploração quer seja na fase de desativação, devido aos potenciais riscos no que respeita às modificações a que o meio ambiente é sujeito. Geralmente, tais impactos variam desde a devastação do coberto vegetal, às mudanças das propriedades físicas, químicas e biológicas do solo, bem com a contaminação das águas superficiais e subterrâneas através da percolação. Para além de que, algumas minas, terminado o seu tempo de vida são usadas para despejos de grandes quantidades de resíduos tóxicos, de várias proveniências, tal como é o caso das indústrias de construção civil e siderúrgica, contribuindo negativamente no aumento dos riscos ambientais e humanos. Segundo a Directiva Europeia 2006/21/EC (EC, 2006), que mantém um grande foco nas indústrias extrativas na Europa, estabeleceu, através do Artigo 20 que: “cada país membro deve elaborar um inventário das antigas instalações das indústrias extrativas, em seu território, que têm o potencial de causar impactos adversos sobre o meio ambiente e a saúde humana”. Deste modo, de acordo com o Artigo 21, devem ser realizadas avaliações de risco seguindo metodologias padronizadas. O objetivo desta dissertação foi de aplicar o esquema Holandês de avaliação de risco ecológico para estimar os riscos de contaminação ecológico na escombreira da Mina de São Pedro da Cova, em Gondomar, seguindo a Tier 1 para no fim integrar os riscos de ambas as linhas de evidência: química e ecotoxicológica. Para a abordagem do problema, a metodologia usada foi a Tier 1: fase de rastreio simples para o processo de avaliação de risco ecológico do Esquema Holandês, com base em uma abordagem tríade da qual foram integrados simultaneamente informações a partir de duas linhas de evidência: química, ecotoxicológica na avaliação de riscos. Foram recolhidas amostras de solo e foram levados para uma bateria de ensaios em condições laboratoriais. Foram avaliados os parâmetros químico-físicos do solo (pH, % da matéria orgânica, capacidade de retenção da água, condutividade e conteúdo total em metais). Os resultados da integração do risco confirmaram que o efeito tóxico foi provavelmente causado por diversos fatores como as características físico-químicas do solo e a elevada concentração em metais. A caracterização de riscos indicou a existência de riscos em especial aos pontos amostrais localizados na parte Sul e Norte da escombreira (T0.0, T1.4, T2.1, T2.2 e T3.2). Contudo, foi registada uma grande diferença entre os riscos calculados com base nas duas linhas de evidência, particularmente para os solos: T1.2 T1.3, T1.4, T2.2 e T2.3, todos pertencentes aos segmentos 1 e 2, respetivamente. Assim, recomenda-se uma investigação mais refinada para a Tier 2 apenas para os pontos de amostragem indicados.

Palavras-chave: Locais de mineração do carvão, Risco ecológico, Metais, Resíduos tóxicos e inertes, Solo e Ecotoxicologia.
CHAPTER I: INTRODUCTION

1.1. Background

At the present time, it is almost impossible to find a natural environment that has not been affected through anthropogenic activities, which spectrum ranges from the exploitation of resources (e.g. mining, forestry practice, fishing, agriculture) up to the construction, manufacturing and processing of diverse materials and compounds (e.g. pharmaceuticals, pesticides, nanomaterials, metallurgy alloys, radioactive materials) among other actions that are not managed properly so that harms human health and the environment.

Within the compartments affected, soil is one of the most important elements in biosphere that fulfils several functions providing ecosystem services via processes and products such as nutrient cycling, climate regulation but also acts as a filter for toxic compounds preventing the contamination of the water resources, and maintains local and global environmental quality, so obviously it is important to human kind and should be protected (Swartjes et al., 2012; Pereira et al., 2007; Römbke et al., 2005). Consequently, these benefit processes (e.g. breakdown of organic matter, element and nutrient cycling, natural attenuation), which are either performed or controlled by soil organisms are good reasons for protecting the soil (Römbke et al., 2005).

The majority of the human activities are responsible for giving rise to a great diversity of contaminant and/or organic-rich wastes which are released and/or dumped in different environmental compartments, in particular soil compartment. Such wastes frequently require a specific characterization and adequate valorisation, remediation or storage/disposal procedures in order to mitigate their risks. Focused on the wastes from extractive industries, and aimed in attaining such objective the European Directive 2006/21/EC (EC, 2006) in the article 20 established that: "each member country should drawn an inventory of the abandoned waste facilities within their territories, which have the potential to cause negative impacts on human health and the environment". Thus, according to article 21, appropriate methodologies like risk assessment procedures should be followed. In this context, emerges the ecological risk assessment (ERA) as a process that can be defined as a technical support for decision making which is commonly used to regulate chemicals, make decisions regarding to remediation of the contaminated sites, the monitoring of exotic organisms, the management of watersheds and others environmental management issues (Suter, 2007). Based on the
different issues, typically ERA can be divided in two main types: i) **predictive ERA**, which is applied to new or existing chemical compounds, aimed in defining risk limits for safe use/application and to support its registration and entrance in the market, and ii) **retrospective ERA** which is applied to evaluate already contaminated sites (van Gestel, 2012).

The first retrospective model of the ecological risk assessment was proposed by the United States Environmental Protection Agency, USEPA, in 1998 (USEPA, 1998). Besides the presentation of a conceptual framework for ERA, the document defined it as a process that evaluates the probability of the adverse ecological effects occur on ecological receptors (organisms, population, communities) as a result of exposure whether to one or more stressors (USEPA, 1998). Therefore, ecological risk assessment, as a tool has a lot of benefits to support environmental decision making process (Oost *et al.*, 2003): providing sound scientific information and a quantitative basis for both, comparisons and establishment of priorities, and can also provide a systematic way to improve the understanding of risks.

The ecological risk assessment process can be split into two parts according to Oost *et al.* (2003): **risk assessment** (scientifically oriented) and **risk management** (politically oriented). Risk assessment is a process that includes elements such as: hazard identification, adverse effect evaluation, exposure evaluation and risk characterization. While environmental risk management deals with the recommendations given by the risk assessors in order to mitigate those risks and integrate actions with existing regulatory processes which support their application (Oost *et al.*, 2003). So, by the focus on the protection of the functional characteristics of soil, clear definitions and purposes for the ecological quality of soil have to be provided by authorities to protect the soil in an understandable and efficient way, in fact, this requires standardised and reproducible methods to determine soil quality, as well as a robust knowledge of soil ecology, to be provided by the scientific community (Römbke *et al.*, 2005).

For the enforcement of European legislation each member state should have already defined their frameworks for ERA, and in fact several have already did that. However, there is a great diversity of tools for risk assessment and some experts have already emphasized that harmonization is required (Carlon *et al.*, 2007). Such variability may correspond to more or less rigorous criteria and evaluations and may result in a great economic disequilibrium between member states. In fact different approaches may lead to great disparities in terms of the number of sites being screened out from the inventory, thus not requiring remediation measures. As Swartjes *et al.* (2008)
suggested, the Dutch framework based on a TRIAD approach, could be adopted by the other European member states, contributing for the necessary harmonization. Nevertheless, as these previous authors emphasize it is necessary to validate this framework, by increasing the number of experimental applications, to different environments and types of soils. The Dutch framework proposed by Jensen and Mesman (2006) integrates information from three different lines of evidence (chemical, ecotoxicological and ecological) in different tiers through which a more site specific evaluation is progressively made decreasing the uncertainty of the risks determined. Portugal is one of the member states that still lack the establishment of a framework for ERA, thus leaving room for excellent opportunities to apply this ERA scheme to different contaminated areas.

1.2. Coal mining and the soil contamination problem due to metals

According to the World Coal Association\(^1\) about 6185 Mt of hard coal and 1042 of brown coal/lignite are presently produced worldwide, being China, the USA, India, Australia and South Africa the larger world coal producers. Hard coal has more carbon, while lignite corresponds to less transformed peat, with low organic material.

The exploration of coal deposits, seen as a source of industrial and domestic fuel, is an important economic activity in developing countries but it may cause serious environmental liabilities (Chiochetta et al., 2013; Cravotta, 2007).

Mining in general, and coal extraction, in particular, tends to have a remarkably adverse impact on the environment, especially on soil compartment, and the impacts can vary depending on the mining methods used and geological conditions of the site (Bell et al., 2001).

The different stages involved in the coal extraction, which include physical, chemical and technological processes undertaken to process the raw material, and separating the element of the interest may result in a degraded landscape, topographic changes (land subsidence) and contamination of the soil compartment and water resources with metals, making these issues common in several regions in the world (Loupasakis et al., 2014; Ribeiro et al., 2010, 2013; Chiochetta et al., 2013, Yenilmez et al., 2010; Bell et

In most places, coal extraction is also responsible for reducing forestry productivity due to once occur changes in the physical, chemical and biological soil properties, even after mine closure (Chiochetta et al., 2013) or due to coal fires. All of these impacts persist after the exploration has stopped especially, for areas explored in the past, or for some regions of the planet, where no legislation enforces particular concerns with the impacts on the environment and/or human health. Soil contamination by metals derived from mining is a global environmental problem that may not only result in environmental hazards and human health, as previously mentioned, but also may have financial implications in terms of remediation and monitoring costs of the site (Chapman et al., 2013). Soil contamination issue is now recognised as a major problem in so far as either the land or aquatic communities are affected due to leaching, drainage and runoff of toxic substances from the local (Fernandez et al., 2006).

In fact drainage from abandoned mines affects the quantity, quality and also the potential uses of the water supply in the coal mining regions worldwide (Cravotta, 2007) and recently there is a very large number of abandoned mining sites due to lower demand for minerals and there is a rapid increase of environmental concerns (Qiu et al., 2004).

In Portugal, the Douro coalfield, the largest Carboniferous coal deposit, with 53 km length and 30-250 width, was explored at different points, between 1795-1994, with a great contribution to the national energy sector (Ribeiro et al., 2010). S. Pedro da Cova (Gondomar, Porto, Portugal) was the biggest centre of coal exploration to the end of the first half of the last century (Ribeiro et al., 2010). A heap occupying an area of 28 000m² persists, located near the village, and between 2001 and 2003, this area was used to dump 320 Mt of wastes from the national steel mill, with serious impacts in terms of groundwater contamination. In 2005, a forest fire was responsible by the ignition of the heap, with the emission of toxic gases to the atmosphere (Ribeiro et al., 2010).

Thus, S. Pedro da Cova coal mine is one within several abandoned mines in Portugal requiring an ecological/environmental risk assessment, to support future decisions regarding the remediation works needed for the area. It is also a good case study to apply the Dutch framework for ERA making the necessary adjustments to validate this framework to different types of contamination existing within the national context, thus supporting the wide use of this framework within the European Union.
1.3. General Objective

Thus, the main objective of the present work was to apply the Dutch framework for the risk assessment of contaminated sites to S. Pedro da Cova abandoned coal mining area, performing the first tier of the evaluation process, integrating both, the chemical and the ecotoxicological lines of evidence. To attain this purpose, the following operational objectives were defined:

1.3.1. Operational objectives

- To collect soil samples from the site following a systematic sampling approach consisting in transects starting from the centre to the periphery of the heap;
  
  a. Chemical line of evidence and physicochemical soil characterization
  
  - To perform the physicochemical characterization of the soil samples, in terms of: pH, electrical conductivity, organic matter content and water holding capacity;
  - To analyse the total content of metals in the soils collected;

  b. Ecotoxicological line of evidence
  
  - To perform ecotoxicological tests with the whole soil matrix: bioluminescence inhibition test with the bacteria Vibrio fischeri; avoidance tests with Eisenia andrei; germination and growth tests with terrestrial plants, namely Brassica rapa (cabbage), Avena sativa (oats) and Zea mays (maize);
  - To test the toxicity of soil elutriates with the freshwater cladoceran Daphnia magna.

  c. Risk Characterisation
  
  - To calculate risks based on the each line of evidence (Chemical and Ecotoxicological) and integrating both lines of evidence following the methodology proposed by Jensen and Mesman (2006).
1.4. Dissertation structure

This thesis was written with the typical structure of a scientific manuscript to be submitted for publication to an international journal of the scientific area. Chapter I is introductory and is essentially concerned with the general considerations in terms of framing under the thematic area/scientific field and the current relevance of the topic; embraces the theoretical framework about ecological risk assessment; Emphasis is given to the worldwide concerns because of metal contamination resulting from opencast mining extraction process, the subsequent closure and the use of these sites for industrial waste dumps; further is also makes a brief presentation of the two main models available for ecological risk assessment of contaminated sites and the European legislation that justifies the present study; This chapter ends with the description of the main objective of the study and the subsequent operational objectives defined to guide the work.

In Chapter II, corresponding to the methodology, describes the methods and procedures used, giving greater emphasis to the physical-chemical characterization of soil samples from the site and ecotoxicological assays performed based on standard protocols; It is also described the sampling design and the study area.

In Chapter III, the main results obtained are described in graphs and tables with summarized data resulting from the statistical analysis performed. Further, calculated risks based on the previous data, are also presented and discussed.

Finally, in chapter IV are announced the general conclusions and recommendations for future research are given.
CHAPTER II: METHODOLOGY

2.1. Tier 1: Simple Screening Stage for ERA Process in S. Pedro da Cova Abandoned Coal Mining Area

In order to achieve the objective outlined in this research, it was followed the tiered approach of the Netherland’s framework for the Ecological Risk Assessment, tier 1: simple screening stage (Jensen and Mesman, 2006). In fact, this is the simplest level of the whole Dutch framework approach for ecological risk assessment and it is typically done to minimise costs, as it can make a previous screen out of areas for a more site-specific evaluation. This framework integrates information from three lines of evidence (LoEs) (thus, receiving the name of TRIAD): the chemical LoE (total concentrations of contaminants in the soils under analysis are compared to soil guideline values), ecotoxicological LoE (standard tests are used to screen the soil samples for presence of harmful effects caused by toxic compounds or its mixtures) and ecological LoE (getting a quick and first impression of the impacts on ecological structure and function of the soil, i.e. to see if there is any visible damage) (Jensen & Mesman, 2006).

2.2. Study area and sampling collection method

The samples were collected in the area of the abandoned coal mine once considered one of the most important mining concession coal basin with an extension of about 300 metres developed longitudinally between the North bank of the Ave and Gafanhão rivers and located in S. Pedro da Cova, Gondomar, in north region of Portugal, depicted on Figure 1, at a distance of about 12 km from Porto city (Vieira, 2007).

For this study, 3 transects were defined, each comprising 3 (T2 e T3) and 4 (T1) sampling points at predefined distances from a gradient that goes from the centre (T0.0) to the periphery of the coalmine heap. Precisely 11 topsoil samples (0-15cm depth) from inside and surrounding area of the abandoned coal mine were collected in a total of eleven sampling points. The samples were homogenised and transported for the laboratory in plastic bags where they were left to dry at room temperature and then they were immediately sieved into 2mm fractions in order to perform physical and chemical characterization and 4-5mm fractions for the bioassays. A GPS (Garmin model 76csx) device was used to georeference the points where the samples were collected.
2.3. Chemical line of evidence and physicochemical characterisation of the soil samples

A sequence of physical and chemical analyses was performed. Soil pH was measured in soil-to-deionised water and 1M KCl suspensions in a proportion of 1:5 (w/v) (Dewis & Freitas, 1984; SPAC, 2000; Tan et al., 1996; ISO 17512-1, 2008). The suspensions were magnetic stirred for 15 minutes and left to rest for 30 minutes to allow the bulk of the soil to settle, before measuring the soil pH with Consort multi-parameter analyser C3030 device. The soil electrical conductivity (Dewis & Freitas, 1984; SPAC, 2000) was measured in the previous soil: water suspension after 12h. The soil moisture content (SPAC, 2000) was determined by weight loss after the dryness of samples at 105-110ºC in the oven. The same samples were kept to determine the percentage of soil organic carbon (SPAC, 2000) by a method based on the loss of weight of a soil sample, after ignition at 450ºC, in a muffle furnace. The water holding capacity was determined according to the ISO/DIS 11268-2.2 method, (ISO/DIS, 1998). For this purpose the soil is placed in polyethylene flasks with the bottom replaced by filter paper. Afterwards, the flasks are placed in water for 2h, for soil saturation by capillarity and then 3h in filter paper to remove the excess of water. The maximum water holding capacity (WHC_max) is then determined by the difference in weight between the
saturated soil and the soil dried at 105-110°C, for 12h. All the parameters were measured in three sub-replicates of each soil sample.

The samples were submitted to metal analysis so that it can be estimated the amount and type of metals found in the samples from the site. The soil samples were dried at 60°C, sieved (100 gr) to 80 meshes, submitted to acid digestion by *aqua regia* and finally the analyses was performed by ICP-MS.

### 2.4. Ecotoxicological line of evidence: ecotoxicological tests with the whole soil matrix and soil elutriates

In order to estimate the potential ecotoxicity of soil samples, a battery of tests was performed following available standard protocols:

#### a) Soil preparation

The OECD artificial soil used for the avoidance with *E. andrei* and plant germination and growth tests was prepared according to OECD guideline 208 (1984), which was comprised of 5% finely ground sphagnum peat, 22.5% kaolin clay, 72.5% industrial sand, with pH about 6.0±0.5. Soil elutriates were prepared by mixing 150g of each soil samples and 450 ml of ASTM hard water in a proportion of 1:4 (w/v), and mixed with a magnetic stir for 8 hours, then allowed the bulk to settle for 8 hours again. The supernatant was drawn off and centrifuged for a period of 15 minutes at 3900 rpm and 6°C. Initially, soil sample T2.2 was selected as a reference soil due to its physicochemical proprieties and its location in north extreme of the transect 2 (T2) and of the mining area. However, the analysis of total metal concentrations of soil samples showed extremely high concentrations of toxic metals (e.g. Hg and As). These values were higher than the HC₅ hazard concentrations for 5% of the species and soil microbial processes proposed by Jänsch *et al.* (2007). Thus, and also based on total metal concentrations (herein described) soil sample T3.1 REF was selected as a local reference soil, despite its location outside, but closer to the mining area.

#### 2.4.1. Avoidance test with *Eisenia andrei* Bouché

The species used in the avoidance tests was the earthworm *Eisenia andrei* obtained from synchronized laboratorial cultures. The overall principle of this test consists in the exposure of this soil living invertebrate to both contaminated and non-contaminated soils (OECD, 1998), adjusted to 40-45% of its maximum soil WHC, in dual-chambers
for 48h, at constant laboratorial conditions (temperature: 20°±2°C; photoperiod:16h:8hD) in dual test chambers. Thus, in two-section chambers (5 replicates per test soil), 200g<sub>dw</sub> of the test soil was placed in one-side of the chamber, and 200g<sub>dw</sub> of OECD soil (used as CTRL soil) were placed in the other side, separated from each other by a removable split card. The animals (ten animals per replicate weight ranging between 225 – 250 g) were introduced in the middle of the two sections of each chamber and the cards were removed. The animals were not fed during the exposure. After 48h of exposure the card was reintroduced and the number of animals in each section of the chamber was counted separately (ISO, 2008a). The validity criteria established by the standard protocol was checked and the average avoidance percentage for each soil was calculated.

2.4.2. Bioluminescence immobilization test with bacteria *Vibrio fischeri* Beijerinck (Microtox®Omni, Basic Solid-Phase test)

The Microtox® Basic Solid-Phase Test was performed according to, standard operating procedures to assess the ecotoxicity of the samples collected at S. Pedro da Cova coal mine area (SDI MicrotoxOmni® 4.1 BSPT, 2009). For this purpose soil suspensions were obtained by magnetic stirring 3.5g of soil with 17ml of solid-phase test diluent (SDI), for 10 minutes. The bacteria were exposed to the suspensions and light emission was recorded after 5, 15 and 30 minutes by using a Microtox model 500 Analyser (SDI MicrotoxOmni® 4.1 BSPT, 2009). The EC<sub>20</sub> and EC<sub>50</sub> values and the corresponding 95% confidence intervals were computed for each elutriates using the Software for Microtox (SDI MicrotoxOmni® 4.1Basic Solid-Phase Test, 2009). The EC50 values obtained for 30 minutes of exposure expressed in mg/L were transformed in percentages and used to calculate the risks of the chemical line of evidence.
2.4.3. Plant germination and growth test

The plant germination and growth tests were performed according to the standard protocol ISO 11269-2 (ISO, 1995) to measure the production capacity of the contaminated soil (dried biomass and fresh biomass above the surface of the soil). This test is based on the emergence and early growth response of a variety of terrestrial plant species added to the test soil. Twenty seeds of selected species obtained from a local supplier (*Avena sativa*, *Zea mays* and *Brassica rapa*) were planted in 4 replicates for each soil sample, containing mine soils or control soil (OECD, 1984). The soils were kept under growing conditions in the chamber following 14 to 21 days, after 50% emergence of the seedlings in the control group has occurred, at a temperature of 20°C±2°C and a photoperiod of 16h:L:8h:D. The emergence and biomass (dry or fresh mass) of the shoots of the plants were weighted and compared with those of the control plants (OECD, 1984). For dry biomass, the plant material above soil from each replicate was harvested, dried at 70°C, for 12h and weighted to the nearest 0.001g. Only the Percentage of seeds germination was used to calculate risks of the ecotoxicological line of evidence.

2.4.4. Mortality or immobilisation acute test

The mortality or immobilisation tests with the cladoceran *Daphnia magna* Straus, (water flea) were performed according to the standard protocol OECD 202 (OECD, 2004) to assess the ecotoxicity of the soil elutriates obtained from each soil collected in S. Pedro da Cova. Neonates with less than 24h, from the 3rd to the 5th progenies of females maintained in synchronized laboratorial cultures, were exposed to a range of dilutions (100-19.8%) of each soil elutriate performed with the ASTM (ASTM, 1980; OECD, 1998) hard-water medium by applying a 1.50 factor. Four replicates were prepared for each dilution plus the CTRL, with 25mL of elutriate and 5 neonates per replicate in glass tubes. The exposure last for 48h, at a temperature of 20°C±2°C and a photoperiod of 16h:L:8h:D. After 24h of exposure the tubes were observed, immobilized animals were counted and removed from the tubes. Whenever as possible,
EC$_{50}$ and EC$_{20}$ values for immobilization were calculated using Probit analysis (Finney, 1978; 1971) by using the Priprobit Software version 1.63. The average percentage of mortality recorded for each non-diluted elutriate was used to calculate the risks for the ecotoxicological line of evidence.
CHAPTER III: RESULTS AND DISCUSSION

3.1. Chemical line of evidence and physicochemical characterisation of the soil

<table>
<thead>
<tr>
<th>Soil samples</th>
<th>Geographic coordinates XY</th>
<th>pH [1M KCl]</th>
<th>Electrical conductivity [µS.cm⁻¹]</th>
<th>Organic matter content [%]</th>
<th>Water holding capacity [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>STDEV</td>
<td>STDEV</td>
<td>STDEV</td>
<td>STDEV</td>
</tr>
<tr>
<td>T0.0</td>
<td>541730; 4556258</td>
<td>3.5</td>
<td>0.01</td>
<td>26.3</td>
<td>2.89</td>
</tr>
<tr>
<td>T1.1</td>
<td>541782; 4556138</td>
<td>4.2</td>
<td>0.06</td>
<td>70.0</td>
<td>5.29</td>
</tr>
<tr>
<td>T1.2</td>
<td>541747; 4556097</td>
<td>2.9</td>
<td>0.01</td>
<td>237.0</td>
<td>5.29</td>
</tr>
<tr>
<td>T1.3</td>
<td>541696; 4555848</td>
<td>4.6</td>
<td>0.01</td>
<td>92.0</td>
<td>4.58</td>
</tr>
<tr>
<td>T1.4</td>
<td>541646; 4555794</td>
<td>3.7</td>
<td>0.01</td>
<td>25.0</td>
<td>5.57</td>
</tr>
<tr>
<td>T2.1</td>
<td>541627; 4556217</td>
<td>3.3</td>
<td>0.01</td>
<td>58.3</td>
<td>8.14</td>
</tr>
<tr>
<td>T2.2</td>
<td>541808; 4556870</td>
<td>3.9</td>
<td>0.02</td>
<td>31.8</td>
<td>1.80</td>
</tr>
<tr>
<td>T2.3</td>
<td>541710; 4556596</td>
<td>4.1</td>
<td>0.02</td>
<td>44.3</td>
<td>0.93</td>
</tr>
<tr>
<td>T3.1REF</td>
<td>541689; 4556656</td>
<td>4.3</td>
<td>0.01</td>
<td>31.4</td>
<td>3.20</td>
</tr>
<tr>
<td>T3.2</td>
<td>541525; 4556024</td>
<td>4.6</td>
<td>0.01</td>
<td>35.8</td>
<td>1.88</td>
</tr>
<tr>
<td>T3.3</td>
<td>541332; 4555852</td>
<td>7.7</td>
<td>0.04</td>
<td>106.9</td>
<td>3.04</td>
</tr>
</tbody>
</table>

The table 1 shows the results of the analysis of physicochemical parameters performed for all samples in which can be noticed relatively low pH values recorded for all samples, ranging between [2.9 – 4.6], except T3.3 sample that had a slightly higher value (pH=7.7). It should be noted that this point lies within the heap, where construction material was dumped, so it is likely to justify this slightly high value. It is also important to highlight the fact that the pH of the reference soil (T3.1REF), used for ecotoxicological tests was relatively low, but similar to the other soils collected in the area. It is worth mentioning that the sample T1.2 had two extreme values, the lowest (2.9) for pH and the highest (237.0) for electrical conductivity, respectively. So, the low values of pH, which characterized the whole set of the soil samples collected in S. Pedro da Cova abandoned coal mining area indicated a lack of neutralizing capacity and the acidic nature of the soil, likely resulting in further dissolution of minerals and the release of toxic metals and other constituents into soil and waterways (Tiwary, 2000). According to Flores et al., (1988) and Howat (2000), when soil pH is low the toxicity of metals becomes more available and often affect plants in terms of nutrient deficiency due to leaching of nutrients from acidic soils and similarly, soil invertebrates and microorganisms handle soil acidity differently and show very different tolerance to low pH. In addition, the mobilization of many metals in soils increases with increasing
acidity (Dempsey et al., 1993). The pH affects the surface charge of metal adsorbents, the degree of ionization, the speciation of surface functional groups and the ionic species of the metal (Guo et al., 2006). A similar study (Tiwary, 2000) in India showed that the overburden dumps around the mines of the coalfields (Jharia Coalfields, Raniganj Coalfields, Central Coalfields Ltd.) were highly acidic having pH values of even 2.65 as a direct result of acid drainage. In terms of the organic matter content (OMC), according to USEPA (2004), soils can be categorised into: i) low organic matter content (<2%) – in this case, there were not recorded sample values that fit into this category; ii) intermediary organic matter content (2% ≤ OMC < 6%) – soil sample T3.2 and iii) high organic matter content (≥6%) – soil samples: T0.0, T1.1, T1.2, T1.3, T1.4, T2.1, T2.2, T2.3, T3.1REF, T3.3. Focused on the OMC, the most notably is the soil T2.1 followed by the soils T1.3, T1.4, T2.3 that recorded high values of the organic matter content favouring soil adsorption and restricts metal bioavailability. Organic matter plays a significant role obfuscating the presence of metals in soils (chemical buffering), the changes in pH and being considered as an indicator of the soil quality (Guo et al., 2006). High quantities of fresh organic matter content were also found by Ribeiro et al. (2010) due to spontaneous combustion of coal. Finally, water holding capacity, which is directly influenced by the soil texture, moisture, salinity and structure, ranged from 11.2% to 43.2% with the exception of the highest value recorded in soil T1.2, which could indicate a higher presence of soluble salts.

<table>
<thead>
<tr>
<th>Tab 2: pH optimum and tolerance of soil test organisms (Chapman et al., 2013)</th>
<th>pH optimum</th>
<th>pH tolerance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. andrei</strong></td>
<td>5.0 – 7.0</td>
<td>4.0 – 9.0</td>
<td>Jänsch et al. (2005a)</td>
</tr>
<tr>
<td><strong>Microtox® V. fischeri</strong></td>
<td>6.0 – 8.0</td>
<td>NA</td>
<td>Environment Canada, 1992</td>
</tr>
<tr>
<td><strong>A. sativa</strong></td>
<td>5.3 – 8.5</td>
<td>&gt;3.8</td>
<td>Bilski and Foy, 1987; US Department of Agriculture, 2012</td>
</tr>
<tr>
<td><strong>B. rapa</strong></td>
<td>5.0 – 8.0</td>
<td>4.2 – 7.8</td>
<td>US Department of Agriculture, 2012; Plants for a future, 1996e2012</td>
</tr>
</tbody>
</table>
Tab. 3: Total metal concentrations (mg/kg) in the soil samples collected in São Pedro da Cova abandoned coal mining area and Hazard concentrations for 5% of species (HC5) proposed by Jänsch et al. (2007).

<table>
<thead>
<tr>
<th>Transect</th>
<th>Cu</th>
<th>Pb</th>
<th>Zn</th>
<th>As</th>
<th>Cd</th>
<th>Cr</th>
<th>Hg</th>
<th>Ni</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0.0</td>
<td>32.63</td>
<td>39.47</td>
<td>49.2</td>
<td>33.3</td>
<td>0.13</td>
<td>6.2</td>
<td>3.927</td>
<td>10.2</td>
<td>175.057</td>
</tr>
<tr>
<td>T1.1</td>
<td>23.39</td>
<td>31.31</td>
<td>32.8</td>
<td>10.5</td>
<td>0.12</td>
<td>8.2</td>
<td>0.319</td>
<td>5.9</td>
<td>112.539</td>
</tr>
<tr>
<td>T1.2</td>
<td>23.57</td>
<td>30.94</td>
<td>51.8</td>
<td>16.4</td>
<td>0.01</td>
<td>4.4</td>
<td>0.583</td>
<td>4.7</td>
<td>132.403</td>
</tr>
<tr>
<td>T1.3</td>
<td>36.09</td>
<td>14.07</td>
<td>46.4</td>
<td>18.1</td>
<td>0.06</td>
<td>25.3</td>
<td>0.084</td>
<td>9.2</td>
<td>149.304</td>
</tr>
<tr>
<td>T1.4</td>
<td>20.63</td>
<td>21.05</td>
<td>80.0</td>
<td>6.2</td>
<td>0.11</td>
<td>28.0</td>
<td>0.093</td>
<td>19.3</td>
<td>175.383</td>
</tr>
<tr>
<td>T2.1</td>
<td>118.13</td>
<td>54.38</td>
<td>90.2</td>
<td>17.9</td>
<td>0.14</td>
<td>30.7</td>
<td>0.4</td>
<td>13.7</td>
<td>325.55</td>
</tr>
<tr>
<td>T2.2</td>
<td>42.83</td>
<td>64.34</td>
<td>166.1</td>
<td>47.6</td>
<td>0.4</td>
<td>13.8</td>
<td>3.226</td>
<td>10.9</td>
<td>349.196</td>
</tr>
<tr>
<td>T2.3</td>
<td>45.76</td>
<td>49.88</td>
<td>84.5</td>
<td>27.8</td>
<td>0.21</td>
<td>7.00</td>
<td>2.136</td>
<td>23.0</td>
<td>240.286</td>
</tr>
<tr>
<td>T3.1REF</td>
<td>45.04</td>
<td>15.05</td>
<td>31.7</td>
<td>19.7</td>
<td>0.05</td>
<td>23.5</td>
<td>0.044</td>
<td>4.9</td>
<td>139.984</td>
</tr>
<tr>
<td>T3.2</td>
<td>51.6</td>
<td>47.14</td>
<td>115.9</td>
<td>17.5</td>
<td>0.21</td>
<td>17.3</td>
<td>0.572</td>
<td>16.3</td>
<td>266.522</td>
</tr>
<tr>
<td>T3.3</td>
<td>32.12</td>
<td>53.03</td>
<td>97.0</td>
<td>14.8</td>
<td>1.26</td>
<td>20.2</td>
<td>0.233</td>
<td>13.6</td>
<td>232.243</td>
</tr>
</tbody>
</table>

HC5[a]  | 55.00| 163.5| 160.3| 5.63 | 6.78 | 5.02 | 1.18 | 64.0 |

Bold letter indicates metal concentrations above HC5 values and also the highest values in terms of total content in metals.

The amount of metals found in soil samples of the abandoned S. Pedro da Cova coal mining area was reported in Tab. 3, based on ICP-MS method. In general, metals were detected in high concentration in all soil samples. Particularly, As and Cr were detected in high concentration for all soil samples, except soil T1.2 in which it was recorded a Cr concentration lower than the HC5 reference value. However, the most worrisome is the fact that all the values recorded exceeded the hazard concentration indicated for the soils (HC5) for 5% of species (Jänsch et al., 2007). Combining the total metal concentration, soil samples T2.1, T2.2, T2.3 and T3.2 presented high level of metal concentration (depicted in Tab. 3). In comparison with others, the most distinctive soil sample in individual metal concentration was T2.2 (the highest concentrations in Zn, As, Cr and Hg), followed by T2.1 (Cu, As, Cr) and T3.2 (Zn, As and Cr). The transect that showed the most contaminated soil samples was T2, in terms of total metals content, followed by T3. The lowest concentration in metals was observed for Pb, Cd and Ni. Whereas for Cu the concentrations were low except for soil sample T2.1. For Zn and Hg slightly lower values were recorded except for soil samples T2.2, T3.2 and T2.2, T2.3, respectively. A gradient of contamination was as expected however, in oppositions with expectations, the concentrations increased from the centre to the extreme. The reference sample T3.1REF, had the lowest metal concentration for this transect and located outside the mining area. In general, it should be assumed that the acidity of soil samples (pH<6.±0.5) recorded might be related to the high metal concentration causing negative toxic effects in plants, soil invertebrates...
and microorganisms, both by direct effects of acidity and by increasing the bioavailability of metals. In a similar case study, Ribé et al. (2012) showed that the toxicity of the samples was most likely caused by the high metal concentration in the soil.

3.2. Ecotoxicological line of evidence: ecotoxicological tests with the whole soil matrix and soil elutriates

3.2.1. Avoidance test with Eisenia andrei Bouché

Due to the geology of the area, it was difficult to find a natural soil from the carboniferous similar to the soils of the contaminated area (Ribeiro et al., 2010) that could be considered from the beginning a reference soil as they were located in a very narrow zone close to the exploitation area. Therefore, the avoidance assay was performed by using OECD as a reference soil, to later select a reference soil for the other assays and for risk calculation. Figure 6 displays the results of the avoidance assay performed with E. andrei. All the validity criteria of the ISO standard protocol (ISO, 2008a) were fulfilled, and no significant avoidance was recorded for the dual control chambers (Fisher’s exact test: p=0.344). All the other soils were highly significantly avoided or preferentially selected by earthworms (Fischer’s exact test: p<0.01), except for T1.4, T2.2 and T3.3 (Fischer’s exact test: p<0.05) which were only significantly avoided. Surprisingly even the soil collected in the mine heap was significantly preferred by earthworms. According to Natal da Luz et al. (2004, 2008), soils with low pH and low organic matter content are avoided by these organisms, which could elucidate the highly significantly avoidance response for soils T0.0, T1.1,
and T1.3 (low pH but high organic matter content, and high levels of As and Cr concentrations, respectively). This could be explained by the fact that *E. andrei* is not a real soil dwelling species, but compost and dung heap species that is easily cultured in laboratory by using horse dung as substrate, thus preferring substrates with a high percentage of organic matter (Hauschild, ). Lock *et al.* (2000); Lock & Janssen (2001) emphasised that organic matter decreases the bioavailability of metals in soil and *earthworms* and other faunal organisms are directly affected by organic matter, so *E. andrei* being a compost worm, is known to prefer soils with high organic matter content because it serves as a source of energy. Thus, the avoidance assay may not be adjusted for soils with such high organic matter content, as it can mask the contamination confounding the selection of soils by earthworms. To check this, the assays could be performed with OECD artificial soil with 10% of OM. In addition to the analysis of total metal contents, T3.1 soil was selected as REF as it was the soil with slightly lower avoidance percentage, and it was located at the South-southwest of the pile. Earthworms are intimately involved in organic matter decomposition (for example, of surface leaf litter and the remineralisation and humification of organic matter) (Smith *et al.*, 2006).
3.2.2. Bioluminescence immobilization test with bacteria *Vibrio fischeri* Beijerinck (Microtox®Omni, Basic Solid-Phase test)

Tab. 4: Shows the results of the acute effects of the bioluminescence assay with *V. fischeri* Beijerinck obtained with the Microtox® BSPT protocol for each soil sample. The highlighted values are those very toxic soil samples.

<table>
<thead>
<tr>
<th>Transect</th>
<th>ECx Values</th>
<th>95% CI</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0.0</td>
<td>EC50&lt;sub&gt;5min&lt;/sub&gt;=4247mg/L</td>
<td>2018 to 8939</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;15min&lt;/sub&gt;=2973mg/L</td>
<td>1476 to 5986</td>
<td>0.8544</td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;30min&lt;/sub&gt;=2064mg/L</td>
<td>1090 to 3908</td>
<td></td>
</tr>
<tr>
<td>T1.1</td>
<td>EC50&lt;sub&gt;5min&lt;/sub&gt;=16910mg/L</td>
<td>13112 to 21808</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;15min&lt;/sub&gt;=17458mg/L</td>
<td>15575 to 19568</td>
<td>0.9761</td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;30min&lt;/sub&gt;=13579mg/L</td>
<td>11675 to 15793</td>
<td></td>
</tr>
<tr>
<td>T1.2</td>
<td>EC50&lt;sub&gt;5min&lt;/sub&gt;=3109mg/L</td>
<td>2622 to 3686</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;15min&lt;/sub&gt;=2274mg/L</td>
<td>1449 to 3570</td>
<td>0.9981</td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;30min&lt;/sub&gt;=2487mg/L</td>
<td>2038 to 3035</td>
<td></td>
</tr>
<tr>
<td>T1.3</td>
<td>EC50&lt;sub&gt;5min&lt;/sub&gt;=22572mg/L</td>
<td>19402 to 26260</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;15min&lt;/sub&gt;=27578mg/L</td>
<td>23548 to 32296</td>
<td>0.9969</td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;30min&lt;/sub&gt;=19526mg/L</td>
<td>14248 to 26758</td>
<td></td>
</tr>
<tr>
<td>T1.4</td>
<td>EC50&lt;sub&gt;5min&lt;/sub&gt;=8747mg/L</td>
<td>5311 to 8570</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;15min&lt;/sub&gt;=8225mg/L</td>
<td>7446 to 9086</td>
<td>0.9959</td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;30min&lt;/sub&gt;=7190mg/L</td>
<td>4390 to 11775</td>
<td></td>
</tr>
<tr>
<td>T2.1</td>
<td>EC50&lt;sub&gt;5min&lt;/sub&gt;=4089mg/L</td>
<td>2153 to 7764</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;15min&lt;/sub&gt;=5708mg/L</td>
<td>3640 to 8952</td>
<td>0.9051</td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;30min&lt;/sub&gt;=4258mg/L</td>
<td>2397 to 7562</td>
<td></td>
</tr>
<tr>
<td>T2.2</td>
<td>EC50&lt;sub&gt;5min&lt;/sub&gt;=16689mg/L</td>
<td>13240 to 21035</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;15min&lt;/sub&gt;=13192mg/L</td>
<td>8750 to 19889</td>
<td>0.9950</td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;30min&lt;/sub&gt;=7374mg/L</td>
<td>4682 to 11613</td>
<td></td>
</tr>
<tr>
<td>T2.3</td>
<td>EC50&lt;sub&gt;5min&lt;/sub&gt;=5604mg/L</td>
<td>5309 to 8215</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;15min&lt;/sub&gt;=5934mg/L</td>
<td>4258 to 8269</td>
<td>0.9924</td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;30min&lt;/sub&gt;=5316mg/L</td>
<td>3919 to 7210</td>
<td></td>
</tr>
<tr>
<td>T3.1REF</td>
<td>EC50&lt;sub&gt;5min&lt;/sub&gt;=14129mg/L</td>
<td>13154 to 15177</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;15min&lt;/sub&gt;=15938mg/L</td>
<td>10783 to 23556</td>
<td>0.9996</td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;30min&lt;/sub&gt;=6541mg/L</td>
<td>3799 to 11262</td>
<td></td>
</tr>
<tr>
<td>T3.2</td>
<td>EC50&lt;sub&gt;5min&lt;/sub&gt;=20432mg/L</td>
<td>12434 to 33579</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;15min&lt;/sub&gt;=21287mg/L</td>
<td>13357 to 33926</td>
<td>0.9251</td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;30min&lt;/sub&gt;=19617mg/L</td>
<td>11640 to 33061</td>
<td></td>
</tr>
<tr>
<td>T3.3</td>
<td>EC50&lt;sub&gt;5min&lt;/sub&gt;=45078mg/L</td>
<td>12258 to 165769</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;15min&lt;/sub&gt;=51546mg/L</td>
<td>8069 to 329829</td>
<td>0.6769</td>
</tr>
<tr>
<td></td>
<td>EC50&lt;sub&gt;30min&lt;/sub&gt;=52101mg/L</td>
<td>9363 to 289932</td>
<td></td>
</tr>
</tbody>
</table>

The classification of the toxicity of soil samples was taken in accordance with that proposed by Kwan & Dutka (1995): samples with values of EC50 = <5000mg/L<sup>-1</sup> (very toxic) – T0.0, T1.2 and T2.1 but, this latter sample showed moderate toxicity to *V. fischeri* at 15 minutes exposure; samples with 5000mg/L<sup>-1</sup> < EC50 = <10,000mg/L<sup>-1</sup> (moderately toxic) – T1.4 and T2.3, and with EC50 > 10,000mg/L<sup>-1</sup> (non toxic) – T1.1, T1.3, T2.2, T3.1REF, T3.2 and T3.3 (Pereira et al., 2011). In this way, samples T0.0, T1.2 and T2.1 displayed lower values of EC50 which means higher level of toxicity which may have been influenced by the physicochemical characteristics of the soil (pH, EC and high levels of Cu, As and Cd). These results came in agreement with the observations reported by Maisto et al. (2011), in which the very toxic soil samples seem to be strongly affected by the soil metal bioavailability, also reported in this study.
Despite the high toxicity that both soils samples T1.2 and T2.1 presented on this assay, they were significantly preferred by earthworms in the avoidance test, probably due to high organic matter content used as source of carbon and food to satisfy its energy requirement, (Niemeyer et al., 2012). The lack of a toxic response observed on the bioluminescence test for soil samples: T1.1, T1.3, T1.4, T2.2, T2.3, T3.1 REF, T3.2 and T3.3 suggested that the contaminants in this soil could not be biologically available or.

As expected, the gradient of contamination on this assay showed that the toxicity varies increasing from the periphery to the centre of the heap.

3.2.3. Plant germination and growth test

According to the standard protocol ISO 11269-2 (ISO, 1995) it was measured the production capacity of the contaminated soil (germination, fresh mass and dried mass). This test was based on the emergence and early growth response of the following terrestrial plant species: *Avena sativa*, *Zea mays* and *Brassica rapa* added to the soil samples from the abandoned coal mine. As the results show, germination, fresh and dry mass parameters were differently influenced by soil contamination (Fig. 7, 8, 9, 10, 11, 12, 13, 14 and 15).

![Avena sativa](image)

**Fig. 7**: Average values of the seeds emergency recorded for *Avena sativa* (oats) after exposition in soil samples and OECD artificial soil. The error bars correspond to standard deviation (Dunnett’s test, p ≤ 0.05).
Fig. 8: Average weight values of fresh mass recorded for *Avena sativa* (oats) after 14 days of exposition in soil samples and OECD artificial soil. The error bars correspond to standard deviation and the significant differences in fresh mass weights are represented by * indicating to significant differences from the control group equal to reference soil (Dunnett’s test, p ≤ 0.05).

Fig. 9: Average weight values of dry mass recorded for *Avena sativa* (oats) after 14 days of exposition in soil samples and OECD artificial soil. The error bars correspond to standard deviation and the significant differences in dry mass weights are represented by * indicating to significant differences from the control group equal to reference soil (Dunnett’s test, p ≤ 0.05).
**Fig. 10:** Average values of the seeds emergency recorded for *Zea mays* (maize) after exposition in soil samples and OECD artificial soil. The error bars correspond to standard deviation (Dunnett’s test, \( p \leq 0.05 \)).

**Fig. 11:** Average weight values of fresh mass recorded for *Zea mays* (maize) after 14 days of exposition in soil samples and OECD artificial soil. The error bars correspond to standard deviation and the significant differences in fresh mass weights are represented by * indicating significant differences from the control group equal to reference soil (Dunnett’s test, \( p \leq 0.05 \)).
**Zea mays**

![Graph showing average weights of dry mass for Zea mays](image)

Fig. 12: Average weight values of dry mass recorded for Zea mays (maize) after 14 days of exposition in soil samples and OECD artificial soil. The error bars correspond to standard deviation and the significant differences in dry mass weights are represented by * indicating significant differences from the control group equal to reference soil (Dunnett’s test, $p \leq 0.05$).

**Brassica rapa**

![Graph showing average values of seeds emergency for Brassica rapa](image)

Fig. 13: Average values of the seeds emergency for Brassica rapa (cabbage) after exposition in soil samples and OECD artificial soil. The error bars correspond to standard deviation and the significant differences of the emergencies are represented by * indicating significant differences from the control group equal to reference soil (Dunnett’s test, $p \leq 0.05$).

**Brassica rapa**

Fig. 14: Average weight values of fresh mass recorded for Brassica rapa (cabbage) after 14 days of exposition in soil samples and OECD artificial soil. The error bars correspond to standard deviation and the significant differences in fresh mass weights are represented by * indicating to significant differences from the control group equal to reference soil (Dunnett’s test, $p \leq 0.05$).

**Brassica rapa**

Fig. 15: Average weight values of dry mass recorded for Brassica rapa (cabbage) after 14 days of exposition in soil samples and OECD artificial soil. The error bars correspond to standard deviation and the significant differences in dry mass weights are represented by * indicating to significant differences from the control group equal to reference soil (Dunnett’s test, $p \leq 0.05$).

**a) Endpoint: Emergency of seeds**

Regarding to this parameter, it was verified that the emergency endpoint of the three terrestrial plant species was the less responsive to the contaminated soil samples. Once analysis of variance (ANOVA) were made regarding to the germination endpoint, significant differences were recorded for Avena sativa ($F=2.0760; P=0.049; \text{d.f. (error; total)} = 36; 47$, depicted in Fig. 7) and Zea mays ($F=4.1110; P<0.01; \text{d.f. (error; total)} = 36; 47$, depicted in Fig. 10). However, the Dunnett's test did not detect a significant reduction in this parameter when compared with T3.1$_{\text{REF}}$ reference soil. There were very significant
differences recorded between soil samples for this parameter and for *Brassica rapa* (F=9.2422; P<0.01; d.f. (error; total) = 36; 47, depicted in fig. 13) and according the Dunnet’s test soils T1.2, T1.3, T1.4 and T2.1 displayed percentages of seed's emergence significantly lower from the T3.1REF reference soil (Dunnett’s test, p ≤ 0.01). And these highly significant differences may be due to the fact that *Brassica rapa* is a very sensitive plant species. No significant differences were recorded for this parameter and for the three species tested between the OECD artificial soil and the T3.1REF, thus meaning that the effects observed were related with seeds quality, but with the phytotoxicity of the soils.

b) Endpoint: Fresh biomass

As far as fresh biomass was considered once again phytotoxic effects were recorded only for soil samples from transect T1. ANOVAs were made regarding to the fresh biomass endpoint and very significant differences were reported for *Avena sativa* (F=7.6519; P<0.01; d.f. (error; total) =36; 47, depicted in Fig. 8) and *Zea mays* (F=11.1901; P<0.01; d.f. (error; total) =36; 47, depicted in Fig. 11) and for *Brassica rapa* (F=16.3739; P<0.01; d.f. (error; total) =36; 47, depicted in fig. 14). Fresh biomass corresponding to soil samples T1.2, T1.4, (*Avena sativa*) T1.2 (*Zea mays*) and T1.2, T1.3, T1.4 (*Brassica rapa*) was very significantly lower when compared with soil T3.1REF (Dunnett’s test, p ≤ 0.01). No significant differences were recorded between the the control group OECD soil, and the reference soil.

c) Endpoint: Dried biomass

Regarding this parameter, the ANOVAs performed showed very significant differences for *Avena sativa* (F=6.9315; P<0.01; d.f. (error; total) = 36; 47, depicted in Fig. 9) and *Zea mays* (F=7.8286; P<0.01; d.f. (error; total) =36; 47, depicted in Fig. 12) and for *Brassica rapa* (F=15.3765; P<0.01; d.f. (error; total) =36; 47, depicted in fig. 15). Again T1.2 for *Avena sativa* and *Zea mays* and T1.2, T1.3, T1.4 for *Brassica rapa* have significantly compromised the production of dry biomass by plants. For these soils the dry biomass produced was significantly very lower from the control group which was equal to reference soil (Dunnett’s test, p ≤ 0.01).

In general lines, *Avena sativa* and *Zea mays* are less sensitive species to contamination, while *Brassica rapa* was more sensitive to contamination. However, very high levels of metals such as As and Cr were found above the HC5 (Table 3) in all
soil samples. Several studies indicate little knowledge regarding to the levels of toxicity of metals in plants (MARQUES et al., 2000).

3.2.4. Mortality or immobilisation acute test with *Daphnia magna* Straus

Regarding the acute assays with *D. magna* with soil elutriates an EC50 value was calculated only for soil T1.3 (EC50=70.48%; 95%CI: 39.59%), which gave rise to the most toxic extract. For the other elutriates, EC50 values greater than 100% were recorded, because percentages of mortality higher than 50% were recorded only for the non-diluted sample (T0.0, T1.1, T1.2, T2.1, T2.2) or because percentages of mortality lower than 50% were recorded even for the non-diluted elutriate (Fig. 16).

3.3. Risk Characterisation

<table>
<thead>
<tr>
<th>Soil Sample</th>
<th>ChemLoE</th>
<th>Ecotox LoE</th>
<th>Integrated Risk</th>
<th>STDEV</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0.0</td>
<td>0.6823</td>
<td>0.5578</td>
<td>0.6252</td>
<td>0.1522</td>
</tr>
<tr>
<td>T1.1</td>
<td>0</td>
<td>0.14992</td>
<td>0.31806</td>
<td>0.18339</td>
</tr>
<tr>
<td>T1.2</td>
<td>0</td>
<td>0.53495</td>
<td>0.75452</td>
<td>0.6544</td>
</tr>
<tr>
<td>T1.3</td>
<td>0</td>
<td>0.49112</td>
<td>0.59558</td>
<td>0.2856</td>
</tr>
<tr>
<td>T1.4</td>
<td>0</td>
<td>0.75452</td>
<td>0.27106</td>
<td>0.81549</td>
</tr>
<tr>
<td>T2.1</td>
<td>0</td>
<td>0.59558</td>
<td>0.19339</td>
<td>0.5755</td>
</tr>
<tr>
<td>T2.2</td>
<td>0</td>
<td>0.923</td>
<td>0.13951</td>
<td>0.015631</td>
</tr>
<tr>
<td>T2.3</td>
<td>0</td>
<td>0.923</td>
<td>0.19339</td>
<td>0.015631</td>
</tr>
<tr>
<td>T3.1/REF</td>
<td>0.49669</td>
<td>0.49669</td>
<td>0.49669</td>
<td>0.015631</td>
</tr>
<tr>
<td>T3.2</td>
<td>0.19846</td>
<td>0.19846</td>
<td>0.19846</td>
<td>0.015631</td>
</tr>
<tr>
<td>T3.3</td>
<td>0.01194</td>
<td>0.01194</td>
<td>0.01194</td>
<td>0.015631</td>
</tr>
</tbody>
</table>

Risk values varying between 0 and 1. Orange colour highlights intermediate risks (0.5=<risk<0.8); red colour for high risk levels (risk>=0.8). Red colour in STDEV highlights values above 0.4.

Finally, the integrated results of the different tests in each line of evidence (LoE) indicated an acceptable (intermediate) environmental risk for sampling points T0.0, T1.4, T2.2 and T3.2, with the exception of T1.1, T2.1, T3.1REF and T3.3. Additionally, it
is recommended further investigation for soil samples in which the standard deviation between the risks calculated for both lines of evidence is above 0.4. This was recorded for sampling points T1.2, T1.3, T1.4, T2.2 and T2.3. At the sampling point T1.4, although there is no chemical risk the calculated integrated risk is higher than 0.5 and the standard deviation between the different LoE is very high also, which suggests that the ecotoxicological effects recorded could have been caused by the physicochemical features of the soil samples, rather the by metals. Unlikely, soil sample T2.2 indicated high calculated risk for Chemical LoE and very low risk for Ecotox LoE giving an indication of the potential low bioavailability of metals in this soil to exert toxic effects. For this soil, the integrated risk was high but the standard deviation was also high. Therefore, further ecotoxicological and chemical analyses, or alternatively a weighting of the data available is required for a more reliable assessment. The acidic pH recorded for T1.2, T1.3 and T1.4 may lie behind the high level of toxic effect observed for this transect.
CHAPTER IV: CONCLUSIONS AND RECOMMENDATIONS

- Anthropogenic actions such as mining, former mining waste piles and other harmful sources can result in negative adverse impacts to both environment and humans due to the toxic effects of the metals and other compounds, so that contaminated soils require a deep evaluation of risks to support remediation actions;
- Bioavailable fractions of metals into soil compartment can usually be toxic to animals, plants and microorganisms and its exposure is often made via food chain and generally can result in chronic or acute effects;
- Triad based ERA is a very useful and standardised tool to estimate the risk in the environment, integrating information from three lines of evidence (i.e., chemistry, ecotoxicology, and ecology) by measuring diverse endpoints that play a key role in the structure and function of the ecosystems (Jensen and Mesman 2006) and demonstrating whether or not the chemical residues found in contaminated sites are biologically significant;
- In terms of the physicochemical parameters, the nature of the soil samples were generally acidic and with a high organic matter content;
- The high values of total metal concentration were recorded in soil samples T2.1, T2.2, T2.3 and T3.2, despite the fact that high values of As and Cr were observed in all soil samples, which indicates an eminent potential hazard to the environment;
- However, the final integrated risks were higher for soil samples: T1.2, T1.3, T1.4 and T2.2.
- However, the variation between the risks calculated for the Chemical and the ecotoxicological lines of evidence, indicate that the evaluation should proceed at least for some soils toward to Tier 2 which could provide more refined data;
- The proposed Dutch framework for ecological risk assessment as proven to be a cheaper, efficient and valuable tool to support the decision making process;
References

13. Fernández, María Dolores; Vega, Maria Milagrosa; Tarazona, José Vicente 2006. Risk-based ecological soil quality criteria for the characterization of contaminated soils: Combination of chemical and biological tools. Science Direct;

27. Kwan, K.K., Dutka, B.J., 1995. Comparative assessment of two solid-phase toxicity bioassays: the direct sediment toxicity testing procedure (DSTTP) and the Microtox Solid Phase Test (MSPT), Bull. Environ. Contam. Toxicol. [55];


31. Maisto, Giulia, Manzo, Sonia; De Nicola, Flavia; Carotenuto, Rita; Rocco, Annamaria; Alfani, Anna 2011. Assessment of the effects of Cr, Cu, Ni and Pb soil contamination by ecotoxicological tests. J. Environ. Monit., 13, 3049


36. OECD 1984. OECD guidelines for testing of chemicals 208: Terrestrial Plants, growth Test;


42. Qiu, Guangle; Feng, Xinbin; Wang, Shaofeng; Shang, Lihai 2004. Mercury and Methylmercury in Riparian Soil, Sediments, Mine-Waste Calcines, and Moss from Abandoned Hg Mines in East Guizhou Province, Southwestern China. Science Direct;


44. Ribé, Veronica; Aulenius, Elisabet; Nehrenheim, Emma; Martell, Ulrika; Odlare, Monica 2012. Applying the triad method in a risk assessment of a former surface treatment and metal industry site. Journal of Hazardous Materials;


52. Swartjes, Frank A., Carlon, Claudio; Wit, Niek H. S. M., 2008. The possibilities for the eu-wide use of similar ecological risk-based soil contamination assessment tools. Science Direct;
54. Tiwary, R. K., 2000. Environmental impact of coal mining on water regime and its management. Central Mining Research Institute, Barwa Road, Dhanbad, Bihar, India
58. Yenilmez, Firdes; Kuter, Nazan; Emil, Mustafa Kemal; Aksoy Aysegul 2010. Evaluation of pollution levels at an abandoned coal mine site in turkey with the aid of GIS. International Journal of Coal Geology;