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Departamento de Biologia

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Derivação de Valores de Triagem para Metais num Solo Natural Português

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Prof. Doutora Ruth Maria de Oliveira Pereira, Professora Auxiliar Departamento de Biologia da Faculdade de Ciências da Universidade do Porto, do Doutor Fernando José Mendes Gonçalves, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro, e do Prof. Doutor Eduardo Anselmo Ferreira da Silva, Professor Catedrático do Departamento de Geociências da Universidade de Aveiro

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palavras-chave   Valores de triagem do solo; urânio; cadmio; cobre; solos naturais; actividade enzimática do solo; testes de reprodução com invertebrados; germinação e crescimento de plantas; factores de avaliação; curvas de distribuição da sensibilidade de espécies.

resumo   O aumento das atividades humanas tem sido responsável por mudanças profundas e por uma degradação contínua do compartimento solo, em todo o território Europeu. Em resposta a este problema, algumas políticas Europeias estão agora a emergir orientadas especificamente para a proteção do solo e para a gestão das áreas contaminadas, a fim de recuperar os solos degradados para outros usos. Para regulamentar a avaliação de risco e a gestão de solos contaminados, muitos Estados-Membros Europeus adoptaram valores de qualidade do solo, como por exemplo os "valores de rastreio ou triagem" (do inglês: soil screening values ou SSVs). Estes valores são particularmente úteis para a primeira etapa dos processos de avaliação de risco ecológico (ARE) de locais contaminados, especialmente para um primeiro rastreio dos locais, destinado a separar aqueles em que os riscos são claramente reduzidos daqueles que exigem uma avaliação mais específica e aprofundada para o local. Assim, a definição de SSVs regionais terá impactos económicos relevantes na gestão dos locais contaminados. Portugal é um dos Estados-Membros Europeus que ainda não definiu SSVs. Neste contexto, este estudo dá uma notável contribuição na geração de dados ecotoxicológicos para parâmetros microbiológicos do solo, plantas terrestres e invertebrados necessários para a obtenção de SSVs para urânio (U), cádmio (Cd) e cobre (Cu), utilizando um solo natural Português, representante de um tipo dominante de solo existente no território nacional.

Assim, foram obtidos SSVs para os metais referidos com base em dois métodos propostos pelo Documento de Orientação Técnica para Avaliação de Riscos da Comissão Europeia, nomeadamente o método dos factores de avaliação (do inglês: assessment factors ou AF) e o método probabilístico da distribuição da sensibilidade espécies (do inglês: species sensitivity distributions ou SSDs) (com algumas adaptações). Os resultados dos dois métodos foram comparados e discutidos. Além disso, este estudo lançou as bases para uma reflexão mais profunda sobre o ponto de corte (concentração de risco para uma determinada percentagem de espécies) a ser estimado a partir das distribuições de sensibilidade das espécies (SSDs), e para ser selecionado para a obtenção de SSVs, com o nível adequado de proteção. Neste estudo foi comprovado que esta seleção pode variar para diferentes metais ou outros contaminantes, no entanto, uma justificação clara deve ser dada, em cada caso.

Os SSvs propostos neste estudo foram de: U (151,4 mg U kg^{-1}_{ms}), Cd (5,6 mg Cd kg^{-1}_{ms}) e Cu (58,5 mg Cu kg^{-1}_{ms}) Estes valores devem agora ser testados quanto à sua capacidade para descriminar solos com diferentes níveis de contaminação. No entanto, este estudo esclarece e sugere a abordagem que deve ser seguida para a derivação de SSVs para outros metais e contaminantes orgânicos, e para outros tipos dominantes de solos naturais portugueses.

Key words

Soil screening values; uranium; cadmium; copper; natural soil; soil enzymes activity; invertebrates reproduction tests; plants seed emergency and growth tests; assessment factors; species sensitivity distributions.

abstract

The increasing human activity has been responsible by profound changes and a constinuos degradation of the soil compartment in all the European territory. Some European policies are appearing focusing soil's protection and the management of contaminated sites, in order to recover land for other uses. To regulate the risk assessment and the management of contaminated soils, many European member states adopted soil guideline values, as for example soil screnning values (SSV).These values are particularly useful for the the first tier of the Ecological Risk Assessment (ERA) processes of contaminated sites,especially for a first screening of sites requiring a more site-specific evaluation. Hence, the approriate definition of regional SSVs will have relevant economic impacts in the management of contaminated sites. Portugal is one of European Member States that still lack these soil guideline values. In this context, this study gaves a remarkable contribution in the generation of ecotoxicological data for soil microbiological parameters, terrestrial plants and invertebrates for the derivation of SSVs for uranium (U), cadmium (Cd) and copper (Cu), using a Portuguese natural soil, representative of a dominant type of soil in the Portuguese territory.

SSVs were derived based on two methods proposed by the the Technical Guidance Document for Risk Assessment of the European Commission; namely the assessment factor method (AF) and the species sensitivity distribution (SSD) method (with some adaptations). The outputs of both methods were compared and discussed. Further, this study laid the foundation for a deeper reflection about the cut-off (hazard concentration for a given percentage of species - HCps) to be estimated from the SSDs, and to be selected for the derivation of SSVs, with the adequate level of protection. It was proven that this selection may vary for different contaminants, however a clear justification should be given, in each case.

The SSvs proposed in this study were for: U (151.4 mg U kg^{-1}_{dw}), Cd (5.6 mg Cd kg^{-1}_{dw}), and Cu (58.5 mg Cu kg^{-1}_{dw}) These values should now be tested for their descriminating power of soils with different levels of contamination. However, this studies clarifies the approach that should be followed for the derivation of SSVs for other metals and organic contaminants, and for other dominant types of Portuguese natural soils.

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General Introduction

General Introduction

1.1 The importance of soil and its protection at international and national level

Soil is the upper layer of the earth crust, with a highly variable thickness, composed of mineral components (mainly silica and several trace metals) resulting from the weathering of bedrock and other parent materials, organic matter , water, air and living organisms (Buyer et al., 2010; Gerrard, 2000), and occupies most of our planet's continental areas. For Singer & Warkentin, (1996), soils are the excited skin of the earth where the atmosphere, hydrosphere, biosphere and pedosphere meet and interact, in an open system. Soil is the ecosystems life substrate, as one finds in it an ample quantity and diversity of living organisms (microorganisms, plants, animals), constituting an abundant biodiversity habitat, with unique genetic patterns. A piece of soil that could be held by our hand may contain hundreds of thousands or even millions of species living in, which benefit from the great number of ecological niches provided by the extremely heterogeneous structure of the soil (Buyer et al., 2010),These organisms are parts of the soil and their activity is vital for its own functioning, as they are responsible for the organic matter decay, nutrient cycling, help in the soil structure formation, thus contributing for the ecosystems stability (Lavelle et al., 2006; Park et al., 2011; Rutgers et al., 2009). The soil is for this reason considered, a living and dynamic medium and represents the main compartment for the global interchange of matter and energy. This compartment plays such necessary functions as the production of a wide range of nourishment and bioenergy (Sparks, 2000), is a physical and nutritive subtract for plants development due to its ability to store water, minerals, organic matter and several chemical substances necessary for the vegetal growth. In addition is an environmental regulator, since besides the storing capacity, it partially transforms compounds flowing between the atmosphere, the hydrosphere and the living organisms, being part of the biochemical and hydrologic cycles. All this characteristics grant it a high buffering capacity strictly related with its organic matter load, which limits the erosion and diffusion of soil contaminants to the water. The soil also performs social economic functions, since it is used by any known society as an important source of raw materials (sands, clays, coal and minerals), as substrate for biomass production, as support for

infrastructures (communication ways, buildings) and for almost all cultural activities (Lavelle et al., 2006; Sampaio, 2004; Swartjes et al., 2008).

However, and despite its importance as a natural resource, the soil keeps being neglected by several human activities (agriculture, industry, mining exploration), which cause deeply adverse effects on its chemical composition, physical structure and its organisms (Cortes et al., 2003; Vaalgamaa and Conley, 2008). Additionally, the soil is a particularly vulnerable medium to external aggressions both from anthropogenic and natural origin, as are the erosion, salinization, acidification, floods and landslides which assume a significant importance on its degradation (Bone et al., 2010; Fullen and Catt, 2004). The decreased fertility, decline in soil organic matter, biodiversity loss, smaller water retention capacity and the interruption of the gaseous cycle and nutrients, are some of the consequences which stems from natural or anthropogenic soil degradation processes. Nevertheless, in the public opinion, air and water are more important than soil, since these are directly used to breathe and drink, while soil only indirectly influences human life. In fact, the relationship between human health and soil quality stills unclear for the general public and the economic value of soil was never perceived even when the conflicts were related with available arable land (Bone et al., 2010). On the other hand, and unlike water and air, the majority of the soil is private property and belongs to people who have their own rights and private interests in the soil use (Römbke et al., 2005). Consequently, and till the nineteen decade, the soil was managed mainly by farmers for increasing fertility and subsequently crop production, without ecological concerns (Postma-Blaauw et al., 2012).

Mainly in the last three decades, the perception of: i) the treats to the soil; ii) the economic and environmental consequences of soil contamination on water and air quality; iii) the loss in biodiversity; iii) the potential impacts of climate change on arable lands and of iv) the non-renewable character of this resource has increasingly revealed its protection a global concern. Despite the efforts to obtain a global assessment of soil degradation, at the end the eighties a great uncertainty persist about the extent, severity and multiple impacts of soil degradation (Hurni et al., 2006). The international documents and agreements specifically dedicated to soil available till then, namely the European Soil

Charter (CE, 1972), the World Soil Charter (FAO, 1982) and the World Soil Policy (UNEP, 1982) did not promote any type of actions on soil protection mainly due to their non-binding nature (Hurni et al., 2006). In 1998, in the 16th World Congress of Soil Sciences (WCSS) of the International Union of Soil Sciences (IUSS) held in Montpelier, specialists agreed on the need of global soil agenda, to the soil on the top of the table of decision-makers and to call the attention of the general public for soil related issues (Hurni et al., 2006). Four years later, a group of specialist was invited to pronounce about the subject, aimed in producing texts for a draft of the soil agenda during the 17th WCSS. The soils specialists agreed about the need of an international soil instrument dedicated exclusively to the soil, to raise awareness about the concerning level of soil degradation worldwide. Nevertheless, they were more confident on the efficiency of international guidelines for soil management rather than on enforceable legislation to protect this resource (Hurni and Meyer, 2002) In 2001, the Convention on Biological Diversity, under the program of work on agricultural biodiversity, recognized the value of the diverse soil biota and of their role in several soil services crucial for the maintenance of all the ecosystems, but especially agro-ecosystems and the maintenance of their production to sustain the growing demand for food (UNEP-CBD, 2000). Despite, highlighting the ecological attributes of soil whose importance go far beyond its production function, the CBD emphasized the benefits of managing soil biodiversity in agricultural systems, in terms of crop production, economic profits and food security (UNEP-CBD, 2000). Under a decision of the Convention of Parties (COP decision IV/6), the parties asked FAO (Food and Agriculture Organization of the United Nations) and other organizations to provide methodologies for the assessment of the biodiversity of agro-systems and tools for their monitorisation (UNEP-CBD, 2000).The CBD, have also highlighted knowledge gaps in terms of soil biodiversity and of the effects of agriculture practices in the soil biota and in their functions (UNEP-CBD, 2000). In 2004, the CBD published a new document describing the context and objectives of the "*International Initiative for the Conservation and Sustainable Use of Soil Biodiversity*" coordinated by FAO, and corresponding strategies and actions to attain these objectives (UNEP-CBD, 2004). Although still mainly focused in agricultural biodiversity, the Convention highlighted the role of soil biota in critical

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ecosystem services and the importance of these services not only for a sustainable agricultural production but also for the functioning of natural ecosystems. In 2006-2007, FAO, had its first external evaluation, and one of the conclusions of the report was that FAO, the organization founded in 1945, was in crisis, mainly due to a shift of research interests to land and climate change research (Hartemink and McBratney, 2008). This report however, considered that land and soils should be a prior area for the allocation of resources, due to the global lack of data required for their management, and the recognition that the pressures on land resources caused by an increasing human population and corresponding demand for agricultural products, urbanization and climate change are expected to persist and to worsen (Hartemink and McBratney, 2008).

Previously to the CBD, only the United Nations Convention to Combat Desertification, established in 1994, focused on a specific soil related problem, the desertification in dry land areas, caused by the combined effect of the over-exploration of resources, inappropriate land use and paucity of rainfall (Fuchs, 2008; UNCCD, 2012)[1]. In fact desertification was the first soil related problem gaining international political attention, after the first United Nations Desertification Conference held in Nairobi, during the seventy decade. However, this convention focused again in the soil as a resource, and its main aim was to restore land and soil productivity to guarantee the sustainability of human populations from these vulnerable areas (UNCCD 2012)[2]. Under the scope of the convention, the parties were also obliged to report the status of land cover, and call the attention of policy makers for the importance of the topic. Despite the efforts the problem was not solved, and a ten year strategic plan (2008-2018) was adopted by the COP-8 (8th Convention of the Parties), to enhance the implementation of the convention (CBD).

Other United Nations networks/organizations, like the UNDP (United Nations Development Program founded in 1965) and the UNEP (United Nations Environment Programme) only after the year 2000, started mentioning soils in their reports, calling the attention, *inter alia*, for the role of global climate changes in the exacerbation of biodiversity loss in this compartment (Hartemink and McBratney, 2008).

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[1] http://www.unccd.int/en/Pages/default.aspx
[2] http://www.unccd.int/en/Pages/default.aspx

Some expectations were placed in the Millennium Ecosystem Assessment (MEA) called by the United Nations Secretary – General Kofi Annan in 2000 - aimed in having information about the consequences of ecosystem change on human well-being and to establish the scientific basis required by decision makers for actions needed to enhance the conservation and sustainable use of ecosystems (MEA, 2005). However, soils did not receive a particular attention, being conceptually considered as an ecosystem service, and once again no meaningful knowledge about the status of soils worldwide was obtained with MEA (Hurni et al., 2006). Meanwhile, countries like the USA, Japan, Canada, Australia and Brazil, have already started establishing soil protection policies (CEC, 2006a). In 2008, the US Congress has adopted a Senate resolution on Soil, which inter alia, emphasized the lack of legislation on soils in USA (Hartemink and McBratney, 2008).

Since 1970 the European Commission was very proliferous in the definition of policies and in the publication of different legal documents aimed in: protecting water resources; managing and reducing solid wastes; landfills management; assess and mitigate the risks of new chemicals produced by the industry, starting by phytopharmaceuticals and then extended to all the chemicals produced by the industry (CEC, 2006a; JRC, 2012). However, any legislation had specifically targeted the protection of soil. The protection of water resources for human consumption was the main focus. In fact this was the priority after the perception of the risks posed to these resources and to the human health (e.g. nitrates contamination of groundwater and eutrophication of surface waters) caused by the intensive application of fertilizers and pesticides by farmers in several European countries (Napier, 1998). Taxes on farm chemicals; the control of their application or even its ban in more sensitive areas, near groundwater resources; education and information programs for farmers; compensations for economic losses caused by the adoption of production systems with lower risks to soil and water resources were some of the measures adopted by some countries and by the Common Agricultural Policy (CAP) of the European Union, after 1992 (Napier, 1998). Several European Member states have also established Water Quality Standards. In 2002 the European Commission took the first step, for the protection of soils, publishing a Communication towards the development of a Thematic Strategy for Soil Protection (CEC, 2002), which was finally published in 2006

(CEC, 2006a), in parallel with a proposal for the Soil Framework Directive (CEC, 2006b). This marks the first central political approaches with the goal to establish a communitarian frame for soil protection and the preservation of its capacity for playing its ecological, economic, social and cultural functions. This Thematic Strategy was published under the scope of the Sixth Environment Action Program of the European Union (2001-2010), whose main priorities were climate change, nature and biodiversity health and quality of life, and natural resources and waste. The strategies are mechanisms for delivering the objectives of the Action Programme, and provide a broad analysis of the issue under consideration, focusing on the pressures and impacts and their link with sectorial policies and suggest a strategy to deal with such pressures and impacts combining market-based approaches, technology and innovation (CEC, 2006a). Each strategy is composed by a former communication (as mentioned above) that highlights the issues and proposes solutions, legislative proposals and an impact assessment.

 In parallel with the a diagnose of the main threats to soils within the European Union (erosion, organic matter decline, compaction, salinization, landslides, sealing contamination), the soil thematic strategy established guideline principles to pursue the objectives of protection and sustainable use of soil, namely: readjust soil uses and management in order to prevent further soil degradation; ii) act directly on sources to reduce the emissions/impacts on soil and to restore degrade soils at least to a level compatible with current or intended uses. In 2012, the EU published a report about the implementation of the Soil Thematic Strategies and ongoing activities (EC, 2012). Since the adoption of the Strategy, the EU has made big efforts for the integration of soil protection on sectorial policies, like the common agricultural policy and the Industrial Emissions Directive (IED, 2010). Further the EU has allocated funds for the rehabilitation of industrial sites and contaminated land as part of the Cohesion Policy, between 2007-2013, as well as for research projects aimed in increasing background knowledge required for action (EC, 2012). However, six years after the adoption of the Soil Thematic Strategy, the main legal document that could arise, addressing systematic tools to monitor and to protect soils across Europe, still without approval (EC, 2012). Without this legal document the foundations to enforce member states to develop legislation on soil protection are

weak, and according to the last thematic report of the European Environment Agency, soil degradation processes have accelerated in many parts of Europe, since 2005, due to inappropriate human uses and due to the lack of harmonized approaches to tackle soil degradation (EEA-JRC, 2010). A number of EU Member States have legislation specific to soil protection. However the majority of this legislation is focused only on soil contamination. Countries like Netherlands, Germany and Belgium, have policies addressing broader soil protection issues. These states are some of the most advanced in questions for soil protection in EU, being the only states with a specific legally binding soil definition (Van Camp et al., 2004).

As previously described global efforts on soil protection have been mainly focused on the view of soil as resource for food production and sustainable development. Research efforts were mainly directed toward the maintenance of soil fertility and more recently on the impacts of climate change on such soil function. The soil as a source for carbon storage, with an important role in the climate control has also received a great attention in the last few years (Lal, 2010; Prechtel et al., 2009).

The UK had a historically dependence on soils for food and fiber production and as a physical support of all activities, especially those related with industrial manufacturing. After the Second World War land-use production was intensified through the use of industrially produced fertilizers and biocides to control pests, and by mechanically based land preparation practices (Haygarth and Ritz, 2009). More recently, one of the main concerns with soil protection in UK (as well as in other member states) is related with their intensive use for construction, since recovery from soil sealing is practically irreversible, representing a definitive loss for any other type of soil use (Haygarth and Ritz, 2009). A postnote document published by the Parliament, in 2006, made a description of the nature and extent of soil degradation in the UK, assuming that about 2.2 million of tons of topsoil were being lost annually, by erosion, in UK (POST, 2006). Despite the perception of the national authorities of the degree of soil degradation, there is no specific UK regulations related with soil protection. Similarly to other member states it is argued that soil has being indirectly protected by other legislation like those related with control of emissions of pollutants, land management and the cleanup of

contaminated sites. The Environment Protection Act from 1990, is an act of the Parliament of the United Kingdom, which establishes for England, Wales and Scotland the structure and the authority that regulates the managements of wastes and the emissions into the environment. The Part II of this act, was added in 1995, and is dedicated specifically to define policies for the identification and compulsory remedial actions of contaminated sites (USEPA, 2009)). According to the Contaminated Land (England) Regulations 2006 (UK Government, 2006), the local authorities are the primary responsible for the identification and management of contaminated lands. The Department of Environment, Food, and Rural Affairs (DEFRA) manages the capital projects program, aimed in assisting local authorities (USEPA, 2009). The work of remediation has been supported carried out by quasi-governmental and non-governmental authorities. Aimed in addressing other soil treats, in 2009 DEFRA published a Soil Strategy for England (DEFRA, 2009), where the main objectives to be attained and the actions previewed are described. With this strategy DEFRA intends to attain a sustainable management of UK soils in 2030, safeguarding their ability to provide essential services for future generations (DEFRA, 2009). According to Haygarth and Ritz, (2009) the challenge will be to optimize the utilization of soils with the variety of demands, identifying the areas more appropriate for specific uses. However, this will require great efforts to obtain detailed soil maps, combining soils data with several other attributes of this resource.

Germany and Netherlands, as stated above, are the most advanced countries in terms of soil protection policies. German government policy recognizes the importance of soils in agriculture, the role soil protection plays in safeguarding other environmental media, and the importance of soil in fighting climate change. Their policies include, the 1998 Federal Soil protection act (Federal Ministry for the Environment Nature Conservation and Nuclear Safety, 1998), and the 1999 Federal Soil Protection and Contaminated Sites Ordinance (Federal Ministry for the Environment Nature Conservation and Nuclear Safety, 1999) which address soil protection and soil remediation and provide the basis for soil policy in Germany. A number of government agencies that have been established for soil protection, like the Federal Institute for Geosciences and Natural Resources formally

established in 1975 and the Federal Environment Agency Soil Protection Commission (KBU) in 2004.The KBU's work focuses on renewable organic resources and soil quality, pollutants and soils, and soil protection and soil awareness. The Federal Institute for Geosciences and Natural Resources conducts research on sustainable soil uses.

Dutch soil policy addresses the long-term protection, management, and sustainable use of soil in the Netherlands. The Dutch soil protection policy includes, The 1987 Dutch Soil Protection Act (revised in 2008), (VROM, 1986), that provides a basis for Dutch soil policy, The 2003 VROM Soil Policy Letter, that articulates an integrated and sustainable approach to soils that incorporates considerations related to land use planning, land conservation, water management, and agriculture and finally, in 2009, the soil remediation circular (VROM, 2009) which establishes remediation objectives and describes soil remediation requirements.

1.2. Contaminated Sites and risk assessment frameworks

Concerns with contaminated areas, started in the nineteen nineties and analyzed in several concerted actions and forums of the European Union (CARACAS, CLARINET and NICOLE), were particularly addressed by the Soil Thematic Strategy and by the proposal of the Soil Framework Directive (SFD), (Swartjes et al., 2008). During this decade several countries started to define risk-based soil quality standards, but mainly related with risks to human health, except for The Netherlands which at this time has already integrated ecological protection objectives (Swartjes et al., 2008). The text of the SFD requests all member states to identify the existing danger zones in their territories, based on common elements which call into question the soil stability, giving them, however, total freedom about how to do it. It is up to each member state the responsibility for the risk zones identification in their own territory and for the goals definition for its mitigation, as well as the measures program to reach. These obligations will allow a better knowledge of the dimension and localization of the soil threats, as well as an integrated adoption, by all member states of the most specific and efficient common measures (CEC, 2006a). For the identification of the threat zones, the commission encourages the usage of the already existent monitoring systems, as well as the development of new methodologies.

However, and according to the Environment European Agency stills difficult to quantify the real extent of soil contamination in Europe, due to the lack of European legislation to oblige member states to make such inventories. This agency estimated in 2007, the existence of about 3 million of contaminated sites in Europe, of which about 250 000 may need urgent remediation (EEA, 2007). The main contaminants are metals and mineral oils (EEA-JRC, 2010). Although progresses have been made in remediation, it also expected an increase in the previous number, as data collection increases.

There is no specific legislation for the soil protection at the national level, the one currently in effect, only contemplates some of its functions and threats to them. The protection of this compartments is dispersed by several strategies connected to the agriculture, to the rural development, territorial planning and to the environment (CEC, 2006a). The Law on Environment of 1987 (law nº 11/87, of April 7, modified by the law nº13/2002, of February 19) is one of the currently used strategies with a positive impact to the matter of soils. This law, which quite recently has undergone a deep revision due to being completely outdated, refers to the need to implement measures for the defense and valorization of the soils and frames itself on the needs and options of the European community in environmental matter. However, and considering this law as a cross-sectoral nature, being the protection of the environment as a whole, it is far from integrating all the problematic of the soil degradation, remediation and protection. It is therefore urgent to implement strategies specially aimed at soil protection, since few where the regulations specifically produced on the matter of soil, to answer these recommendations requested by the Soil Framework Directive. In this sense, the methodology of Ecological Risk Assessment (ERA), proposed by USEPA, (1989), has been recognized as powerful tool for the decision-making process in contaminated sites management, and therefore capable of meet the required framework directive on soil (Critto et al., 2007; O'Halloran, 2006; Solomon and Sibley, 2002; Suter et al., 2000). This methodology, quite flexible and multifunctional is based on the collection, organization and analysis of environmental data in order to determine the acceptable level of risk and to set priorities for action (Jensen and Mesman, 2006a). ERA has as goal to estimate the adverse effects that may occur or are already underway in natural communities, in sites

exposed to physical, chemical and biological agents (Solomon and Sibley, 2002; USEPA, 1989). The Ecological Risk assessments may be used to predict the likelihood of future adverse effects, the prospective risk, or may be used to evaluate environmental problems arising from historical and ongoing activities, the retrospective risk assessment (Gorsuch et al., 2006; Newman and Unger, 2003). The prospective risk analysis, allows the production of information for generic or hypothetical contamination scenarios. This method proceeds to the estimation of the foreseen effects, studying the behavior and the toxicological/ecological impacts which the chemical substances may have in the different ecological receptors. The prospective analysis has been assuming great importance in the soil contamination prevention, since it starts to become required for the notification of new chemical, dangerous, biocides substances [Directive 93/67/EEC (EEC, 1993) and the Regulation 1488/94 (EEC, 1994)]. On the other hand, the retrospective risk analysis, evaluates the chemical effects after they have been freed on the environment, allowing the acknowledgment of contaminated sites, with past origins, but with serious consequences in the present. This type of analysis is essential for the contaminated sites management, once given its diversity, it allows to define the true problem dimension, giving a more accurate evaluation of the real risks for the ecological receptors potentially affected by the contaminant.

Frequently, ERA is performed in phases or tiers, which may include the retrospective and prospective analyses. This European method of contaminated sites risk analysis designated by TRIAD, was proposed by Chapman, (1990) for the assessment of sediment quality and posterior adopted for the ecological risk analysis of contaminated soils (Rutgers et al., 2000). The TRIAD is based in an organization by steps, being therefore slightly different of the one proposed by (USEPA, 1989). With the adoption of this assessment model, the risks characterization mixes data received from three evidence lines: chemistry (chemical-physical properties and bioavailability of pollutants), ecotoxicology (laboratory-based toxicity testing) and ecology (indigenous biota community characterization), (Critto et al., 2007; Niemeyer et al., 2010; Swartjes, 2011). This multidisciplinary combination provides a reduction of uncertainty associated with risk assessment, since it allows a more detailed and accurate than an approach that relies

solely on one evidence line (Alvarenga et al., 2012; Fernández et al., 2005). ERA framework allows thus to get a more reliable evaluation of the contamination and its effects over the ecological receptors. Nevertheless, an ecological assessment based on this kind of methodology, as a rule of thumb, requires however more time, effort and money. In this sense, it is important an initial stage of screening, which allows screening the sites which do not need a more detailed analysis, reducing therefore the costs and simplifying the decision during the risk assessment.

1.3. Soil screening values (SSV) in the assessment of contaminated soils

In most European countries, the first step of European Risk Assessment frameworks for contaminated soils, characterized as screening phase, consists in a quite simplified first approach, which includes preliminary evaluation of risks based on the total concentration of contaminants. These concentrations are compared with soil screening values (SSVs), from dose–response relationships, to assess the likelihood of harm (Jensen and Mesman, 2006). SSVs are concentration thresholds of a given soil contaminant, which when exceeded, It is highly advisable to submit the site to a set of new risk evaluations more specific. These values should provide a level of protection for the terrestrial species and ecological soil functions and be practical in the contaminated soils evaluation, they should, however be reasonable and not so low that even at trivial concentrations no chemical is ever screened out from further risk assessment (Fishwick, 2004). SSVs, with the help of a decision support system, will allow the risk managers to identify contaminated sites, as well as decide about the necessity of an additional intervention in those same sites (Provoost et al., 2008). In the United States of America, the US Environmental Protection Agency (USEPA), has developed SSVs to be used in evaluation processes of ecological risk (USEPA, 1989). Similar work has been done in Europe, in which several countries like Denmark, Germany, Netherlands, Spain, have been deriving their own SSVs from natural soils, for the contaminants present in soil and using them to define environmental quality standards (Crommentuijn et al., 2000; Scott-Fordsmand and Pedersen, 1995; Vega et al., 1999; Wilke et al., 2004). Although the great work already done in other European countries, thresholds concentrations have never been

established for any metal in natural Portuguese soils, being that, in Portugal, these guidance values only exist for the application of sewage sludge in agricultural soils by the law by decree 118/2006. 37 (MAOTDR, 2006). As expected, these values are not appropriate to be used on site specific evaluations, being needed to use other countries values on the evaluation of contaminated sites (Pereira et al., 2008)). It is known, through several studies (Amorim et al., 2005; Criel et al., 2008; Kuperman et al., 2006; Peijnenburg and Jager T, 2003), that the soil physical and chemical properties combination influence the contaminants toxicity over the natural soils organisms, as well as their transport for other compartments. Amorim et al., (2005); Rooney et al., (2006) confirms the influence of soil proprieties in the toxicity of metals in plants and invertebrates of soil (respectively), when different soils were used. Thereby, if a great uncertainty associated with SSVs derivation, due to use of different soil types for their derivation, a multiplicity of SSVs be expected for a same species. Additionally, different countries, such the Netherlands, Germany, Canada, have derived these values based on scientific data for human and/or ecological receptors but also integrating societal values (O'Halloran, 2006). Assessment of contaminated sites using values indicatives of soil quality from other countries should therefore be discouraged. For these reasons, regulatory agencies both from the United Stated and European Union have been reinforcing the importance for each country using their own SSVs, so as to minimize, as much as possible, the variability sources. So, it is important that each country get their own SSVs from reference natural soils, representative of the main soil types from each region. The SSVs are usually determined through extrapolation methods from species sensitivity curves or by applying safety factors, using available published results from laboratory toxicity tests on single species or microbial mediated processes (Rooney et al., 2006; Vega et al., 1999).

1.4. Use of natural reference soils in the ERA framework

If from one hand, the use of reference natural soils become determining on obtaining the ecotoxicological data for the regional relevance SSV`s derivation, on the other hand, the use of this soil type in ecotoxicological essays with terrestrial organisms, has been gaining more and more relevance. The natural soils, when used in ecotoxicological tests, increase

the ecological importance of the ecotoxicological evaluations, leading to a reduction of the evaluation uncertainties, thus being highly recommended in this type of evaluation (Ardestani and van Gestel, 2013; Kuperman et al., 2006; Römbke et al., 2006; van Assche et al., 2002; Van Gestel and Weeks, 2004). Portugal, as well as in other European countries, where the characterization/use of natural soils is still quite premature, both the toxicity evaluation of chemical substances in the terrestrial compartment, as well as the contaminated soils evaluation, is often based in ecotoxicological tests made in OCDE artificial soil. This artificial soil consists of a mixture of sand (70%), kaolinite clay (20%) and ground peat (10%) with the addition of $CaCO_3$ to maintain pH of 6±0.5. Organic matter corresponds to 6.17% and total nitrogen to 0.11% (giving C:N of 32.6) with the water holding capacity (WHC) of about 56% (OCDE, 1984).

The use of this artificial soil is clearly advantageous for reasons of standardization and comparability of results throughout the world (Lokke et al., 2002), being commonly used in toxicity essays with a wide range of soil invertebrates (ISO, 2004, 1999) despite being developed for earthworms. There are however well known differences in terms of physical and chemical properties of these artificial soils and natural soils (Chelinho et al., 2011a; Criel et al., 2008a; Rooney et al., 2007; van Gestel et al., 2011). Some recent studies have indicated that the use of artificial soils may not yield the same results as natural soils in ecotoxicological essays which evaluate the toxicity of different chemicals (Amorim et al., 2005; Domene et al., 2011; Lock and Janssen, 2001; Rhodes et al., 2008; Römbke et al., 2006). The disparities between the artificial and natural soil are particularly accentuated in relation to organic matter content, which in artificial soil OECD presents a percentage too high, due to the excessive content (10%) of peat, when compared with natural soils. The existence of a greater organic matter in soil, generally results in a greater contaminants absorption capacity and, thus, in their lower mobility and bioavailability to exercise toxic effects, for instance, a lower toxicity has been reported for many test species in the OCDE artificial soil in relation to the natural soil (Lock and Janssen, 2001) which can lead to a under- or overestimation of the toxicity (Amorim et al., 2005). As a result, the data arising from studies using artificial soils may lead to wrong conclusions and mean that any risk assessment may be erroneous, and the data

extrapolation of chemical substances toxicity, obtained with artificial soils, for natural reference soils becomes difficult and not advisable (Hofman et. al., 2008). Additionally, several problematic issues relative to the OECD artificial soil have been recently revealed as a standard reference soil (Bielská et al., 2012; Hofman et al., 2009). A major criticism is related to inter-laboratory variability of toxicity results, even when the same chemical is tested, and the same standardized procedure is used (Bielská et al., 2012; Rombke and Moser, 2002). The absence of detailed specifications concerning the characteristics of the components constituting it leads to a great diversity of such soil, varying between manufacturers and countries (Bielská et al., 2012). A greater consistency should exist in the soil preparation between different laboratories concerning the properties of used compounds and the final mixture, possibly specified by international standards (range of the total organic carbon content, the peat particles size etc.), in order to bridge these gaps.

Thus, the use of natural soil continues to increasingly gain importance in ecotoxicological risk assessment of pollutants, being its integration into the ERA one of the major objectives of ecotoxicologists (Domene et al., 2010; Kuperman et al., 2009; Niemeyer et al., 2010; Rocheleau et al., 2010). Some attempts have emerged in order to increment the use of standardized natural soil, selected as German reference soil, the LUFA 2.2 natural soil (Agrarian Research center; Speyer / Germany), there are many studies, especially at European level, which has involved the use of this soil as reference soil in ecotoxicity tests (Gomes et al., 2011; Lourenço et al., 2012; Pereira et al., 2010; Vijver et al., 2001). Although widely used, this has not yet been recommended in international guidelines test, although it is a natural soil, is incapable of representing all natural European soils and therefore should not be considered exclusively a standard soil (Rombke and Amorim, 2004).

In this sense, various European initiatives have been promoting the selection and characterization of reference natural soils, representatives of different lithologies of each country, which act as control and substrate for the dilution of contaminated soils in ecotoxicological assays performed to evaluate the ecotoxicity of contaminated soils, in tier 2 risk assessment frameworks (Jensen and Mesman, 2006). A set of reference soils

known as EUROSOILS was introduced by the European Commission in 1990, where the selected soils intended to be representatives of different European soils, in order to create a common basis for better comparison and quality control of soil sorption data (Kuhnt and Muntau, 1992). Therefore, six regionally representative soils aimed to cover a wide range of pH, organic matter and cation exchange capacity values were identified and characterized as reference soils for chemical testing in the EU (Gawlik et al., 2004, 2001; Kuhnt and Muntau, 1992). However, the use of those soils appears to not have been successful, not only due to the limited available soil, since for ecotoxicological essays, a large substrate quantity is needed, as also the lack of knowledge relatively to the behavior of some of those soils test species. Besides, upon the EUROSOILS proposal, no Portuguese soil was included, making Portugal keeping struggling with the lack of natural soils. As a result, Rombke and Amorim, (2004) suggested a natural soil type called the SIM-SOILS, having main properties, texture, organic matter content and C : N, similar to EUROSOILS, aimed to reflect ecological condition for soil organisms and to control environmental availability of contaminants in soils. Other works have been developed with Portuguese soils, (Chelinho et al., 2011a) four soils from the Southern country region were tested to be validated as reference soils for the Mediterranean region, in a joint work with solid from Spain and Italy. Despite that work contribution in the identification of natural soils of reference for the national context, a gap exists between the northern and Southern country regions, which were not included in this characterization. Being Portugal a country with a wide lithological diversity and consequently high soil diversity, it makes perfect sense a characterization / validation of different natural soils representatives of the different country geological contexts, namely of the center zone, in which soils coming from granitic areas, the most abundant at a national level, prevails.

1.5. Aim and scope of the thesis

The final outcome of this thesis is to generate ecotoxicological data, for the derivation of Soil Screening Values (SSVs) with regional relevance, to be used in Ecological Risk Assessment frameworks aiming to protect the soil ecosystem, and ultimately the human health. For this purpose, natural reference soils will be used from the center of the

country, previously identified, characterized and shown in this paper, which can also to be used as substrate in future ecotoxicological evaluations.

At national level, this study may provide an important contribution with regards to the identification/characterization of natural soils for later usage as a reference soil in ecotoxicological essays, made on the second tier of ERA frameworks, over contaminated sites. The use of these soils, will contribute to an increase on the ecological relevance of the risk analysis in Portuguese territory. Besides, these soils will allow to define SSVs for some metals as Uranium, Copper and Cadmium, to be used in the first tier of ERA processes applied to contaminated sites, which will fill the gap of a serious lack of soil quality criteria that occurs at this level, in the national context.

Thus, this thesis is structured into seven chapters, the first and seven chapters concern the general introduction and final remarks of the thesis, respectively, while the other five are related with research work. Below is a brief description of each chapter:

[Chapter II] The natural soil PTRS1, representing one of the dominant types of soil from a granitic region, was been contaminated with different Uranium concentrations and used as substrate for ecotoxicological tests, to obtain NOEC, LOEC, EC_{20} and EC_{50} for uranium, to be used in the derivation of SSVs for national assessments of contaminated sites. For this purpose, the reproduction ecotoxicological tests of invertebrate (*Eisenia andrei*, *Folsomia candida, Enchytraeus crypticus*) and seed germination/grow of plants species (*Avena sativa, Lycopersicon esculentum, Zea mays, Lycopersicon esculentum*) was assessed. The effect of uranium on soil enzymatic activity was also tested.

[Chapter III] In order to deriving SSVs cadmium, for national assessments of contaminated sites, and similarly to previous chapter, the performance of invertebrate and plant species commonly used in standard ecotoxicological assays, as well as the activity of soil enzymes was tested, used as substrate the PTRS1 soil, contaminated with a different copper concentrations.

[Chapter IV] This chapter intend to determine NOEC, LOEC, EC_{20} and EC_{50} values for copper, to be used in the derivation of SSVs, using a natural reference soil from the centre of Portugal. The same ecotoxicological assays described in both previous chapters was

performed, used as substrate the PTRS1 soil contaminated with different cadmium concentrations.

[Chapter V] In this chapter, using toxicity data obtained in chapters II, III and IV applying appropriate extrapolation methods, SSV for Uranium, Copper and Cadmium, and for a dominant type of soil from a granitic region, the PTRS1, was derived taking into consideration the total and the bioavailable concentrations of metals.

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Chapter II

Contribution for the derivation of a soil screening value (SSV) for Uranium, using a natural reference soil.

Contribution for the derivation of a soil screening value (SSV) for Uranium, using a natural reference soil

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Abstract

In order to regulate the management of contaminated land, many countries have been deriving soil screening values (SSV). However, the ecotoxicological data available for uranium in European countries is still insufficient and incapable to generate a SSVs. In this sense, and so as to make up for this shortcoming, a battery of ecotoxicological assays aimed in evaluating a wide range of endpoints (soil enzymes activity, reproduction of invertebrates, emergence/growth of plants) were carried out, in a natural soil artificially spiked with uranium. In terrestrial ecotoxicology, given the fact that soils have different properties that can influence the bioavailability and the toxicity of chemicals, which in turn may lead to unfeasible environmental risk assessment, the use of natural regional representative soils is of great importance. Thus, the Portuguese natural reference soil PTRS1, collected in a granitic region, was used as test substrate. This study allowed the determination of NOEC, LOEC, EC_{20} and EC_{50} values for uranium. Soil enzyme activities were the most sensitive parameters, followed by the reproduction of invertebrates and emergence/germination of plants. In particular, dehydrogenase and UR enzymes displayed the lowest EC_{20} values (34.9 and <134.5 mg U Kg^{-1}_{dw}, respectively). *E. andrei* and *E. crypticus* revealed to be more sensitive to uranium than *F. candida*. EC_{50} values of 631.00, 518.65 and 851.64 mg U Kg^{-1}_{dw}, were recorded for the three species, respectively. Concerning plants, only *L. sativa* was affected by U at concentrations up to 1000 mg U

kg[1]. The outcomes of the study may be in part be constrained by physical and chemical characteristics of soils, hence contributing for the discrepancy between the data generated in this study and that available in the literature. A predicted no effect concentration (PNEC) value of 15.5 mg kg^{-1} of soil dry weight was obtained for U, following the assessment factor method.

Key-words: uranium (uranyl ion) toxicity; natural reference soil; soil enzymes activity; *Eisenia andrei*; *Enchytraeus crypticus*; *Folsomia candida*; *Avena sativa*; *Zea mays*; *Lacuta sativa*; *Lycopersicon esculentum*.

2.1 Introduction

Uranium (U) is a natural soil component, being originated from rocks in the Earth's crust, where it mainly occurs in the form of oxides. Natural processes acting on rocks and soils, such as wind, water erosion, dissolution, precipitation and volcanic activity contribute for U dispersal in the environment (Gavrilescu et al., 2009). The use of U as fuel in nuclear power plants has driven to its large-scale exploration worldwide. The U exploration became significantly important in the world during the Second World War, and later on during the Cold War, in both cases to supply military needs of the greatest potencies. Recently, the World Nuclear Association estimated worldwide reserves of U at 5.4 million tons in 2009, of which Australia had about 31%, followed by Kazakhstan (12%), Canada and Russia with 9% (http://www.world-nuclear.org/info/inf75.html). The remarkable energy crisis that is currently faced worldwide due to the exhaustion of carbon based energy resources is demanding further extraction of U, as nuclear energy arises as a potential solution. Hence, it is expected that the mining and milling of U will increase in the next decades, contributing for its widespread in the environment (Malyshkina and Niemeier, 2010).

During the last century, Portugal has actively explored radioactive ores and was for some time ranked as one of the main U producers. The extraction of U ore in Portugal started in 1908, first driven by the interest in radium (being U a by-product) and then by the interest in its military applications, till 2001 (Carvalho et al., 2009; Pereira et al.,

2014). Most of the old U mines were located in the granitic regions of the Iberian Meseta, in the centre-north of Portugal (Beiras), (Carvalho et al., 2007). Nowadays, although the mining activities ceased, like in several other places in the world, the old U mines represent a serious environmental problem, due to waste accumulation (mainly tailings and sludge) and improper disposal of radioactive material, composed by U and its daughter radionuclides (Arogunjo et al., 2009; Carvalho, 2011; Carvalho et al., 2007; Figueiredo et al., 2011; Gavrilescu et al., 2009; Momčilović et al., 2010; Niemeyer et al., 2010; Patra et al., 2011; Pereira et al., 2008, 2006; Scheele, 2011; Vandenhove et al., 2006; Wang et al., 2007). Soils and water are the two major environmental matrices affected by U contamination.

U has a long half-life, persisting in nature as different isotopes, with different chemical and radiological characteristics (ASTDR, 2011). The toxic effects induced by this metal are caused by both properties. However, since U isotopes mainly emit alpha particles, with little penetration capacity, the main radiation hazards only occur after ingestion or inhalation of these isotopes and daughter radionuclides (ASTDR, 2011). Once in the soil, U interacts with all the components of this matrix, such as clay minerals, aluminum and iron oxides, organic matter and microorganism, in a very complex system, where pH and organic matter seem to have the major role in controlling U mobility (pH ≥ 6) and leaching (pH <6), (Vandenhove et al., 2007a). The high mobility/availability of U, will in turn increase the ecological risks posed to soil and water compartments (Geng et al., 2011; Geras'kin et al., 2007; Gongalsky, 2003; Islam and Sar, 2011; Joner et al., 2007; Kenarova et al., 2010; Lourenço et al., 2012; Pereira et al., 2009)

The soil has been recognized as an important compartment that provides crucial ecosystem services (e.g. filter of contaminants, reservoir of carbon and a bank of genes) and is the support of agro-sylvo-pastoral production (Lavelle et al., 2006; O'Halloran, 2006) and of several other human activities. The soil compartment offers raw materials (e.g., peat, clay, ore) and contributes for climate regulation and biodiversity conservation, as well as other cultural services (Barrios, 2007; Dominati et al., 2010). The recognition of the importance for maintaining the provision of such services has increased the necessity to create appropriate legal tools to correctly and effectively protect this

resource. In this sense, the Soil Framework Directive proposed by the Commission of the European Communities (CEC), aims to establish a common strategy for the protection and sustainable use of soils (CEC, 2006c). For that end, this proposal defines measures for the identification of the main problems faced by soils, the adoption of strategies to prevent their degradation, as well as for the rehabilitation of contaminated or degraded soils (Bone et al., 2010). The Soil Framework Directive will fill in the gap regarding soil protection, since this compartment has never been a target of specific protection policies at the European Community level, (CEC, 2006c). Many countries, committed in regulating the management of contaminated land, have adopted generic quality standards, the soil screening values (SSVs), (Jensen and Mesman, 2006a). SSVs are concentration thresholds above which, more site-specific evaluations are required to assess the risks posed by soil contamination (Fishwick, 2004). The SSVs should provide a level of protection to terrestrial species and ecological functions of the soil (Carlon, 2007; Fishwick, 2004; USEPA, 2003). SSVs are particularly useful for the first tier of Ecological Risk Assessment (ERA) processes applied to contaminated sites, supporting the decision-making at this initial stage of assessment (Provoost et al., 2008), which at the end is aimed in setting priorities for remediation and risk reduction measures (van Gestel, 2012). In the case of Portugal, SSVs for soils have never been established for metals or organics. Only threshold concentrations of metals on sewage sludge were legally established to regulate the application of this solid waste on agricultural soils (MAOTDR, 2006). However, but they are not appropriate for soil ERA purposes, once they represent different matrices with unequal characteristics and uses.

he use of natural reference soils in ecotoxicological tests has been recommended by several authors (Kuperman et al., 2006; Römbke et al., 2006; van Assche et al., 2002). This is because the properties of the OECD artificial soil besides varying between batches prepared in different laboratories, they are also not representative of the great majority of natural soils (Hofman et al., 2009). Different levels of toxicity, for each contaminant, can be expected in soils with different properties (Domene et al., 2011; Rooney et al., 2007; Song et al., 2006; van Gestel et al., 2011), hence it is important each country derives their own SSVs using natural reference soils representing the main types of soils

within their territories. In this context, the main aim of this work was to obtain ecotoxicological data for U, performing soil enzymes activity tests, invertebrates and plant tests, using for that a Portuguese natural reference soil (PTRS1), that represents one of the dominant types of soil from a granitic region (cambisol) of the country (Caetano et al., 2012), to make a first proposal of a SSV for this metal.

2.2 Material and methods

2.2.1 Test soil

The natural soil (PTRS1) used as test substrate in this study was collected in Ervas Tenras [Pinhel, Guarda, Portugal center; geographical coordinates: $40^o44'4.27''$N and $7^o10'54.3''$W)], at 655m altitude, in a granitic region.

A composite soil sample was collected and immediately brought to the laboratory where it was air dried. Another portion of the soil, was immediately sieved through a 2 mm mesh size and the sieved fraction (<2 mm) was stored in polyethylene bags, at -20 °C, until further analysis of soil microbial parameters. For the tests with soil organisms and plants, the soil was passed through a 4 mm mesh sieve and the sieved fraction (<4 mm) and defaunated through two freeze–thawing cycles (48 h -20 °C followed by 48 h at 25 °C) for the ecotoxicological tests.

The physical and chemical properties (including total metal contents) of the PTRS1 soil were presented in a preliminary study by Caetano et al., (2012), (*c.f.,* table in annex) aimed in characterizing this soil as a reference substrate for ecotoxicological purposes. Nevertheless, the main properties of the PTRS1 are described in Table II.1, of the results section.

2.2.2. Test substance

For all the test organisms, the natural soil was spiked with a stock solution of uranyl nitrate 6-hydrate, $UO_2(NO_3)_26H_2O$, (98%, PANREAC) prepared with deionized water filtered in a Milli-Q equipment (hereinafter referred as deionized water), in order to obtain a range of concentrations, which were ascertained by range finding tests performed with the different test species.

For soil enzyme tests, the PTRS1 soil was spiked with the following concentrations: 0.0, 134.6, 161.5, 193.8, 232.5, 279.0, 334.8, 401.8, 482.2, 578.7, 694.4, 833.3, 1000 mg U Kg$^{-1}_{dw}$. To obtain these concentrations, the stock solution of uranyl nitrate was diluted in the amount of water required to adjust the soil moisture at 80% of its maximum water holding capacity (WHC$_{max}$).

The following U concentrations were used to expose the earthworms in the reproduction tests: 0.0, 113.1, 124.4, 136.9, 150.5, 165.6, 231.9, 324.6, 454.5, 500.0, 550.0, 605.0, 665.5 mg U Kg$^{-1}_{dw}$. For potworms, collembolans and terrestrial plant assays the same range of concentrations was tested: 0.0, 167.4, 192.5, 221.4, 254.6, 292.7, 336.6, 420.8, 526.0, 657.5, 756.1, 869.6, 1000 mg U Kg$^{-1}_{dw}$.

The amount of water required to adjust the WHC of the soil to 45% of its maximum value was used to dilute the stock solution for these tests.

2.2.3 Ecotoxicological assessment

2.2.3.1 Soil microbial activity

Ten grams of sieved PTRS1 soil per replicate and concentration were spiked with different U concentrations; a total of three replicates were used per treatment. Six replicates with the same amount of soil only moistened with deionized water were also prepared for the control. The soil was incubated for 30 days, at 20±2ºC, and a photoperiod of 8h^L: 16h^D. During the incubation period, the soil moisture was weekly monitored by weighing the pots, and whenever needed it was adjusted to 80% of its WHC$_{max}$ by adding deionized water. After the incubation period, 1g of soil per replicate and concentration was weighted and placed in falcon tubes, and then frozen to -20ºC, until analysis. Thereby, a total of 9 sub-replicates were made for each concentration. The soil was thawed at 4°C before analysis.

The activity of arylsulphatase (ARYL), dehydrogenase (DHA), urease (UR), and cellulase (CELL) enzymes and changes in the nitrogen mineralization (NMIN) and potential nitrification (PN) were measured in the soil samples spiked with the different uranium concentrations.

For the determination of ARYL activity, the method proposed by Tabatabai and Bremner, (1970) and Schinner et al., (1996) was followed. After addition of p-nitrophenylsulfate, soil sub-samples were incubated for one hour, at 37°C. The nitrophenyl liberated by the activity of ARYL was extracted and colored with sodium hydroxide and determined photometrically at 420 nm. The results were expressed as µg p-nitrophenylsulfate (p-NP) g^{-1} soil $_{dw}$ h^{-1}. The method proposed by (Öhlinger, 1996) was used to assess the DHA. The samples were suspended in a solution of trifeniltetrazol chloride (TTC) and incubated, at 40°C, for 24 hours. The triphenyl formazan (TPF) produced was extracted with acetone and measured spectrophotometrically, at 546 nm, and the results were expressed as µg triphenylformazan (TPF) g^{-1} soil $_{dw}$ h^{-1}.

The CELL activity was tested according to the method proposed by Schinner et al., (1996) and Schinner and von Mersi, (1990). The reducing sugars produced during the incubation period causes the reduction of hexacyanoferrate (III) potassium to hexacyanoferrate (II) potassium in an alkaline solution. The complex ferric hexacyanoferrate (II) has a blue coloration and is formed by the reaction of potassium hexacyanoferrate (II) with ferric ammonium sulphate in acid solution. The activity of CELL was then measured colorimetrically at 690 nm and expressed as µg glucose g^{-1} soil $_{dw}$ 24 h^{-1}. NMIN activity was measured according to Schinner et al., (1996). For this purpose, the soil samples were incubated for 7 days, at 40°C. During this period, the organic forms of nitrogen are converted in inorganic nitrogen (NH_4^+), which is determined by a modification of the Berthelot reaction, after extraction with potassium chloride. The reaction of ammonia with sodium salicylate in the presence of sodium dichloroisocyanurate formed a green colored complex, in alkaline pH that was measured at 690 nm. NMIN was expressed as µg nitrogen (N) $.g^{-1}$ soil$_{dw}$ d^{-1}. The UR activity was assayed according to the method proposed by Kandeler and Gerber, (1988) and Schinner et al., (1996). The samples were incubated for 2h, at 37°C, after addition of a buffered solution of urea. The released ammonia was extracted with a solution of potassium chloride and determined by the Berthelot reaction modified. The determination was based on the reaction of sodium salicylate with ammonia in the presence of chlorinated water, producing a green colored complex in alkaline pH. UR was detected at 690 nm and

expressed as µg nitrogen (N) g^{-1} soil$_{dw}$ 2 h^{-1}. The quantification of potential nitrification was determined by the method of Kandeler, (1996), which is a modification of the technique proposed by Berg and Rosswall, (1985). The ammonium sulphate was used as substrate, and soil samples were incubated for 5h, at 25°C. Nitrate released during the incubation period was extracted with potassium chloride and determined colorimetrically at 520nm. This reaction was expressed as µg nitrite (N) g^{-1} soil $_{dw}$ h^{-1}.

2.2.3.2 Invertebrate and plant tests

2.2.3.2.1 Test organisms and culture conditions

The earthworm *Eisenia andrei* (Oligochaeta: Lumbricidae), the potworm *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae) and the springtail *Folsomia candida* (Collembola: Isotomidae) were used as invertebrate test organisms. All organisms were obtained from laboratorial cultures, kept under controlled environmental conditions (temperature: 20±2ºC; photoperiod: 16h^L: 8h^D). The earthworms (*E. andrei*) are maintained in plastic boxes (10 to 50 L) containing a substrate composed by peat, dry and defaunated horse manure (through two freeze–thawing (48h at -20 ºC followed by 48h at 65 ºC), and deionized water. The pH of the culture medium is adjusted to 6.0 - 7.0 with $CaCO_3$. The organisms are fed, every 2 weeks, with six tablespoon oatmeal previously hydrated with deionized water and cooked for 5 min. The potworms (*E. crypticus*) are cultured in plastic containers (25.5 cm length; 17.4 cm width; 6.5 cm height), which are filled with pot soil moistened to the nearest 60% of its WHC$_{max}$ and with pH adjusted to 6.0 ± 0.5. The organisms are fed twice a week with a teaspoon of macerated oat. The collembolans (*F. candida*) are maintained in plastic containers filled with culture medium composed by moistened Plaster of Paris mixed with activated charcoal 8:1 (w:w). They are fed with granulated dry yeast, twice a week, which is added half a teaspoon small amounts to avoid spoilage by fungi.

Seeds from four plant species (two dicotyledonous and two monocotyledoneous), purchased from a local supplier, were used for seed germination and growth tests: *Avena sativa, Zea mays*, *Lacuta sativa* and *Lycopersicon esculentum.*

2.2.3.2.2 Reproduction tests with invertebrates

The reproduction tests with *E.andrei*, *E. albidus* and *F. candida* were carried out according to the ISO guidelines 11268-2 (ISO, 1998), 16387 (ISO, 2004) and 11267 (ISO, 1999), respectively. Each replicate of the invertebrate tests contained 10 individuals in a certain developmental stage: the earthworms had a fully developed clitellum and an individual fresh weight between 250 and 600 mg; the potworms were 12-mm size; and the springtails were 10–12 days old. Five hundred grams of dry soil were weighted per test vessel for earthworms. For the tests with potworms and collembolans 20 g and 30 g of soil were weighted per replicate, respectively. Following an ECx sampling design, which considers more concentrations and less number of replicates, two replicates per concentration and five replicates for the control were prepared in the reproduction tests with *E. andrei*. Adult earthworms were removed from the test containers after 28 days. The produced cocoons persisted in the soil until 56 days have been completed. After this period, the juveniles from each test container were counted. During the test, organisms were fed once a week, with 5 g per box of defaunated horse manure (using the same procedure above described), and the soil moisture content was weekly monitored (following the procedures outlined in ISO guideline (ISO, 1998).

The *E. albidus* reproduction test was held for 28 days and the adults were left in the vessels until the end of the test. About 2mg of rolled oats were placed on the soil surface, weekly to feed the animals. At the end of the test, the potworms were killed with alcohol, colored with Bengal red and counted according to the Ludox Flotation Method, as described in ISO 16387 (ISO, 2004). The reproduction tests with *F. candida* took four weeks to be completed. The collembolans were fed with granulated dry yeast, obtained from a commercial supplier, being weekly added (about 2 mg of yeast per test glass vessel container) to the soil surface. At the end of the test, the containers were filled with water and the juveniles were counted after flotation. The addition of a few dark ink drops provided a higher contrast between the white individuals and the black background. The organisms were then counted through the use of the *ImageJ* software (http://imagej.nih.gov/ij/). The exposure was carried out at 20±2ºC and a photoperiod of 16[L]: 8[D]. For both species five replicates of uncontaminated natural PTRS1 soil were

prepared for the control. The same ECx sampling design applied for earthworms was followed. However, in order to reduce the variability of the results, three replicates were prepared per test concentration (instead of two for the earthworms).

2.2.3.2.3 Seed germination and plant growth tests

Germination and growth tests with terrestrial plants were performed following standard procedures described by the ISO guideline 11269–2 (ISO, 2005). For this purpose, 200 g_{dw} of the spiked soil with the concentrations described above were tested. In this case, the amount of water required to adjust the WHC_{max} of the soil to 45% was used to dilute the stock solution and to moist the soil at the beginning of the test. The soil was placed in the plastic pots (11.7 cm diameter, 6.2 cm height) and twenty seeds were added to each pot and gently covered with the spiked soil. In the bottom of each plastic pot a hole was previously made to let a rope passing through. This rope made the communication with a cup filled with deionized water and placed under the test pot. The level of water in the lower recipient was adjusted whenever needed, as to guarantee the necessary conditions of moisture according to, the recommendations specified in (ISO, 2005). Five replicates of uncontaminated natural PTRS1 soil were prepared for the control, while three replicates were tested per concentration, in order to minimize the variability of the results, and to follow the ECx sampling design, similarly used for the invertebrate tests.

At the beginning of the test, nutrients (Substral® - Plants fertilizer using 1 bottle cap for 2 L of water proportion according to the manufacturer recommendation; Fertilizer NPK: 6-3-6; nitrogen (N): 6%; phosphate (P_2O_5): 3%; potassium (K$_2$O): 6%; iron (Fe): 0,03%; trace elements: Cu, Mn, Mo and Zn), were added in each lower recipient containing the water. Pots were maintained at constant conditions of temperature (20 ± 2°C), photoperiod (16h^L: 8h^D) and light intensity (25.000 lux). The endpoints seed germination, and fresh and dry biomass, above soil, were assessed for each species at the end of the exposures according to the methods outlined in ISO, (2005).

For this work, a battery of enzymes involved in different biogeochemical cycles [S (sulfur cycle), N (Nitrogen cycle), C (Carbon cycle)], as well as enzymes more indicative of the good physiological conditions of the whole microbial community (e.g. dehydrogenase)

were selected. The species of invertebrates and plants were selected based on the availability of standard protocols. Since we aimed to obtain data for the derivation of SSVs, for regulatory purposes, this procedure is recommended.

2.2.4 Statistical Analysis

A one-way analysis of variance (one-way ANOVA) was performed to test significant differences between the uranium concentrations tested for each endpoint analyzed: the activity of enzymes, the number of juveniles produced by potworms and collembolans, the number of emerged seeds, and the fresh and dry mass of the plants. The Kolmogorov-Smirnov test was applied to check data normality, whereas homoscedasticity of variances was checked by the Levene's test. When these two assumptions of the one-way ANOVAs were not met, a Kruskal-Wallis analysis was performed. The statistical analysis was run in the SigmaPlot 11.0 software for Windows. When statistical significant differences were recorded, the Dunnett's (for parametric one-way ANOVA) or the Dunn's test (for non-parametric ANOVA) was carried out to perceive which concentrations were significantly different from the respective control. Based on the outcomes of the multiple comparison tests the NOEC (no-observed-effect-concentration) and LOEC (low-observed-effect-concentration) values were determined. The EC_{20} and EC_{50} values for each endpoint were calculated whenever possible, after fitting the data to a logistic model using the STATISTICA 7.0 software.

2.3 Results and Discussion

2.3.1 Soil microbial activity

As far as authors are aware, this study gathers for the first time more extensive data regarding the ecotoxicity of spiked soils with U on soil microbial parameters. Only a study from Sheppard and Evenden, (1992) has analyzed the effect of uranium on soil phosphatase activity in eleven different Canadian soils (including an agricultural, a boreal forest and a garden soil). This study recorded a significantly depressed activity only at the highest concentration tested (1000 mg U/Kg^{-1}_{dw}) for all the soils. These results suggested that probably, soil phosphatase activity was one of the less sensitive soil microbial

parameters to uranium. In fact Pereira et al., (2006) also reported the low sensitivity of this parameter in mine soils contaminated with metals.

The variation in soil enzyme activities, NMIN and PN in the PTRS1 soil, spiked with different U concentrations, is shown in Figure II.1, and the Table II.1 summarizes toxicity values obtained for each biochemical parameter.

U had a clear inhibitory effect in almost all functional parameters tested. Overall, DHA and UR were the most affected soil enzymes by U, being their activity significantly inhibited at concentrations equal or lower than 134.5 mg U kg^{-1} (Table II.1). DHA have a relevant role in the oxidation of soil organic matter (SOM), being a good indicator of the active microbial biomass in the soil compartment (Taylor et al., 2002). As such, U (in the form of uranyl) strongly affected the normal microbial activity in PTRS1 soil. Indeed, the inhibition of UR activities indicates that U had a deleterious effect on soil N-cycle (Figure II.1, Table II.1). The reduction in the activity of this enzyme may have been caused by a negative effect of U on the overall microbial biomass, which in turn was also translated in a reduction in the oxidation rate of organic N into ammonium (Kandeler, 1996; Wang et al., 2011). ARYL is regularly involved in the S-cycle by catalyzing hydrolysis reactions in the biogeochemical transformation of S (Taylor et al., 2002). This parameter was significantly affected by U, at a LOEC of 279.0 mg U kg^{-1}. On its turn, the CELL activity was significantly inhibited at intermediate U concentrations. However in the highest concentrations the tendency was reversed and the activity increased, but not for levels significantly different from the control (Figure II.1). Thereby, we can conclude that the C-metabolism associated with the degradation of soil organic matter and catalyzed by these extracellular enzymes (Alvarenga et al., 2008) was constrained by U. NMIN and PN are indicators of the functioning of the N-cycle, hence providing an overview of the activity of specific microbial groups (nitrifying bacteria) directly involved in both processes (Winding et al., 2005). The general pattern of response observed for these two parameters corresponded to stimulation at the lower U concentrations and inhibition under the highest ones (Figure II.1), leading to EC_{50} values of 347.0 and 610.0 mg U kg^{-1} (Table II.1), respectively. It has been stated that NMIN is normally less sensitive than potential nitrification, since the

former is carried out by a wider diversity of microorganisms (Winding et al., 2005). However, our data showed the opposite (Figure II.1).

The sensitivity of soil microbial parameters to metals has already been demonstrated by several authors, either in metal-polluted or in artificially spiked soils (e.g., Coppolecchia et al., 2011; Hu et al., 2013; Khan et al., 2007; Lee et al., 2009, 2011; Papa et al., 2010; Pereira et al., 2013). DHA and UR had generally been referred as the most affected enzymes for different metals (e.g., Cu, Pb, Zn, Cd, Fe, Cr, Ni), (e.g. Gülser and Erdoğan, 2008; Khan et al., 2007; Lee et al., 2009; Thavamani et al., 2012). ARYL and CELL, however, have shown contradictory responses in different studies. Some authors observed negative correlations between ARYL and CELL activities and Zn (Coppolecchia et al., 2011) and Cu concentrations respectively e.g. (Alvarenga et al., 2012; Antunes et al., 2011), ; while others observed positive correlations between ARYL and Cd (Antunes et al., 2011), and no changes on CELL activities in the presence of metals in urban soils was observed (Sivakumar et al., 2012). Usually, PN is negatively influenced by the presence of metals and metalloids such as Pb, Cu and As (Antunes et al., 2011; Pereira et al., 2006). The inhibitory effect of some metals like Zn, Cd and Pb on NMIN was also observed by (Dai et al., 2004).

However, there are no available studies on the toxicity of U on soil microbial enzymes, except one (Antunes et al., 2011) that evaluated the effect of soils from an abandoned U mine (presenting a mixture of metals) on these microbial parameters. These author's found negative correlations (based on the Spearman coefficient) between U levels in soil and the activities of CELL enzymes. For DHA, PN and ARYL no significant correlations were detected. Nevertheless and as previously mentioned, this study analyzed mining contaminated soils, where the mixture of metals, may cause either synergistic or antagonistic effects, and where a well-adapted and functional microbial community was likely established, since a more active disturbance of soils has stopped for some decades.

The inhibition of soil enzyme activities recorded could has been caused by toxicological effects of metals on soil microorganisms with subsequent decrease in their abundance and/or biomass; and/or by the direct inactivation of extracellular enzymes by metals (Kızılkaya and Bayraklı, 2005). Although the toxicological mechanisms of metals on

enzyme activities are yet to be unraveled, their effect may either occur through complexation with the substrate or with the active binding sites of enzymes, or by reaction with the enzyme–substrate complex (Hinojosa et al., 2004). Notwithstanding, the levels of metals may be not the sole effect on soil microbial activity. Soil properties (e.g. pH, organic matter content, nutrients and soil texture) may also interfere and modulate the bioavailability and, consequently, the influence of metals on soil enzyme activities and mineralization processes (Papa et al., 2010a; Turner et al., 2002). According to the literature, clays can retain and protect extracellular hydrolases, namely UR (Lee et al., 2009). But the low clay content, of the PTRS1 soil (3.32%) (Table II.1), could has been responsible for a high bioavailability of U, leading to the impairment of soil microbial community through cytotoxic effects, which reduced the metabolic activity of microorganisms. Other investigations also showed the same evidence (Antunes et al., 2011). Additionally, the low pH of PTRS1 soil (Table II.1.) has likely increased the availability of U and subsequently its impact on enzyme processes, PN and NMIN, particularly at higher U concentrations. On the other hand Coppolecchia et al., (2011) suggested that a decrease in pH under higher Zn concentrations might have enhanced the inhibition of ARYL activity recorded on their study. Although the interaction between uranium and abiotic factors was not tested in our study, the acidic pH of PTRS1 probably had some influence on ARYL response to uranium concentrations. Nevertheless, the PTRS1 is a common type of soil in the Portuguese territory, thus the results obtained will allow the derivation of more adjusted and ecologically relevant risk levels.

The above results illustrated well the effects of U in the performance of soil enzymes, reinforcing the importance of these parameters as bioindicators of soil quality. Indeed, the EC_{20} values calculated for DHA (34.9 mg U kg^{-1}), UR (<135.5 mg U kg^{-1}), NMIN (152.2 mg U kg^{-1}) and ARYL (155.3 mg U kg^{-1}) are within the environmental concentrations quantified in soils from an abandoned U mine, following extractions with *aqua regia* or with rainwater (Pereira et al., 2008). In this sense, the data herein generated represent a great asset for the derivation of SSVs, since they have a great ecological representativeness.
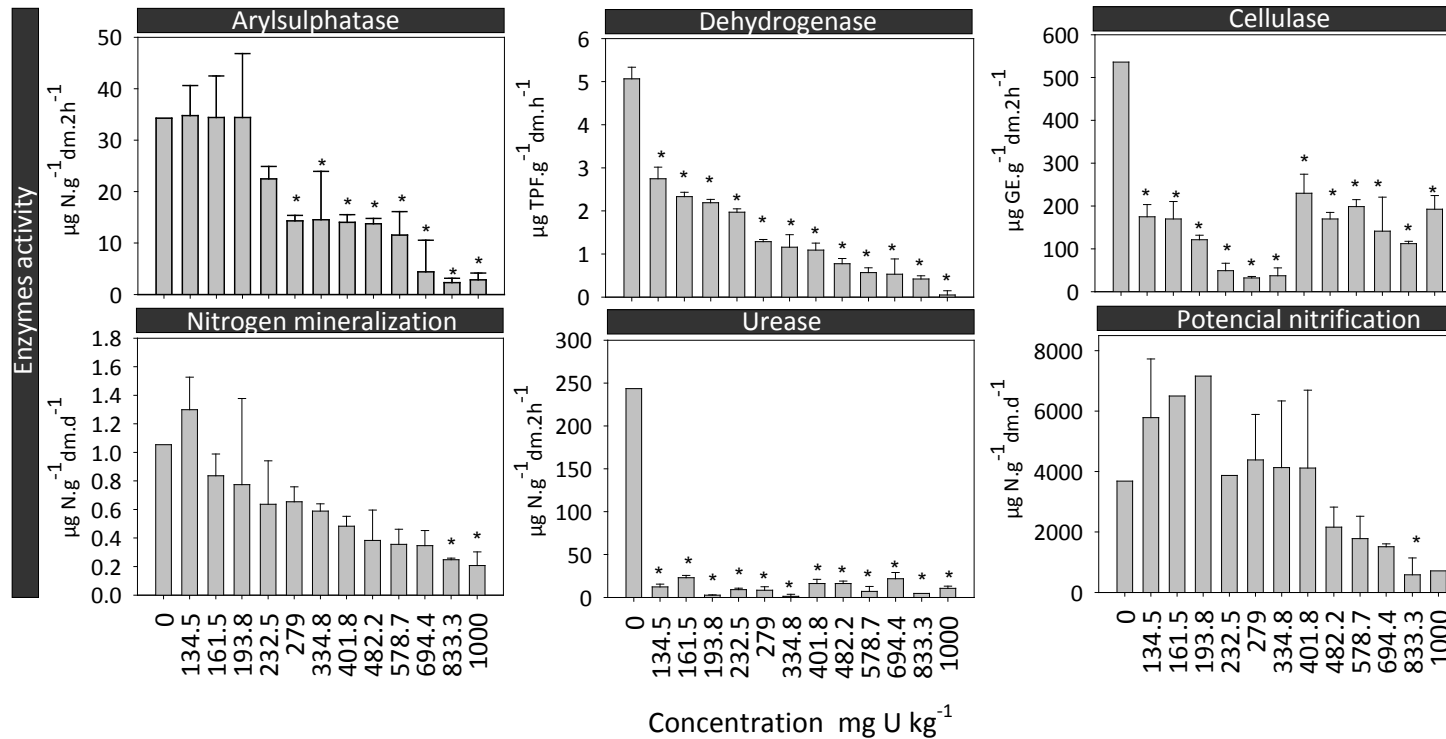
Figure II.1 Response of the arylsulphatase, dehydrogenase, cellulase urease, activity, N mineralization and potential nitrification to soils spiked with a range of uranium concentrations. The error bars indicate the standard deviation. The asterisks sign out significant differences relatively to the control (0 mg U Kg^{-1}_{dw}), ($P < 0.05$).

2.3.2 Uranium toxicity to the reproduction of soil invertebrates

The reproduction tests with the three invertebrate species revealed that *E. andrei, E. crypticus and F. candida* were quite sensitive to U in the PTRS1 soil. Tests fulfilled the validity criteria established by the standard guidelines (ISO, 2004, 1999, 1998). The resulting NOEC, LOEC, EC_{20} and EC_{50} values obtained in this study and toxicity data available in the literature are summarized in the Table II.1.

The effects of U in the reproduction of *E. andrei* were evident, since statistical significant differences were found between the control and the highest tested concentrations of U for this organism (F = 5.218, d.f. = 23, p = 0.002), (Figure II.2). The tested metal did not significantly affect the reproduction of *E. andrei* at concentrations up to 500.0 mg U Kg^{-1} (NOEC) but compromised this endpoint for concentrations above 550.0 mg U Kg^{-1} (LOEC). EC_{20} and EC_{50} values of U for *E. andrei* reproduction were 474.83 mg U Kg^{-1} and 631.00 mg U Kg^{-1}, respectively (Table II.1). The results obtained in our study, did not support the conclusions from other works (Sheppard and Sheppard, 2005), in which most of the organisms were not affected by U concentrations lower than 1000 mg U Kg^{-1}. Likewise, Sheppard and Stephenson, (2012) recorded no toxic effects for *E. andrei* below the same concentration in basic soils (carbonated) (pH 7.5, 18% organic matter, 18% clay). However, in the same work, the production of juveniles was compromised when organisms were exposed to U in two soils with a low percentage of organic matter (2.2% and 1%) and with a pH of 7.5 and 6.2, respectively. According to the literature, the adsorption of metals to soil components is dependent on its physical and chemical properties. Several studies demonstrated, that soil properties such as e.g. pH, cation exchange capacity (CEC), $CaCO_3$, Fe, manganese oxides, clay and organic matter content can influence the bioavailability and therefore the toxicity of chemicals to soil organisms (Domene et al., 2011; Römbke et al., 2006; van Gestel et al., 2011). Equally important, is the potential influence of soil properties in the behavior and performance of the test species. In their study, Sheppard and Stephenson, (2012) attributed the reduced rate of reproduction of *E. andrei* to the low organic matter content of the test soils, as *E. andrei* is an epigeic species with a high preference for organic matter rich soils. Further, Chelinho et al., (2011), observed that soils with an

organic matter content below 4% reduced or completely inhibited earthworms reproduction. However, the PTRS1 natural soil, has a high organic matter content, 6.2% (according to the classification provided by Murphy et al., (2012). Besides, as previously checked, the intrinsic properties of this soil did not compromise the performance of earthworms (Caetano et al., 2012). A high organic matter content of soils is usually related with a decrease in the toxicity of the contaminants for the organisms, due to the high adsorption of chemicals to this soil constituent, resulting in a low bioavailable fraction of the chemical (Kuperman et al., 2006; Natal-da-Luz et al., 2011; Römbke et al., 2006). However, this was not the case in the study. In fact, Lourenço et al., (2011a) and Lourenço et al., (2011b) exposed *E. andrei* to a uranium mine contaminated soil with a concentration of uranium of 215.72±8.50 mg U Kg^{-1}, a pH of 7.79±0.01, and 7.71±0.60% of organic matter and observed that the bioaccumulation of uranium and of daughter radionuclides was in tandem with loss of DNA integrity of coelomocyte cells, changes in the frequency of cells of immune system and also with histopathological changes (especially of the epidermis and chloragogenous tissue and intestinal epithelium). A high bioavailability of uranium and other metals was also not expected with such soil properties however the effects observed in the epidermis and in the intestinal tract of earthworms suggested that not only soil properties governed the exposure of the organisms and the uptake of metals. In fact, some other authors (Hobbelen et al., 2006) had also suggested that the direct dermal exposure of the earthworms to metals in the soil pore water, the ingestion of water, polluted food and/or soil particles may strongly favor the bioaccumulation of metals. Since pH is variable in the different compartments of gastrointestinal tract of earthworms, it can increase the mobilization of contaminants from soil after its ingestion (Li et al., 2009; Peijnenburg and Jager T, 2003).

Although, other metals were present in the contaminated soil tested by Lourenço et al., (2011a) and Lourenço et al., (2011b) uranium likely had a crucial role in the toxic effects observed, because it's content in whole body of the earthworms has significantly increased after 14 days of exposure and persisted till 56 days. These authors also suggested that the changes observed in DNA integrity were likely early warning indicators of effects on the growth and reproduction of the organisms. And in fact, effects on

reproduction were observed in our study, in organisms exposed to high uranium concentrations, as previewed by these authors. Further, conclusions made in the studies of Lourenço et al., (2011a) and Lourenço et al., (2011b) about the role of uranium in the biological effects recorded are reinforced by the work of Giovanetti et al., (2010). These authors exposed earthworms, from the species *E. fetida,* to a soil (no information provided about the soil) contaminated with both natural and depleted uranium for 7 and 28 days. Regarding natural U no mortality or significant changes in weight were observed for both exposure periods at U concentrations up to 600 mg $kg^{-1}_{dw.}$ The chloragogeneous tissue, the main storage tissue of U, presented meaningful changes after 7 days of exposure for concentrations $\geq$ 300mg U Kg^{-1}, while DNA strand breaks were recorded, increasing in a dose dependent manner for concentrations above 150 mg U Kg^{-1}. These concentrations of U were both close to the one quantified in the soil tested by Lourenço et al., (2011b) supporting the likely dominant role of U in the toxic effects observed in their study.

Regarding to *E. crypticus*, significantly differences in reproduction were obtained (F = 31.05, d.f.= 12, p < 0.05), (Figure II.2). The reproduction of potworms was not significantly affected at concentrations of U up to 420.8 mg U Kg^{-1} (NOEC), and was significantly reduced above 526.0 mg U Kg^{-1} (LOEC), (Table II.1). The EC_{20} value estimated was 469.7 mg U Kg^{-1} and a 50% reduction in the number of juveniles produced (EC_{50}) was estimated at a concentration of 518.6 mg U Kg^{-1}. Although no toxicity values are reported for the lowest concentrations tested, enchytraeids showed considerable sensitivity to U, since the number of juveniles were minimal or no juveniles were produced by *E. crypticus* at concentrations above 657.5 mg U Kg^{-1} (Figure II.2). Despite enchytraeids are commonly used in standardized toxicity tests, to the best of our knowledge, no data are available in the literature regarding the effects of U on the reproduction of this test species. The available information concerns only the toxic effects caused by others metals, or by natural soil properties in the reproduction of this species (Amorim et al., 2005b; Domene et al., 2011; Kuperman, 2004; Kuperman et al., 2006; Peijnenburg et al., 1999). Thus, taking into account this literature review pH and CEC were the most important parameters controlling the high sensitivity of enchytraeids

to metals. Additionally, and according to Kuperman et al., (2006), adults survival and juveniles production by *E. crypticus* can be maximized in natural soils with properties within the following ranges: 4.4 – 8.2 pH; 1.2 – 42% OM; 1 – 29% clay. The PTRS1 natural soil used as test substrate fell into in these ranges (Table II.1), and similarly to *E. andrei,* the reproduction of this species was not compromised during the validation of the PTRS1 natural soil as a reference soil (Caetano et al., 2012), meaning that the soil properties did not limit the performance of *E. crypticus*.

Concerning to *F. candida*, U affected the production of juveniles, as shown by a significant decrease of this endpoint along the concentrations tested (F = 11.6, d.f. = 12, p < 0.05) (Figure II.2). The number of juveniles was not significantly affected up to a U concentration of 675.50 mg U Kg^{-1} (NOEC), but it was significantly decreased for U concentrations equal to or greater than 756.10 mg U Kg^{-1} (LOEC). The EC$_{20}$ value estimated for reproduction in our study was 343.41 mg U Kg^{-1} which is considerably lower than the toxicity data reported by Sheppard and Sheppard, (2005), EC$_{20}$ >710 mg U Kg^{-1} in two loam soils with pH 7.5. The low sensitivity of *F. candida* to U was also observed by Sheppard and Stephenson, (2012) which tested 3 soils amended with a range of uranium concentrations and aged for 10 years before testing. In this study, the lowest EC$_{20}$ value obtained was 840 mg U kg^{-1} in a loam soil (pH 7.5, 24% clay, 2.2% OM). Despite this, *F. candida* was more sensitive in the study of Sheppard and Stephenson (since their EC$_{20}$ value was similar to the EC$_{50}$ recorded in our study 851.64 mg U Kg^{-1}) but these authors did not discard the impact of soil properties in the performance of the species. When considering the number of juveniles produced, U was less toxic to *F. candida* comparatively to *E. andrei* and *E. crypticus*. The lower sensitivity of *F. candida* is also consistent with other studies, when the effects of other metals in the reproduction of the three species was investigated (Kuperman, 2004; Koen Lock and Janssen, 2001), or even when other species of collembolans are tested (Sheppard and Stephenson, 2012). Besides other reasons, the exposure of *F. candida* to chemicals in soil is apparently lower than for earthworms, which are exposed both by ingestion of contaminated soil (mineral particles, organic matter and chemicals in the soil solution) and also through direct dermal contact (Layinka et al., 2011). Despite the widely known influence of soil

parameters on the bioavailability of chemicals and their influence on the reproduction of soil organisms, less is known about the intrinsic effects of physicochemical parameters of the soils in the reproduction of *F. candida*. In generally, several authors have reported a high tolerance of *F. candida* reproduction, to a wide range of soil textural classes, organic matter contents and soil pH (M Amorim et al., 2005; Domene et al., 2011; Jänsch et al., 2011). Once again the performance of this species was not compromised by the intrinsic properties of the PTRS1 soil, hence the effects observed can undoubtedly be attributed to uranium exposure.
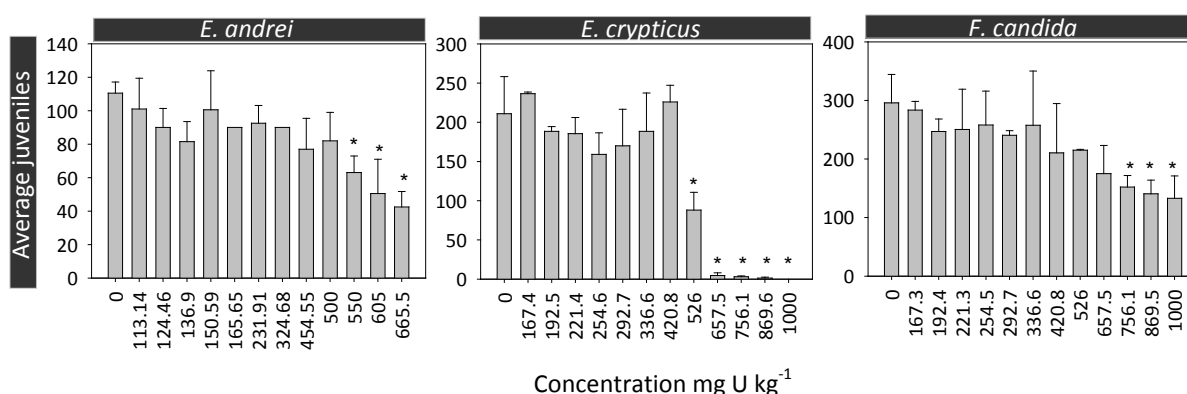
Figure II.2 Reproductive output of *Eisenia andrei*, *Enchytraeus crypticus* and *Folsomia candida* exposed the natural soil PTRS1 spiked with different concentrations of Cu. Error bars indicate the standard error and asterisks sign out significant differences between the treatment and the control (0 mg U kg$^{-1}_{dw}$), ($p<0.05$).

2.3.3. Phytotoxicity of uranium

Relatively to terrestrial plants, tests fulfilled all the validity criteria as described by the standard guidelines (ISO, 2005). Data obtained showed no adverse effects on seed's emergence of all species tested. No significant differences in seeds germination were recorded between treatments ($p > 0.05$) for all the species tested. In opposition, it was possible to observe a relatively high rate of germination, either in monocotyledonous and dicotyledonous species (Figure II.3). An apparent hormetic effect was also recorded for the other endpoints measured for almost all the species tested. Such occurrence was also recorded by other author's and it was attributed to the use of uranyl nitrate, as uranium test compound, which corresponds to a supplementary dose of N given to plants (Sheppard and Sheppard, 2005). The lack of sensitivity of seeds emergence endpoint was

somewhat expectable, based on previous results from other studies (e.g. Pereira et al., 2009); seed coats form a barrier which protects embryos from exposure to a wide range of contaminants, especially metals. Thus, the germination relies almost exclusively on the seed reserves making it a less sensitive endpoint to the toxicity of soil pollutants toxicity (Liu et al., 2007).
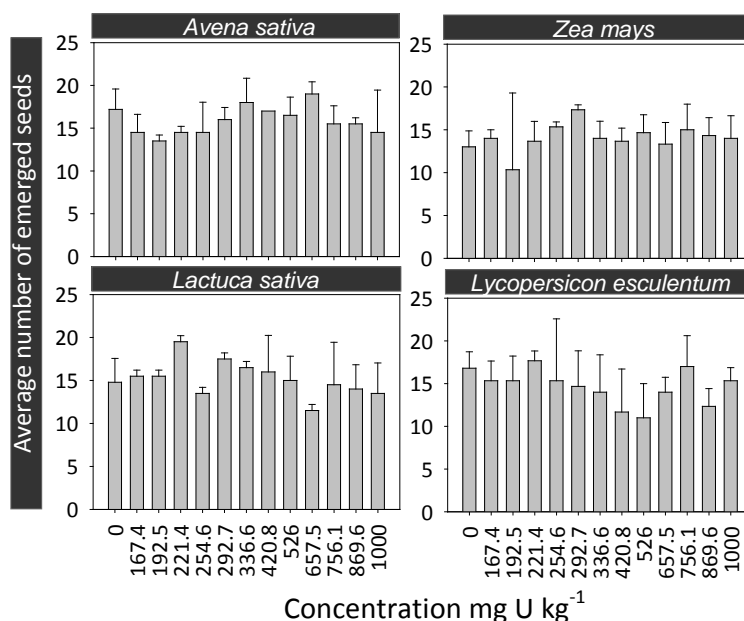
Figure II.3 Average number of emerged seeds in monocotyledonous, *Avena sativa and Zea mays*, and dicotyledonous species, *Lycopersicon esculentum and Lactuca sativa* exposed to PTRS1 soil contaminated with Cu. Error bars indicate the standard error and asterisks represent significant differences between the treatments and the control (0 mg U kg^{-1}_{dw}), (p <0.05).

With regard to production of fresh and dry-mass, it was possible to perceive that the tested plants displayed different sensitivities to this metal. However, no significant differences were generally observed comparatively to the control with except for *L. sativa* dry mass (H = 22.8, d.f. = 12, p = 0.029). Thus, and according to Figure II.4, *L. sativa* was the most sensitive terrestrial plant to U comparatively with all the other tested species in terms of dry mass yield. The high sensitivity of *L. sativa* was also found by Hubálek et al., (2007) and Soudek et al., (2011). This was probably caused by the high capacity of this species to bioaccumulate high concentrations of metals, including uranium (Pereira et al., 2009).

The exposure of plants to metals, was already extensively studied, showing that these contaminants can induce biological effects on germination, growth and development, as well as, alterations in the nutrient profile of plants (Gopal and Rizvi, 2008; Pereira et al., 2009). However, only some studies (e.g. Sheppard and Evenden, 1992; Sheppard et al., 1992) and others reviewed (Sheppard and Sheppard, 2005) have assessed the ecotoxicological effects of U on terrestrial plant species.
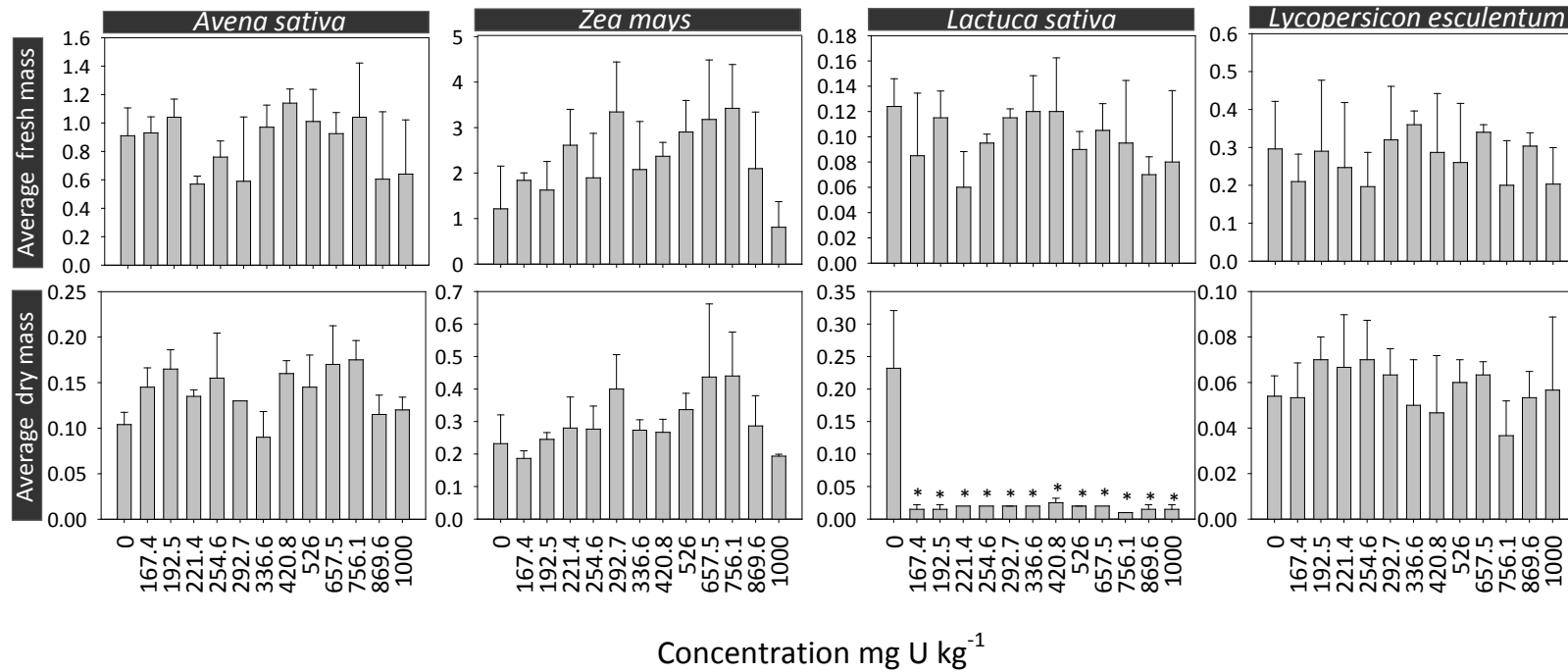
Figure II.4 Average values of fresh and dry mass measurements in monocotyledonous, *Avena sativa and Zea mays*, and dicotyledonous species, *Lycopersicon esculentum* and *Lactuca sativa* grown in PTRS1 soil artificially spiked with Cu. Error bars represent the standard error and the asterisks indicate significant differences between the treatments and the control (0 mg U kg^{-1}_{dw}) (P<0.05).

Based on our study, once again was proved the diverse ecotoxicological outcomes for U effects on plant species, since no effects were observed, in the range of tested concentrations for the three evaluated endpoints (in three out of four species), in the PTRS1. Similar results were obtained by Sheppard and Stephenson, (2012) in acidic soils (Table II.1), when testing the emergence and growth of wheatgrass *Elymus lanceolatus*. Like in our study, these authors did not observe any effect on this species in concentrations up to 1000 mg U Kg^{-1}, including. In opposition, Sheppard and Sheppard, (2005) revised data on U toxicity to terrestrial plants and reported concentration effects (EC$_{25}$) ranging from 300 to 500 mg U Kg^1, considering only the most reliable studies. Stojanović et al., (2009) also reported phytotoxic effects of U on *Zea mays* exposed, on different types of soils, to concentrations of 250, 500 and 1000 mg U kg^{-1}, but especially at the highest concentration tested and in the most acidic soil. However, no statistical analysis of the data was performed in this study.

Soil properties are also the factors that most strongly affect U uptake and phytotoxic effects, (Soudek et al., 2011; Tunney et al., 2009; Vandenhove et al., 2007b). Parameters like pH, organic matter, clay minerals, carbonates, as well as Fe, Al and Mn oxides contents in soil, affect the bioavailability and toxicity of U to plants (Bednar et al., 2007; Vandenhove et al., 2007a). The bivalent uranyl ion (UO$_2^{2+}$) is sorbed to the negatively charged surfaces of clay minerals and organic compounds. In acidic soils, but with the increase of pH, more negatively charged binding sites are available on mineral surfaces due to the progressive reduction of protons occupying these binding sites. However, pH values close to 6, like the one of the PTRS1, tends to favor the availability of U, since the concentrations of carbonates tends to increase, and U is released to the soil solution in the form of U-carbonate complexes (Vandenhove et al., 2007a). The natural soil PTRS1 besides being an acidic soil, had a lower clay content, which thus means lower adsorption binding sites for the bivalent uranyl ion (UO$_2^{2+}$), hence constraining the bioavailability of U in the test soils.

Despite the likely availability of U in the PTRS1, other soil properties and plant mechanisms may explain the reduced sensitivity of the plants in comparison with soil microbial parameters and invertebrates (except for lettuce). In a study published by

(Viewweger and Geipel, 2010) a substantial increase in U absorption by *Arabidopsis halleri*, was attributed to Fe deficiency in the medium of hydroponically grown plants. With respect to this metal, in the natural PTRS1 soil, the analyses done by Caetano et al., (2012) showed that Fe surpassed the soil benchmark values proposed by two EPA regions (http://rais.ornl.gov/tools/eco_search.php). In this sense, it is hypothesized that the high Fe content of the PTRS1 natural soil, may have also contributed for reducing the absorption of U by plants. As far as plant mechanisms are considered, in several studies reviewed by Mitchell et al., (2013) the transport of uranium within plants was reduced and higher uranium concentrations were consistently found in the roots. Using X-ray absorption spectroscopy (XAS) and transmission electron microscopy (TEM), Laurette et al., (2012) observed that when plants are exposed to U and phosphates, needle-like U-phosphates are formed and precipitate, both outside and inside the cells, or persist in the subsurface of root tissues. The precipitation of U-phosphate complexes acts as a protective mechanism preventing U translocation to the shoots and leaves. This can also occur when the culture medium of the plants has no phosphate, since some plants are able to exudate phosphates. Further, U may be also absorbed like UO_2^{2+} and linked to endogenous organophospate groups (Laurette et al., 2012). In opposition, when translocation occurs within plants, U has mainly formed U-carboxylated complexes. Plants can also exudate organic acids to the rhizosphere environment or UO_2^{2+} may form complexes with endogenous compounds like malic, citric, oxalic and acetic acid (Laurette et al., 2012). In summary, the different mechanisms described above could explain the lack of toxic effects observed for *A. sativa*, *Z. mays* and *L. esculentum*, in opposition to *L. sativa*. Most concerning is the fact that the majority of studies testing the phytotoxicity of uranium, including those performed by us, were made with the addition of nutrients solution, which increased the availability of phosphates to the test soil, likely decreasing the sensitivity of plants to U. Hence, to enhance the protection level of SSVs derived for plants, more assays with different plant species should be performed and the addition of nutrients should be prevented, or at least the tests may include replicates with and without nutrients.

Table II.1 Toxicity data for microbial processes, soil invertebrates and plants with effect concentrations as mg U kg$^{-1}_{dw}$ soil, with indication of the 95% confidence intervals

Biota	Endpoint	Soil type	pH	OM	WRC (%)	Clay	(mg U Kg$^{-1}_{dw}$)				Reference
							NOEC	LOEC	EC$_{20}$	EC$_{50}$	
Arylsulphatase			5.91±0.098	6.5±0.004	23.94±1.839	3.3	232.5	279	155.3 (84.76-255.87)	295.6 (216.09-375.17)	present study
Dehydrogenase			5.91±0.098	6.5±0.004	23.94±1.839	3.3	< 134.5	≤134.5	34.9 (20.52-59.35)	110.3 (83.25-137.47)	present study
Nitrogen mineralization			5.91±0.098	6.5±0.004	23.94±1.839	3.3	694.4	833.3	152.2 (46.66-257.79)	347.0 (211.25-482.91)	present study
Celulase	enzim. act.	natural soil	5.91±0.098	6.5±0.004	23.94±1.839	3.3	≤134.5	≥ 134.5	n.d.	n.d.	present study
Urease			5.91±0.098	6.5±0.004	23.94±1.839	3.3	< 134.5	≤134.5	< 134.5	< 134.5	present study
Potencial nitrification			5.91±0.098	6.5±0.004	23.94±1.839	3.3	< 134.5	≤134.5	429.5 (229.53-629.46)	610.0 (459.07-761.11)	present study
Eisenia andrei			5.91±0.098	6.5±0.004	23.94±1.839	3.3	500.0	550.0	474.8 (391.47-558.04)	631.0 (532.78-699.21)	present study
Eisenia fetida			6.2	1.0	n.d.	2.0	n.d.	n.d.	>1000	n.d.	Sheppard and sheppard, 2012
Eisenia fetida	rep.	natural soil	6.2	1.0	n.d.	2.0	n.d.	n.d.	>1120	n.d.	Sheppard and sheppard, 2012
Eisenia fetida			7.5	2.2	n.d.		>838	n.d.	n.d.	n.d.	Sheppard and sheppard, 2005
Eisenia fetida			7.5	18.4	n.d.		>994	n.d.	n.d.	n.d.	Sheppard and sheppard, 2005
Enchytraeids crypticus	rep.	natural soil	5.91±0.098	6.5±0.004	23.94±1.839	3.3	420.8	526.0	469.7 (355.47-584.04)	518.6 (480.40-556.90)	present study
Folsomia candida			5.91±0.098	6.5±0.004	23.94±1.839	3.3	675.5	756.1	343.4 (172.23-514.60)	851.64 (606.10-1097.18)	present study
Folsomia candida	rep.	natural soil	7.5	2.2	n.d.	24	n.d.	n.d.	840.0	n.d.	Sheppard and sheppard, 2012
Folsomia candida			7.5	n.d.	n.d.	n.d.	n.d.	n.d.	>720	n.d.	Sheppard and sheppard, 2005
Avena sativa			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≥1000	>1000	n.d.	n.d.	present study
Zea mays			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≥1000	>1000	n.d.	n.d.	present study
Lactuca sativa			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≥1000	>1000	n.d.	n.d.	present study
Lycopersicon esculentum	germ.	natural soil	5.91±0.098	6.5±0.004	23.94±1.839	3.3	≥1000	>1000	n.d.	n.d.	present study
Elymus lanceolatus			6.2	1.0	n.d.	2.0	n.d.	>1000	n.d.	n.d.	Sheppard and sheppard, 2012
Elymus lanceolatus			7.5	2.2	n.d.	24.0	n.d.	>1001	n.d.	n.d.	Sheppard and sheppard, 2012
Avena sativa			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≥1000	>1000	n.d	n.d.	present study
Zea mays			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≥1000	>1000	n.d	n.d.	present study
Lactuca sativa	f. m.	natural soil	5.91±0.098	6.5±0.004	23.94±1.839	3.3	≥1000	>1000	n.d	n.d.	present study
Lycopersicon esculentum			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≥1000	>1000	n.d	n.d.	present study
Avena sativa			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≥1000	>1000	n.d.	n.d.	present study
Zea mays			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≥1000	>1000	n.d.	n.d.	present study
Lactuca sativa	d. m.	natural soil	5.91±0.098	6.5±0.004	23.94±1.839	3.3	< 167.4	≤ 167.4	n.d.	n.d.	present study
Lycopersicon esculentum			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≥1000	>1000	n.d.	n.d.	present study
Zea mays			5.2	2.5	n.d.	n.d.	n.d.	>100	n.d.	n.d.	Stojanivic et al.,2009

Average ± STDEV: pH (H$_2$O); OM-organic matter (%) and WHCmax – maximum water holding capacity (%); Clay %; rep.-reproduction; germ.-germination; f.m.- fresh mass; d.m- dry mass; Enz. act.- enzyme activity; n.d.-not determined.

2.3.4. Derivation of a Soil Screening Value (SSV) for uranium applying assessment factors

Following the approach suggested by the Technical Guidance Document published by the European Commission (EC, 2003) in support of the Commission Directive 93/67/EEC on Risk Assessment for new notified substances, of the Regulation nº 1488/94 on Risk Assessment for existing substances and Directive 98/8/EC of the European Parliament and the Council, concerning the placing of biocidal products on the market a predicted no effect concentration for U in the PTRS1 soil was determined, based on the endpoint for which both the lowest NOEC and EC_{20} values were obtained. These values corresponded to arylsulfatase activity. Further, since more than three NOEC values were obtained in this study for at least three species, an assessment factor of 10 was applied, giving a PNEC value changing between 15.5 (EC_{20} based) and 23.3 (NOEC based) mg Kg^{-1}. This value was six to four times lower than the PNEC value suggested by Sheppard and Sheppard [81], which was 100 mg U kg^{-1}.

2.4 Conclusion

With the present study it was possible to generate a set of important ecotoxicological data for the derivation of a SSV for U using a Portuguese natural soil representative of a granitic region, where this type of mine exploration occurred.

Soil Enzyme activities were clearly inhibited by U, namely in the highest concentration tested. The obtained results depended not only on the concentrations of U but also on the properties of soil, which were likely responsible for the great bioavailability of U and by the effects in soil microbial population and, consequently, in their activity. With the exception of CELL activity, it was possible to calculate the effect concentration values for the remaining enzyme activities, some of which were particularly sensitive to U (namely DHA and UR). Further, and comparatively, to the remaining effect concentrations obtained/estimated for invertebrates and plants, the soil microbial parameters were more sensitive to U contamination. Regarding DHA and UR activities, no NOEC values were obtained, hence it is possible that EC_{20} could be even lower than 100 mg U $kg^{-1}_{dw.}$

The toxic effects of U in soil invertebrates were also confirmed, but the tested species showed a variable sensitivity to this metal in soil. The increasing order of species sensitivity to U based on EC_{50} values for reproduction was *E. crypticus > E. andrei > F. candida*. However, if EC_{20} values are considered *F. Candida* is the most sensitive invertebrate, since its EC_{20} value was 343.41 mg U Kg^{-1}, compared to 474.83 mg U Kg^{-1} and 469.76 mg U Kg^{-1} EC_{20} values estimated for *E. andrei* and *E. crypticus*, respectively. The EC_{20} value showed to be much more protective for *F. candida* comparatively to the EC_{50} value obtained for the same species. Additionally, the EC_{20} values estimated were lower than the NOEC values for *E. andrei* and *F. candida*. Thus, the EC_{20} values estimated using the logistic model should be selected for the derivation of more protective SSVs. Relatively to plants the tested species showed no adverse effects caused by U in soil, with the exception of *L. sativa* in terms of dry mass yield. Considering the results obtained, it was possible to verify a great variability between the EC_x values estimated in this study and those reported in the scientific literature. Multiple factors can contribute to this discordance, but probably at least for some species, soils physical and chemical properties were the main factors responsible for such differences. Although, this reinforces, at least in part, the importance of using natural soils representatives of the main types of soil from each region in ecotoxicological evaluations and in their use in the derivation of SSVs, the data generated suggests that the SSV derived for uranium, for Portuguese regions with soils similar to the PTRS1, was six times lower than the PNEC value proposed by Sheppard and Sheppard, (2005), (without including soil microbial community data). Nevertheless, as mentioned previously, more data with other plant species should be obtained following standard protocols. This SSV value is near the background values found in non-contaminated soils (Caetano et al., 2012; Pereira et al., 2008), but not in some areas with naturally occurring uranium anomalies in soils, where concentrations ranging between 13-724 mg U Kg^{-1} can be found (Pereira and Neves, 2012) .

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Chapter III

Contribution for the derivation of a SSV for Cadmium using a natural

reference soil

Contribution for the derivation of soil screening values (SSVs) for cadmium using a natural reference soil.

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Abstract

In order to generate a set of ecotoxicological data useful in derivation of cadmium SSVs, a battery of ecotoxicological tests was carried out, in a Portuguese natural soil contaminated with cadmium. The toxicity of this metal was studied for microbial parameters, reproduction of invertebrates, seed germination and growth of terrestrial plants.

We observed that cadmium slightly compromises enzymatic activity, with the most enzymes being affected at concentrations above 100 mg Cd kg^{-1}_{dw}. Only, acid phosphatase and N mineralization were less tolerant to Cd, reducing their activity at concentrations equal or greater than 13.4 mg Cd kg^{-1}_{dw}. The production of juveniles by soil invertebrates was constrained at low Cd concentrations, with *E. crypticus* proving to be the most sensitive species to this metal. The EC_{50} values obtained for these organisms were 8.3, 76.4 and 64.8 mg Cd Kg^{-1}_{dw}, respectively. Seed germination in *A. sativa* and *Z. mays* was not compromised by tested concentrations being the EC_{50} values obtained for *L. sativa* and *L. esculentum,* 460, 919.04 mg Cd kg^{-1}_{dw}, respectively. Dry mass was the most sensitive endpoint analyzed, with values among 20.4 mg Cd kg^{-1}_{dw} for *L. sativa* and 185.1 mg Cd k^{-1}_{dw} for *Z. mays*.

With data obtained in this work, we propose PNEC values to be used as a SSVs for Cd changing between 3.7 (EC_{20} based) and 3.5 (NOEC based) mg Kg^{-1}_{dw}. This work is a good contribution for the establishment of national soil quality guideline values.

Key-words: cadmium, natural reference soil, toxicity values, soil enzymes activity; *Eisenia andrei*; *Enchytraeus crypticus*; *Folsomia candida*; *Avena sativa*; *Zea mays*; *Lacuta sativa*; *Lycopersicon esculentum*.

3.1 Introduction

Persistent contaminants like metals are present in many soils worldwide, affecting human health and ecosystems. The contamination of soils and the subsequent effects in their quality, led to a growing concern regarding the protection of the diversity and ecological functions of this environmental compartment (CEC 2006a; CEC 2006b). During the last decade in several European countries new regulations for soil protection and conservation have been developed, and the basis and emphasis of these regulations changed progressively from the simple quantification of pollutants, as a measure of soil quality, to ecologically risk-based limits (Frank et al., 2008). In this context, many countries developed soil screening values (SSVs) or other risk-based quality criteria to be used in the risk assessment of contaminated sites. SSVs are defined as threshold concentrations of chemicals in soils which, when attained, a more detailed analysis is needed (Fishwick, 2004; Carlon, 2007). SSV was not the designation used by all the countries, but such variation also reflects the variability in the assumptions and in the procedures followed for the derivation of such values. The Netherlands, for example, defined the Target Values which are the levels below which, the risks to the ecosystems are negligible and no further investigation was required (Swartjes, 1999). The Basque country on its turn named their risk-based soil quality values as Indicative Values for Assessment (IVAs), with three different levels (A, B and C), being the level B the one below which the risk is acceptable (Urzelaiet et al., 2000). Outside Europe, USEPA defined the Ecological Soil Screening Levels (ECO-SSLs) to identify the chemicals of potential concern (COPCs) in soils (USEPA, 2003). Despite the designation (herein in this paper the generic term SSVs will be used), all of these benchmark values are particularly useful for the screening phase of the ecological risk assessment (ERA), aimed in identifying the soils that require an additional evaluation, based on more site-specific ecotoxicological and ecological data. The clear advantages of using SSVs rely on the speed and ease of application and the clarity of the conclusions for regulators and non-specialist

stakeholders (Carlon, 2007). Such advantages make these values a very useful tool for the management of contaminated land, even economically, since they can help to screen out several sites from more time consuming and expensive evaluations.

In the Portuguese context, and in opposition to what was done for water resources, especially now, enforced by the Water Framework Directive (EC, 2000) and daughter directives, no soil protection values or risk limits for metals or other contaminants were defined or even legally established. Therefore, when the assessment of contaminated sites is performed, values from other countries have to be used (e.g. Pereira et al., 2006).

In Europe the attainment of the final text of the soil framework directive and its final approval has been delayed, mainly due to the lack of agreement between member states regarding the best approach to identify risk areas and/or prior areas for intervention within their territories (ENDS Europe, 2007). In fact the lack of a national risk assessment framework, or at least of a common accepted procedure, and the lack of legally established SSVs at the European level or at each member state level, is also contributing for the inexistence of a more broad soil protection policy within the European Union.

SSVs are determined based on toxicity data for plants, invertebrates, soil microbial processes (Swartjes, 1999; Sheppard and Sheppard, 2005; Kuperman, 2004) and sometimes data for mammals and birds, like the ECO-SSLs from USEPA (USEPA, 2003), or even based on background concentrations, like the Target Values from The Netherlands. These values were first defined based on ecotoxicological data, but later re-derived based on the 95% percentile of the background concentrations measured for 24 different contaminants on top soils, from agricultural areas and nature reserves from different regions of the country (Swartjes et al., 2012). Sometimes, they are also limited for soils falling within a given range of specific soil parameters (USEPA, 2003). In fact, knowing that soil properties influence metal partitioning and speciation and, therefore, their bioavailability and ecotoxicity (Amorim et al., 2005; Rooney et al., 2006; Criel et al., 2008; Domene et al., 2010; van Gestel et al., 2011) it is easy to accept that data acquisition for the derivation of SSVs should be conducted in natural soils, representatives of geological heterogeneity of each country, which is particularly high within the European Union (Rombke and Amorim, 2004). This will reduce the uncertainty associated with risk

characterization in the former tier of the ERA process which relies on these values. In fact, as it was summarized by Bone et al., (2010) there is a straight link between several soil properties and the ability of soils to act as a source of contaminants, affecting both water resources and the exposed biota.

Cadmium was chosen for this study because, is one of the most toxic metals (van Gestel & Mol 2003) and is also one of the top pollutants associated with battery recycling sites, improper dumpsites, mining and smelter areas and chemical manufacturing areas all over the world (Blacksmith Institute and Green Cross, 2012). Soils may also be polluted with Cd by agricultural activities like soil applications of commercial phosphate fertilizers, sewage sludge, manure and lime (Adriano, 2001; Adams et al., 2004; Kidd et al., 2007; Monteiro et al., 2009; Nagajyoti et al., 2010). This metal, like the others, occurs naturally in the earth's crust, and may also enter the atmosphere due to the weathering of rocks, windblown soil, and volcanoes. However, more than 90% of Cd in the surface environment is from anthropogenic sources (Pan et al., 2010; Roca-Perez et al., 2010). As far as the mining activity is considered Cd usually occurs in association with Zn ore sphalerite and is recovered as byproduct of Zn mining. In the soil solution, Cd form complex ions with chloride ($CdCl^+$, $CdCl^{3-}$, $CdCl_4^{2-}$), hydroxyl groups $CdOH^+$, $Cd(OH)^{3-}$, $Cd(OH)_4^{2-}$], and bicarbonate, as well as neutral soluble species such as cadmium sulphate ($CdSO_4$) and cadmium chloride ($CdCl^2$) (Alloway, 1995; Kabata-Pendias and Mukherjee, 2007).

Toxicity of Cd in soils has been assessed by several authors with several bioassays and different species (Lock and Janssen, 2001; An et al., 2004; Sagardoy et al., 2009; Bur et al., 2010; Novais et al., 2011), and the behavior of this metal in the soil matrix could be explained mainly by variations in soil pH, organic matter content and by other soil properties (van Gestel and van Diepen, 1997; Sauvé et al., 2000; Barančíková et al., 2004; Kirkham, 2006). Therefore, the soils used to derive ecotoxicological data, may influence the bioavailability of Cd and subsequently its toxicity.

In this context, the purpose of our study was to obtain ecotoxicological data for Cd, performing soil enzymes activity tests, invertebrates and plants tests, using a Portuguese natural reference soil (PTRS1) as test substrate.

3.2 Material and methods

3.2.1 Test soil

A Portuguese natural soil (PTRS1), already characterized as a reference substrate for ecotoxicological purposes by Caetano et al., (2012), (*c.f.,* table in annex), was used in this study. This soil, whose physical and chemical properties (including total metal contents) was from a granitic region located in the center of the country, Ervas Tenras [(Pinhel, Guarda: $40^{o}44'4.27''$N and $7^{o}10'54.3''$W)]. The soil was collected and immediately brought to the laboratory. A portion of the soil, was immediately sieved through a 2 mm mesh size and the sieved fraction (<2 mm) was stored in polyethylene bags at -20°C until further use for soil enzymes activity measurements. For the tests with soil organisms and plants, the soil was air-dried and then passed through a 4 mm sieve and the sieved fraction (<4 mm), and defaunated through two freeze–thawing cycles (48 h -20 °C followed by 48 h at 25 °C) at room temperature.

3.2.2. Test substance

For all the tests, the natural soil was spiked with a stock solution of cadmium sulfate $CdSO_4$, (99%, Sigma) prepared with Milli-Q water, in order to obtain the different ranges of concentrations tested which were ascertained by range finding tests performed with the different test species.

For soil enzyme tests, the PTRS1 soil was spiked with the following concentrations: 0.0, 13.4, 16.1, 19.3, 23.2, 27.9, 33.4, 40.1, 48.2, 57.8, 69.4, 83.3, 100.0 mg Cd kg^{-1}_{dw}. The stock solution of cadmium sulfate was diluted in the amount of water required to adjust the WHC to 80% of its maximum value.

The following Cd concentrations were used to expose the earthworms, collembolans and four terrestrial plant species: 0.0, 35.0, 42.0; 50.4, 60.4, 90.7, 136.0, 204.1, 306.1, 459.2, 688.9, 826.6, 992.0 mg Cd kg^{-1}_{dw}. In the case of potworms the following concentrations were used: 0.0, 7.0, 7.7, 8.4, 9.3, 1.6, 14.5, 18.2, 22.7, 28.4, 35.5, 39.1, 43.0 mg Cd kg^{-1}_{dw}. The amount of water required to adjust the WHC of the soil to 45% of its maximum value was used to dilute the stock solution for tests with invertebrates and plants.

3.2.3 Ecotoxicological assessment

3.2.3.1 Soil microbial activity

Three replicates per test concentration were prepared for each enzyme assay. For the control, six replicates, only spiked with deionized water filtered in a Milli-Q equipment (hereinafter referred as deionized water) were prepared. The replicates were incubated for 30 days, at 20±2°C; photoperiod: 16h^L: 8h^D. During the incubation period, the soil moisture was continuously monitored and adjusted to 80% of its WHC$_{max}$. After the incubation period, 1g of soil per replicate and concentration was weighted and placed in falcon tubes, and then frozen to -20ºC, until analysis. Thereby, a total of 9 sub-replicates were made for each concentration. The soil was thawed at 4°C before analysis. The activity of urease (UA), cellulase (CEL), dehydrogenase (DHA), and acid phosphatase (ACP) were tested, as well as changes in the nitrogen mineralization (NMIN).

The UA activity was assayed according to the method proposed by Kandeler and Gerber, (1988) and Schinner et al., (1996) The samples were incubated for 2h, at 37°C, after the addition of a buffered solution of urea. Ammonia released was extracted with a solution of potassium chloride and determined by the modified Berthelot reaction. The determination was based on the reaction of sodium salicylate with ammonia in the presence of chlorinated water, producing a green colored complex in alkaline pH. UR was detected at 690nm and expressed as µg nitrogen g^{-1} soil$_{dw}$ 2 h^{-1}. The CELL activity was tested according to the method proposed by Schinner and von Mersi, (1990) and Schinner et al., (1996). The reducing sugars produced during the incubation period caused the reduction of hexacyanoferrate (III) potassium to hexacyanoferrate (II) potassium in an alkaline solution. This last compound reacts with with ferric ammonium sulphate in acid solution to form a ferric complex of hexacyanoferrate (II), of blue staining. The activity of CELL was measured colorimetrically, at 690 nm, and expressed as µg glucose g^{-1} soil $_{dw}$ 24 h^{-1}. The method proposed by Öhlinger, (1996) was used in order to assess the DHA. The samples were suspended in a solution of trifeniltetrazol chloride (TTC) and incubated at 40°C for 24 hours. The triphenyl formazan (TPF) produced was extracted with acetone and measured spectrophotometrically at 546 nm and the results were expressed as µg triphenylformazan (TPF) g^{-1} soil$_{dw}$ h^{-1}. The acid phosphomonoesterase activity was tested

according to, the method proposed by Schinner et al., (1996). After addition of the buffered solution of p-nitrophenyl phosphate soil samples were incubated for 2h, at 35°C. The p-nitrophenol released by the phosphomonoesterase activity was extracted with sodium hydroxide, producing a yellow color that was measured spectrophotometrically at 405nm and expressed as µg nitrophenol (NP).g^{-1}. NMIN activity was measured according to Schinner et al., (1996).The soil samples were incubated for 7 days, at 40°C. During this period, the organic forms of nitrogen led to nitrogen in inorganic form (mainly ammonium ion, NH_4^+), which was determined by a modification of the Berthelot reaction after extraction with potassium chloride. The reaction of ammonia with sodium salicylate (NH_3) in the presence of sodium dichloroisocyanurate formed a green colored complex with the addition of alkaline pH and it was measured at 690 nm. NMIN was expressed as µg nitrogen (N).g^{-1} $soil_{dw}$ d^{-1}

3.2.3.2 Invertebrate and plant tests

3.2.3.2.1 Test organisms and culture conditions

The toxicity of Cd was assessed using the earthworm *Eisenia andrei* (Oligochaeta: Lumbricidae), the potworm *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae) and the springtail *Folsomia candida* (Collembola: Isotomidae). All organisms were obtained from laboratorial cultures, kept under controlled environmental conditions (temperature: 20±2ºC; photoperiod: $16h^L$: $8h^D$). The earthworms (*E. andrei*) were maintained in plastic boxes (10 to 50 L) containing a substrate composed by peat, dry and defaunated horse manure (through two freeze–thawing, 48h at -20 ºC followed by 48h at 65 ºC), water and $CaCO_3$ to adjust the pH between 6 and 7. The organisms were fed every 2 weeks with six tablespoon oatmeal previously hydrated with deionized water filtered in a Milli-Q equipment (hereinafter referred as deionized water) and cooked for 5 minutes. The potworms (*E. crypticus*) were cultured in a box (25.5 cm length; 17.4 cm width; 6.5 cm height), which was filled with pot soil moistened to the nearest 60% of its water holding capacity (WHC_{max}) and with a pH adjusted to 6.0 ± 0.5. The organisms are fed twice a week with a teaspoon of macerated oat. The collembolans (*F. candida*) were maintained in plastic containers filled with a culture medium composed by moistened Plaster of Paris

mixed with activated charcoal 8:1 (w:w). They are fed with granulated dry yeast, twice a week, which is added half a teaspoon small amounts to avoid spoilage by fungi.

Seeds from four plant species (two dicotyledonous and two mocotyledoneous) were purchased from a local supplier and used for seed germination and growth tests: *Avena sativa, Zea mays*, *Lacuta sativa* and *Lycopersicon esculentum.*

3.2.3.2.2 Reproduction tests with invertebrates

The reproduction tests with invertebrates were carried out according to the ISO guidelines 11268-2 (ISO, 1998) for *E. andrei*, 16387 (ISO 2004) *for E. crypticus* and 11267 (ISO 1999) in case of *F. candida*. Each replicate of invertebrate tests contained 10 individuals in a certain developmental stage: the earthworms had a fully developed clitellum and an individual fresh weight between 250 and 600 mg, the potworms were of 12-mm size and the springtails were 10–12 days old. Five hundred grams of dry soil was weighted per test vessel for earthworms. For the tests with potworms and collembolans 20 and 30 g of soil were weighted per replicate, respectively. Following an ECx sampling design, which considers more concentrations and less number of replicates, two replicates per concentration and five replicates for the control were prepared in the reproduction tests with *E. andrei*. Adult earthworms were removed from the test containers after 28 days. The produced cocoons persisted in the soil until 56 days have been completed. After this period, the juveniles from each test container were counted. During the test, organisms were fed once a week, with 5 g per box of defaunated horse manure (following the same procedure above described), grounded and sieved horse manure and the soil moisture content was adjusted.

The duration of the *E. crypticus* reproduction tests was 28 days and the adults were left in the vessels until the end of the test. About 2mg of rolled oats were placed on the soil surface weekly to feed the animals. At the end of the test, the potworms were sacrificed with alcohol, colored with Bengal red and counted according to the Ludox Flotation Method, as described in 16387 (ISO, 2004). The reproduction tests with *F. candida* took four weeks to be completed. The collembolans were fed with granulated dry yeast, obtained from a commercial supplier, being weekly added about 2 mg of yeast per

test vessel. At the end of the test, the test containers were filled with water and the juveniles were counted after flotation. The addition of a few dark ink drops provided a higher contrast between the white individuals and the black background. Organisms were counted afterwards by using ImageJ software (online available, http://rsb.info.nih.gov/ij/download.html). The exposure was carried out at 20±2°C and a photoperiod of 16^L: 8^D. For both species five replicates of uncontaminated natural PTRS1 soil were used as controls. The same ECx sampling design applied for earthworms was followed. However, in order to reduce the variability of the results, we prepared three replicates per test concentration. Controls in tests also included five replicates of calcium sulphate controls ($CaSO_4.2H_2O$), and the concentrations were based on the highest sulphate concentrations tested, namely 3380.70, 8.24 and 285.4 mg $CaSO_4.2H_2O$ kg^{-1}_{dw} for *E. andrei, E. crypticus* and *F. candida*, respectively.

3.2.3.2.3 Seed germination and plant growth tests

The standard procedure described by the ISO guideline 11269–2 (ISO 2005) was used to assess the effect of Cd in the germination and growth of four species of terrestrial plants. For this purpose, $200g_{dw}$ of the spiked soil with the same concentrations previously mentioned were tested (cf. section 3.2.2). In this case, the amount of water required to adjust the WHC_{max} of the soil to 45% was used to dilute the stock solution and moist the soil at the beginning of the test. The soil was placed in the plastic pots (11.7 cm diameter, 6.2 cm height) and twenty seeds were added to each test pot being afterwards gently covered with soil. In the bottom of each plastic pot a hole was previously made to let a rope passing through, hence allowing communication with the pot filled with distilled water and the test pot on it. The level of water in the lower recipient was adjusted whenever needed, to keep soil moisture constant. Five replicates of uncontaminated natural PTRS1 soil were used for the control, while three replicates were performed per concentration in order to minimize the variability of the results, and by following the same ECx sampling used for the invertebrates. For each plant test five replicates of calcium sulphate with 1913.6 mg of $CaSO_4.2H_2O$ kg^{-1} were also included as an additional control. At the beginning of the test, nutrients (Substral® - Plants fertilizer using 1 bottle

cap for 2 L of water proportion according to the manufacturer recommendation; Fertilizer NPK: 6-3-6; nitrogen (N): 6%; phosphate (P_2O_5): 3%; potassium (K$_2$O): 6%; iron (Fe): 0,03%; trace elements: Cu, Mn, Mo and Zn), were added to each water containing recipient.. Pots were maintained at constant conditions of temperature (20 ± 2°C), photoperiod (16h^L: 8h^D) and luminosity (25.000 lux). Seed germination, fresh and, dry biomass above soil, were the endpoints assessed for each species at the end of the exposures.

For this work, a battery of enzymes involved in different biogeochemical cycles, N (Nitrogen cycle), C (Carbon cycle)], as well as enzymes more indicative of the good physiological conditions of the whole microbial community (e.g. DHA) were selected. The species of invertebrates and plants were selected based on the availability of standard protocols. Since we aimed to obtain data for the derivation of SSVs, for regulatory purposes, this procedure is recommended.

3.2.4 Statistical Analysis

To determination of significant differences between treatments for each endpoint analysed (activity of enzymes, number of juveniles produced by potworms and collembolans, number of emerged seeds, the fresh and dry mass of the plants), a one-way analysis of variance (one-way ANOVA) was carried out. When ANOVA assumptions were not met a Kruskal-Wallis analysis was performed (SigmaPlot 11.0 for Windows). To assess significant differences between the control and spiked soils, Dunnet method was performed (SigmaPlot 11.0 for Windows). The NOEC (no-observed-effect-concentration) and LOEC (low-observed-effect-concentration) values were determined, based on the outcomes of the multiple comparison tests. The logistic model was used for calculate the ECx values of metal concentration producing a 20% (EC$_{20}$) or 50% (EC$_{50}$) reduction in the tested endpoints. The statistical analyses were performed using STATISTICA 7.0 software.

3.3 Results and discussion

3.3.1 Soil microbial activity

The variation in soil enzyme activities and NMIN in the PTRS1 soil, contaminated with a range of Cd concentrations, is given in Figure III.1. Table III.1 summarizes the toxicity values obtained for each biochemical parameter.

Some of the functional parameters tested were clearly inhibited by Cd added to the soil being ACP activity and NMIN the less tolerant microbial parameters to Cd, (Table III.1). The general pattern of response observed for these parameters corresponded to an inhibition, starting on the lowest Cd concentrations (LOEC= 13.4 mg Cd kg^{-1}_{dw}), (Figure III.1), and leading to an EC_{50} value of 40.2 mg Cd kg^{-1}_{dw} in case of ACP (Table III.1). ACP is one of the many phosphatases in soils and it is an extracellular enzyme secreted by plants and microorganisms, being largely responsible for the mineralization of organic phosphate compounds to inorganic forms, in more acidic soils, which are the only forms taken up by plants and microorganisms (Rao et al., 2000; Huang and Shindo, 2000). Similarly, previous researches also showed a significant inhibition of ACP activity with an increasing concentration of Cd. Dar, (1996) also reported a significant decrease in activity of this enzyme at 50 mg Cd kg^{-1} in a sandy-loam soil (pH 7.9), while Khan et al., (2010) observed a 30.6 % inhibition for the highest Cd concentration tested (5 mg Cd kg^{-1}) 2 weeks after the contamination of a soil, with pH 7.9 and 9.3% of clay. Tejada et al., (2011), observed no significant inhibition of alkaline phosphatase activity for Cd concentrations up to 250 mg kg^{-1}, in a soil with low organic matter content (4.1±0.8).

The inhibition of enzymatic activity in soil could be explained either by metal induced changes in the expression of enzymes or with changes in the viability of soil microorganisms (Papa et al., 2010a). A combination of both factors is also possible. In our work, the decrease in ACP activity may be associated to Cd-promoted changes in the overall microbial community structure. This hypothesis is supported by a concomitant decrease in the mineralization of nitrogen compounds for all the concentrations tested, which was an indication of disturbances in specific microbial groups and subsequently on the N-cycle (Winding et al., 2005). Dai et al., (2004) also observed a negative correlation

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between NMIN and Cd content of soil samples, which reinforces the sensitivity of the microorganisms specifically involved in N-cycle to this metal.

The UA activity was affected by Cd in our study, although no significant differences between tested concentrations and control were recorded for this microbial parameter ($p > 0.05$). The response observed corresponded to a stimulation at the lowest Cd concentrations and an inhibition for the highest ones (Figure III.1). An EC_{20} of 47.8 mg Cd Kg^{-1}_{dw} was calculated while the EC_{50} value calculated was greater than the highest tested concentration (Table III.1). Similar sensitivity for this soil microbial parameter was observed by Pan and Yu, (2011) that registered an inhibition of 23% in UA activity in a brown soil exposed to 50 mg kg^{-1}_{dw} of Cd, for 10 days. Hassan et al., (2013) recorded the minimum UA activity at the utmost Cd level (200 mg kg^{-1}), after 30 days of incubation of a sandy loam soil.

UA is an extracellular enzyme closely related to the N-cycle since it is involved in the hydrolysis of urea to ammonium and carbon dioxide (Kandeler et al., 1996; Wang et al., 2011) and their activity is mainly associated with soil clay, being well known the protective role of this soil component. This is reflected in the work of Dar, (1996) and Hassan et al., (2013) who found more prominent effect of Cd in sandy loam than in clay loam and loam soils. This could be explained by the existence of more cation-exchange sites in clay loam and loam textured soils, particularly due to the presence of clay minerals and organic complexes, which can bind the Cd^{2+} rendering it less available (Hassan et al., 2013). Accordingly, the physical-chemical characteristics of soils play a fundamental role in metal complexation and, subsequently, in their effects on the soil biochemical parameters (Papa et al., 2010; Tejada et al., 2011). Thus, soil enzyme activities and the mineralization process depend from both metal levels and soil properties. Thereby the combination of both, the low clay content of the PTRS1 soil (3.2%), and the high Cd concentrations tested may have been responsible for the impairment in the activity of this enzyme.

DHA activity displayed highly variable results on available studies, probably due to differences in the tested soils in terms of physical and chemical properties and in the diversity of corresponding soil microbial communities (Moreno et al., 2009), the period of

incubation, as well as other factors. Pan and Yu, (2011) for example observed an inhibition of 37.8% in the DHA activity in a brown soil exposed to 100 mg kg^{-1} of Cd, for 10 days. Tejada et al., (2011) only observed 26.2% of inhibition in the activity of DHA for the highest Cd concentration tested (250 mg kg^{-1}), after 120 days of exposure of a Plagic Antrosol, with a very low organic matter percentage. Moreno et al., (2009) observed a highly significant inhibition, after 7 days of incubation, in the activity of DHA and UA activity in a forest and shrubland soil contaminated with 12.5 mg Kg^{-1} of Cd. In our study no significant inhibition of DHA activity was observed in comparison with the control, for all the Cd concentrations tested. The EC$_{20}$ value obtained for this enzyme was greater than 100 mg Cd kg$^{-1}_{dw}$, the highest tested concentration (Figure III.1). Since DHA is an intracellular enzyme, from the electron transport system, found in viable cells (Nielsen and Winding, 2002) it gives an indication of the impact on the viability of the soil microbial community (Taylor et al., 2002). Therefore, we can hypothesize that either concentrations of Cd up to 100 mg kg^{-1} did not affect the overall soil microbial community in the PRS1, or that after one month, the most affected *taxa* from the community, were replaced by more tolerant ones, mimicking the potential impairment on DHA activity. In fact, the second hypothesis seems more acceptable, especially when a joint analysis of the results, for all the biochemical parameters analysed, is made. This is also reinforced by previous studies which demonstrated more severe effects of Cd on the soil microbial activity of a forest soil, after 7 days of exposure, rather than after 60 days (Moreno et al., 2009).

CEL was lightly inhibited, but not significantly, at some intermediate Cd concentrations and no inhibition occurred at higher concentrations. The activity of this enzyme in our study may have been more related with fungi rather than soil bacteria. Besides bacteria, also fungi organisms are capable to produce cellulolytic enzymes (Baldrian and Valásková, 2008). Additionally, several studies have shown that bacteria are more sensitive to metals than fungi (Stefanowicz et al., 2008), namely to Cd (Vig et al., 2003). Thereby, in case of soil contamination, it is hypothesized that the fungi community may have ensured the production of these enzymes due to their low sensitivity to soil contamination.

According to Hinojosa et al., (2004b) organic matter also modulates the nature and degree of inhibition of soil enzymes by metals. Low organic matter reduces the potential of the soil to inactivate metals via complexation or sorption reactions and increases the availability of metals and their impact on enzymatic processes (Speir et al., 1995). The PTRS1 soil used in our study has a high percentage of organic matter (6.2%), which can also contribute for explaining the low sensitivity of the majority of soil microbial parameters evaluated in this study, at least up to the concentration of 100 mg kg^{-1} of Cd, which can already be considered an extremely high concentration in an environmental perspective.

In summary, as it was possible to perceive, with the analysis of published data, the sensitivity of the soil microbial parameters is quite variable, depending on the metal tested (e.g. Caetano et al., (a,b, c, submitted)) and the soil properties, therefore their integration in the derivation of soil screening values (SSVs) is a good justification for using natural soils for obtaining SSVs with more regional relevance. Nevertheless, doubts still persist about the best exposure period for enzyme assays. A general analysis of the results obtained by different authors, suggest that lower exposure periods may be overprotective, as they do not reflect possible adjustments of the soil microbial community that can overcome the former impacts on soil functions.
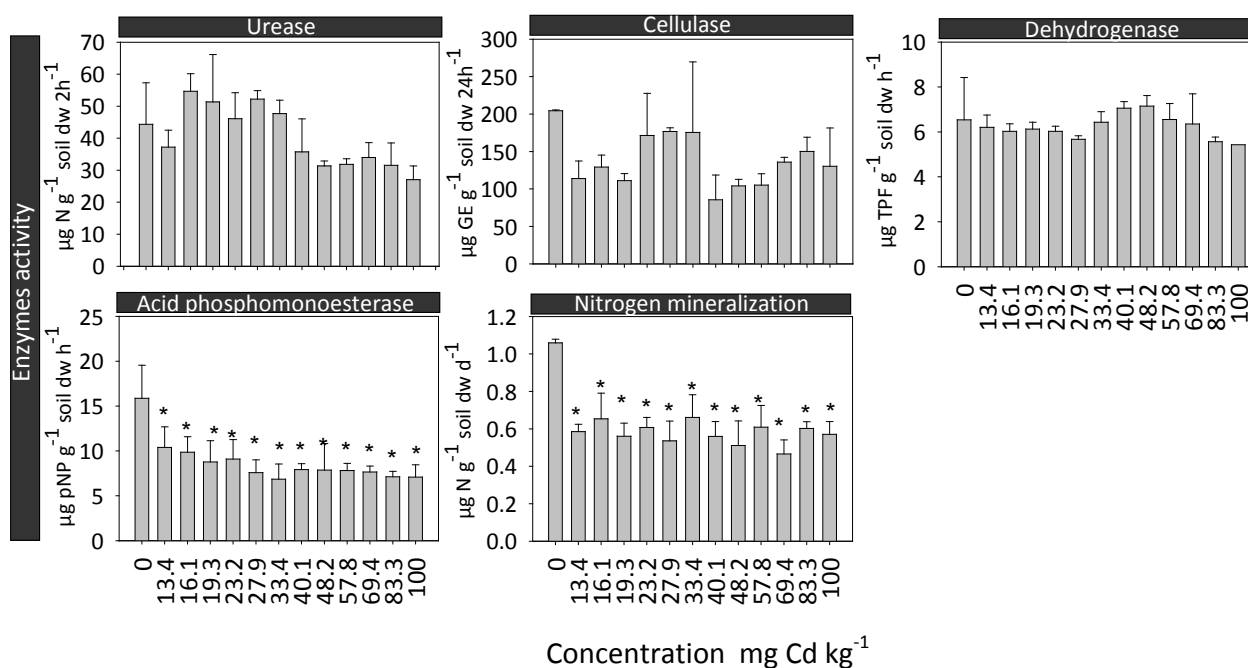
Figure III.1 Soil enzyme activities and N mineralization in PTRS1 soil spiked with a range of cooper concentrations. The error bars indicate the standard deviation. The asterisks sign out significant differences relatively to the control (0 mg Cd Kg$^{-1}_{dw}$), ($P < 0.05$).

3.3.2 Cadmium toxicity in soil invertebrate's reproduction

All the reproduction tests reported complied with the validity criteria for negative controls defined in the respective standard guidelines (ISO 11268, 1998, ISO 16387, 2004; and ISO 11267, 1999) for the three invertebrate species. The reproduction of all invertebrates was clearly compromised by Cd, being the number of juveniles significantly lower than the numbers recorded in the controls, even at the lowest concentrations tested (Figure III.2). Sulphate controls for all the tests showed no statistically significant effects on reproduction endpoints compared with distilled water controls (P > 0.05).

Significantly differences in reproduction of E. andrey were obtained between control and the tested concentrations of (F = 68.2, d.f. = 12, p < 0.001). For this invertebrate, EC$_{20}$ and EC$_{50}$ values calculated were 37.2 and 76.4 mg Cd kg$^{-1}_{dw}$, respectively (Table III. 1). Comparison of effect concentrations determined in this study with literature values indicated that the sensitivity of *E. andrei* was in good agreement with previous studies reporting the toxicity of Cd for the close related *E. fetida* species, using natural soils. Lock and Janssen, (2001) investigated the toxicity of Cd in two natural soils using the

earthworm *E. fetida* and obtained EC_{50}= 73.1 mg Cd kg^{-1}_{dw} (Table III. 1), a value very similar to those obtained in this study (Table III. 1). It should be noted that this natural soil tested by Lock and Janssen, (2001) had a low pH (4.4), clay (1%), and a very high organic matter content (4.8%) closely resembling the PTRS1 soil. The same authors also recorded a lower EC_{50} value (EC_{50}= 55.4 mg Cd kg^{-1}), for a soil with low organic matter content (1.5%) and a high percentage of clay (17%) (Table III. 1). A higher EC_{50} value (108.01 mg Cd kg^{-1}) was obtained by the same authors when using OCDE artificial soil. A similar response was reported by Spurgeon and Hopkin, (1995) in the OCDE soil, obtaining a EC_{50} value for *E. fetida* reproduction of 295.0 mg Cd Kg^{-1}. Therefore, although the PTRS1 natural soil had a high organic matter content, 6.2% (Murphy et al., 2012), it was not sufficient to reduce the bioavailability of Cd and consequently its toxicity to the *E. andrei* reproduction.

In addition to the influence of soil properties in the toxicity of chemicals to soil organisms (Adhami et al., 2008; Domene et al., 2011; van Gestel et al., 2011), also species-specific regulation and detoxification mechanisms can influence the toxicity of metals to organisms. In opposition to essential metals, for which regulation mechanisms are available allowing organisms to maintain homeostasis, under variable environmental conditions, organisms also have to activate detoxification mechanisms for Cd as it is a non-essential element, (Lock and Janssen, 2001). Thus, fault in detoxification mechanisms, at the concentrations tested, were likely responsible by the effects of Cd on *E. andrei.* Cd can affect demography and reproduction, neurosecretory processes immunity and osmoregulation of earthworms (Reinecke and Reinecke, 2002; Siekierska, 2003; Homa et al., 2003; Reinecke et al., 1999). Further, strong DNA damage on earthworm *E. fetida* was observed by Li et al., (2009) at 10 mg Cd kg^{-1}. Thus, we hypothesized that changes in DNA integrity of *E. andrei* may have led to effects on the growth and reproduction of the organisms, translated in a significant decrease in the number of juveniles, even for the lowest concentrations tested. This process may have been favored by the direct exposure of the earthworms to the metal through dermal contact in the soil solution and by soil ingestion, which strongly favors the bioaccumulation of Cd (Hobbelen et al., 2006; Vijver et al., 2005).

Concerning to *E. crypticus* this study showed very high levels of sensitivity of this species to different Cd concentrations, resulting in a significant reduction in the number of juveniles comparatively to the control (F = 50.9 d.f. = 12, p < 0.001), (Figure III.2)*. The number of juveniles was significantly affected for Cd concentrations above 7.7 mg Cd Kg$^{-1}_{dw}$ (LOEC). The EC$_{50}$ value for this species was estimated at the concentration 8.2 mg Cd Kg$^{-1}_{dw}$, which is in agreement the value reported by Novais et al., (2011) of 6.2 mg Cd Kg^{-1}, in the standard natural soil Lufa 2.2 (Table III.1). Lock and Janssen, (2001), obtained EC$_{20}$ and EC$_{50}$ values for reproduction of 19.9 and 72.4 mg Cd Kg$^{-1}_{dw}$, respectively, using a natural loamy soil (17% clay) for testing (Table III. 1). These authors also reported an even higher EC$_{50}$ value for the OCDE artificial soil (10% organic matter), EC$_{50}$= 158.0 mg Cd Kg^{-1}. A joint analysis of all the results obtained by different authors suggest once again that differences in organic matter and clay content of the different test soils explained the differences in toxicity data gathered. Cd was reported as responsible by significant changes in the activity of antioxidant enzymes and subsequent increase in lipid peroxidation in *Enchytraeus albidus* at concentrations above 1 mg Cd kg^{-1} (Novais et al., 2011). Thereby, cadmium may have induced oxidative stress and membrane damage above 7.7 mg Cd Kg$^{-1}_{dw}$ causing effects in the reproduction of *E. crypticus*, as observed in our study.

Regarding to *F. candida*, significantly differences in reproduction were obtained between control and the tested concentrations of Cd (F = 39.6, d.f. = 12, p < 0.001), (Figure 2). Cadmium has constrained the reproduction of organisms for concentrations above 42.0 mg Cd Kg^{-1} (LOEC). EC$_{20}$ and EC$_{50}$ values of Cd for *F. candida* reproduction were 31.7 and 64.8 mg Cd Kg$^{-1}_{dw}$, respectively (Table III. 1). Similarly, van Gestel and Mol, (2003) found EC$_{50}$ values for the effect of Cd on reproduction of *F. candida*, in two natural soils, of 57.9 and 53.7 mg Cd Kg^{-1} (Table III. 1). The EC$_{50}$ value (193.0 mg Cd Kg^{-1}) obtained by the same authors when using the artificial OECD soil, showed a drastic decrease in toxicity of Cd, probably caused by the high OM content of this test soil (10.9 % organic matter). Bur et al., (2010) tested three natural soils with different pH (8.2, 4.5, 6.5) and organic matter contents (2, 16.5, 1.6 %) and also reported lower toxicity values for these soils, EC$_{50}$= 182.0, 111.1 and 117.0 mg Cd Kg^{-1} respectively, compared to the result

obtained in our study (Table III.1). These results suggested that high organic matter content may compensate the potential effect of a low pH, and *vice-versa*, in the bioavailability of cadmium and subsequently in its toxicity to collembolans. Based on EC_{20} and EC_{50} values for the effect of Cd on the reproduction of the three tested invertebrates, *E. crypticus* proved to be the most sensitive organism to Cd followed by *F. candida* and *E. andrei,* both with a very similar sensitivity to this metal (Table III. 1).
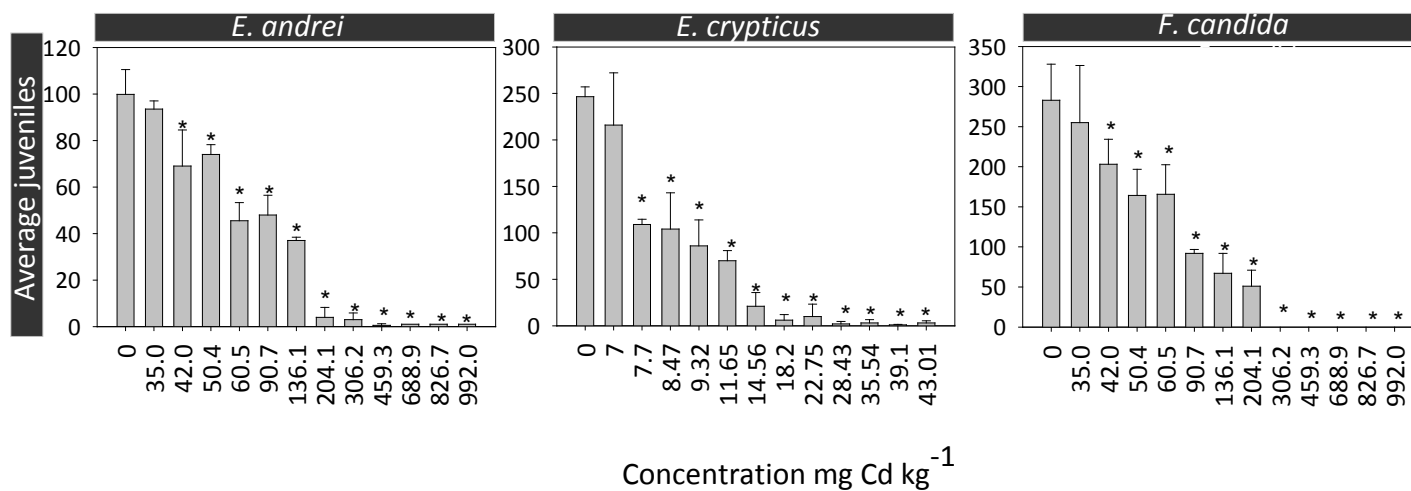
Figure III.2 Reproductive output of *EisenIa andrei*, *Enchytraeus crypticus* and *Folsomia candida* exposed the natural soil PTRS1 spiked with different concentrations of Cu. Error bars indicate the standard error and asterisks sign out significant differences between the treatment and the control (0 mg Cd kg^{-1}_{dw}), ($p<0.05$).

3.3.3 Cadmium toxicity in seed germination and plant growth

The physiological effects of Cd in plants were well evident in our study as can be seen in Figures III.3 and III.4 and in the data described in Table III.1. The effects of this metal in plants are well described in the literature and in fact it is one of the most concerning non-essential metals since, as it was reviewed by Clemens (2006), it can be easily taken up by plants and in some species it can be translocate from roots to the upper plant tissues, posing serious risks to higher trophic levels. Therefore, ecotoxicological data for plant endpoints is of crucial relevance for the derivation of risk limits aimed in protecting the whole terrestrial ecosystems from Cd contamination. In our study, seed germination of *Lactuca sativa* and *Lycopersicon esculentum* was significantly inhibited by high concentrations of Cd (F = 39.1, d.f. = 12, $p < 0.001$ and F = 36.3, d.f. = 12, $p < 0.001$, respectively), (Figure III. 3) namely for Cd concentrations equal to or greater than 459.2 mg Cd Kg^{-1}_{dw} and 826.9 mg Cd Kg^{-1}_{dw} (LOEC), respectively (Table III. 1). The emergence of lettuce seeds was more sensitive, since an EC_{20} value of 279.3 mg Cd Kg^{-1}_{dw} was recorded, while the EC_{20} value estimated for *L. esculentum* was 644.9 mg Cd Kg^{-1} EC_{50} (Table III.1). For maize and oat no significant differences were recorded for this endpoint ($p > 0.05$) for all the concentrations tested (Figure III.3). Indeed, negligible effects of Cd on seed germination were found in some previous studies, showing that seed germination is resistant to Cd in soils (An, 2004; Cao et al., 2007). The differences in the sensitivity of this endpoint to Cd contamination were expectable, since it is related with properties of the own seeds coating structure that protects the embryo from external hazards (Lin and Xing, 2007), which varies among plant species. In fact, seeds germination depends almost exclusively from internal food reserves for the supply of metabolites for respiration and other anabolic reactions (Liu et al., 2007; Lin and Xing, 2007). Therefore, only those coatings more permeable to water and water soluble contaminants can give less protection to their embryos.
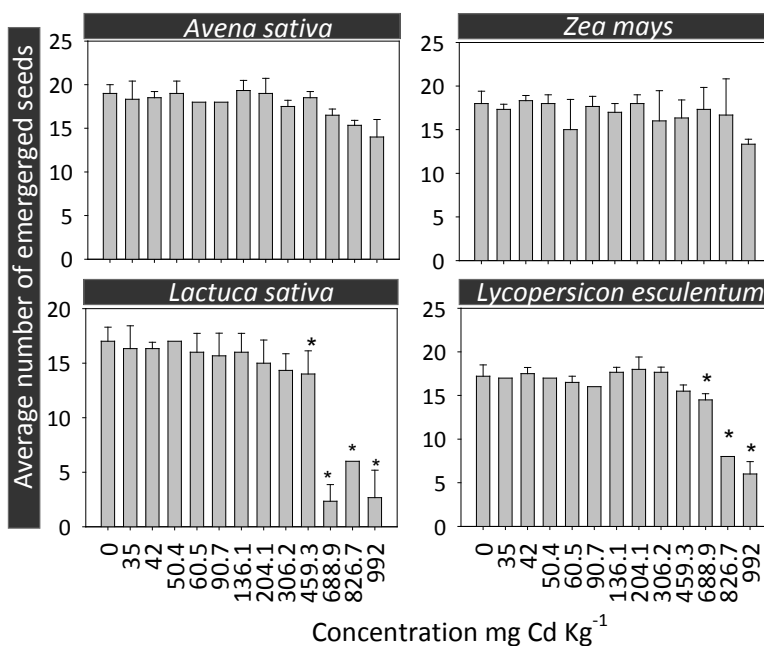
Figure III.3 Average number of emerged seeds in monocotyledonous, *Avena sativa and Zea mays*, and dicotyledonous species, *Lycopersicon esculentum and Lactuca sativa* exposed to PTRS1 soil contaminated with Cu. Error bars indicate the standard error and asterisks represent significant differences between the treatments and the control (0 mg Cd kg^{-1}_{dw}), ($p < 0.05$).

The toxicity of Cd resulted essentially in a significant inhibition in the growth (fresh and dry mass) of the plant species tested. Significant effects in both endpoints were found between the control and almost all tested concentrations (fresh mass, $F = 16.5$, d.f. $= 12$, $p < 0.001$, $F = 34.2$, d.f. $= 12$, $p < 0.001$); $F = 22.4$, d.f. $= 12$, $p < 0.001$; $F = 20.1$, d.f. $= 12$, $p < 0.001$) and dry mass, $F = 10.2$, d.f. $= 12$, $p < 0.001$; $F = 20.6$, d.f. $= 12$, $p < 0.001$, $F = 10.3$, d.f. $= 12$, $p < 0.001$; $F = 19.0$, d.f. $= 12$, $p < 0.001$), *A. sativa, Z. mays, L sativa and L. esculenteum*, respectively. Dry biomass was the most sensitive endpoint to Cd, being the LOEC value for all the species 35 mg Cd Kg^{-1}_{dw}. Lettuce was also the most sensitive species for this endpoint. The EC_{20} and EC_{50} values obtained for this species were lower than the lowest Cd concentration tested (35.0 mg Cd Kg^{-1}_{dw}). The high sensitivity of *Lactuca sativa* comparatively to other plant species has been observed by other authors, both testing soils contaminated only with cadmium (da Rosa Corrêa et al., 2006; Lamb et al., 2010) or with a mixture of metals, with Cd included (e.g. Pereira et al., 2009). The high capacity of this species to bioaccumulate high concentrations of metals, including Cd is a good

explanation for such sensitivity (Pereira et al., 2009). In fact, it was already demonstrated that Cd^{2+} is easily taken up into cells by Fe^{2+}, Zn^{2+} and Ca^{2+} transporters/ channels, with growth inhibition and leaf chlorosis as the most evident effects of exposure (Clemens, 2006; Kirkham, 2006; Rodríguez-Serrano et al., 2009). Due to the similarities among Cd^{2+} and Fe^{2+}, Ca^{2+} and Zn^{2+} cations, the main mechanisms of toxicity are likely related with changes in the homeostasis of these essential cations or with their displacement from proteins (Verbruggen et al., 2009). Thereby, this metal is rapidly taken up by plant roots and can be loaded into the xylem for its transport into leaves. Once in plants cadmium alters the chloroplast ultrastructure, photosynthesis rate, disturbs the Calvin cycle, nitrogen, sulfur and antioxidant enzymes and the uptake and distribution of macronutrients and micronutrients (Mobin & Khan, 2007; Khan et al., 2007; Iqbal et al., 2010; Márquez-García et al., 2011).

The same mechanisms may explain the sensitivity, although lower, of the other tested plants. In case of *L. esculentum,* the EC_{20} values obtained for fresh mass *and* dry mass were 78.0 and 76.0 mg Cd Kg^{-1}, respectively (Table III.1). Both monocotyledonous showed to be more sensitive than *L. esculentum*, since lower EC_{20} values were obtained: 4.7 mg Cd Kg^{-1} (fresh mass) for *A. sativa* and 37.5 and 24 mg Cd Kg^{-1}_{dw} for *Z. mays* (fresh and dry mass, respectively).

Similar to invertebrates, also distinct toxicity values were reported in the literature for terrestrial plants. The US EPA Draft Ecological Soil Screening Level (Eco-SSL) for Cadmium, Interim Final (USEPA, 2005) identified more than 700 papers on plant toxicity studies related to Cd exposures. For example, da Rosa Corrêa et al., (2006) studied the phytotoxic effects of cadmium in some crop terrestrial plants growing in natural loamy soil (pH=6.6, clay 26%) and found an EC_{50} value of 80 mg Cd Kg^{-1}_{dw} for fresh mass of *L. sativa* (Table III.1). The same authors reported an EC_{50} value of 11.5 mg Cd Kg^{-1}_{dw} for *A. sativa* (fresh mass), which is lower than the value obtained in our study for the same species $EC_{50}=$ 36.5 mg Cd Kg^{-1}_{dw} (Table III.1). Once again the great variability between toxicity data obtained for different soils, using the same test species (from plants to invertebrates), as can be observed on table (Table III.1), justify the definition of regional SSVs based on ecotoxicological data obtained for dominant types of natural soils.

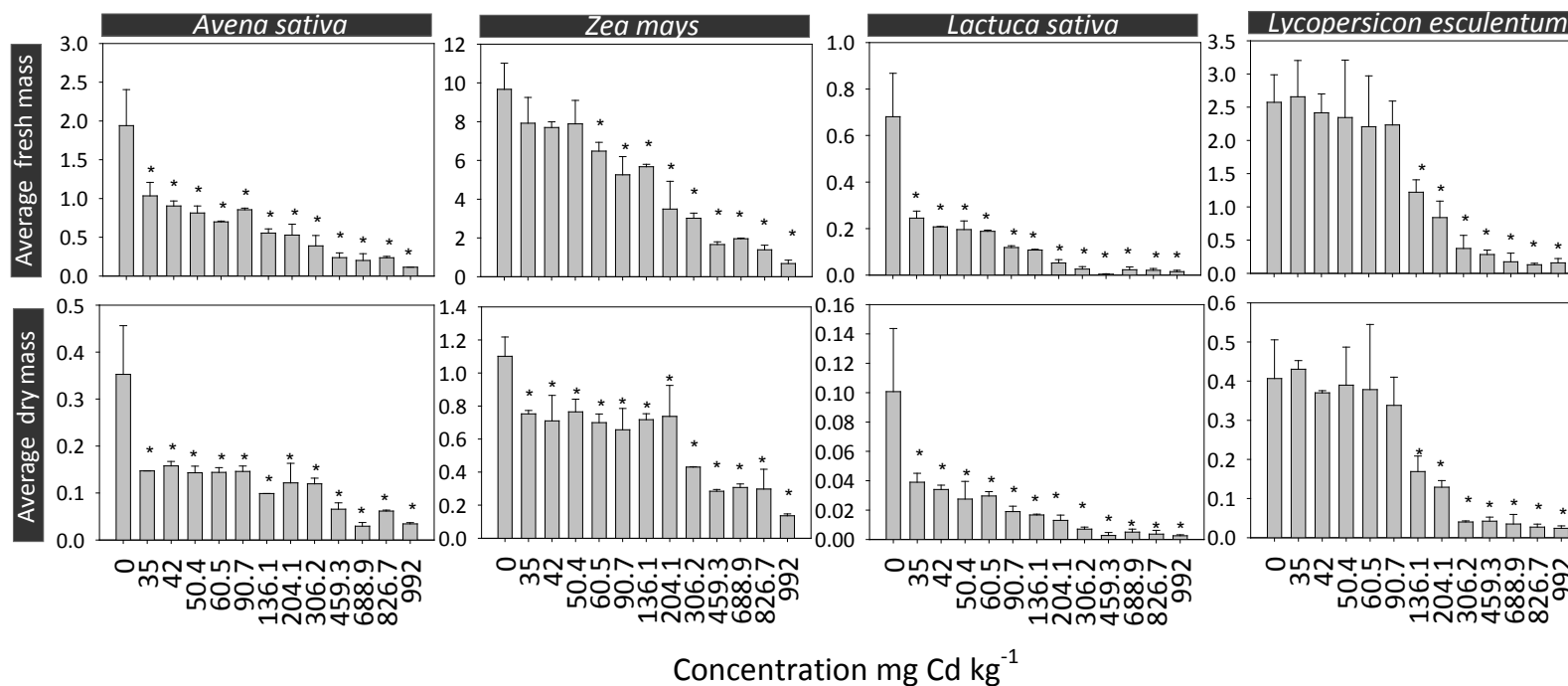**Figure III.4** Average values of fresh and dry mass measurements in monocotyledonous, *Avena sativa and Zea mays*, and dicotyledonous species, *Lycopersicon esculentum* and *Lactuca sativa* grown in PTRS1 soil artificially spiked with Cu. Error bars represent the standard error and the asterisks indicate significant differences between the treatments and the control (0 mg Cd kg^{-1}_{dw}) ($P<0.05$).

Table III.1 Toxicity data for microbial processes, soil invertebrates and plants with effect concentrations as mg U kg-1dw soil, with indication of the 95% confidence interval.

Biota	Endpoint	Soil type	pH	OM	WRC (%)	Clay	NOEC	LOEC	EC$_{20}$	EC$_{50}$	Reference
									(mg Cd Kg^{-1})		
Urease			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≥100	>100	47.8 (15.67-80.04)	> 100	
Cellulase			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≥100	> 100	99.9 (75.17-124.67)	> 100	
Dehydrogenase	enzim. act.	natural soil	5.91±0.098	6.5±0.004	23.94±1.839	3.3	≥100	> 100	≥100	> 100	present study
Acid phasphatase			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≤13.4	13.4	< 13.4	40.2 (0.88-79.5)	
Nitrogen mineralization			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≤13.4	13.4	n.d.	n.d.	
Eisenia andrei	rep.	natural soil	5.91±0.098	6.5±0.004	23.94±1.839	3.3	35	42	37.3 (26.60-47.95)	76.4 (62.69-90.12)	present study
Eisenia fetida	rep. (21 days)	OCDE	6.3	10	n.d.	20	152	n.d.	n.d.	295 (n.d)	Spurgeon and Hopkin, 1995
Eisenia fetida	rep.	OCDE	6	10	n.d.	20	58	100	n.d.	108(92.1-121)	Lock and Janssen, 2001
Eisenia fetida	rep.	natural soil	6.3	1.5	n.d.	17	32	56	n.d.	55.4 (43.5-67)	Lock and Janssen, 2001
Eisenia fetida	rep.	natural soil	4.4	4.8	n.d.	1	32	56	n.d.	73.1 (67.8-78.8)	Lock and Janssen, 2001
Enchytraeids crypticus		natural soil	5.91±0.098	6.5±0.004	23.94±1.839	3.3	7.0	7.7	< 7	8.3 (7.54-8.87)	present study
Enchytraeids albidus		Lufa 2.2 soil	5.5	4.4	n.d.	6	1	3.2	n.d.	6.2 (n.d)	Novais et al., 2011
Enchytraeids albidus	rep.	natural soil	6.3	1.5	n.d.	17	56	100	19.9 (14.50-25.30)	72.4 (61.9-83.3)	Lock and Janssen, 2001
Enchytraeids albidus		OCDE	6	10	n.d.	20	100	180		158.0 (140.0-174.0)	Lock and Janssen, 2001
Folsomia Candida		natural soil	5.91±0.098	6.5±0.004	23.94±1.839	3.3	35.0	42.0	< 35	64.8 (54.47-75.20)	present study
Folsomia Candida		OCDE	6	10	n.d.	20	32	56	158.0 (137.01-184.02)	n.d.	Lock and Janssen, 2001
Folsomia Candida		OCDE	6.1	10.9	n.d.	5.2	n.d.	n.d.	n.d.	193.0 (101.0-369.0)	van Gestel et al., 2003
Folsomia Can,dida		Lufa	5.5	4.2	n.d.	3.6	n.d.	n.d.	n.d.	57.9 (38.2-87.6)	van Gestel et al., 2003
Folsomia Candida	rep.	natural soil	6.1	3.0	n.d.	1.4	n.d.	n.d.	n.d.	53.7 (19.0-152.0)	van Gestel et al., 2003
Folsomia Candida		natural soil	8.2	2.0	n.d.	37.2	n.d.	400.0	n.d.	182.0 (134.0-254.0)	Bur et al., 2010
Folsomia Candida		natural soil	4.5	16.5	n.d.	19.4	n.d.	1.7	n.d.	111 (96.0-133.0)	Bur et al., 2010
Folsomia, Candida		natural soil	6.1	1.6	n.d.	24.8	n.d.	100.0	n.d.	107.0 (n.d)	Bur et al., 2010
Avena sativa			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≥992.0	>992.0	n.d.	n.d.	present study
Zea mays			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≥992.0	>992.0	n.d.	n.d.	present study
Lactuca sativa			5.91±0.098	6.5±0.004	23.94±1.839	3.3	306.2	459.3	279.3 (202.24-356.46)	460.0 (386.36- 533.66)	present study
Lycopersicon esculentum	germ.	natural soil	5.91±0.098	6.5±0.004	23.94±1.839	3.3	459.3	689.7	644 (547.74-742.07)	919.04 (8414.24-996.84)	present study
Avena sativa			6.6	3.0	n.d.	26.0	n.d.	50.0	n.d.	400.0 (n.d)	Corrêa et al., 2006
Lactuca sativa			6.6	3.0	n.d.	26.0	n.d.	25.0	n.d.	150.0 (n.d)	Corrêa et al., 2006
Zea mays			4.3	3.0	n.d.	21.0	n.d.	640.0	n.d.	n.d.	An Y. 2004
Avena sativa			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≤35	35.0	< 35	36.5 (19.40-53.63)	present study
Zea mays			5.91±0.098	6.5±0.004	23.94±1.839	3.3	50.4	60.5	37.5 (22.20-52.87)	135.1 (101.52-168.80)	present study
Lactuca sativa			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≤35	35.0	< 35	< 35	present study
Lycopersicon esculentum	f. m.	natural soil	5.91±0.098	6.5±0.004	23.94±1.839	3.3	90.7	161.0	78.03 (46.03-110.03)	145.5 (111.17- 179.85)	present study
Avena sativa			6.6	3.0	n.d.	26.0	n.d.	12.5	n.d.	11.5 (n.d)	Corrêa et al., 2006
Lactuca sativa			6.6	3.0	n.d.	26.0	n.d.	6.25	n.d.	80.0 (n.d)	Corrêa et al., 2006
Avena sativa			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≤35	35.0	n.d.	< 35	
Zea mays			5.91±0.098	6.5±0.004	23.94±1.839	3.3	≤35	35.0	< 35	185.1 (100.30-269.90)	
Lactuca sativa	d. m.	natural soil	5.91±0.098	6.5±0.004	23.94±1.839	3.3	≤35	35.0	< 35	< 35	present study
Lycopersicon esculentum			5.91±0.098	6.5±0.004	23.94±1.839	3.3	90.7	161.0	76.0 (43.88-108.06)	137.4 (102.74-172.07)	

Average ± STDEV: pH (H2O); OM-organic matter (%) and WHCmax – maximum water holding capacity (%); Clay %; rep.-reproduction; germ.-germination; f.m.- fresh mass; d.m- dry mass; Enz. act.- enzyme activity; n.d.-not determined.

3.3.4. Derivation of a Soil Screening Level (SSV) for cadmium applying assessment factors

A former attempt to derive a risk limit for Cd, for a dominant Portuguese soil was made following the approach suggested by the Technical Guidance Document published by the European Commission (EC, 2003), in support of the Commission Directive 93/67/EEC on Risk Assessment for new notified substances, of the Regulation nº 1488/94 on Risk Assessment for existing substances and Directive 98/8/EC of the European Parliament and the Council. This value was determined based on the endpoint, for which, both the lowest NOEC and EC_{20} values were obtained, which was the average number of juveniles produced by *E. andrei*. For this purpose and given that more than three NOEC and EC_{20} values were obtained for at least three different species, an assessment factor of 10 was applied. The PNEC (predicted no effect concentration) obtained changed between 3.7 (EC_{20} based) and 3.5 (NOEC based) mg Cd Kg^{-1}_{dw}. Both values are higher than background values found in non-contaminated soils by André et al.,(2009) and Caetano et al.,(2012), which are usually below 1 mg Cd kg^{-1}_{dw}. Hence they can be accepted as former SSVs for Portuguese natural soils similar to PTRS1.

3.4 Conclusion

This study confirmed the high sensitivity of the great majority of tested organisms to Cd in the PTRS1 soil. ACP activity and NMIN were the less tolerant microbial parameters to Cd, (Table III.1), being their activity inhibited at Cd concentrations equal or greater than 13.4 mg Cd kg^{-1}_{dw}. The remaining microbial parameters were significantly affected at concentrations above 100 mg Cd kg^{-1}_{dw}. However, we cannot discard the hypothesis that changes in the soil microbial community structure may have masked the effects of Cd on these functional microbial parameters, after one month of exposure.

Soil invertebrates were apparently more sensitive to Cd than the soil microbial parameters evaluated. *E. andrei* and *F. candida* showed very similar sensitivities to this metal presenting EC_{50} values of 76.4 and 64.8 mg Cd Kg^{-1} respectively, whereas *E. crypticus was* the most sensitive invertebrate species showing an EC_{50} value of 8.3 mg Cd Kg^{-1}_{dw}.

Growth of terrestrial plants analyzed by dry mass yield was the most sensitive endpoint, showing NOEC$_S$ values lower than the lowest Cd concentration tested, for all the test species. *L. sativa* was the most sensitive plant tested for all the endpoints.

Data generated in this study were used to calculate a PNEC value for Cd, based on the application of assessment factors. The PNEC values obtained indicate that terrestrial organisms will only be affected at concentrations that are higher than background concentrations. This value is much lower than the Eco-SSL value suggested by USEPA (2005) for cadmium, that range from 32 mg Cd kg$^{-1}_{dw}$ for plants and 140 mg Cd kg$^{-1}_{dw}$ for soil invertebrates. Comparing with the Canadian Soil Quality Guideline for Cd, our values are between the guideline value for agricultural and residential parkland soils (1.4 and 10 mg.Kg^{-1}), (CCME, 1999a). These last values have been suggested by national authorities for national evaluations, therefore they were defined for both human health and ecosystems protection, usually assuming the lowest value obtained. Considering, only ecosystems protection the same values changed between 3.8 and 10 mg.Kg^{-1}Cd, which are now in perfect agreement with our values.

The present work represents an important contribution for the establishment of national soil quality guidelines and for the processes of ecotoxicological risk assessment in the Portuguese context.

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Chapter **IV**

Copper toxicity in a natural reference soil – ecotoxicological data for the future derivation of soil screening levels

Copper toxicity in a natural reference soil – ecotoxicological data for the future derivation of soil screening levels

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Abstract

The risk assessment of potential pollutants is conventionally done resorting to soil screening values (SSVs) which define threshold levels above which further action should be taken. Since SSVs are still unavailable for the Portuguese context, standardized toxicity tests should be urgently undertaken to fill the data gaps used in their derivation. Thus, the present study intended to evaluate the toxicity of copper for terrestrial species, using a Portuguese natural reference soil (PTRS1), in order to generate toxicity values to be used in the derivation of Cu SSVs. The soil biochemical parameters and reproduction of invertebrates were tested, as well as the seed germination and growth of terrestrial plant species. Cu was responsible for the reduction in some enzyme activities, invertebrate reproduction and growth of plants. We found significant negative relationships between Cu and urease, cellulase and nitrogen mineralization activity ($P < 0.01$). The EC_{50} values calculated for the reproduction of invertebrates were 130.9, 165.1 and 191.6 mg Cu kg$^{-1}_{dw}$ for *E. andrei*, *E. crypticus* and *F. Candida*, respectively. Only the seed germination of *L. sativa* was conditioned by copper in soil; whilst the growth of all plants was compromised by this metal to an EC_{50} for fresh mass within the range of 89 and 236.3 mg Cu kg^{-1}, and for dry mass within the range of 93.1 and 290.5 mg Cu kg$^{-1}_{dw}$. The overall results and their comparison with previous data confirmed the direct influence of soil properties on copper toxicity, which underline the importance of using regional natural soils in the

derivation of screening values. A predicted no effect concentration (PNEC) obtained for Cu when a factor of 10 was used varied between 6.5 (EC_{20}-based) and 7.9 (NOEC-based) mg Cu Kg^{-1}_{dw}, which are lower than background Cu concentration quantified in Portuguese soils. Thus, we suggest a PNEC value of 65 mg Cu Kg^{-1}_{dw}, obtained without application of any factor.

This study also describes the procedure that could be easily followed by other countries for the derivation of SSVs adjusted to their soils.

Key-words: Copper, natural soil, toxicity values, soil enzymes activity; *Eisenia andrei*; *Enchytraeus crypticus; Folsomia candida*; *Avena sativa; Zea mays; Lacuta sativa; Lycopersicon esculentum*.

4.1 Introduction

The overuse of metals in diverse activities and their extraction processes to supply many needs, has been leading to their overspread and accumulation in different environmental compartments, particularly in the terrestrial ecosystems (Kakkar and Jaffery, 2005; Mackie et al., 2012). As a consequence, several adverse effects on terrestrial species, and ecosystem functions and services have been reported (Anderson et al., 2009; Pereira et al., 2009; Lourenço et al., 2011b; Macdonald et al., 2011). In response to the recognized need to deal with metal-contaminated sites, many countries have been committed with the development and regulation of benchmarks for metals, in order to facilitate the evaluation and the management of contaminated land (Carlon, 2007; Crommentuijn et al., 2000b; Fishwick, 2004; O'Halloran, 2006). These benchmarks are designated as soil screening values (SSVs), which should guarantee the protection of terrestrial elements and ecosystem functioning (Fishwick, 2004). By definition SSVs are the highest concentrations of a given contaminant (e.g., metal) in the soil, above which an additional evaluation or risk remediation measure is mandatory (Fishwick, 2004; Carlon, 2007). These values are strongly needed in the Tier 1 screening phase of Ecological Risk Assessment (ERA) frameworks (Jensen and Mesman, 2006; Merrington et al., 2006; Weeks and Comber, 2005), as a reference basis to compare with the content of chemical

residues in the soil, and thus provide the first evaluation of risks, based on a chemical line of evidence.

In general, the current procedures for the derivation of SSVs are based on toxicity data obtained from several standard ecotoxicological tests performed with different terrestrial species and targeting a wide range of biological responses (e.g., soil microbial activity and diversity, growth and reproduction of invertebrates, emergence and growth of plants). If possible, it would be important to derive SSVs from data obtained with natural reference soils defined in each country (Kuperman et al., 2006). The physical and chemical properties of natural soils have been widely referred as constraining factors of the bioavailability of metals, what in turn influences the responses of organisms (Criel et al., 2008; Römbke et al., 2006; van Gestel et al., 2011; Chelinho et al., 2011). Thereby, the use of a limited number of soils, like the artificial (OCDE, 1984) or the natural LUFA soils, with a limited range of properties, in the derivation of SSVs can lead to under or over estimations of risks for metal-contaminated areas and corresponding nearby terrestrial ecosystems, which soils can present clearly distinct properties. As a result, inappropriate risk management decisions might be taken (Jänsch et al., 2007; Vijver et al., 2001).

Metals are meaningful environmental pollutants, since their toxicity is a problem of increasing significance for ecological, evolutionary, nutritional and environmental reasons (Nagajyoti et al., 2010). The derivation of SSVs for metals is especially important when assessing the environmental risks of essential metals such as Cu, for which not only toxicity but also possible deficiency effects should be considered (Janssen et al., 2000). In fact, Cu is directly involved in a wide range of physiological processes in soil organisms (Pavel et al., 2013). This metal can be found in soils under many forms, but it is mainly available as a free cation (Cu^{2+}) on the surface of clay particles or in association with organic matter (Schulte and Kelling, 2004). The free form Cu^{2+} is responsible for Cu activity and bioavailability (Sauvé et al., 1996), which is in turn mostly influenced by soil organic matter content and pH (Schulte and Kelling, 2004). Notwithstanding, the increased concentration of Cu^{2+} in soils, essentially triggered by industrial (Kabata-Pendias, 2010; Halim et al., 2003) and agricultural wastes (Horswell et al., 2003; Giller et al., 1998), together with its toxicity for soil organisms (An, 2006; Criel et al., 2008;

Wyszkowska et al., 2009), reinforces the relevance of evaluating its effects in natural soils, in order to obtain ecotoxicological data for the future derivation of SSVs. Furthermore, considering the heterogeneity of soil properties within and between geographical areas, it is quite relevant to derive SSVs for natural reference soils representative of the main soil types in each territory. In the case of Portugal, SSVs were not derived yet for dominant natural soils, which should be legally enforced to comply with soil protection policies (e.g. CEC, 2006). Hence, with the intention of facilitating site-specific ecological and ecotoxicological evaluations in the Portuguese context, the main objective of the present work was to assess the effects of Cu toxicity in terrestrial species and functions, using a Portuguese natural soil. For this purpose, a battery of sub-lethal ecotoxicological tests was performed in a Portuguese reference soil (PTRS1) to test the influence of Cu on soil biochemical parameters, reproduction of invertebrates, seed germination, and growth of terrestrial plants. The dataset generated in this study was used to derive PNECs for Cu based on assessment factors.

4.2 Material and methods

4.2.1 Soil sampling and processing

The soil used in this study was collected from the top 20 cms of a field in Ervas Tenras [(Pinhel, Guarda: $40^o44'4.27''$N and $7^o10'54.3''$W)], center of Portugal. This soil is representative of a granitic region, and was previously characterized selected as a natural reference soil, after being validated and characterized for this ecotoxicological purposes (Caetano et al., 2012), (*c.f.,* table in annex). The main properties of the PTRS1 are described in Table IV.2, of the results section. After sampling, the batch of soil was immediately brought to the laboratory, sieved through a 2mm mesh size and the sieved fraction (< 2 mm) was stored in polyethylene bags, at -20 °C, until further use for soil enzymatic activity measurements. For the tests with soil invertebrates and plants, the soil was air-dried and then sieved through a 4 mm sieve, and the < 4 mm fraction was defaunated through two freeze–thawing cycles (48 h -20 °C followed by 48 h at 25 °C), until the beginning of the tests.

4.2.2. Test substance and concentration ranges

For all the tests the natural soil was spiked with a stock solution of copper (II) sulfate
pentahydrate ($CuO_4S.5H_2O$; Merck Ensure) prepared with deionized water filtered in a
Milli-Q equipment (hereinafter referred as deionized water), in order to obtain the
different ranges of concentrations. These concentrations were defined based on the
results of range finding tests performed with all test species and are presented in Table
IV.1. The amount of water required to adjust the WHC of the soil to 45% of its maximum
value was used to dilute the stock solution for tests with invertebrates and plants and
80% of its maximum value in case of enzymes. In order to discard the potential effect of
sulfate on the highest concentrations of copper sulfate, controls with calcium sulfate
($CaSO_4.2H_2O$) were additionally performed at 2303.2, 366.3 and 182.4 mg $CaSO_4.2H_2O$ Kg^-
$^1{}_{dw}$ for *E. andrei, E. crypticus* and *F. candida*, respectively. In case of plants, the calcium
sulfate was added in the following concentrations, 1314.3, 1546.7, 1256 and 808.6 mg
$CaSO_4.2H_2O$ $Kg^{-1}{}_{dw}$ for *A. sativa, Z. mays, L. sativa and L. esculentum*, respectively. The
calcium sulphate concentrations mentioned above corresponded to the highest sulphate
concentrations added to soils through the spiking with Cu sulphate.

Table IV 1. Copper concentrations used in ecotoxicological tests (mg Cu kg$^{-1}{}_{dw}$)							
Microorganisms	**Invertebrates**			**Plants**			
Biochemical parameters	*E. andrei*	*E. crypticus*	*F. candida*	*A. Sativa*	*Z. mays*	*L. sativa*	*L.esculentum*
80.8	35	150.0	46.3	168.0	235.3	64.3	20.0
96.9	40.3	172.5	53.2	184.8	258.8	77.2	25.0
116.2	46.3	198.4	61.2	203.2	284.7	92.6	31.2
139.5	60.2	238.1	79.6	243.9	341.7	120.4	43.8
167.5	78.2	285.7	103.5	292.7	410.0	156.5	61.3
200.9	101.7	342.8	134.5	351.3	492.0	203.4	85.8
241.1	132.2	411.4	174.8	421.5	590.4	264.5	120.1
289.4	171.9	493.6	227.3	505.8	649.4	343.8	168.1
347.2	223.4	592.3	295.5	607.0	714.4	446.9	235.3
416.7	256.9	681.2	339.8	667.7	785.8	536.3	294.1
500.0	295.5	783.4	390.8	734.5	864.4	643.6	382.4
600.0	339.8	900.9	449.4	807.9	950.8	772.3	497.1

4.2.3 Ecotoxicological assessment

4.2.3.1 Soil microbial activity

Three replicates per test concentration were prepared for each soil microbial parameter. For the control, six replicates were prepared with deionized water filtered in a Milli-Q equipment (hereinafter referred as deionized water). The replicates were incubated for 30 days at 20±2°C, under the photoperiod 16h^L: 8h^D. During the incubation period, the soil moisture was weekly monitored by weighing the pots, and

whenever needed it was adjusted to 80% of its WHC$_{max}$ by adding deionized water. After the incubation period, 1g of soil per replicate and concentration was weighted and placed in falcon tubes, and then frozen to -20ºC, until analysis. Thereby, a total of 9 sub-replicates were made for each concentration. The soil was thawed at 4°C before analysis.

The activities of urease (UR), cellulase (CELL), acid phosphatase (ACP), dehydrogenase (DHA) and nitrogen mineralization (NMIN) were tested. The UR activity was assayed according to the method proposed by Kandeler and Gerber, (1988) and, Schinner et al., (1996). The samples were incubated for 2h, at 37°C, after the addition of a buffered solution of urea. The ammonia released was extracted with a solution of potassium chloride and determined by the modified Berthelot reaction. The quantification was based on the reaction of sodium salicylate with ammonia in the presence of chlorinated water, producing a green complex in alkaline pH. UR was detected at 690 nm and expressed as µg nitrogen (N) g^{-1} soil$_{dw}$ 2 h^{-1}. The CELL activity was tested according to, the method proposed by Schinner and von Mersi, (1990) and, Schinner et al., (1996). The reducing sugars produced during the incubation period caused the reduction of hexacyanoferrate (III) potassium to hexacyanoferrate (II) potassium in an alkaline solution. This last compound reacts with ferric ammonium sulphate in acid solution to form a ferric complex of hexacyanoferrate (II), of blue staining, which is measured colorimetrically, at 690 nm, and expressed as µg glucose g^{-1} soil $_{dw}$ 24 h^{-1}.

NMIN activity was measured according to Schinner et al., (1996). The soil samples were incubated for 7 days, at 40°C. During this period, the organic forms of nitrogen were converted to inorganic forms (mainly ammonium ion, NH$_4^+$), which were determined by a modification of the Berthelot reaction, after extraction with potassium chloride. The

reaction of ammonia with sodium salicylate (NH_3) in the presence of sodium dichloroisocyanurate formed a green complex at an alkaline pH and it was measured at 690 nm and expressed as µg nitrogen (N).g^{-1} $soil_{dw}$ d^{-1}.

The acid phosphomonoesterase activity was tested according to the method proposed by Schinner et al., (1996). After addition of the buffered solution of p-nitrophenyl phosphate, soil samples were incubated for 2h, at 35°C. The p-nitrophenol released by the phosphomonoesterase activity was extracted with sodium hydroxide, producing a yellow color that was measured spectrophotometrically at 405 nm and expressed as µg nitrophenol (NP).g^{-1}.

The method proposed by Öhlinger, (1996) was used in order to assess DHA. The samples were suspended in a solution of trifeniltetrazol chloride (TTC) and incubated at 40°C, for 24 hours. The triphenylformazan (TPF) produced was extracted with acetone and measured spectrophotometrically at 546 nm and the results were expressed as µg triphenylformazan (TPF) g^{-1} $soil_{dw}$ h^{-1}.

4.2.3.2 Invertebrate and plant tests

4.2.3.2.1 Test organisms and culture conditions

The earthworm *Eisenia andrei* (Oligochaeta: Lumbricidae), the potworm *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae) and the springtail *Folsomia candida* (Collembola: Isotomidae) were used to assessed the toxicity of Cu. All the organisms used for this study were age-synchronized from a culture kept in the laboratory, under controlled environmental conditions (temperature: 20±2ºC; photoperiod: $16h^L$: $8h^D$). The earthworms (*E. andrei*) were maintained in plastic boxes (10 to 50 L) containing a substrate composed by peat, dry and defaunated horse manure (through two freeze–thawing, 48h at -20 ºC followed by 48h at 65 ºC), water and $CaCO_3$ to adjust the pH, between 6 and 7. The earthworms were fed every 2 weeks with about six tablespoon oatmeal previously hydrated with deionized water filtered in Milli-Q equipment (hereinafter referred as deionized water) and cooked for 5 minutes. The potworms (*E. crypticus*) were cultured in boxes (25.5 cm length,17.4 cm width, 6.5 cm height), filled with pot soil moistened to the nearest 60% of its water holding capacity (WHC_{max}) and

with a pH adjusted to 6.0 ± 0.5. The organisms are fed twice a week with a teaspoon of macerated oat. The collembolans (*F. candida*) were maintained in plastic containers filled with a culture medium composed by moistened Plaster of Paris mixed with activated charcoal 8:1 (w:w). They are fed with granulated dry yeast, twice a week, which is added half a teaspoon small amounts to avoid spoilage by fungi.

Seeds from four plant species (two dicotyledonous and two monocotyledoneous), purchased from a local supplier, were used for seed germination and growth tests: *Avena sativa, Zea mays*, *Lactuca sativa* and *Lycopersicon esculentum.*

4.2.3.2.2 Reproduction tests with invertebrates

The standard protocols ISO 11268-2 (ISO 1996), 16387 (ISO 2004) and 11267 (ISO 1999) were followed for performing the reproduction tests with *E. andrei*, *E. crypticus* and *F. candida*, respectively. Ten earthworms with a developed clitellum and an individual fresh weight between 250 and 600 mg were introduced into each container with 500 g of dry soil. Worms were fed weekly with 5 g of defaunated horse manure (following the same procedure above described) grounded and sieved per box and the soil moisture content was adjusted. Adult earthworms were removed from the test containers after 28 days. The produced cocoons were left in the soil until 56 days have been completed. After this period, the juveniles from each test container were counted. Ten potworms with 12-mm size were introduced in each test vessel containing 20 g of dry soil. The adults were left in the vessels during 28 days, until the end of the test. About 2mg of rolled oats were placed on the soil surface weekly to feed the animals. At the end of the test, the potworms were sacrificed with alcohol, colored with Bengal red and counted according to the Ludox Flotation Method, as described in ISO 16387 (ISO, 2004). Ten 10–12 days old springtails were placed *per* test container previously filled with 30 g of soil. The collembolans were fed with about 2 mg of granulated dry yeast that was weekly added to the soil surface. The reproduction tests with *F. candida* took four weeks to be completed. At the end of the experiment, the test containers were filled with water and the juveniles were counted after flotation. The addition of a few dark ink drops provided a higher contrast between the white individuals and the black background. Organisms were counted afterwards by

using the ImageJ software. All invertebrates were kept under a 16h^L: 8h^D photoperiod and at 20±2ºC. Following an ECx sampling design, two replicates per concentration and five replicates for the control were prepared in the reproduction tests with *E. andrei*. For potworms and collembolans assays, three replicates were prepared *per* concentration.

4.2.3.2.3 Seed germination and plant growth tests

The effect of Cu in germination and growth of terrestrial plants was assessed in accordance to the ISO 11269–2 protocol (ISO, 2005). For this purpose, 200 g$_{dw}$ of uncontaminated (control) and spiked soil (test treatments; *cf.* section 3.2.2) were used per replicate, in a total of five replicates for the control and three for each Cu concentration. As such, the same ECx sampling design used for the invertebrates was followed in the plant tests.

The amount of water required to adjust the WHC$_{max}$ of the soil to 45% was used to dilute the stock solution and moist the soil at the beginning of the test. The soil was placed in the plastic pots (11.7 cm diameter, 6.2 cm height) and twenty seeds were added to each test pot and gently covered with soil. In the bottom of each pot a hole was previously made to let a rope passing through, hence allowing communication with another pot placed below and filled with distilled water. The level of water in this latter recipient was adjusted whenever needed to guarantee a continuously supply of water to the soil above by capillarity.

At the beginning of the tests, nutrients (Substral® - Plants Fertilizer; using 1 bottle cap for 2 L of water proportion, according to the manufacturer recommendation; Fertilizer NPK: 6-3-6 with 6% nitrogen (N), 3% phosphate (P$_2$O$_5$), 6% potassium (K$_2$O), 0.03% iron (Fe) and trace elements as Cu, Mn, Mo, Zn) were supplied. Pots were maintained at constant conditions of temperature (20 ± 2°C), photoperiod (16h^L: 8h^D) and light intensity (25.000 lux). The endpoints seed germination, and fresh and dry biomass, above soil, were assessed for each species at the end of the exposures according to the methods outlined in ISO, (2005).

For this work, a battery of enzymes involved in different biogeochemical cycles [S (sulfur cycle), N (Nitrogen cycle), C (Carbon cycle)], as well as enzymes more indicative of

the good physiological conditions of the whole microbial community (e.g. dehydrogenase) were selected. The species of invertebrates and plants were selected based on the availability of standard protocols. Since we aimed to obtain data for the derivation of SSVs, for regulatory purposes, this procedure is recommended.

4.2.4 Statistical Analysis

The soil microbial parameters, number of juveniles produced by potworms and collembolans, number of emerged seeds, fresh and dry mass of plants were compared to that of the respective controls by a one-way ANOVA, (SigmaPlot 11.0 for Windows). When statistical significant differences were recorded, the Dunnett's (for parametric one-way ANOVA) or the Dunn's test (for non-parametric ANOVA) was carried out to perceive which concentrations were significantly different from the respective control. The Kolmogorov-Smirnov test was applied to check data normality, whereas homoscedasticity of variances was checked by the Levene's test. Whenever the ANOVA assumptions were not met, a Kruskal-Wallis analysis was performed (SigmaPlot 11.0 for Windows). The NOEC (no-observed-effect-concentration) and LOEC (low-observed-effect-concentration) values were determined based on the outcomes of the multiple comparison tests. The metal concentration producing a 20% (EC_{20}) and a 50% (EC_{50}) reduction in the tested endpoints was calculated after fitting the data to a logistic model. The ECx determinations were performed using the STATISTICA version 7.0 software.

4.3 Results and discussion

4.3.1 Soil biochemical parameters

The enzyme activities and N mineralization determined in PTRS1 soil artificially spiked with Cu are shown in Figure IV.1. Table IV.2 presents the toxicity values calculated for each parameter.

Overall, the N and C cycles were the most affected ones under Cu toxicity. For the activity of UR, which is an extracellular enzyme involved in the N-cycle, it was observed a negative relationship with increasing Cu concentrations. A LOEC of 167.4 mg Cu Kg^{-1}_{dw} and

an EC_{50} of 171.8 mg Cu Kg^{-1}_{dw} were determined. This finding is in accordance with previous studies, which also reported a negative correlation between Cu levels and UR activity (e.g., Wyszkowska et al., 2005, 2006; Alvarenga et al., 2008; Gülser and Erdoğan, 2008; Lee et al., 2009; Zeng et al., 2011). Hu et al., (2013) observed that UR was one of the most sensitive soil enzymes to Cu, for which they calculated EC_{50} values of 505 mg total Cu Kg^{-1}_{dw} and 64 mg available Cu Kg^{-1}_{dw}, in a soil presenting a higher pH (7.95) and clay content (39.5%), and lower organic matter content (16.33 mg Kg^{-1}_{dw}) comparatively to PTRS1 soil. Indeed, Wightwick et al., (2013) had also obtained a significant reduction in UR activity in vineyard soils (pH 8.2, 1.3% of total organic C and 16.0 % of clay) containing around 60 mg Cu Kg^{-1}_{dw}; whilst Ge and Zhang, (2011) verified that different soils (pH between 6.85-7.77 and organic C between 23.8-25.8 g Kg^{-1}_{dw}) with increasing concentrations of Cu (67.5 – 2712.1 mg Kg^{-1}_{dw}) evidenced successively lower UA activities (45.5 – 10.3 mg NH_4^+ Kg^{-1} soil h^{-1}). UA is mainly synthesized by microorganisms and is implied in the catalysis of organic N oxidation into ammonia (Kandeler et al., 1996; Wang et al., 2011). Thus, the decrease of UA activity in our study may be due to a negative effect of Cu on specific microbial biomass pool (Kandeler et al., 1996; Wang et al., 2011).

Besides UA, another parameter that is also an indicator of N-cycle functioning – NMIN - was severely affected under Cu exposure. The lowest concentration at which was observed a significant inhibition on NMIN (80.7 mg Cu Kg^{-1}_{dw}) was not considered the LOEC due to the high variability recorded between replicates. As such, the LOEC considered for NMIN was 139.5 mg Cu Kg^{-1}_{dw}; while the EC_{20} and EC_{50} values calculated for this enzyme were of 90.7 and 146.5 mg Cu Kg^{-1}_{dw}, respectively. The behavior of NMIN in the presence of metals has not been very coherent across the studies. Dai et al., (2004) determined non-significant negative correlations between this parameter and Cu in metal-polluted soils. On the contrary, Płaza et al., (2010) attained increased levels of N mineralization (15 – 2 μg NH_4^+) under metal-polluted soils presenting 26 – 39 mg Cu Kg^{-1}_{dw}, respectively. Still, Hassen et al., (1998) did not identify considerable changes in NMIN of clayey loamy soils artificially-spiked with 50 and 250 mM Cu. This parameter provides an overview of the biomass of specific microbial groups (nitrifying bacteria), which are directly involved in the mineralization of organic N into ammonia (Winding et al., 2005).

Although it does not usually provide the most accurate and sensitive outcome comparatively to other N-cycle indicators (Winding et al., 2005), in our study it was achieved a clear negative dose-response relationship between NMIN and Cu levels in PTRS1 soil.

The activity of soil CELL was significantly depleted at 500 mg Cu kg$^{-1}_{dw}$, being calculated EC$_{20}$ and EC$_{50}$ values of 457.6 and 571.4 mg Cu Kg$^{-1}_{dw}$, respectively (Table IV.2). Thus, the C-metabolism associated with the degradation of soil organic matter including cellulosic constituents that are specifically catalyzed by these extracellular enzymes (Alvarenga et al., 2008) was compromised under the highest Cu concentrations. Some authors reported negative correlations between CELL activity and Cu concentrations quantified in mine soils (Alvarenga et al., 2008; Antunes et al., 2011), while others (e.g., Sivakumar et al., 2012) did not observe changes in its normal activity in urban soils contaminated with Cu.

Both ACP and DHA were not significantly impaired by Cu despite the decreasing trend observed for the DHA along increasing concentrations of the metal. Previous research has shown that ACP is the least affected enzyme by metals (Alvarenga et al., 2008; Kandeler et al., 1996; Pereira et al., 2006), such as Cu (Wyszkowska et al., 2005; Santiago-Martín et al., 2013). However, other authors observed a significant negative correlation between phosphatase and Cu levels in urban soils (24-36.7 mg Cu Kg$^{-1}_{dw}$; Papa et al., 2010) and polymetallic mine soils (0.14 – 1.88 mg Cu Kg$^{-1}_{dw}$; Antunes et al., 2011). Unlike our outcome, Wyszkowska et al., (2005) obtained near to 50% inhibition of ACP activity under 600 mg Cu Kg$^{-1}_{dw}$ in heavy loamy sand and silt light loam soils (pH 6.9). In our study, however, this extracellular enzyme involved in the mineralization of organic P was not considerably constrained.

Likewise, DHA proved to be very tolerant to Cu in PTRS1 soil, although its response allowed the calculation of an EC$_{20}$ of 425.9 mg Cu Kg$^{-1}_{dw}$. Dehydrogenases DHA are intracellular enzymes that are involved in soil organic matter oxidation, and their degradation in soil occurs immediately after cell death (Pereira et al., 2006). Thereby, their activity has been pointed out as a valuable indirect indicator of soil microbial activity and cell viability (Taylor et al., 2002; Wyszkowska et al., 2005). Contrary to the profile obtained in this study, it has been broadly stated that DHA is highly sensitive to soil metal

pollution (e.g., Lee et al., 2009; Wyszkowska et al., 2005; de Santiago-Martín et al., 2013). Nevertheless, some authors pointed out that an increased activity of dehydrogenase may be forged by high soil Cu concentrations (e.g., Rossel et al., 1996; Taylor et al., 2002), which can explain our results. This could be associated to Cu reduction of the TPF produced upon dehydrogenase catalysis, what would consequently interfere with the spectrophotometer measurement of TPF (Trasar-Cepeda and Gil-Sotres, 1988).

Additionally, the absence of effects in the activity of this enzymes was also reported in previous study by Caetano et al., b), (*submited*) when tested the effects of cadmium. Taking in account that dehydrogenase are an enzymes of lower specificity, an apparent lack of effect can be related with the time of exposure. In both works, the time of exposure was of one month, which could be too long allowing an adaptation of affected microbial community being replaced by more tolerant ones, mimicking the potential impairment on activity of this enzyme. In this sense, we can conclude that for risk assessment procedures, aimed in defining risk limits for chemicals, erroneous conclusions can be drawn about the effects of soil microbial community, if a reduced number of parameters are tested. Further, if f on the one hand, too long exposure times can mask the effects in the overall microbial community on the other hand, short exposures can falsely magnify the effects in the microorganisms. Therefore, exposure time should be the targeted in future studies, aimed in standardizing the use of soil enzyme activities for risk assessment purposes.

The mode of action of Cu, as well as its toxicity, may vary depending on the targeted enzyme (Papa et al., 2010; Ge and Zhang, 2011). Although the interaction mechanisms between metals and enzymes were not unraveled yet, the inhibition of enzymatic reactions by metals can be explained by a direct (*i.e.,* inhibition or inactivation of enzymes by metal reaction with their sulphydral groups; reaction with the substrate or the substrate-enzyme complex) and/or an indirect effect (*i.e.,* the changing of microbial community that synthesizes the enzymes), or still a combination of both (Kızılkaya and Bayraklı, 2005; Lee et al., 2009; Papa et al., 2010).

Besides, soil properties such as pH, soil texture, organic matter and nutrient contents may often interfere and modulate the bioavailability and, consequently, the toxicity of metals

on soil enzymes and mineralization processes (Turner et al., 2002; Papa et al., 2010). According to the literature, the extracellular hydrolases such as UR, CELL and ACP may be retained and protected by organic matter, humic colloids and clays (e.g., Trasar-Cepeda et al., 2008; Lee et al., 2009), thereby preventing conspicuous inhibitions of enzyme activities. On the other hand, those soil properties may indeed decrease metal bioavailability by complexation, and consequently reduce its toxicity to the enzymes (Papa et al., 2010; Tejada et al., 2011). Hence, we can hypothesize that the low clay content in PTRS1 soil (3.32%; *cf.* Table IV.2) together with its acidic pH may be responsible for a higher bioavailability and toxicity of Cu to UR and CELL activities and N mineralization.

Copper induced negative effects in some biochemical parameters of PTRS1 soil. Nevertheless, it is quite remarkable how contradictory the profiles of these parameters may be throughout different studies, despite being mentioned as good bioindicators of soil quality (Trasar-Cepeda et al., 2000; Shen et al., 2005). Trasar-Cepeda et al., (2008) pointed out some explanations for such occurrence, which may be linked to the lack of standard protocols, to the spatio-temporal changes in soil biochemical and intrinsic geochemical properties, and to the absence of reference soils that may represent optimal quality conditions and help defining threshold values. Focusing the study of Cu toxicity on the biochemical parameters of a reference Portuguese natural soil (Caetano et al., 2012) will enlarge the knowledge of their response under different soil properties. Ultimately, it may serve as a comparison mean between studies, and also help in the future definition of ecotoxicological thresholds for these parameters based on certain properties or types of natural reference soils.
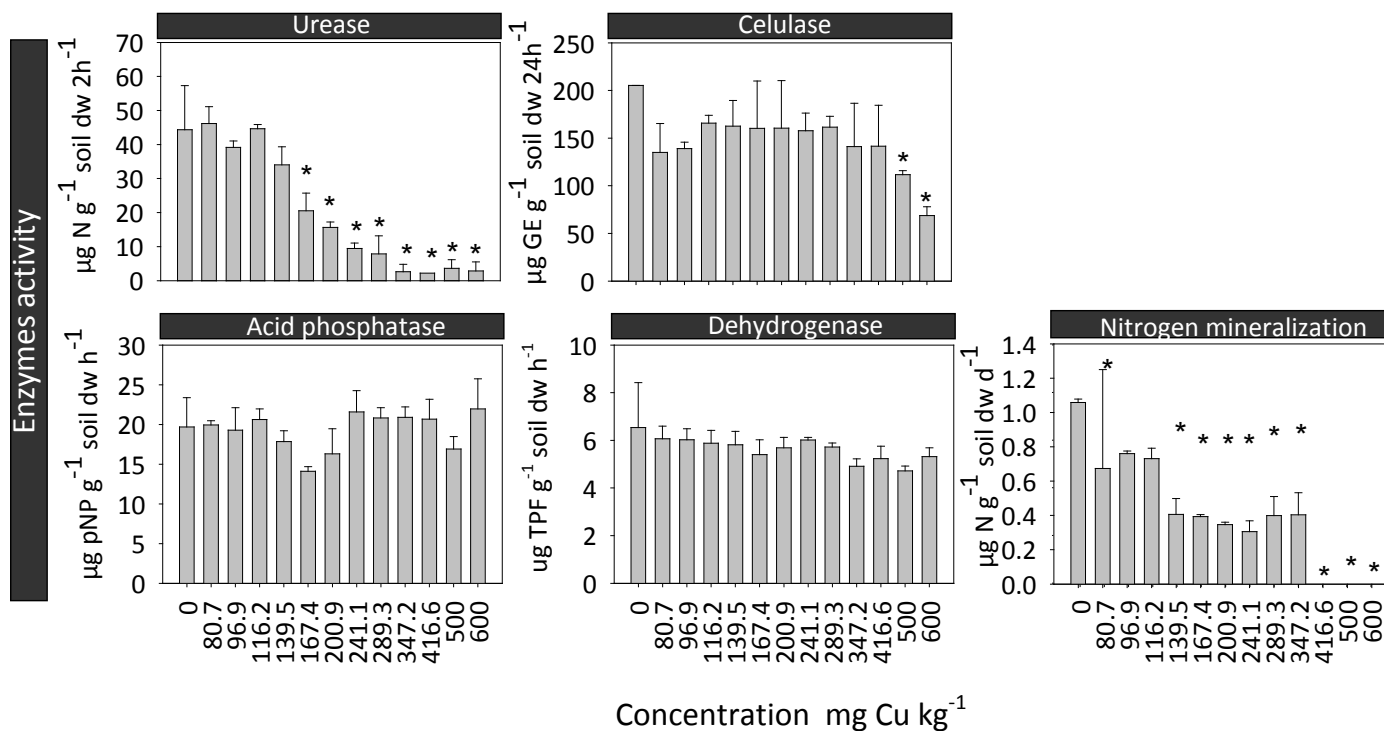
Figure IV.1 Soil enzyme activities and N mineralization in PTRS1 soil spiked with a range of cooper concentrations. The error bars indicate the standard deviation. The asterisks sign out significant differences relatively to the control (0 mg Cu Kg$^{-1}_{dw}$), ($P < 0.05$).

4.3.2 Reproduction of soil invertebrates

The values of NOEC, LOEC, EC_{20} and EC_{50} determined for the reproduction of invertebrates under Cu exposures are summarized in Table IV.2. The validity criteria outlined in the respective guidelines were fulfilled in all experiments. Besides, the sulphate control treatments showed no statistically significant ($P > 0.05$) effects on the reproductive output of the three invertebrates relatively to the negative controls.

A significant impairment on the reproduction of all invertebrates was recorded, under Cu exposure, ($F = 11.3$, d.f. = 12, $p < 0.05$), ($F = 15.9$, d.f. = 12, $p < 0.05$) and ($F = 29.6$, d.f. = 12, $p < 0.05$), for *E. andrei*, *E. crypticus* and *F. candida,* respectively (Figure IV.2). The reproduction of three invertebrates, and was significantly decreased for Cu concentrations of 132.2 mg Cu Kg^{-1}_{dw} for *E. andrei*, 150.0 mg Cu Kg^{-1}_{dw} for *E. crypticus* and 103.5 mg Cu Kg^{-1}_{dw} in case of *F. candida*. Moreover, potworms exposed to Cu above 681.1 mg Cu Kg^{-1}_{dw} did not produce any juvenile (Figure IV.2; Table IV.2). Through the concentration-effect relationships, estimated by fitting a logistic model to the data, 20% effect on the reproduction rate (EC_{20}) of invertebrates was obtained at concentrations of 73.0, 89.9 and 65.8 mg Cu kg^{-1}_{dw} for *E. andrei*, *E. crypticus* and *F. candida*, respectively (Table IV.2). The EC_{50} values obtained for *E. andrei* indicated, as well, that this species was slightly more sensitive to the metal (130.9 mg Cu kg^{-1}_{dw}) in comparison to *E. crypticus* and *F. candida*, for which were derived the respective EC_{50} values of 165.1 and 191.6 mg Cu kg^{-1}_{dw} (Table IV.2).

The results obtained in our study for the three soil invertebrates, in general, were partially supported by the ones reported in the literature (Table IV.2). Criel et al., (2008) tested Cu toxicity in the reproduction of *E. fetida* and *F. candida* in various European natural soils and found different toxicity values between samples. The authors reported 28-day EC_{50} values for *E. fetida* cocoon production that ranged between 349 and 778 mg Cu kg^{-1}_{dw} in soils with high clay content (20 – 24%), and between 72 and 192 mg Cu kg^{-1}_{dw} in soils presenting low clay content (7.0 – 9.0%), irrespective of the acidic pH (3.0 – 6.5) and organic matter load (0.8 – 51%) of the natural soils. Spurgeon and Hopkin, (1995) determined a 21-day EC_{50} for *E. fetida* reproduction in OCDE artificial soil of 716 mg Cu kg^{-1}_{dw}, while others observed a 28-day an EC_{50} of 309 mg Cu kg^{-1}_{dw} (Owojori et al., 2009) in

the same soil, being both however above the toxicity value herein determined for *E. andrei*. Although most of the soils are European, their great spatial heterogeneity justifies the great variability among Cu toxicity data obtained for different samples. It is widely accepted that the toxicity of metals may be increased when they are under more bioavailable forms (Lock and Janssen, 2003). In turn, this is favored by low soil pH, as well as reduced organic matter and clay contents, which may complex with metals and retain them (Amorim et al., 2005). PTRS1 soil presents a reduced percentage of clay and a low pH that may enhance the bioavailability of Cu and, hence, increase its toxicity along 56 days of *E. andrei* exposure (EC$_{50}$ = 130.9 mg Cu kg$^{-1}_{dw}$; Table IV.2).

Concerning enchytraeid's reproduction, Amorim et al., (2005) reported a higher toxicity of Cu for the *E. luxuriosus* and obtained EC$_{50}$ values of 91 and 48 mg Cu kg$^{-1}_{dw}$ in natural European soils (EUROSoil), and an EC$_{50}$ of 97 mg Cu kg$^{-1}_{dw}$ for *E. albidus* in the standard natural soil LUFA 2.2. Notwithstanding, the same authors observed that the effect of Cu on the reproduction of that species was lower when using the OCDE artificial soil as a substrate (EC$_{50}$ > 320 mg Cu kg$^{-1}_{dw}$; Table IV.2).

For *F. candida* it was determined a LOEC of 103.5 and an EC$_{50}$ of 191.6 mg Cu kg$^{-1}_{dw}$. In the literature, both lower and higher EC$_{50}$ values were obtained for the same species. Criel et al., (2008) found EC$_{50}$ values of 50.6 and 12.6 mg Cu kg$^{-1}_{dw}$ in two natural soils with a low pH and organic matter content comparatively to PTRS1 soil (Table IV.2), while lower toxic effects (418 and 863 mg Cu kg$^{-1}_{dw}$) were observed for *F. candida* reproduction when tested in natural soils with a pH of 4.2 and 7.5, respectively, both presenting higher organic matter and/or clay contents than our tested soil (Table IV.2). Amorim et al., (2005) also determinate, lower Cu toxicity (EC$_{50}$s of 262 and 948 mg Cu kg$^{-1}_{dw}$) for the same species in natural European soils with more organic matter than the PTRS1 soil. On the other hand, Sandifer and Hopkin, (1996) tested the effect of OECD artificial soil pH (4, 5, 6) on Cu toxicity for *F. candida* reproduction and concluded that lower pH values strongly impair *F. candida* reproductive output (EC$_{50}$ of 1480, 710, 700 and mg Cu kg$^{-1}_{dw}$, respectively). In our study, although the high organic matter percentage may have contributed to decrease the bioavailable fraction of the metal, due to the great sensitivity

of these organisms to Cu, reproduction was still affected. Thus, both soil organic matter and pH are interfering factors for Cu effects on *F. candida*.
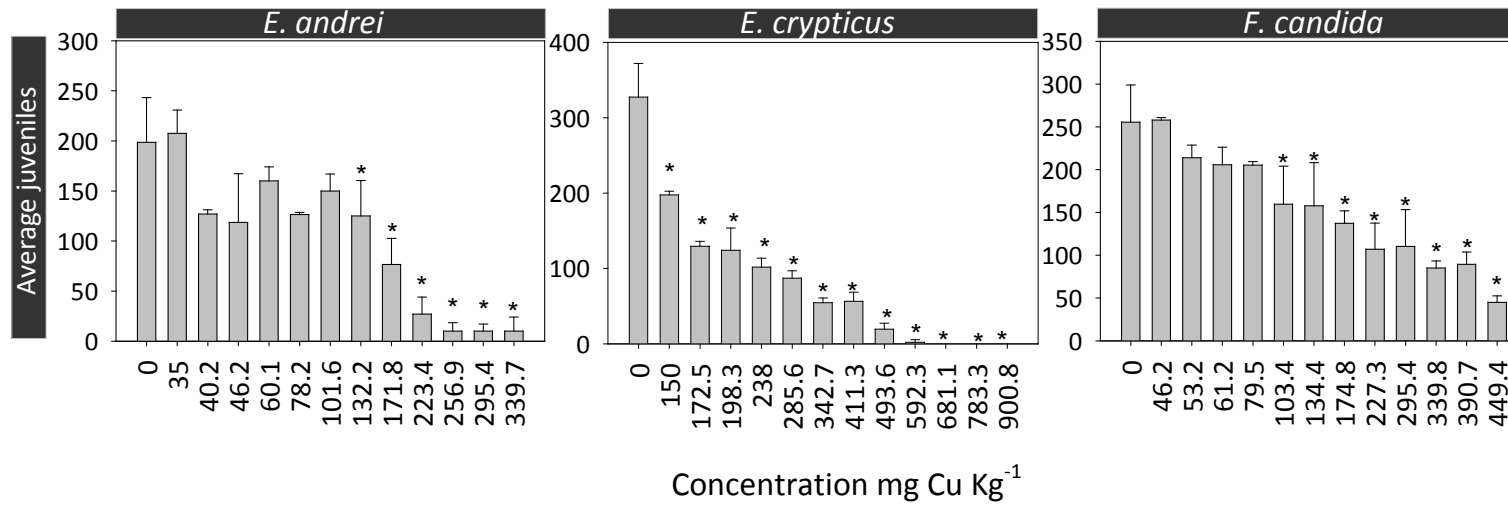
Figure IV.2- Reproductive output of *Eisenla andrei*, *Enchytraeus crypticus* and *Folsomia candida* exposed the natural soil PTRS1 spiked with different concentrations of Cu. Error bars indicate the standard error and asterisks sign out significant differences between the treatment and the control (0 mg Cu kg^{-1}_{dw}), ($p < 0.05$).

4.3.3 Seed germination and plant growth

Seed germination was not severely constrained for most of the plant species exposed to Cu in PTRS1 soil (Figure IV.3, Table IV.2), except for *L. sativa*. The LOEC value calculated for this species was ≤ 64.3 mg Cu Kg^{-1}_{dw}, and the Cu concentrations causing a 20% (EC_{20}) and 50% (EC_{50}) inhibitory effect on lettuce germination were 83.3 mg Cu Kg^{-1}_{dw} and 179.1 mg Cu Kg^{-1}_{dw}, respectively. *Z. mays* germination was only slightly affected at a LOEC of 864.4 mg Cu Kg^{-1}_{dw}, being the EC_{20} of 868.2 mg Cu kg^{-1}_{dw} and the $EC_{50} > 950.8$ 4 mg Cu Kg^{-1}_{dw} (Table IV.2). On the other hand, Cu was not toxic for the germination of *A. sativa* and *L. esculentum*. Previous studies had already observed that germination is a less sensitive endpoint for a range of soil pollutants (An, 2004a,b; An, 2006b; Lamb et al., 2010). The effects of metals on seed germination are related with their ability to reach embryonic tissues across physical and physiological barriers, such as the seed coats (Munzuroglu and Geckil, 2002). This ability is directly dependent on the structure of seeds coat, which varies according to the plant species, and the physical and chemical properties of the metal ions themselves (Munzuroglu and Geckil, 2002; Seregin and Kozhevnikova, 2005; Lin and Xing, 2007; Liu et al., 2007). In this way, the high toxicity of Cu to *L. sativa* germination can be linked to the high seed coat permeability to this metal, thereby leading to the accumulation of Cu in seeds and consequent inhibition of their germination.
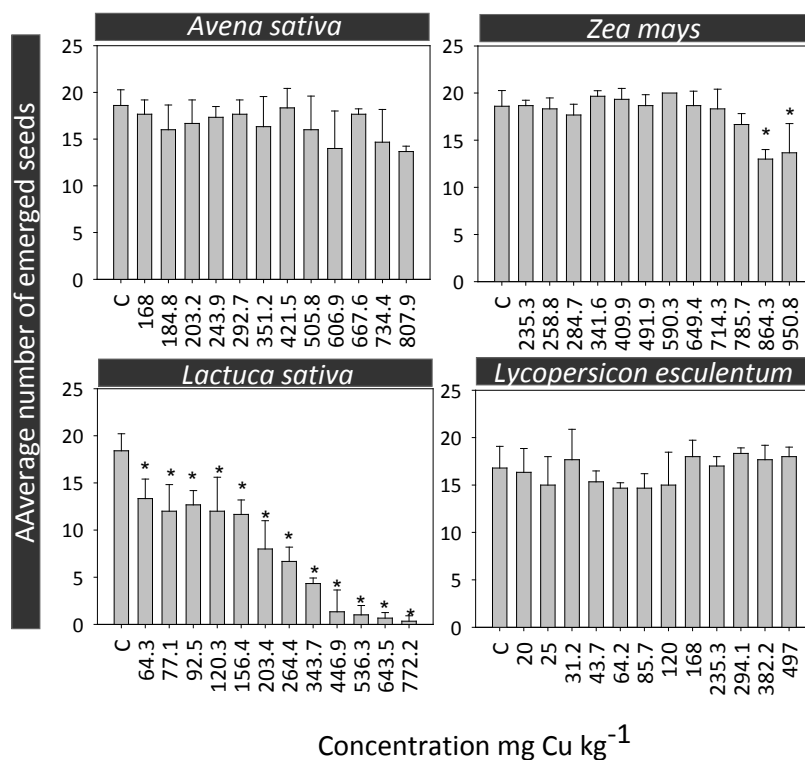
Figure IV.3- Average number of emerged seeds in monocotyledonous, *Avena sativa and Zea mays*, and dicotyledonous species, *Lycopersicon esculentum and Lactuca sativa* exposed to PTRS1 soil contaminated with Cu. Error bars indicate the standard error and asterisks represent significant differences between the treatments and the control (0 mg Cu kg^{-1}_{dw}), ($p < 0.05$).

Contrary to germination, plants growth was strongly inhibited by Cu (Figure IV.4, Table IV.2). Dose-response relationships were clearly obtained for the fresh and dry mass of both monocotyledonous and dicotyledonous species subjected to increasing Cu concentrations (Figure IV.4). This outcome was not influenced by the amount of sulphate as far as no statistically significant differences were measured between sulphate controls and the negative control with water ($P > 0.05$), for all the recorded endpoints.

The toxicity data presented in Table IV.2 suggests slight differences in the tolerance of plant species to free-metal concentrations. Most of all, dicotyledonous species were more sensitive than monocotyledonous, irrespective of the endpoint. The fresh and dry mass endpoints of monocotyledonous were significantly decreased for Cu concentrations of 203.2 and 292 mg Cu kg^{-1}_{dw}, respectively, for *A. sativa;* whereas for *Z. mays* the LOEC value for both endpoints was 235.3 mg Cu kg^{-1}_{dw}. In the case of dicotyledonous, the fresh and dry masses of *L. sativa* were significantly decreased for Cu concentrations ≤64.3 mg

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Cu kg$^{-1}_{dw}$, while for *L. esculentum* the LOEC was = 120.0 mg Cu kg$^{-1}_{dw}$ (Figure IV.4; Table IV.2). Based on the EC$_{50}$ values the plant species can be arranged in the following decreasing order of sensitivity for both measuring endpoints: *L. sativa > L. esculentum ≥ Z. mays > A. sativa* (*cf.* Table IV.2).

Copper is an essential element for plant growth and plays a significant role in many physiological processes, such as photosynthesis, respiration, carbohydrate distribution, N reduction and fixation, protein metabolism (Chatterjee et al., 2006; Xu et al., 2006; Nagajyoti et al., 2010). However, for the growth of the four species considered in this study, Cu was generally very toxic, even at low concentrations. The great toxicity of Cu for plants is in agreement with other studies, according to which shoot and root growth of maize and lettuce was more sensitive to Cu than to other metals like Pb, Zn or Cd (An, 2006; Lamb et al., 2010). Excess of Cu in soil plays cytotoxic role, induces stress and can unfavorably cause injury and symptoms to plant including growth retardation and leaf chlorosis (Verma et al., 2011; Thounaojam et al., 2012).

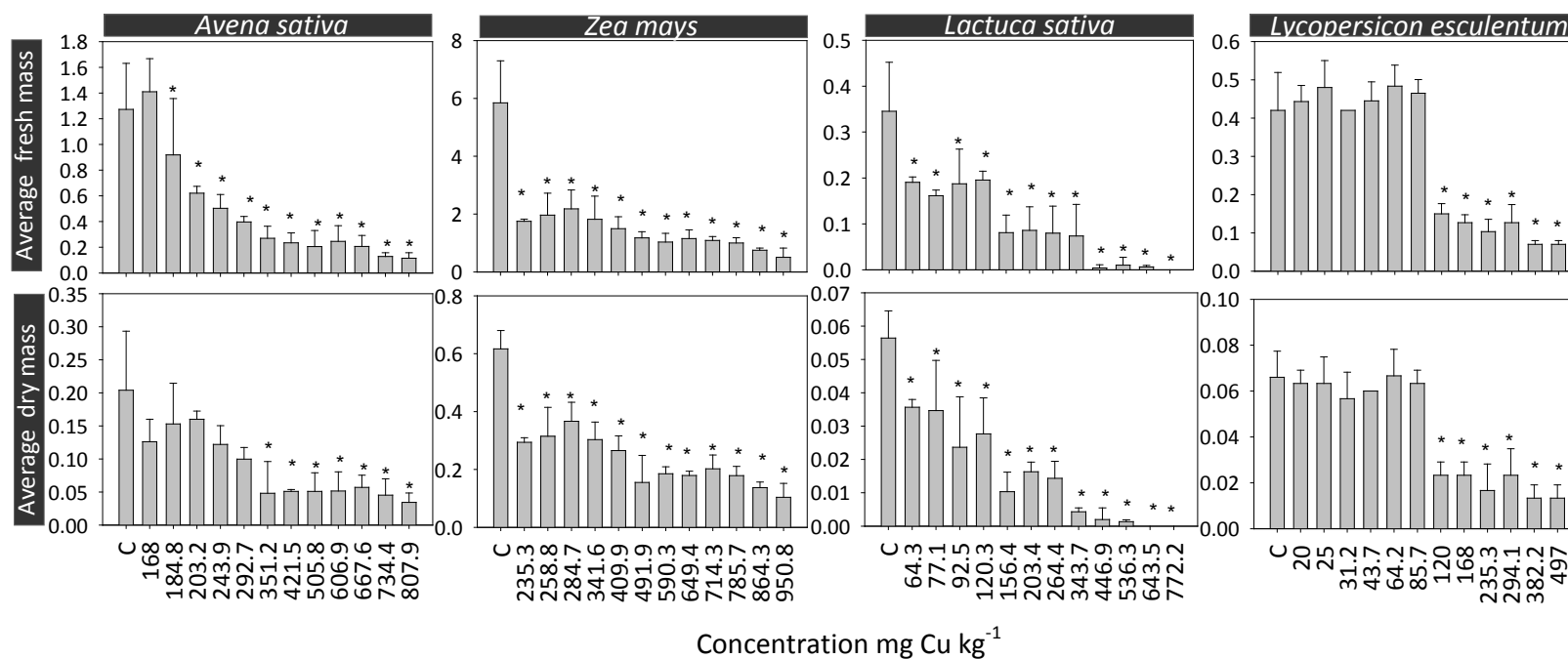**Figure IV.4-** Average values of fresh and dry mass measurements in monocotyledonous, *Avena sativa and Zea mays*, and dicotyledonous species, *Lycopersicon esculentum* and *Lactuca sativa* grown in PTRS1 soil artificially spiked with Cu. Error bars represent the standard error and the asterisks indicate significant differences between the treatments and the control (0 mg Cu kg^{-1}$_{dw}$) (P<0.05).

The uptake of metals by plants strongly depends on their physiology, nutritional status, capacity for regulation of internal metal concentrations, as well as of the ability of roots to interfere with local soil chemical conditions through the release of protons and organic acids (Ginocchio et al., 2002). However, soil physical and chemical properties, such as texture, pH, and organic matter content, are known to be important factors in determining the mobility of metals in soils, such as Cu, hence affecting their phytotoxicity (Alva et al., 2000; Daoust et al., 2006; Rooney et al., 2006). In particular, organic matter supplies organic chemicals to the soil solution, which may serve as chelators and increase metal availability to plants (Vega et al., 2004; McCauley et al., 2009; Schaub et al., 2007; Laing et al., 2009). This can partially explain the results obtained in our study, since the PTRS1 soil contain an elevated percentage of this constituent 6.5% according to the classification provided by Murphy et al., (2012). And such organic matter can provide components that can increase Cu bioavailability to plants. On the other hand, the pH of PTRS1 soil was hardly interfering with Cu phytotoxicity. Soil pH was referred as the most important factor influencing metal speciation, solubility from mineral surfaces, mobility, and bioavailability (Muehlbachova et al., 2005; Zhao et al., 2010). A negative correlation between soil pH and metal mobility and bioavailability to plants has been well documented in numerous studies, (Badawy et al., 2002; Wang et al., 2006; Du Laing et al., 2007). An increase in pH usually reduces the ion activity in solution by complexation (Römkens et al., 1999). This occurs especially with metals like Cu, which are known to have a great ability to form very stable metal–organic complexes (Stevenson, 1994), thereby reducing its toxic effect on plants. Considering that PTRS1 is an acidic soil, this might have enhanced Cu bioavailability, and therefore the high toxicity for plants. On other hand, the addition of nutrients in test containers may promote their uptake by plants and stimulate soil microbial activity, what may in turn contribute to soil acidification and the consequent metal toxicity (Römkens et al., 1999).

Chapter IV – Copper toxicity in a natural reference soil – ecotoxicological data for the future derivation of soil screening levels

Table IV.1 Toxicity data for microbial processes, soil invertebrates and plants with effect concentrations as mg U kg-1dw soil, with indication of the 95% confidence intervals

Biota	Endpoint	Soil type	pH	OM	WRC	Clay	NOEC (mg Cu Kg^{-1})	LOEC	EC$_{20}$	EC$_{50}$	Reference
Urease			5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	139.5	167.4	124.8 (104.08 - 145.59)	171.8 (156.18-187.57)	
Cellulase			5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	500.0	600.0	429.9 (-19.90-871.9)	571.4 (485.17-657.76)	
Acid fosfatase	enzim. act.	natural soil	5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3			n.d.	n.d.	
Dehydrogenase			5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	≥ 600	>600	425.9 (-19.9- 871.9)	n.d.	present study
Nitrogen mineralization			5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	116.2	139.5	90.7 (28.39-153.15)	146.5 (87.00-206.02)	
Eisenia andrei	rep.(56 days)	natural soil	5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	101.6	132.2	73.0 (34.94- 111.14)	130.9 (91.69-170.14)	present study
Eisenia fetida	rep.(21 days)	natural soil	4.7	23.3		24.0	119.0	162.0	n.d.	778.0 (497.0-1.2)	Criel et al. 2008
Eisenia fetida	rep.(21 days)	natural soil	5.2	0.8		9.0	58.4	99.4	n.d.	72.0 (105.0-171.0)	Criel et al. 2008
Eisenia fetida	rep.(21 days)	natural soil	3.0	51.0		7.0	179.0	245.0	n.d.	192.0 (154.0-238.0)	Criel et al. 2008
Eisenia fetida	rep.(21 days)	Lufa 2.2	5.0	2.1		7.9	87.5	159.0	n.d.	155.0 (130.0-184.0)	Criel et al. 2008
Eisenia fetida	rep.(21 days)	OCDE	6.5	4.7		20.0	188.0	363.0	n.d.	349.0 (301.0-406.0)	Criel et al. 2008
Eisenia feoida	rep.(21 days)	OCDE	6.0	10.0		20.0		n.d.	n.d.	309.0 (224.0-400.0)	Oeojori et al. 2008
Eisenia fetida	rep.(21days)	OCDE	6.3	10.0		20.0	29.0	n.d.	n.d.	716.0 n.d	Spurgeon and Hokin 1995
Enchytraeids crypticus		natural soil	5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	< 150	150.0	< 150	165.1(146.84-183.27)	present study
Enchytraeids albidus		OCDE	6.2	8.0		10.0	> 320	n.d.	n.d.	> 320.0	Amorim et al. 2005
Enchytraeids albidus	rep.	Lufa 2.2	5.8	4.4		6.0	100.0	n.d.	n.d.	97.0 n.d.	Amorim et al. 2005
Enchytraeids albidus		natural soil	5.4	4.1		23.0	10.0	n.d.	n.d.	48.0	Amorim et al. 2005
Enchytraeids albidus		natural soil	6.7	6.5		26.0	32.0	n.d.	n.d.	91.0 n.d.	Amorim et al. 2005
Folsomia Candida		natural soil	5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	79.6	103.5	65.8 (36.87-94.84)	191.6 (147.12-236.12)	present study
Folsomia Candida		natural soil	3.0	5.1		7.0	< 32	n.d.	n.d.	50.6 36.6- 70.0	Criel et al. 2008
Folsomia Candida		natural soil	4.2	12.9		13.0	290.0	544.0	n.d.	418 (235.0-745.0)	Criel et al. 2008
Folsomia Candida		natural soil	3.4	1.9		0.0	30.1	51.9	n.d.	12.6 (4.07-38.7)	Criel et al. 2008
Folsomia Candida	rep.	natural soil	7.5	1.3		26.0	472.0	725.0	n.d.	863.0 (752.0-990.0)	Criel et al. 2008
Folsomia Candida		natural soil	3.2	9.2		10.0	100.0	n.d.	n.d.	262.0 n.d.	
Folsomia Candida		natural soil	6.2	12.9		6.0	320.0	n.d.	n.d.	948.0 n.d.	Amorim et al. 2005
Folsomia Candida		natural soil	4.0	10.0		20.0	n.d.	n.d.	n.d.	1480.0 n.d.	Sandifer et al 1996
Folsomia Candida		OCDE	5.0	10.0		20.0	n.d.	n.d.	n.d.	710.0 n.d.	Sandifer et al 1996
Folsomia Candida		OCDE	6.0	10.0		20.0	n.d.	n.d.	n.d.	700.0 n.d.	Sandifer et al 1996
Avena sativa			5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	≥ 807.9	>807.9	n.d.	n.d.	
Zea mays	germ.	natural soil	5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	785.8	864.4	868.2 (811.17-925.28)	>1000	present study
Lactuca sativa			5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	≤ 64.3	64.3	83.3 (59.11-110.58)	179.1 (144.53-213.74)	
Lycopersicon esculentum			5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	≥497.7	>497.7	n.d.	n.d.	
Avena sativa			5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	184.8	203.2	< 168	236.3 (191.86-280.82)	
Zea mays	f.m.	natural soil	5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	≤ 235.3	235.3	< 235.5	< 235.5	present study
Lactuca sativa			5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	≤ 64.3	64.3	< 64.3	89.0 (58.89-119.10)	
Lycopersicon esculentum			5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	85.7	120.0	78.8 (50.57-107.65)	135.0 (106.70-163.34)	
Avena sativa			5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	243.9	292.7	< 168	290.5 (197.03-384.04)	
Zea mays	d. m.	natural soil	5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	≤ 235.3	235.3	< 235.5	285.4 (213.34-357.40)	present study
Lactuca sativa			5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	≤ 64.3	64.3	< 64.3	93.1 (71.30-115.03)	
Lycopersicon esculentum			5.91±0.098	6.5±0.004 (65.3 g/Kg)	23.94±1.839	3.3	85.7	120.0	70.4 (35.37-105.46)	151.1 (109.86-192.36)	

Average ± STDEV: pH (H2O), OM-organic matter(%), WHCmax – maximum water holding capacity (%); Clay %; rep.-reproduction; germ.-germination; f.m.- fresh mass; d.m- dry mass; Enz. act.- enzyme activity; n.d.-not determined.

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4.3.4. Derivation of Soil Screening Values (SSV) for copper applying assessment factors

The predicted no effect concentration (PNEC) value for Cu was derived based on the data obtained in this work. A risk limit for Cu in a dominant Portuguese soil was defined following the approach suggested by the Technical Guidance Document published by the European Commission (EC, 2003), in support of the Commission Directive 93/67/EEC on Risk Assessment for new notified substances, of the Regulation nº 1488/94 on Risk Assessment for existing substances and Directive 98/8/EC of the European Parliament and the Council. The toxicity values selected for PNEC derivation corresponded to the lowest NOEC and EC_{20} values obtained across all endpoints, which in this case occurred for the average number of juveniles produced by *F. candida*. An assessment factor of 10 was applied, since more than three NOEC values were used for at least three different test species. However, the PNEC values obtained for Cu when this factor was applied, varied between 6.5 (EC_{20}-based) and 7.9 (NOEC-based) mg Cu Kg^{-1}_{dw}, which were lower than the background concentrations reported by Inacio et al. (2008) for the national context (18.6 mg Cu Kg^{-1}_{dw}), or even lower than the background Cu concentration quantified in PTRS1 soil (9 mg Cu Kg^{-1}_{dw}; Caetano et al., 2012). However, and as verified during the validation of PTRS1 soil by the same authors, the combined background concentrations of metals found in this soil did not compromise its habitat function for terrestrial species.

Besides, the PNEC values were much lower than the Eco-SSL values suggested by USEPA (2007) for Cu, which ranged between 70 mg Cd kg^{-1}_{dw} for plants and 80 mg Cd kg^{-1}_{dw} for soil invertebrates. Comparing with the Canadian Soil Quality Guideline values for ecosystems protection (63 mg Kg^{-1}Cu; CCME, 1999), the PNEC herein derived was also considerably lower. However, if any factor is applied and based in EC_{20} value we obtain a PNEC value of 65 mg Cu $Kg^{-1}_{dw,}$ which is in perfect agreement with Canadian values proposed for this metal. Due to the nonexistence of screening values at national level, the Canadian values are recommended in assessment of contaminated soils, though they never had been validated prior for any Portuguese soil. In this way, the value proposed by Canadian Soil Quality Guideline for Cu is validated in this work for soils with similar characteristics to PTRS1 soil. Thereby, we propose a PNEC of 65 mg Kg^{-1}_{dw} for Cu.

4.4 Conclusion

At the light of the results herein generated it was reinforced the toxicity of Cu to different soil constituents. This metal produced negative impairments on soil biochemical traits given by a significant reduction in the activity of UR and CELL enzymes and on NMIN. The high inhibitory effects of Cu were also verified on the reproduction of soil invertebrates following the increasing order of sensitivity: *E. andrei* < *F. candida* < *E. crypticus*.

Although seed germination was not severely constrained by this metal, except for *L. sativa*, plant growth measured as fresh and dry mass showed to be the most sensitive endpoint to Cu phytotoxicity. In general, the growth of dicotyledonous species (*L. escolentum* and *L. sativa*) was more affected than that of monocotyledonous plants (*A. sativa* and *Z. mays*).

Unequivocally, the large variation in toxicity values obtained with invertebrates and plants in literature clearly demonstrates the influence of soil properties on the bioavailability of Cu and consequent toxicity to soil organisms.

From the ecotoxicological data obtained in this study, and applied an Assessment Factor approach with a factor of 10, it was possible to calculate a PNEC value for Cu, which was lower than the background concentrations already reported for the national context. The Assessment Factors proved to be excessively protective when the objective is the derivation of screening values of copper for soils.

The data obtained in the present study represent an important breakthrough in the definition of national soil quality guidelines that are extremely useful for the screening of soil contamination and its protection within the national context.

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Chapter **V**

Derivation of Soil Screening Values (SSV) for U, Cd and Cu, from a natural reference soil

Derivation of Soil Screening Values (SSV) for U, Cd and Cu, using a natural reference soil

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Abstract

Ecotoxicological data for uranium (U), cadmium (Cd) and copper (Cu), was obtained for soil invertebrates and plants and for soil microbial parameters, following standard protocols and well known protocols from the literature. The obtained sensitivity values (EC_{20} and EC_{50}s) were used to derive generic soil screening values (SSVs) for these metals. The SSVs were derived following the species sensitive distribution (SSD) approach and HCp values (HC_5 or HC_{50}) for each metal were estimated. The selection of the best HCp to support the derivation of SSVs was discussed, based on the statistical confidence of the estimations, the sensitivity data in left side of the curves, below the HCps selected, and on available data for field and laboratorial studies with Portuguese natural contaminated soils. All the criteria was considered with the aim of preventing the selection of over-protective HCp values. The, following SSVs were proposed: uranium 151.4 mg U kg^{-1}_{dw}, cadmium 5.6 mg Cd kg^{-1}_{dw}, copper and 58.5 mg Cu kg^{-1}_{dw}.

A comparative analysis with other European and international soil quality guideline values was made. The present work represents an important contribution for setting a national approach for deriving soil screening values for a generic use, but soil type-based, for the environmental risk assessment of contaminated areas. The approach proposed will take benefit from the tools and methodologies developed by North European countries with great expertise in the area, thus contributing for the harmonization of procedures within Europe.

Keywords: Soil screening values, uranium, cadmium, cooper, natural soil.

5.1 Introduction

Formerly developed, in The Netherlands, in 1983, to protect human health, from groundwater contamination caused by the leaching of buried wastes, (Nason et al., 2003), soil screening values (SSVs) are more recently defined as threshold concentrations below which communities of organisms can be chronically exposed without adverse consequences, or, when exceeded, additional risk assessment procedures are recommended for a detailed site-specific assessment (Fishwick, 2004; Carlon, 2007). The SSVs have a remarkable importance at a screening level of Ecological Risk Assessment (ERA) procedures aimed in assessing contaminated areas, as they allow to screen out rapidly and with minimal costs those sites (or at least sub-areas within the areas) for which risks are too low and a deep evaluation can be discarded (Provoost et al., 2008). SSVs should therefore be distinguished from soil guideline values, which represent a limit above which an intervention is required, or from limit values for emissions more adjusted to regulate sludge deposition (Ferguson et al., 1998).

The comparison of SSVs with the total measurable concentrations of contaminants, helps to infer whether these contaminants are present at concentrations that can pose a risk to the ecological receptors both, individually or combined, through the calculation of the toxic pressure of the mixtures by applying the multi-substance potentially affected fraction ms-PAF method (De Zwart and Posthuma, 2005; Jensen and Mesman, 2006a; Weeks and Comber, 2005). These comparisons, performed for each chemical, present at a contaminated site, are also useful to identify the most likely contaminants responsible by the effects observed (Jensen and Pedersen, 2006).

Although, ecological risk assessment tends to be relegated to a second plan it is extremely important to support remediation plans and future uses of the sites (Ferguson et al., 1998; Moreno-Jiménez et al., 2011). In this context, the derivation of SSVs has become a critical need in recent years, due to the generalized and recognized problem of soil contamination in Europe, as expressed in the text of the Thematic Strategy for Soil Protection published by the European Community (CEC, 2006a). Panoply of contaminants released from diverse human activities, are harmful to biota, ecosystems, and in the last instance to human health, both directly and indirectly (by affecting several ecosystem

services), and have attained concerning concentrations in the soil compartment, preventing the use of meaningful areas of soil for other uses. In this sense, SSVs are an extremely important component of ERA Frameworks, and for the application of present and future soil protection policies, aimed in dealing with contaminated areas, as they can be more comprehensible for all the professionals' involved, stakeholders and general public. For this reason several European countries such as Austria, Flanders, Finland, France, Norway, The Switzerland, Denmark, Germany, The Netherlands, UK, The Basque Country and Spain (Carlon, 2007; Crommentuijn et al., 2000a; Fishwick, 2004; O'Halloran, 2006) have already derived their own SSVs.

The SSVs are derived based on ecotoxicological data obtained from laboratorial tests, with different species relevant to soil ecosystems, and whenever as possible following standard protocols. And, for this purpose, three widely recognized methods have been applied, depending on data available, and were adopted by many countries already applying   risk assessment frameworks: i) the assessment factor (AF) method; ii) the statistical distribution method, as the species sensitivity distributions (SSDs) and, iii) the equilibrium partitioning method (Ferguson et al., 1998; Posthuma et al., 2002; Fishwick, 2004). Although transparent, easy-to-use and applicable to small data sets, the AF approach, is based on the lowest available toxicity value, usually obtained from single species-laboratorial test, which is divided by a given assessment factor ranging from 1000 to 1, depending of the amount and type (acute *versus* chronic) of available toxicity data (Fishwick, 2004). The AF method is advisable for a former definition of toxicity-based SSVs based on the precautionary principle, and they should be revised as soon as more ecotoxicological data is obtained (Fishwick, 2004). Further they should not be confounded with uncertainty factors which are used to extrapolate from acute to chronic data, from laboratorial to field data etc., (Smrchek et al., 1993). But for more detail, Chapman et al., (1998) has made a critical evaluation of the application of both, assessment and uncertainty factors in the ERA process. In contrast, the SSDs, when used correctly, they can introduce greater statistical confidence into risk assessment processes when compared to the assessment factor approach (EC, 2003). This methodology is based on the recognition that species are not equally susceptible to toxicants, thus representing

the variation in sensitivity of species toward certain contaminant by a statistical or empirical distribution function of responses, for a set of species (Posthuma et al., 2002). For this purpose toxicity data, of a given number of selected species, or from a natural community is fitted to a cumulative distribution function (e.g. lognormal or log-logistic) that can be then used, in an "inverse mode", to determine the hazard concentration for a given percentage of species (HCp), depending of the level of protection required (Posthuma et al., 2002). Usually, a point estimate or a cut-off known as the HC_5 (hazardous concentration for 5% of species), or the 95% protection level is extrapolated from the curve (Forbes & Calow, 2002; Wheeler et al., 2002; Domene et al., 2008), i.e. the concentration for which no more than 5% of the species will be affected. However, some authors suggest that when all of this data (from species, to communities and functions) is available and is used, a new definition for HCp should be proposed, since measures are no more at species level (Wheeler et al., 2002). This new definition was already introduced (e.g. Jänsch, et al., 2007) and SSVs can in fact be developed for several receptors such as soil-dwelling invertebrates, soil functions, the microbial community, terrestrial plants and even wildlife (Nason et al., 2003).

There are cases where hazardous concentration affecting 50% of the species (HC_{50}) are also used (Rutgers et al., 2008). Both HC_5 or HC_{50} values are normally derived from a dataset of NOEC (chronic no observed effect concentrations) values for soil organisms, EC_{20} or EC_{50} values can also be used, depending again on the protection level required (Kapustka, et al., 2006; Jänsch et al., 2007), which in turn may depend from present and future soil uses. However, and as far as NOEC values are considered several recommendations pointed out for their replacement by low EC values (EC_5 or EC_{10}), which requires the adoption of the ECx sampling design in standard tests (Chapman et al., 1996; OCDE, 1998; Warne and Dam, 2008). Further, although some authors argue that the p percentage of species is frequently a policy decision (Fishwick, 2004) the more protective 5th percentile should be selected especially when optimal sample sizes (number of species sensitivity values) are low (Newman et al., 2000) Despite the advantages of the SSD approach, highlighted by Fishwick, (2004), several disadvantages were also pointed out, as for example the exclusion of interactions between species, which may compromise the

use of HCp values derived from single species toxicity data to protect ecosystems. A criticism that was minimized by the results of Maltby et al., (2005) which showed that the SSDs were similar for both laboratorial and field exposures, and that the lowest HC_5 estimate (95% protection with 95% confidence) was protective of the freshwater ecosystems, for the pesticides under evaluation, when single applications were considered

The equilibrium partitioning method (Eq-P method) formally developed for sediments, could also be used to compensate the lack of toxicity data for terrestrial species of certain compounds, by converting a PNEC (Predicted No Effect Concentration) value for the aquatic compartment (usually derived using SSDs) in a PNEC for soil, by using a soil/water partition coefficient (Fishwick, 2004). However, and as showed by van Beelen et al., (2003) , the Eq-P method can lead both to over- and underestimation of terrestrial HC_5, being preferable to use terrestrial toxicity data to derive HC_5 values for soil, when more than four values are available.

In Europe, risk assessment methods for new and existing chemicals are described in the technical guidance document (TGD) developed by the European Commission (EC, 2003) and the inclusion of statistical extrapolation methods using SSDs is increasingly recommended (Posthuma et al., 2002; Fishwick, 2004; Wheeler et al., 2002) for the soil compartment and for regulatory purposes, despite some of the disadvantages pointed out like the accuracy-dependency from the amount and quality of data available and model used (Newman et al., 2000; Wheeler et al., 2002). Some countries like Portugal, are making temporary use of foreign values (Ferguson, 1999; Pereira et al., 2008), however is widely recognized the great variability of soils within the European territory, since at least 320 main types of soils were identified, with great differences in terms of their physical, chemical and biological properties (CEC, 2006a). Such differences are expected to account for differences in the behavior of chemicals in the soil and consequently it's toxicity will also be constrained by different soil types (Semenzin et al., 2007; Rombke & Amorim, 2004). Thereby, the main objective of this work was the derivation of Portuguese SSVs for Uranium (U), Cadmium (Cd) and Copper (Cu) using a set of ecotoxicological data previously obtained for the soil microbial community, invertebrates and plants using a

Portuguese natural reference soil, representative of dominant soils within the continental territory, by the application of the SSD method, following the criteria and procedures outlined in the European Union (EU) Technical Guidance Document (EC, 2003). HCp values estimated from SSDs for U, Cd and Cu will be compared with the SSVs values previously determined by the assessment factor (AF) approach by Caetano et al., (a,b, c, submitted). Further, HCp values estimated from SSDs, based on EC_{20} and EC_{50} values, will be discussed in terms of their degree of protection, after comparison with soil background values for the three metals as well as with the SSVs obtained for other countries. The values estimated by the SSD method will also be compared with previous SSVs determined by using the assessment factor (AF) (Caetano et al., (a,b, c, submitted)) for each metal. Portuguese SSVs for uranium, copper and cadmium will be suggested for regulatory purposes.

5.2 Material and methods

5.2.1 Toxicity data of U, Cd and Cu

Uranium (U), cadmium (Cd) and copper (Cu) were selected to derive SSVs as they are some of the top pollutants associated with industrial and agricultural activities, with consequent relevant toxicity in the soil compartment (van Gestel & Mol 2003; Pereira et al., 2008; Kabata-Pendias, 2010). Additionally, in case of uranium it represents a serious environmental problem in Portugal, due to the accumulation of uranium mining wastes for several decades of exploration of radioactive ore, in the last century (Carvalho, 2011; Pereira et al., 2013). Further, the definition of SSVs for this metal is extremely important, since to the best of our knowledge any other European country has made such attempt. Copper and cadmium are also found in several other mining contaminated areas (Pereira et al., 2006; Pereira et al., 2008) and copper containing fungicides have been widely over-applied in agriculture, especially in vineyards, for more than one century (Ruyters et al., 2013) being a problem for wine producing countries like Portugal. A finding that led the European Commission to restrict the annual application of copper (EC, 2002). Ecotoxicological data generated in previous studies for the concerned metals by Caetano et al., (a,b, c, submitted) were used to derive SSVs for these elements, using a Portuguese

natural soil. Data was gathered from ecotoxicological tests, performed according to internationally standardized guidelines to assess sub-lethal effects on invertebrates and plants such as the reproduction of *Eisenia andrei* (ISO, 1998), *Folsomia candida* (ISO, 1999) and *Enchytraeus crypticus* (ISO, 2004), and the emergence/growth of terrestrial plants (*Avena sativa, Lycopersicon esculentum, Zea mays, Lycopersicon esculentum*), (ISO, 2005). Soil microbial activity was also tested measuring a range of soil enzyme activities (arylsulphatase, dehydrogenase, urease, and cellulase) as well as changes in the nitrogen mineralization and potential nitrification, on soils spiked with a range of metal concentrations, after one month of exposure Caetano et al., (a,b, c, submitted for more details). Although no standard protocols are available for these parameters, the protocols used are clearly published in the literature (e.g. Schinner et al., 1996) and have been used in several studies, including some studies published by our team (Pereira et al., 2006; Antunes et al., 2011). All tests were conducted using a Portuguese natural reference soil PTRS1, as test substrate, previously characterized and validated by Caetano et al. (2012) as a natural reference soil.

Although NOEC values are recommended when SSD approach is applied for the derivation of SSVs (Fishwick, 2004), these values have been widely criticized because they depend from the range of concentrations tested, the variability of the data, the selected significance level and the sample size (OCDE, 1998; Warne & Dam, 2008; Meng et al., 2010)

Therefore, the model used in this study was applied both to EC_{20} and EC_{50} values (Table V.1) since they are statistical estimated toxicity concentrations and therefore more reliable (Jänsch et al., 2007). The authors decided not to use EC_{10} values (advised to replace NOEC values), (OCDE, 1998) because it will be possible to perceive they would give rise to extremely overprotective values, which in turn could result in several false positives, reducing the importance of the tier 1 of risk assessment procedures for contaminated lands.

5.2.2 Development of SSVs values

5.2.2.1 SSVs calculated using species sensitivity distributions (SSD)

The SSDs were obtained using two recognized tools for SSDs generation : (1) Microsoft Excel template for SSD generator applied by USEPA (http://www.epa.gov/caddis/da_software_ssdmacro.html); (2) ETX 2.0 program (Van Vlaardingen et al., 2003) internationally applied (e.g. in the Netherlands (VROM, 2002) and Denmark (Scott-Fordsmand & Pedersen, 1995), (http://www.rivm.nl/rvs/Risicobeoordeling/Modellen_voor_risicobeoordeling/ETX_2_0). Both software's were used in this work in order to compare and confirm the output data obtained. The concentration affecting a given proportion of species and microbial functions (HCp) was estimated, after fitting a linearized log-normal distribution to ECx values, by the SSD generators. Therefore, for each metal we generated SSDs based in EC_{20} to estimate both HC_5 and HC_{50} cut-offs (i.e. hazardous concentration affecting 5 and 50% of the species and microbial processes at their EC_{20} effect level, respectively) and EC_{50}-based HC_5 values (i.e. hazardous concentration affecting 5% of the species and microbial processes at their EC_{50} effect level). Since both tools used the same statistical model to fit the data, HCp values and graphs generated by the SSD generator from USEPA are presented, because this tool was more user-friendly for this purpose.

5.3 Results and discussion

5.3.1 Data set used for U, Cd and Cu

The datasets used in this study include a total of 6, 8, 10 EC_{20} values and 6, 14, 15 EC_{50} values for the effects of U, Cd and Cu, respectively, on three different trophic levels (microorganisms, invertebrates and plants) (Table V.1, Table V.2). All the toxicity data reported in this study are expressed in terms of soil dry mass. Both 5th (HC_5) and 50th (HC_{50}) percentiles of a chronic toxicity distribution were chosen assuming that at these metal concentrations no more than 5% and 50% respectively, of all species and microbial processes will show a detrimental effect.

Table V.1 Toxicity data for soil enzymes activity, reproduction of invertebrates, seed germination and growth of terrestrial plants with effect concentrations of U, Cd and Cu (mg.kg$^{-1}_{dw}$), (Caetano et al., *a,b,c submited),* with indication of the 95% confidence between brackets.

Metals	Biota		Endpoint	EC$_{20}$	EC$_{50}$
Uranium (mg U Kg^{-1})	Microbial processes	Arylsulphatase	enz.act.	155.3 (84.76-255.87)	295.6 (216.09-375.17)
		Nitrogen mineralization		152.2 (46.66-257.79)	347.0 (211.25-482.91)
		Potencial nitrification		429.5 (229.53-629.46)	610 (459-761.1)
	Invertebrates	*Eisenia andrei*	rep.	474.8 (391.47-558.04)	631 (532.78-699.21)
		Enchytraeids crypticus		469.7 (355.4-584.0)	518.6 (480.40-556.90)
		Folsomia candida		343.4 (172.23-514.60)	851.64 (606.10-1097.18)
Cadmium (mg Cd Kg^{-1})	Microbial processes	Celulase	enz.act.	47.8 (15.67-80.04)	-
		Urease		99.9 (75.17-124.67)	-
		Acid phasphatase		-	40.2 (0.88-79.5)
	Invertebrates	*Eisenia andrei*	rep.	37.3 (26.60-47.95)	76.4 (62.69-90.12)
		Enchytraeids crypticus		-	8.3 (7.54-8.87)
		Folsomia candida		-	64.8 (54.47-75.20)
	Plants	*Lactuca sativa*	germ.	279.3 (202.24-356.46)	460.0 (386.36- 533.66)
		Lycopersicon esculentum		644 (547.74-742.07)	919.04 (8414.24-996.84)
		Avena sativa	f.m.	-	36.5 (19.40-53.63)
		Zea mays		37.5 (22.20-52.87)	135.1 (101.52-168.80)
		Lactuca sativa		-	20.2 (10.11-10.60)
		Lycopersicon esculentum		78.03 (46.03-110.03)	145.5 (111.17- 179.85)
		Avena sativa	d.m.	-	27.48 (1.70-53.25)
		Zea mays		-	185.1 (100.30-269.90)
		Lactuca sativa		-	20.4 (5.32-35.48)
		Lycopersicon esculentum		76.0 (43.88-108.06)	137.4 (102.74-172.07)
Copper (mg Cu Kg^{-1})	Microbial processes	Dehydrogenase	enz. act.	124.8 (104.0 - 145.5)	-
		Nitrogen mineralization		90.7 (28.39-153.15)	146.5 (87.00-206.02)
		Celulase		429.9 (-19.90-871.9)	571.4 (485.17-657.76)
		Urease		124.8 (104.08 - 145.59)	171.8 (156.18-187.57)
	Invertebrates	*Eisenia andrei*	rep.	73.0 (34.94- 111.14)	130.9 (91.69-170.14)
		Enchytraeids crypticus		-	165.1(146.84-183.27)
		Folsomia candida		65.8 (36.87-94.84)	191.6 (147.12-236.12)
	Plants	*Zea mays*	germ.	868.2 (811.17-925.28)	-
		Lactuca sativa		83.3 (59.11-110.58)	179.1 (144.53-213.74)
		Avena sativa	f.m.	-	236.3 (191.86-280.82)
		Zea mays		-	126.1 (56.22-195.99)
		Lactuca sativa		-	89.0 (58.89-119.10)
		Lycopersicon esculentum		78.8 (50.57-107.65)	135.0 (106.70-163.34)
		Avena sativa	d.m.	-	290.5 (197.03-384.04)
		Zea mays		-	285.4 (213.34-357.40)
		Lactuca sativa		-	93.1 (71.30-115.03)
		Lycopersicon esculentum		70.4 (35.37-105.46)	151.1 (109.86-192.36)

rep.-reproduction; germ.-germination; f.m.- fresh mass; d.m- dry mass; enz. act.- enzyme activity; (n.d)-not determined.

Table V.2 Summary table of the distribution of toxicity data, per throphic level; N = Total number of species/microbial processes.

Metals	Total number $EC_{20}s$	Total number $EC_{50}s$	Microbial processes	Animal processes	Plant species	N
Uranium	6	6	3	3	0	6
Cadmium	8	14	3	3	4	10
Copper	10	15	4	3	4	11

5.3.1 SSDs generation and HCps estimation and selection

For all the metals, the number of toxicity values available were above the minimum of four, recommended by van Beelen et al. (2003), but below the recommended number of data points (10-15) required to stabilize log-logistic models, (Table V.2). According to Table V.3 the model fitted better to EC_{20} values, rather than to EC_{50} values, except for copper. Further, HC_5 values derived from EC_{50} values were 2.5 greater (the maximum recorded for copper) than the HC_5 values derived from EC_{20}. But, as far as uranium is considered all the EC_{20} and EC_{50} values recorded for this metal were above these HC_5s, suggesting that both values will have the same degree of protection. However, since both HC5s fall in the lower limit of the cumulative curve, where no more toxicity data exists below them, on a statistical point of view they are a less strong estimation. The same was not true for the HC_{50} based on EC_{20} values (Figure V.1c and Table V.3), suggesting that it could be a more confident estimation to be proposed as a SSV for uranium. Nevertheless, if selected, an effect level of about 50% should be expected in some important microbial parameters, related with the nitrogen cycle on soils at concentrations close to this cut-off. In fact, and looking for the SSD for uranium, the soil microbial enzymes were the most sensitive parameter to U, as they are located in left part of the SSD-curve. Hence it is important to guarantee that soil contamination with U will not compromise nutrient's cycling to a level that will result in the subsequent limitation of the net primary productivity.

Furthermore, and comparing the different HCp values obtained for uranium in this study, with field ecotoxicological data or with data from Portuguese natural soils contaminated with uranium reported in literature, we can notice the occurrence of effects at both higher and lower environmental concentrations of U. For example, effects in the feeding activity of soil fauna, measured by the bait lamina assay, were recorded by André et al., (2009), in soils containing 210.6 mg U kg^{-1}. Antunes et al., (2011) observed the inhibition of several soil enzyme activities in soils with concentrations of U ranging between 99.2 and 289.10 mg U kg^{-1}. A concentration of U of 215.7 mg U kg^{-1} was present in soils that caused effects in reproduction, growth reduction, DNA damages, cytotoxicity, and changes in the populations of cells from the immunity system (Lourenço et al., 2011).

Further, the avoidance of natural soils by *E. andrei* was also recorded for concentrations above 103 mg U kg^{-1} (Antunes et al., 2008). Pereira et al., (2009) observed an inhibition in the growth of *Lactuca sativa* (measured in terms of wet mass) in natural soils from mining area with U concentrations ranging between 103.3 and 1408.0 mg U kg^{-1}. In fact some of the reported toxic effects on soil microbial enzymes activity, invertebrates and plants occurred at concentrations of U below the HC_{50} (based on EC_{20} values) estimated in this study, i.e. below 303.2 mg U Kg^{-1}. Nevertheless, all the effects were recorded on soils contaminated with complex mixtures of metals, and the effects observed cannot be exclusively attributed to uranium. This was reinforced by Lourenço et al., (2011), which observed that earthworms from the species *E. andrei* exposed to the contaminated soil have significantly accumulated not only uranium, but all the metals analysed (Be, Al, Mn, Fe, Ni, Zn. Se, Sr, Cd, Ba and Pb) as well as radionuclides like ^{226}Ra, after 14 and 56 days of exposure. In summary and based on all of these evidences the HC_{50} EC_{20}-based for uranium of 303.2 mg U kg^{-1} (Table V.4), seemed to be the most appropriate cut-off to support the definition of a SSV for this metal. The intention is not to accept the impairment of these important processes for the biogeochemical cycles, as only a 20% of effect is accepted, but rather to prevent many false positives in the screening step of the risk assessment procedure. This estimation was also selected based on two main aspects: i) the uranium toxicity data fitted better to EC_{20} values (r^2=0.904) and, ii) the best 95% confidence intervals were obtained for both the HC_{50} EC_{20}-based and the HC_5 EC_{50}-based estimations (which span for a factor of about 4). However, more data should be collected for other species and endpoints to confirm the estimation of the SSV for U. While that data is not available the lower limit of the 95% confidence interval of the HC_{50} EC_{20}-based estimation (151 mg U kg^{-1}) is suggested as a SSV for uranium and for soils similar to the PTRS1.

As far as cadmium is considered, and following the above described rationale for selecting the HCp for this metal based on the statistical confidence of the estimate, the HC_{50} EC_{20}-based should be selected (Figure V.2 c; Table V.3). However, selecting a risk limit of 95.8 mg Cd kg^{-1} all the invertebrate species will be seriously under protected, as well as several microbial functions, as can be perceived from Table (V.I). Further, although

the EC_{20} data for cadmium fitted better to the log-normal distribution (r^2=0.777), the estimation of the HC_{50} EC_{20}-based did not show the best 95% confidence interval. In alternative we propose to select the HC_5 EC_{50}-based corresponding to a Cd concentration of 8.4 mg kg^{-1} of soil, since at this level a 50% inhibitory effect on reproduction was recorded only for enchytraeids. This HCp was also lower and upper bounded by the toxicity data used to generate the SSD cumulative distribution, in opposition to the HC_5 EC_{20}-based cut-off (Figure V.2 a; Table V.3). Comparing with field data and with data obtained for Portuguese soils, André et al., (2009) observed the impairment soil fauna feeding activity at a maximum Cd concentrations of 4.3 mg Cd kg^{-1}. Alvarenga et al., (2012) found effects in the growth of *Avena sativa* in a natural soil, coming from one mining area, with 3.38 mg Cd kg^{-1}. Effects in the activity of soil dehydrogenases and changes in potential nitrification were observed by Pereira et al., (2006), in soils from a mining area with concentrations of Cd equal to 2 and < 1 mg Cd kg^{-1} for dehydrogenase and < 1 mg Cd kg^{-1} for potential nitrification. Although from a mining area explored in the past for cupreous pyrites, the Cd concentrations recorded by these authors were always below 3 mg Cd kg^{-1}. In turn, Natal da Luz et al., (2004) observed that the invertebrates *E. andrei* and *F. candida* avoided soils with 0.01 and 0.07 mg Cd kg^{-1}, respectively, from the same abandoned mining area referred above. (Table V.3). Once again data obtained from Portuguese natural contaminated soils pointed for Cd toxicity, always occurring at concentrations lower than the HC_5 EC_{50}-based estimated, but once again these effects were observed on soils contaminated with cadmium mixed with several other metals. Hence, the lower limit of the 95% confidence interval of this cut-off corresponding to 5.6 mg Cd kg^1, seems to be appropriate as a trigger value for more site-specific assessments of soils similar to the PTRS1 (Table V.4). In this case we also considered better to be overprotective, since the mathematical model did not fitted so well to both EC_{20} and EC_{50} values.

Concerning to Cu, the HC_5 EC_{20}-based (Figure V.3a; Table V.3), can be immediately eliminated, since it could be an over-protective cut-off, because all the ecotoxicological data obtained for this metal (Caetano et al., (c, submitted)), (Table V.1) were above the concentration of 23.4 mg Cu kg^{-1}, and even the lowest EC_{20} obtained was 2.8 times higher

than the HC_5 EC_{20}-based. As far as the other HCp values are considered, the EC_{50} values for copper fitted better to the statistical model (Table V.3), and selecting the HC_{50} EC_{20}-based (145 mg Cu kg-1) we are accepting a 20% level of effect on some soil microbial parameters, invertebrates and plants, and in some cases, especially for plants, we are also accepting a 50% level of effect (Table V.1), since some toxicity data recorded were below this concentration.

The comparison of these values with data, on Portuguese natural soils, can help us to decide which value is wiser to propose. Alvarenga et al., (2012) observed the mortality of *E. andrei* exposed to a natural soil with 434 mg Cu kg^{-1}. The same invertebrate species avoided a mining soil, with 6.31 mg Cu kg^{-1} in a study carried by Natal da Luz et al., (2004). *F. candida* avoided a soil from the same area with 0.81 mg Cu kg^{-1} in the same study. As far as soil microbial processes are considered, Pereira et al., (2006) reported effects in activity of soil dehydrogenase in natural soils from a cupreous pyrite mining area with 30, 55 and 80 mg Cu kg^{-1}. Likewise, Antunes et al., (2011), verified a significant negative correlation between phosphatase activity and Cu levels in polymetallic mine soils with concentrations of copper ranging between 0.14 and 1.88 mg Cu Kg^{-1}. In summary, and once again, data available are from soils contaminated with a complex mixture of metals, and they support the choice of the HC_5 EC_{50}-based for being used as a SSV for copper, since although effects were sometimes recorded at very low concentrations of copper, this metal was not the sole element exerting toxic effects in the soils under evaluation. In this case, the selection of the lower limit of the 95% confidence interval was not necessary, since it was very close to the cut-off estimated. Thereby the proposed SSV for copper and for soils similar to the PTRS1 is 58.5 mg kg^{-1}. No toxicity data obtained by Caetano et al., (c, submitted)) fell below this concentration.

The SSVs proposed for each metal, are summarized in table V.4. All the values were above background values known for these metals. Regarding uranium background values of 6.1 and 7.8 mg U kg^{-1} were recorded by André et al., (2009) and Caetano et al., (2012), respectively, in reference soils, including the PTRS1. Similarly, lower background values for Cd were obtained by the same authors, which were usually below 1 mg Cd kg$^{-1}_{dw}$ in soils and also by Pereira et al. (2006) that found a background level of Cd of 2 mg kg^{-1} for

a natural reference soil. The background concentrations reported for Cu in Portuguese soils changed between 9 mg Cu kg^{-1} reported by Caetano et al., (2012), 18.6 mg Cu kg^{-1} reported by Inácio et al., (2008) and 30 mg kg^{-1} (Pereira et al., 2006), which are also lower than the cut-off selected for this metal.

The HCps selected in this study varied according to, the metal, from HC$_5$ EC$_{50}$ based (Cd and Cu) to HC$_{50}$ EC20 based (U). Although, some authors argue that the selection of cut-off should be a political decision, we have shown that in fact, we cannot always select the same cut-off, and that the decision has to be based on the statistical confidence of the data, a critical analysis of the main groups of organisms and functions affected, the level of effect considered admissible, and the comparative analysis with data from natural soils. Furthermore, the analysis of field or laboratorial data and of all the toxicity values produced to generate the SSDs could help us to prevent the selection of an extremely overprotective limit that will lead to false positives. Nevertheless, data available for Portugal related with natural soils is limited, and is also impossible to find a natural soil, with only one metal at environmental concerning concentrations. In the case, of the HCps selected in this study to derive SSV values we clearly assumed that a high level of effect (50%) can be accepted for only 5% of the species, while for a high percentage of species only 20% level of effect, should be accepted. This decision could be made in a more generic basis i.e. for all the soils independently of their use. We also consider these cut-offs and these argumentation will also be better accepted by policy makers, rather than presenting the extremely overprotective HC$_5$ EC$_{20}$ based cut-off. However, more toxicity data for other species and endpoints is clearly needed to increase the confidence in the estimation of the HCp value selected for uranium. As suggested by Boekhold, (2008) soil ecological parameters should also be integrated in SSDs. Endpoints like litter decomposition or changes in soil microbial structural and functional diversity, could be additional endpoints to be considered in the future, as well as data obtained from mesocosm studies. However, some of these parameters will make the application of the SSD-method more laborious and time consuming, limiting our ability to apply this method to a wide array of contaminants. It will also be important to discuss in the future, the use of toxicity data obtained for soil elutriates, tested with aquatic organisms. As far as Cd

SSV derived in this study is considered, new models can be tested to fit the data already available. Nevertheless, we can assume that the SSVs proposed in this study are conservative, since they were derived from spiked soils, where the ageing process did not occur. Hence they can be used safely.

The comparison of the SSVs derived in this study with previous SSVs based on PNECs (predicted no effect concentrations) estimations (Caetano et al., (a,b, c, submitted)), through the application of the assessment factor's method (Table V.4), (EC, 2003), shows that the PNEC values were several times lower than the SSVs estimated on this study, and almost similar, or even lower than the soil background concentrations. Therefore, although the AF method can in fact be used when almost no data exists about the toxicity of a given compound, efforts must be done for collecting more data as soon as possible, for replacing the trigger limits obtained by this method, as they will reduce the utility of the screening step of the risk assessment process. For this reason, and also considering that SSDs demands more and superior quality data, when the aim is the derivation of SSVs this approach must be definitely considered for this purpose (Wheeler et al., 2002). Further, and as it was perceived by the analysis of HC_5EC_{20} values made above, the approach recommended by the European Technical Guidance Document (EC, 2003) for the derivation of PNEC values, which considers the quotient between the lower limit of the 50% confidence interval of the 5th percentile of the SSD (based on NOEC values) and an assessment factor ranging between 1 and 5 (depending the overall quality of the data) it will be extremely overprotective, at least based on data generated in this study, and for the purpose of obtaining SSVs, to trigger more detailed evaluations of contaminated soils. Therefore a new approach is proposed and discussed in this study, also based on SSD distributions.

Table V. 3 HCp values obtained from SSD method for U, Cd and Cu using EC_{20} and EC_{50} values (mg.kg$^{-1}_{dw}$), 95% confidence intervals between brackets.

Metals	$HC_5 EC_{20}$	r^2	$HC_5 EC_{50}$	r^2	$HC_{50} EC_{20}$	r^2
Uranium	110.5 (45.93-266.02)	0.904	122.2 (61.79-242.00)	0.874	303.2 (151.44-606.99)	0.904
Cadmium	15.8 (5.08-43.34)	0.777	8.4 (5.59-12.76)	0.716	95.8 (41.03-223.89)	0.777
Copper	24.3 (7.42-75.58)	0.778	58.5 (58.57-101.78)	0.931	145.4 (52.5-403.12)	0.778
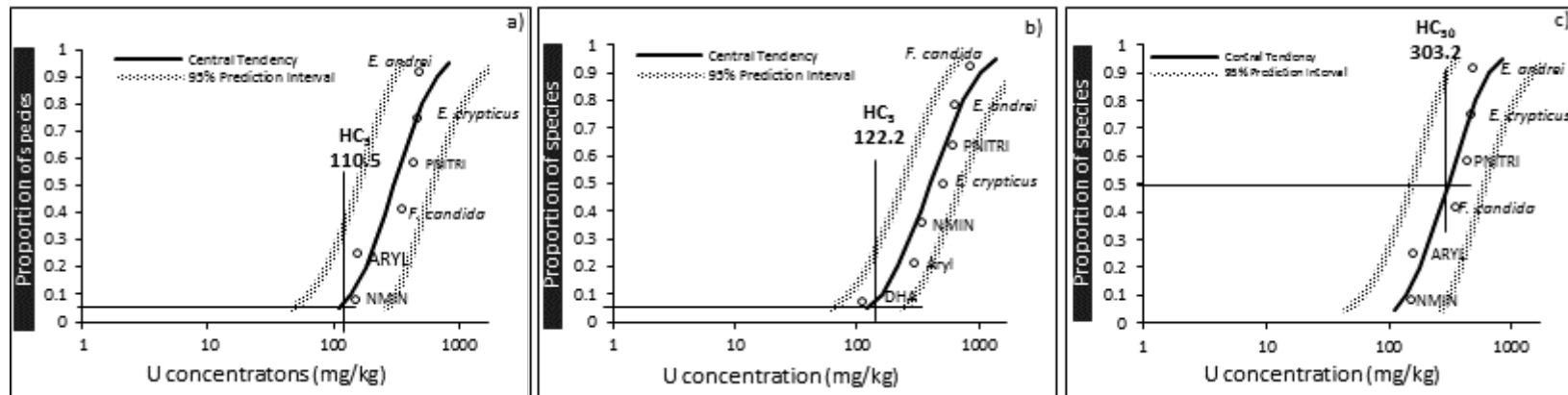
Figure V.1 SSDs for uranium based on EC_{20} values (a,c) and EC_{50} values (b) for different microbial processes, invertebrates and plants and used to estimate HC5 (a,b) and HC_{50}cut-offs.

Figure V.2 SSDs for cadmium based on EC_{20} values (a,c) and$_5$ EC_{50} values (b) for different microbial processes, invertebrates and plants and used to estimate HC5 (a,b) and HC_{50} HC_{50}cut-offs.

Figure V.3 SSDs for copper based on EC_{20} values (a,c) and$_5$ EC_{50} values (b) for different microbial processes, invertebrates and plants and used to estimate HC5 (a,b) and HC_{50}cut-offs.
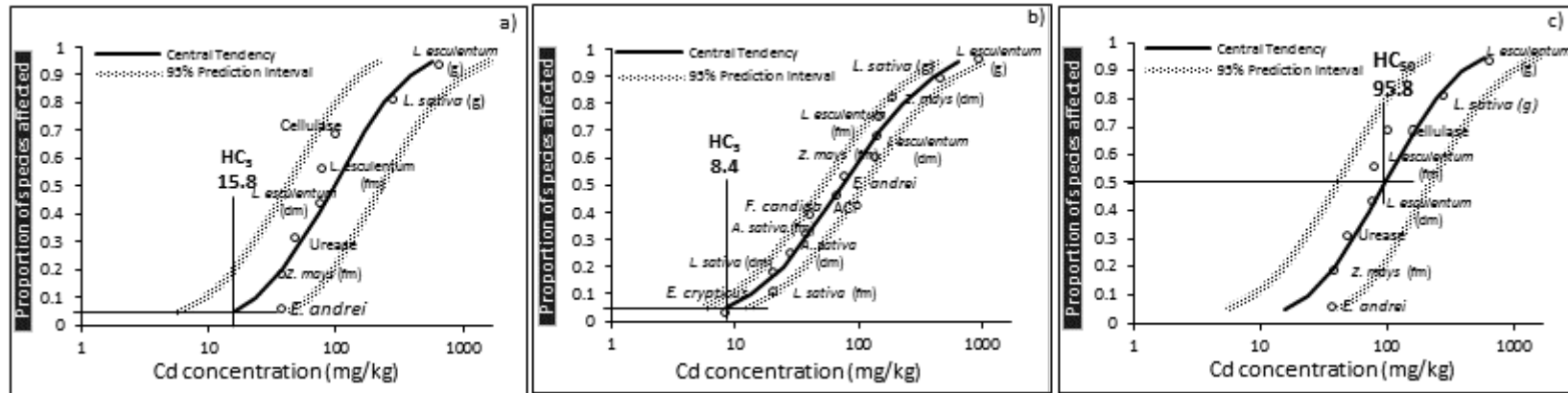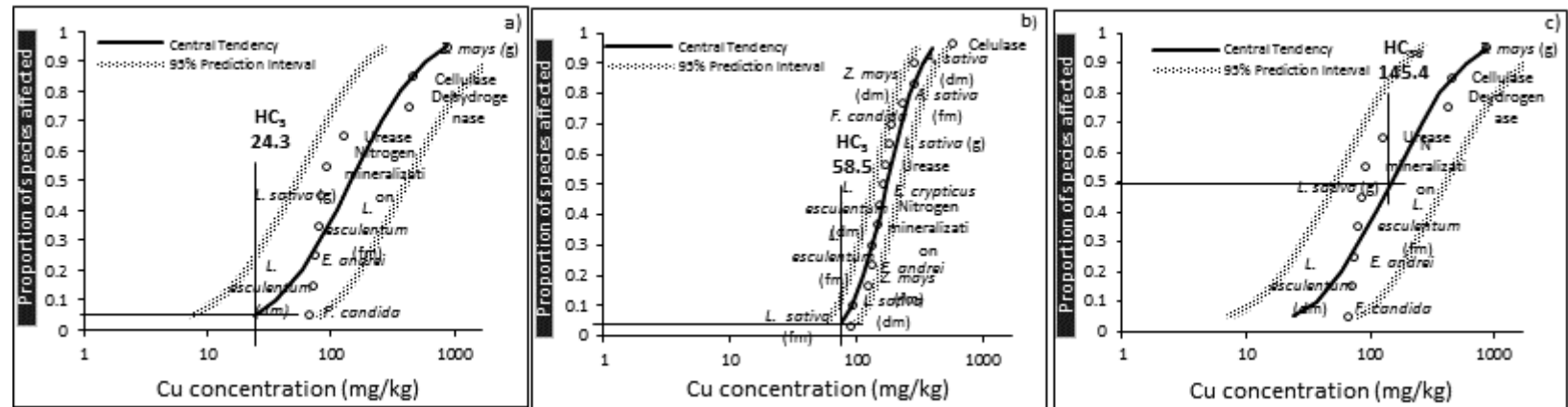
Table V.4 Summary table of SSV obtained for U, Cd and Cu using the SSD method and the AF approach.		
Method/ SSV (mg.kg^{-1}$_{dw}$)	SSD distribution method	Assessment Factor (AF)
Uranium	151.4	15.2
Cadmium	5.6	3.7
Copper	58.5	6.5

5.4 Comparison of Portuguese SSVs with other European and International values

The SSVs obtained in this study, for a given type of Portuguese natural soil, and for Cd, Cu and U, were compiled together with SSVs available for other European countries (e.g., Denmark, Germany, The Netherlands, Italy, France, Belgium, Poland, Finland, Austria and Sweeden) as well as with Soil Quality Guideline (SQG) Values for Canada and ECO-SSLs for USEPA. Table V.5. shows the great variation among SSVs available as well as of the criteria used in their definition (e.g. soil type, land use). An exhaustive analysis and discussion of the reasons underlying such variation was already made by Carlon and Swartjes, (2007) and Provoost et al., (2008), which include: i) the legal frameworks supporting the derivation of SSVs; ii) the scientific basis; iii) the transparency in the methodology applied; iv) revisions already made to previous derived SSVs; v) receptors included (human and/or non-human receptors); vi) the integration of economic and social factors; vii) toxicological/ecotoxicological data expressed in terms of total versus leaching/extractable concentrations; viii) background concentrations taken into account or not in the derivation of SSVs; ix) soil type, soil fraction and soil depth used; x) data sources; xi) normalization of ecotoxicological data for a standard soil; xii) the use of terrestrial versus aquatic toxicity data; xiii) application of SSDs *versus* AF methods; xiv) level of protection of the SSD application; xv) the probabilistic model selected; xvii) applied assessment factors etc. Acting together, and with different levels of influence all these factors determine the differences recorded in table V.5. The great variability in physical and chemical properties of test soils used for the derivation of SSVs, was probably one the meaningful factors responsible for such differences between countries. This was in fact confirmed by several studies, demonstrating the influence of soil properties in the

bioavailability of contaminants and hence in their capacity to promote different toxic effects on soils organisms (Criel et al., 2008; Domene et al., 2010; van Gestel et al., 2011). An aspect that reinforces the need of deriving such values based on different types of natural soils. The variation recorded in this study was greater for Cd, whose values varied by a factor of 33.3 (i.e. 0.3 and 10mg Cd kg^{-1}), while for Cu values varied by a factor of 6.6 (i.e. 30 and 200 mg Cu kg^{-1}). Moreover, as it is possible to verify, and to the best of our knowledge, any European country derived till now a SSV for U, probably because this metal was not perceived as a problem in their countries. Only SQG values are available for Canada, which is the world's leading producer of U (for more details please see: http://www.nrcan.gc.ca/energy/uranium-nuclear/7693). In Portugal, although the exploration of this metal finished, and the most concerning uranium mines are already suffering remediation works, several other abandoned mines require a risk evaluation (Pereira et al., 2014), for which the SSV derived in this study will be of crucial importance. In all the cases, our values fell within the ranges of available values, except for U, since our SSV for U was about 4.5 times higher than the Canadian Soil Quality Guideline proposed for both, the protection of the environment and human health (Table V.5). Canada develops their guideline values based on land use, assuming that lands don't need the same level of protection (e.g. an industrial soil use does not require the same level of protection as an agricultural use), (CCME, 1997). Nevertheless, Canadian values are usually determined for human health and the environment, and usually the most protective value is selected as the generic SQG, and this was the case of U, since the values for human health were lower than the environmental values, as showed by table V.5. However, one environmental SQG values (the SQG$_{Environ}$ based on residential parkland soil) was significantly higher than the SSVs proposed in this study, something that is apparently contradictory. The SSV for uranium derived in our study is similar to the PNEC value derived by Sheppard and Sheppard, (2005) of 100 mg U kg^{-1}, (Table V.5), which is based on data obtained for several invertebrate and plant species, and soils, but no soil microbial data was included. However, as it was possible to perceive from table V.1 the inclusion of data from the soil microbial community is of particular importance, since it has shown to be more sensitive than invertebrates and plants, to this metal. Although

apparently less protective, our SSV has taken into account soil microbial data, accepting a 20% level of effect, but in spiked soils where the soil microbial community was exposed to the uranyl ion which is one the most soluble and toxic forms of uranium (ASTDR, 2011). Hence once again, we considered that the SSV proposed is conservative value.

As far as Cd and Cu SSVs are considered, and has previously mentioned our values fell within the range of available values for other countries, and this is really demonstrative how problematic could be the selection of SSVs from other countries, when a country does not have their own values to be used in risk assessment procedures. Clearly an over or an underestimation of risks can occur depending on the choice. Within this scenario several authors are discussing for several years the economic implications and the inequalities generated by the existence of different SSVs between countries (Provoost et al., 2008; Sauvé et al., 1996)

The costs of risk assessment processes and of cleaning up contaminated areas have a significant impact in national economies; hence it is important to have SSVs that can discriminate soils that really require a deeper evaluation, from those that are relatively safe. But using Portugal as an example, Canadian Soil Quality Guidelines have been recommended by public authorities for being used (Inácio et al., 2008). For two, out of three, of the metals analysed in this study (U and Cd) Canadian values are highly conservative comparing to our SSVs, and then if used in the evaluation of national scenarios will likely conduct to an overestimation of risks, especially for those areas located far away from human populations, but that cannot be neglected because of that, and kept without any evaluation. In this case the costs of using such values seemed to be clearly high than those of using SSVs derive based on similar types of natural soils, even if our SSVs are in some cases more conservative than other's available for other countries (like the case Cu or even Cd, when compared with USEPA Eco-SSLs and other SSVs from other countries) (Table V.5). This is true, especially if our SSVs are derived following the rationale described in this study aimed in balancing the protection of species and soil functions, with the attempt to reduce the occurrence of false positives, which also contribute for increasing the costs of the risk assessment of contaminated lands.

Is almost generally accepted that soil quality standards should be derived based on the utilization of natural soils (Provoost et al., 2008), the discussion now is if we should harmonize the procedures between European countries. As demonstrated in our study such harmonization is possible, at least to a certain degree, especially when software models are available to help in the application of the SSD-method and in the derivation of the HCp values. Some degree of variability should be accepted in the selection of the HCp values, however the rational followed should be clearly explained.

Table V.5 SSVs derived for Portugal, Canadian soil quality guideline values, soil guideline values for different European countries, ECO-Soil Screening Levels from USEPA and other soil threshold values for metals available in the literature (mg/Kg$_{dw}$).

	Portugal – Natural soil	Portugal – Agricultural soils (SQGV[a])	Residential parkland soil (SQGV[a])	SQG$_E$[b]	Denmark[e]	Germany[f]	The Netherlands[g]	Italy[h]	France[i]	Belgium[j]	Poland[k]	Finland[l]	Austria[m]	Sweeden[n]	Eco-SSL USEPA[o] Plants	Eco-SSL USEPA[o] Invertebrates	Other References
U	151.4	23	23	33[c] - 500[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	100[p]
Cd	8.4	1.4	10	3.8[c] -10[d]	0.3	1.5[f1] - 1.0[f2]	0.8	2	20	2	1	10	1	0.4	32	140	6.7[q]
Cu	58.8	63	63	63[c,d]	30	100[f1] - 60[f2]	36	120	95	200	30	150	100	100	70	80	55[q]

NA—Not Available; [a] Canadian Soil Quality Guidelines Values for the Protection of Environmental and Human Health; [b] SQGE soil quality guideline for environmental health; [c] based in agricultural soil; [d] based in residential parkland soil (CCME, 2007 (U), 1999 (Cd), 1999 (Cu)); [e] Ecotoxicological soil quality criteria (Scott-Fordsmand and Pedersen, 1995 *in* Carlon, 2007); [f] precaution values for clay soil (f1) and loam soil (f2) (Carlon, 2007); [g] Dutch target values (Carlon, 2007); [h] Limit values for surface soils in residential public green use (Carlon, 2007); i -VDSS - valeur de definition de source sol (Carlon, 2007); [j] SSVs for a special area, green area with high biological value, areas for the protection of groundwater, agricultural areas (Carlon, 2007); [k] guideline values for soil in protected areas (Carlon, 2007); [l] Lower guideline value based on ecotoxicological risks (Carlon, 2007); [mG]uideline values triggering more evaluation for soil used for agricultural and garden purposes and non-agricultural soils (carlon, 2007); [n] Guideline values for contaminated soils of sensitive use (Carlon, 2007); [o] USEPA Values established for soil invertebrates (http://www.epa.gov/ecotox/ecossl/); [p] PNEC$_{values}$ (Sheppard and Sheppard, 2005); [q] HC$_5$ -EC$_{50 \text{ based}}$ values (Jänsch et al. 2007).

5.5 Conclusion

Based in the SSD-method, soil screening values for uranium, cadmium and copper, were derived in this study for Portuguese soils similar to the PTRS1. The SSVs derived were based on EC_{20} or EC_{50} and HCp values (HC_5 or HC_{50}) were selected taking into account: i) the fit of the statistical model used to the toxicity data used (r^2); ii) the best 95% confidence intervals obtained for HCps; iii) the existence of toxicity data on the left part of the SSD curve, iv) a comparative analysis with field and laboratorial data available for contaminated Portuguese natural soils. The SSVs derived in this study are proposed for a generic use, since in the author's opinion, at this screening step, aimed in selecting sites requiring a more site-specific risk evaluation, such decision must be based only on the degree of contamination. Future land uses should be later considered, by local authorities, supported by scientific expertise of risk assessors, in the definition of the level of risk that can be considered acceptable or not. In fact, this strategy is already followed by the decision support system of the Dutch risk assessment framework (Jensen and Mesman, 2006b). This work has shown that the harmonization of procedures to derive SSVs between European countries it is possible, especially when software tools are available to obtain SSD curves, but some variability should be accepted in the selection of the cut-offs to support the derivation of SSVs. A role of thumb could be to keep the process transparent but as simplest as possible.

5.6 References

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Chapter VI

Final remarks

Final remarks

After the publication of Rachel's Carson book "Silent Spring" in 1962, the general public, scientists and politicians were awakened for the risks posed to soil ecosystems by the intentional use of chemicals (pesticides) to increase crop production (van Gestel, 2012). Forty years later, more threats to soils were identified and only in 2006, the European Commission published the final version of the Soil Thematic Strategy for Soil Protection (CEC, 2006a). However, till now the EU Union still lacks appropriate legal documents to enforce an adequate soil management, in order to prevent a further degradation of this environmental compartment and of the services provided. Meanwhile, several European countries, with a high economic development and with a great awareness of the problems caused by their past and actual industrial and agricultural activities, started the development of their own soil protection policies, as well as the development of guidelines and frameworks for soil monitoring and assessment. In Portugal, some actions were taken only related with most concerning abandoned mine areas, mainly enforced by the public opinion, manifested after the perception of risks posed to human health, by the exposure to wastes (mainly radiological) left in these areas. Nevertheless, any attempt was made to develop national legislation related with soil protection and with the evaluation and clean-up of contaminated areas. Concerns with soils have been integrated only in other sectoral policies (e.g. agriculture, forestry) and national programs like the one related with desertification combat (Rosas et al., 2009). To the best of our knowledge, any survey was made to get information about the extent of soil degradation or about the number of contaminated sites within the national territory, or at least this information is not public.

Due the expanding perception of soil degradation and of the resulting economic and environmental consequences and the recognized need to recover the extensive area of degraded soils for new land-uses, the Soil Thematic Strategy is being applied and new European policies are emerging (like the Soil Framework Directive that stills in debate in the Council and European Parliament[3]) focusing soil's protection and the management of contaminated sites. In order to be prepared for accomplishing the objectives proposed,

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[3] http://europa.eu/rapid/press-release_IP-12-128_en.htm?locale=en, on-line available: March 2014.

European member states must develop and validate their own tools and frameworks for assessing soil contamination, in a conservative and cost effective manner. Such evaluation has to include both human health and ecological risks, if they are made for mitigating the risks to humans, but also for recovering soils for new land-uses. Portugal is one of the European member states that still lack soil quality guideline values, like for example soil screening values (SSVs) which are crucial for screening evaluations of soil's contamination. These values, if supported by sound scientific information collected for Portuguese natural soils, if representative of the dominant soils existing within the national territory and if derived based on expert judgment, could be adequately balanced to protect ecosystems, but also to make a safe preliminary evaluation of contaminated areas. Such evaluations may reduce the number of sites requiring a deeper and a more expensive risk assessment evaluation. For this purpose Portugal may take benefit from all the experience gained by countries from the North of Europe, like Germany and The Netherlands, with a great experience in environmental risk assessment (ERA) of contaminated lands. Therefore it may start producing data for the derivation of their own SSVs and for the adaptation of existing ERA frameworks to different types of contamination and for different climatic regions. In this context, this work appeared as a first attempt to derive SSVs for metals, using a Portuguese natural reference soil, representative of a dominant type of soil in the territory (Caetano et al., 2012). A set of ecotoxicological data for soil microbial parameters, invertebrates and terrestrial plants was obtained for developing SSVs for U, Cd and Cu. The selection of these metals was justified, but as far as uranium was considered, any other country has made an attempt to obtain a soil guideline value for this metal and it assumes a special regional importance, since in Portugal, during the 20th century, the exploration of uranium containing ore, caused serious environmental impacts that still persist nowadays (Carvalho et al., 2005).

Chapter II, III, IV, included a description of all the assays performed with soils spiked with U, Cu and Cd containing solutions, following standard protocols and well known protocols for microbial parameters.

The results from chapter II, showed clear inhibitory effects of U in almost all tested invertebrate species. The microbial parameters tested were also clearly inhibited by U, being the most sensitive parameters to this metal, comparatively to the remaining effect concentrations obtained for invertebrates and plants. This study gathers for the first time more extensive data regarding the ecotoxicity of spiked soils with U on soil microbial functional parameters. The results also revealed that the exposure to U in spiked soils inhibited the reproduction of invertebrates, namely of *E. crypticus,* which was the most sensitive species to this metal in soil. In opposition, plants reveled to be the less sensitive species, since except for *Lactuca sativa* any other species was affected by concentrations of uranium up to 1000 mg/kg$_{dw.}$ Due to the lower sensitivity of plants, less toxicity data (EC$_{20}$ and EC$_{50}$) was obtained and used for constructing a species sensitivity distribution (SSD) for this metal (Chapter V). Although the data available fitted well to the log-logistic models more data should be obtained in the future, especially for other plant species and soil microbial parameters, aimed in improving the robustness of the HCps estimated. Nevertheless, the SSV derived can be safely used, since we consider that it is a conservative risk limit, because of the following aspects: i) it was obtained for a natural soil, hence taken into account the role of soil physical and chemical properties in the availability of uranium, in real scenarios of contamination; ii) it was obtained from ecotoxicological data generated with soils spiked in laboratory, and hence with an overestimated bioavailability and, iii) it was estimated based on ecotoxicological data obtained for the uranyl ion (UO$_3^-$) (ASTDR, 2011), which is one of the most toxic forms of uranium, but is not the only form of uranium in contaminated soils.

In the Chapter III, the high toxicity of Cd for the great majority of soil organisms was confirmed. Some of microbial parameters tested reveled a great sensitivity to the contaminated soil, whereas others proved to be less affected. As far as the soil enzymes activity are considered, we hypothesized that the results obtained (which pointed out for less sensitivity of this parameter, despite the high toxicity of cadmium) can be related to the exposure time (one month) selected in our studies. Structural changes in the soil microbial community may have masked or compensated the effects of Cd on these functional microbial parameters. In our opinion, although soil microbial enzymes have

been shown to be quite responsive to soil contamination with metals (Lee et al., 2009; Thavamanier al., 2012; Zeng et al., 2011), and although well established and quite simple protocols are available it is urgent to perceive which is the most appropriate exposure period for these assays. On one hand, low periods of exposure may overestimate the effects, on the other hand high exposure periods may allow the soil microbial community to accommodate the impacts, replacing more sensitive *taxa* while keeping the same functions. In parallel, and to reduce potential over or underestimations of SSVs, caused by soil microbial parameters, endpoints related with soil microbial structural diversity should also be integrated in the ecotoxicological data set required. Concerning to invertebrates, the production of juveniles was strongly constrained by Cd for all the tested species, even at the lowest concentrations tested. Regarding the plants, the effects of Cd were also evident, and resulted essentially in a significant growth inhibition of all the species tested. Seed germination was only slightly affected. *L. sativa* was the most sensitive plant species tested for all the endpoints.

As above mentioned Chapter IV reports all the ecotoxicological data obtained for Cu using PTRS1 as a test substrate. A significant inhibition in the activity of some soil enzymes and in nitrogen mineralization was verified. The reproduction of invertebrates was also constrained by Cu, namely in *E. crypticus.* Plant seed's germination proved once again to be few sensitive to copper like it was for Cd and U. In opposition, plant's growth was strongly inhibited by Cu, whit evident effects in both fresh and dry mass.

For all the metals, the influence of PTRS1 physical and chemical properties in the availability of metals was discussed in comparison with other studies using different types of soil. The effects observed were attributed only to the test metals, since no effects of the intrinsic soils properties on the test species were expected based on Caetano et al. (2012).

Finally, in chapter V, describes the derivation of SSVs for U, Cd and Cu based in all the ecotoxicological data obtained in Chapter II, III and IV, using the SSD-method. Thus, we propose for uranium a SSV of 154.1 mg Cu kg^{-1}_{dw}, for cadmium mg Cu kg^{-1}_{dw} and for copper 58.5 mg Cu kg^{-1}_{dw}. The soil screening values proposed in this study are for a generic use, but should be based on soil type, similarly to the precautionary values from

Germany (Carlon, 2007). Two of the SSVs (U and Cd) derived in this study were higher than the Canadian Soil Quality Guideline Values, which are the guideline values that have been recommended for use in Portugal (Inácio, et al., 2008)This finding triggered the discussion about the economic impacts of using SSVs from other countries, which will conduct to false positives and hence in higher number of areas requiring more site specific evaluations. This is also a good argument for the derivation of our SSVs based on national dominant soil types.

This study has also proved that the harmonization of procedures is possible if we take benefits from the methodologies already available. It also laid the foundation for the discussion about the best cut-off (from SSDs) to be selected for deriving SSVs. Further, a new approach is proposed to replace the one presented by the European Technical Guidance Document (EC, 2003) for the determination of PNEC values for the soil compartment, as in the author's opinion these values will be extremely conservative reducing the importance of the screening step of the ERA process. In opposition to what has been proposed by other author's the selection of the best cut-off should be not made, only based on political decisions, but also based on an expertise judgment which should be clearly explained to the other parties involved. Although with regional importance, the approach followed in this work could be useful for other European Member states that still lack the definition of soil quality guideline values.

6.2 Future perspectives

Even though the great effort in developing SSVs for three metals, unfortunately this work was only the first step in the derivation of these values for environmental risk assessment in Portugal. Nevertheless, this study and the work previous published by Caetano et al. (2012) has made clear which is the best approach to be followed for other metals and organic contaminants. Future work should be focused in characterizing at least more two natural soils, as reference soils, and use them as test substrates for the derivation of SSVs. The map of Portuguese soils[4] already available could be used for this purpose, and the reference soils tested by Chelinho et al. (2011), from the south of country may also be an

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[4] http://www.igeo.pt/atlas/cap1/Cap1d_6.html

alternative. Moreover, these values must validated in the future, through their application in different national soils of the same type, both contaminated and non-contaminated, to check for their discrimination power.

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Annex

Annex

Table presenting the general physical and chemical properties of PTRS1 soil.

| | pH (KCL, 1M) | pH (H$_2$O) | Conductivity mS cm^{-1} | OM (%) | WHC (%) | Size of particles/mm | | | |
						Clay (< 4mm)	Silt (4-6 mm)	Sand (63 mm- 2mm)	Gravel (>2mm)
PTRS1	4.31 ± 0.02	5.91 ± 0.1	4.86 ± 0.23	6.5 ± 0.004	23.9 ±1.84	3.32	22.87	46.99	23.99

OM- organic matter; WHC- water retention capacity.