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Ecotoxicological bioassays as complementary tools for the risk assessment of contaminated soils

Jaume Bori Dols

ECOTOXICOLOGICAL BIOASSAYS AS COMPLEMENTARY TOOLS FOR THE RISK ASSESSMENT OF CONTAMINATED SOILS

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Jaume Bori Dols

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ECOTOXICOLOGICAL BIOASSAYS AS COMPLEMENTARY TOOLS FOR THE RISK ASSESSMENT OF CONTAMINATED SOILS

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Jaume Bori Dols

Centre de Recerca i Innovació en Toxicologia (CRIT)

Innotex Center, Universitat Politècnica de Catalunya (UPC)

Thesis Director

Ph.D. Maria Carme Riva - UPC Research Director

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“No heretem la Terra dels nostres avantpassats, l'agafem prestada dels nostres fills”

Proverbi natiu americà

Resum

Els impactes ambientals associats a l'imparable creixement de la població humana estan amenaçant els ecosistemes de tot el món. D'entre ells, els ecosistemes terrestres es veuen cada vegada més degradats degut a un ús i administració insostenibles, els quals estan conduint a la pèrdua d'un recurs fonamental per a la vida al planeta. Com a interfase entre la terra, l'aire i l'aigua, els ecosistemes terrestres realitzen multitud de funcions culturals, econòmiques, ambientals i socials que cal protegir. A tal efecte, durant les últimes dècades s'han creat diverses eines legislatives enfocades a assegurar la protecció dels sòls, les quals han tingut un èxit variable. No obstant això, els sòls són un dels principals destins dels contaminants antropogènics i, en conseqüència, la contaminació induïda per l'home encara representa una seriosa amenaça per als ecosistemes terrestres a causa de l'alliberament massiu de metalls, hidrocarburs i plaguicides (entre d'altres). En aquest context, l'aplicació de metodologies adequades per a l'avaluació i remediació de sòls contaminats ha esdevingut obligatòria si es vol preservar la seva capacitat per a desenvolupar les seves funcions. Tradicionalment els riscs associats a la contaminació del sòl han estat avaluats a través de quantificacions químiques de contaminants. Malauradament, aquestes tècniques han demostrat ser insuficients per a una adequada valoració de la contaminació del sòl ja que només poden centrar-se en les concentracions de contaminants específics i obvien les interaccions entre contaminants, la matriu del sòl i els organismes que l'habiten. D'altra banda, els bioassajos d'ecotoxicitat integren totes aquestes interaccions i poden esdevenir eines valuoses per a una avaluació millor i més realista dels efectes dels contaminants en els ecosistemes terrestres. En aquest treball s'han aplicat anàlisis químics conjuntament amb bioassajos d'ecotoxicitat terrestre i aquàtica a fi d'avaluar els riscs ecològics associats a mostres d'emplaçaments contaminats i a sòls contaminats artificialment. La idoneïtat dels diferents tests d'ecotoxicitat s'ha avaluat d'acord a la naturalesa del contaminant del sòl i s'han analitzat els paràmetres responsables de la toxicitat vers els organismes. Els bioassajos seleccionats inclouen mesures de diferents paràmetres (mortalitat, creixement reduït, etc.), temps d'exposició (aguda o crònica), respostes efectives (letal o subletal), i organismes (cucs de terra, col·lèmbols, plantes, bacteris, algues, dàfnids i peixos). Aquest estudi demostra que l'aplicació de bioassajos d'ecotoxicitat no només és útil sinó desitjable com a eina complementaria per a una avaluació fidedigna dels sòls contaminats.

En el *Capítol 1* s'introdueix breument la problemàtica de la contaminació del sòl, els seus principals contaminants i els mètodes disponibles per a l'avaluació del risc associats als sòls. També es presenten la hipòtesi d'aquest treball i els seus principals objectius. Finalment es resumeix la metodologia aplicada durant la realització d'aquesta tesi.

En el *Capítol 2* s'estudia l'amenaça ambiental que representen els sòls situats al voltant d'una mina de mercuri abandonada a la Vall del Azogue (Almeria, Espanya).

Al *Capítol 3* s'avaluen els riscs associats a una zona minera de F-Ba-Pb-Zn abandonada a l'àrea d'Osor (Girona, Espanya).

Al *Capítol 4* s'avaluen els impactes ecològics dels sòls del districte miner de mercuri abandonat a Almadén (Ciudad Real, Espanya).

En el *Capítol 5* s'avalua el procés de remediació d'un sòl contaminat amb hidrocarburs a través d'assajos d'ecotoxicitat i anàlisis químics i se n'estudia la seva idoneïtat com a eines de monitoratge de la degradació d'hidrocarburs.

En el *Capítol 6* s'estudien els riscs que representen per als ambients terrestres i aquàtics les dosis d'aplicació d'imidacloprid (formulació comercial Confidor®), un insecticida fins fa poc aplicat massivament.

El *Capítol 7* presenta un procediment alternatiu per a testejar la resposta conductual dels col·lèmbols *Folsomia candida* en assajos d'allunyament.

El *Capítol 8* inclou la informació més rellevant i presenta les principals conclusions d'aquesta tesi.

Abstract

Environmental impacts associated to the unstoppable growth of human population are threatening ecosystems worldwide. Among them, soil ecosystems are becoming increasingly degraded due to their unsustainable use and management, which is leading to the loss of a key resource that is fundamental to life on the planet. As the interface between land, air and water, soil ecosystems perform many cultural, economic, environmental, and social functions that are worthy of protection. During the last decades, several legislative tools have been created with varying success aiming to ensure soil protection. Even so, soils are major sinks of anthropogenic pollutants and, in consequence, human-induced contamination still represents a serious threat for soil ecosystems due to the massive release of metals, hydrocarbons and pesticides (among others). In this context, the application of methodologies for the proper assessment and remediation of contaminated soils has become mandatory if their ability to perform their functions is to be preserved. The risks associated to soil contamination have been traditionally evaluated through chemical quantification of pollutants. Unfortunately, such techniques have proven insufficient to properly assess soil pollution because they can only focus on concentrations of specific contaminants and they obviate the interactions between pollutants, soil matrix and soil inhabiting organisms. Ecotoxicity bioassays, on the other hand, do integrate all these interactions and can become very valuable tools for a better and more realistic assessment of the effects of contaminants in soil ecosystems. In this work, chemical analysis together with terrestrial and aquatic ecotoxicity bioassays are applied to samples from contaminated sites and to artificially-contaminated soils in order to evaluate their associated ecological risks. The suitability of different ecotoxicity tests is assessed according to the nature of the soil contaminant, and the parameters responsible of the toxicity to organisms are analyzed. The selected bioassays include measurements on different endpoints (mortality, reduced growth, etc.), exposure times (acute or chronic), effective responses (lethal or sublethal), and organisms (earthworms, collembolans, plants, bacteria, algae, daphnids and fishes). This study proves that the application of ecotoxicity bioassays is not only useful but also desirable as a complementary tool for a reliable assessment of contaminated soils.

In *Chapter 1*, the problem of soil contamination, the main soil pollutants and the available tools for soil risk assessment are briefly introduced. The hypothesis of this work and its main objectives are also presented. Finally, the methodology applied during the performance of this work is summarized.

In *Chapter 2*, the environmental threats of soils surrounding and abandoned mercury mine in Valle del Azogue (Almería, Spain) are studied.

Chapter 3 assesses the risks associated to an abandoned F-Ba-Pb-Zn mining area in Osor (Girona, Spain).

Chapter 4 evaluates the ecological impacts of soils from the abandoned mercury mining district of Almadén (Ciudad Real, Spain).

In *Chapter 5*, the remediation procedure of a hydrocarbon-contaminated soil is assessed through ecotoxicity tests and chemical analysis and their suitability as monitoring tools of hydrocarbon degradation is studied.

In *Chapter 6*, the risks that field doses of the (until recently) massively-applied insecticide imidacloprid (commercial formulation Confidor®) pose for the terrestrial and aquatic compartments are studied.

Chapter 7 presents an alternative procedure to test the behavioral response of collembolans *Folsomia candida* in avoidance tests.

Chapter 8 includes the most relevant information and presents the main conclusions of the thesis.

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Finalment sembla que arriba la fi d'aquesta etapa que tant m'ha aportat i exigit. Vull aprofitar aquestes línies per dedicar unes paraules a totes aquelles persones i entitats que d'una manera o altra han participat i ajudat al desenvolupament d'aquesta tesi, tant directament com indirectament.

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Abbreviations

AAS: Atomic Absorption Spectroscopy

AC50: Half maximal avoidance concentration

BTEX: Benzene, toluene, ethylbenzene, and xylenes

CEC: Cation Exchange Capacity

CHC: Chlorinated Hydrocarbon

dw: dry weight

EC: Electrical Conductivity

EC50: Half maximal effective concentration

EDS: Energy Dispersive X-Ray Spectroscopy

EU: European Union

FTIR: Fourier Transform Infrared Spectroscopy

GC-FID: Gas Chromatography - Flame Ionization Detector

Hg-TDC: Hg Thermo Desorption Curves

HPLC-MS: High Performance Liquid Chromatography - Mass Spectrometry

IC50: Half maximal inhibitory concentration

ICP-AES: Inductively Coupled Plasma - Atomic Emission Spectrometry

ICP-MS: Inductively Coupled Plasma - Mass Spectrometry

ICP-OES: Inductively Coupled Plasma - Emission Spectroscopy

INAA: Instrumental Neutron Activation Analysis

LC50: Half maximal lethal concentration

LOEC: Lowest Observed Effect Concentration

LOI: Loss On Ignition

NOEC: No Observed Effect Concentration

PAHs: Polycyclic Aromatic Hydrocarbons

PCBs: Polychlorinated Biphenyls

SEM: Scanning Electron Microscopy

SOM: Soil Organic Matter

SPTD: Solid-phase-Hg-Thermo-Desorption

TD-ICP: Total Digestion - Induced Coupled Plasma

TOC: Total Organic Carbon

TPHs: Total Petroleum Hydrocarbons

WHC: Water Holding Capacity

XRD: X-ray diffraction

CHAPTER I. Introduction, hypothesis, objectives and methodology

1.1. Introduction

1.1.1. Soil functions and state

Soil is defined as the top layer of the Earth's crust and is composed by varying proportions of mineral particles, organic matter, water, air and living organisms. Soil-forming processes tend to be slow and occur over long periods of time (typical rates of soil formation are in the order of only 1-2 cm per 100 years). Compared to the lifespan of human beings, soil loss is not recoverable which means that soil must be regarded as a non-renewable resource (JRC 2012). As a interface between earth (lithosphere), air (atmosphere) and water (hydrosphere), soil (pedosphere) performs multitude of key cultural, economic, environmental, and social functions that are worthy of protection (EC 2002b):

a) *Food and other biomass production*. Almost all agriculture production needs soil to provide water and nutrients as well as for roots fixation.
b) *Storing, filtering and transformation*. Soil stores and partially transforms minerals, organic matter, water, energy, and diverse chemical substances. It functions as a natural filter for groundwater and releases CO_2, methane and other gases in the atmosphere.
c) *Habitat and gene pool*. Soil is the habitat for a huge amount and variety of organisms living in and on the soil, all with unique gene patterns. It therefore performs essential ecological functions.
d) *Physical and cultural environment for mankind*. Soil is the platform for human activity and is also an element of landscape and cultural heritage.
e) *Source of raw materials*. Soils provide raw materials such as clay, sands, minerals and peat.

Although mentioned separately, soil functions are interdependent. Competition between them occurs when the ability of a soil to develop those functions is reduced or compromised, thus leading to a threat in the sustainability of the soil (EC 2002b). Soil threats are complex and frequently inter-linked. When occurring simultaneously, effects tend to increase and lead to soil degradation when it has lost the capacity to carry out its functions.

The degradation of soil functions can sometimes be appreciated at land surface (e.g. poor crop yields). However, evidences of low soil functionality frequently need to be collected through field sampling and laboratory analysis (JRC 2012). Even more, the buffering capacity of a soil, its capability to filter and absorb contaminants and its resilience usually hidden soil damage until it is far advanced (EEA 2000b). After many years of misuse, warning signs are nowadays appearing more clearly both locally (e.g. soil contamination in cities) and regionally (e.g. loss of agricultural productivity).

The unsustainable use and management of land is leading to increased soil degradation and to the loss of a key resource that is fundamental to life on the planet. In the European Union, an estimated 52 million hectares of land, representing more than 16% of the total land area, are affected by some kind of degradation process (EC 2002b). Globally, nearly 2 billion hectares of land are affected by human-induced degradation of soils (UN 2000).

Nowadays, the unstoppable increase of human population is requiring an even greater intensification of agriculture that at the same time is jeopardizing the capacity of soils to release and absorb nutrients and chemicals. Besides, continuous expansion of built-up areas and infrastructure, particularly in large urban agglomerations, is sealing off the soil from productive uses. Each year, an additional 20 million hectares of agricultural land become too degraded for crop production or are lost to urban sprawl (EEA 2000b). In the years to come, soil sustainable use and management will be a great challenge to both users and policy-makers in order to preserve its long-term availability and viability.

1.1.2. Soil protection policies

Despite its importance and unlike air and water, soil protection is not specifically targeted by any EU legislation. However, different EU policies for water, wastes, chemicals, industrial pollution, nature protection, pesticides and agriculture indirectly contribute to soil protection. Unfortunately, these policies have other aims and they are not sufficient to ensure an adequate level of protection for all European soils. Additionally, the prevention of soil degradation is seriously limited by the scarcity of data (JRC 2012).

Aiming to build political commitment to soil protection in the coming years, the European Commission published in the year 2002 the communication 'Towards a Thematic Strategy for Soil Protection'. This communication outlined the first steps for the development of a Thematic Strategy to protect soils in the European Union from several threats (EC 2002b):

- *Erosion.* Erosion is a naturally-occurring phenomenon consisting in the removal of soil particles by water or wind. However, human activities are known to increase erosion rates.
- *Decline in organic matter.* Soil organic matter assures the binding and buffering capacity of soil, thus contributing to limit the diffusion of pollution from soil to water and air.
- *Sealing*. When land is sealed through the construction of buildings, roads or other land developments, it losses the capacity of rainwater absorption and filtering.
- *Compaction.* Compaction occurs when soil is subjected to mechanical pressure through the use of heavy machinery or overgrazing, reducing the pore space within the soil and therefore losing absorptive capacity.
- *Decline in biodiversity.* Soil organisms play an essential role in maintaining the physical and biochemical properties needed for soil fertility.
- *Salinization.* Is the result of the accumulation of soluble salts in soils to the extent that soil fertility is severely reduced.
- *Landslides*. Can be triggered by factors such as land abandonment and land use change.
- *Contamination.* The introduction of contaminants in the soil above certain levels may result in negative consequences for all types of ecosystems and organisms including humans. Although

there has been a reduction in emissions and use of some hazardous substances due to application of policy measures, they are countered by a general increase in economic activity.

The approval in the year 2004 of the Directive 2004/35/EC on environmental liability with regard to the prevention and remedying of environmental damage represented the first attempt to incorporate soil protection to European legislation. Two years later, an amendment of this directive established a framework for the protection of soils across the European Union. Under the overall objective of soil protection and sustainable use, the framework was based on the following guiding principles (COM 2006b):

1) Preventing further soil degradation and preserving its functions.
2) Restoring degraded soils to a level of functionality consistent at least with current and intended use, thus also considering the cost implications of the restoration of soil.

At the Environment Council celebrated in March 2010, a minority of Member States blocked further progress of the Soil Framework Directive on grounds of subsidiarity, excessive costs and administrative burden. Taking note that the proposal had been pending for almost eight years without a qualified majority in the Council in its favor, the Commission on 30 April 2014 took the decision to withdraw the proposal for a Soil Framework Directive, opening the way for an alternative initiative in the next mandate.

As in the EU, soil degradation in Spain is confronted through several laws that indirectly protect it. Among them, the Law 10/1998 (BOE 1998) on residues became the first legal binding and enforceable instrument to regulate waste management and disposal. More recently, the Royal Decree 9/2005 (BOE 2005) established a list of potentially soil contaminating activities as well as criteria and standards for declaring sites as contaminated. For the first time in Europe, this legislation made it possible to determine soil pollution based on results from biological toxicity tests (Tarazona et al. 2006).

1.1.3. Soil contamination

Soil contamination is recognized as a major threat by the soil framework directive (COM 2006b). However, as the directive was not approved, soil contamination in the EU can only be faced individually at a state level. Even so, in many countries the prevention of soil contamination has strong links with national policies on chemical substances, on environmental protection for water and air (EC 2000), on waste management (EC 2008a), and on certain land uses like agriculture (Van-Camp et al. 2004). Although the creation of new contaminated sites is limited by regulation, a wide number of contaminated sites exists which require or are likely to require management in order to reduce their associated risks.

Soil contamination can be distinguished between that from clearly defined sources (local contamination) and that from diffuse sources (diffuse contamination). Local contamination is widespread throughout the EU and it is generally associated with past and present commercial, industrial and mining activities and with waste disposal and treatment (JRC 2012). Besides soil, these activities can potentially contaminate

ground water and surface waters through leaching and run-off of pollutants from contaminated sites. A study by Panagos et al. (2013) estimated in 2.5 million the number of potentially contaminated sites in the EU and in 342,000 the number of identified contaminated sites. The management of those contaminated sites was estimated to cost around 6 billion Euros (€) annually. Mineral oil and heavy metals are the main contaminants, contributing to approximately 60% of soil contamination and 53% of ground water contamination (Figure 1).

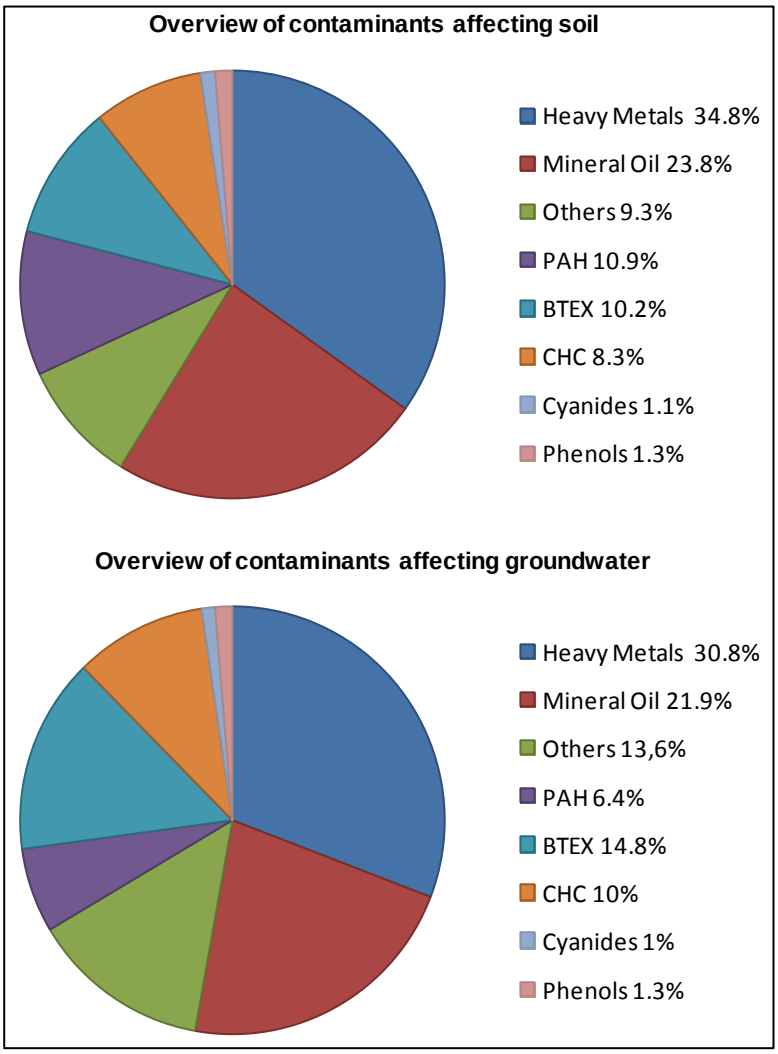

Figure 1. Distribution of contaminants affecting soil and groundwater in Europe. PAH: Polycyclic aromatic hydrocarbons; BTEX: Benzene, toluene, ethyl benzene, and xylene; CHC: Chlorinated hydrocarbons. Adapted from Panogos et al. (2013).

Diffuse contamination can have its origins in atmospheric deposition, farming practices and inadequate waste and wastewater recycling and treatment. Acidifying compounds (e.g. SO_2, NOx), heavy metals (e.g. cadmium, lead, arsenic, mercury), and organic compounds (e.g. dioxins, PCBs, PAHs) are likely to reach soils through atmospheric deposition, thus causing detrimental effects to soils and their inhabitants.

Acidifying pollutants are especially troublesome since they can decrease the buffering capacity of soils, which can lead to an overwhelming of the soil storage capacity and to a massive release of toxic metals. Additionally, acidification promote the leaching out of nutrients from soil resulting in a possible loss of soil fertility, eutrophication problems in water and excess of nitrates in drinking water (EC 2002b).

Major soil problems related with inadequate farming practices are the presence of heavy metals in fertilizers and animal feed as well as the widespread use of pesticides. The presence of metals in products for agricultural practices involves unknown effects on soil organisms, a possible risk of metal uptake throughout the food chain and unknown effects of antibiotics contained in animal feed. On the other hand, pesticides can pollute different environmental compartments by accumulating in soil, leaching to the groundwater or through volatilization. They may also affect soil biodiversity and enter the food chain. Although proper studies are demanded by authorities prior to the release of pesticides into the environment, information on their combined effects remains limited. Even more, according to FAO (2008) and Eurostat (2010a), pesticides consumption in Europe has continued to grow steadily during recent years.

Regarding waste management, concerns are raising due to to the field application of sewage sludge (final product of the treatment of wastewater), which is potentially contaminated by a wide range of pollutants such as heavy metals and organic compounds. Due to their high persistence (heavy metals) and poor biodegradability (trace organic compounds), an inadequate treatment of sewage sludge may involve an increase in the concentration of such pollutants in the soil that can pose a threat to soil microorganisms, plants, fauna and human beings (EC 2002b).

1.1.3.1. Heavy metals

Trace levels of heavy metals can be naturally found in soils as a result of pedogenetic processes involving weathering of parental materials. Such naturally-occurring metal concentrations are usually lower than 1000 mg kg^{-1} and non-toxic (Pierzynski et al. 2000; Kabata-Pendias and Pendias 2001). However, human activities tend to promote the accumulation of some metals in concentrations above background levels. Heavy metals in soils can be considered a threat if (i) their rates of generation via man-made cycles are more rapid relative to natural ones, (ii) they become transferred to random environmental locations where higher potentials of direct exposure occur, (iii) metal concentrations in discarded products are relatively high compared to those in the receiving environment, and (iv) the chemical form (species) in which a metal is found in the receiving environmental system may make it more bioavailable (D'Amore et al. 2005).

Anthropogenic emissions of heavy metals and metalloids are abundant and have different origins: rapidly expanding industrial areas, mine tailings, disposal of metal wastes in improperly protected landfills, leaded gasoline and lead-based paints, land application of fertilizers, animal manures, biosolids (sewage sludge), compost, and pesticides, coal combustion residues, spillage of petrochemicals, and atmospheric

deposition (Basta et al. 2005; Khan et al. 2008; Zhang et al. 2010). In addition, heavy metals released from the aforementioned anthropogenic sources tend to be more mobile and, consequently, more bioavailable than pedogenic or lithogenic ones (Kuo et al. 1983; Kaasalainen and Yli-Halla 2003).

Heavy metals can be divided into those that are required by organisms (essential heavy metals) and those that do not participate in any normal biological function (non-essential heavy metals). Regardless of their group, an excess of metals will lead to toxicity due to their interaction with biomolecules and the following disruption of critical biological processes. Additionally, both types of metals may accumulate in tissues and magnify through the food web, thus becoming a serious threat to human and environmental health (Gall et al. 2015). Other risks associated with metal contamination of soils include direct ingestion or contact with contaminated soil, water contamination, reduction in crop production and food quality, and land tenure problems (McLaughlin et al 2000a; McLaughlin et al 2000b; Ling et al. 2007).

Unlike organic pollutants, metals cannot be degraded through microbial or chemical mechanisms and their presence in soil is expected to persist for long periods of time. Several reactions are believed to control their distribution: (i) mineral precipitation and dissolution, (ii) ion exchange, adsorption, and desorption, (iii) aqueous complexation, (iv) biological immobilization and mobilization, and (v) plant uptake (Levy et al. 1992).

Several essential and non-essential metals and metalloids released from anthropogenic sources are known to reach soils frequently. Among them, arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), and zinc (Zn) are most commonly found in contaminated sites (GWRTAC 1997). Their fate, bioavailability, mobility and toxicity within the soil compartment will be ultimately determined by their chemical form and speciation (Shiowatana et al. 2001).

Arsenic

Arsenic is a naturally present metalloid that can be found in a wide variety of minerals and is usually recovered from processing of ores containing Cu, Pb, Zn, Ag and Au (Wuana and Okieimen 2011). Anthropogenic sources of arsenic include ashes from coal combustion, mining activities and soil application of fertilizers and pesticides (Basu et al. 2001). Arsenic exhibits a complex chemistry and it can be found in several oxidation states in soils. As(V) dominates in aerobic environments, behaves as a chelate and can precipitate in the presence of metal cations although arsenate complexes are only stable under certain circumstances. As(III) is the dominant form in reducing environments and it is the most toxic and water soluble form of arsenic (USEPA 1992). Elemental arsenic may be found under extremely reducing conditions. Arsenic compounds are usually strongly adsorbed to soils and their migration to groundwater and surface waters is therefore limited. Arsenic is associated with skin damage, increased risk of cancer, and problems with the circulatory system (Scragg 2006).

Cadmium

Cadmium in soils is found as the divalent Cd(II) ion and it is not associated with any essential biological function. However, Cd can substitute Zn (an essential micronutrient for soil organisms) due to their chemical similarity and may be responsible for the malfunctioning of metabolic processes (Campbell 2006). Cadmium is widely used in Ni/Cd batteries and in anticorrosive coatings and it can reach soils as an inevitable by-product of Zn refining, after application of fertilizers, pesticides, and biosolids (sewage sludge), due to disposal of industrial wastes or the deposition of atmospheric contaminants (Wuana and Okieimen 2011). As with many metals, Cd distribution is controlled by pH. Under acidic conditions, cadmium mobility increases and it is little adsorbed by soil colloids whereas its concentration in the soil solution decreases at pH greater than 6 (USEPA 1992). Despite its high biopersitence (once absorbed it has a half-life of several years), few toxicological properties are associated with Cd. The major threat to human health is chronic accumulation in kidneys, which can lead to kidney dysfunction.

Chromium

Chromium is less common in nature than other metals and it can be found as Cr(VI) and Cr(III) but not in the elemental form. Major releases of Cr in soil occur during the extraction of chromite ($FeCr_2O_4$), in electroplating processes and in the disposal of Cr-containing wastes (Smith et al. 1995). Cr(VI) predominates in contaminated sites and it is the most toxic and mobile form of chromium whereas Cr(III) is the dominant form at low pH (<4) and under certain redox conditions. Cr mobility in soils is highly influenced by clay, iron oxide and organic matter contents. Water contamination by chromium usually occurs through surface run-off of its soluble or precipitated forms as well as through leaching of soluble and un-adsorbed chromium complexes. Once in the water compartment, chromium is ultimately deposited into sediments due to its association with particles (Smith et al. 1995). Cr(VI) is considered as carcinogenic for humans and it is associated with certain hepatic, pulmonary and digestive disorders as well as with allergic dermatitis (Scragg 2006).

Copper

Copper is an essential micronutrient required for the proper growth of plants and animals. It is among the top used metals worldwide (VCI 2011), with applications in agriculture, fossil fuels, electrical industry and metallurgy. Copper release into the environment usually occurs through water contamination from Cu pipes and after the application of algaecides, and through soil contamination after spraying of Cu-containing pesticides. Cu is adsorbed by soils and soil constituents to a greater extent than other metals and it is rapidly stabilized to a form that does not represent an environmental threat. However, copper affinity for soluble organic ligands and the formation of these complexes may greatly increase Cu mobility in soils (EPA 1992). In addition, the solubility of Cu is drastically increased at pH 5.5 (Martínez and Motto 2000). Despite not being magnified in the body or bioaccumulated in the food chain, high doses of copper are known to cause damage to different organs.

Lead

Lead is an unessential element that naturally occurs as a mineral combined with other elements like sulphur (i.e. PbS, $PbSO_4$) or oxygen ($PbCO_3$). Average Pb concentration in surface soils worldwide reaches 32 mg kg^{-1}, and ranges from 10 to 67 mg kg^{-1} (Kabata-Pendias and Pendias 2001). Lead is the fifth most industrially-produced metal and it has many applications both alone and in alloys with other metals and metalloids. Some applications of Pb include the manufacture of ammunition, pigments and plumbing and, in alloys with other metals, it is used in storage batteries, solders or anodes (Manahan 2003). Pb in soils, ground waters and surface waters is usually found in the form of ionic lead, Pb(II), lead oxides and hydroxides, and lead metal oxyanion complexes, being Pb(II) the most common and reactive form. Once in soil, soluble lead reacts with clays, phosphates, sulfates, carbonates, hydroxides, and organic matter, thus greatly reducing its solubility (USEPA 1992). Lead can form organic (organolead) compounds, whose toxicities and environmental effects are of special concern due to their former massive use as a gasoline additive. The toxicity of lead will be driven by the level and duration of exposure.

Mercury

Mercury is a unique metallic element due to its particular characteristics: it is the only liquid metal at standard temperature and pressure, it can volatilize and it is capable for methylation. The primary source of mercury is the sulphide ore cinnabar. Mercury release to the environment is associated with chlor-alkali plants, coal combustion, gas/oil pipelines and as a byproduct of ore processing (Smith et al. 1995). Once in the environment, Hg exists in mercuric (Hg^{2+}), mercurous (Hg_2^{2+}), elemental (Hg^0), or alkylated forms (methyl/ethyl mercury). The distribution of mercury species in soils is dependent on soil pH and redox potential. Mercurous and mercuric mercury are more stable and are adsorbed by clay minerals, oxides, and organic matter. Sorption to soils, sediments, and humic materials as well as coprecipitation with sulphides are important mechanisms for Hg removal from solution (EPA 1992). Under certain conditions, organic or inorganic Hg may be reduced to elemental Hg, which may then be alkylated by biotic or abiotic processes to the more toxic methylmercury form (Wang et al. 2012). The toxicity of mercury is driven by its form although neurotoxic effects are common after exposure.

Nickel

Nickel can be found in all soils either naturally-occurring in very low concentrations or after release from anthropogenic sources (Iyaka 2011). Nickel is mostly used as an ingredient in steel and other metal products and it ends up in soil, air and water due to anthropogenic emissions. Soil contamination by nickel is associated with metal plating industries, combustion of fossil fuels and nickel mining and electroplating (Khodadoust et al. 2004). Ni release into air is related to power plants and trash incinerators whereas water contamination is associated with wastewaters (Budavari 1996). Nickel exists in various forms depending of the environmental pH. Nickeloneus ion, Ni(II), predominates in low pH and it precipitates as the stable nickelous hydroxide, $Ni(OH)_2$ in neutral to slightly alkaline solutions. Nickel is

usually immobilized after adsorption to clays, iron and manganese oxides, organic matter and sediments. Since nickel does not accumulate in plants or animals, it is not expected to biomagnify. However, despite being an essential element in small doses, it is associated with cancer development when certain concentrations are exceeded.

Zinc

Zinc is an essential trace element that can be naturally found in soils (approximately 70 mg kg^{-1} in crustal rocks)(Davies and Jones 1988). However, Zn production and environmental release is continuously rising due to anthropogenic additions. Most Zn emissions are related with industrial activities such as mining, coal and waste combustion, and steel processing (EPA 1992). Once Zn reaches soils, it is readily adsorbed by clay minerals, carbonates, or hydrous oxides in a pH-driven reaction. Zn retention in soils through precipitation is not common due to the relatively high solubility of its compounds. Zinc contamination is known to negatively influence the breakdown of soil organic matter due to its detrimental effects to populations of microorganisms and earthworms. Additionally, water-soluble zinc in soils is likely to contaminate groundwater. Water contamination by zinc is associated with metal release from industrial sources and toxic waste sites, either through waste waters disposal or leaching. Zinc can increase the acidity of waters and biomagnify up the food web and can cause health problems.

1.1.3.2. Petroleum hydrocarbons

Total petroleum hydrocarbons (TPHs) include the bulk of components in nearly all crude oils. They consist in a complex mixture of substances including chain and cyclic hydrocarbon molecules, heteroatomic compounds, and high-molecular weight polycondensation compounds (resins and asphaltenes)(Tang et al. 2012), each presenting specific physical, chemical and toxicological properties (Mao et al. 2009). Several of these compounds are acutely toxic (Heitkamp et al. 1988) and are considered priority pollutants by the US Environmental Protection Agency (Bojes and Pope 2007). Even more, total petroleum hydrocarbons are considered persistent pollutants and include some compounds able to bioconcentrate and bioaccumulate (McElroy et al. 1989) as well as recognized mutagens (Mortelmans et al. 1986) and carcinogens (IARC 1982).

Petroleum hydrocarbons can be naturally found in soils as a result of biogeochemical processes as well as after migration from deep oil-bearing strata. However, the contamination by petroleum hydrocarbons that is nowadays affecting vast areas of the Earth's surface has its origin in anthropogenic sources, mainly in the extraction and transportation of raw hydrocarbons and their derivatives (Gennadiev et al. 2015).

The anthropogenic introduction of total petroleum hydrocarbons into soil can occur from pipeline blow-outs, waste dumping, disposal after drilling oil and gas wells, road accidents, leakage in underground storage tanks, and uncontrolled landfill activities (Chaineau et al. 2003). At the same time, petroleum-contaminated soils can also pollute local groundwater, thus rendering potable water unsafe, limiting

ground water use, causing enormous economic loss and ecological disaster, and even destroying agricultural production (Wang et al. 2008).

Once petroleum hydrocarbons reach the terrestrial compartment, their fate will be driven by different reactions:

- *Sorption*. Petroleum hydrocarbons can be fixed by soil organic and mineral components (Barnes and Chuvilin 2009). Soil sorption is usually enhanced at high organic matter contents, which at the same time reduce the susceptibility of petroleum hydrocarbons to microbial biodegradation (Liu et al. 2013). Soil texture also influences the accumulation of hydrocarbons in soils, with lower retention levels detected in sandy soils (Zhang et al. 2012).
- *Photodegradation*. This process consists in the decomposition of a compound by radiant energy. The photodegradation of petroleum hydrocarbons is most active in soils from tropical latitudes where solar radiation is more intense.
- *Biodegradation*. Microorganisms able to transform hydrocarbons into energy, cell mass and biological waste products are widely distributed in soil habitats (Rahman et al. 2002). Many components of raw petroleum and diesel fuel can be decomposed by microorganisms although chain components are most usually subjected to biological degradation (Pandey et al. 2016). Besides microorganisms, plant enzymes are known to participate in the degradation of hydrocarbons (Khan et al. 2013), which is therefore more intensively performed in the rhizosphere (Martin et al. 2014). The decomposition rate of petroleum hydrocarbons varies among soils and it is influenced by parameters like the presence of oxygen, the temperature range and the aggregate composition of soils, among others (Chang et al. 2013).
- *Evaporation*. Under certain conditions, some fractions of petroleum hydrocarbons (especially the lighter ones) are susceptible to evaporation (Salanitro 2001). Such process relies on the soil porosity and in the character of the aggregates and can increase in wetted soils (Fine and Yaron 1993).
- *Migration*. Petroleum hydrocarbons can migrate from the soil surface to deeper regions of the soil profile and even reach the groundwater level. While migrating, hydrocarbons can displace water and air from soil pores, thus reducing their availability to microorganisms (Huesemann et al. 2014). Some compounds can even dissolve in soil pore water during the course of the migration although the hydrosolubility of hydrocarbons is generally low.

1.1.3.3. Pesticides

Although natural pesticides were used for centuries, current pesticides consist in synthetic compounds that are massively applied in agricultural production to control unwanted pests and weeds in an attempt to reduce yield losses while keeping the quality of the product. Only in the European Union, 320,000 tons of active substances are sold annually (EC 2010). Despite the strict regulations on pesticide development

and use, serious concerns have been raising during the last decades due to risks associated to manufacturing, handling and application of pesticides, and their aerial dispersion, leaching and run-off from treated fields (Stoate et al. 2001; Berny 2007).

Pesticides can be classified either according to their target pest or to their chemical identity. Roughly, the classification by target pest includes herbicides, fungicides, insecticides and rodenticides that are specifically aimed at weeds, fungi, insects and rodents respectively. In terms of chemical identity, pesticides are classified in groups that share a common chemistry (e.g. organophosphates, organochlorines, etc.). It is important to note that, when discussing a pesticide, you can refer either to the pesticide compound itself (i.e. the active ingredient) or to the pesticide product or formulation (which contain several other components).

Soil properties and soil-occurring processes play an important role in the environmental distribution of pesticides. The following are considered the main soil processes affecting pesticide fate:

- *Volatilization*. Pesticide volatilization from soils depend on physicochemical properties of the compound together with the application technique (spraying is more susceptible to volatilization than soil incorporation) and it is one of the main causes of pesticide dispersion.
- *Adsorption/desorption*. Soil particles can retain pesticides, thus determining their bioavailability, leachability, degradability and dispersion throughout the soil profile.
- *Run-off*. Run-off of pesticides from soils is considered one of the main sources of surface waters contamination. Pesticides will be transported either dissolved or associated with colloids.
- *Leaching*. Similarly to run-off, leaching of pesticides from soils usually lead to groundwater contamination.
- *Plant uptake*. The uptake by plants can sometimes become the main objective of pesticides application and their incorporation rate depends on plant species, growth stage and the intended use of the pesticide.
- *Degradation*. It is the major cause of pesticide disappearance after application. Pesticide breakdown can be driven by radiation (photochemical degradation), chemical processes (chemical degradation) or microorganisms (biodegradation).

Contamination by pesticides is known to negatively affect both human and environmental health. Effects on humans are associated to the exposure of workers during pesticide application and of consumers to pesticide residues in fresh fruit, vegetables and drinking water (Damalas and Eleftherohorinos 2011). Detrimental effects after acute or chronic exposures to these compounds include carcinogenesis (Blair et al. 1985), neurotoxicity (Tanner and Laangston 1990) and effects on cell development (Gray et al. 1994) among others. Environmental impacts are associated with air and water contamination and with detrimental effects on wildlife, fish, plants and other non-target organisms (Burger et al. 2008; Mariyono 2008). These effects, however, depend on the toxicity of the pesticide, the way it has been applied, the

dosage, the adsorption on soil colloids, the weather conditions, and the environmental persistence of the pesticide (Eleftherohorinos 2008).

1.1.4. Soil health assessment

Pankhurst et al. (1997) defined ‘soil health’ as "the continued capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain biological productivity, promote the quality of air and water environments, and maintain plant, animal, and human health". Soil contamination involves risks at different levels and can lead to the ecological imbalance of soils, thus becoming a threat for the sustainability of the whole soil ecosystem (Cortet et al. 1999; Edwards 2002). The proper evaluation of soil contamination and its associated risks has therefore become essential for the preservation of soil health.

Soil contamination can be evaluated through physical, chemical, and biological parameters. However, the impacts of anthropogenic contamination on soil ecosystems have been primary evaluated through physical-chemical indicators rather than through biological ones, which were considered more difficult to measure (Parisi et al. 2005). Following a chemical-specific approach, the pollutant or pollutants of interest are quantified and their concentrations are compared with threshold values in order to determine their associated risks.

However, several restrictions of chemical methodologies were reported that limited their suitability for the assessment of soil contamination. First of all, chemical analyses require previous knowledge on the compounds of interest and their intermediary metabolites because they are unable to detect all soil contaminants (Loibner 2003). Furthermore, they provide no information on the bioavailable fractions of pollutants (Alexander 2000), their synergisms and antagonisms, and their interactions with the soil matrix and organisms (Gruiz 2005). In this context, it has become clear that biological indicators can turn into valuable tools for the evaluation of dynamic soil systems (Blair et al. 1996) due to the high sensitivity of biological processes and the capacity of organisms to detect and rapidly respond to contaminant concentrations in soil.

Once released into the environment, the risks associated to pollutants cannot be solely determined according to their toxicity. In fact, very toxic substances can pose a little threat if they are not easily available to organisms. The term bioavailability refers to the biologically active fraction of a contaminant, i.e. the ability of an environmental pollutant to reach an organism (or a target part of it) and cause some effect (Landis et al. 2011). The bioavailability of an environmental contaminant is driven by factors related with the compound itself (concentration, chemical structure, etc.) and by the receiving environmental matrix. The interactions between them will ultimately define the environmental fate of polluting compounds and, in consequence, their associated risks (Figure 2).

Since soil is a very complex environmental matrix, many soil-occurring phenomena exist that markedly modify the characteristics of soil pollutants, which will ultimately trigger a huge diversity of detrimental

effects on soil ecosystems. These effects range from direct acute toxicity to particular taxa or trophic groups of invertebrates, microorganisms or plants to indirect effects like alteration of predator/prey relationships or effects on soil food webs.

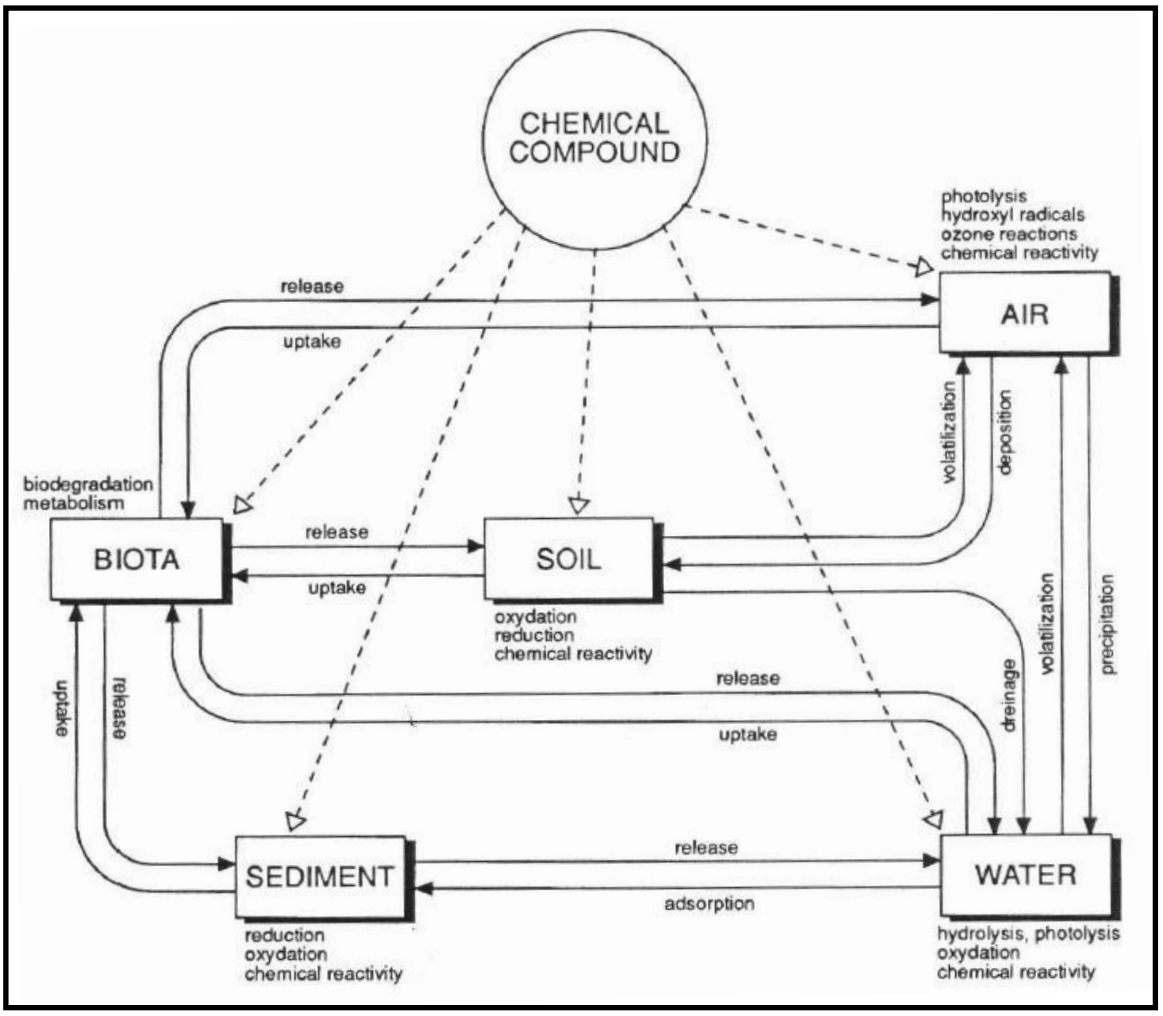

Figure 2. Scheme of the main transport and transformation processes for chemical compounds in environmental compartments. Taken from Vighi and Calamari (1993).

1.1.5. Ecotoxicology

Ecotoxicology is a scientific discipline that deals with understanding the origins and endpoints of chemical products in the environment with the aim of protecting the structure and functioning of ecosystems (Connell et al. 1999; Van Gestel 2012). Its origin dates back to 1969, when the term was first coined by R. Truhaut (Truhaut 1977). As a truly multidisciplinary field, it incorporates elements of ecology, toxicology, chemistry, epidemiology, and pharmacology.

Due to its relevance at both regional and global scales, the development of ecotoxicology stimulated the creation of several organizations dedicated to environmental safety like the International Academy of Environmental Safety (IAES) in 1971 and the International Society of Ecotoxicology and Environmental Safety (SECO-TOX) in 1972. At the same time, these organizations raised their concerns for the lack of

suitable scientific tools that could help in the regulation of the environmental release of pollutants (Twardowska 2004).

Nowadays, ecotoxicology involves a variety of scientific principles and methods that aim to better anticipate the environmental impacts related to the anthropogenic release of pollutants. These methods usually rely in exposing selected test organisms to polluted environments and extrapolating the observed effects to population and community levels in order to assess potential risks for exposed ecosystems.

Ecotoxicology has traditionally evolved faster for aquatic ecosystems than for soil. While several harmonized aquatic tests were developed in the 1970s, only one method for soil organisms was internationally accepted by 1995. Since the appearance of the first soil tests, data on terrestrial ecotoxicity has grown considerably (Løkke and Van Gestel 1998).

1.1.5.1. Terrestrial ecotoxicology

Ecotoxicology can be divided into subfields according to the environmental compartment in which it is focused. Terrestrial ecotoxicology is the subfield that studies, evaluates and quantifies the effects of toxic substances on the diversity and function of soil-based plants and animals (Garcia 2004). The ecotoxicological assessment of soils is strongly influenced by the complexity of the soil matrix and its interactions with polluting substances. After reaching the terrestrial compartment, pollutants are usually bound to the solid phase of the soil but their bioavailable fractions can be dissolved in the soil pore water. Consequently, soil ecotoxicologists have to deal with at least three compartments (soil, pore water and organisms). Additionally, soil-occurring phenomena like sorption, partitioning, and speciation strongly affect soil contaminants and must be taken into consideration since they will ultimately determine changes in toxicity and biodegradation rates of pollutants (Van Straalen 2002).

The risk assessment of contaminated soils can be performed through different tests, which are classified according to their duration, the number of species involved and their complexity (Römbke et al. 1996, Landis and Yu 2004)(Figure 3). At the same time, the ecological relevance of each test is correlated with its complexity, costs and duration (Römbke and Notenboom 2002). Because of these limiting parameters, the most complex tests are only seldom applied.

The first steps of such studies are single species laboratory tests, where organisms from selected species are exposed to polluted soils under controlled environmental conditions and acute toxicity, chronic toxicity and behavioral responses are measured. Standard short-term acute toxicity tests are widely applied for the identification of highly toxic compounds although they are markedly less sensitive than other tests. In contrast, chronic tests offer high sensitivity and the opportunity to study the toxicity at different stages of the organism's life cycles in exchange for higher costs and time consumption. Finally, avoidance tests provide a faster assessment that is usually applied as a first screening tool. When applied together, soil laboratory tests can provide much more valuable information on the risks associated to a polluted soil (Heupel 2002).

While laboratory tests focus on representative species of the most important functional groups within the soil ecosystem (Garcia 2004), microcosm and mesocosm studies explore the effects of pollutants at community level, thus providing more environmentally-relevant results. Microcosms are usually filled with natural soils, study the interactions between a few species of animals and/or plants and are carried out under the controlled environmental conditions of a laboratory. On the other hand, mesocosms are larger, include multiple species, are carried out under natural conditions and can report effects at population and community levels (Crossland 1994).

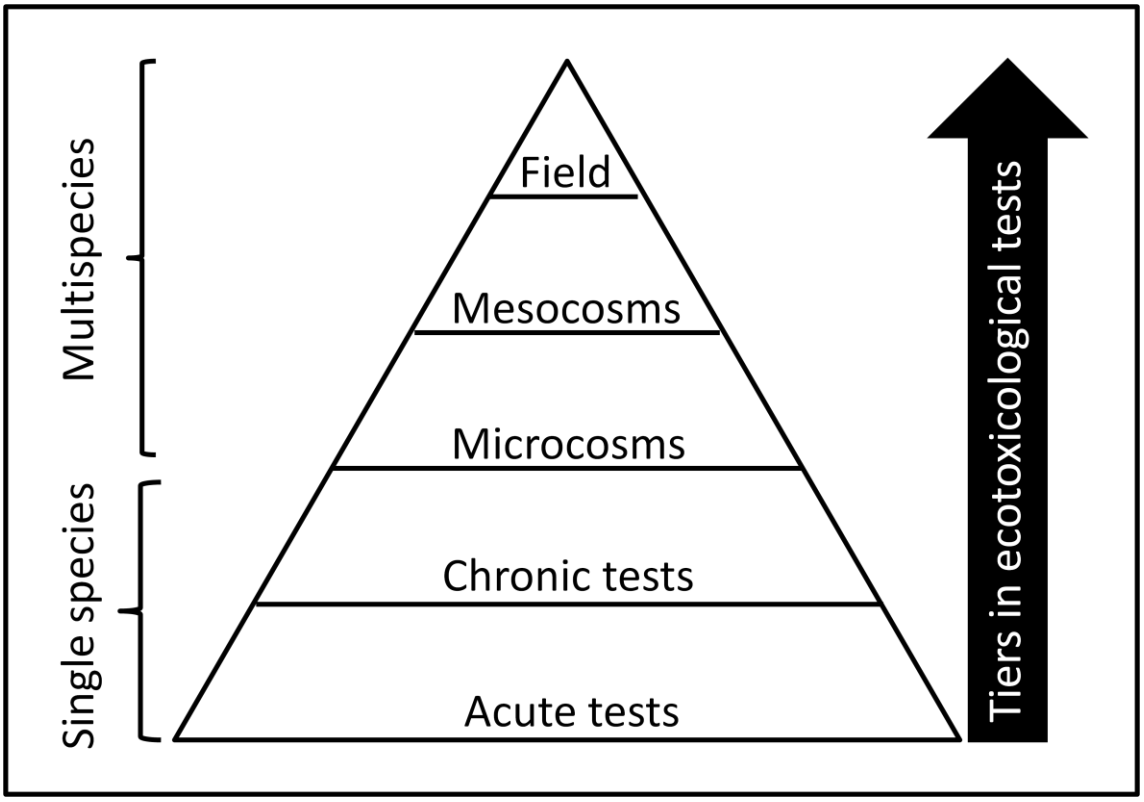

Figure 3. Sequence of tiers used in Environmental Risk Assessment (ERA). From basic tests (acute) to most complex systems (field) of evaluation in soil ecotoxicology. According to the increases of tiers levels there is an increase of the bioassays complexity and costs. Adapted from Landis and Yu (2004).

The final step in ecotoxicological risk assessments involves field studies. Such studies provide the most precise evaluation of soil contamination because they are performed under natural ecosystem and climate conditions. Field tests allow the measurement of additional parameters like bioaccumulation, diversity and abundance of species, and microbiological analyses (Cardoso and Alves 2012). However, the broad array of available tools implies a challenge when interpreting their results due to the dynamism of soil ecosystems, which complicates establishing cause-effect relationships.

Stimulated by the needs of numerous regulatory agencies worldwide, present terrestrial ecotoxicology has evolved towards more precise quantitative and qualitative assessment of the effects of pollutants in soils (Shugart 2009). At the same time, efforts have also focused in developing more ecologically relevant tests as a response to authors who argued that ecotoxicology was too simplistic (Calow and Forbes 2003).

Special attention is nowadays given to evaluating the effects of mixtures of chemicals (Van Gestel et al. 2011), the influence of stress factors (Holmstrup et al. 2010), the application of available tests to emerging chemicals (Van Gestel 2012), and the application of biomolecular tools (Bradley and Theodorakis 2002).

1.1.5.2. Laboratory tests

Laboratory single-species tests are usually applied during the initial stages of soil risk assessment. Parameters like soil properties, test organisms and conditions, and type and concentration of contaminants play a major role in the performance of such tests and must be carefully selected in accordance with the purpose of the test. At the same time, soil laboratory ecotoxicity tests can be classified in terms of exposure time (acute or chronic), observed effect (mortality, reduced growth, etc.) or effective response (lethal or sublethal)(Kapanen and Itävaara 2001).

Laboratory ecotoxicological tests can be applied following two different approaches (Van Gestel 2012). The prognostic approach aims at predicting the potential effects that the release of chemicals might have in the environment, thus helping in the regulation of their use and introduction into the market. In contrast, the diagnostic approach aims to determine the status and ecological risks associated to already contaminated natural soils, thus providing relevant data for the proper management of polluted sites.

Ecotoxicological laboratory tests present many advantages that make them suitable as a main hazard assessment technique or as complements to other methodologies (e.g. chemical analysis). These tests are relatively quick, simple, replicable and inexpensive to perform. Moreover, they provide an insight into complex biological functions that is rarely obtained by other methodologies. Additionally, soil ecotoxicity tests can also be easily used to compare the relative sensitivities of soil organisms to particular chemicals or chemical mixtures. They are particularly useful in comparing chemicals of concern or in identifying and isolating spatial and temporal distributions of soil toxicity. Finally, they can also greatly assist in understanding the effects of soil characteristics (e.g. pH, clay content, organic matter content, salinity, etc.) on soil toxicity and bioaccumulation (OEHHA 2009). Due to their multiple strengths, laboratory tests are routinely applied to provide data required by different regulatory authorities prior to authorizing the sale of pesticides (EC 2013) or the application of residues on agricultural soils (EC 2008b).

Despite their widespread use, ecotoxicological laboratory tests also present major drawbacks. They are limited to species suitable for culture and use in laboratory facilities, which may not have ecological significance in terms of functional importance in soil processes nor be key indicator species (Moore and Ruiter 1997). Additionally, laboratory tests are performed under optimal conditions and therefore ignore several variables that may play an important role in interactions between organisms and their environment, thus leading to difficulties in extrapolating the results (Van Gestel and Van Straalen 1994). When testing natural soils, any disruption of their associated redox gradients and physicochemical and biological processes during handling can markedly influence the performance of the tests. Besides, the

use of ‘control’ or ‘reference’ site soils is considered nearly impossible (Giller et al. 1998), which led to the application of artificial and less naturally-resembling soils.

Nowadays several laboratory tests have been standardized for the evaluation of soil quality, many of them by guidelines from the Organization for Economic Cooperation and Development (OECD) or by the standards from the International Organization for Standardization (ISO). Following these protocols, lethal and sublethal effects can be assessed in terrestrial plants, earthworms, collembolans, enchytraeids and other insects. Due to their standardization, results from such tests can be compared, thus increasing their reliability. However, for a better characterization of the risks associated to polluted soils, such tests should be applied in batteries rather than individually (DECHEMA 1995; Keddy et al. 1995). Test batteries should include taxonomically different species that are representative of the studied ecosystem, play different roles in it and have different routes of exposure (Van Straalen and Van Gestel 1993).

Besides testing the whole soil, soil contamination can also be evaluated in the laboratory through aquatic tests where organisms are exposed to aqueous extracts from polluted soils. Such methods can also be useful in the estimation of the risk that soil contaminants pose to the water compartment as well as in studying the impacts of chemicals on the filter function of soils (DECHEMA 1995, Hammel et al. 1998; Bispo et al. 1999; Van Gestel et al. 2001; Robidoux et al. 2004). Despite providing quick and low-cost data, such tests are considered less ecologically relevant (Van Gestel et al. 2001).

Regardless of the environmental matrix tested, the basis for the application of ecotoxicological laboratory tests is the progressive and measurable relationship expected to exist between concentration of contaminants and effects on organisms under controlled test conditions (dose-response model)(Figure 4). Some toxicity parameters can be derived from this model in order to provide useful instruments for the evaluation of ecological risks and consequently to establish protection limits for ecosystems.

A common toxicity parameter is the EC50 (median effective concentration), which is the concentration of test substance (e.g. chemical compound, contaminated soil or water sample) in test medium that is estimated to cause some defined toxic effect on 50% of the test organisms. In most instances, the EC50 and its 95% confidence limits are statistically derived by analyzing the percentages of organisms affected at various test concentrations after a fixed period. Depending on the study objectives, an effective concentration other than EC50 (e.g. an EC20) might be calculated instead of or in addition to the EC50. Other parameters like LC50 or IC50 can be applied for specific measured effects (lethality and inhibitory effects respectively). Other useful parameters for policy-makers are NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration).

1.1.5.3. Test organisms

Prior to its release, the toxicity of a substance should be tested in all the species inhabiting the ecosystems that are receiving or expected to receive the compound. However, such goal is virtually unachievable in the laboratory and consequently the hazards for terrestrial ecosystems are assessed in a range of

representative soil-inhabiting species that satisfy certain requirements (Ronday and Houx 1996; Fountain and Hopkin 2005; Alves and Cardoso 2016):

- Sensitivity to a range of impacts
- Ecological relevance
- Easy and inexpensive maintenance in the laboratory
- Short generation time
- Well-known biological parameters

Unfortunately, the current number of soil species meeting all these requirements (i.e. standard soil species) is rather limited. Among them, soil invertebrates like earthworms, collembolans, mites, and enchytraeids play a major role in the batteries of soil ecotoxicological tests as they offer a wide range of morphological and physiological characteristics as well as feeding and behavioral habits (Peijnenburg et al. 2012).

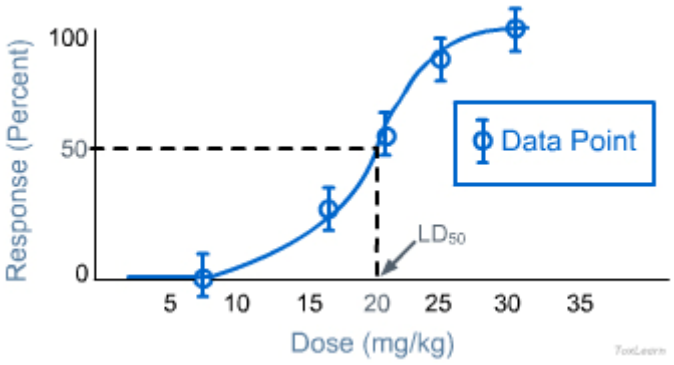

Figure 4. Dose-Response graph. Adapted from https://toxlearn.nlm.nih.gov.

Earthworms are considered very valuable components of the soil biota due to their role in maintaining soil structure and fertility through breakdown and transformation of organic matter (Bouché 1988). They also constitute up to 92% of the total soil biomass, can be found in a broad variety of soils, regions, and climates, and are important to food webs (Edwards 2004). Morphologically, earthworms present two main routes of exposure to soil contaminants: through the absorption of water across their skin and through the ingestion of contaminated soil.

Among earthworm species available for toxicity testing, ISO standards and OECD guidelines have traditionally focused on the genus *Eisenia* (*Eisenia fetida* and *Eisenia andrei*) due to their worldwide distribution, natural tolerance to organic substrates, and ease of acquisition and handling (Alves and Cardoso 2016). Nevertheless, concerns exist due to the fact that both species are usually found in soils rich in organic matter rather than in agricultural soils.

The order Collembola consists in diverse and abundant groups of terrestrial arthropods that can be found widespread on earth (Coleman et al. 2004). Although direct effects of collembolans on ecosystem

processes do not stand out (they represent a modest contribution to soil biomass and respiration), they influence microbial ecology and soil fertility through decomposition and nutrient cycling processes (Culik and Zeppelini 2003; Coleman et al. 2004; Jänsch et al. 2005a). The exposure of collembolans to pollutants is associated with water ingestion or absorption, food consumption and inhalation of soil pore air (Peijnenburg 2012).

Folsomia candida is the collembolan species most frequently used in standard ecotoxicological tests due to its high sensitivity, short generation time, high reproduction rate and ease of culturing (Crouau et al. 2012). It is distributed worldwide although larger populations usually occur in sites rich in organic matter (Fountain and Hopkin 2005). Apart from *Folsomia candida*, *Folsomia fimetaria* is also used as test organism due to its presence in agricultural soils where *F. candida* is not usually found.

Besides faunal species, terrestrial plants have been usually applied in standardized soil ecotoxicity tests because of their essential role in healthy ecosystems. Within the edaphic system, plants produce O_2 and energy for almost all other life forms and changes in their diversity and abundance may influence the distribution and abundance of several dependent species (OEHHA 2009). Plant exposure to pollutants mostly occurs either by absorption through their aerial parts or through their roots. Once exposed, phytotoxicity endpoints include rates of seedling emergence and germination, time until emergence, survival rate, root length and visual observations of abnormalities.

Several plant species are suitable for ecotoxicity testing although most assays have been traditionally performed with crop and other domesticated species. Even so, protocols can be easily adapted to native or undomesticated species. Among plant groups, standard tests have focused on monocotyledons and dicotyledons. When selecting species within these groups, it is important to consider their sensitivity, requirements and relevance (OEHHA 2009). Additionally, many soil properties (pH, grain size, organic matter, texture, water-holding capacity, etc.) can negatively influence seedling emergence and growth.

1.2. Hypothesis

Soil contamination has reached such high levels that many soil ecosystems are becoming seriously threatened. The inherent complexity of the soil matrix together with the massive accumulation of mixtures of pollutants with unknown effects further complicates the proper assessment of the risks associated to field contaminated soils and soil contaminants. In addition, traditional tools for soil assessment have been questioned due to their associated limitations. In this context, ecotoxicity bioassays can become a suitable complementary tool to better evaluate the risks that soil contamination pose for ecosystems.

1.3. Objectives

Main objective

The main objective of this work is to assess the suitability of ecotoxicological bioassays as complementary tools for the evaluation of soil contamination and its associated ecological effects, thus improving soil risk assessment methodologies and establishing criteria that allow a better characterization of soil ecosystems.

Specific objectives

- To evaluate the suitability of different terrestrial ecotoxicity tests for the assessment of soil contamination. To do so, ecotoxicological bioassays are carried out in field soils heavily contaminated with metals, hydrocarbons or pesticides and study lethal and sublethal effects on earthworms *Eisenia fetida*, collembola *Folsomia candida* and plant species *Brassica rapa*, *Trifolium pratense* and *Lolium perenne*.
- To study the relevance of aquatic ecotoxicity tests as evaluation tools of soil contamination through the performance of bioassays with aquatic bacteria *Vibrio fischeri*, algae *Raphidocelis subcapitata*, crustacean *Daphnia magna* and fish *Danio rerio* in aqueous extracts from contaminated soils.
- To help in the development of contaminant-specific batteries of ecotoxicological bioassays.
- To identify interactions between soil physicochemical parameters, pollutants, and toxicity to test organisms that help in understanding the risks associated to contaminated soils.
- To evaluate the suitability of ecotoxicity tests as monitoring tools of soil remediation procedures.
- To develop new procedures that improve current methodologies for the ecotoxicological assessment of contaminated soils.

1.4. Methodology

1.4.1. Sample collection

1.4.1.1. Field Soils

The proper collection of field soils is of greatest importance in terrestrial ecotoxicology because it can markedly influence the overall results of a study. During sampling, main efforts should focus in obtaining samples that are as representative as possible of the site and in ensuring that soils undergo minimum changes before examination.

In this work, composite samples are collected in each site. A specific number of sub-samples are chosen so that representativeness of the site is ensured. Soil samples are collected from the top soil (0-25cm), thus covering the "A horizon" of the soil. When necessary, the surface layer of decomposing organic matter on top of the soil is removed prior to sampling. Samples are obtained through manual excavation and stored in plastic bags or previously-cleaned plastic containers. The volume of each sample varies between studies depending on the accessibility to the site, sampling limitations, and the aim of the study.

1.4.1.2. Water samples

Aqueous extracts from field soils are obtained immediately after sample pretreatment and in accordance with a protocol based on the British Standard EN 12457-2 (2002). This methodology requires previous sieving (<4 mm) and moisture determination of the soil. A portion of sample containing 100 g dw of soil is placed into a 2-L glass Erlenmeyer and 1 L of deionized water is poured so that a ratio of 10 L kg^{-1} is achieved. The Erlenmeyer is then sealed with a plastic lid, covered with aluminum foil and placed on an orbital platform shaker Unimax 2010 (Heidolph, Germany). The glass vessel is shaken for 24±0.5 h at 120 rpm and at 20±5 ºC. Careful must be taken to prevent the sedimentation of soil particles. After a settling period of 15±5 min, the water extract is centrifuged for 10 minutes at 2000 g in a Digicen 21R centrifuge (Ortoalresa, Spain). The supernatant is then filtered through a 0.45 µm membrane filter in a vacuum filter system. In those cases where the sample cannot be filtered through a 0.45 µm membrane filter due to the plugging of the membrane, a 1 µm filter is used. Water extracts are stored at 4±2 ºC and in the dark if the tests are expected to be performed within 7 days. Otherwise, extracts are frozen at -20 ºC.

1.4.2. Sample Analysis

1.4.2.1. Pretreatment

Once in the laboratory, soil samples undergo a pretreatment procedure prior to storage or analysis. First of all, living material (roots, soil macroinvertebrates, etc.), stones and other large objects are removed by hand. Afterwards, samples are spread in a thin layer and air dried until constant mass. Soils are then thoroughly homogenized, soil aggregates are crushed, and the resulting samples are sieved through a 2 mm sieve and homogenized again. Soils with high silt and/or loam contents are sieved through a 4 mm sieve to avoid plugging the mesh (ISO 2008). Samples are kept cool (4±2 ºC) and in the dark for up to 3 months. Both soil and water samples are taken to room temperature prior to analysis.

1.4.2.2. Physicochemical characterization

The physical-chemical properties of soils have a major impact on the fate and toxicity of the contaminants that reach them. Therefore, measuring and understanding those parameters have become essential for a proper risk assessment of soil contamination.

pH

The measurement of pH is performed based on the ISO 10390 (2005a) standard. Following this standard, the pH of the soil is potentiometrically measured in the supernatant of a 1:5 soil:liquid (v/v) suspension. Five grams of air-dried and sieved soil are placed in a suitable glass vessel. A solution of potassium chloride (1 mol KCl L^{-1})(reagent grade, Scharlau) is prepared and 25 ml of suspension are added into the glass vessel. The soil-liquid suspension is mixed vigorously for 5 min using a mechanical shaker and left resting for 2 h. A Microph 2001 ph-meter (Crison, Spain) is calibrated according to the recommendations of the manufacturer and pH is measured. For water samples, pH is directly measured in a suitable volume of sample.

Electrical Conductivity

Electrical conductivity (EC) is determined following a procedure based on the ISO 11265 (1994) standard in which the content of water-soluble electrolytes is measured in an aqueous extract of soil. A sample containing 20 g of air-dried and sieved soil is placed in a suitable glass vessel at room temperature (20±1 ºC). 100 ml of deionized water with a specific electrical conductivity ≤ 0.2 mS m^{-1} are added and the suspension is vigorously mixed for 30 min in a mechanical shaker. The suspension is then filtered directly through a filter paper. A Ecoscan Con 5 conductivity meter (Eutech Instruments, UK) is calibrated with a potassium chloride conductivity standard (0.01 mol L^{-1}; 1413 µS cm^{-1})(reagent grade, Scharlau) and the conductivity of the filtrate is measured. Electrical conductivity in water samples is directly measured in a suitable volume of sample.

Water holding capacity

Water holding capacity (WHC) of soils is calculated on the basis of the Annex F of the ISO 17512 (2008) standard. A definite quantity of soil sample (35 to 40 g) is dried to constant mass at 105 ºC. The bottom of a suitable polyethylene plastic tube (2 cm diameter, 10 cm height) is closed with filter paper and the tube is weighed. The tube is then filled up to a mark with dried soil, weighed again, and placed in a water bath for about 3 hours. Care should be taken that the water level is above the mark of the tube. After the specified time, the tube is placed on filter paper for 2 h and it is weighed again once the water that cannot be retained by capillarity has been released. The water holding capacity is calculated according to the following equation:

$$WHC = [(m_S - m_T - m_D)/ m_D] \times 100$$

Where:

m_S is the mass of the water-saturated substrate plus the mass of the tube plus the mass of the filter paper;

m_T is the tare (mass of tube plus mass of filter paper);

m_D is the dry mass of substrate.

Moisture

The ISO standard 11465 (1993) is taken as reference for the determination of soil moisture. A sample containing 5 to 15 g of air-dried fine soil (fraction < 2 mm) is transferred to a dried, tared vessel and weighed. The sample is dried at 105±5 °C until constant mass is achieved. The vessel is then removed from the oven, closed with a lid, cooled in a desiccator, and weighed. Percentage of moisture content is obtained by the equation:

$$Moist\ \% = [(A - B)/(B - Tare)] \times 100$$

Where:

A : Weight of tared moisture vessel and air-dried soil sample;

B : Weight of tared moisture vessel and oven-dried soil sample.

Soil Organic Matter

The soil organic matter (SOM) content is determined through the Loss On Ignition (LOI) method, which is based on ignition (550±25 ºC) of a dried (105 ºC) soil sample until mass constancy is achieved. The dry mass (m_S) of the soil is calculated according to the procedure for the determination of the moisture content of a soil. A bowl is heated in the muffle furnace at 550±25 ºC for 20 min and cooled in a desiccator. Its tare mass (m_t) is determined to 0.1 g. A sample of 5 to 20 g of oven-dried (105 ºC) soil is

weighed in the bowl and placed in a cold HK-11 muffle furnace (Hobersal, Spain). The muffle furnace is heated gradually to 550±25 ºC for 2-4 h until mass constancy is achieved. After that time, the muffle furnace is cooled down to 100 ºC and the bowl is placed in the desiccator and cooled to room temperature (approx. 1 h).

The mass of the filled bowl (m_c + m_t) is measured twice and the difference of each individual measurement from the mean should not exceed 5% of the mean. The loss of mass after ignition is calculated as follows:

$$\Delta m = (m_s + m_t) - (m_c + m_t) = m_s - m_c$$

The LOI corresponds to the SOM content and can be calculated using the following equation:

$$\text{LOI (\%)} = (\Delta m / m_s) \times 100$$

Where:

Δm: loss of mass of the soil after ignition at 550 ºC (g);

m_s: mass of the soil dried at 105 ºC (g);

m_t: mass of the crucibles/bowls ignited to 550 ºC (g);

m_c: mass of the soil ignited to 550 ºC (g).

Total Organic Carbon

Total organic carbon (TOC) in water samples is determined in accordance with the UNE-EN 1484 (1998) guideline. Determinations are performed with a Total Organic Carbon Analyzer TOC-V_{CSH} (SHIMADZU, Japan) as specified by the manufacturer. Briefly, the equipment burns the sample and subsequently analyzes the CO_2 released from the combustion using a non-dispersive infrared detector. Separate measures determine Total Carbon (TC) and Inorganic Carbon (IC) concentrations. Total Organic Carbon is calculated as follows:

$$\text{TOC (mg L}^{-1}) = \text{TC (mg L}^{-1}) - \text{IC (mg L}^{-1})$$

Texture

The measure of soil texture is adapted from the ISO 11277 (1998) for the determination of the particle size distribution in mineral soil through sieving and sedimentation. This method is based on Stokes' Law. This law states that denser (larger, usually) particles sink farther than less dense (smaller) particles when suspended in a liquid. There are two critical assumptions: (1) the particles all have the same density and (2) the particles are spherical. Actually, neither of these assumptions can be perfectly satisfied. The procedure is divided into the following steps:

1. 20 to 50 g of dried (105 ºC) and sieved (< 2 mm) soil are placed in a 250 ml beaker. 10 ml of 30% H_2O_2 are poured into the vessel for the destruction of organic matter.
2. When the reaction diminishes, approximately 50-ml of distilled water are added and brought to boil for 15-20 minutes. The sample is then removed from heat source and let cool.
3. A solution of sodium hexametaphosphate is prepared by dissolving 40 g of the reagent salt (Calgon®) in one liter of distilled water. This solution acts as a deflocculating agent i.e. separates and suspends the colloidal particles.
4. 125 ml of deflocculating solution are poured in the beaker. The beaker is then covered with Parafilm and left overnight on an orbital shaker.
5. After standing overnight, the sample is gently poured through a 62.5 mm sieve placed over a large funnel that sets in a 1000 ml cylinder. Sample spillage should be carefully avoided. All silt and clay (< 62.5 mm) is washed through the sieve using distilled water.
6. The entire sand fraction (very fine to very coarse) is now in the sieve. Sand is carefully transferred to a tared beaker, drought and weighed.
7. The cylinder is filled to the 1000 ml mark with distilled water and another beaker is weighed.
8. The temperature of the water is recorded and the settling time chart is checked to determine the time at which the 0.002 mm size fraction must be withdrawn (Table 1).

Temperature (ºC)	Time of sampling
20	7 h 44 min 16 s
21	7 h 34 min 04 s
22	7 h 23 min 53 s
23	7 h 13 min 13 s
24	7 h 03 min 02 s
25	6 h 52 min 50 s
26	6 h 44 min 02 s
27	6 h 35 min 42 s
28	6 h 26 min 53 s
29	6 h 18 min 33s
30	6 h 09 min 45 s

Table 1. Pipette sampling time.

9. The cylinder is vigorously agitated for 20 seconds and immediately after the time count begins. At the required time, the sample is collected from a depth of 10 cm using a 20 ml pipette. The sediment sample is expelled into the beaker and the pipette is washed into the same beaker with distilled water. The sample is dried in the oven, cooled in the desiccator and weighed. The sample draw time corresponds to the settling time of a specific particle size. Therefore, the mass of the 0.002 mm sample corresponds to the mass of sediment finer than this size fraction (i.e. clay).
10. When measuring the mass of the samples, it must be taken into account the mass of deflocculating agent. To determine the amount of dispersant in the aliquot, the total mass of dispersant in the graduated cylinder is divided by 50 (the 20 ml aliquot is 1/50th of the original

1000 ml solution in the cylinder). This value must be subtracted from the mass of each sample. In our case, 125 ml of a solution that is made dissolving 40 g of sodium hexametaphosphate in 1 L of distilled water would contain 5 g of dispersant. Five grams divided by 50 means that each sample aliquot should contain 100 mg of sodium hexametaphosphate.

11. The total weight of sand and clay fractions in the soil sample is calculated by multiplying the mass of the sample by 50. The mass of silt is calculated by subtracting the mass of sand and clay fractions from the initial weight of the sample.
12. The percentage of each fraction is estimated and soil texture is determined according to USDA-FAO texture classification (FAO 1990).

Others

Besides the above-mentioned characteristics, other physicochemical parameters of test soils have been analyzed by external institutions under proper quality standards:

- Bulk density and Total porosity: The bulk density gives a rough estimation of the aeration and permeability of a soil. The lower the bulk density, the higher the permeability. Bulk density is needed for converting water percentage by weight to content by volume, calculating the porosity and void ratio when the particle density is known (Blake and Hartge 1986).
- Cation exchange capacity (CEC): CEC is the number of exchangeable cations per dry weight that a soil is capable of holding, at a given pH, and available for exchange with the soil water solution (Robertson et al. 1999). CEC is used as a measure of soil fertility, nutrient retention capacity, and capacity to protect groundwater from cation contamination.
- Field capacity: Field Capacity is the amount of soil moisture or water content held in the soil after excess water has drained away and the rate of downward movement has decreased. After the drainage has stopped, the large soil pores are filled with both air and water while the smaller pores are still full of water. At this stage, the soil is said to be at field capacity. At field capacity, the water and air contents of the soil are considered to be ideal for crop growth.
- $N\text{-}NO_3$: The three main sources of nitrogen in agriculture are urea, ammonium and nitrate. Most plants prefer nitrate ($N\text{-}NO_3$) to ammonium ($N–NH_4$). However, high $N\text{-}NO_3$ in soil and water systems is a cause of concern for human and environmental health.
- Organic carbon: Carbon is the chief element (48–58%) in SOM. Therefore, organic C determination is used as a basis of SOM estimates in soils.
- Total nitrogen and C/N ratio: Analysis of total N, C/N ratio, and inorganic N (ammonium, nitrate) provides an insight into the nitrogen supply to soil microflora and plants.

1.4.2.3. Chemical analysis

Chemical analyses required for the performance of this work have been requested to external institutions, who have applied specific extraction and analysis techniques in accordance with the type of contaminant and environmental matrix. Quality control is guaranteed by the contracted institutions through the application of the corresponding sample blanks and certificate reference materials. Solid soil samples are analyzed after undergoing the pretreatment process described in 1.4.2.1. Water samples for metal analysis are filtered through a 0.45 μm pore size cellulose nitrate membrane and acidified prior to sending for chemical determinations.

Metals and metalloids

Metals and metalloids in soil samples have been quantified through instrumental neutron activation analysis (INAA), inductively coupled plasma emission spectroscopy (ICP-OES), and atomic absorption spectroscopy (AAS). Metals and metalloids in water samples have been determined through inductively coupled plasma mass spectrometry (ICP-MS) and atomic absorption spectroscopy (AAS).

Organic compounds

Total petroleum hydrocarbons (TPH) in soils have been quantified through gas chromatography using a flame ionization detector (GC-FID) whereas their presence in the water compartment has been analyzed through Fourier transform infrared spectroscopy (FTIR). Pesticides in water extracts have been studied through high performance liquid chromatography-mass spectrometry (HPLC-MS).

1.4.2.4. Terrestrial ecotoxicity tests

Terrestrial laboratory tests are performed using whole soils. When dilution of a field soil is needed, it is performed by mixing the test soil with the standard artificial substrate recommended by ISO and OECD guidelines. This substrate, which also acts as control soil, consists of a mixture of 70% industrial sand (with more than 50% particles between 0.05 and 0.2 mm), 20% kaolinite clay, and 10% peat (ground and dry). After mixing, the pH of the substrate is adjusted to 6.0±0.5 through the addition of $CaCO_3$. The environmental conditions of the tests vary in accordance with the corresponding test requirements and the number of replicates per treatment is adjusted to the availability of sample. All soil bioassays are carried out at 40% to 60% of the water holding capacity of the test soil. EC50 and LC50 values are expressed either as the percentage of contaminated soil mixed with artificial soil (w/w) or as the concentration of test substance (mg kg^{-1}) in test soil.

Rearing and maintenance of test organisms

Earthworms *Eisenia fetida* are reared in 30-L plastic breeding boxes containing a 1:1 mixture of horse manure and peat. On a weekly basis, the pH (6 to 7) and moisture content (moist but not too wet) of the

breeding substrate is controlled and earthworms are fed with a mixture of oat and water. Breeding substrate is renewed every 4 to 6 months. Organisms are kept at 20±2 ºC and in the dark. Synchronized cultures are prepared by putting adult worms into fresh substrate and removing them after 14 to 28 days. Mature organisms (fully-developed clitellium; 3 to 12 months old) weighing between 300 and 600 mg are selected for the performance of the tests. Earthworms are acclimated in control soil for up to 72 hours prior to beginning the tests.

Collembolans from the species *Folsomia candida* are reared in 145/20 mm Petri dishes filled with a substrate of plaster of Paris and activated charcoal (8:1, w/w) to a height of approximately 10 mm. Deionized water is added to almost saturation. Moisture content is maintained weekly by supplying water with a pipette and individuals are fed twice a week with granulated dry yeast added in small amounts to avoid spoilage by fungi. Springtails are transferred to fresh substrate every two months. Organisms are kept in a climatic chamber with controlled temperature (20-22 °C) and relative humidity (65-70 %) and in the dark. Juvenile springtails of standard age (10 to 12 days old) are used in tests. Juveniles are obtained by placing a number of adult organisms in fresh substrate and allowing them to lay eggs. Adult springtails are removed once eggs have been laid and juveniles are used 12 days after hatching.

Earthworms, Acute Toxicity Tests

The objective of acute toxicity tests is to assess whether a substance causes the death of test organisms. These tests are useful for short-term identifications of highly toxic contaminants and as preliminary evaluations ("range-finding tests") to determine concentration ranges to be used in definitive acute toxicity tests and/or in sublethal tests. In this work, Acute Toxicity Tests with earthworms are adapted from the OECD 207 (1984) guideline.

Briefly, ten organisms per replicate are exposed to a range of concentrations of polluted soils (500 g dw per replicate) in plastic containers (140x140x80 mm). Test containers are kept under constant light (400-800 lux) at a temperature of 20±2 ºC. Mortality and biomass loss is assessed after 7 and 14 days of exposure. One concentration resulting in no mortality and one resulting in total mortality are usually included. When two consecutive concentrations result in 0 and 100% mortality, these two values are considered sufficient to indicate the range within which the LC50 fell (OECD 1984).

Earthworms, Reproduction Tests

Chronic toxicity tests are medium-term tests that measure sublethal effects of potentially toxic substances, such as changes in reproduction and growth. They are considered more suitable for assessing effects at population level (Hoffman et al. 2003; Van Gestel 2012). Reproduction tests with earthworms are adapted from the OECD 222 (2004) guideline, in which adult organisms are exposed to a range of sublethal concentrations of contaminated soil. Test concentrations are defined according to preliminary tests or to results from the literature. Shortly, 10 adult earthworms are placed per replicate in rectangular plastic containers (140x140x80 mm) filled with 500 g dw of the corresponding contaminated soil. Test vessels

are incubated in a controlled chamber at 20±2 ºC and under a 16:8 h light:dark cycle. Earthworms are fed dried horse manure (≈5 g per replicate) on a weekly basis. After 28 days of exposure, adult earthworms are removed by hand and the percentage of adult body biomass relative to the initial weight is calculated to assess the effects of contaminants on the growth of earthworms. Test containers are incubated for 28 additional days and, after 56 days from the beginning of the assay, the number of juveniles is counted to determine the effect of the treatment on the reproductive output of earthworms.

Earthworms and Collembolans, Avoidance Tests

Behavioral tests are based on the ability of animals to avoid potentially toxic substances (Hund-Rinke et al. 2003). Avoidance tests with soil invertebrates are gaining popularity due to their capability of providing preliminary and ecologically relevant responses to soil pollution after a shorter period of time relative to that of other toxicity tests (Cardoso and Alves 2012). However, the application of such tests is usually recommended alongside with acute and/or chronic toxicity tests because certain substances are known to cause high mortality rates without triggering an avoidance response (Yearcley et al. 1996; Heupel 2002). Avoidance tests with *E. fetida* and *F. candida* are adapted from ISO 17512-1 (2008) and ISO 17512-2 (2011) standards respectively. Rectangular (220x140x50 mm; test with earthworms) and round (diameter 8 cm, depth 8 cm; test with collembolans) plastic containers are divided into two equal compartments by a vertically introduced plastic divider. Each section of the container is filled with the corresponding soil (control or test; 250 g dw per section in tests with earthworms and 30 g wet weight in tests with collembolans respectively). The divider is then removed and ten adult earthworms or twenty adult collembolans are carefully placed on the line separating both soils. Test containers are covered with a transparent plastic lid and incubated for 48 hours in an environmental chamber at 20±2 ºC and under a 16:8 h light:dark photoperiod. At the end of the test period, the divider is reinserted and the number of individuals in each section is counted. In tests with collembolans, the soil from each section is carefully emptied into two different vessels and flooded with water. After gentle stirring, the animals floating on the water surface are counted. Results are expressed as percentage of individuals in the control section at the end of the test or as percentage avoidance according to the equation:

$$x = [(n_c - n_t) / N] \times 100$$

Where:

x = percent avoidance;

n_c = number of individuals in the control soil;

n_t = number of individuals in the test soil;

N = total number of individuals.

Additionally, a double control test is performed with control soil in both compartments to determine whether the organisms are randomly distributed between the two compartments in the absence of contaminants.

When applying avoidance tests, concerns exist if the control and test soils differ in other parameters than the presence of contaminants. In that case, statistical calculations to assess effects of contaminants only are not encouraged because soil properties can mask an effect of the pollutants and the application of fixed threshold for the assessment of the habitat function is recommended as alternative. Even so, in this work we apply both strategies (habitat function assessment and EC50 calculation) to contaminated field soils. We apply a diagnostic approach in which the field soil is confronted to a soil of known suitability (artificial control soil) and we estimate the tendency of organisms to avoid the test soil as a whole. Thus, EC50 estimates are expressed in terms of the percentage of test soil that cause 50% of avoidance, rather than focusing on the concentration of contaminants (mg kg^{-1}) corresponding to this percentage of test soil. In other words, what EC50 means in those cases is the suitability of the studied soil as a habitat, regardless of the parameters that determine its suitability (i.e. the presence of pollutants or specific physicochemical properties). The impact of differing soil properties is however reduced by the dilution of test soils with artificial control soils when preparing the selected test concentrations.

Plants, Seedling Emergence and Growth Tests

These tests are designed to assess effects on seedling emergence and early growth of higher plants following exposure to a contaminated soil. Tests are adapted from the OECD 208 (2006) guideline, which benefits from the higher sensitivity of plants during the first days of seedling growth and provides data as to whether a test substance or site soil either inhibits or enhances the growth of terrestrial plants. According to the aim of the study, the test can be conducted in order to determine the dose-response curve or at a single concentration/rate i.e. as a limit test. Several species of monocotyledon and dicotyledon plants have been historically used as tests organisms (OECD 2006). Among them, monocotyledon species *Lolium perenne* (perennial ryegrass) and dicotyledon species *Brassica rapa* (turnip) and *Trifolium pratense* (red clover) are selected. Briefly, groups of five seeds are sown in plastic containers containing 100 grams (wet weight) of soils from contaminated sites that have not been diluted (i.e. limit test). Tests are performed for up to 28 days in an environmental chamber at 24±2 ºC and under a 16:8 hours light:dark photoperiod (350±50 µE $m^{-2}s^{-1}$). The moisture content and the number of sprouts are checked daily. After the observation period, i.e. 14 to 21 days after 50% of emergence is detected in the controls, plants are harvested and weighed. Results are expressed as percentage of seed emergence and fresh biomass.

1.4.2.5. Aquatic ecotoxicity tests

In this work, aquatic bioassays are used to i) indirectly study soil contamination through the evaluation of the toxicity associated to water extracts from test soils and to ii) assess the risks that polluted soils may pose to the aquatic compartment. All aquatic tests are carried out without adjustment of pH and, when required, water samples are diluted with the corresponding test medium. The environmental conditions of the tests vary in accordance with the corresponding test requirements. Toxicity results are expressed as the percentage of water extract in test medium (V/V) reducing by 50% the endpoint measured (EC50, LC50 or IC50).

Rearing and maintenance of test organisms

Raphidocelis subcapitata is a unicellular, curved-shaped microalga. Due to its ubiquitous distribution, sensitivity to pollutants, and ease of culture under laboratory conditions, it is widely used as bioindicator species to assess nutrient levels or toxic substances in freshwater environments as well as a standard test organism in laboratory ecotoxicological tests (Geis et al. 2000). Cultures of *Raphidocelis subcapitata* are kept agitated (100 rpm) in a culturing shaking table under sterile conditions, constant illumination (4000-5000 lux) and temperature (20±2 ºC), and a pH between 6.9 and 7.2. Cultures are re-inoculated periodically in fresh medium so that only populations in the exponential phase are used in tests.

Daphnia magna is a freshwater cladocera widespread throughout the globe. It shows many desirable characteristics for test organisms to be used in ecotoxicological studies (Anderson 1944): representativeness, easy to culture, relatively short life span, reproduction by parthenogenesis, and high offspring. Bulk cultures of 15 daphnids are kept in plastic aquariums containing 2.5 liters of ASTM hard synthetic water as culture medium (pH 7.8-8)(ASTM 1988). Cultures are maintained at 20±2 ºC in a 16:8h light:dark cycle. Culture medium is changed three times per week and enriched with an organic extract. Additionally, a concentrate of *Chlorella vulgaris* is supplied as food. Neonates are removed daily. Only neonates from the second brood and onwards and less than 24 hours old are used for toxicity testing.

Fish species *Danio rerio* is a very common and useful model organism that has contributed to advances in fields like developmental biology, oncology, toxicology, ecotoxicology and many others since the decade of 1970s. Several characteristics make the zebra fish an ideal model organism for environmental assessment: sensitivity to contaminants, small size, ex-utero development of the embryo, short reproductive cycle, and transparent embryos (Dai et al. 2014). *D. rerio* are supplied by P&S Piscicultura Superior SL (Barcelona, Spain). Individuals with the same age and between 2 and 4 cm long are kept at 21-24 ºC under a 16:8 light/dark photoperiod and 80% of air saturation for at least 12 days before starting the test. Organisms are fed daily with commercial fish food until 24 h before starting the test.

Bacteria, Luminescence Inhibition Test

This test is based in the inhibition of the light emitted by the bioluminescent bacteria *Vibrio fischeri* after exposure to a toxic sample. Due to its short duration, simplicity and reproducibility, this assay is widely used in routine screening of water bodies as well as in the ecotoxicological assessment of toxic substances in different substrates such as water, air, soils and sediments (USEPA 2014). *V. fischeri* are globally-spread marine bacteria with bioluminescent properties i.e. in optimal conditions (pH 7 to 7.2, saline environment) they emit light as a result of their cellular respiration. The emitted light can be measured and a decrease in the light output means that bacteria have been negatively affected by the sample. In this work, *V. fischeri* bioluminescence inhibition is evaluated using the Microtox® *in vitro* testing system in accordance with the ISO 11348-3 (2007) standard. Analyses are performed in a temperature-controlled, self-calibrating photometer Model 500 Analyzer (SDI, USA). In short, freeze-dried bacteria are reconstituted and incubated in test medium at 15 ºC. After incubation, light emission is measured and bacteria are exposed for 15 minutes to different concentrations of test sample. Light emission is measured again at the end of the exposure period and differences in light output are used to calculate the toxicity of the sample.

Algae, Growth Inhibition Tests

Tests with algae are performed in accordance with the OECD 201 (2011) guideline. This test is based in the reduction of *Raphidocelis subcapitata* growth rate as a response to the exposure to toxic samples. A major benefit of this test is that, in spite of the relatively brief duration, effects over several generations can be assessed. Assays are carried out in tubes containing 9 mL of test solution (test medium plus the corresponding volume of test sample) and 1 mL of algal inoculums of known concentration. Tubes are placed in a controlled room at 20±2 ºC and under constant illumination (4000-5000 lux) and agitation. After 72 hours of incubation, the absorbance of each replicate is measured at 665 nm with a CECIL CE9200 spectrophotometer and algal concentration is estimated. When testing aqueous extracts from soils, interferences in the spectrometric measure due to the presence of suspended particles are eliminated by measuring the absorbance of each test solution without the addition of algae. Algal growth in each replicate is calculated and compared with the growth in controls. Results are expressed as percentage of algal growth inhibition.

Daphnia sp., Acute Immobilization Tests

For many years, the cladoceran *Daphnia magna* has been used as a standard aquatic test species. Chronic and acute tests with *D. magna* are among the most frequently performed studies in aquatic toxicology (Martins et al. 2007). Acute tests with *D. magna* are performed according to the indications of the OECD 202 (2004) guideline. Briefly, assays are carried out in glass tubes containing 10 mL of test solution (test medium plus the corresponding volume of test sample) and 5 daphnids. Test vessels are kept in an

incubator at 21±2 ºC and in the dark. Immobilization is visually recorded after 24 and 48 hours of exposure and compared with control values. Mortality at the end of the test is expressed as a percentage.

Fish, Lethality Tests

Acute tests with *D. rerio* are adapted from the OECD 203 (1992) guideline. In short, seven individuals are placed in 5-L aquariums containing 3 L of test medium (1 g fish L^{-1}). Dissolved oxygen is kept above 60% of air saturation and a 16:8-h light/dark photoperiod and a temperature of 20-24 ºC are set. Test organisms are not fed during the assay and mortality is recorded after 24, 48, 72 and 96 hours of exposure. Mortality at the end of the test is expressed as percentage.

1.4.3. Statistical analysis

Statistical analysis is performed using SPSS software (SPSS 15.0 for Windows; SPSS Inc., Chicago, IL, USA), Minitab Statistical Software (Minitab 15.0; Minitab Inc., State College, PA, USA) and STATISTICA software (STATISTICA 8.0; OK, USA).

Data are checked for their homogeneity of variances and normality. Differences between means are tested with one-way ANOVA. Whenever significant differences are found ($P < 0.05$), Tukey and/or Dunnett post hoc tests are applied to further elucidate differences. Non-normal data are log-transformed. When the assumption of normality is not reached, non-parametric Kruskall-Wallis tests alongside with Mann-Whitney post hoc tests are performed.

Median effective, lethal, and inhibitory concentrations (EC50, LC50, and IC50 respectively) and their 95% confidence intervals are calculated by Probit regression using appropriate distribution models. Estimated values are compared using the Confidence Interval Ratio Test recommended by Wheeler et al. (2006)

The significance of avoidance responses is tested with Fisher's exact test using a two-tailed test for the double control conditions and a one-tailed test for the contaminated soil combination conditions (Zar 1999).

Relationships between parameters are examined through scatterplots. Pearson and Spearman correlations are used to measure linear and monotonic relationships respectively. Complex combinations of variables are analyzed by Principal Component Analysis (PCA), which facilitates the reduction, transformation and organization of the original data creating a new set of uncontrolled variables which are the linear combinations of the original ones.

CHAPTER II. Geochemistry and environmental threats of soils surrounding an abandoned mercury mine[1]

[1] Published in *Environmental Science and Pollution Research* (2016) In press.

Abstract

The closure of mercury mining areas is generally associated with a release of Hg and other metals into the environment due to the abandonment of mining wastes. Because of their potential toxic properties, the mobilization of particulate and soluble metal species is of major concern. In the present study, the environmental risks posed by soils surrounding an abandoned mercury mining area in Valle del Azogue (Almeria, Spain) are assessed through the determination of physical-chemical parameters, the quantification of metal concentrations, and the application of aquatic and terrestrial ecotoxicity bioassays. Chemical analysis of soil samples revealed concentrations of Hg, As, Ba, Pb, Sb and Zn above international intervention values. Results from terrestrial tests showed detrimental effects in all studied organisms (*Eisenia fetida*, *Folsomia candida* and different plant species) and revealed the avoidance response of earthworms as the most sensitive endpoint. Surprisingly, the most toxic samples were not the ones with higher metal contents but those presenting higher electrical conductivity. Aquatic ecotoxicity tests with *Vibrio fischeri*, *Raphidocelis subcapitata*, *Daphnia magna* and *Danio rerio* were in accordance with terrestrial tests, confirming the need to couple environmental chemistry with ecotoxicological tools for the proper assessment of metal-contaminated sites. In view of the results, a remediative intervention of the studied area is recommended.

2.1. Introduction

One of the main deleterious effects of already closed mines is usually associated with the abandonment of large volumes of wastes (Dudka and Adriano 1997). Tailings formed during the processing of the mineral ore are frequently stored in steep stock piles where they are prone to erosion (Henriques and Fernandes 1991), thus becoming a potential source of pollution to the surrounding environment. Such residues are frequently dispersed by atmospheric emissions, mechanical dispersion or water-leaching from waste deposits (Johnson et al. 1994; Adriano 2001) and are likely to contaminate soils, ground waters, surface waters and stream sediments of the surrounding area. In this context, one of the worst scenarios can occur if the dispersed residues reach agricultural or urban soils and expose humans to heavy metals either directly by suspended dust in air, or indirectly, by transfer into the food chain (Torres and Johnson 2001).
In SE Spain, the Valle del Azogue mine was the main mercury mine in the Betic Range during the 19th century. It was active approximately between 1873 and 1890 and produced about 1000 tons of Hg by means of underground works and small open pits located near two smelter sites. The only existing references to this deposit are by Cortazar (1875) and Becker (1888), who reported the presence of Hg mineralization associated with exhalative deposits. Cinnabar (HgS) was the main ore although high contents of Sb, As, Au, Ag, Pb, Zn and Ba were also reported in the mineralized veins (Navarro et al. 2006). Calcines and secondary Hg and Fe minerals (mainly metacinnabar and Fe oxides) produced during the roasting of the mineral ore were dumped near the metallurgical facilities, where they have become a

potential source of particulate and soluble Hg species (Rytuba 2005) that might be transported as Hg^0 vapor (Navarro et al. 2000; Gustin et al. 2002), ionic soluble phases or colloid particles (Shaw et al. 2001; Lowry et al. 2004).

The environmental risks of metal-contaminated sites were traditionally assessed by means of chemical analysis of metal concentrations and the subsequent comparison with values from quality guidelines. More recently, it was concluded that chemical extractions of metals from multi-contaminated soils did not provide enough information about their bioavailable fractions (Alexander 2000; Ehlers and Luthy 2003; Semple et al. 2004; Harmsen 2007) and were not able to reflect the toxicity of all substances in soil, their synergic and antagonistic effects and their interactions with the soil matrix and organisms (Gruiz 2005). In this context, the application of batteries of terrestrial ecotoxicity tests gained special relevance as complementary, inexpensive, simple and quick tools able to report realistic and non-overestimated effects of contaminated sites to soil organisms (Leitgib et al. 2007; Alvarenga et al. 2008; Maisto et al. 2011; Alvarenga et al. 2012; Agnieszka et al. 2014; Bes et al. 2014; Bori and Riva 2015, Bori et al. 2015). At the same time, aquatic bioassays traditionally applied for the toxicity determination of aquatic pollutants (Lopez-Roldan et al. 2012), industrial effluents (Riva et al. 1993; Riva and Valles 1994; Riva et al. 2007) or extracts of sediments (Pereira-Miranda et al. 2011) were incorporated to assess the impacts of soil composition and run-off on receiving waters (Loureiro et al. 2005a; Rocha et al. 2011).

The first environmental concerns about the Valle del Azogue mine and its residues were reported by Martínez et al. (1998), Viladevall et al. (1999) and Navarro et al. (2000), who documented the release of Hg vapor into the atmosphere through volatilization as well as the transport of metallic Hg^0 contained in the underground mineralization, soils and mine wastes (calcines, low ore stockpiles and slags). The natural release of Hg into the atmosphere facilitated a near-surface deposition of Hg^0 in soils and sediments, which was added to the Hg^0 accumulated from the furnaces. The threats posed by this area due to the high contents of heavy metals and their potential mobilization were confirmed by geochemical studies (Navarro et al. 2006; Navarro et al. 2009a). Despite those potential threats, to date the risk assessment of the area has only been performed through chemical and mineralogical analysis.

With this in mind, the major purpose of this work was to assess the risk that the area surrounding the Valle del Azogue mine poses to the environment due to the presence of mercury and other metals. To attain this goal, this study aimed the following: (1) to characterize the area by means of physicochemical and mineralogical determinations; (2) to quantify metal concentrations in soils and in their water extracts; (3) to study the toxicity of the area through the application of aquatic and terrestrial ecotoxicity tests and (4) to establish relationships between physicochemical parameters, metal contents and toxicity to organisms.

2.2. Methodology

2.2.1. Study area and sampling sites

Samples of soils and mine wastes were collected in the Valle del Azogue mine (SE Spain). The sampling area, comprising the North of Sierra Almagrera, is located 90 km NE of the city of Almería, in a semi-arid and intensively cultivated region (Figure 1).

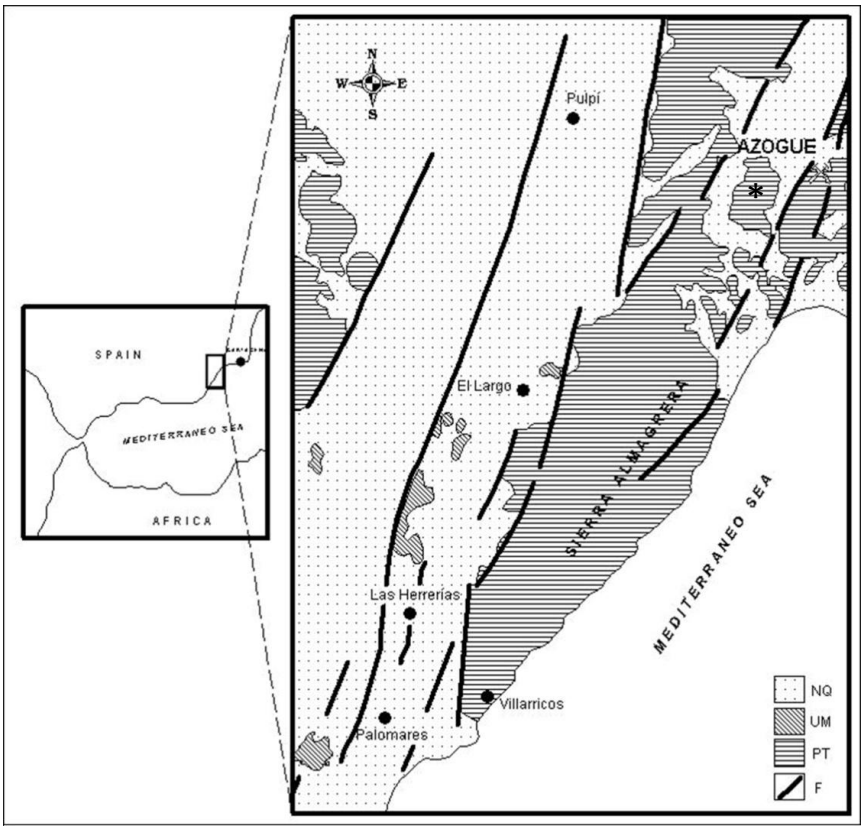

Figure 1. Location map and synthetic geology of the study area. NQ: Quaternary and Tertiary sediments; UM: Volcanic tertiary rocks ; PT: Metamorphic basement; F: Main fractures; *: Study area. Adapted from Navarro et al. (2009).

Due to mining and metallurgical activities, plants have disappeared from the area or have been severely affected by high contents of mercury and other metals (Viladevall et al. 1999). The main ore is composed of stibnite, cinnabar, arsenic minerals (realgar and orpiment), sphalerite, siderite, chalcopyrite, pyrite, quartz, calcite and barite (Navarro et al. 2006). Together with the Iberian Pyrite Belt and the Cartagena mining district, this abandoned mining area is one of the oldest metallurgical and mining areas in the Iberian Peninsula (Navarro et al. 2006).

Samples were collected from seven sites spread throughout the mining district in order to have a representative characterization of the area (Figure 2). Samples A1, A2 and A6 comprised soils mixed with mining wastes originated by ore extraction and located close to the main open pits. Samples A4, A5 and

A7 were soils mixed with calcines derived from metallurgical ore processing and located near the main furnace location. Sample A3 consisted in an anthropogenic soil.

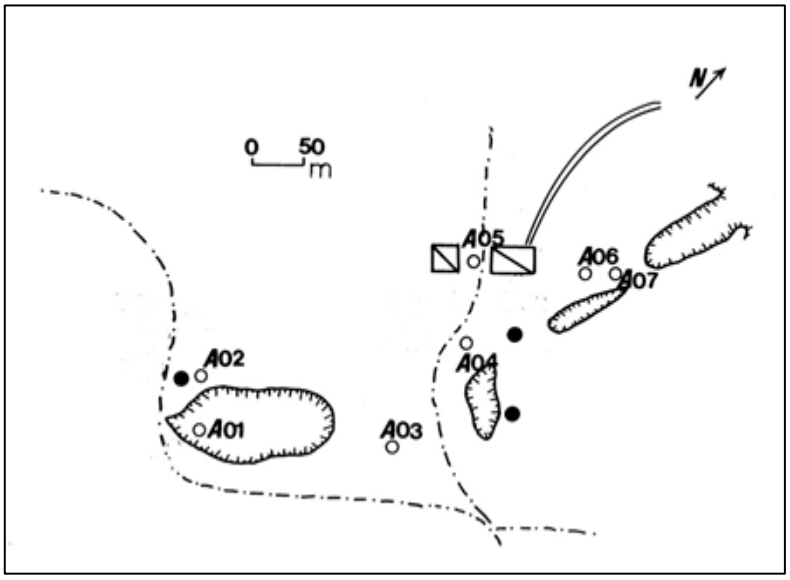

Figure 2. Sampling sites. White circles: Sampling points; Black circles: Mine shafts; Striped figures: Open pits. Adapted from Navarro et al. (2009).

2.2.2. Soils and mine wastes sampling and analysis

Soil samples were collected and pretreated as previously specified. The following parameters were evaluated: pH, electrical conductivity, soil organic matter, pore size distribution, porosity, bulk density, and field capacity. Additionally, sulphur and calcium contents were determined by Actlabs (Ontario, Canada) through Total Digestion-Induced Coupled Plasma (TD-ICP).

Samples for chemical analysis were sent to Actlabs (Ontario, Canada) for metal quantification. Au, Ag, As, Ba, Br, Ca, Ce, Co, Cr, Cs, Eu, Fe, Hf, Hg, Ir, La, Lu, Na, Ni, Nd, Rb, Sb, Sc, Se, Sm, Sn, Sr, Ta, Th, Tb, U, W, Y and Yb were quantitatively analyzed by instrumental neutron activation analysis (INAA) and Mo, Cu, Pb, Zn, Ag, Ni, Mn, Sr, Cd, Bi, V, Ca, P, Mg, Tl, Al, K, Y and Be were analyzed by inductively coupled plasma emission spectroscopy (ICP-OES). Hg phases were determined by solid-phase-Hg-thermo-desorption (SPTD) based on the specific thermal desorption or decomposition of Hg compounds from solids at different temperatures (Biester and Scholz 1997; Navarro et al. 2006). Mercury thermo-desorption curves were determined by means of an in-house apparatus, consisting of an electronically controlled heating unit and an Hg detection unit. Measurements were carried out at a heating rate of 0.5 °C s^{-1} and a nitrogen-gas flow of 300 mL min^{-1}. The lowest level of detection under the given conditions is in the range of 40-50 ng if all Hg is released within a single peak (Biester and Scholz 1997). Results are depicted as Hg thermo desorption curves (Hg-TDC) that show the release of Hg^0 versus temperature. Mine wastes samples were studied using transmitted and reflected light microscopy, X-ray diffraction (XRD) and scanning electron microscopy (SEM) with an attached Energy Dispersive X-Ray

Spectroscopy system (EDS) at the Electronic Microscopy Laboratory of the Universitat Autònoma de Barcelona.

2.2.3. Water extracts collection and analysis

Water extracts from each test soil were obtained as previously specified. The physicochemical analysis of the samples included measurements of pH, electrical conductivity and total organic carbon. A subsample of each water extract was sent to Actlabs (Ontario, Canada) for metal quantification through inductively coupled plasma mass spectrometry (ICP-MS).

2.2.4. Terrestrial ecotoxicity tests

E. fetida acute toxicity tests

Each test ran with 6 concentrations (1-10-18-32-57-100%) plus a control and three replicates per treatment.

Avoidance tests with E. fetida and F. Candida

Tests with earthworms ran with 5 concentrations (1-3.1-10-31-100%) plus a control and three replicates per treatment whereas 4 concentrations (17-31-56-100%) plus a control and 5 replicates per treatment were prepared for collembolans. Due to the high toxicity of sample A6, both assays required lower test concentrations (1-1.5-2-2.5-3.1% for earthworms and 10-17-31-56% for collembolans).

Seedling emergence and growth tests

Twenty seeds of the corresponding plant species were sown in each test soil and in the control artificial soil (four replicates per soil i.e. 5 seeds per test container).

2.2.5. Aquatic toxicity tests

Bacteria luminescence inhibition tests

Test organisms were exposed to 4 concentrations of water extracts (5.63-11.25-22.5-45%). Three replicates per treatment were measured.

Algal growth inhibition tests

Tests ran with three replicates per treatment and 7 concentrations (0.1-0.32-1-3.2-10-32-90%) plus a control that consisted in algae culture medium. Lower test concentrations (0.001-0.0032-0.01-0.032-0.1%) were applied to the sample A4 due to its high toxicity.

Daphnia magna acute immobilization tests

Daphnids were exposed to 7 dilutions of water-extracts (1-2.2-4.8-10-22-48-100%) plus a control in four replicates per treatment. Lower test concentrations were required for samples A4 (0.01-0.022-0.048-0.1-0.22-0.48-1%) and A6 (0.82-1-1.3-1.7-2.2-2.9%).

Fish, acute toxicity tests

Four concentrations (10-22-48-100%) were tested with most samples except for A2, A4 and A6, which required additional lower test concentrations (1-2.2-4.8-10-22-48-100%).

2.3. Results and Discussion

2.3.1. Physicochemical parameters and geochemistry of soils and mine wastes

Soil physicochemical characteristics markedly varied between sites (Table 1). Samples A2 and A4 were very strongly acidic (pH < 3.9) while the remaining samples presented pH values closer to neutrality (6.83 to 7.55). Electrical conductivity ranged from moderate (0.59 mS cm^{-1} in A5) to rather high values (8.25 mS cm^{-1} in A6). Organic matter contents remained below 10% (5.20 to 9.82%), which corresponds to the contents usually found in mineral soils. Soil pore sizes (in terms of equivalent diameter) ranged from 0.8 mm in soil A5 to 3.4 mm in A1, revealing that the studied soils were largely comprised of a sandy material fraction. The coarser sample A1 was associated to mining wastes and, possibly, to overburden ore deposit. Average values of porosity, bulk density and field capacity of the sampled area were 0.40, 1450 kg m^{-3} and 8% respectively. Sulphur content ranged from 0.28% (A7) to 2.81% (A5) while that of calcium ranged from 0.12% (A7) to 7.33% (A5).

	A1	A2	A3	A4	A5	A6	A7
pH	6.84 ± 0.06c	2.90 ± 0.01a	7.35 ± 0.03cd	3.83 ± 0.09b	7.55 ± 0.14d	6.83 ± 0.05c	7.33 ± 0.18cd
EC	1.14 ± 0.04b	2.47 ± 0.03cd	2.28 ± 0.07c	2.64 ± 0.04d	0.59 ± 0.04a	8.25 ± 0.08f	4.75 ± 0.04e
SOM	6.40 ± 0.09bc	9.82 ± 0.12f	7.35 ± 0.20d	6.81 ± 0.09cd	5.42 ± 0.25a	5.88 ± 0.16ab	5.20 ± 0.06a
d_e	3.4	1.8	1.0	0.8	0.5	1.9	1.3
Porosity	-	0.34	0.45	-	-	-	0.41
BD	1330	1430	1310	1410	1470	1400	1490
FC	8	8	10	10	7	8	8
S	1.8	2.05	0.47	2.02	2.81	1.49	0.28
Ca	1.34	0.27	2.49	3.09	7.33	2.28	0.12

Table 1. Physical-chemical characteristics (mean ± sd; N=3 when possible) of sampled soils. Values within the same row followed by the same letter are not significantly different ($P > 0.05$). EC: electrical conductivity (mS cm^{-1}); SOM: soil organic matter (%); d_e: equivalent diameter (mm); BD: bulk density (kg m^{-3}); FC: field capacity (%); S: Sulphur content (%); Ca: calcium content (%).

Total contents of most studied metals were extremely high in the sampled soils, calcines and mining wastes (Figure 3), and were similar to values reported by Navarro et al. (2000) and Navarro et al. (2006) for the same area. Contents of As ranged from 142 mg kg^{-1} dw (A3) to 1550 mg kg^{-1} (A4) and exceeded up to two orders of magnitude the intervention values for soil remediation of Dutch regulations (55 mg kg^{-1})(VROM 2000). Ba presented the highest concentrations among the studied metals and its values ranged from 7350 mg kg^{-1} (A3) to 110000 mg kg^{-1} (A1), thus exceeding by three orders of magnitude the Dutch values for soil remediation (625 mg kg^{-1}). Hg concentrations markedly varied between sites. Total Hg contents were below 25 mg kg^{-1} in A5 and reached 4000 mg kg^{-1} in A1, exceeding the Dutch regulations values (10 mg kg^{-1}) by different orders of magnitude depending on the site. Similarly, Sb presented varying concentrations (from 357 mg kg^{-1} in A3 to more than 10000 mg kg^{-1} in A4 and A5) and a low intervention value for soil remediation (15 mg kg^{-1}) that was markedly exceeded in all soils. Pb and Zn were the only metals whose intervention values (530 mg kg^{-1} and 720 mg kg^{-1} respectively) were not surpassed in all sites. The lowest Pb contents were quantified in A3 (134 mg kg^{-1}) while the highest were determined in A5 (1820 mg kg^{-1}). The lowest Zn concentration was also quantified in A3 (424 mg kg^{-1}) while the highest exceeded the intervention value by one order of magnitude in A2 (3190 mg kg^{-1}).

The studied soil samples presented physical-chemical characteristics typical from tailings usually found surrounding mining sites: neutral to acidic pH, high EC, low fertility and high total concentrations of heavy metals (Conesa et al. 2006; Navarro et al. 2008; Carmona et al. 2009). The low pH of soils A2 and A4 was explained by pyrite oxidation and could explain their high Zn contents, which showed a negative significant correlation with soil pH ($r = -0.96$, $P < 0.01$). Thus, the possible main hydrogeochemical reactions associated to sulphide oxidation are pyrite and sphalerite oxidation:

$$FeS_2 + 7/2\ O_2 + H_2O \rightarrow Fe^{2+} + 2\ SO_4^{2-} + 2\ H^+ \quad [1]$$

$$ZnS + 8\ Fe^{3+} \rightarrow Zn^{2+} + 8\ Fe^{2+} + SO_4^{2-} + 8\ H^+ \quad [2]$$

Such low pH represented one of the main threats of the area since it can lead to the solubilization of metals and consequently to the spread of contamination towards the water compartment (Navarro Flores and Martínez Sola 2010). The samples with lower pH (A2 and A4) showed high S contents, possibly associated with arsenian pyrite, whereas samples with the highest pH value (A3 and A5) showed high Ca contents that could be associated with significant amounts of calcite in the soil. Additionally, statistically significant positive correlations were found between Sb concentrations and As ($r = 0.97$, $P < 0.01$), Ba ($r = 0.93$, $P < 0.05$) and Hg ($r = 0.97$, $P < 0.01$) contents, which might be indicative of a common origin. The particular structure of the soils comprising the study area was another major cause of concern since the high percentage of sand and the absence of a proper soil structure due to the high presence of mine tailings and wastes can further increase the leaching of heavy metals (Conesa et al. 2006).

Hg-thermodesorption curves (Hg-TDC) of mining wastes, soils and calcine samples showed predominant release of Hg in two temperature ranges: 200-250 ºC and 300-330 ºC. The first temperature range was assigned to a release of Hg from the soil matrix components based on the Hg-TDCs of standard materials

(Biester and Scholz 1997). Thus, we assume that most Hg present in the calcine material is bound to mineral components mainly by iron oxides, which were formed when the cinnabar-bearing ore was being roasted. Earlier studies already suggested that Hg^0 formed during thermal breakdown of cinnabar is re-condensed during cooling of the material and adsorbed to iron oxide surfaces (Biester et al. 2000). In addition to matrix-bound Hg, some calcine samples contained traces of cinnabar. This could be explained by an incomplete breakdown of cinnabar ore during the roasting process. The second temperature range was assigned to Hg release from cinnabar, which was the predominant Hg mineral in contaminated soils and mining wastes (host rock and low grade stockpiles). Cinnabar and Hg sulphates were also detected in several samples (Navarro et al. 2006). No free metallic Hg, which is typically released at temperatures below 100°C, was found in any of the samples studied.

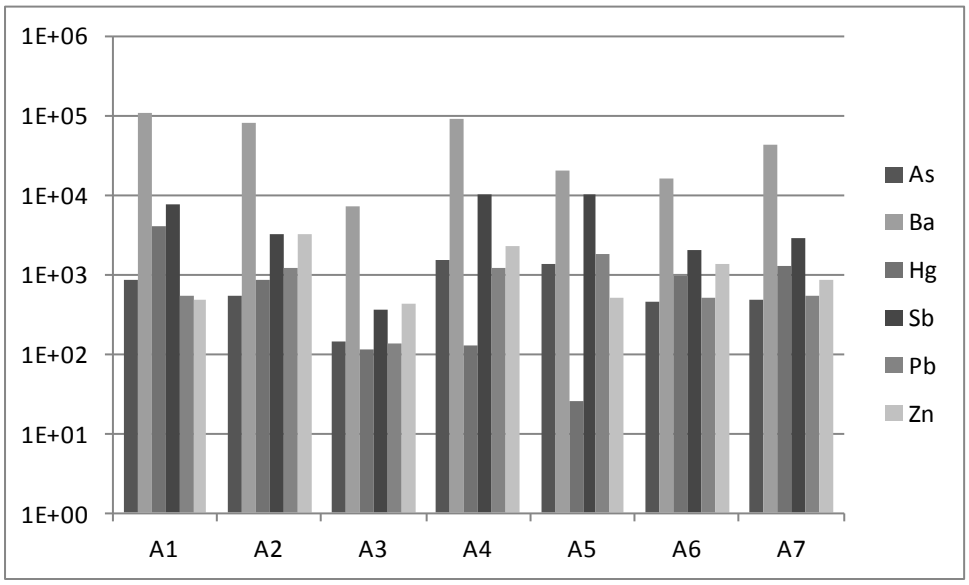

Figure 3. Total metal concentrations (mg kg^{-1} dry weight) in test soils.

Mercury phase characterization by X-ray showed the presence of cinnabar (HgS), corderoite ($Hg_3S_2Cl_2$), laffittite ($AgHgAsS_3$), metacinnabar ((Hg)S,), shakhovite ($Hg_4SbO_5(OH)_3$), schuetteite ($Hg_3(SO_4)O_2$) and tiemannite (HgSe)(Table 2). The proportionally Hg predominant phase was cinnabar, which was concordant with the SPTD analyses. The detailed SEM and EDS systems study of mine wastes samples showed the presence of primary and secondary cinnabar associated with barite, pyrite and botryoidal pyrite. Also, SEM observations showed several small particles containing both Hg and Cl that may be associated to calomel (Hg_2Cl_2). Moreover, some particles containing both Hg and Br were observed and may be associated to kuzminite ($Hg_2(Br,Cl)_2$)(Navarro et al. 2009b; Navarro et al. 2012). Additionally, main minerals in the gangue were quartz, barite and silicates.

	Minerals		Secondary minerals	
Calcines	Quartz*[a]	SiO_2	Hematite*	Fe_2O_3
	Barite*[a]	$Ba(SO_4)$	Hg^0	Hg
	Illite*[a]	$KAl_2Si_3AlO_{10}(OH)_2 \cdot 3H_2O$		
	Calcite*	$Ca(CO_3)$		
	Cinnabar	HgS		
	Orthoclase	$K(Al, Fe)Si_2O_8$		
Mineralization, wastes and soil	Quartz*[b]	SiO_2	Hg^0	Hg
	Barite*[b]	$Ba(SO_4)$	Goethite	FeOOH
	Cinnabar-Metacinnabar*	HgS	Jarosite	$KFe_3(SO_4)_2(OH)_6$
	Dolomite*[b]	$CaMg(CO_3)_2$	Hematite	Fe_2O_3
	Calcite*[b]	$Ca(CO_3)$	Inyoite[b]	$CaB_3O_3(OH)_5 \cdot 4H_2O$
	Huntite*[b]	$Mg_3Ca(CO_3)_4$	Ferrihydrite	$Fe(OH)_3$
	Stibnite*	Sb_2S_3	Kaolinite	$KAl_2Si_3AlO_{10}(OH)_2 \cdot 3H_2O$
	Realgar*	AsS	Gypsum	$Ca(SO_4) \cdot 2H_2O$
	Oripment	As_2S_3	Schuetteite	$Hg_3(SO_4)O_2$
	Calcopyrite	$CuFeS_2$	Tiemannite	HgSe
	Arsenian pyrite*	$Fe(S_{1-x}As_x)_2$	Corderoite	$Hg_3S_2Cl_2$
	Esfalerite	ZnS	Shakhovite	$Hg_4SbO_5(OH)_3$
	Orthoclase	$K(Al, Fe)Si_2O_8$	Calomel	Hg_2Cl_2
	Au	Au	Kuzminite	$Hg_2(Br, Cl)_2$
	Illite*[b]	$Al_4(Si_4O_{10})(OH)_8$		

Table 2. Minerals identified by DRX in the Valle del Azogue soil and mine wastes. Modified from Navarro et al. (2012). *: high-medium abundant minerals. [a]: detected by DRX in calcines; [b]: detected by DRX in mining wastes

2.3.2. Analysis of water extracts

Data from water extracts are presented in Table 3. Similarly to soils, pH differed significantly from the strong acidity of samples A2 and A4 to the neutrality of the rest. All sampling sites were clearly differentiated by the salinity of their extracts, with the highest values determined in samples A6 (5.29 mS cm^{-1}) and A7 (3.53 mS cm^{-1}). Organic matter content in soils was not mirrored in their water extracts, where the lowest value of total organic carbon was determined in sample A5 (1.36 mg L^{-1}) and the highest in the sample A3 (6.47 mg L^{-1}).

	A1	A2	A3	A4	A5	A6	A7
pH	6.90 ± 0.08c	3.08 ± 0.02a	7.14 ± 0.12cd	4.18 ± 0.06b	7.46 ± 0.05d	7.58 ± 0.04d	8.16 ± 0.14e
EC	1.57 ± 0.01a	2.70 ± 0.02d	2.07 ± 0.01b	2.51 ± 0.03c	2.01 ± 0.01b	5.29 ± 0.03f	3.53 ± 0.02e
TOC	1.48 ± 0.02a	2.28 ± 0.04c	6.47 ± 0.06f	1.95 ± 0.02b	1.36 ± 0.08a	4.16 ± 0.02e	2.54 ± 0.12d
As	571	69.4	25.9	4.83	5.53	4.61	4.84
Ba	79.2	111	140	87.4	280	306	304
Hg	2.1	2.8	0.3	42.9	19.1	1220	28.7
Sb	154	24.1	24.4	32.3	78.7	62.5	68.4
Pb	0.22	297	1.68	199	1	1.17	0.6
Zn	27.4	>5000	871	>50000	1040	345	181

Table 3. Physical-chemical characteristics (mean ± sd; N=3) and total concentrations of metals (in µg L^{-1}) in water samples extracted from test soils. Values within the same row followed by the same letter are not significantly different ($P > 0.05$). EC: electrical conductivity (mS cm^{-1}); TOC: total organic carbon (mg L^{-1}).

Metal concentrations in water extracts markedly varied depending on the metals and samples. With the exception of Zn, metal concentrations in all extracts represented less than 1% of their soil contents, thus revealing the low concentration of water-soluble metal species in soils. In the case of Zn, the average recovery rate in water extracts was 4%. Even so, high metal concentrations were detected in water extracts due to their extremely high contents in test soils. Arsenic, mercury and zinc concentrations were markedly higher in samples A1 (571 µg L^{-1}), A6 (1220 µg L^{-1}) and A4 (> 50000 µg L^{-1}) respectively. Ba and Sb contents were similar among samples, with concentrations that ranged between 79.2 µg L^{-1} (A1) and 306 µg L^{-1} (A6) for Ba and between 24.1 µg L^{-1} (A2) and 154 µg L^{-1}(A1) for Sb. Lead concentrations markedly varied from 0.22 µg L^{-1} in soil A1 to 297 µg L^{-1} in soil A2. The acidity of the extracts A2 and A4 was associated to their markedly higher contents of Pb and Zn, whose concentrations were negatively correlated ($r = -0.977$, $P < 0.01$ for Pb and $r = -0.572$, $P = 0.18$ for Zn) with pH of aquatic samples. At the same time, soluble Hg was found responsible of the higher electrical conductivity of extracts A6 and A7 ($r = 0.88$, $P < 0.01$) whereas As and Sb contents correlated as in soil samples ($r = 0.83$, $P < 0.05$).

Pb concentrations in the extracts A2 and A4 were associated with high amounts of lead in soils (Figure 3), which was possibly originated by galena weathering:

$$PbS + 8Fe^{3+} + 4H_2O \rightarrow 8\ H^+ + SO_4^{=} + Pb^{2+} + 8Fe^{2+} \qquad [3]$$

The higher concentrations of Zn in water samples A2 and A4 were also associated with high contents of Zn in soils. Zn may be mobilized by sphalerite weathering [2]. Higher As concentrations in water extracts (samples A1 and A2) was associated with moderate As contents in soils (Figure 3), while high Sb concentrations (samples A1 and A5 to A7) could be associated with elevated contents of Sb in soil (Figure 3). Thus, the mobilization of Sb in water extracts could be originated by stibnite weathering.

2.3.3. Ecotoxicological evaluation

Terrestrial and aquatic ecotoxicological bioassays presented different sensitivities depending on the test endpoints and organisms. Within terrestrial assays, *E. fetida* mortality test showed the lesser sensitivity, was unable to estimate median lethal concentration values for soils A1 and A5 and provided the highest EC50s (LC50) (i.e less toxicity detected)(Table 4). Despite its low sensitivity, the mortality of *E. fetida* was significantly and positively correlated with sublethal effects (avoidance response) observed in earthworms ($r=0.833$; $P < 0.05$) and collembolans ($r=0.838$; $P < 0.05$). In contrast, the behavioral test with *E. fetida* presented an extreme sensitivity, was able to estimate median effective concentration values for all soils and provided EC50s as low as 0.33% (sample A6). According to Hund-Rinke and Wiechering (2001), all tested soils should be considered to have a limited habitat function because the percentage of earthworms in control sections was higher than 80% at the end of the tests. Results from avoidance tests with earthworms were in agreement with previous studies that highlighted the higher sensitivity of sublethal endpoints in general (Hund-Rinke et al. 2002; Davies et al. 2003) and of avoidance tests with earthworms in metal-contaminated soils in particular (Alvarenga et al. 2012). EC50s

estimated for avoidance tests with earthworms were significantly and positively correlated (r = 0.984, $P < 0.01$) with those from collembolans, thus indicating the potential and suitability of behavioral responses as endpoints in terrestrial ecotoxicology. However, sensitivity differed between species (Table 4) and only four soils (A2, A3, A6 and A7) showed a limited habitat function when soil arthropods were used as test organisms. Thus, our study points out the higher sensitivity of avoidance tests with earthworms, which is in agreement with results from Hentati et al. (2013) and Da-Luz et al. (2012) after exposing earthworms and collembolans to soils contaminated with petroleum compounds and the pesticide diazinon respectively.

	E. fetida Acute Toxicity	*E. fetida* Avoidance Behavior	*F. candida* Avoidance Behavior
A1	-	19.61 (14.13-27.20)	-
A2	74.54 (-)	5.29 (3.63-7.70)	17.93 (12.02-26.75)
A3	61.92 (49.17-83.91)	4.04 (2.91-5.63)	38.33 (26.48-55.48)
A4	74.54 (-)	20.92 (15.33-28.55)	75.65 (44.54-128.48)
A5	-	79.60 (44.41-142.66)	-
A6	16.00 (12.51-22.34)	0.33 (0.24-0.45)	4.21 (3.40-5.21)
A7	24.48 (18.43-32.13)	4.95 (3.54-6.92)	42.43 (27.42-65.66)

Table 4. LC50s and EC50s (95% confidence intervals) of terrestrial ecotoxicity tests with soil invertebrates. Results expressed as percentage weight of soil sample mixed with artificial soil (w/w). '-': non-applicable.

Seed germination and growth rates in test soils are presented in Figure 4. Emergence and growth of the three studied species was totally inhibited in soils A2, A4, A6 and A7, which was expected due to the absence of a plant cover in the sampling area. It is important to emphasize that toxic effects of soils contaminated by mining tailings should not be exclusively associated to the presence of metals, but also to the fact that these anthropogenic soils are the product of a relatively rapid accumulation of mine wastes and consequently have not been formed through the complex and long process of rock erosion and materials accumulation that supplies the parameters needed for the proper development of flora (Dudka and Adriano 1997). Only *Lolium perenne* was able to germinate and grew in sample A3 (average of 15% and 12% as percentage of the controls respectively)(data not shown). Results from tests with plants confirmed those from ecotoxicity tests with invertebrates, revealing soils A1 and A5 as the least toxic. Both A1 and A5 soils presented similar percentages of emergence when compared with the controls, with values that ranged from 26 to 70% in soil A1 and from 32 to 85% in soil A5 depending on the test species. Among species, the highest inhibition was found in *T. pratense* while no statistical differences were found between the germination of *B. rapa* and *L. perenne*. These results are in accordance with those from Ramírez et al. (2008), who estimated lower EC50s (i.e higher sensitivity) for *T. pratense* than for *B. rapa* and *L. perenne* when exposed to different sewage sludge. Regarding plant growth, it was significantly higher in soil A5 (48 to 61%) than in A1 (32 to 36%). No significant statistical differences in growth rate were appreciated between test species within the same soil.

In this study, total metal concentrations indicated soils A4, A1 and A2 respectively as the most contaminated. However, results from bioassays identified soils A6, A3, and A7 as the most toxic to terrestrial organisms. The low toxicity of samples A4, A1 and A2 was explained by differences in the toxicity exerted by each metal and by metal bioavailability. The sample A1, for instance, was composed of soil mixed with mining wastes and presented high mercury contents. However, results from the mineralogical analysis pointed out that Hg was mainly found in the form of cinnabar, thus becoming inaccessible to soil organisms. On the other hand, the high toxicity of soils A6 and A7 was attributed to their high electrical conductivity, which was positively and significantly correlated with the toxicity to soil invertebrates (r = 0.98, $P < 0.01$ for earthworms mortality; r = 0.89, $P < 0.01$ for earthworms avoidance; r = 0.89, $P < 0.05$ for collembolans avoidance). According to Alvarenga et al. (2012), the high salinity of metal-contaminated soils could be indicative of a high bioavailability of metals. Since EC was positively correlated with Hg concentrations and the mineralogical analysis detected the presence of mercury sulfates, we attributed the high electrical conductivity and consequently high toxicity of samples A6 and A7 to the concentration of mercury in the form of salts. No clear explanation was found for the high toxicity shown by sample A3.

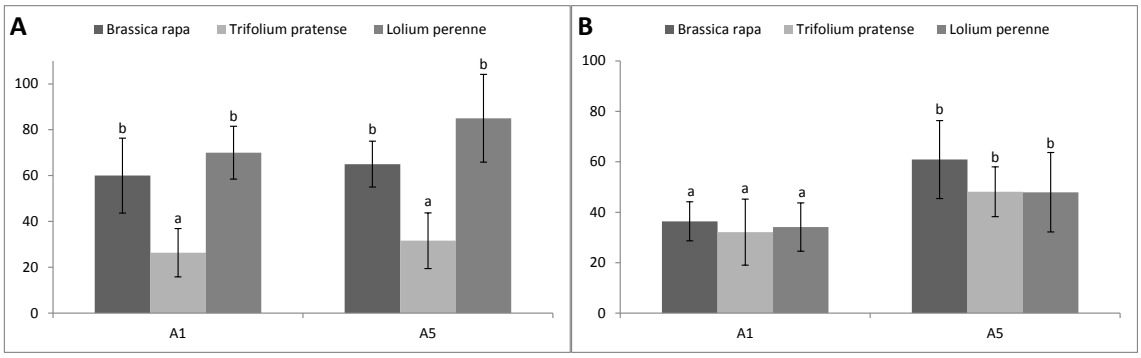

Figure 4. *Brassica rapa, Trifolium pratense* and *Lolium perenne* seedling emergence (A) and fresh biomass (B) as percentage of the controls. Means and standard deviations from four replicates. Values presenting the same letter are not statistically different ($P > 0.05$; ANOVA test).

Toxicity of water extracts to aquatic organisms is shown in Table 5. The observed toxic effects in the bacterial bioluminescence inhibition assay were not sufficient to estimate EC50 values for samples A1, A3, A5 and A7 whereas extracts A2 and A4 were very toxic and A6 presented moderate toxicity. The lesser sensitivity of *V. fischeri* luminescence towards leachates from mine soils was previously documented (Alvarenga et al. 2008; Maisto et al 2011). In contrast, the growth inhibition of the microalgae *R. subcapitata* showed an extreme sensitivity that correlated significantly with Zn content in water extracts (r = 0.996, $P < 0.01$). Consequently, tests with algae estimated the lowest EC50 values for all water samples and became the most metal-sensitive among the aquatic ecotoxicity tests applied, as previously reported by Maisto et al. (2011) and De Paiva Magalhães et al. (2014). *D. magna* was moderately affected by water extracts, showing significant correlations with their pH (r = -0.913, $P < 0.01$) and Pb contents (r = 0.94, $P < 0.01$). Samples A3 and A5 caused no mortality to the crustaceans

while extremely high toxicity was observed for samples A2, A4 and A6, thus becoming more sensitive than *V. fischeri* to this type of contamination (Alvarenga et al. 2013) but not as metal-sensitive as algal growth rate (Maisto et al. 2011). As expected, bioassays with fish were the least sensitive among all tested species due to their higher resistance to most metals (De Paiva Magalhães et al. 2014). Even so, fish lethality was significantly correlated with pH ($r = -0.855$, $P < 0.05$) and Pb content ($r = 0.904$, $P < 0.01$) and occurred in the samples that proved more toxic to the other aquatic species tested (A2, A4 and A6).

	Bacteria Luminescence Inhibition	Algal Growth Inhibition	*Daphnia magna* Immobilization	*Danio rerio* Acute Toxicity
A1	>45	29.1 (20.6-44.3)	69.6 (50.1-120.7)	>100
A2	0.71	5.7 (4.7-6.8)	0.39 (0.29-0.52)	14.8 (-)
A3	>45	36.6 (25.3-58.2)	>100	>100
A4	1.2	0.015 (0.009-0.025)	0.47 (0.38-0.59)	52.7 (-)
A5	>45	9.2 (5.2-16.1)	>100	>100
A6	20.3	1.2 (1-1.4)	1 (0.9-1.1)	69.3 (-)
A7	>45	26.6 (20.7-35.6)	24 (17-34)	>100

Table 5. EC50 and LC50 values (95% confidence limits) of aquatic ecotoxicity tests. Results expressed as percentage volume of water extract mixed with test medium (v/v). '-': non-applicable.

Results from ecotoxicological bioassays with water extracts confirmed the high toxicity of sample A6 and detected remarkable deleterious effects by samples A2 and A4, which were the ones that presented higher metal contents. In addition, leachates from samples A2 and A4 showed the greater mobilization of Pb and Zn, which may indicate that aquatic toxicity was directly related with these metals. The toxicity of sample A6 was therefore attributed to its high salinity caused by the solubilization of mercury salts, while that of samples A2 and A4 resulted from their high acidity and metal concentrations.

2.3.4. Multivariate analysis

Principal component analysis for the terrestrial compartment was carried out on 10 variables: As, Ba, Hg, Pb, Sb, Zn, pH, Earthworms survival (ES), Earthworms avoidance (EA) and Collembola avoidance (CA). Variables were reduced to 4 principal components that explained 96.8% of the total variance (Table 6). Principal component 1 (PC1) was responsible for 47.4% of the total variance and was best represented by As and inversely related with Sb, Zn, pH, ES and EA. PC1 is explanatory of the toxicity of samples A6, A3 and A7, reflecting the role of pH and As content in soil toxicity and the detrimental effects observed through the survivability and avoidance of earthworms. Component 2 was responsible for 25.9% of the total variance and showed a direct correlation between Ba, Hg and Zn, which is frequent in soils contaminated by mining activities. It was also related with ES and CA. Component 3 explained 18.7% of the total variance and was positively represented by Hg and inversely by Pb, indicating the influence of

lithogenic geochemistry over soil composition. Component 4 explained 4.6% of the total variance and was represented by As and inversely by CA, indicating the possible influence of As in the behavior of collembolans.

Variables	PC1	PC2	PC3	PC4
As	0.4215	0.2244	0.0267	0.4459
Ba	0.0697	0.3228	-0.0752	-0.0077
Hg	-0.0874	0.5042	0.6851	0.0078
Sb	-0.5006	-0.1134	-0.0034	-0.3401
Pb	0.1603	0.0601	-0.5955	0.0391
Zn	-0.4002	0.4398	-0.2821	-0.257
pH	-0.3788	0.1143	-0.0322	0.3457
ES	-0.2364	0.3505	-0.2441	0.4297
EA	-0.3561	-0.174	-0.0055	0.4764
CA	0.2171	0.4638	-0.1713	-0.2945

Table 6.- Principal components loadings of soils.

Principal component analysis of leachates was applied using the same 6 geochemical variables of the soil multivariate analysis plus pH and electrical conductivity. The following ecotoxicological variables were also considered: *V. fischeri* luminescence inhibition (VFLI), Algal growth inhibition (AGI), *D. magna* immobilization (DMI) and *D. rerio* acute toxicity (DRAT). The loadings of the first four principal components are shown in Table 7 and explain 93.4% of the total variance. PC1 was responsible for 48% of the total variance and was directly related with Ba, Hg, and DRAT and inversely with DMI, indicating a geochemical factor associated with the ecotoxicity of Hg. Component 2 explained 27.4% of the total variance and was associated with Ba, Pb and Zn and inversely with AGI and DMI, suggesting the ecotoxicity of these dissolved metals. Component 3 explained 11.9% of the total variance and was represented by Hg, Zn, VFLI and inversely by Ba. Component 4 explained 6% of the total variance and was associated with As (possibly), Hg, Pb and EC indicating the effect of metal concentration in electrical conductivity (EC). Since component 4 is inversely associated with DRAT, it may explain the ecotoxicity related with *D. rerio*.

Variables	PC1	PC2	PC3	PC4
As	0.1584	0.1385	-0.0421	0.2613
Ba	0.3089	-0.4734	-0.4621	0.1034
Hg	0.6116	-0.1533	0.3274	0.5841
Sb	-0.0714	-0.0054	0.0149	0.1826
Pb	-0.0745	0.3910	-0.1628	0.2960
Zn	0.0286	-0.3847	0.6000	-0.2446
VFLI	0.1183	0.3159	0.3114	-0.0248
AGI	-0.2993	-0.4079	-0.1852	0.2804
DMI	-0.4572	-0.3531	0.2472	0.1607
DRAT	0.3453	-0.1935	-0.2609	-0.4260
pH	0.1066	-0.0489	0.1617	-0.0634
EC	-0.2255	-0.0396	-0.0644	0.3294

Table 7. Principal components loadings of water extracts.

2.4. Conclusions

The environmental risks posed by the studied area were successfully evaluated. The Valle del Azogue mining area presented physical-chemical parameters typical from abandoned mining areas. High concentrations of mercury (mainly bound to the matrix or released from cinnabar) were detected throughout the area. Besides mercury, several other metals were quantified in amounts exceeding international intervention values. The risk of metals leaching towards the surrounding aquatic compartment due to the particular characteristics of the studied soils was identified.

The application of a battery of bioassays with organisms from different species proved to be a very valuable tool for the assessment of metal-contaminated sites. Most soil samples exerted severe toxic effects to terrestrial organisms, including the death of soil invertebrates and the total inhibition of plant growth. The avoidance test with earthworms was the most sensitive terrestrial bioassay, identifying almost all test soils as toxic after only 48 hours of exposure. To a lesser extent, aquatic bioassays confirmed the high toxicity detected by terrestrial tests and the growth inhibition of microalgae was identified as the most sensitive test.

This study successfully helped in the interpretation of the complexity associated to metal-contaminated soils. Relationships between physical-chemical parameters of soils and water extracts, heavy metals concentrations, and toxicity were established. Interestingly, the most contaminated soils were not identified as the most toxic by terrestrial tests, thus emphasizing the importance of ecotoxicological tests as complementary tools for the reliable risk assessment of contaminated sites. Furthermore, the electrical conductivity of terrestrial and aquatic samples was established as one of the main source of toxicity. In view of the results, an intervention on the studied area is encouraged due to the threat presented by the contaminated soils and the risk of spreading the contamination to agricultural areas located close to the studied site and/or towards the groundwater systems.

CHAPTER III. Ecotoxicological risks of the abandoned F-Ba-Pb-Zn mining area of Osor (Spain)[2]

[2] In revision by *Environmental Geochemistry and Heath* (2016).

Abstract

Due to its potential toxic properties, metal mobilization is of major concern in areas surrounding Pb-Zn mines. In the present study, metal contents and toxicity of soils, aqueous extracts from soils, and mine drainage waters from an abandoned F-Ba-Pb-Zn mining area in Osor (Girona, NE Spain) were evaluated through chemical extractions and ecotoxicity bioassays. Toxicity assessment in the terrestrial compartment studied lethal and sublethal effects on earthworms *Eisenia fetida*, arthropods *Folsomia candida* and several plant species whereas aquatic tests involved bacteria *Vibrio fischeri*, microalgae *Raphidocelis subcapitata* and crustaceans *Daphnia magna*. Metal quantifications revealed high concentrations of Ba (250-5110 mg kg^{-1}), Pb (940 - > 5000 mg kg^{-1}) and Zn (2370-11300 mg kg^{-1}) that exceeded intervention values for human health protection. Risks for the aquatic compartment were identified through the release of drainage waters and by leaching and run-offs from metal-contaminated soils. Cd (1.98-9.15 µg L^{-1}), Pb (2.11-326 µg L^{-1}) and Zn (280-2900 µg L^{-1}) concentrations in water samples surpassed international values of aquatic life criteria. Terrestrial ecotoxicity tests were in accordance with metal quantifications and identified the most polluted soils as the most toxic. Avoidance and reproduction tests with earthworms showed the highest sensitivity to metal contamination. Aquatic bioassays with extracts from soils confirmed the results from terrestrial tests and detected severe toxic effects caused by the mine drainage waters. Algal growth inhibition was the most sensitive aquatic endpoint. In view of the results, the application of a containment or remediative procedure in the area is encouraged.

3.1. Introduction

Once released into the environment, most metals cannot be degraded and are distributed through the different environmental compartments according to their mobility and bioavailability (Misra et al. 1994; Jung et al. 2002; Liu et al. 2003). Soils are considered major sinks for heavy metals, whose release is associated with the anthropogenic application of fertilizers, animal manures, sewage sludge or pesticides, and with inadequate disposal of mine wastes among others (Khan et al. 2008; Zhang et al. 2010). The ecological impact of mining activities on a given site is ultimately controlled by climate, mining methods, geological conditions, and whether the mine is active or abandoned (Bell et al. 2001). However, some common procedures like the accumulation of large volumes of tailings (residues formed during the processing of the mineral ore) in steep stock piles can increase the environmental risks posed by a mining area. Under these storage conditions, those residues are prone to erosion (Henriques and Fernandes 1991) and might be dispersed to soils, surface and ground waters, and stream sediments of the surrounding area through atmospheric emissions, mechanical dispersion or water-leaching (Johnson et al. 1994; Adriano 2001).

The abandoned Osor mining district lies some 35 km SE of Girona, in the La Selva basin and Montseny-Guilleries massif, which is part of the Catalonian Coastal Range (CCR) in the NE section of the Iberian Peninsula. In this area, the exploitation of F-Ba-Pb-Zn ores until 1980 generated important amounts of mine-waste impoundments with high contents of cadmium, lead, zinc and other metals. Due to the lack of proper containment of mining wastes prior to the closure of the mine, metal contamination is affecting surface waters, groundwater, sediments and soils located in the vicinity (Navarro et al. 2011; Navarro et al. 2015). The generation of neutral mine drainage waters within the area is also a major cause of concern due to their potential to mobilize metalloids such as As, Sb, Se and metals such as Cd, Pb, and Zn (Heikkinen et al. 2009; Jang and Kwon 2011; Plante et al. 2011). High Fe, Mn, Ni, Pb and Zn concentrations were already reported by Navarro et al. (2015) in the main dewatering system of the Osor area, which discharges its waters directly into a natural creek.

The environmental risks of metal-contaminated sites were traditionally assessed by chemical extractions. However, it was concluded that this approach did not provide enough information about the bioavailability of metals and was not able to reflect the toxicity of all substances in soil, the synergic and antagonistic effects of contaminants and their interactions with the soil matrix and test organisms (Gruiz 2005). In this context, the application of batteries of terrestrial ecotoxicity tests gained special relevance as complementary tools able to report realistic, non-overestimated effects of contaminated sites to soil organisms (Alvarenga et al. 2012; Bes et al. 2014; Bori and Riva 2015, Bori et al. 2015). At the same time, aquatic bioassays traditionally applied for the toxicity determination of aquatic pollutants (Lopez-Roldan et al. 2012) or industrial effluents (Riva et al. 1993; Riva and Valles 1994; Riva et al. 2007) were incorporated to assess the impacts of soil composition and run-offs on receiving waters (Loureiro et al. 2005a; Rocha et al. 2011).

With this in mind, the aim of this study was to help in the assessment of the environmental risks posed by the abandoned mining site of Osor. To do so, metal quantifications and ecotoxicological bioassays were applied to soils from the area, to their water extracts, and to water samples from the mine drainage system. Terrestrial tests studied the mortality, the inhibition of reproduction and the avoidance response of *Eisenia fetida*, the avoidance response of *Folsomia candida* and the germination and growth rates of different plant species. Impacts on the aquatic compartment were measured through the luminescence inhibition of bacteria *Vibrio fischeri*, the growth inhibition of microalgae *Raphidocelis subcapitata* and the mortality of crustacean *Daphnia magna*.

3.2. Methodology

3.2.1. Study area and sampling sites

The Osor vein deposit is located 4 km SE of Anglès town (NE Spain) and includes several geologically similar and thick (1-4 m) fluorite-barite-sphalerite-galena veins exploited to a depth of 300 m. Gangue minerals include quartz, barite, calcite, pyrite, and silicates (mainly muscovite, albite and biotite). The exploitation of these veins concluded in 1980, after reaching yearly productions of 20000-30000 t of fluorite, 2000 t of Pb concentrates, and 3000 t of Zn concentrates. The Osor flotation tailings are homogeneous in grain size and composition and occupy an area of 3150 m² with a mean thickness of 15 m. Mine drainage is performed through the Coral adit, which drains the Osor vein system with an estimated discharge into the Osor creek between 300 and 1100 m^3 day^{-1} of metal-contaminated, near-neutral mine waters (Navarro et al. 2015). In addition, episodic discharges of contaminated sediments and draining waters from the Osor tailings area also occur.

Soil samples were collected from three different sites within the Osor mining area: a sample of soil from a mine waste dump (sample EM-1) located close to the main extraction area (Leonor shaft), a sample of flotation tailings (TOS sample), and a sample of alluvial soil (sample OS-6) collected near Osor creek (Figure 1). Each sample was composed of 4 sub-samples collected within the same site and thoroughly mixed.

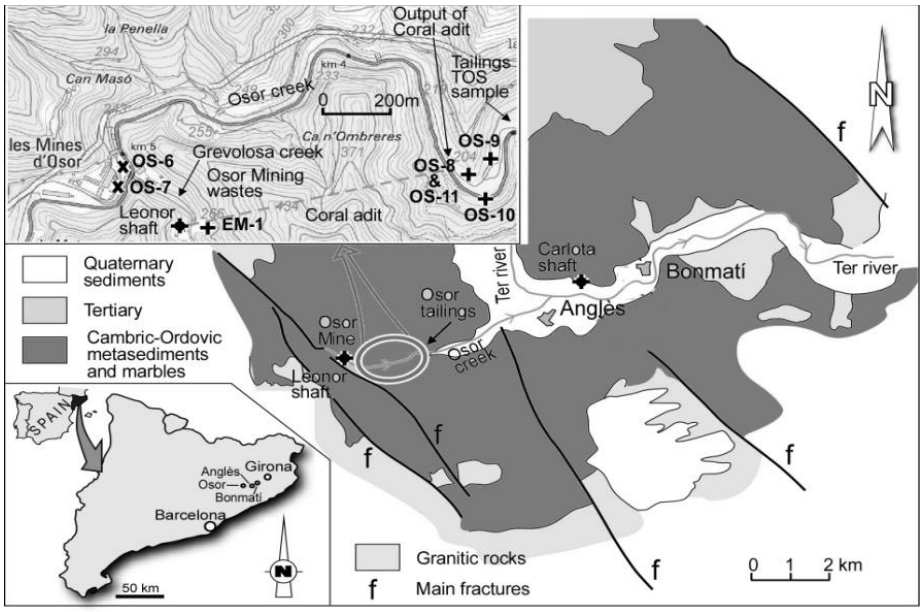

Figure 1. Location map and synthetic geology of the study area. Adapted from Navarro et al. (2015).

3.2.2. Soils and mine wastes sampling and analysis

Soil samples were collected and pretreated as previously specified. The following physical-chemical characteristics were evaluated: pH, electrical conductivity, soil organic matter and texture (N=3). Identification and analysis of mineral phases from selected samples were performed in the laboratories of the University of Barcelona (UB) by X-ray diffraction (XRD). Once the materials had been dried and ground, their geochemical compositions were analyzed by Actlabs (Ontario, Canada) using instrumental neutron activation analysis (INAA). The following elements were studied: Au, Ag, As, Ba, Br, Ca, Co, Cr, Cs, Fe, Hf, Hg, Ir, Mo, Na, Ni, Rb, Sb, Sc, Se, Sn, Sr, Ta, Th, U, W, Zn, La, Ce, Nd, Sm, Eu, Tb, Yb, and Lu. In addition, the concentrations of the following elements were determined by acid digestion and subsequent analysis by inductively coupled plasma atomic emission spectrometry (ICP-AES): Ag, Cd, Cu, Mn, Mo, Ni, Pb, Zn, Al, Be, Bi, Ca, K, Mg, P, Sr, Ti, V, Y, and S.

3.2.3. Water extracts collection and analysis

Water samples were obtained as previously reported. Additionally, a sample from the output of the Coral adit (CA sample) was collected in a high-density polypropylene bottle, sealed with a double cap and stored in a refrigerator until analysis. Samples for metal analysis were filtered through a 0.45 μm pore size cellulose nitrate membrane, acidified and sent to Actlabs (Ontario, Canada). The following elements were analyzed by ICP-MS: Li, Na, Mg, Al, Si, K, Ca, Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ge, As, Se, Br, Rb, Sr, Y, Zr, Ag, Cd, Sn, Sb, Te, I, Cs, Ba, Hg, and Pb. These determinations were compared to the reference sample NIST 1640 to confirm accuracy. The pH, electrical conductivity (EC) and total organic carbon (TOC) of all water samples were also determined (N=3).

3.2.4. Terrestrial ecotoxicity tests

E. fetida acute toxicity tests

Each test ran with 4 concentrations (12.5-25-50-100%) plus a control and three replicates per treatment.

E. fetida reproduction tests

Tests ran with 5 concentrations (1-2.56-6.4-16-40% for samples EM-1 and TOS and 1.28-3.2-8-20-50% for OS-6) and three replicates per treatment. Six replicates with artificial control soil were tested.

Avoidance tests with E. fetida and F. Candida

Avoidance tests with both species ran with 4 concentrations plus a control and three replicates per treatment. All assays with collembolans as well as the assay with earthworms in OS-6 were performed in 30-45-67-100% of test soil mixed with artificial soil whereas tests with earthworms in EM-1 and TOS required lower test concentrations (7.5-15-30-60%).

Seedling emergence and growth tests

Twenty seeds of the corresponding plant species were sown in each test soil and in the control artificial soil (four replicates per soil i.e. 5 seeds per test container).

3.2.5. Aquatic toxicity tests

Bacteria luminescence inhibition tests

Test organisms were exposed to 4 concentrations of water extracts (5.63-11.25-22.5-45%). Three replicates per treatment were analyzed.

Algal growth inhibition tests

Tests ran with three replicates per treatment and 7 concentrations (0.1-0.32-1-3.2-10-32-90%) plus a control that consisted in algae culture medium.

Daphnia magna acute immobilization tests

Daphnids were exposed to 7 dilutions of water extracts (1-2.2-4.8-10-22-48-100%) plus a control in four replicates per treatment.

3.3. Results and Discussion

3.3.1. Physicochemical characteristics and geochemistry of soils and mine wastes

All the studied soils presented similar physicochemical parameters. The pH was slightly acid in OS-6 (6.12), neutral in EM-1 (7.22) and moderately alkaline in TOS (8.12). The electrical conductivity was low in all the studied sites, with values of 278.5 µS cm^{-1}, 338.5 µS cm^{-1} and 439.5 µS cm^{-1} in OS-6, EM-1 and TOS respectively. Organic matter contents remained below 10% in all sites (3.40% in EM-1, 3.69% in TOS and 3.76% in OS-6), thus classifying the studied samples as mineral soils. Sand was the main component of all soils, which presented a loamy sand texture.

Total concentrations of metals and metalloids for which the Waste Agency of Catalonia has established General Reference Levels to protect human health (WAC 2015) are shown in Table 1. The results from chemical extractions revealed that, in some sites, Ba, Pb, Sb and Zn contents exceeded up to one order of magnitude the intervention values for soils under industrial use. The mine waste sample from the Osor sector (EM-1) was the most heavily polluted. High amounts of Pb (> 5000 mg kg^{-1}) in this site may be linked to the presence of argentiferous galena whereas Sb contents (56.5 mg kg^{-1}) might be linked to galena or undetected sulfosalts (Navarro et al. 2015). High concentrations of Zn (11300 mg kg^{-1}) and Cd (24.9 mg kg^{-1}) were also detected and associated with sphalerite. The Osor flotation tailings sample

(TOS) contained lower amounts of Pb (940 mg kg^{-1}) and Zn (2370 mg kg^{-1}) although both metals still exceeded the intervention values (550 mg kg^{-1} and 1000 mg kg^{-1} respectively). On the other hand, TOS showed the highest Ba content of the area (5110 mg kg^{-1}), probably due to the accumulation of gangue material in flotation processes. Concentrations of Ba (2200 mg kg^{-1}), Pb (>5000 mg kg-1), and Zn (2730 mg kg^{-1}) in the alluvial soil (OS-6) also exceeded catalan intervention values, which was unexpected due to its distance from the main mining areas.

	As	Ba	Be	Cd	Co	Cr	Cu	Hg	Ni	Pb	Sb	Se	Ti	V	Zn	Sn
EM-1	15.2	250	<1	24.9	6	<2	11	3	3	>5000	56.5	<0.1	0.03	4	11300	<0.01
TOS	12.5	5110	2	7.6	14	39	47	<1	18	940	1	<0.1	0.24	45	2370	0.02
OS-6	5.7	2200	3	11.5	9	44	24	<1	19	>5000	6.1	<3	0.21	48	2730	<0.01
CAL	30	1000	90	55	90	1000	1000	30	1000	550	30	70		1000	1000	

Table 1. Total metal concentrations (mg kg^{-1} for all metals with the exception of Ti and Sn, which are expressed as %) in sampled soils. CAL: Catalonia intervention values for soils under industrial use.

3.3.2. Physicochemical characteristics and hydrochemistry of aquatic samples

Water samples from the Osor mining area showed similar physicochemical parameters. All samples presented neutral pH, with values of 7.56, 7.77, 7.83 and 8.08 for EM-1, TOS, OS-6 and CA respectively. Total contents of organic carbon were low in the extracts from EM-1 and TOS (1.41 mg L^{-1} and 1.87 mg L^{-1} respectively) and increased in CA (2.96 mg L^{-1}) and in the extract from OS-6 (8.71 mg L^{-1}). Electrical conductivity was significantly lower in the extracts from test soils (63.50 µS cm^{-1}, 125.55 µS cm^{-1} and 156.45 µS cm^{-1} for OS-6, TOS and EM-1 respectively)($P < 0.05$; Mann Whitney U Test) than in the sample from the Coral adit (958 µS cm^{-1}).

Metal concentrations in water samples are shown in Table 2. Total contents of most metals in water extracts represented less than 1% of their soil contents, thus revealing their relatively immobility in soils. Even so, Ba, Pb and Zn presented high concentrations in water that were attributed to their remarkable contents in soils. Ba concentration was similar between water extracts and ranged from 306 µg L^{-1} (OS-6) to 450 µg L^{-1} (TOS). The contents of Pb in EM-1 and OS-6 reached significant values of 183 µg L^{-1} and 326 µg L^{-1} respectively despite Pb tendency to be adsorbed by Fe and Mn oxyhydroxides and the low solubility of Pb sulfate and hydroxycarbonate. Zn presented the highest concentrations in water extracts (280 µg L^{-1} to 901 µg L^{-1}), which was in accordance with its greater abundance in soils. As seen in soils, the TOS sample presented markedly lower Pb and Zn concentrations (3.33 µg L^{-1} and 280 µg L^{-1} respectively) than EM-1 (183 µg L^{-1} and 498 µg L^{-1} respectively). In contrast, the highest Pb and Zn concentrations (326 µg L^{-1} and 901 µg L^{-1} respectively) were detected in the extract from the alluvial soil (OS-6), suggesting a higher risk of metal leaching from this site. Such risk was associated to the higher acidity and sand content of the site, which can facilitate metal solubilization and leaching (Navarro Flores and Martínez Sola 2010). The sample collected from the mine dewatering system (CA) presented metal

contents that fell within the range quantified in water extracts. The only exceptions were Ba and Zn, whose concentrations in CA (34.1 µg L^{-1} and 2900 µg L^{-1} respectively) differed by one order of magnitude with their contents in the extracts from contaminated soils. The low concentration of barium in the water from the Coral adit was attributed to Ba sedimentation throughout the mine drainage system whereas the high concentration of Zn was associated to its precipitation as a secondary phase (carbonate, hydroxide, etc.). In view of the results from metal quantifications, Cd, Zn and Pb were considered the contaminants of greatest environmental concern because they exceeded the aquatic life criteria of the US Environmental Protection Agency (2016) in most water samples. These criteria establish the highest concentration of specific pollutants that are not expected to pose a significant risk to the majority of species in a given aquatic environment.

	As	Ba	Be	Cd	Co	Cr	Cu	Hg	Ni	Pb	Sb	Se	Ti	V	Zn	Sn
EM-1	0.76	340	<0.1	9.15	0.4	<0.5	3.8	1.1	0.3	183	1.7	<0.2	5.1	0.2	498	<0.1
TOS	0.24	450	<0.1	3.64	0.36	<0.5	3.7	0.8	<0.3	3.33	0.52	<0.2	<0.1	<0.1	280	<0.1
OS-6	1.4	306	<0.1	2.34	2.03	1.7	18.4	2	3	326	1.52	1	15.6	3.6	901	<0.1
CA	1.59	34.1	-	1.98	19.1	-	7.3	-	17.9	2.11	-	4.4	-	-	2900	-
US EPA	340	-	-	2		570	-	-	-	65	-	-	-	-	120	-

Table 2. Total metal concentrations (µg L^{-1}) in water extracts from test soils and in the Coral adit (CA). US EPA: Aquatic Life Criteria for acute exposures by the United States Environmental Protection Agency (in µg L^{-1}).

3.3.3. Ecotoxicological evaluation

Terrestrial and aquatic ecotoxicological bioassays were successfully applied. Marked differences in sensitivity were appreciated between test endpoints and organisms. Within the bioassays with earthworms, lethality tests were not sensitive enough to estimate LC50s for any of the studied soils whereas reproduction and avoidance tests detected toxicity in all samples (Table 3). The exposure of *E. fetida* to EM-1 and TOS in acute tests caused 40% of mortality when the sample was not diluted, with 69.76% and 74.31% average decrease in the body mass of test organisms respectively. Mortality was not appreciated when earthworms were exposed to OS-6. In contrast, reproduction tests with earthworms presented an extreme sensitivity and estimated EC50s of 1.05%, 1.48% and 1.09% for EM-1, TOS and OS-6 respectively. The avoidance behavior of earthworms was slightly less sensitive than reproduction and estimated EC50s of 2.75%, 7.99% and 31.32% for EM-1, TOS and OS-6 respectively. Even so, avoidance tests proved to be a very valuable tool for the risk assessment of metal-contaminated soils because they were able to reduce the duration of the assays to 2 days. Additionally, the studied samples were considered to have a limited habitat function because more than 80% of the test organisms preferred the control soil instead of the test soils at the end of the assay (Hund-Rinke and Wiechering 2001). Results from terrestrial bioassays with earthworms were in agreement with previous studies that highlighted the higher sensitivity of sub-lethal endpoints in the ecotoxicological evaluation of

contaminated soils (Hund-Rinke et al. 2002; Davies et al. 2003; Bori et al. 2016). On the other hand, avoidance tests with the soil arthropod *F. candida* were not able to detect toxicity in any of the studied samples. The lower sensitivity of collembolans in comparison with earthworms was already reported by Da-Luz et al. (2012), Hentati et al. (2013), and Bori et al. (2016) after studying avoidance responses in soils contaminated with pesticides, petroleum compounds and metals respectively. Even more, collembolans showed a significant attraction towards the contaminated section of the containers that increased while increasing test concentrations. Such response was previously reported after exposure to the pesticide Dimethoate although it was attributed to the incapability of collembolans to escape from the contaminated soil (due to the effects of the pesticide in their nervous system) rather than an attraction towards it (Pereira et al. 2013).

	EM-1	TOS	OS-6
Reproduction inhibition	1.05 (0.48-1.56)	1.48 (0.57-2.52)	1.09 (0.11-1.94)
Avoidance response	2.75 (1.61-7.12)	7.99 (4.84-11.15)	31.32 (24.66-37.9)

Table 3. EC50s (95% confidence intervals) of terrestrial ecotoxicity tests with *E. fetida* expressed as percentage of soil sample mixed with artificial soil (w/w).

Percentages of seedling germination and growth of selected plant species in undiluted test soils are depicted in Figure 2. Seedling emergence was high in controls (95% to 100%) and was not completely inhibited in any of the tested soils. Even so, statistically significant differences in germination rates ($P < 0.05$; Tukey test) were detected between species and soils. The emergence of *B. rapa* and *T. pratense* was statistically inhibited in EM-1 (germination rates of 60% and 20% respectively) and TOS (60% and 40% respectively) whereas only *T. pratense* was significantly inhibited in OS-6 (60% of seedling emergence). *L. perenne* germination was not inhibited in any site. No statistical differences in emergence rates were detected between EM-1 and TOS while germination of *B. rapa* and *T. pratense* was significantly higher in OS-6 than in EM-1. The observed inhibition of plant emergence was associated to As contents, which showed a negative and statistically significant correlation with germination rates ($r = -0.999$; $P < 0.01$). Despite remaining below the intervention value established by the Waste Agency of Catalonia, As concentrations in the studied sites were within EC10 and EC50 ranges (1.95-568.12 mg As kg^{-1} and 14.86-795 mg As kg^{-1} respectively) derived from emergence experiments conducted with different plant species in As-contaminated soils (Sun et al. 2012). Between species, *T. pratense* was most sensitive to the contaminated soils, followed by *B. rapa* and *L. perenne*. The same sensitivity range was reported by Ramírez et al. (2008) and Bori et al. (2016) after exposing the same species to sewage sludge and metal-contaminated soils respectively. Regarding plant growth, all species were significantly inhibited in EM-1 and TOS whereas only *L. perenne* growth was inhibited in OS-6. No statistical differences in the average fresh biomass of *B. rapa* and *T. pratense* were detected between sites EM-1(28.45 mg and 1.95 mg

respectively) and TOS (35.91 mg and 5.93 mg respectively) whereas the growth of *L. perenne* was significantly higher in TOS (17.86 mg) than in EM-1 (10.93 mg). Similarly to seedling emergence, seedling growth in OS-6 was statistically higher than in EM-1 and TOS. Among the studied species, *B. rapa* showed the highest growth rate in all sites although *T. pratense* was the most sensitive species to the presence of metals.

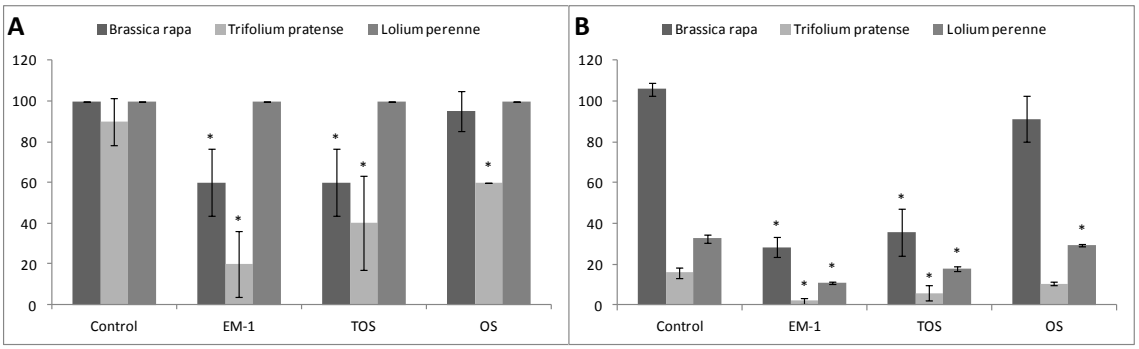

Figure 2. *Brassica rapa, Trifolium pratense* and *Lolium perenne* seedling emergence (in %)(A) and fresh biomass (in mg)(B). Means and standard deviations from four replicates. .'*' means statistically different from control ($P < 0.05$).

Toxicity of water samples to aquatic organisms is shown in Table 4. The bioluminescence inhibition of *V. fischeri* was the least sensitive endpoint and showed no detrimental response to any of the water samples (Alvarenga et al. 2008; Teodorovic et al. 2009; Maisto et al 2011; Bori et al. 2016). As previously reported by several authors (Maisto et al. 2011; De Paiva Magalhães et al. 2014; Bori et al. 2016), algal growth inhibition showed the highest sensitivity towards metal-contaminated water samples. Half maximal inhibitory concentrations (IC50) were estimated for all samples and were significantly higher ($P < 0.05$)(i.e less toxicity detected) in TOS, OS-6, and in the Coral adit (13.2%, 14.1%, and 20% respectively) than in the extract from EM-1 (3.7%). The toxicity of the different samples to *R. subcapitata* was explained by their Cd content, which showed a positive and statistically significant correlation (r=0.99; $P < 0.05$) with algal growth inhibition. *D. magna* showed moderate sensitivity to metal contamination and estimated similar LC50s for samples EM-1 and CA (67% and 57% respectively). The exposure of daphnids to water extracts from TOS and OS-6 caused no mortality to test organisms.

	Water samples			
	EM-1	TOS	OS-6	CA
Algal Growth Inhibition (IC50)	3.7 (2.7 - 5.2)	13.2 (10.0 - 17.9)	14.1 (7.6-28.1)	20.4 (9.2-46.5)
Daphnia Immobilization (LC50)	67 (49 - 101)	-	-	57 (39.3-101)

Table 4. IC50 and LC50 (95% confidence intervals) of aquatic ecotoxicity tests expressed as percentage of water sample in test medium (V/V). "-": non-applicable.

The ecotoxicological evaluation of water extracts from sampled soils confirmed the results from terrestrial tests and identified the sample EM-1 as the most toxic. Among the metals of greatest environmental concern (Cd, Pb, and Zn), Cd was considered to have a major role in the toxicity exerted by water extracts due to its concentration (surpassing the US EPA criterion in all samples) and the significant correlation found with the toxicity to algae. However, cadmium alone was not sufficient to explain the observed toxicity since Cd contents in the extracts were markedly lower than the IC50 and LC50 estimated for *R. subcapitata* (67 µg L^{-1}) and *D. magna* (101.17 µg L^{-1}) by Rodgher et al. (2012) and Shaw et al. (2006) respectively. Nonetheless, previous studies by Biesinger et al. (1986) and Barata et al. (2002) reported additive effects in Cd-Zn mixtures that led to an increase in toxicity, which could better explain the effects of the extracts to test organisms. Additionally, Pb contents might have contributed to algal growth inhibition in the water extracts from TOS and OS-6, where Pb concentrations were one order of magnitude higher than the IC50 (83.9 µg L^{-1}) reported by De Schamphelaere et al. (2014). On the other hand, the influence of Pb in *D. magna* lethality was considered negligible since Pb concentrations were markedly lower than the LC50 established by Fagašová (1994)(19498 µg Pb L^{-1}). The toxicity of the sample from the mine dewatering system (CA sample) was attributed to Zn, whose concentration widely exceeded the IC50 (100 µg L^{-1}) and LC50 (819.99 µg L^{-1}) estimated for *R. subcapitata* and *D. magna* by Kasemets et al. (2003) and Shaw et al. (2006) respectively.

3.4. Conclusions

The application of chemical extractions and ecotoxicity tests to soils from the Osor mining area revealed that this abandoned mine site poses an important risk to the surrounding environment due to its high contents of metals. Metal contamination derived from past mining activities was high in those sites where mine wastes (EM-1) and flotation tailings (TOS) were abandoned although threatening contamination levels were also reached in sites where no mining-related activities were expected (OS-6). Ba (250-5110 mg kg^{-1}), Pb (940 - >5000 mg kg^{-1}) and Zn (2370-11300 mg kg^{-1}) concentrations in soils were of greatest environmental concern because they all exceeded the General Reference Levels to protect human health established by the Waste Agency of Catalonia. Besides soils, metal contamination also affected or is likely to affect the aquatic compartment either through the leaching of metals towards the mine dewatering system or through run-off from metal-contaminated soils. The studied draining waters and water extracts from contaminated soils presented patterns of metal contamination similar to soils, with concentrations of Cd (1.98-9.15 µg L^{-1}), Pb (2.11-326 µg L^{-1}) and Zn (280-2900 µg L^{-1}) that surpassed international values of aquatic life criteria.

The application of terrestrial ecotoxicity tests confirmed the results from chemical extractions and linked metal concentrations with toxicity to soil organisms. All the studied soils caused detrimental effects to earthworms although toxicity was mainly attributed to Pb and Zn contents. Additionally, As contents had

a negative impact in the development of plants. Among terrestrial ecotoxicity tests, sublethal endpoints with *E. fetida* and emergence and growth of plant species were the most sensitive endpoints and should be prioritized when aiming to directly assess the toxicity of metal-contaminated soil samples. Aquatic bioassays were in accordance with terrestrial tests and identified the sample from the main waste dump (EM-1) as the most toxic. Toxicity in the aquatic compartment was again related with Pb and Zn contents although Cd also played an important role in algal growth inhibition. The water sample collected from the drainage system was heavily polluted by Zn (2900 μg L^{-1}) and toxic to aquatic organisms. The liberation of contaminated mine waters through the adit is of special concern because they reach the Osor creek and can further spread metal contamination downstream. Among the aquatic bioassays applied, the growth inhibition of *R. subcapitata* was the most sensitive endpoint whereas the luminescence inhibition of *V. fischeri* showed no responses and is not recommended in further analysis of metal-contaminated water samples. In view of the results from our study, the abandoned mining area of Osor is considered to pose an important environmental threat and the application of a containment or remediative procedure in the area should be encouraged.

CHAPTER IV. Ecotoxicological evaluation of contaminated soils from the abandoned mercury-mining area of Almadén (Spain)

Abstract

Ecosystems in the mining district of Almadén (Spain) are among the most mercury-contaminated worldwide, but little is known about their toxic effects on biota. In the present study, the toxicity of soils collected from Almadén was examined through different terrestrial and aquatic ecotoxicological laboratory tests. Soil toxicity testing involved *Eisenia fetida*, *Folsomia candida* and multiple plant species as test organisms and covered a wide range of long-term and short-term lethal and sublethal endpoints. Aquatic tests used aqueous extracts from test soils to assess the luminescence inhibition of *Vibrio fischeri*, the growth inhibition of *Raphidocelis subcapitata* and the morality of *Daphnia magna*. Despite very high mercury concentrations in some soils, results from ecotoxicity tests showed no heavy detrimental effects on test organisms. The lack of response was explained by mercury speciation in the area, where Hg has been long documented to be mainly found in the low bioavailable and non-toxic form of cinnabar. Nevertheless, significant responses were detected in avoidance tests with *Folsomia candida*, in seedling emergence and growth tests with *Trifolium pratense* and *Brassica rapa* and in aquatic tests with *Raphidocelis subcapitata*, which suggested these tests as the most appropriate choice in further biomonitoring programs of metal-contaminated soils from this area.

4.1. Introduction

Ecotoxicity tests have become a common complementary tool in the environmental risk assessment of metal-contaminated mining areas (Alvarenga et al. 2008; Alvarenga et al. 2012; Bes et al. 2014; Bori et al. 2016). Traditionally, the risks posed by these sites were evaluated by chemical analysis of metals that were not able to quantify all pollutants in soil, their synergic and antagonistic effects and their interactions with the soil matrix and organisms (Gruiz 2005). In this context, terrestrial bioassays were needed to report realistic and non-overestimated effects of the bioavailable fraction of metals to soil organisms (Alvarenga et al. 2008; Maisto et al. 2011; Alvarenga et al. 2012; Bes et al. 2014; Bori and Riva 2015; Bori et al. 2015; Bori et al. 2016). Additionally, ecotoxicological tests usually applied in the toxicity determination of aquatic pollutants (Riva et al. 1993; Riva and Valles 1994; Riva et al. 2007) allowed the assessment of the impacts associated with metal leaching and run-offs from metal-contaminated soils (Loureiro et al. 2005; Rocha et al. 2011).

Located in central Spain, the mining district of Almadén (Ciudad Real) hosts the largest mercury deposits of Earth, which accounted for approximately one third of total Hg resources (Hernández et al. 1999). After centuries of mining activity, the extraction of mercury (mainly in the form of cinnabar: HgS) left a legacy of soil contamination due to the anthropogenic dispersion of Hg from abandoned mineral dumps and the deposition as gaseous mercury (Hg^0) by furnace emissions. Consequently, total mercury contents in the order of hundreds to thousands of parts per million were reported in soils from this area (Higueras et al. 2006; Millán et al. 2006; Conde Bueno et al. 2009; Colacevich et al. 2011; Millán et al. 2011).

To date, the majority of risk assessment studies performed on the Almadén mining district have focused on chemical quantifications of mercury species. Among them, insoluble mercuric sulfide (naturally occurring cinnabar) is the most common form of mercury and it is scarcely mobile and non-toxic. However, weathering processes can redistribute Hg in other chemical forms like mercury salts, which can be soluble in water and bioavailable, and are considered toxic (Boening 2000). To the best of our knowledge, studies on the toxicity of these soils to organisms have been limited to evaluate Hg accumulation by terrestrial plants (Higueras et al. 2006; Millán et al. 2006; Millán et al. 2011), river crustaceans (Higueras et al. 2006) and earthworms (Colacevich et al. 2011), while many other lethal and sublethal effects have been unattended.

This study aims to evaluate the toxicity that mercury-contaminated soils from the Almadén mining district exert to aquatic and terrestrial organisms in order to contribute in the assessment of the environmental risk posed by this area. To do so, we applied a battery of standard ecotoxicological tests to four soils collected from different areas within the mining district. Metal concentrations were determined in soils and in their water extracts. Terrestrial bioassays were performed to evaluate lethal and sublethal effects to the soil invertebrates *Eisenia fetida* and *Folsomia candida* as well as the impacts on germination and growth rates of different plant species. Tests on the aquatic compartment focused on acute effects of water extracts from contaminated soils to bacteria *Vibrio fischeri*, microalgae *Raphidocelis subcapitata* and cladocerans *Daphnia magna*.

4.2. Methodology

4.2.1. Collection and analysis of soils and water extracts

Test soils were collected from four sites within the mining district of Almadén: the mine of Almadén, the village of Almadenejos, the mine of El Entredicho and the mine of Las Cuevas (Figure 1). Sampling sites were selected according to previous studies that reported high Hg concentrations in soils (Colacevich et al. 2011). Samples were collected and pretreated as previously specified. The following parameters were evaluated: pH, electrical conductivity (EC), soil organic matter (SOM), soil texture and Water Holding Capacity (WHC). Metal contents in soils were quantified by Actlabs (Ontario, Canada). Cr, Hg and Ni were quantitatively analyzed by instrumental neutron activation analysis (INAA) and Cd, Cu, Ni, Pb and Zn were analyzed by inductively coupled plasma emission spectroscopy (ICP-OES).

Water extracts from test soils were obtained as previously mentioned. Electrical conductivity, pH and contents of total organic carbon were measured. A subsample of each water extract was filtered through a 0.45 µm pore size membrane and sent to Analiza Calidad (Barcelona, Spain) for the quantification of Cd, Cr, Cu, Hg, Ni, Pb and Zn through Graphite Furnace Atomic Absorption Spectroscopy (AAS).

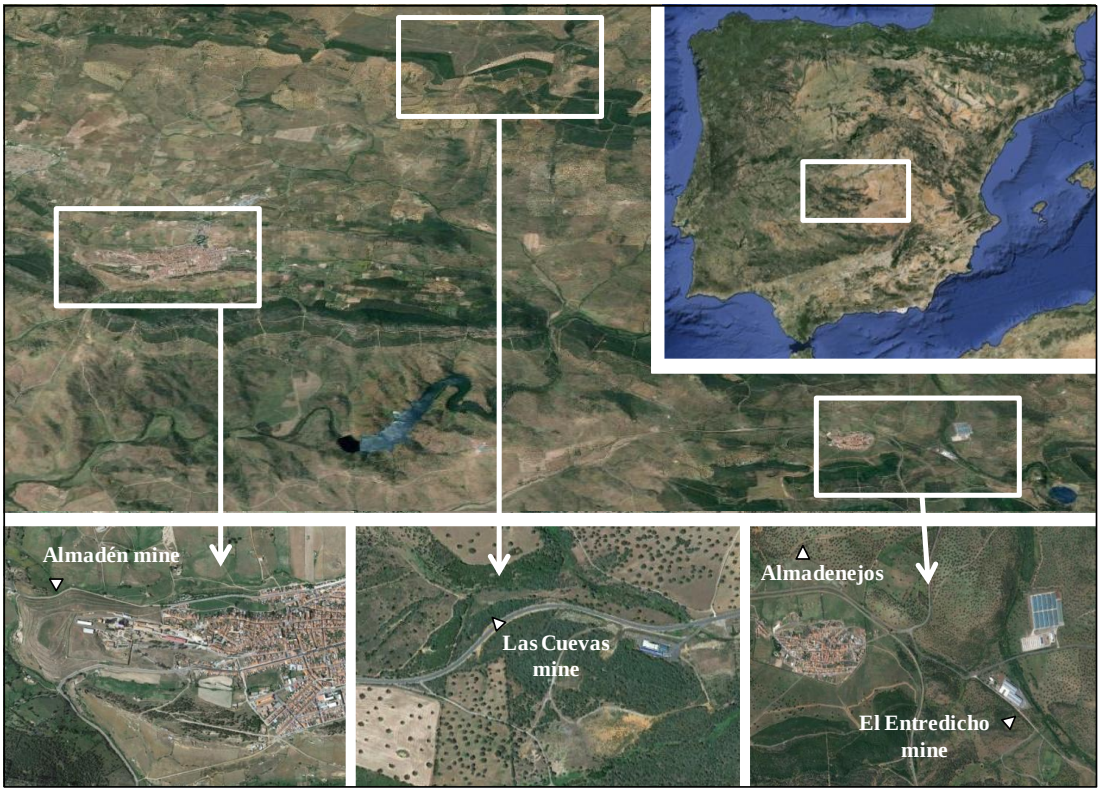

Figure 1. Location of sampling sites within the mining district of Almadén.

4.2.2. Terrestrial ecotoxicity tests

Terrestrial bioassays were performed using the whole sampled soils without dilution.

E. fetida acute toxicity tests

Four replicates were analyzed for each test soil.

E. fetida reproduction tests

Tests ran with three replicates per test soil and six replicates with artificial control soil.

Avoidance tests with E. fetida and F. Candida

Avoidance tests studied 5 replicates per test soil plus 5 dual-control replicates with each species.

Seedling emergence and growth tests

Twenty seeds of the corresponding plant species were sown in each test soil and in the control artificial soil (four replicates per soil i.e. 5 seeds per test container).

4.2.3. Aquatic ecotoxicity tests

Bacteria luminescence inhibition tests

Test organisms were exposed to 4 concentrations of water extracts (5.63-11.25-22.5-45%). Three replicates per treatment were measured.

Algal growth inhibition tests

Tests ran with 7 concentrations (0.1-0.32-1-3.2-10-32-90%) plus a control (consisting in algae culture medium) and three replicates per treatment.

Daphnia magna acute immobilization tests

Daphnids were exposed to 7 dilutions of aqueous extracts (1-2.2-4.8-10-22-48-100%) plus a control in four replicates per treatment.

4.3. Results

4.3.1. Physicochemical properties of test soils and water extracts

The physicochemical parameters of the studied soils are shown in Table 1. Values of pH were low in all soils and ranged from the moderate acidity of Almadén (5.91) to the extreme acidity of El Entredicho (3.83). Electrical conductivity was low in Almadén, Almadenejos and Las Cuevas (138.65 to 204 µS cm^{-1}) and markedly lower in El Entredicho (47.70 µS cm^{-1}). Las Cuevas and Almadén presented higher percentages of organic matter (10.83% and 13.44% respectively) than El Entredicho and Almadenejos (5.89% and 6.66% respectively). Soils from Almadén, Almadenejos and Las Cuevas presented a loamy texture while the sample from El Entredicho was mainly composed of clay. Values of Water Holding Capacity ranged from 32.64% (El Entredicho) to 43.13% (Las Cuevas).

Metal quantifications revealed the presence of varying concentrations of Hg and other metals (Table 1). Cd contents were below the detection limit of the analytical method in all sites. Cr and Cu concentrations ranged from 77 to 120 mg kg^{-1} and from 14 to 44 mg kg^{-1} respectively and were higher in the mines of Almadén and El Entredicho. Mercury contents were markedly lower in El Entredicho and Almadenejos (7 mg kg^{-1} and 14 mg kg^{-1} respectively) than in Las Cuevas (91 mg kg^{-1}) and Almadén (261 mg kg^{-1}). Ni concentrations were similar between soils and ranged from 31 to 46 mg kg^{-1}. The lowest concentration of Pb was detected in El Entredicho (17 mg kg^{-1}) whereas the highest contents were quantified near the village of Almadenejos (73 mg kg^{-1}). Zn contents ranged from 41 mg kg^{-1} (El Entredicho mine) to 127 mg kg^{-1} (Almadén mine).

Physicochemical parameters of the water extracts are summarized in Table 2. All samples presented neutral pH values (from 7.38 in Las Cuevas to 7.86 in El Entredicho). As seen in soil samples, electrical

conductivity was low in extracts from Almadén (190.33 µS cm^{-1}), Almadenejos (102.33 µS cm-^{1}) and Las Cuevas (121.30 µS cm^{-1}) and markedly lower in El Entredicho (53.07 µS cm^{-1}). Similarly, total organic carbon content was lower in the sample extracted from El Entredicho (9.14 mg L^{-1}) than in the other extracts (77.9 mg L^{-1} in Almadenejos to 128.40 mg L^{-1} in Almadén).

Samples	Almadén mine	Almadenejos village	El Entredicho mine	Las Cuevas mine
pH	5.91 ± 0.02	5.65 ± 0.03	3.83 ± 0.02	5.11 ± 0.11
EC	204 ± 4.24	138.65 ± 26.23	47.70 ± 1.56	178.35 ± 24.96
SOM	13.44 ± 0.21	6.66 ± 0.05	5.89 ± 0.01	10.83 ± 0.23
Texture	Loam	Sandy Loam	Clay	Loam
WHC	36.62 ± 0.01	38.26 ± 1.93	32.64 ± 1.72	43.13 ± 4.60
Cd [12]	< 0.3	< 0.3	< 0.3	< 0.3
Cr [380]	120	77	107	78
Cu [190]	44	14	23	22
Hg [10]	261	14	7	91
Ni [210]	46	31	32	36
Pb [530]	61	73	17	41
Zn [720]	127	46	41	88

Table 1. Physicochemical characteristics (mean ± standard deviation; N=3) and metal contents (in mg kg^{-1} dw) of sampled soils. EC: Electrical Conductivity (µS cm^{-1}); SOM: Soil Organic Matter (%); WHC: Water Holding Capacity (%); [ ]: Intervention values for soil remediation according to Dutch regulations (VROM 2000).

Metal concentrations in water extracts markedly varied. Hg and Ni contents were below the detection limit of the analytical method in all samples. Similarly, Cd and Pb concentrations in water extracts could not be determined except for Cd in Almadenejos (1.27 µg L^{-1}) and Pb in Almadén and Almadenejos (5.73 and 6.80 µg L^{-1} respectively). Low concentrations of Cr (from 3.66 to 10.74 µg L^{-1}) and Cu (< 5 to 17.75 µg L^{-1}) were detected in all samples. Zn could only be quantified in the sample from Almadén (250 µg L^{-1}).

Samples	Almadén mine	Almadenejos village	El Entredicho mine	Las Cuevas mine
pH	7.40±0.02	7.40±0.02	7.86±0.07	7.38±0.06
EC	190.33±2.29	102.33±3.09	53.07±1.46	121.30±1.25
TOC	128.40±0.01	77.9±0.44	9.14±0.48	112.15±0.07
Cd	<0.50	1.27	<0.50	<0.50
Cr	10.74	4.41	3.66	4.27
Cu	16.53	16.28	<5.00	17.75
Hg	<1.00	<1.00	<1.00	<1.00
Ni	<2.50	<2.50	<2.50	<2.50
Pb	5.73	6.80	<1.00	<1.00
Zn	250	<100	<100	<100

Table 2. Physicochemical characteristics (mean ± standard deviation; N=3) and total concentrations of metals (in µg L^{-1}) in water samples extracted from test soils. EC: Electrical Conductivity (in µS cm^{-1}); TOC: Total Organic Carbon (in mg L^{-1}).

4.3.2. Ecotoxicological evaluation

Mortality of earthworms was only reported after exposure to the soil from El Entredicho, which caused 5% of mortality after 14 days and an average decrease of 19.8% in body weight. Detrimental effects on reproduction tests with *E. fetida* were again detected in organisms exposed to the sample from El Entredicho, with a significant reduction of 43.23% in the average body weight of adult organisms after 28 days and a total inhibition of juvenile production at the end of the test period (Figure 2). In contrast, the average production of juveniles per replicate was stimulated ($P < 0.05$) in sols from Almadén, Las Cuevas, and Almadenejos (59, 64.33, and 84.67 respectively) when compared with the ISO control soil (36.8).

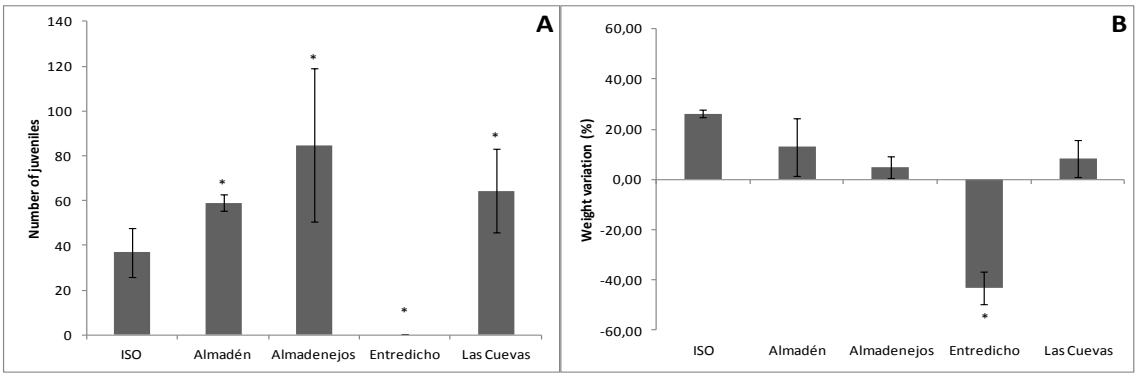

Figure 2. Juvenile production (A) and weight variation (B) of *E. fetida* in reproduction tests. Mean values ± standard deviations of 3 replicates. '*': Significantly different from control ($P < 0.05$; Mann Whitney U Test).

Results from dual-control avoidance tests with earthworms and collembolans showed an even distribution of individuals between sections, with a percentage of organisms per section between 40 and 60%. All test soils triggered a significant response on earthworms ($P < 0.05$; Fisher Exact Test). Earthworms significantly avoided the soil from El Entredicho, with an average of 85% of individuals located in the control section at the end of the test (Figure 3). In contrast, *E. fetida* were significantly attracted ($P < 0.05$) to soils from Almadén, Almadenejos and Las Cuevas (93% to 94% of organisms in test sections at the end of the test). Tests with *F. candida* detected significant avoidance responses ($P < 0.05$; Fisher Exact Test) in soils from Almadén, El Entredicho and Las Cuevas. The average percentage of individuals in the control section of test chambers containing the sample from El Entredicho (91.92%) was significantly higher than the average values in soils from Almadén, Las Cuevas and the ISO artificial control soil (58.72%, 62.71% and 55.87% respectively). Collembolans neither avoid nor were attracted by the soil from Almadenejos.

Figure 4 shows the percentages of plants' seed germination and growth in test soils. All species presented high germination rates in the ISO artificial soil (95 to 100%). When compared with the controls, the emergence of *B. rapa* and *T. pratense* was statistically inhibited ($P < 0.05$; Mann Whitney U Test) in samples from Almadén (germination rates of 75% and 35% respectively), Almadenejos (70% and 45%

respectively) and El Entredicho (25% and 0% respectively), while emergence rates of *L. perenne* showed no differences between soils. The sample from Las Cuevas showed no significant detrimental effects on the germination of the studied plants. Between species, *T. pratense* emergence showed statistically higher sensitivity than *B. rapa* and *L. perenne* in Almadén and El Entredicho.

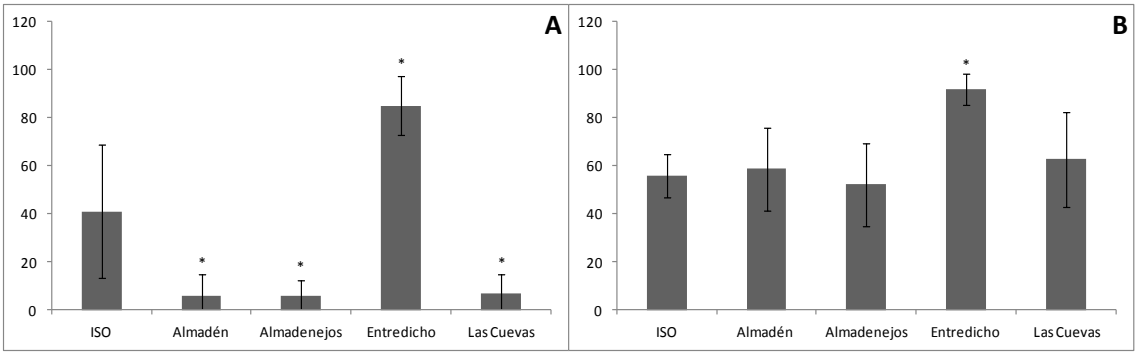

Figure 3. Mean percentage of earthworms (A) and collembolans (B) in control sections of test containers in avoidance tests. Mean values ± standard deviations of 5 replicates. '*': Significantly different from control ($P < 0.05$; Mann Whitney U Test).

Marked differences in plant growth were detected between test soils and species. The growth of all species was statistically inhibited ($P < 0.05$) in El Entredicho (average fresh biomass < 20 mg) while no affectations were observed in Las Cuevas (average fresh biomass of 0.60, 0.03 and 0.24 g for *B. rapa*, *T. pratense* and *L. perenne* respectively). When compared with controls, the growth of *T. pratense* was statistically inhibited ($P < 0.05$) in Almadén (average fresh biomass of 0.05 g) while that of *L. perenne* was stimulated in Almadenejos (0.39 g). On average, *B. rapa* presented higher growth rates, followed by *L. perenne* and *T. pratense*.

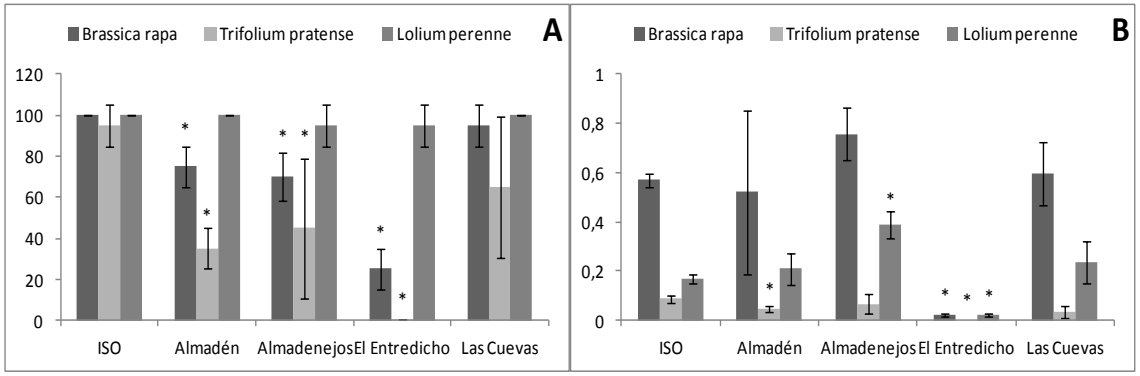

Figure 4. *Brassica rapa, Trifolium pratense* and *Lolium perenne* A) seedling emergence (in %) and B) fresh biomass (in grams). Means and standard deviations from four replicates. '*' means statistically different from control ($P < 0.05$; Mann Whitney U Test).

Aquatic bioassays presented marked differences in the sensitivity to the water extracts from contaminated soils. On the one hand, *V. fischeri* Luminescence Inhibition Test and *D. magna* Acute Immobilization Test were not able to detect toxicity in any of the samples. On the other hand, microalgae *R. subcapitata* were extremely affected by the exposure to undiluted aqueous extracts, with percentages of growth

inhibition ranging from 72.1% in the sample from Almadenejos to 100% in Almadén, El Entredicho and Las Cuevas. Higher half maximal inhibitory concentrations (i.e. less toxicity detected) of 18.8% and 13.9% were estimated for samples from Almadenejos and El Entredicho while significantly lower values ($P < 0.05$; Confidence Interval Ratio Test) of 7.8% and 4.5% were estimated for water extracts from Almadén and Las Cuevas (Figure 5).

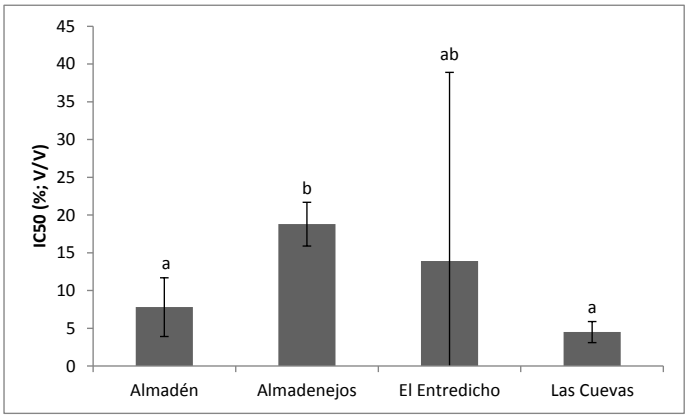

Figure 5. IC50s of aqueous extracts from test soils in algal growth inhibition tests. Values with the same letter are not statistically different ($P > 0.05$; Confidence Interval Ratio Test).

4.4. Discussion

4.4.1. Impacts on the terrestrial compartment

Despite specific variations detected in the soil from El Entredicho, all the studied sites presented quite similar physicochemical properties. On the other hand, metal contents markedly differed between locations. Mercury concentrations were within the range reported by previous surveys on this area (Sánchez et al. 2005; Millán et al. 2006; Colacevich et al. 2011; Millán et al. 2011) and exceeded the intervention values for soil remediation of Dutch regulations (VROM 2000) in all sites except for El Entredicho. Such concentrations were very high for uncontaminated soils but can be expected in an area like the mining district of Almadén, rich in mercury deposits and subjected to prolonged mining activity (Higueras et al. 2003). Even so, sequential extraction procedures applied by other authors (Sánchez et al. 2005; Millán et al. 2006; Colacevich et al. 2011) revealed that Hg in soils from the Almadén mining district is not easily bioavailable, thus presenting a limited risk for terrestrial organisms. In fact, the chemical analysis performed by Colacevich et al. (2011) in soils from the same sites than ours revealed that less than 0.18% of the total soil Hg was attributed to the chemically available Hg fraction (the sum of water soluble and exchangeable fractions). Cd, Cr, Cu, Ni, Pb and Zn concentrations did not reach the intervention values of Dutch regulations (VROM 2000) in any site and consequently were not considered a major threat to terrestrial organisms.

Results from ecotoxicological assays revealed that most soils did not exert severe detrimental effects to terrestrial invertebrates and plants. Lethality of earthworms was almost negligible, which was also reported by Colacevich et al. (2011) after a 44-day exposure of *Lumbricus terrestris* to soils collected from the same sites. Sublethal endpoints evaluated on earthworms showed opposite responses depending on the test soil. On the one hand, the sample from El Entredicho totally inhibited the reproduction of earthworms and was considered to have a limited habitat function since more than 80% of the test organisms remained in the control section at the end of avoidance tests (Hund-Rinke and Wiechering 2001). On the other hand, soils from Almadén, Almadenejos and Las Cuevas markedly stimulated the reproduction of *E. fetida* and attracted earthworms in avoidance tests. Such responses were unexpected in earthworms exposed to metal-contaminated soils, although Abassi and Soni (1983) reported a significant increase in juvenile production of *Octochaetus pattoni* exposed for 60 days to soils freshly spiked with $HgCl_2$. An explanation for the sub-lethal responses observed in our study may be that soils from Almadén, Almadenejos and Las Cuevas were better able to fulfill the ecological requirements of *E. fetida* than the ISO artificial soil thanks to their natural pedological properties and the fact that mercury was scarcely bioavailable. Other authors previously reported that artificial soils were rejected by earthworms in standardized avoidance tests when confronted with natural soils (Chelinho et al. 2011; Frankenbach et al. 2014). Among the invertebrate species studied, collembolans showed higher sensitivity than earthworms. Lock and Janssen (2001) already documented that *F. candida* were more sensitive than *E. fetida* to soils freshly spiked with $HgCl_2$. However, the low bioavailability of mercury in test soils together with the negative and statistically significant correlation found between soil pH and avoidance response of collembolans ($r = -0.96$, $P = 0.042$) points out that soil acidity instead of mercury concentration would be the main responsible for the observed responses. The impact of the studied soils in the germination rates of *Trifolium pratense* and *Brassica rapa* was high in Almadén and Almadenejos and extreme in El Entredicho, while severe effects on growth were only observed in El Entredicho. The selected plant species presented the same ranking of sensitivity reported by Ramírez et al. (2008) and Bori et al. (2016).

Among sites, El Entredicho represented the main threat for terrestrial organisms. However, metal concentrations in El Entredicho were the lowest among the studied soils and therefore cannot explain the toxicity exerted by this site. Consequently, we considered that toxicity was associated to the extreme acidity of the site, parameter that showed a significant correlation with the avoidance response of *F. candida* and is known to cause a detrimental impact in the behavior and reproduction of earthworms (Jänsch et al. 2005; Römbke et al. 2006; Chelinho et al. 2011, Scheffczyk et al 2014) and in the growth of *B. rapa* (Römbke et al. 2006) in natural acid soils. Furthermore, pH in the soil from El Entredicho was below the tolerance range of *E. fetida* (4.0-9.0; Jänsch et al. 2005), *T. pratense* (4.5-8.2; US Department of Agriculture 2015) and *B. rapa* (4.2-7.8; US Department of Agriculture 2015). A similar situation was reported by Bowers et al. (1997) after observing that, despite high metal concentrations in 25 different metal-contaminated sites, pH accounted for the greatest amount of variation in the response of lettuce

(*Lactuca sativa*) and ciliates (*Colpoda inflata*). The lower toxicity of the heavily mercury-polluted soils, especially those from the mines of Almadén and Las Cuevas, was attributed to the very limited bioavailability of mercury.

4.4.2. Impacts on the aquatic compartment

The physicochemical analysis of water samples revealed that electrical conductivities and organic matter contents in the aqueous extracts presented the same patterns than in test soils, with the sample from El Entredicho presenting significantly lower values than the rest. The pH of this sample, however, remained neutral like the others. Mercury concentrations were below the detection limit of the analytical method in all water extracts, which could be explained by the very low Hg concentrations reported in the water-soluble fractions of soils from Almadén (0.02 to 0.04 mg kg^{-1} dw)(Colacevich et al. 2011). With the exception of Zn, the concentrations of all other studied metals were undetectable or low in most samples and remained beneath the aquatic life criteria of the US Environmental Protection Agency (2015) that establish the highest concentration of specific pollutants that are not expected to pose a significant risk to the majority of species in a given aquatic environment. Zn concentration, on the other hand, doubled the US EPA criterion (120 µg L^{-1}) in the sample from Almadén.

Aquatic bioassays showed the lesser sensitivity of *V. fischeri* and *D. magna* towards water extracts from metal-contaminated soils, as previously reported by Alvarenga et al. (2008) and Maisto et al. (2011). The lack of response in these species was explained by the low concentrations of metals in the extracts. Mercury contents were markedly below the EC50 for *V. fischeri* (46 µg Hg^{2+} L^{-1}) (Dutka and Kwan 1982) and *D. magna* (18.6 µg Hg^{2+} L^{-1})(Fagašová 1994) respectively. Similarly, the concentrations of chromium and copper (the only metals that could be quantified in almost all extracts) were up to three orders of magnitude lower than EC50s estimated for *V. fischeri* (Quershi et al. 1984) and *D. magna* (Fagašová 1994; Kungolos et al. 2009). On the other hand, *R. subcapitata* proved extremely sensitive to metal contamination (Maisto et al. 2011; De Paiva Magalhães et al. 2014) and detected toxicity in all extracts. Furthermore, Zn and Cu contents in some extracts exceeded or were close to the IC50 of algal growth tests estimated by De Paiva Magalhães et al. (2014).

As in terrestrial tests, high Hg concentrations in soils were not translated into severe toxicity to aquatic organisms. However, the toxicity derived from each site differed between environmental compartments. On the one hand, the soil from El Entredicho was extremely toxic to terrestrial organisms but gave a relatively low-toxic water extract. In contrast, samples from Almadén and Las Cuevas were scarcely toxic to terrestrial organisms but strongly inhibited algal growth. The inhibitory effects exerted by the sample from Almadén were explained by its high content of Zn, which exceeded the US EPA criterion and was significantly correlated with the growth inhibition of *R. subcapitata* in water extracts from mine soils (Bori et al. 2016). The explanation for the low IC50 estimated in Las Cuevas was unclear although it might be related with Hg concentration in the exchangeable fraction of test soils, which would also partly

explain the toxicity of the extracts from Almadén mine since those were the most mercury-contaminated sites. Besides Zn and Hg, the role of the additive joint action of other dissolved metals (especially copper) should be taken into consideration when analyzing the toxicity in the aquatic compartment.

4.5. Conclusions

This study successfully assessed the toxicity of soils from the Almadén mining district and highlighted the need of combining chemical analysis with ecotoxicological bioassays for a better assessment of contaminated sites. Very high concentrations of mercury were identified throughout the area although the low availability of the metal (commonly found as insoluble cinnabar) limited the toxicity of the samples. Even so, some sites proved moderately to acutely toxic to terrestrial and aquatic organisms. *E. fetida* were not threatened by most soils while collembolans *F. candida* and terrestrial plant species *T. pratense* and *B. rapa* showed significant detrimental effects. Ecotoxicological risks of the water samples were only identified for *R. subcapitata*, who was extremely sensitive to water extracts from the contaminated soils. In view of the results, the avoidance response of *F. candida*, the seedling emergence and growth rates of *T. pratense* and *B. rapa* and the growth inhibition of *R. subcapitata* are the most appropriate endpoints in further monitoring programs of Hg-contaminated soils.

Despite the low concentrations of available Hg and the moderate toxicity observed, the Almadén mining district presents several ecological threats. The extreme acidity of sites like El Entredicho is likely to jeopardize the survival of many terrestrial invertebrate and plant species. Also, contents of metals other than mercury might relate with the high toxicity of water extracts to algae. Finally, weathering processes could redistribute Hg contents within the mining district in more bioavailable and toxic forms. Because of this, a regular monitoring of the area with the aforementioned ecotoxicological tools together with a remediative intervention is advisable.

CHAPTER V. Bioassays with soil invertebrates as monitoring tools of hydrocarbon degradation[3]

[3] Submitted to *Environmental Science and Pollution Research* (2016).

Abstract

In this study, chemical analyses and ecotoxicity tests were applied for the assessment of a heavily hydrocarbon-contaminated soil prior and after the application of a remediation procedure. Terrestrial bioassays studied the survival and reproduction of *Eisenia fetida* and the avoidance response of *E. fetida* and *Folsomia candida* whereas effects on aquatic organisms were studied by means of acute tests with *Vibrio fischeri*, *Raphidocelis subcapitata* and *Daphnia magna*. The bioremediation procedure led to a significant reduction in the content of hydrocarbons and in toxicity although bioassays were not able to report a percentage decrease of toxicity as high as the percentage reduction in the concentration of hydrocarbons (from 34264 mg kg^{-1} to 3074 mg kg^{-1} i.e. 91% decrease). Sublethal tests proved the most sensitive terrestrial bioassays and avoidance tests with earthworms and collembolans showed potential as monitoring tools of hydrocarbon remediation due to their high sensitivity and short duration. The concentration of hydrocarbons in water extracts from test soils were 130 µg L^{-1} and 100 µg L^{-1}. Similarly to terrestrial tests, most aquatic bioassays detected a significant reduction in toxicity, which was almost negligible at the end of the treatment. *D. magna* survival was the most affected by water extracts although toxicity to the crustacean was associated to the electrical conductivity of the samples rather than to the concentration of hydrocarbons. Ecotoxicity tests with water extracts proved less relevant in the assessment of hydrocarbon-contaminated soils due to the low hydro solubility of hydrocarbons and the influence of the physicochemical parameters of the water extracts.

5.1. Introduction

Total petroleum hydrocarbons (TPHs) have become a worldwide cause of concern due to their environmental persistence, bioconcentration and bioaccumulation (McElroy et al. 1989) as well as their potential toxicity, mutagenicity and carcinogenicity (Brown et al. 1999; White and Claxton 2004). The environmental release of TPHs into soils is known to occur through several ways: pipeline blow-outs, waste dumping, disposal after drilling oil and gas wells, road accidents, leakage in underground storage tanks, or uncontrolled landfill activities among others (Chaineau et al. 2003). Once in soils and depending on the solubility and hydrophobicity of hydrocarbon fractions, TPHs can reach the water compartment through leaching (Stroo et al. 2000). Furthermore, highly-mobile TPHs might reach ground waters and at the same time become more toxic to soil organisms (Cvancarova et al. 2013).

Hydrocarbon-contaminated sites require the application of proper management and remediation procedures to render their soils environmentally acceptable. To achieve this goal, autochthonous populations of hydrocarbon degraders can be stimulated under certain environmental conditions (temperature, soil moisture, nutrients, etc.) and their success in reducing the contents of hydrocarbons can be monitored through chemical quantifications. However, those analyses have proved insufficient for a proper characterization of the overall soil quality because they are unable to identify all compounds in

soils (Fernandez et al. 2005). Moreover, they cannot detect intermediary metabolites of increased toxicity (Haeseler et al. 2001; Loibner et al. 2003) nor provide information on bioavailability, synergic and antagonistic phenomena (Juvonen et al. 2000). On the other hand, ecotoxicological tests do integrate all soil-occurring phenomena and are therefore recommended for the ecological risk assessment of polluted soils (Bori and Riva 2015; Bori et al. 2015; Bori et al. 2016) and as monitoring tools of hydrocarbon remediation (Salanitro et al. 1997; Saterbak et al. 1999; Mendonça and Picado 2002; Lors et al. 2009; Megharaj et al. 2011). In order to obtain useful information on potential ecological risks, such tests are usually applied in batteries that include species from different taxonomical groups and routes of exposure (Békaert et al. 1999; Bispo et al. 1999; Rila and Eisentraeger 2003; Fernandez et al. 2005).

In the evaluation of the environmental risk posed by contaminated soils, most efforts have focused in the study of the effects to soil-dependent organisms (Keddy et al. 1995; Walker et al. 2006). Within this group, soil invertebrates (earthworms and collembolans) are most frequently used for the assessment of lethal and sublethal responses. Among the available endpoints, chronic studies have the advantage of being more sensitive than acute tests and providing information on potential effects on the habitat function of the soil (DECHEMA 1995). However, their higher costs and time consumption make them less suitable for the assessment of polluted soils and as monitoring tools. On the other hand, sublethal tests that evaluate the tendency of earthworms and collembolans to avoid contaminated soils proved quick and sensitive tools for soil quality assessment. Due to their relatively recent standardization (ISO 2008; ISO 2011), the application of such tests for the evaluation of remediation procedures is scarcer. At the same time, aquatic ecotoxicity tests traditionally used for the assessment of water contamination (Riva 1991; Riva et al. 1993; Riva and Lopez, 2001; Riva et al. 2007) can be used as indicators of soil quality through their application on water extracts from polluted soils. However, those tests are considered less relevant from an ecological point of view (Van Gestel et al. 2001).

The aims of this study were: (i) to apply chemical analyses in combination with ecotoxicity tests for the evaluation of a hydrocarbon-contaminated soil prior and after applying a bioremediation procedure, and (ii) to compare the sensitivity of bioassays carried out directly in soils and in their water extracts in order to determine the most suitable battery of tests to evaluate a hydrocarbon-contaminated soil and to monitor its remediation.

5.2. Methodology

5.2.1. Soil samples collection and analysis

Test soils were collected prior (untreated sample; UTR) and after (treated sample; TR) applying a bioremediation process to a heavily hydrocarbon-contaminated soil from an industrialized area in Getafe (Spain). The remediation procedure lasted 120 days and consisted in a stimulation of autochthonous

populations of hydrocarbon degraders in static, ventilated biopiles. Each biopile was 37 m length, 28 m width and 2 m high and contained 1800 m^3 of soil. Composite samples were collected and pretreated as previously specified. The following physicochemical parameters were evaluated: pH, electrical conductivity, soil organic matter, texture and water holding capacity. TPHs in soils (C10–C40) were analyzed by Geotecnia 2000 (Madrid, Spain) in accordance with a method accredited by the Spanish National Accreditation Organization (ENAC). Briefly, hydrocarbons were extracted with hexane, purified with Florisil (reagent grade, Sigma Aldrich) and quantified through gas chromatography using a flame ionization detector (GC-FID). Cd, Cr, Cu, Hg, Ni, Pb and Zn contents were analyzed through atomic absorption spectroscopy by Analiza Calidad (Barcelona, Spain). Reference materials were used for quality control.

5.2.2. Water samples collection and analysis

Water extracts from test soils were obtained as previously described. Values of pH, electrical conductivity and total organic carbon were determined. A subsample of each water extract was sent to Analiza Calidad (Barcelona, Spain) for the quantification of metals and total hydrocarbons through atomic absorption spectroscopy (AAS) and Fourier transform infrared spectroscopy (FTIR) respectively.

5.2.3. Terrestrial ecotoxicity tests

E. fetida acute toxicity tests

Each test ran with 5 concentrations (10-18-31-54-100% for UTR and 41-51-64-80-100% for TR) plus a control and five replicates per treatment.

E. fetida reproduction tests

Tests ran with 5 concentrations (0.25-0.5-1.0-2.0-4.0% for UTR and 3.13-6.25-12.5-25-50% for TR) and three replicates per treatment. Six replicates with artificial control soil were also analyzed.

Avoidance tests with E. fetida and F. Candida

Avoidance tests ran with 5 concentrations plus a control and 5 replicates per treatment. Assays with earthworms were performed at 0.16-0.31-0.63-1.25-2.5% (UTR) and 1.25-2.5-5.0-10-20% (TR) of test soil mixed with artificial soil whereas test concentrations in assays with collembolans were 2.5-5.0-10-20-40% (UTR) and 5.0-10-20-40-80% (TR).

5.2.4. Aquatic ecotoxicity tests

Bacteria luminescence inhibition tests

Test organisms were exposed to 4 concentrations of water extracts (5.63-11.25-22.5-45%). Three replicates per treatment were measured.

Algal growth inhibition tests

Tests ran with 6 concentrations (10-17-29-49-84-90%) plus a control (consisting in algae culture medium) and three replicates per treatment.

Daphnia magna acute immobilization tests

Daphnids were exposed to 10 dilutions of water-extracts (0.1-0.22-0.48-1.0-2.2-4.8-10-22-48-100%) plus a control in four replicates per treatment.

5.3. Results and Discussion

5.3.1. Physicochemical analysis of soil samples

As expected, physicochemical properties were very similar between soils (Table 1). Both samples had a slightly acidic pH (6.54 in UTR and 6.79 in TR) and showed moderate conductivity (1355 µS cm^{-1} in UTR and 1249 µS cm^{-1} in TR). Both soils presented low organic matter contents (1.99% in UTR and 1.08% in TR) and a silt loam texture. The water holding capacity was markedly higher in the treated soil (40.56%) than in the untreated one (13.13%). Both sites were heavily contaminated by petroleum hydrocarbons although their concentration before treatment (34264 mg kg^{-1} dw) was one order of magnitude higher than afterwards (3074 mg kg^{-1} dw). Aliphatic compounds predominated over aromatic ones and represented approximately 73% (UTR) and 89% (TR) of the quantified hydrocarbons. At the same time, both fractions (aliphatic and aromatic) were almost exclusively composed by C16-C21 and C21-C35 compounds. The lower presence of lighter hydrocarbons was associated to volatilization. On the other hand, metal concentrations on both soils were very low and similar to the local geochemical background (BOCM 2006). Furthermore, metal contents were at least one order of magnitude lower than the intervention values for soil remediation established by Dutch regulations (VROM 2000) and were not considered to pose a risk to soil organisms.

5.3.2. Physicochemical analysis of water samples

The physicochemical characteristics of the water extracts are shown in Table 2. The sample from UTR was slightly acidic (pH of 6.45) whereas the extract from TR presented a neutral pH (7.91). Both extracts

presented high conductivity (2263 µS cm^{-1} and 2410 µS cm^{-1} respectively) and organic carbon contents (18.37% to 21.97%). The standard water extraction procedure gave low extraction yields (expressed as the ratio of pollutant concentration in water extract to concentration in soil: [µg/L]/[µg/kg]). Ratios of extraction were in the range of 10^{-8} and 10^{-9} for hydrocarbons and 10^{-2} and 10^{-3} for metals, which were in accordance with the hydrosolubility of each substance.

	Untreated soil (UTR)	Treated soil (TR)
Physicochemical parameters		
pH	6.54±0.12	6.79±0.15
EC (µS cm^{-1})	1355±57	1249±39
SOM (%)	1.99±0.09	1.08±0.21
Texture	Silt Loam	Silt Loam
WHC (%)	13.13±0.18	40.56±2.14
Hydrocarbons (mg kg^{-1} dw)		
Total	34264	3074
Aliphatic fraction		
C10-C12	< 50	< 50
C12-C16	64	< 50
C16-C21	3100	450
C21-C35	22000	2300
Aromatic Fraction		
C10-C12	< 50	< 50
C12-C16	< 50	< 50
C16-C21	1000	54
C21-C35	8100	270
Heavy metals (mg kg^{-1} dw)		
Cd	0.03	0.09
Cr	0.84	0.21
Cu	8.01	9.39
Hg	<0.05	<0.05
Pb	17.56	12.85
Zn	5.88	9.63
Ni	0.43	0.30

Table 1. Physicochemical properties (mean ± sd; N=3), contents of hydrocarbons and contents of metals in soils before and after treatment. EC: Electrical Conductivity; SOM: Soil Organic Matter; WHC: Water Holding Capacity.

Total contents of hydrocarbons reached 130 µg L^{-1} and 100 µg L^{-1} in UTR and TR respectively and did not correlate with soil contents, where a difference of one order of magnitude was detected between sites. Such low difference in hydrocarbon contents between water extracts was explained by the fact that UTR presented a markedly higher concentration of heavier petroleum hydrocarbons (C16-C21 and C21-C35), which are less soluble in water than lighter ones (Brassington et al. 2007), while the concentration of lighter and more hydrosoluble petroleum hydrocarbons was very similar between sites. Among the

analyzed metals, only Cd, Cr and Cu could be quantified in the extracts (concentrations ranging from 1.27 to 18.76 µg L^{-1}).

	Untreated soil (UTR)	Treated soil (TR)
pH	6.45±0.08	7.91±0.11
EC (µS cm^{-1})	2263±91	2410±65
TOC (mg L^{-1})	18.37±0.03	21.97±0.02
Hydrocarbons (µg L^{-1})	130	100
Cd (µg L^{-1})	1.27	<0.5
Cr (µg L^{-1})	4.46	4.20
Cu (µg L^{-1})	18.76	16.26
Hg (µg L^{-1})	<1	<1
Pb (µg L^{-1})	<1	<1
Zn (µg L^{-1})	<100	<100
Ni (µg L^{-1})	<2.5	<2.5

Table 2. Physicochemical properties (mean ± sd; N=3), contents of hydrocarbons and contents of metals in water extracts from test soils. EC: Electrical conductivity; TOC: Total Organic Carbon.

5.3.3. Toxicity to terrestrial organisms

Both test soils proved toxic to soil invertebrates (Table 3). Even so, all terrestrial bioassays detected higher toxicity in UTR than in TR, thus confirming that toxicity to terrestrial organisms was related with hydrocarbons content.

In acute tests, *Eisenia* survival rate decreased throughout time. LC50s of 81.90% and 56.16% were estimated after 7 days and 14 days of exposure to UTR. Similar values (83.13% and 71.07% respectively) were estimated for TR. The body mass of earthworms decreased in controls, which was associated to the lack of food supply during tests. Likewise, body mass loss increased while increasing test concentrations and reached 100% (i.e. 100% mortality) at the highest test concentrations. Despite being recommended for the assessment of hydrocarbon-contaminated soils (Saterbak et al. 1999; Son et al. 2003; Van Gestel and Weeks 2004; Eom et al. 2007; Lors et al. 2009), mortality of earthworms was the least sensitive terrestrial endpoint and the only direct test that was not able to distinguish between soils according to their toxicity.

Earthworms' survival in reproduction tests was only affected in the exposure to the highest concentration of UTR soil (10% mortality). However, a marked decrease in body weight was appreciated (Figure 1). After 28 days of exposure, the lowest concentration of UTR soil caused 18.5% decrease in the weight of earthworms, which further decreased to 36.70% in the highest test concentration. In the treated soil, the slight increase in biomass observed at low test concentrations (14.6% to 17.6%) was followed by an abrupt decrease at higher ones. Both soils caused a significant decrease in the average number of juvenile production. When compared with controls, the inhibition of juvenile production was statistically

significant in concentrations higher than 1% of UTR soil and in all tested concentrations with TR. EC50s for the inhibition of juvenile production in UTR soil and TR soil were estimated at 0.83% and 2.45% of test soil in test substrate respectively and confirmed the higher sensitivity of sub-lethal endpoints suggested by other authors (Hund-Rinke et al. 2002; Davies et al. 2003).

	Eisenia fetida			*Folsomia candida*
	Survival	Reproduction	Avoidance	Avoidance
Untreated soil (UTR)	56.16 (29.62-73.42)	0.83 (0.69-0.99)	1.25 (0.85-1.83)	10.33 (7.05-15.11)
Treated soil (TR)	71.07 (51.25-85.78)	2.45* (1.36-3.27)	6.53* (4.85-8.79)	51.74* (33.21-80.61)

Table 3. LC50 and EC50 values (95% confidence limits) from terrestrial tests. '*': statistically different from the same test performed in untreated soil ($P < 0.05$; Confidence Interval Ratio Test).

Dual-control avoidance tests with *E. fetida* and *F. candida* showed an equal distribution of individuals between sections of the test containers. Mortality was not detected in tests with earthworms and a clear preference for the artificial control soil was observed. Statistically significant avoidance responses ($P < 0.05$; Fisher Exact test) were detected at concentrations of UTR soil higher than 0.31%, with avoidance responses ranging from 32% to 80% (Figure 2). Statistically significant avoidance responses were also observed in exposures to concentrations of 5% to 20% of TR soil (40% to 80% of avoidance). EC50 for UTR soil was estimated at 1.25% whereas that for the TR soil was slightly higher (6.53% i.e. less toxicity detected). Despite the reduction of soil toxicity due to the remediation treatment, both tested soils were considered to present a limited habitat function because the percentages of avoidance reached values higher than 60% (Hund-Rinke and Wiechering 2001).

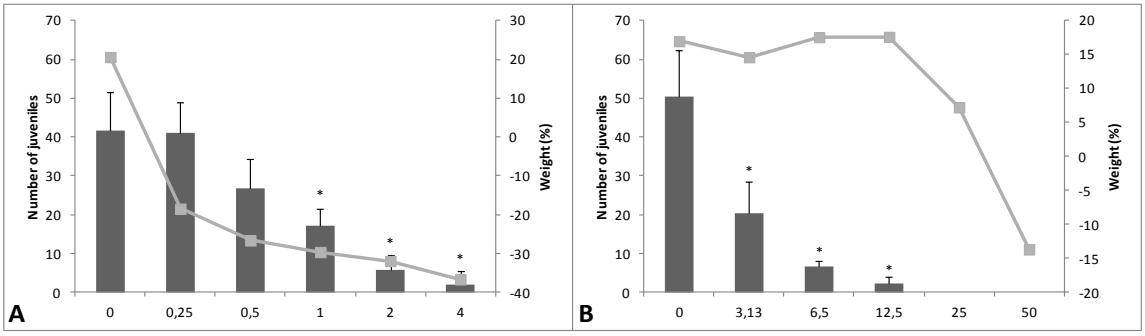

Figure 1. Number of juveniles (bar; left *Y*-axis) and weight variation (curve; right *Y*-axis) of *E. fetida* exposed to test concentrations of the untreated (A) and treated (B) soils in reproduction tests. Mean values ± standard deviations of 3 replicates. '*': Statistically different from control ($P < 0.05$; Mann Whitney *U* Test).

The number of dead or missing collembolans in avoidance tests never reached values higher than 20% per treatment, thus accomplishing with the requirements of the ISO standard. The results were in agreement with those from tests with *E fetida* although significant responses occurred at higher hydrocarbon concentrations (Figure 3). Statistically significant avoidance responses (Fisher Exact test; $P < 0.05$) of *F.*

candida were detected at concentrations higher than 5% of UTR soil and 10% of TR soil. EC50s were estimated at 10.33% for UTR and 51.74% for TR. Despite the high sensitivity of *F. candida* to hydrocarbon-contaminated soils (Bori and Riva 2015), our results were in accordance with those from Da-Luz et al. (2008) and Hentati et al. (2013) which suggested the higher sensitivity of *E. fetida* to soil contamination by hydrocarbons.

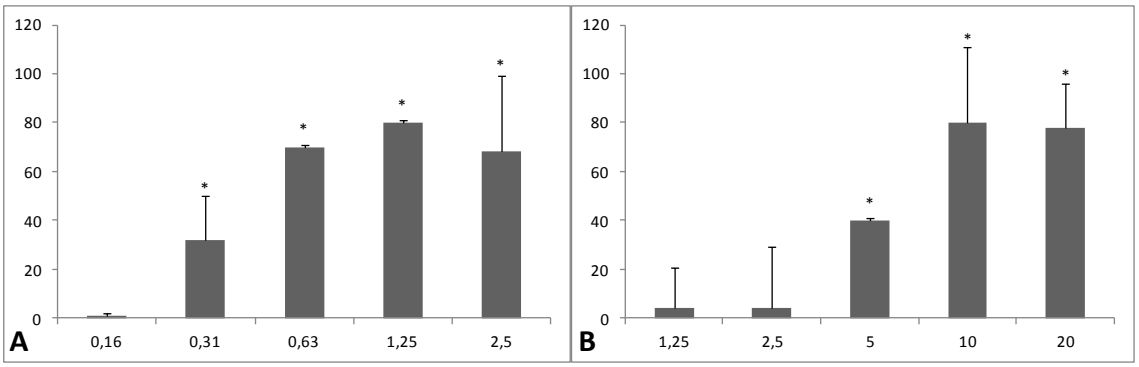

Figure 2. Percentage of avoidance of earthworms *E. fetida* exposed to the untreated (A) and treated (B) soils. Mean values ± standard deviations of 5 replicates per treatment. '*': Significantly different from control ($P < 0.05$; Fisher Exact Test).

In this study, several soil bioassays recommended for the assessment of contaminated soils (Cortet et al. 1999) were successfully performed. The sensitivity ranking according to the detected toxicity was as follows (in decreasing order): Earthworm reproduction > Earthworm avoidance > Collembola avoidance > Earthworm survival. In an attempt to evaluate the suitability of soil ecotoxicity tests with invertebrates as complementary tools for soil remediation monitoring, the percentage decrease in the concentration of hydrocarbons throughout the treatment was calculated and compared with the percentage decrease in toxicity (i.e. the increase in LC50 or EC50 values). Calculations were performed as follows: % decrease = 100 - [(X_A / X_B) x 100], where X_A = value after remediation, and X_B = value before remediation. Results showed a 91% decrease in the total concentration of hydrocarbons, which was similar to microbial degradation rates reported in previous studies (Bossert and Bartha 1984; Morgan and Watkinson 1989; Atlas and Bartha 1992; Salanitro et al. 1997; Suguira et al. 1997). Between fractions, the average degradation of aliphatic compounds (87.52%) was slightly lower than that of aromatic ones (95.6%). None of the terrestrial ecotoxicity tests was able to report a toxicity decrease as high as the percentage reduction in the contents of hydrocarbons although avoidance tests were close (80.86% and 80.06% decrease in toxicity for tests with earthworms and collembolans respectively). Earthworms' survival was the least sensitive to changes in hydrocarbon contents (21% toxicity decrease) whereas earthworms' reproduction reported 66.08% decrease of toxicity despite presenting the highest sensitivity to hydrocarbons. Discrepancies between reduction of contamination and of toxicity after remediation of hydrocarbon-contaminated soils were previously reported by Hubálek et al. (2007) and Al-Mutairi et al. (2008). Such discrepancies were attributed to the presence of toxic intermediate metabolites and to their synergic or antagonistic behavior, which are difficult to detect with chemical methodologies.

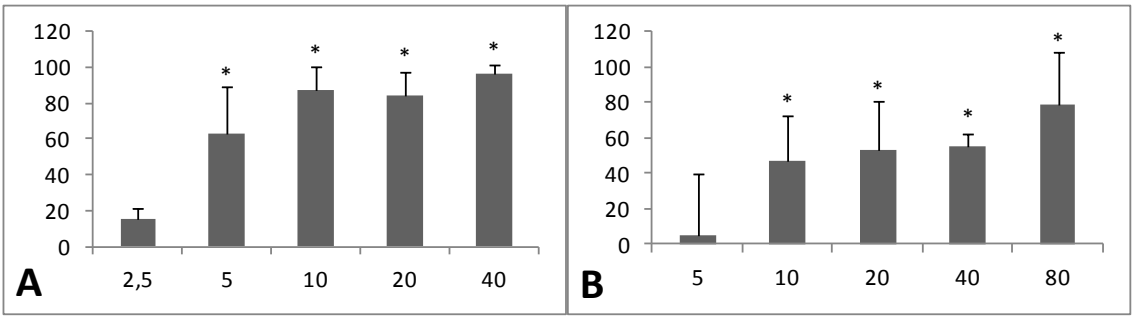

Figure 3. Percentage of avoidance of collembolans *F. candida* exposed to the untreated (A) and treated (B) soils. Mean values ± standard deviation of 5 replicates per treatment. '*': Significantly different from control ($P < 0.05$; Fisher Exact Test).

5.3.4. Toxicity to aquatic organisms

Results of bioassays carried out with water extracts from test soils are summarized in Table 4. All aquatic bioassays estimated significantly lower EC50s (i.e. higher toxicity detected) for the sample extracted from the untreated soil. Even more, the extract obtained after soil remediation proved innocuous to most test organisms.

	V. fischeri Luminescence inhibition	*R. subcapitata* Growth inhibition	*D. magna* Acute immobilization
Untreated soil	47.84 (39.51-56.18)	49 (44-56)	2.30 (1.0-4.7)
Treated soil	>100*	>100*	91* (70-139)

Table 4. LC50 and EC50 (95% confidence limits) of aquatic bioassays performed with water extracts from test soils. '*': statistically different from the same test performed in the extract from the untreated soil ($P < 0.05$; Confidence Interval Ratio Test).

The elutriate from the untreated soil was moderately toxic to aquatic microorganisms *V. fischeri* and *R. subcapitata*. The concentration of water extract reducing bacterial luminescence by 50% after 15 min was 47.84%, and that inhibiting algal growth after 72 hours was 49%. *D. magna* survival was more severely affected by water-extracted pollutants and LC50 was estimated at a concentration of 11.9% after 24 hours and of 2.3% after 48 hours. The water extract from the treated soil was toxic to *D. magna* after 48 hours of exposure (EC50 of 91%) but not after 24 hours (EC50 > 100%).

Although all the studied endpoints were focused on acute responses, aquatic bioassays showed marked differences in sensitivity to the water extracts (in decreasing order): *D. magna* immobilization > *R. subcapitata* growth inhibition ≈ *V. fischeri* luminescence inhibition. These results were not in agreement with previous studies that reported the markedly higher sensitivity of *R. subcapitata* and *V. fischeri* in comparison with *D magna* towards water extracts from hydrocarbon-contaminated soils (Rojíčková-

Padrtová et al. 1998; Bispo et al. 1999; Mendonça and Picado 2002; Eom et al. 2007). The higher toxicity to *D. magna* was associated with the conductivity of the extracts, which was already reported by Thavamani et al. (2015) after assessing the toxicity of a leachate from a hydrocarbon-contaminated soil to *Daphnia carinata*. Aquatic bioassays were successfully applied to water extracts from test soils and were able to detect a decrease in toxicity. However, their performance was markedly influenced by the physicochemical parameters of the water extracts and by the limited hydrosolubility of hydrocarbons. Consequently, they were considered less relevant than direct tests for the assessment of hydrocarbon-contaminated soils (Van Gestel et al. 2001).

5.4. Conclusions

The bioremediation procedure applied to a heavily hydrocarbon-contaminated soil led to a significant reduction in the content of hydrocarbons as well as in toxicity. Even so, the treated soil still presented toxic contents of hydrocarbons. Our study confirmed the higher sensitivity of sublethal endpoints in comparison with lethal ones, with reproduction tests with earthworms showing the highest sensitivity to hydrocarbon contamination. Due to their short duration and high sensitivity, avoidance tests represent a promising tool for routine assessment of hydrocarbon-contaminated soils. Despite their sensitivity, none of the bioassays showed a reduction in toxicity as remarkable as the reduction in the contents of hydrocarbons, thus demonstrating the need to complement chemical analysis with ecotoxicological tools in the evaluation of contaminated soils.

The concentration of hydrocarbons in water extracts and their toxicity to aquatic organisms also decreased after bioremediation. Most aquatic bioassays detected a significant reduction in toxicity, which was almost negligible at the end of the treatment. However, the low hydrosolubility of hydrocarbons and the influence of water physicochemical parameters to some aquatic test organisms limited the performance of aquatic bioassays for the assessment of hydrocarbon-contaminated soils and their remediation.

CHAPTER VI. Environmental impacts of an imidacloprid-containing formulation: from soils to waters[4]

[4] Published in *Afinidad* (2015), Vol 72, No 571, pp 169-176.

Abstract

The neonicotinoid pesticide imidacloprid is among the top sold agrochemicals worldwide. Due to its widespread use in mixtures with different solvents and co-adjuvants, studying the environmental impact of derived commercial formulations has become mandatory. In this study we applied laboratory ecotoxicological tests to evaluate the impact of the imidacloprid-containing formulation Confidor® 20SL on the terrestrial and aquatic compartments. Lethal and sublethal effects of recommended application doses of the product were assessed on standard terrestrial invertebrates *Eisenia fetida* and *Folsomia candida* whereas the toxicity of water extracts from contaminated soils was evaluated in the aquatic model organisms *Daphnia magna* and *Raphidocelis subcapitata*. The exposure to environmentally relevant concentrations of imidacloprid caused no mortality to earthworms (LC50 of 4.23 mg imidacloprid kg^{-1} dry soil) but altered their behavior and reproduction patterns (EC50s for avoidance and reproduction tests of 0.43 and 1.40 mg imidacloprid kg^{-1} dry soil, respectively). Effects on collembolans *F. candida* were negligible. Imidacloprid presented moderate extractability in water, with recovery rates that ranged from 25.4 to 50.4% of the amount in soils and concentrations in water extracts from 13.05 to 71.8 µg L^{-1}. Standard aquatic ecotoxicity tests were not able to detect chronic or acute toxicity in standard test organisms. Nonetheless, concentrations of the insecticide in water extracts were high enough to pose a lethal threat to several other non-standard aquatic organisms.

6.1. Introduction

Despite the potential harmful effects of pesticides, the massive application of plant protection products seems necessary in order to provide enough food to satisfy the demands of the increasing human population. Neonicotinoids are a relatively new group of systemic insecticides developed in the 1980s and first commercially available in the form of imidacloprid in early 1990s (Kollmeyer et al. 1999). They bind to the post-synaptic nicotinic acetylcholine receptors (nAChRs) in the central nervous system of insects, thereby disrupting their nerve impulses. Due to their systemic activity, high toxicity to insects, low toxicity to vertebrates and versatile application, neonicotinoids are among the largest selling and most used pesticides worldwide (Elbert et al. 2008; Jeschke et al. 2011; Main et al. 2014). Within this group of insecticides, imidacloprid-containing formulations account for up to 41% of the neonicotinoids market, becoming the second most used agrochemical worldwide (Jeschke et al. 2011; Pollack 2011).

The prophylactic use of imidacloprid during the last decades has led to serious environmental concerns because of its chemical properties. Regardless of the application route of imidacloprid-containing formulations, the bulk of the active ingredient ends up in soil, where it is subjected to various transformation and transportation processes. Due to its high persistence because of a generally long half-life in soils, non-target soil organisms and terrestrial pollinators are usually exposed to fluctuating concentrations of the insecticide. During the last decades, detrimental effects after exposure to

imidacloprid have been documented in terrestrial snails (Radwan and Mohamed. 2013), beetles (Russell et al. 2010), earthworms (Luo et al. 1999; Capowiez et al. 2003; Dittbrenner et al. 2010; Dittbrenner et al. 2011), collembolans (Idinger 2002; Alves et al. 2014) and bees (Decourtye et al. 2004; Dively et al. 2015) among others. Furthermore, its high water solubility, high partitioning and low soil sorption enhance the movement of the neonicotinoid from the terrestrial to the aquatic compartment by spray drift, leaching or surface runoff (Roessink et al. 2013). Concentrations of imidacloprid have been measured in surface and ground waters worldwide (Lamers et al. 2011; Starner and Goh 2013) and toxic effects have been documented in many aquatic non-target organisms (Tisler et al. 2009; LeBlanc et al. 2012, Roessink et al. 2013; Pérez-Iglesias et al. 2014 among others).

In the European Union, ecotoxicological laboratory tests are used as a preliminary step in the assessment of the environmental impacts of pesticides and are required prior to the commercialization of plant protection products (EC 2009). Most laboratory tests follow standardized guidelines to study the toxic effects that pesticides cause to a set of non-target model organisms that play key roles in ecosystem structure and function. Among the invertebrate species mostly recommended for terrestrial ecotoxicological assays, acute and chronic effects of imidacloprid have been reported in *Eisenia fetida* (Dittbrenner et al. 2011; Alves et al. 2013) and *Folsomia candida* (Idinger 2002; Alves et al. 2014). Similarly, aquatic ecotoxicology have been traditionally applied for the toxicity determination of aquatic pollutants (Lopez-Roldan et al. 2012), industrial effluents (Riva et al. 1993; Riva and Valles 1994; Riva et al. 2007) or elutriates of sediments (Pereira-Miranda et al. 2011) among others. Effects of imidacloprid on the aquatic environment have been mostly studied through standard aquatic toxicity tests with the model organisms *Daphnia magna* (Crustacea) and *Raphidocelis subcapitata* (Chlorophyta) (Pavlic et al. 2005; Jemec et al. 2007; Tisler et al. 2009; Malev et al. 2012). Unfortunately, the application of ecotoxicity tests for the regulation of pesticides have traditionally focused on parental compounds, passing over the fact that are commercial formulations instead of pure active ingredients the ones applied in the environment. This approach neglects the effects of some co-formulants and solvents present in commercial formulations that can be more important than the active substances to non-target organisms (Anderson and Roberts 1983; Neves et al. 2001) due to its own toxicity or through the modification of the toxicity and bioavailability of the pesticide (Malev et al. 2012). Furthermore, it is known that the leaching potential of pesticides is affected by the type of formulation, surfactants and adjuvants (Camazano et al. 1995; Hall et al. 1998).

Despite the amount of available data regarding the impacts of imidacloprid to non-target organisms, data on the toxicity of imidacloprid-containing formulations like Confidor® 20SL is scarcer. Data on such commercial products is required since some studies revealed a higher toxicity and leaching potential of the commercial formulation in comparison with the active ingredient (Gupta et al. 2002; Jemec et al. 2007; Malev et al 2012). In order to widen the available information on this formulation, we studied the environmental impacts associated to field application rates of Confidor® 20SL. Effects on the terrestrial compartment were assessed through standard ecotoxicity tests that evaluated the mortality, inhibition of

reproduction and avoidance behavior of earthworms *E. fetida* as well as the avoidance of collembolans *F. candida* after exposure to treated soils. Impacts on the aquatic compartment were assessed by extracting water samples from treated soils and evaluating the effects of the aqueous extracts to non-target aquatic invertebrate *D. magna* and microalgae *R. subcapitata*. Following this methodology, the main objective of this study was to characterize via lower-tier standard ecotoxicological tests the risk that the application of the recommended field rates of the commercial formulation Confidor® 20SL poses to the aquatic and terrestrial compartments.

6.2. Methodology

6.2.1. Soil sampling and analysis

A soil from a known natural uncontaminated area near the laboratory was selected for the performance of the tests. Samples were collected and pretreated as usual. Several soil parameters were analyzed: moisture, pH, electrical conductivity, organic carbon, organic matter, total nitrogen, C/N ratio, $N\text{-}NO_3$, cation exchange capacity and texture (Table 1).

Moisture (%)	pH	EC ($\mu S\ cm^{-1}$)	Organic carbon (%)	Organic matter (%)	Total nitrogen (%)	C/N	$N\text{-}NO_3$ (mg/kg)	CEC (meq/100g)	Textural class
3.0	7.2	192.65	6.2	10.7	0.4	16.9	15	22.8	Loamy

Table 1. Physical-chemical parameters of the sampled soil. EC: electrical conductivity; C/N: carbon-nitrogen ratio; CEC: cation exchange capacity.

6.2.2. Soil contamination

The insecticide Confidor® 20SL (soluble concentrate, 20% imidacloprid (w/v)) was purchased from Bayer (Germany). Toxicity tests were performed in a range of concentrations that included the lowest and highest application rates recommended by the manufacturer (0.5 and 4 L Confidor ha^{-1}, respectively), two intermediate concentrations (1 and 2 L Confidor ha^{-1}) and a concentration of 8 L Confidor ha^{-1} to cover the worst case scenario of an excessive application of the insecticide. Assuming a depth of incorporation in the soil profile of 0-5 cm and a soil density of 1.5 g cm^{-3}, the application rates of Confidor amounted to 0.78-1.56-3.1-6.20-12.4 mg per kg of soil dw, which corresponded to 0.13-0.26-0.5-1-2 mg of imidacloprid kg^{-1} dry soil respectively. The application of the formulation consisted in preparing a stock solution of 1000 mg Confidor L^{-1} in deionized water. Different spiking solutions were applied to the soil in order to provide the desired concentrations of test substance and a moisture content corresponding to 60% of the WHC. Soils were carefully mixed to ensure an evenly distribution of the pesticide and were left overnight for equilibration. Only deionized water was added to the controls.

6.2.3. Water extracts collection and analysis

Water extracts from imidacloprid-contaminated soils were obtained as previously described. Imidacloprid contents were analyzed by SAILab (Barcelona, Spain) through High Performance Liquid Chromatography – Mass spectrometry (HPLC/MS)(Agilent 1200 LC/ Applied Biosystems 3200 LMS).

6.2.4. Terrestrial ecotoxicity tests

E. fetida acute toxicity test

Four replicates were prepared per test concentration. Since no mortality was expected at field application rates of the pesticide, higher concentrations of Confidor (0.1-1-10-100 mg kg^{-1} dw) were included in order to estimate the LC50.

E. fetida reproduction test

Four replicates per treatment and 6 replicates for the control were prepared.

Avoidance tests with E. fetida and F. Candida

Five replicates per treatment were applied following each methodology and in dual control tests.

6.2.5. Aquatic ecotoxicity tests

Algal growth inhibition test

The test ran with 3 replicates for each water extract from contaminated soils plus the extract from the control soil and the additional control with algae culture medium.

Daphnia magna acute immobilization test

The test ran with four replicates per water extract.

Daphnia magna chronic toxicity test

Chronic toxicity to *D. magna* was evaluated following the OECD Guideline 211 (1998) for a semi static exposure system. Ten replicates per extract were prepared, each consisting of a 250 mL glass vessel filled with 75 mL of the corresponding sample and one daphnid. During the assay, test solutions were replaced and enriched with seaweed extract three times per week. Animals were fed with a concentrate of *Chlorella vulgaris* (0.1-0.2 mg per day). The assay was carried out in a controlled room for 21 days at a temperature of 20±2 ºC and a light:dark cycle of 16:8 hours.

6.3. Results and Discussion

6.3.1. Impacts on the terrestrial compartment

The exposure of soil invertebrates to field doses of Confidor in standard ecotoxicity tests showed marked differences in sensitivity between endpoints and test species. Mortality of earthworms occurred at concentrations higher than 19.77 mg Confidor kg^{-1} (soil dw) (LOEC)(Table 2) and the LC50 was estimated at 24.71 mg kg^{-1} dry soil (corresponding to 4.23 mg imidacoprid kg^{-1} dry soil), thus indicating that the recommended doses of the formulation did not represent a lethal threat to *E. fetida*. Similar toxicity values were reported by Luo et al. (1999) and Gomez-Eyles et al. (2009) using pure imidacloprid as test substance (LC50s of 2.30 mg kg^{-1} soil dw and 2.36 mg kg^{-1} soil dw respectively). On the other hand, studies by Kreutzweiser et al. (2008) and Alves et al. (2013) reported LC50s 10 times higher (25 and 25.53 mg imidacloprid kg^{-1} soil dw respectively) after applying the commercial imidacloprid-containing formulations Merit Solupak® and Gaucho®. Differences in LC50s between studies may be partly explained by variations in experimental parameters like soil organic matter, texture or time of exposure (Kula and Larink 1997) and by the influence of certain components from the commercial formulations to the overall toxicity of the product.

	EC50(LC50)	Lower limit (95%)	Upper limit (95%)	LOEC	NOEC
Mortality	24.71/4.23	23.30/3.99	26.20/4.48	19.77/3.38	15.21/2.6
Reproduction	8.41/1.40	5.38/0.90	12.87/2.15	12.40/2	6.20/1
Avoidance	2.57/0.43	1.86/0.31	3.21/0.54	0.78/0.13	<0.78/<0.13

Table 2. EC50, LC50, confidence intervals (95%), LOEC and NOEC of Confidor / Imidacloprid estimated for earthworm mortality, reproduction and avoidance tests. Values presented in [mg Confidor /kg soil dw] / [mg Imidacloprid /kg soil dw].

The reproduction test gave varying results depending on the concentration of pesticide in soil. *E fetida* produced a significantly higher number of juveniles (Dunnet's test; $P < 0.05$) in soils treated with the lowest application rate of imidacloprid than in the control soil (Figure 1). On the other hand, significant detrimental effects on the reproductive output occurred at twice the highest recommended dose (12.4 mg Confidor kg^{-1} soil dw)(LOEC). The EC50 for the reproduction test was estimated at 8.41 mg Confidor kg^{-1} soil dw (corresponding to 1.40 mg imidacloprid kg^{-1} soil dw)(Table 2), a concentration that could be easily reached if the formulation is not properly employed in terms of applied concentrations or time between applications. A similar EC50 (1.41 mg kg^{-1} soil dw) was reported by Gomez-Eyles et al. (2009) using pure imidacloprid as test substance. On the other hand, a study by Alves et al. (2013) observed a significantly lower toxicity (EC50 of 4.07 mg imidacloprid kg^{-1} soil dw) of an imidacloprid-containing formulation. Luo et al. (1999) and Capowiez and Berard (2006) linked the decrease in the reproductive

output to the damage exerted by imidacloprid to spermatozoa of earthworms. However, it was not concluded whether differences in toxicity between studies were due to the experimental conditions or to the nature of the test substance (active ingredient versus commercial formulation). Additionally, it is noteworthy the hormetic response that Confidor triggered in the reproductive output of exposed earthworms. An enhanced reproduction rate was previously documented by Senapati et al. (1992) and Suthar (2014) after exposing earthworms to low concentrations of the pesticides malathion and methyl parathion respectively although the biochemical mechanism of this response is not clear yet. Similar results have not been reported for other neonicotinoids or neonicotinoid-based formulations. Regarding the reduction of body weight, it followed the same pattern than juvenile production, with an average weight loss lower than controls at low application rates of insecticide and significantly higher at higher test concentrations (Figure 1).

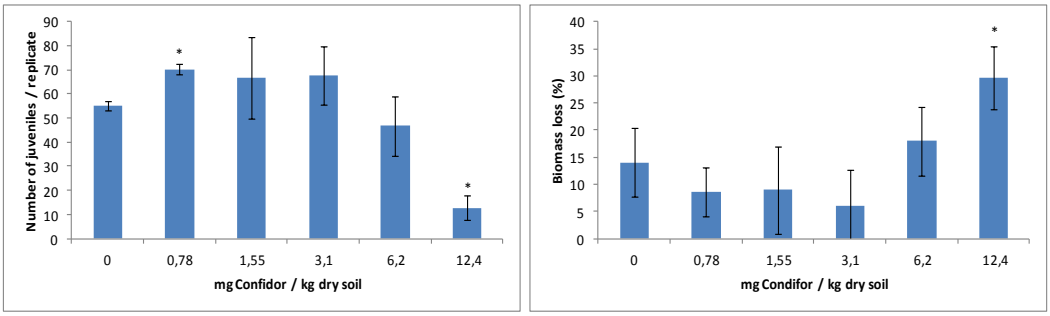

Figure 1. Effects of varying concentrations of Confidor on the reproductive output and weight loss of *E. Fetida* in reproduction tests. Data presented as treatment means ± sd (N=4). Asterisks indicate significant differences with controls (Dunnet's test, $P < 0.05$).

Earthworms exhibited a significant avoidance behavior in response to the presence of all test concentrations of the formulation (Figure 2). The LOEC value was established at the lowest tested concentration, corresponding to the minimum application rate recommended by the manufacturer (Table 2). Furthermore, the EC50 was estimated at 2.57 mg Confidor kg^{-1} soil dw and within the range of recommended application doses. According to Hund-Rinke and Wiechering (2001), soils contaminated with concentrations of Confidor higher than 1.56 mg kg^{-1} soil dw presented a reduced habitat function and should be considered as toxic to earthworms since they presented avoidance responses higher than 60% (i.e. more than 80% of individuals remained at the control section of the test chamber). Our results were in accordance with those from Alves et al. (2013) who estimated an EC50 of 0.11 mg kg^{-1} in *Eisenia andrei* for a commercial formulation of imidacloprid. In contrast, Capowiez and Bérard (2006) reported no avoidance response of earthworm species *Aporrectodea nocturna* and *Allolobophora icterica* after exposure to 0.5 and 1 mg kg^{-1} (soil dw) of Confidor® 200 SL. Even so, previous studies documented behavioral alterations in burrow length, overall distance travelled and rate of burrow reuse under the same experimental conditions (Capowiez et al. 2003). Similarly, earthworms exposed to the pesticide in our study presented an altered locomotion pattern. After the increase in the avoidance response observed at

0.78 and 1.56 mg Confidor kg^{-1} soil dw, the behavioral response turned stable while increasing test concentrations. A study by Pereira et al. (2010) reported that the exposure of *E. andrei* to the carbamate insecticide methomyl induced an inhibition of the Acetylcholine esterase activity that led to hyperactivity in test organisms and in consequence to the adoption of an irregular avoidance behavior. Similar conclusions were postulated by Martínez Morcillo et al. (2013) after exposing earthworms from the species *Lumbricus terrestris* to chlorpyrifos, another insecticide known to affect the nervous system of soil invertebrates. Based on behavioral alterations reported by Capowiez et al. (2003) and according to the mechanism of action of imidacloprid, we hypothesized that the exceeding of certain toxicity threshold somehow altered the locomotive ability of the test organisms and led to an erratic movement pattern, thus causing the stabilization of the avoidance response. In the case of collembolans, no avoidance behavior was detected in response to the application of Confidor recommended doses. Furthermore, a significant preference for the contaminated soil (Fisher exact test, $P < 0.05$) was observed at concentrations of 3.1 and 12.4 mg Confidor kg^{-1} dw (data not shown).

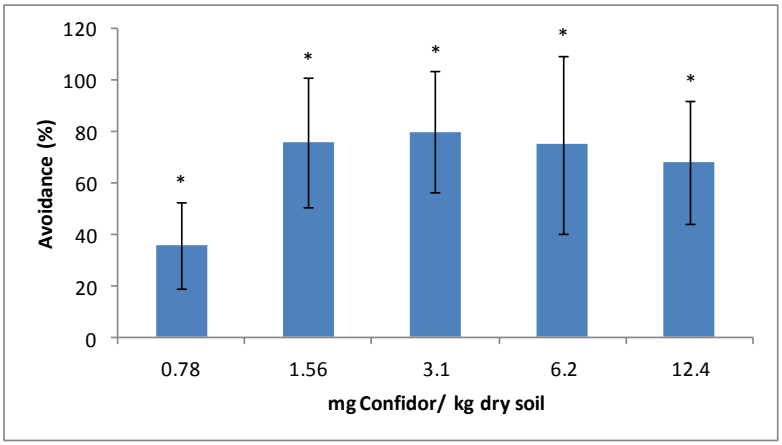

Figure 2. Avoidance response (%) of *E. fetida* (mean ± sd)(N=5) to varying concentrations of Confidor in avoidance tests. Asterisks indicate significant differences with the control (Dunnet's test; $P < 0.05$).

6.3.2. Impacts on the aquatic environment

Concentrations of imidacloprid were determined in water extracts from contaminated soils (Table 3). The concentrations of active ingredient in leachates ranged from 13.05 µg L^{-1} (corresponding to the soil treated with 0.26 mg imidacloprid kg^{-1} dw) to 71.8 µg L^{-1} (2 mg imidacloprid kg^{-1} soil dw) and were positively correlated with concentrations in test soils ($r = 0.910$, $P < 0.05$; Spearman). The concentrations of imidacloprid in water extracts were within the range estimated by Fossen (2006) for chronic and acute surface water exposures (17.24 and 36.04 µg L^{-1} respectively) or after accidental direct spray in a pond or stream (22 µg L^{-1})(SERA 2005). Pesticide recovery ranged from 25.4% to 50.4% of the total amount previously spiked in soil. Recovery rates were in accordance with the relatively high water solubility (0.5

to 0.6 g L^{-1}) and low octanol-water partitioning coefficient (Log (P_{ow})=0.57) of imidacloprid reported by other authors (Gupta et al. 2002; Kurdwadkar et al. 2014).

Although the highest concentration of imidacloprid determined in water extracts was almost 10^3 times lower than the LC50 for *D. magna* found in bibliography (85 mg L^{-1}) (Fossen 2006), mortality tests were performed since previous studies reported the higher toxicity of imidacloprid-containing commercial formulations to *D. magna* due to the presence of toxic adjuvants (Jemec et al. 2007). The exposure to the extracts caused no mortality after 48 hours in the acute toxicity test or after 21 days in the reproduction test. Similarly, differences with the control in the number of neonates per adult, brood size, day of first brood and number of broods per adult in the chronic test were not detected (LOEC in chronic tests estimated between 2.5 and 10 mg L^{-1} (Jemec et al. 2007)). Regarding the effects on the microalgae *R. subcapitata*, algal growth rates in water extracts from all soils (including the untreated soil) were significantly lower than in algal culture medium (data not shown). However, no significant differences in growth inhibition were found between water extracts. Consequently, algal growth inhibition was related to the fact that water parameters deviated from the standard test medium and not to the presence of the insecticide. Results with this model organism were expected based on the insecticidal type of action of imidacloprid and its estimated IC50 (> 600 mg L^{-1})(Daam et al. 2013) although previous studies reported the high toxicity to algae of some Confidor® 200 SL co-formulants (Malev et al. 2012). We hypothesized that the lower toxicity detected in our study was related to the fact that in previous studies the commercial formulation was directly spiked into water while we used aqueous extracts from contaminated soils. Since the purpose of adjuvants is associated to the fixation of the pesticide in soil, a lower extractability of potentially toxic co-adjuvants should be expected.

mg Confidor kg^{-1} soil (dw)	mg imidacloprid kg^{-1} soil (dw)	Water extract (µg L^{-1} leachate)	Recovery rates (%)
0.78	0.13	< QL	-
1.56	0.26	13.05±3.04	50.35±11.95
3.1	0.5	16.35±4.60	32.70±9.19
6.2	1	25.4±8.21	25.4±8.21
12.4	2	71.8±0	35.9±0

Table 3. Concentration of imidacloprid in water extracts from contaminated soils. Means ± standard deviations (N=3). QL (quantification limit): 1 µg L^{-1}. Recovery rates: (µg imidacloprid L^{-1} leachate)/(µg imidacloprid kg^{-1} soil dw)*100. Recovery rates calculated considering the 1/10 dilution in the water extraction procedure).

Despite the low toxicity of water concentrations of imidacloprid to the standard organisms *D. magna* and *R. subcapitata*, the presence of the active ingredient in the water extracts was high enough to represent a lethal or sublethal threat to several other non-standard, freshwater macroinvertebrate species. Daam et al. (2013) reported that a concentration of 52 µg of imidacloprid L^{-1} (value that could be easily extracted from soils where Confidor is improperly applied) was expected to produce 50% affection to 25% and 79% of the crustacean and insect taxa respectively. Furthermore, Roessink et al. (2013) documented LC50s and EC50s close or below 25 µg imidacloprid L^{-1} for the non-standard insect species *Notonecta*

spp., *Micronecta* spp., *Limnephilidae*, *Caenis horaria* and *Cloeon dipterum* and the macrocustacean *Gammarus pulex*, a concentration of active ingredient that was reached in our aqueous extracts.

6.4. Conclusions

Our study pointed out that the application of recommended field doses of the imidacloprid-containing formulation Confidor® 20SL represents a potential threat for the environment. Although mortality was not reported, the exposure to the pesticide caused sublethal effects to *E. fetida*. The influence of some co-adjuvant and solvents to the overall toxicity of pesticide formulations was hypothesized after comparing results from terrestrial ecotoxicity tests using pure imidacloprid with those from tests using commercial formulations. Confidor® 20SL presented toxicity levels in terrestrial standard ecotoxicity tests closer to those from the active ingredient alone than to other commercial formulations. Additionally, reproduction and avoidance tests with earthworms showed responses that had not been previously reported, highlighting the need to keep studying the impacts of massively-applied pesticides.

The application of Confidor® 20SL to agricultural soils posed a risk to the aquatic compartment. Despite the low response of aquatic standard ecotoxicity tests to the presence of the pesticide and to other components of the formulation, final concentrations of the insecticide in the aquatic compartment were high enough to represent a lethal threat to many other non-standard, non-target aquatic organisms, thus emphasizing the need for testing organisms from different taxonomical groups when assessing the environmental risks posed by pesticides.

CHAPTER VII. An alternative approach to assess the habitat selection of *Folsomia candida* in contaminated soils[5]

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Abstract

Avoidance tests with collembolans provide a quick assessment of soil quality. However, some parameters of the procedure can be modified in order to increase its performance. In this study we assessed the tendency of *Folsomia candida* to avoid soils contaminated with boric acid (350-700-1400-2800-5600 mg kg^{-1} soil dry weight), phenmedipham (35-70-140-280 mg kg^{-1} dw) and petroleum hydrocarbons (1312-1838-2625-3675-5250 mg kg^{-1} dw) by preferring an untreated soil. Two separate methodologies were applied, the one presented in the ISO standard 17512:2 and a modified version of the Petri dish method that allowed data acquisition after 2, 24 and 48 hours of exposure. After combining data from three separate trials, effective median concentrations (EC50) estimated with the presented method were lower and showed similar or less variability than those from the ISO procedure, thus suggesting the modified protocol as a suitable alternative screening tool.

7.1. Introduction

Ecotoxicological bioassays became an essential tool for the assessment of risks associated with soil contaminants (Loureiro et al. 2005). In this context, some laboratory ecotoxicological tests follow standardized guidelines to study the effects that soil contaminants cause to a well-defined set of non-target model organisms. Also for collembolans, which contribute to the fertility of soils through decomposition and nutrient cycling processes (Culik and Zeppelini 2003), standardized test guidelines have been developed assessing their potential avoidance of a contaminated soil by preferring a control soil as habitat (ISO 2011). This procedure provides information comparable to the one obtained with other more complex ecotoxicological soil tests but requires less experimental efforts (Domene et al. 2011).

The suitability of the standard avoidance test with Collembola as screening tool of soil contamination relies on its ecological relevance and sensitivity. Additionally, it also benefits from exposure times shorter than acute or reproduction tests and can therefore be routinely applied in 'on site' procedures (Eisenträger et al. 2005). Despite those benefits, avoidance tests present a high variability in their results, which is at least partly explained by the gregarious behavior of collembolans and by unexplained shifts in avoidance responses over time (Filser et al. 2013). According to Filser et al. (2000), the aggregation of individuals in the test containers can be controlled by reducing their density (for instance performing single specimen tests). Regarding temporal variations, Filser and Hölscher (1997) suggested involving sufficient replication and assessing the behavior regularly during the bioassay. Additionally, Van Gestel (2012) highlighted the need to review existing test guidelines for soil contamination assessment in order to make them applicable to new chemicals. Such revision should involve the miniaturization of test systems since many new materials can only be produced in small amounts.

In this study we present an alternative approach that aims to strengthen the use of avoidance tests with Collembola as an early-warning tool of soil contamination through the simplification of the test preparation and data collection. Current avoidance tests with collembolans allow test organisms to dig into soils. Consequently, a destructive and time-consuming analysis of soil samples by flooding and counting the floating individuals is required. Similarly to the study by Aldaya et al. (2006), the presented alternative procedure uses 55 mm Petri dishes, requires fewer resources and involves a slight compression of the soils to prevent collembolans from hiding, thus allowing the observation of test organisms through the transparent lid of the vessel. The major purpose of this work is to study whether the presented procedure can provide information equivalent to the one obtained following the ISO standard 17512 (ISO 2011). Additionally, we aimed at assessing whether a reduction in exposure times can be performed while ensuring reliable data collection. To attain these goals, several tests following the ISO standard and the Petri dish procedure were performed. Data from the ISO standard was collected after 48 hours of exposure whereas exposure times in the Petri dish procedure were 2, 24 and 48 hours. Manifold concentrations of the two reference chemicals recommended by the ISO standard 17512-2 (boric acid and phenmedipham) as well as a soil contaminated with petroleum hydrocarbons sampled from the field were selected as test items.

7.2. Methodology

7.2.1. Experimental design

In this study, avoidance tests with collembolans were carried out following two different experimental procedures: a) using the ISO standard 17512-2 (ISO 2011) and b) using 55 mm Petri dishes as test containers. The selected exposure times were 48 hours with the ISO procedure and 2, 24 and 48 hours for the Petri dish methodology. Median effective concentrations (EC50s) were determined 3 times for each test substance and exposure time in independent experimental runs. Five replicates per test concentration were prepared. Additionally, dual-control tests (10 replicates) with control soil at both sides of the test container were performed with each experimental run in order to validate the tests by checking the homogeneity in the distribution of collembolans. Tests were performed in an environmental chamber at 20±2 ºC and under a 16:8 h light:dark cycle.

According to the procedure described in the ISO standard 17512-2 (ISO 2011), cylindrical plastic containers (diameter 8 cm; depth 8 cm) were divided into two equal sections. Approximately 30 g (wet weight) of control and contaminated soils were placed into the corresponding section and the divider was removed. Twenty organisms were carefully placed on top of the soils. After 48 hours of exposure, the divider was introduced again and the soil from each section was carefully emptied. Each soil was flooded with water and after gentle stirring the animals floating on the water surface were counted. Missing

individuals were considered as dead and discarded for the subsequent calculations. The alternative method used plastic Petri dishes (55 mm diameter, 14 mm height) as test vessels. Petri dishes were divided into two sections filled with 6 g (wet weight) of the corresponding soil. Wet soils were pressed by hand in order to obtain a suitable texture that prevented collembolans from hiding into soil. Ten collembolans were carefully placed on top of the line dividing the two sections. The distribution of individuals was recorded after 2, 24 and 48 hours of incubation.

7.2.2. Soil contamination

A soil from a known natural uncontaminated area near the laboratory (Pereira Miranda et al. 2011) was collected to act as control soil. Soil sampling and pretreatment was performed as usual. Several parameters were analyzed: texture, pH, water holding capacity, organic matter, moisture, and cation exchange capacity (CEC)(Table 1).

	Texture	pH_{KCl}	WHC (%)	Organic matter (%)	Moisture (%)	CEC (meq/100g)	Petroleum hydrocarbons (C10-C40)(mg kg^{-1})
Control Soil	Clay loam	7.6	41.4	10.7	18.6	22.8	-
Field soil	Silty loam	7.9	24.9	8.3	7.5	23.4	5250

Table 1. Physical-chemical characteristics of the control and field soils.

The control soil was spiked with the reference chemicals boric acid (Scharlab, 99.8% pure) and phenmedipham (Sigma-Aldrich, 99.7% pure)(ISO 2011). A stock solution of each substance was prepared with the proper solvent (deionized water for boric acid and methanol (Labkem, 99.5% pure) for phenmedipham). Spiking solutions providing the desired concentration of test substance in soil and a moisture content between 40 and 60% of the water holding capacity of the soil were obtained diluting the stock solutions. Batches of control soil were homogeneously contaminated with the corresponding solution and divided into two sub-batches (one for the application of each methodology). The control soil was treated with five concentrations of boric acid corresponding to 350.0, 700.0, 1400.0, 2800.0 and 5600.0 mg kg^{-1} dry soil and was left for equilibration before starting the tests. In the case of phenmedipham, the control soil was spiked with the corresponding solution, thoroughly mixed and left overnight until methanol was evaporated. Final concentrations of phenmedipham in soils were 35.0, 70.0, 140.0 and 280.0 mg kg^{-1} soil dw. Additionally, a soil from a site (hereinafter field soil) contaminated with petroleum hydrocarbons was selected to ensure the transferability of the proposed test design to a more realistic scenario. Sampling and pre-treatment of the field soil were carried out as usual. Physical-chemical properties of the field soil can be seen in Table 1. Hydrocarbons in soil (C10–C40) were determined through gas chromatography and flame ionization detector (GC-FID)(Table 1). Final test concentrations were 25, 35, 50, 70 and 100% of field soil mixed with control soil, corresponding to 1312, 1838, 2625, 3675 and 5250 mg of petroleum hydrocarbons per kg dw. When dilution of the field soil was

needed, it was achieved by mixing it with the control soil (w/w). Prior to the start of the tests, soils were hydrated with deionized water until the desired moisture content was reached.

7.3. Results and Discussion

Dual-control tests with both methodologies showed an even distribution of collembolans, with a number of organisms per section between 40 and 60% of the total. Additionally, the number of dead or missing organisms never reached values higher than 20% per treatment, thus meeting the requirements of the ISO standard (ISO 2011)(Table 2). Results from avoidance tests revealed the high variability inherent in the procedures, with estimated EC50s that markedly varied with the trial within each test substance and experimental procedure. In some cases effective median concentrations could not be reported. In order to improve the results of the avoidance tests, data from the three available trials were combined. To do so, the mean avoidance percentage of all replicates per treatment (N = 15) was used for the calculation of Probit regressions. After combining the results, EC50s were successfully calculated for both experimental procedures (Table 2).

Effective median concentrations estimated after the exposure to the reference substances boric acid and phenmedipham were in some cases higher than those found in literature. For boric acid, previous studies reported an EC50 of 1440 mg kg^{-1} (Becker et al. 2011) after applying the ISO standard in OECD artificial soil and questioned the suitability of boric acid as reference substance in avoidance tests with collembolans due to the low sensitivity of the organisms (Amorim et al. 2012). Our results agreed with those studies, reporting an EC50 of 3397.58 mg kg^{-1} for the ISO test (Table 2). Differences in EC50s between studies might be partly explained by the soil typology since the percentage of organic matter and clay, soil constituents related with the binding of boron (Goldberg 1997), were higher in our soil (10.7 and 29.1% respectively) than in the OECD artificial soil (approximately 8 and 20% respectively).

Test substance	Procedure	X^2	*P*	EC50	Confidence limits (95%)	Mortality (%)
Boric acid (mg/kg)	ISO 48h	3.45	0.179	3397.58a	2521.10 – 4578.68	4.8±1.9
	Pd. 2h	1.30	0.730	1124.63b	893.26 – 1415.92	0.5±0.9
	Pd. 24h	1.04	0.792	1034.24b	836.78 – 1290.21	1.3±1.6
	Pd. 48h	4.51	0.105	1729.90b	1017.15 – 2692.90	2.3±2
Phenmedipham (mg/kg)	ISO 48h	4.67	0.097	289.76a	225.14 – 372.92	7.9±4.3
	Pd. 2h	5.08	0.079	127.93b	97.51 – 167.85	1.7±0.7
	Pd. 24h	2.79	0.248	155.14ab	83.28 – 289	4.3±2.2
	Pd. 48h	1.25	0.263	201.49ab	121.31 – 334.66	7.3±4.1
Petroleum hydrocarbons (mg/kg)	ISO 48h	0.42	0.810	2744.70a	2276.93 – 3308.55	11±1.8
	Pd. 2h	1.45	0.485	1392.30b	1195.43 – 1621.73	1.9±2
	Pd. 24h	3.08	0.214	1487.85b	1326.15 – 1669.50	2.5±1.6
	Pd. 48h	5.44	0.066	1615.95b	1463.70 – 1780-43	4±1.6

Table 2. EC50 avoidance values (mg kg^{-1}), confidence limits (95%) and percentage of mortality per replicate (mean ± sd) estimated with the data combined from the available trials (N=15 replicates per treatment). EC50s within the same test substance followed by the same letter are not significantly different ($P > 0.05$; Confidence Interval Ratio Test). Pd: Petri dish.

Regarding the exposure to phenmedipham, EC50s from both tested methodologies were two orders of magnitude higher than those calculated by Diogo et al. (2007)(4.14-8.01 mg phenmedipham kg^{-1}) after applying Betosip® (active ingredient phenmedipham) to OECD artificial soil following the ISO standard. In this case, differences in the results between studies were attributed to soil typology and to the form in which the test substance was applied. The contents of organic matter and silt, soil constituents known to reduce the bioavailability of phenmedipham (Domene et al. 2012), were again higher in our soil than in the OECD artificial soil. More importantly, since the ISO standard 17512-2 only requires a reference substance that has phenmedipham as the unique active ingredient, several products that fulfill this requirement can be applied. While we used pure phenmedipham as test substance, the study by Diogo et al. (2012) applied the commercial formulation Betosip®, thus complicating the comparison of results due to the presence of co-formulants of unknown effect to the test organisms. No previous studies were found where avoidance EC50s were estimated after exposing collembolans to pure phenmedipham. Nonetheless, results from the present study suggest that the pure compound is not the best choice as reference substance due to the low sensitivity shown by collembolans.

In exposures to petroleum hydrocarbons, the detected avoidance responses were similar to those documented by Hentati et al. (2013) and Aldaya et al. (2006) after assessing hydrocarbon-contaminated field soils with the ISO standard and with a procedure involving Petri dishes respectively, thus confirming the sensitivity of the test organisms towards the presence of hydrocarbons.

For all tested substances, results from the Petri dish procedure presented similar or lower variability and EC50s (i.e. higher sensitivity) than the ISO method. In the exposure to boric acid and the hydrocarbon-contaminated field soil, EC50 estimates from the Petri dish procedure after all exposure times were significantly lower than those from the ISO methodology (Table 2). In the case of phenmedipham, a statistically lower EC50 was only found after two hours of exposure due to the higher variability observed at longer exposure times. The higher sensitivity of avoidance tests with collembolans performed in Petri dishes was also reported by Boiteau et al. (2011) after applying modified versions of the plastic cup test (ISO 2005b) and of the Petri dish avoidance test (Aldaya et al. 2006) in the assessment of the avoidance response of *F. candida* to copper. No clear explanation for the higher sensitivity of the Petri dish procedure was found although we hypothesized that it might be related to the disposal of soil in the test chambers. Due to the much lower volume of soil available for collembolans in the Petri dishes, test organisms had fewer chances to find a suitable spot in the contaminated section and more often migrated to the non-contaminated soil.

The application of the Petri dish procedure allowed the observation of temporal trends in avoidance responses. EC50s for all tested substances tended to increase throughout time (i.e. decrease in sensitivity) although no statistically significant differences were found between exposure times ($P < 0.05$; Confidence Interval Ratio Test). Therefore, for the tested substances, an exposure of 2 hours may be sufficient when an early screening of soil contamination is required. A shortening of the exposure time was already suggested by Da-Luz et al. (2008) after finding consistent avoidance responses after 24 hours. Aldaya et

al. (2006) and Lors et al. (2006) also established shorter exposure times of 20 to 100 minutes in their avoidance tests with collembolans. Even so, caution must be taken since the absence of significant differences between exposure times might be explained by the high variability of the results, especially in the case of phenmedipham.

7.4. Conclusions

Findings of our study suggest that the presented procedure could become a valuable tool for an initial screening of soil contamination supplying rapid information for future decision-taking. Despite the suboptimal sensitivity of the test organisms to some of the tested substances, the Petri dish method provided information equivalent or even more sensitive than the ISO standard and represented an improvement in terms of time and resources needed for the performance of the test. Additionally, data recorded in this study pointed out that an exposure time of two hours with the Petri dish avoidance test may be enough for an early warning tool. Despite the potential benefits of the presented test, further research is required in order to reduce the high variation of results inherent in avoidance tests. At the same time, the performance of the test and the reduction of the exposure time from 48 to 2 hours should be validated with other soils and chemical substances. Finally, a revision of the reference substances is suggested due to the low sensitivity of *F. candida* to boric acid and pure phenmedipham.

CHAPTER VIII. General discussion and conclusions

8.1. General discussion

Soil contamination is among the main responsible of the worldwide degradation of edaphic systems. The massive release of environmental pollutants in an attempt to satisfy the demands of the increasing human population is close to overwhelm soil ecosystems, thus limiting the performance of their functions. Under these circumstances, the application of the better available tools for the assessment of soil contamination has become essential if contaminated soils are expected to be properly characterized and treated.

In this context, this thesis has shown the suitability of standard ecotoxicological bioassays for the risk assessment of contaminated field soils (diagnostic approach) and for the evaluation of the threats associated to compounds that are likely to reach the edaphic system (prognostic approach). Ecotoxicity tests have provided an alternative and more realistic insight on the ecological impacts associated to soil contamination. Their application has been easy and relatively inexpensive and they have detected effects on exposed organisms that cannot be identified through other methodologies. Ecotoxicological bioassays have supplied a wide range of sensitivities to soil contaminants through the application of several endpoints and test organisms, thus being suitable to assess soil contamination by different types of pollutants. Furthermore, the detected toxicity often correlated between tests and, more importantly, they have allowed the identification of the main toxicity sources from soils. In this regard, bioassays excel due to their capacity to respond to the bioavailable fractions of pollutants, which are the main responsible of the threats posed by contaminated soils. Ecotoxicological evaluations have also been able to identify different dose-response models, which are essential for the proper interpretation of the toxicity exerted by some pollutants. Finally, the availability of test species have allowed studying soil effects in species representative of most organisms inhabiting soil ecosystems.

Due to their many benefits, ecotoxicological bioassays have proved to be a very valuable complementary tool of chemical analysis since the combined application of both approaches have provided a much more accurate assessment of the risks associated to contaminated soils, especially in field contaminated sites. The importance of combining both approaches relies in the fact that it has allowed accurately link environmental risks posed by contaminated sites (in the form of toxicity to organisms) with specific pollutants and soil physicochemical parameters. Such links cannot be established if a single approach (either chemical or ecotoxicological) is solely applied.

In the metal-contaminated area of the Valle del Azogue, chemical analysis clearly identified the quantitatively more polluted sites. However, despite the overall soil quality within the area was rather low, ecotoxicological bioassays detected higher toxicity in some of the lesser-polluted sites. The interpretation of the results from both approaches attributed the observed toxicity to the presence of some metals in specific chemical forms, which modified the salinity of soils from certain sites causing severe detrimental effects to organisms (Bori et al. 2016).

Similarly, chemical analysis quantified mercury contents above international remediation values in several sites located within the mining district of Almadén. However, ecotoxicity tests identified the only

site where mercury concentration did not reach the intervention value as the one presenting the most severe ecological risks. The analysis of the chemical forms of mercury within the studied area together with the results from bioassays revealed that toxicity in the area was associated to high soil acidity rather than to mercury content, which was in an almost unavailable form. On the other hand, the ecotoxicological evaluation confirmed chemical determinations in the study performed on the abandoned mining area of Osor and identified the site with the highest contents of metals as the most toxic to test organisms.

Combining chemical and ecotoxicological analysis for the evaluation of soils from hydrocarbon-contaminated sites allowed a better understanding of the risks associated to hydrocarbon degradation in soils. In this manner, ecotoxicological bioassays validated the chemical analysis and associated the observed toxicity with soil contents of hydrocarbons.

8.1.1. Suitability of different terrestrial ecotoxicity tests for the assessment of soil contamination

In this work, several bioassays with terrestrial organisms that are considered as representative of the edaphic system have been successfully applied. Despite differing in their suitability as assessment tools due to the specific characteristics of each test, results from most terrestrial bioassays are positively correlated (Table 1 - Annex II).

The mortality test with *E. fetida* is among the terrestrial ecotoxicity tests most often applied for soil contamination assessment (Alves and Cardoso 2016). In terms of the resources required for its performance, it is an intermediate between avoidance and reproduction tests. Its sensitivity, however, is rather low when compared with other tests with *E. fetida*. Even so, such tests also allow measurements on weight loss of organisms, which is a more sensitive sublethal parameter. In this work, results from mortality tests with *E. fetida* are positively correlated with sublethal effects observed in earthworms and collembolans (Table 1 - Annex II) and are considered suitable for soil contamination assessment.

The reproduction test with *E. fetida* shows an extreme sensitivity to soil contamination that is not reached by any other test applied directly on soil samples. At the same time, it provides information on the effects of soil contaminants at two life stages of earthworms (adults and juveniles). As in acute toxicity tests with earthworms, measurements in adult organisms include mortality (which is not expected) and weight variation. It correlates positively with the avoidance response of *E. fetida* (Table 1 - Annex II), with whom it shares a high sensitivity towards soil contamination due to the fact that both bioassays test sublethal effects (Hund-Rinke et al. 2002; Davies et al. 2003). Additionally, reproduction tests were able to detect unexpected increases on the reproductive output of earthworms exposed to mercury-contaminated field soils from the Almadén mining district and to a natural soil artificially polluted with low contents of the neonicotinoid insecticide imidacloprid (Bori et al. 2015). On the other hand, reproduction tests are clearly the most labor-intensive and time-consuming assays, which may seriously limit their application and suitability.

Avoidance tests with earthworms are a relatively new tool within soil ecotoxicological bioassays and their application is still lower than that of other soil ecotoxicity tests. They are very useful as early screening tools for the assessment of the habitat function of field soils after only 48 hours of exposure. In addition, avoidance tests show a very high sensitivity to soil contamination that is only matched by reproduction tests and they are the only bioassays that are positively and significantly correlated with all other terrestrial tests (Table 1 - Annex II), thus pointing out its suitability for the assessment of soil quality. Nevertheless, it is important to bear in mind their limitations due to differences in soil properties between control and test soils, what make them inappropriate when aiming to test the toxicity of specific compounds. In this regard, some avoidance tests performed on metal-contaminated field soils showed that earthworms were attracted by test soils due to the low bioavailability of pollutants and their preference for natural soils rather than artificial ones (Chelinho et al. 2011; Frankenbach et al. 2014). Also, the exposure to a neonicotinoid pesticide altered the avoidance pattern of earthworms maybe due to effects on the nervous system of test organisms (Bori et al. 2015). Such effects should be carefully attended when performing avoidance tests to study certain field soils and types of pollutants.

Avoidance tests with collembolans are among the most recently standardized terrestrial ecotoxicity bioassays and share the same benefits and limitations than avoidance tests with earthworms (i.e. quick response and relative influence of soil properties in their performance) although some differences can be appreciated. On average, avoidance tests with *F. candida* present a lesser sensitivity towards contaminated soils that is usually found between that from acute and avoidance tests with earthworms respectively, with whom they are statistically and positively correlated (Table 1 - Annex II). However, collembolans have shown attraction towards natural soils contaminated with metals and with a neonicotinoid insecticide that had been avoided by *E. fetida* and, at the same time, they have significantly avoided metal-contaminated soils that have attracted earthworms. Therefore, applying avoidance tests with both species may be the most suitable approach for the proper evaluation of the effects of contaminated soils in the behavior of soil-dwelling organisms. When compared with avoidance tests with earthworms, assays with collembolans offer the main advantages of requiring a markedly lower amount of sample and presenting a lower sensitivity to soil properties (Da-Luz et al. 2008; Domene et al. 2011), what may make them more suitable to test the toxicity of chemical compounds.

Tests with plants provide an insight on the effects of contaminated soils to soil organisms other than invertebrate species. Although EC50s for tests with plants were not estimated, their results when performed as limit tests usually confirmed the results from terrestrial bioassays with invertebrates. Tests with terrestrial plants usually showed similar sensitivity than lethality and avoidance tests with earthworms and collembolans towards samples from metal-contaminated sites. In addition, the wide range of available test species provide sensitive organisms to many different soil pollutants. Because of this, tests with terrestrial plants are very useful for the assessment of soil contamination

8.1.2. Relevance of aquatic ecotoxicity tests as measurement tools of soil contamination

Aquatic ecotoxicity tests are commonly applied for the assessment of contaminated soils. The basis for their application is the assumption that the bioavailable fraction of soil pollutants matches their water-soluble fraction. However, the results obtained during the performance of this thesis point out that aquatic ecotoxicity tests performed on water extracts from contaminated soils are not representative of the toxicity to soil organisms since no significant correlations have been found between results from terrestrial and aquatic tests (Table 1 - Annex II). Possible explanations for the lack of correlations include the low extractability of contaminants from soils, differences in the interactions between pollutants and environmental matrices (soil or water) and variations in the sensitivity of aquatic and terrestrial organisms towards the same pollutants due to taxonomic divergences.

Extraction yields of soil pollutants in aqueous solutions have been very low regardless of the type of pollutant. Generally, metal contents in aqueous extracts from contaminated soils accounted for approximately 1% to 5% of the total metal concentrations in soils and markedly varied depending on the metal species found in each site. Even so, when heavily metal-polluted areas were studied, such concentrations were high enough to pose a serious threat for aquatic organisms. Extraction rates in aqueous extracts from hydrocarbon-contaminated soils were lower due to the limited solubility of hydrocarbons in water whereas the neonicotinoid pesticide imidacloprid was moderately extracted because of its relatively high hydrosolubility and low soil sorption.

In the study of the metal-contaminated area of the Valle del Azogue, aquatic and terrestrial ecotoxicity assays were agree in the identification of the most toxic sample, whose toxicity was attributed to its salinity. However, two sites with moderate contents of metals that presented low toxicity to terrestrial organisms gave markedly toxic aqueous extracts due to a higher metal solubilization originated by their lower soil pH (Bori et al. 2016). In the mining area of Osor, results from aquatic ecotoxicity tests were in accordance with those from terrestrial bioassays whereas the site that presented highest toxicity to terrestrial organisms within the Almadén mining district was among the least detrimental to aquatic organisms and its toxicity was associated to its pH rather than to metal contents.

The limited hydrosolubility of hydrocarbons (and even lower solubility of their heavier fractions in comparison with the lighter ones (Brassington et al. 2007)) made aquatic ecotoxicity tests less relevant to study hydrocarbon-contaminated field soils (Van Gestel et al. 2001). Even so, aquatic bioassays agreed with terrestrial tests in the identification of the most toxic sample despite differences in the concentration of hydrocarbons between aqueous extracts were almost negligible in comparison with differences in their corresponding soil contents.

Aqueous extracts from soils contaminated with recommended application rates of imidacloprid presented higher recovery rates of the insecticide (25% to 50% of the total content in the corresponding soil), probably due to its specific chemical properties (Gupta et al. 2002; Kurdwadkar et al. 2014). In addition, the concentrations of active ingredient in the extracts were positively correlated with its soil contents.

However, the aqueous extracts showed no toxicity in any of the applied tests whereas their corresponding soils were markedly toxic to earthworms.

Despite their low representativeness of the toxicity to soil organisms, aquatic bioassays proved very valuable to assess the ecological threats that contaminated sites pose to the aquatic compartment through the potential leaching and run-off of soil pollutants. The suitability of aquatic ecotoxicological bioassays for the risk assessment of water samples is shown by the multiple significant and positive correlations established between results from aquatic tests (Table 1 - Annex II).

Generally, bacterial luminescence inhibition tests are less sensitive to contaminants and their results are significantly and positively correlated with those from assays with *D. magna* and *D. rerio* (Table 1 - Annex II). Tests with *V. fischeri* were among the least sensitive to metal-contaminated aqueous extracts from mining sites and in several cases they were not able to report toxicity. Such low sensitivity was previously documented by other authors although it was attributed to the pH correction of water samples suggested by standard methods (Alvarenga et al. 2013), which was not carried out during the performance of this work. Similarly, the sensitivity of the bacteria bioluminescence inhibition test towards contamination by hydrocarbons was lower than that of other aquatic bioassays applied.

The growth inhibition test with algae shows an extreme sensitivity towards aquatic pollutants and is correlated with *D. magna* immobilization (Table 1 - Annex II). Algal growth inhibition assays proved the most metal-sensitive tests (Maisto et al. 2011; De Paiva Magalhães et al. 2014), thus becoming the best tool to assess the risks that metal-contaminated sites from mining areas pose to the aquatic compartment. On the other hand, *R. subcapitata* showed a markedly lower sensitivity towards hydrocarbons.

D. magna immobilization tests show an intermediate sensitivity to pollutants, what make them correlate positively with all other aquatic tests (Table 1 - Annex II). When exposed to metal contamination, *D. magna* immobilization reported higher toxicity than bacterial tests and lower than algal ones (Maisto et al. 2011; Alvarenga et al. 2013). On the other hand, *D. magna* showed the highest sensitivity towards aqueous extracts from hydrocarbon-contaminated sites. Finally, bioassays with *D. rerio* were the least sensitive among all tested species due to their higher resistance to most metals (De Paiva Magalhães et al. 2014).

8.1.3. Development of contaminant-specific batteries of bioassays

The application of ecotoxicological bioassays in batteries including different test species and endpoints has been strongly recommended by several authors (Van Straalen and Van Gestel 1993). In this thesis, the tests included in each battery were selected according to several factors (sensitivity, availability of sample, etc.) although lethal and sublethal endpoints were always included. In general, test batteries performed on each contaminated soil were very similar and successfully assessed soil contamination by metals, hydrocarbons and insecticides.

The sensitivity ranking of the terrestrial tests applied was as follows (in decreasing order): earthworms' reproduction > earthworms' avoidance > collembolans' avoidance > earthworms' mortality, and scarcely varied regardless of the soil contaminant. Therefore, according to their sensitivity, none of the applied tests was specially recommended nor censored for the assessment of a specific group of pollutants. Nonetheless, care must be taken when applying avoidance tests to soils contaminated with substances that can affect the locomotive ability of test organisms since they can alter the responses of the tests.

In contrast, aquatic bioassays were more affected by the nature of the soil contaminant since it ultimately determines its solubility in water and, consequently, its toxicity to aquatic organisms. Therefore, testing the toxicity of water extracts was more useful when studying metal-contaminated sites due to the higher hydrosolubility of metal species than when studying sites contaminated with hydrophobic substances (e.g. hydrocarbons). On average, the sensitivity ranking of the aquatic tests applied was: algae reproduction > daphnids immobilization > bacteria luminescence inhibition > fish lethality and was only altered in water extracts from hydrocarbon-contaminated sites.

8.1.4. Interactions between soil physicochemical parameters, pollutants, and test organisms

Due to the complexity of the soil matrix, many interactions occur between soils, pollutants and organisms that are essential for the proper interpretation of the risks associated to soil contamination. Only metals have been included as soil pollutants for the study of such interactions since not enough data have been collected to properly establish correlations with soil contents of hydrocarbons and insecticides.

Table 2 (Annex II) points out several physicochemical properties from the studied field soils that are markedly related with the toxicity reported in terrestrial and aquatic test organisms. Among them, soil pH partly explains the toxicity observed on some aquatic organisms since it is negatively correlated with detrimental effects appreciated in bioassays with *D. rerio* and *V. fischeri*¸ which are the least sensitive aquatic tests. Thus, toxicity to these organisms increases while increasing the acidity of soils.

On the other hand, soil electrical conductivity (which is a measure of soil salinity) is among the main responsible of the detrimental effects observed in terrestrial organisms since it shows significant correlations with several parameters measured in terrestrial bioassays (Table 2 - Annex II). Soil EC is positively correlated with the toxicity detected in avoidance tests with soil invertebrates and in lethality tests with earthworms and, at the same time, it negatively affects the emergence of all studied plants. However, it is important to bear in mind that pH and salinity of certain field soils may indicate a high bioavailability of pollutants (e.g. metals) (Alvarenga et al. 2012). Therefore, soil contamination should be considered indirectly responsible of the toxicity reported in those sites were pH and EC play a major role in the observed toxicity.

Regarding soil organic matter, high contents benefit earthworms in reproduction tests as well as *B. rapa* growth probably due to its binding capacity of soil pollutants. At the same time, SOM is positively correlated with the lethality of *D. rerio*. Finally, the water holding capacity of test soils shows a

significant negative correlation with the toxicity observed in reproduction tests with earthworms and a positive correlation with the growth of all studied plants (Table 2 - Annex II).

In view of the results from Table 3 (Annex II), relationships between total metal contents in soils and toxicity to organisms is rather limited. Consequently, chemical quantifications of individual metals may not be adequate to estimate lethal and sublethal risks that contaminated sites pose to terrestrial and aquatic species (Gruiz 2005).

According to Table 3, significant relationships between total concentrations of metals and detrimental effects to terrestrial organisms are only established between soil contents of chromium and nickel and toxicity detected by the highly-sensitive reproduction test with earthworms. Also, Ba contents significantly stimulate the growth of *L. perenne.* The lack of additional significant correlations is explained by the major role that the chemical form and speciation of metals have in their bioavailability and toxicity (Shiowatana et al. 2001). Since metals are usually found in different chemical forms among sites (and consequently with different associated toxicity), it is difficult to establish correlations with total contents of metals that are quantified in different areas. Thus, correlations should be better established with specific chemical forms of metals.

In contrast, several significant positive correlations are detected between soil contents of metals and toxicity to aquatic species (Table 3 - Annex II). According to Table 3, mortality of *D. magna* is associated with soil contents of Ba whereas that of *D. rerio* significantly correlates with Zn and Cd concentration in test soils. Regarding the effects on aquatic microorganisms, As content in soil is correlated with the inhibition of *R. subcapitata* growth whereas Ba significantly inhibits the luminescence of *V. fischeri*. Despite the detected correlations, it is still complicated to estimate the risks that metal-contaminated sites pose to the aquatic compartment through the quantification of total metal contents in soils because, as previously mentioned, metal solubility will rely on the specific chemical form in which the metal is found within the site of study.

Even though soil physicochemical parameters like pH and EC are known to be strongly influenced by the presence of metals (Alvarenga et al. 2012), few significant interactions are reported between them (Table 4 - Annex II). Soil contents of Cr are reported to significantly increase the acidity of the studied field soils whereas Cr and Ni contents positively correlate with soil organic matter content in soils, which may be explained by the binding action of organic matter.

8.1.5. Suitability of ecotoxicity tests as monitoring tools of soil remediation procedures

As suitable complements of chemical analysis, ecotoxicity tests show potential for routinely monitoring of remediation procedures of hydrocarbon-contaminated soils. In this regard, ecotoxicological bioassays successfully associated toxicity to organisms with hydrocarbon contents. However, only avoidance tests with earthworms were able to report a percentage decrease in toxicity close to the percentage reduction of hydrocarbons content throughout the remediation process. Such discrepancy may be explained by the

formation of toxic intermediaries during the degradation of hydrocarbons (Haeseler et al. 2001; Loibner et al. 2003), which are more difficult to quantify through chemical analysis. Due to their low cost and time-consumption, avoidance tests with *E. fetida* may be routinely applied to assess the extent of remediation of a hydrocarbon-contaminated soil. Regarding aquatic bioassays, the low hydrosolubility of hydrocarbons limits their suitability.

8.1.6. New procedures that could help in the ecotoxicological assessment of contaminated soils

The alternative procedure presented in this thesis for the assessment of the avoidance response of collembolans enhanced the strengths of the ISO test while keeping its sensitivity and a similar or even lower variability in the results (Bori and Riva 2015). The limitation in the variability of the results may be explained by the reduction of the organism's density in tests containers and by a more regular assessment of the behavior of test organisms during the assays (Filser and Hölscher 1997; Filser et al. 2000). Additionally, this miniaturized test system may be especially useful to test samples of limited availability (Van Gestel 2012) and for an initial 'on-site' assessment of soil contamination prior to the definitive sampling of test soils.

8.2. Conclusions

1. Ecotoxicological bioassays present several specific characteristics that make them unique and very suitable tools to realistically evaluate the risks associated to contaminated soils.

2. The combined application of ecotoxicity tests and chemical analysis for the assessment of soil contamination provides a more accurate interpretation of the threats posed by contaminated sites since it allow linking concentrations of pollutants to effects on organisms.

3. Terrestrial ecotoxicity tests are the most relevant tool for the ecological risk assessment of contaminated soils although the available tests present different suitability.

4. Lethality tests with earthworms show lesser sensitivity than other tests but their results are positively correlated with those from sublethal assays and are therefore considered suitable assessment tools.

5. Reproduction tests with earthworms show the highest sensitivity to all soil pollutants, what make them able to report some responses that are not appreciated through lesser-sensitive tests. However, the resources and efforts required for their performance limit their routine application.

6. Avoidance tests with earthworms are very useful as quick assessment tools of the habitat function of contaminated soils but they are less appropriate to evaluate the toxicity of specific compounds due to the influence of soil properties on the observed response. Additionally, they should be avoided in the evaluation of soils contaminated with substances known to affect the nervous system of test organisms.

7. Avoidance tests with collembolans show similar benefits than tests with earthworms and a lesser influence of soil properties in exchange for a lower sensitivity to most pollutants. They may be better applied in combination with tests with earthworms.

8. Tests with terrestrial plants are a good complement to assays with soil invertebrates, providing organisms from a different taxonomic group that show a wide range of sensitivities to soil contamination.

9. Aquatic ecotoxicity tests performed on aqueous extracts from contaminated soils are not representative of the toxicity observed in terrestrial tests.

10. Aquatic bioassays are very useful to assess the ecological threats that contaminated sites pose for the aquatic compartment through the risks of pollutants' leaching and run-off.

11. In general terms, the sensitivity of terrestrial ecotoxicity tests is little influenced by the nature of the soil contaminant whereas that of aquatic tests is affected by the hydrosolubility of the pollutant.

12. The toxicity reported by aquatic and terrestrial ecotoxicity tests is strongly correlated with some soil physicochemical properties like pH, electrical conductivity, organic matter contents and water holding capacity.

13. Chemical quantifications of soil pollutants are not adequate to estimate toxicity from contaminated sites because total concentrations of metals in soils are little representative of their detrimental effects to terrestrial organisms. In contrast, some effects on aquatic organisms are explained by metal contents in soil.

14. The chemical form in which a metal is found within a contaminated site plays a key role in the interpretation of the ecological risks associated to such area.

15. Avoidance tests with earthworms may become an alternative to chemical analysis for the routine assessment of the remediation success in hydrocarbon-contaminated soils.

16. The alternative procedure presented for the assessment of the avoidance response of collembolans equals and may even improve current methodologies and therefore shows potential to become a first screening tool of soil contamination.

CHAPTER IX. References

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ANNEX I. Total metal contents in soil and water samples

Table 1. Total concentrations of metals quantified through INAA in soils from the “Valle del Azogue” mining district.

		A1	A2	A3	A4	A5	A6	A7
Au	ppb	-40	-19	-5	-65	-125	-20	-10
As	ppm	864	550	143	1550	1320	462	477
Ba	ppm	110000	78900	7350	93000	20400	15800	42800
Br	ppm	-2,5	-1,1	7,4	-3,6	86,4	15,8	11,1
Ce	ppm	-15	51	70	-18	-36	68	41
Co	ppm	-5	-1	-1	-4	25	8	4
Cr	ppm	-30	83	64	-43	-60	44	-9
Cs	ppm	-3	7	2	-4	-5	4	-1
Eu	ppm	-0,5	-0,2	1,6	-0,8	-1,6	-0,2	-0,2
Fe	%	1,28	2,27	3,44	3,06	2,72	2,49	2,34
Hf	ppm	-2	-1	7	-3	-7	-1	-1
Hg	ppm	4000	865	116	130	-25	935	1240
Ir	ppb	-20	-18	-5	-64	-50	-5	-5
La	ppm	16,9	31,7	29,5	28,1	21,6	28,4	19,5
Na	%	0,4	0,64	1,03	0,69	2,23	1,18	1,24
Nd	ppm	-23	10	32	191	-58	55	-5
Rb	ppm	-15	99	126	-58	-78	-44	-15
Sb	ppm	7460	3290	357	>10000	>10000	2000	2850
Sc	ppm	4,3	10,4	12,6	9,7	7,8	9,7	8,3
Se	ppm	-15	-10	-6	-31	-37	-9	-3
Sm	ppm	-0,1	2,9	5,1	3,1	1,7	3,6	2,4
Sn	%	-0,3	-0,12	-0,05	-0,43	-0,86	-0,14	-0,09
Ta	ppm	-2,8	-0,5	-0,5	-3,3	-6	-2,5	-0,5
Tb	ppm	-1,1	-0,5	-0,5	-1,4	-2,6	-0,5	-0,5
Th	ppm	-3,2	6,5	11,6	-3,8	-7	7,2	4,8
U	ppm	-7,5	-2,9	-0,9	-8,9	-15	-3	-1,9
W	ppm	-3	-1	-1	-6	-13	-4	-1
Yb	ppm	-1,1	2,3	2,7	-1,7	-3,4	4,3	-0,2

Table 2. Total concentrations of metals quantified through ICP-OES in soils from the “Valle del Azogue” mining district.

		A1	A2	A3	A4	A5	A6	A7
Ag	ppm	50	25,7	1,1	34,5	58,7	15,7	15,3
Cu	ppm	8	31	27	42	32	38	28
Cd	ppm	2	10,6	1	6,6	5	1,4	0,8
Mo	ppm	7	2	< 1	< 1	< 1	1	< 1
Pb	ppm	549	1210	134	1190	1820	512	536
Ni	ppm	7	20	55	23	20	34	18
Zn	ppm	465	3190	424	2230	503	1330	854
S	%	1,808	2,05	0,47	2,02	2,81	1,49	0,28
Al	%	2,26	6,57	6,58	5,09	3,11	6,4	5,74
Be	ppm	-1	2	3	2	1	3	2
Bi	ppm	-2	< 2	< 2	< 2	< 2	< 2	< 2
Ca	%	1,34	0,27	2,49	3,09	7,33	2,28	0,12
K	%	1,22	1,87	1,98	1,45	1,14	2,07	1,49
Mg	%	0,14	0,23	1,28	1,16	0,94	0,69	0,54
Mn	ppm	10	53	823	171	64	108	31
P	%	0,019	0,042	0,052	0,035	0,027	0,047	0,054
Sr	ppm	741	871	454	721	569	852	2200
Ti	%	0,21	0,36	0,38	0,28	0,16	0,35	0,31
V	ppm	22	57	91	30	25	63	53
Y	ppm	6	18	12	13	7	12	16

Table 3. Total concentrations of metals quantified through ICP-MS in aqueous extracts from soils collected in the “Valle del Azogue” mining district.

		A1	A2	A3	A4	A5	A6	A7
Na	µg/L	16100	2850	> 100000	8120	> 100000	> 100000	> 100000
Li	µg/L	3	258	13	103	7	10	7
Be	µg/L	< 0.1	19,5	< 0.1	1,9	< 0.1	< 0.1	< 0.1
Mg	µg/L	7330	44400	83000	49100	65300	184000	64700
Al	µg/L	2	> 100000	1080	11100	48	12	6
Si	µg/L	9400	1700	2200	3500	1500	1700	1300
K	µg/L	5450	1140	7900	810	5230	7540	5350
Ca	µg/L	> 200000	> 200000	> 200000	543000	60300	43000	54500
Sc	µg/L	< 1	30	< 1	< 10	< 1	< 1	< 1
Ti	µg/L	30,8	5,2	8,3	12,8	0,6	< 0.1	< 0.1
V	µg/L	2,6	42,2	0,4	< 1	< 0.1	< 0.1	< 0.1
Cr	µg/L	2,5	137	1,1	< 5	< 0.5	< 0.5	< 0.5
Mn	µg/L	6,2	3940	69,3	1280	8	5,8	3,3
Fe	µg/L	< 10	10800	40	640	< 10	< 10	90
Co	µg/L	0,077	649	2,89	71,1	0,412	0,21	0,086
Ni	µg/L	< 0.3	1310	6,4	267	1,4	0,5	< 0.3
Cu	µg/L	2	1780	13,5	577	4,9	3,5	5,4
Zn	µg/L	27,4	> 5000	871	> 50000	1040	345	181
Ga	µg/L	< 0.01	5,93	0,03	2,08	0,01	< 0.01	< 0.01
Ge	µg/L	0,01	0,71	0,01	0,39	0,01	0,02	0,01
As	µg/L	571	69,4	25,9	4,83	5,53	4,61	4,84
Se	µg/L	0,6	7,5	0,3	3,4	3,2	6,8	3,1
Rb	µg/L	3,48	3,78	3,95	8,17	11,2	17,5	10,7
Sr	µg/L	463	994	226	1420	1020	933	953
Y	µg/L	0,006	61	0,268	23,8	0,1	0,013	0,011
Zr	µg/L	0,01	0,08	0,04	< 0.1	0,02	0,03	0,02
Nb	µg/L	< 0.005	< 0.005	< 0.005	< 0.05	< 0.005	0,005	< 0.005
Mo	µg/L	1,3	< 0.1	1	< 1	3,2	2,9	2,6
Ag	µg/L	< 0.2	0,4	< 0.2	< 2	1,2	74,6	10,5
Cd	µg/L	0,12	450	1,97	264	1,07	0,39	0,15
In	µg/L	< 0.001	0,094	< 0.001	< 0.01	< 0.001	< 0.001	< 0.001
Sn	µg/L	< 0.1	0,1	< 0.1	< 1	< 0.1	< 0.1	0,4
Sb	µg/L	154	24,1	24,4	32,3	78,7	62,5	68,4
Te	µg/L	< 0.1	< 0.1	< 0.1	< 1	< 0.1	< 0.1	< 0.1
Cs	µg/L	0,073	0,44	0,034	13	0,458	0,641	0,288
Ba	µg/L	79,2	111	140	87,4	280	306	304
La	µg/L	0,008	30,7	0,116	20,8	0,084	0,015	0,009
Ce	µg/L	0,016	112	0,41	41,2	0,166	0,025	0,019
Pr	µg/L	< 0.001	12,5	0,044	3,65	0,011	< 0.001	< 0.001
Nd	µg/L	0,004	54,7	0,209	14,9	0,061	0,01	0,006

Sm	µg/L	< 0.001	15,1	0,054	3,37	0,012	< 0.001	< 0.001
Eu	µg/L	< 0.001	3,49	0,013	0,777	0,004	0,002	0,002
Gd	µg/L	< 0.001	16,4	0,07	4,17	0,015	< 0.001	< 0.001
Tb	µg/L	< 0.001	2,84	0,008	0,592	< 0.001	< 0.001	< 0.001
Dy	µg/L	< 0.001	17,2	0,069	3,54	0,011	< 0.001	< 0.001
Ho	µg/L	< 0.001	3,09	0,011	0,67	0,001	< 0.001	< 0.001
Er	µg/L	< 0.001	7,8	0,032	1,76	0,006	< 0.001	< 0.001
Tm	µg/L	< 0.001	1	0,002	0,193	< 0.001	< 0.001	< 0.001
Yb	µg/L	< 0.001	6,53	0,024	1,29	0,002	< 0.001	< 0.001
Lu	µg/L	< 0.001	0,879	< 0.001	0,121	< 0.001	< 0.001	< 0.001
Hf	µg/L	< 0.001	0,041	< 0.001	< 0.01	< 0.001	< 0.001	< 0.001
Ta	µg/L	< 0.001	0,003	< 0.001	< 0.01	< 0.001	< 0.001	< 0.001
W	µg/L	< 0.02	0,02	< 0.02	< 0.2	< 0.02	< 0.02	< 0.02
Hg	µg/L	2,1	2,8	0,3	42,9	19,1	1220	28,7
Tl	µg/L	0,418	0,152	0,34	106	6,32	7,65	4,11
Pb	µg/L	0,22	297	1,68	199	1	1,17	0,6
Bi	µg/L	< 0.3	< 0.3	< 0.3	< 3	< 0.3	< 0.3	< 0.3
Th	µg/L	< 0.001	15,3	0,033	< 0.01	< 0.001	< 0.001	< 0.001
U	µg/L	0,465	57,6	0,545	0,45	0,343	0,105	0,283

Table 4. Total metal concentrations in soils from the mining area of Osor.

		EM-1	TOS	OS-6
Al	%	0,61	4,46	6,48
Ag	ppm	29,9	0,6	3,5
As	ppm	15,2	12,5	5,7
Au	ppb	6	<2	<2
Ba	ppm	250	5110	2200
Be	ppm	<1	2	3
Bi	ppm	0,4	0,3	<2
Ca	%	29,8	7,33	4,62
Cd	ppm	24,9	7,6	11,5
Co	ppm	6	14	9
Cr	ppm	<2	39	44
Cu	ppm	11	47	24
Fe	%	0,35	1,78	2,54
Hg	ppm	3	<1	<1
K	%	0,28	1,86	3,09
Mg	%	0,03	0,49	0,58
Mn	ppm	89	684	418
Mo	ppm	<1	1	2
Na	%	0,07	1	1,51
Ni	ppm	3	18	19
P	%	0,008	0,041	0,078
Pb	ppm	>5000	940	>5000
Rb	ppm	23	103	146
S	%	2,43	0,23	0,28
Sb	ppm	56,5	1	6,1
Se	ppm	<0,1	<0,1	<3
Sr	ppm	17	105	109
Ta	ppm	<0,5	<0,5	4
Ti	%	0,03	0,24	0,21
Th	ppm	1	5,2	8,7
U	ppm	<0,5	2,4	3,1
V	ppm	4	45	48
W	ppm	<1	<1	<1
Zn	ppm	11300	2370	2730
Y	ppm	82	30	22
La	ppm	7,5	27,8	23,4
Ce	ppm	13	55	38
Nd	ppm	<5	24	13
Sm	ppm	2	5	4,8
Sn	%	<0,01	0,02	<0,01
Tb	ppm	<0,5	<0,5	<0,5

Table 5. Total concentrations of metals in aqueous extracts from soils sampled in the mining area of Osor.

		EM-1	TOS	OS-6
Na	µg/L	2750	1700	1690
Li	µg/L	1	< 1	2
Be	µg/L	< 0.1	< 0.1	0,4
Mg	µg/L	1130	1090	1290
Al	µg/L	108	10	1750
Si	µg/L	1100	700	3400
K	µg/L	940	660	1500
Ca	µg/L	25000	20500	7700
Sc	µg/L	< 1	< 1	< 1
Ti	µg/L	5,1	< 0.1	15,6
V	µg/L	0,2	< 0.1	3,6
Cr	µg/L	< 0.5	< 0.5	1,7
Mn	µg/L	9,8	12,8	203
Fe	µg/L	120	< 10	1450
Co	µg/L	0,4	0,362	2,03
Ni	µg/L	0,3	< 0.3	3
Cu	µg/L	3,8	3,7	18,4
Zn	µg/L	498	280	901
Ga	µg/L	0,28	0,04	1,67
Ge	µg/L	0,06	0,01	0,16
As	µg/L	0,76	0,24	1,4
Se	µg/L	< 0.2	< 0.2	1
Rb	µg/L	0,816	0,536	2,26
Sr	µg/L	46,6	30,6	20,5
Y	µg/L	0,522	0,023	4,33
Zr	µg/L	0,09	0,02	0,46
Nb	µg/L	0,024	< 0.005	0,049
Mo	µg/L	0,6	1,5	0,1
Ag	µg/L	< 0.2	< 0.2	< 0.2
Cd	µg/L	9,15	3,64	2,34
In	µg/L	< 0.001	< 0.001	0,002
Sn	µg/L	< 0.1	< 0.1	< 0.1
Sb	µg/L	1,7	0,52	1,52
Te	µg/L	< 0.1	< 0.1	< 0.1
Cs	µg/L	0,036	0,007	0,104
Ba	µg/L	340	450	306
La	µg/L	0,525	0,014	7,35
Ce	µg/L	1,11	0,033	23,8
Pr	µg/L	0,139	< 0.001	1,64
Nd	µg/L	0,557	0,013	6,29

Sm	µg/L	0,137	0,002	1,35
Eu	µg/L	0,033	0,004	0,219
Gd	µg/L	0,125	0,002	1,29
Tb	µg/L	0,014	< 0.001	0,178
Dy	µg/L	0,089	0,002	0,922
Ho	µg/L	0,014	< 0.001	0,155
Er	µg/L	0,034	< 0.001	0,389
Tm	µg/L	0,003	< 0.001	0,045
Yb	µg/L	0,024	< 0.001	0,292
Lu	µg/L	< 0.001	< 0.001	0,033
Hf	µg/L	0,003	< 0.001	0,022
Ta	µg/L	0,003	0,002	0,003
W	µg/L	< 0.02	< 0.02	0,04
Hg	µg/L	1,1	0,8	2
Tl	µg/L	< 0.001	< 0.001	0,044
Pb	µg/L	183	3,33	326
Bi	µg/L	< 0.3	< 0.3	< 0.3
Th	µg/L	0,042	< 0.001	0,305
U	µg/L	0,047	0,005	0,595

ANNEX II. Pearson correlation coefficients

Table 1. Pearson's correlation coefficients among terrestrial and aquatic ecotoxicity tests. "*": significant at $P < 0.05$ level (two-tailed); "**": significant at $P < 0.01$ level (two-tailed). For a more convenient interpretation of toxicity data, all median toxicity values (AC50, EC50, IC50 and LC50) are converted into Toxic Units (i.e. the inverse of the EC50 expressed in percentage, using the formula: TU = (100/EC50)) prior to calculating correlation coefficients.

	D. magna LC50	*D. rerio* LC50	*E. fetida* AC50	*E. fetida* EC50	*E. fetida* LC50	*F. candida* AC50	*R. subcapitata* IC50	*V. fischeri* EC50
D. magna LC50	1,000	**0,881****	0,198	0,592	0,218	0,359	**0,578***	**0,944****
D. rerio LC50	**0,881****	1,000	0,017	-	-0,014	0,184	0,083	**0,934****
E. fetida AC50	0,198	0,017	1,000	**0,661***	**0,762****	**0,957****	-0,091	-0,065
E. fetida EC50	0,592	-	**0,661***	1,000	0,018	0,576	0,020	0,571
E. fetida LC50	0,218	-0,014	**0,762****	0,018	1,000	**0,733****	0,014	0,074
F. candida AC50	0,359	0,184	**0,957****	0,576	**0,733****	1,000	-0,053	0,096
R. subcapitata IC50	**0,578***	0,083	-0,091	0,020	0,014	-0,053	1,000	0,468
V. fischeri EC50	**0,944****	**0,934****	-0,065	0,571	0,074	0,096	0,468	1,000

Table 2. Pearson's correlation coefficients among ecotoxicity tests and physicochemical properties of test soils. "*": significant at $P < 0.05$ level (two-tailed); "**": significant at $P < 0.01$ level (two-tailed). For a more convenient interpretation of toxicity data, all median toxicity values (AC50, EC50, IC50 and LC50) are converted into Toxic Units (i.e. the inverse of the EC50 expressed in percentage, using the formula: TU = (100/EC50)) prior to calculating correlation coefficients.

	pH	EC	SOM	WHC
D. magna LC50	-0,478	0,408	0,288	-0,105
D. rerio LC50	**-0,878****	0,043	**0,863***	0,176
E. fetida AC50	0,141	**0,803****	-0,129	-0,370
E. fetida EC50	0,583	0,579	**-0,728***	**-0,891****
E. fetida LC50	-0,109	**0,823****	0,012	-0,226
F. candida AC50	0,111	**0,840****	-0,141	-0,446
R. subcapitata IC50	-0,297	0,139	0,035	0,142
V. fischeri EC50	**-0,716****	0,179	0,301	0,006
*B. rapa*_emergence (%)	0,216	**-0,710****	0,060	0,457
*B. rapa*_growth (mg)	-0,133	-0,406	**0,540***	**0,779****
*T. pratense*_emergence (%)	0,190	**-0,600***	0,087	0,487
*T. pratense*_growth (mg)	-0,070	-0,413	0,489	**0,710****
*L. perenne*_emergence (%)	0,191	**-0,811****	-0,050	0,443
*L. perenne*_growth (mg)	-0,141	-0,386	0,468	**0,751****

Table 3. Pearson's correlation coefficients among ecotoxicity tests and metal contents in test soils. "*": significant at $P < 0.05$ level (two-tailed); "**": significant at $P < 0.01$ level (two-tailed). Median toxicity values (AC50, EC50, IC50 and LC50) are converted into Toxic Units (TU = 100/EC50) prior to calculating correlation coefficients.

L. peremne growth (mg)	*L. perenne* emergence (%)	*T. pratense* growth (mg)	*T. pratense* emergence (%)	*B. rapa* growth (mg)	*B. rapa* emergence (%)	*V. fischeri* EC50	*R. subcapitata* IC50	*F. candida* AC50	*E. fetida* LC50	*E. fetida* EC50	*E. fetida* AC50	*D. rerio* LC50	*D. magna* LC50	
-0,396	-0,485	-0,361	-0,377	-0,389	-0,365	0,419	**0,642***	0,086	0,169	-0,396	0,018	-0,055	0,491	As
-0,386	**-0,550***	-0,373	-0,410	-0,391	-0,414	**0,604***	0,491	0,031	0,128	0,466	-0,080	0,330	**0,598***	Ba
-0,305	-0,143	-0,244	-0,062	-0,291	0,003	-0,040	-0,160	0,068	0,092	-0,367	0,058	-0,256	-0,066	Hg
-0,357	-0,435	-0,329	-0,321	-0,356	-0,284	0,273	-0,007	0,162	0,223	0,562	0,097	-0,034	0,286	Sb
-0,515	-0,271	-0,451	-0,187	-0,506	-0,186	0,490	0,341	-0,035	-0,013	0,292	-0,051	0,302	0,455	Pb
-0,306	0,098	-0,310	-0,071	-0,313	0,036	0,162	0,062	-0,079	-0,119	0,495	0,035	**0,936****	0,116	Zn
-0,312	-0,209	-0,257	-0,127	-0,257	-0,146	-0,093	-0,069	0,382	0,343	0,414	0,392	-0,025	0,072	Be
0,314	0,516	0,124	0,314	0,255	0,429	0,188	0,029	-0,321	-0,455	0,224	-0,206	**0,853***	0,084	Cd
0,131	0,168	0,183	0,449	0,231	0,443	-0,241	-0,203	-0,241	-0,241	-0,330	-0,283	-1,000	-0,232	Co
0,358	0,016	0,315	-0,108	0,351	-0,104	0,202	0,065	-0,124	-0,125	**-0,782***	-0,119	0,578	0,220	Cr
-0,195	-0,279	-0,120	-0,171	-0,159	-0,278	0,280	0,341	0,131	0,190	-0,282	0,140	0,290	0,419	Cu
0,357	-0,122	0,342	-0,054	0,371	-0,148	-0,036	0,008	0,048	0,070	**-0,804****	0,062	-0,121	0,024	Ni
0,299	-0,015	0,467	0,202	0,306	0,144	-	-0,700	-	-	-0'312	-0,617	-	-0,796	Ti
-0,104	-0,306	-0,063	-0,328	-0,089	-0,382	0,019	-0,184	0,278	0,321	-0,435	0,185	0,111	0,110	V

Table 4. Pearson's correlation coefficients among soil physicochemical parameters and metal contents in test soils. "*": significant at $P < 0.05$ level (two-tailed); "**": significant at $P < 0.01$ level (two-tailed).

	pH	EC	SOM	WHC
As	-0,122	0,277	-0,064	-0,110
Ba	-0,309	0,249	0,062	-0,131
Hg	0,270	0,178	-0,133	-0,302
Sb	0,012	0,320	-0,005	-0,313
Pb	-0,010	0,136	-0,018	-0,250
Zn	0,081	-0,066	-0,212	-0,316
Be	0,095	0,334	0,183	-0,281
Cd	-0,151	-0,392	0,012	-0,072
Co	0,299	-0,205	0,372	0,303
Cr	**-0,605***	-0,083	**0,672***	0,365
Cu	-0,086	0,283	0,440	0,035
Ni	-0,179	0,118	**0,713****	0,288
Ti	0,062	-0,007	0,405	0,264
V	-0,092	0,272	0,432	-0,067

ANNEX III. List of contributions related with this thesis

List of publications directly and indirectly related with this work:

- Bori J and Riva MC (2015) An Alternative Approach to Assess the Habitat Selection of *Folsomia candida* in Contaminated Soils. *Bulletin of Environmental Contamination and Toxicology* 95(5): 670-674.
- Bori J, Ribalta C, Domene X, Riva MC, Ribó JM (2015) Environmental effects of an imidacloprid-containing formulation: from soils to waters. *Afinidad* 571(72): 169-176.
- Bori J, Vallès B, Navarro A, Riva MC (2016) Geochemistry and environmental threats of soils surrounding an abandoned mercury mine. *Environmental Science and Pollution Research International*. In Press.
- Bori J, Vallès B, Navarro A, Riva MC (2016) Ecotoxicological Risks of the Abandoned F-Ba-Pb-Zn Mining Area of Osor (Spain). In revision by *Environmental Geochemistry and Health*.
- Bori J, Vallès B, Ortega L, Riva MC (2016) Bioassays with soil invertebrates as monitoring tools of hydrocarbon degradation. Submitted to *Environmental Science and Pollution Research International*.
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- Bori J, Vallès B, Navarro A, Riva MC: Heavy metal toxicity of mine tailings to aquatic and terrestrial organisms. *26th SETAC Europe Annual Meeting*. Nantes, 22nd-26th May 2016.
- Bori J, Vallès B, Navarro A, Riva MC: Ecotoxicological risks of soils surrounding an abandoned mercury mine. *25th SETAC Europe Annual Meeting*. Barcelona, 3rd-7th May 2015.
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ANNEX IV. Publications

Environ Sci Pollut Res
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RESEARCH ARTICLE

Geochemistry and environmental threats of soils surrounding an abandoned mercury mine

Jaume Bori [1] & Bettina Vallès [1] & Andrés Navarro [1,2] & Maria Carme Riva[1]

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Abstract The closure of mercury mining areas is generally associated with a release of Hg and other metals into the environment due to the abandonment of mining wastes. Because of their potential toxic properties, the mobilization of particulate and soluble metal species is of major concern. In the present study, the environmental risks posed by soils sur- rounding an abandoned mercury mining area in Valle del Azogue (Almeria, Spain) are assessed through the determination of physical-chemical parameters, the quantification of metal concentrations, and the application of aquatic and ter- restrial ecotoxicity bioassays. Chemical analysis of soil samples revealed concentrations of Hg, As, Ba, Pb, Sb, and Zn above international intervention values. Results from terrestrial tests showed detrimental effects in all studied organisms (*Eisenia foetida*, *Folsomia candida*, and different plant species) and revealed the avoidance response of earthworms as the most sensitive endpoint. Surprisingly, the most toxic samples were not the ones with higher metal contents but the ones presenting higher electrical conductivity. Aquatic ecotoxicity tests with *Vibrio fischeri*, *Raphidocelis subcapitata*, *Daphnia magna*, and *Danio rerio* were in accordance with terrestrial tests, confirming the need to couple environmental chemistry with ecotoxicological tools for the proper assessment of metal-contaminated sites. In view of the results, a remediative intervention of the studied area is recommended.

Environmental impacts of an imidacloprid-containing formulation: from soils to waters

Jaume Bori[1]*, Carla Ribalta[1], Xavier Domene[2,3], Maria Carme Riva[1], Juan Manuel Ribó[1]

[1]Center for Research and Innovation in Toxicology, INNOTEX, Technical University of Catalonia (UPC). Ctra. Nac. 150; Km 15, zona IPCT ed.TR23, 08227 Terrassa (Barcelona) Spain. [2]CREAF, Cerdanyola del Vallès 08193, Spain. [3]Univ Autònoma Barcelona, Cerdanyola del Vallès 08193, Spain

Impactos ambientales de una formulación que contiene imidacloprid: de los suelos a las aguas

Impactes ambientals d'una formulació que conté imidacloprid: dels sòls a les aigües

Recibido: 26 de junio de 2015; aceptado: 1 de julio de 2015

RESUMEN

El pesticida neonicotinoide imidacloprid se encuentra entre los agroquímicos más vendidos en todo el mundo. Debido a su amplio uso en mezclas con diferentes disolventes y co-adyuvantes, estudiar el impacto ambiental de las formulaciones comerciales derivadas se ha convertido en obligatorio. En este estudio se utilizaron ensayos ecotoxicológicos de laboratorio para cuantificar el impacto del Confidor® 20SL (formulación que contiene imidacloprid) en los compartimentos terrestre y acuático. Los efectos letales y subletales de las dosis recomendadas de aplicación del producto fueron evaluadas en los invertebrados terrestres *Eisenia foetida* y *Folsomia candida* mientras que la toxicidad de los lixiviados de los suelos contaminados se evaluó en los organismos acuáticos modelo *Daphnia magn*a y *Raphidocelis subcapitata* (anteriormente *Selenastrum capricornutum*). La exposición a concentraciones ambientalmente relevantes de imidacloprid no causó mortalidad en las lombrices de tierra (CL_{50} de 4.23 mg de imidacloprid por kg de suelo seco) pero alteró los patrones de comportamiento y reproducción (valores de CE_{50} de 0.43 y 1.40 mg de imidacloprid por kg de suelo seco en los ensayos de alejamiento y reproducción respectivamente). Los efectos en los colémbolos *F. candida* fueron despreciables. El imidacloprid presentó una lixiviabilidad moderada, con tasas de recuperación en los extractos acuosos que fueron del 25.4 al 50.4% de la cantidad presente en los suelos y concentraciones de 13.05 a 71.8 µg por litro. Las pruebas estándar de ecotoxicidad acuática no fueron capaces de detectar toxicidad aguda o crónica en los organismos de ensayo. Sin embargo, las concentraciones de insecticida en los extractos fueron lo suficientemente grandes como para representar una amenaza letal para otros organismos acuáticos no estándar.

Palabras clave: Imidacloprid; ecotoxicidad; extractos acuosos; lombrices de tierra.

RESUM

El pesticida neonicotinoide imidacloprid es troba entre els agroquímics més venuts a tot el món. Degut al seu ampli ús en mescles amb diferents dissolvents i co-adjuvants, estudiar l'impacte ambiental de les formulacions comercials que en deriven ha esdevingut obligatori. En aquest estudi es van utilitzar assajos ecotoxicològics de laboratori per a quantificar l'impacte del Confidor® 20SL (formulació que conté imidacloprid) en els compartiments terrestre i aquàtic. Els efectes letals i subletals de les dosis recomanades d'aplicació del producte van ser avaluades en els invertebrats terrestres *Eisenia foetida* i *Folsomia candida* mentre que la toxicitat dels lixiviats dels sòls contaminats es va avaluar en els organismes aquàtics model *Daphnia magna* i *Raphidocelis subcapitata* (anteriorment *Selenastrum capricornutum*). L'exposició a concentracions ambientalment rellevants d'imidacloprid no va causar mortalitat en els cucs de terra (CL_{50} de 4.23 mg d'imidacloprid per kg de sòl sec) però en va alterar els patrons de comportament i reproducció (valors de CE_{50} de 0.43 i 1.40 mg d'imidacloprid per kg de sòl sec en els assajos d'allunyament i reproducció respectivament). Els efectes en els col·lèmbols *F. candida* van ser menyspreables. L'imidacloprid va presentar una lixiviabilitat moderada, amb taxes de recuperació en els extractes aquosos que van anar del 25.4 al 50.4% de la quantitat present en el sòls i concentracions de 13.05 a 71.8 µg per litre. Les proves estàndard d'ecotoxicitat aquàtica no van ser capaces de detectar toxicitat aguda o crònica ens els organismes d'assaig. No obstant això, les concentracions d'insecticida en els extractes van ser prou grans com per a representar una amenaça letal per a altres organismes aquàtics no estàndard.

Paraules clau: Imidacloprid; ecotoxicitat; extractes aquosos; cucs de terra

SUMMARY

The neonicotinoid pesticide imidacloprid is among the top sold agrochemicals worldwide. Due to its widespread use in mixtures with different solvents and co-adjuvants, studying the environmental impact of its derived commercial formulations has become mandatory. In this study we used laboratory ecotoxicological tests to quantify the impact of the imidacloprid-containing formulation Confidor®

*Corresponding author. jaume.bori@crit.upc.edu / +34 937398396

20SL on the terrestrial and aquatic compartments. Lethal and sublethal effects of recommended application doses of the product were assessed on standard terrestrial invertebrates *Eisenia fetida* and *Folsomia candida* whereas the toxicity of leachates from contaminated soils was evaluated in the aquatic model organisms *Daphnia magna* and *Raphidocelis subcapitata*. The exposure to environmentally relevant concentrations of imidacloprid caused no mortality to earthworms (LC_{50} of 4.23 mg imidacloprid kg^{-1} dry soil) but altered their behavior and reproduction patterns (EC_{50} values for avoidance and reproduction tests of 0.43 and 1.40 mg imidacloprid kg^{-1} dry soil, respectively). Effects on collembolans *F. candida* were negligible. Imidacloprid presented moderate leachability, with recovery rates that ranged from 25.4 to 50.4% of the amount present in soils and concentrations in water extracts from 13.05 to 71.8 µg L^{-1}. Standard aquatic ecotoxicity tests were not able detect chronic or acute toxicity in standard test organisms. Nonetheless, concentrations of the insecticide in water extracts were high enough to pose a lethal threat to several other non-standard aquatic organisms.

Keywords: *Imidacloprid, ecotoxicity, water-extracts, earthworms*

1. INTRODUCTION

Despite the potential harmful effects of pesticides, the massive application of plant protection products is necessary in order to provide enough food to satisfy the demands of an increasing human population. Neonicotinoids are a relatively new group of systemic insecticides developed in the 1980s and first commercially available in the form of imidacloprid since early 1990s (Kollmeyer et al. 1999). They bind to the post-synaptic nicotinic acetylcholine receptors (nAChRs) in the central nervous system of insects, thereby disrupting their nerve impulses. Due to their systemic activity, high toxicity to insects, low toxicity to vertebrates and versatile application, neonicotinoids are among the largest selling and most used pesticides worldwide (Elbert et al. 2008; Jeschke et al. 2011; Main et al. 2014). Within this group of insecticides, imidacloprid-containing formulations account for up to 41% of the neonicotinoids market, becoming the second most used agrochemical worldwide (Jeschke et al. 2011; Pollack 2011).

The prophylactic use of imidacloprid during the last decades has led to serious environmental concerns because of its chemical properties. Regardless of the application route of imidacloprid-containing formulations, the bulk of the active ingredient ends up in soil, where it is subjected to various transformation and transportation processes. Due to its high persistence because of a generally long half-live in soils, non-target soil organisms and terrestrial pollinators are usually exposed to fluctuating concentrations of the insecticide. During the last decades, detrimental effects after exposure to imidacloprid have been documented in terrestrial snails (Radwan and Mohamed. 2013), beetles (Russell et al. 2010), earthworms (Luo et al. 1999; Capowiez et al. 2003; Dittbrenner et al. 2010; Dittbrenner et al. 2011), collembolans (Idinger 2002; Alves et al. 2014) and bees (Decourtye et al. 2004; Dively et al. 2015) among others. Furthermore, its high water solubility, high partitioning and low soil sorption enhance the movement of the neonicotinoid from the terrestrial to the aquatic compartment by spray drift, leaching or surface runoff (Roessink et al. 2013). Concentrations of imidacloprid have been measured in surface and ground waters worldwide (Lamers et al. 2011; Starner and Goh 2013) and toxic effects have been documented in many aquatic non-target organisms (Tisler et al. 2009; LeBlanc et al. 2012, Roessink et al. 2013; Pérez-Iglesias et al. 2014 among others).

In the European Union, ecotoxicological laboratory tests are used as a preliminary step in the assessment of the environmental impacts of pesticides and are required prior to the sale of plant protection products (EC 2009). Most laboratory tests follow standardized guidelines to study the toxic effects that pesticides cause to a set of non target model organisms that play key roles in ecosystem structure and function. Among the invertebrate species mostly recommended for terrestrial ecotoxicological assays, acute and chronic effects of imidacloprid have been reported in *Eisenia fetida* (Dittbrenner et al. 2011; Alves et al. 2013) and *Folsomia candida* (Idinger 2002; Alves et al. 2014). Similarly, aquatic ecotoxicology have been traditionally applied for the toxicity determination of aquatic pollutants (Lopez-Roldan et al. 2012), industrial effluents (Riva et al. 1993; Riva and Valles 1994; Riva et al. 2007) or elutriates of sediments (Pereira-Miranda et al. 2011) among others. Effects of imidacloprid on the aquatic environment have been mostly studied through standard aquatic toxicity tests with the model organisms *Daphnia magna* (Crustacea) and *Raphidocelis subcapitata* (Chlorophyta) (Pavlic et al. 2005; Jemec et al. 2007; Tisler et al. 2009; Malev et al. 2012). Unfortunately, the application of ecotoxicity tests for the regulation of pesticides have traditionally focused on parental compounds, passing over the fact that are commercial formulations instead of pure active ingredients the ones applied in the environment. This approach neglects the effects of some co-formulants and solvents present in commercial formulations that can be more important than the active substances to non-target organisms (Anderson and Roberts 1983; Neves et al. 2001) due to its own toxicity or through the modification of the toxicity and bioavailability of the pesticide (Malev et al. 2012). Furthermore, it is known that the leaching potential of pesticides is affected by the type of formulation, surfactants and adjuvants (Camazano et al. 1995; Hall et al. 1998).

Despite the amount of available data regarding the impacts of imidacloprid to non-target organisms, data on the toxicity of imidacloprid-containing formulations is scarcer. Data on such commercial products is required since some studies revealed a higher toxicity and leaching potential of the commercial formulation in comparison with the active ingredient (Gupta et al. 2002; Jemec et al. 2007; Malev et al 2012). In order to widen the available information on this formulation, we studied the environmental impacts associated to the field application rates of Confidor® 20SL.

Table 1. *Physical-chemical parameters of the test soil. C/N: carbon-nitrogen ratio; CEC: cation exchange capacity*

Moisture (%)	pH	Organic carbon (%)	Organic matter (%)	Total nitrogen (%)	C/N	$N\text{-}NO_3$ (mg/kg)	CEC meq/100g	Textural class
3.0	7.2	6.2	10.7	0.4	16.9	15	22.8	Loamy

Effects on the terrestrial compartment were assessed through standard ecotoxicity tests that evaluated the mortality, inhibition of reproduction and avoidance behavior of earthworms *E. fetida* and avoidance of collembolans *F. candida* after exposure to treated soils. Impacts on the aquatic compartment were assessed through the leaching of treated soils and the evaluation of the acute effects of the water extracts to the non-target aquatic invertebrate *D. magna* and the microalgae *R. subcapitata*. Following this methodology, the main objective of this study was to characterize via lower-tier standard ecotoxicological tests the risk that the application of the recommended field rates of the commercial formulation Confidor® 20SL poses to the aquatic and terrestrial compartments.

2. MATERIALS AND METHODS

A soil from a known natural uncontaminated area near the laboratory was selected for the performance of the tests. Samples were collected from the topsoil (0-20 cm depth), air-dried and sieved through a 2 mm mesh. Several soil parameters were analyzed: moisture, pH, organic carbon, organic matter, total nitrogen, C/N ratio, N-NO_3, cation exchange capacity and texture (Table 1).

The insecticide Confidor® 20SL (soluble concentrate, 20% imidacloprid (w/v)) was purchased from Bayer (Germany). Toxicity tests were performed in a range of concentrations that included the lowest and highest application rates recommended by the manufacturer (0.5 and 4 L Confidor ha^{-1}, respectively), two intermediate concentrations (1 and 2 L Confidor ha^{-1}) and a concentration of 8 L Confidor ha^{-1} to cover the worst case scenario of an excessive application of the insecticide. Assuming a depth of incorporation in the soil profile of 0-5 cm and a density of 1.5 g/cm^3, the application rates of Confidor amounted to 0.78-1.56-3.1-6.20-12.4 mg per kg of soil dry weight (dw) and corresponded to 0.13-0.26-0.5-1-2 mg of imidacloprid kg^{-1} dry soil respectively. The application of the formulation into the soil consisted in preparing a stock solution of 1000 mg Confidor L^{-1} in deionized water. Different spiking solutions were applied to the soil in order to provide the desired concentrations of test substance and a moisture content of 60% of the WHC. Soils were carefully mixed to ensure an evenly distribution of the pesticide and left overnight for equilibration. Only deionized water was added to the controls.

Water-extracts were obtained from each soil following the British Standard EN 12457-2 (2002). Soil samples were incorporated to 2-L glass vessels at a ratio of 1 kg/10 L, corresponding to 0.1 kg of soil per liter of deionized water. Vessels were placed at a rotating apparatus and mixed during 24 hours at a temperature of 20±2ºC. After a settling period of 15 minutes, samples were centrifuged (2000g, 10 minutes) and filtered. The supernatant was kept refrigerated until use. The concentration of imidacloprid in the leachates was analyzed by SAILab (Cerdanyola del Vallès, Barcelona, Spain) by High Performance Liquid Chromatography/MS (Agilent 1200 LC/ Applied Biosystems 3200 LMS).

Synchronized cultures of earthworms *E. fetida* and collembolans *F. candida* were obtained from the Centre for Research and Innovation in Toxicology of the Technical University of Catalonia (UPC) in Terrassa (Spain). Earthworms were bred in a cow manure–peat mix (1:1, w/w) at a temperature of 20±2 ºC and under a 16:8 light:dark photoperiod and were fed once a week with moistened bread. Forty-eight hours prior to starting the tests, adult clitellate animals were acclimated to the untreated soil. Only individuals weighting between 300 and 600 mg were selected. Collembolans were cultured in vessels filled with a substrate of plaster of Paris and charcoal (8:1 w/w) at 20±2ºC. Individuals were fed twice a week with granulated dry yeast added in small amounts to avoid spoilage by fungi. Organisms between 10 and 20 days old were selected for avoidance tests. Terrestrial bioassays were performed in a climate-controlled room at 20±2ºC and under a 16:8 light-dark photoperiod except for the acute toxicity test with earthworms that was carried out under constant illumination (400-800 lux).

Lethal effects to *E. fetida* were assessed following the recommendations by the OECD guideline 207 (OECD 1984). Ten individuals were placed in plastic containers containing 500 g of spiked soil (dw). Four replicates were prepared per test concentration. The percentage of mortality and pathological symptoms were monitored after 7 and 14 days of exposure. As no mortality was expected at field application rates of the pesticide, higher concentrations of Confidor were included in order to estimate the LC_{50}.

Effects on the reproduction of earthworms were studied by means of the OECD 222 (2004) guideline. Ten earthworms were placed in 1-L plastic containers filled with 500 grams of dry soil. Four replicates per test concentration and 6 replicates for the control were prepared. Animals were fed weekly with 2 grams of moistened bread during 4 weeks. After 28 days of exposure, surviving earthworms were sorted by hand and the mortality and changes in biomass were recorded. Juvenile worms and cocoons remained in the test vessels for another 28 days. The number of juveniles was recorded after 56 days by heating the soils in a warm bath at 60ºC for 20-25 minutes and waiting for the juveniles to emerge.

Avoidance tests with *E. foetida* and *F. candida* were carried out according to the ISO 17512 (2008) and ISO 17512 (2011) standards respectively. Tests were performed in plastic containers divided into two equal sections by a vertically introduced plastic card. In the test with earthworms, each side of the vessel (control and test) was filled with 350g (dw) of the corresponding soil and the divider was removed. Ten adult earthworms were placed in the line separating both soils. In the test with collembolans, 25 g (dw) of soil were filled into the corresponding section and twenty springtails were carefully placed on top of the soils. In both cases tests ran with five replicates per concentration. At the end of the test period the plastic card was reinserted and the number of individuals at each section counted. In tests with collembolans, the soil from each section was carefully emptied into two different vessels and flooded with water. After gentle stirring the animals floating on the water surface were counted. Missing animals were considered as dead organisms and discarded for the later calculations. Dual-control tests were carried out with both methodologies (5 replicates each) to guarantee the homogeneous distribution of the organisms in the absence of the test substance.

Toxicity in the aquatic compartment was tested in two model species, the cladocera *D. magna* and the microalgae *R. subcapitata*. Cultures of 15 daphnids were maintained in 2.5 L ASTM hard synthetic water kept at 20±2ºC in a 16:8h light:dark cycle. Culture media were changed

three times per week and an organic extract and a concentrate of *Chlorella vulgaris* were added as food. Neonates were collected daily and only those less than 24 hours old were used in tests. Cultures of the algae *R. subcapitata* were kept under a constant illumination of 4000-5000 lux at 20±2ºC. Only populations in the exponential phase were used for the assays. The acute toxicity test with *D. magna* was carried out according to the OECD Guideline 202 (1984). Four replicates were prepared per leachate. Each replicate consisted in a glass tube with 10 mL of the corresponding leachate and 5 daphnids. The test was performed in an incubator at 21ºC and in the dark. Immobilization was visually recorded after 24 and 48 hours of exposure. Chronic toxicity to *D. magna* was evaluated following the OECD Guideline 211 (1998) for a semi static exposure system. Ten replicates per leachate were prepared, each consisting of a 250 mL glass vessel filled with 75 mL of test solution and one daphnid. During the assay, test solutions were replaced and enriched with seaweed extract three times per week. Animals were fed with a concentrate of *Chlorella vulgaris* (0.1-0.2 mg per day). The assay was carried out in a controlled room for 21 days at a temperature of 20±2ºC and a light:dark cycle of 16:8 hours. The growth inhibition test with *R. subcapitata* was carried out following the recommendations of the OECD Guideline 201 (1984). The test ran with 3 replicates for each water extract from contaminated soils plus the leachate from the control soil and an additional control with algae culture medium. Each replicate consisted in 9 mL of test solution and 1 mL of algal inoculum of known concentration. In order to avoid interferences in the spectrometric measure of the leachates at the end of the test, one extra tube was prepared with 9 mL of leachate, 1 mL of culture medium and no algae. The tubes were placed in a controlled room at 20±2 ºC under constant light (4000-5000 lux) and agitation. After 72 hours of incubation, the absorbance of each replicate was measured at 665 nm with a CECIL CE9200 spectrophotometer in order to determine the final algal concentration.

Results of toxicity tests were calculated as percentages. Differences between treatment means (i.e., different concentrations of Confidor) were tested through Analysis of Variance (ANOVA)(P<0.05). When significant differences were detected, the Dunnet post-hoc test was applied to compare treatment means with the control using SPSS 19.0 (NY, USA) software. NOEC (No observed effect concentration) and LOEC (Lowest observed effect concentration) values were established through this procedure. The percentage of avoidance was calculated following the equation presented in the ISO standards 17512 (2008) and 17512 (2011):

$$x = \left(\frac{n_c - n_t}{N}\right) \times 100$$

where x is avoidance, expressed as a percentage; n_c is the number of individuals in the control soil; n_t is the number of individuals in the test soil and N is the total number of individuals. The significance of the avoidance responses were analyzed using the Fisher Exact test (Zar 1998). A two-tailed test were used in the analysis of the dual-control test and a one-tailed test was used for the polluted soils. The null hypothesis assumed an even distribution of individuals between both soil sections and was rejected for a probability equal or lower than 0.05. Median lethal concentration (LC_{50}) values and effective median concentration values (EC_{50}) were estimated by the Probit method following logistic regressions with Statistica software version 8.0 (OK, USA) and Minitab 13.20 software (PA, USA) respectively.

3. RESULTS AND DISCUSSION

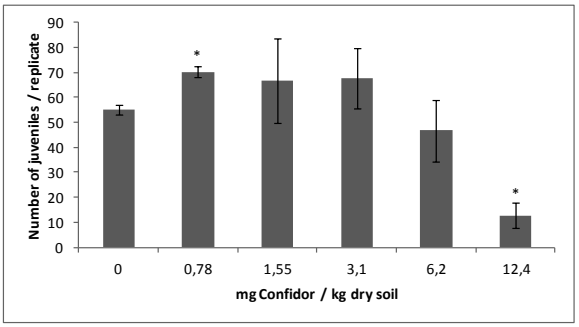
The exposure of soil invertebrates to field doses of Confidor in standard ecotoxicity tests showed marked differences in sensitivity between endpoints and test species. Mortality of earthworms occurred at concentrations higher than 19.77 mg Confidor kg^{-1} (soil dw) (LOEC) (Table 2) and the LC_{50} was estimated at 24.71 mg kg^{-1} dry soil (corresponding to 4.23 mg imidacoprid kg^{-1} dry soil), indicating that the recommended doses of the formulation did not represent a lethal threat to *E. fetida.* Similar toxicity values were reported by Luo et al. (1999) and Gomez-Eyles et al. (2009) using pure imidacloprid as test substance (LC_{50} values of 2.30 mg kg^{-1} soil dw and 2.36 mg kg^{-1} soil dw respectively). On the other hand, studies by Kreutzweiser et al. (2008) and Alves et al. (2013) reported LC_{50} values 10 times higher (25 and 25.53 mg imidacloprid kg^{-1} soil dw respectively) after applying the commercial imidacloprid-containing formulations Merit Solupak® and Gaucho®. Differences in LC_{50} values between studies were partly explained by variations in experimental parameters like soil organic matter, texture or time of exposure (Kula and Larink 1997) although the influence of certain components from commercial formulations to the overall toxicity of the product was not discarded.

Table 2: *EC_{50} (effect concentration 50%), LC_{50} (lethal concentration 50%), confidence intervals (95%), LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration) values of Confidor / imidacloprid estimated for earthworm mortality, reproduction and avoidance tests. Values presented in [mg Confidor /kg soil dw] / [mg Imidacloprid /kg soil dw]*

Test	EC_{50}(LC_{50})	Lower limit (95%)	Upper limit (95%)	LOEC	NOEC
Mortality	24.71/4.23	23.30/3.99	26.20/4.48	19.77/3.38	15.21/2.6
Reproduction	8.41/1.40	5.38/0.90	12.87/2.15	12.40/2	6.20/1
Avoidance	2.57/0.43	1.86/0.31	3.21/0.54	0.78/0.13	<0.78/<0.13

The reproduction test gave varying results depending on the concentration of pesticide in soil. *E fetida* produced a significantly higher number of juveniles (Dunnet's test, P < 0.05) in soils treated with the lowest application rate of imidacloprid than in untreated soils (Fig. 1). On the other hand, significant detrimental effects on the reproductive output occurred at twice the highest recommended dose (12.4 mg Confidor kg^{-1} soil dw)(LOEC). The EC_{50} for the reproduction test was estimated at 8.41 mg Confidor kg^{-1} soil dw (corresponding to 1.40 mg imidacloprid kg^{-1} soil dw) (Table 2), a concentration that could be easily reached if the formulation is not properly employed in terms of applied concentrations or time between applications. A similar EC_{50} value (1.41 mg kg^{-1} soil dw) was reported by Gomez-Eyles et al. (2009) using pure imida-

cloprid as test substance whereas a study by Alves et al. (2013) observed a significantly lower toxicity (EC_{50} value of 4.07 mg imidacloprid kg^{-1} soil dw) of a imidacloprid-containing formulation. Luo et al. (1999) and Capowiez and Berard (2006) linked the decrease in the reproductive output to the damage exerted by imidacloprid to spermatozoa of earthworms. It was not concluded whether differences in toxicity between studies were due to the experimental conditions or to the nature of the test substance (active ingredient or commercial formulation). Additionally, it is noteworthy the hormetic response that Confidor triggered in the reproductive output of exposed earthworms. An enhanced reproduction rate was previously documented by Senapati et al. (1992) and Suthar (2014) after exposing earthworms to low concentrations of the pesticides malathion and methyl parathion respectively although the biochemical mechanism of this response is not clear yet. Similar results have not been reported for other neonicotinoids or neonicotionid-based formulations. Regarding the reduction of body weight, it followed the same pattern than juvenile production, with an average weight loss lower than controls at low application rates and significantly higher at high test concentrations (Fig. 1).



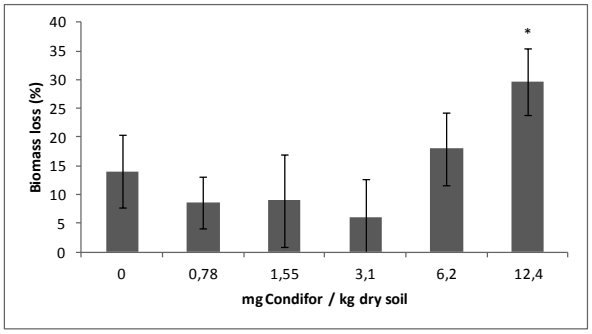


Figure 1: *Effects of varying concentrations of Confidor on the reproductive output and weight loss of E. Fetida in reproduction tests. Data presented as treatment means ± SD(N=4). Asterisks indicate significant differences with controls (Dunnet's test, P < 0.05).*

Earthworms exhibited a significant avoidance behavior in response to the presence of all test concentrations of the formulation (Figure 2). The LOEC value was established at the lowest tested concentration, corresponding to the minimum application rate recommended by the manufacturer (Table 2). Furthermore, the EC_{50} value was estimated at 2.57 mg Confidor kg^{-1} soil dw, within the range of recommended doses. According to Hund-Rinke and Wiechering (2001), soils contaminated with concentrations of Confidor higher than 1.56 mg kg^{-1} soil dw presented a reduced habitat function and should be considered as toxic to earthworms since they presented avoidance responses higher than 60% (i.e more than 80% of individuals remained at the control section of the test chamber). Our results were in accordance with those from Alves et al. (2013) who estimated an EC_{50} value of 0.11 mg kg^{-1} in *Eisenia andrei* for a commercial formulation of imidacloprid. In contrast, Capowiez and Bérard (2006) reported no avoidance response of earthworm species *Aporrectodea nocturna* and *Allolobophora icterica* after exposure to 0.5 and 1 mg kg^{-1} (soil dw) of Confidor® 200 SL despite previous studies documented behavioral alterations on burrow length, overall distance travelled and rate of burrow reuse under the same experimental conditions (Capowiez et al. 2003). Similarly, earthworms exposed to the pesticide in our study presented an altered locomotion pattern. After the increase in the avoidance response observed at 0.78 and 1.56 mg Confidor kg^{-1} soil dw, the behavioral response turned stable while increasing test concentrations. A study by Pereira et al. (2010) reported that the exposure of *E. Andrei* to the carbamate insecticide methomyl induced a inhibition of the Acetylcholine esterase activity that led to hyperactivity in the test organisms and in consequence to the adoption of an irregular avoidance behavior. Similar conclusions were postulated by Martínez Morcillo et al. (2013) after exposing earthworms from the species *Lumbricus terrestris* to chlorpyrifos, another insecticide known to affect the nervous system of soil invertebrates. Based on behavioral alterations reported by Capowiez et al. (2003) and the mechanism of action of imidacloprid, we hypothesized that the exceeding of certain toxicity threshold somehow altered the locomotive ability of the test organisms and led to an erratic movement pattern, thus causing the stabilization of the avoidance response. In the case of collembolans, an avoidance behavior in response to the application of Confidor recommended doses was not detected at any test concentration. Furthermore, a significant preference for the contaminated soil (Fisher exact test, $P < 0.05$) was observed at concentrations of 3.1 and 12.4 mg Confidor/kg dw (data not shown).

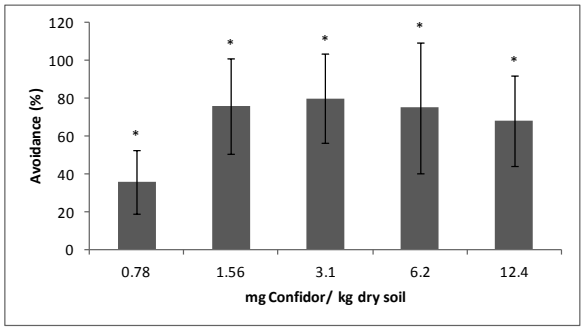


Figure 2: *Avoidance response (%) of E. fetida (mean ± SD)(N=5) to varying concentrations of Confidor in avoidance tests. Asterisks indicate significant differences with the control (Fisher's test, P < 0.05).*

To determine the leaching potential of imidacloprid and its risk for aquatic organisms, concentrations of imidacloprid were determined in water extracts from contaminated soils (Table 3). The concentrations of active ingredient in leachates ranged from 13.05 µg L^{-1} (corresponding to the soil treated with 0.26 mg imidacloprid kg^{-1} dw) to 71.8 µg L^{-1} (2 mg imidacloprid kg^{-1} soil dw) and were positively correlated with concentrations in test soils ($r = 0.910$, $P < 0.05$,

Spearman). The concentrations of imidacloprid in water extracts were within the range estimated by Fossen (2006) for chronic and acute surface water exposures (17.24 and 36.04 µg L^{-1} respectively) or after accidental direct spray in a pond or stream (22 µg L^{-1})(SERA 2005). The recovery of the pesticide ranged from 25.4% to 50.4% of the total amount previously spiked in soil. Recovery rates were in accordance with the relatively high water solubility (0.5 to 0.6 g L^{-1}) and low octanol-water partitioning coefficient (Log (Pow)=0.57) of imidacloprid reported by other authors (Gupta et al. 2002; Kurdwadkar et al. 2014) but were higher than expected according to the high organic carbon content of our soil, a parameter positively correlated with imidacoprid sorption in soils (Cox et al. 1998).

Table 3: *Concentration of imidacloprid in water extracts from contaminated soils. Means ± Standard deviations (N=3).*

mg Confidor / kg soil (dw)	mg imidacloprid / kg soil (dw)	Water extract (µg/L leachate)	Recovery rates (%)
0.78	0.13	< QL	-
1.56	0.26	13.05±3.04	50.35±11.95
3.1	0.5	16.35±4.60	32.70±9.19
6.2	1	25.4±8.21	25.4±8.21
12.4	2	71.8±0	35.9±0

QL (quantification limit): 1 µg/L

Although the highest concentration of imidacloprid determined in water extracts was almost 10^3 times lower than LC_{50} values found in bibliography for *D. magna* (85 mg L^{-1}) (Fossen 2006), mortality tests were performed since previous studies reported the higher toxicity of imidacloprid-containing commercial formulations to *D. magna* due to the presence of toxic adjuvants (Jemec et al. 2007). The exposure to the leachates caused no mortality after 48 hours of exposure in the acute toxicity test and 21 days in the reproduction test. Similarly, differences with the control in the number of neonates per adult, brood size, day of first brood and number of broods per adult in the chronic test were not detected (LOEC value in chronic tests estimated between 2.5 and 10 mg L^{-1} (Jemec et al. 2007)). Regarding the effects on the microalgae *R. subcapitata,* algal growth rates in water extracts from all soils (including the untreated soil) were significantly lower than in algal culture medium (data not shown). However, no significant differences in growth inhibition were found between soil leachates. Consequently, algal growth inhibition was related to the fact that water parameters deviated from the standard test medium and not to the presence of the insecticide in soil leachates. Results with this model organism were expected based on the insecticidal type of action of imidacloprid and its estimated EC_{50} values (> 600 mg L^{-1})(Daam et al. 2013) although previous studies reported the high toxicity to algae of some Confidor® 200 SL co-formulants (Malev et al. 2012). We hypothesized that the lower toxicity detected in our study was related to the fact that in previous studies the commercial formulation was directly spiked into water while we used leachates from contaminated soils. Since the purpose of adjuvants is associated to the fixation of the pesticide in soil, we expected a lower leachability of potentially toxic co-adjuvants.

Despite the low toxicity of leachate concentrations of imidacloprid to the standard organisms *D. magna* and *R. subcapitata*, the presence of the active ingredient in the water extracts was high enough to represent a lethal or sublethal threat to several other non-standard, freshwater macroinvertebrate species. Based on the available bibliography, Daam et al. (2013) reported that a concentration of 52 µg of imidacloprid L^{-1} (value that could be easily reached in soils if Confidor is improperly applied) was expected to produce 50% affection to 25% and 79% of the crustacean and insect taxa respectively. Furthermore, Roessink et al. (2013) documented LC_{50} and EC_{50} values for the non-standard insect species *Notonecta* spp., *Micronecta* spp*., Limnephilidae*, *Caenis horaria* and *Cloeon dipterum* and the macrocustacean *Gammarus pulex* close or below 25 µg imidacloprid L^{-1} , a concentration of active ingredient reached in our leachates.

4. CONCLUSION

Our study pointed out that the application of recommended field doses of the imidacloprid-containing formulation Confidor® 20SL represents a potential threat for the environment. Although mortality was not reported, the exposure to the pesticide caused sublethal effects to *E. fetida* earthworms. The influence of some co-adjuvant and solvents to the overall toxicity of pesticide formulations was observed after comparing results from terrestrial ecotoxicity tests with imidacloprid with those from commercial products. Confidor presented toxicity levels in terrestrial standard ecotoxicity tests closer to those from the active ingredient than to other commercial formulations. Additionally, reproduction and avoidance tests with earthworms showed responses that had not been previously reported, highlighting the need to keep studying the impacts of massively-applied pesticides.

The application of Confidor® 20SL to agricultural soils posed a risk to the aquatic compartment due to the high leachability of imidacloprid. Despite the low response of aquatic standard ecotoxicity tests to the presence of the pesticide or to other components of the formulation, final concentrations of the insecticide in the aquatic compartment were high enough to represent a lethal threat to many other non-standard, non-target aquatic organisms, thus emphasizing the need for testing organisms from different taxonomical groups when assessing the environmental risks posed by pesticides.

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An Alternative Approach to Assess the Habitat Selection of *Folsomia candida* in Contaminated Soils

Jaume Bori[1] · **Maria Carme Riva**[1]

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Abstract Avoidance tests with collembolans provide a quick assessment of soil quality. However, some parameters of the procedure can be modified in order to increase its performance. In this study we assessed the tendency of *Folsomia candida* to avoid soils contaminated with boric acid [350–700–1400–2800–5600 mg/kg soil dry weight (dw)], phenmedipham (35–70–140–280 mg/kg dw) or petroleum hydrocarbons (1312–1838–2625–3675–5250 mg/kg dw) by preferring an untreated soil. Two separate methodologies were applied, the one presented in the ISO standard 17512:2 and a modified version of the Petri dish method that allowed data acquisition after 2, 24 and 48 h of exposure. After combining data from three separate trials, effective median concentration values (EC_{50}) from the presented method were lower and showed similar or less variability than those from the ISO procedure, suggesting the modified protocol as a suitable alternative screening tool.

Keywords Avoidance · Screening · Collembola · Soil contamination

Ecotoxicological bioassays became an essential tool for the assessment of risks associated with soil contaminants (Loureiro et al. 2005). In this context, some laboratory ecotoxicological tests follow standardized guidelines to study the effects that soil contaminants cause to a well defined set of non-target model organisms. Also for collembolans, which contribute to the fertility of soils through decomposition and nutrient cycling processes (Culik and Zeppelini 2003), standardized test guidelines have been developed assessing their potential avoidance behavior of a contaminated soil by preferring a control soil as habitat [ISO standard 17512:2 (ISO 2011)]. This procedure provides information comparable to the one obtained with other more complex ecotoxicological soil tests but requires less experimental efforts (Domene et al. 2011).

The suitability of the standard avoidance test with Collembola as screening tool of soil contamination relies on its ecological relevance and its sensitivity, while it also benefits from exposure times shorter than in acute or reproduction tests and can therefore be routinely applied in 'on site' procedures (Eisenträger et al. 2005). Despite those benefits, avoidance tests present a high variability in their results, which is at least partly explained by the gregarious behavior of collembolans and unexplained shifts in the cultures avoidance responses over time (Filser et al. 2013). According to Filser et al. (2000), the aggregation of individuals in the test containers can be controlled by reducing their density (for instance performing single specimen tests). Regarding temporal variations, Filser and Hölscher (1997) suggested involving sufficient replication and assessing the behavior regularly during the bioassay. Additionally, Van Gestel (2012) highlighted the need to review existing test guidelines in order to make them applicable to new chemicals. Such revision should involve the miniaturization of test systems since many new materials can only be produced in small amounts.

In this study we present an alternative approach that aims to strengthen the use of avoidance tests with Collembola as early-warning tool of soil contamination through the simplification of the test preparation and data

✉ Jaume Bori
jaume.bori@crit.upc.edu

[1] Center for Research and Innovation in Toxicology (CRIT-Innotex Center), Technical University of Catalonia (UPC), Ctra. Nac. 150 km 15, 08227 Terrassa, Barcelona, Spain

collection. Current avoidance tests with collembolans allow test organisms to dig into soils. Consequently, a destructive and time-consuming analysis of soil samples by flooding and counting the floating individuals is required. Similarly to the study by Aldaya et al. (2006), the presented alternative procedure uses 55 mm Petri dishes, requires fewer resources and involves a slight compression of the soils to prevent collembolans from hiding, thus allowing the observation of test organisms through the transparent lid of the vessel. The major purpose of this work is to study whether the presented procedure can provide information equivalent to the one obtained following the ISO standard 17512:2. Additionally, we aimed at assessing whether a reduction in exposure times can be realized while still ensuring reliable data. To attain these goals, several tests following the ISO standard and the Petri dish procedure were performed. Data from the ISO standard was collected after 48 h of exposure whereas exposure times with the Petri dish procedure were 2, 24 and 48 h. Manifold concentrations of the two reference chemicals recommended by the ISO standard 17512:2 (boric acid and phenmedipham) as well as a soil contaminated with petroleum hydrocarbons sampled from the field were selected as test items.

Materials and Methods

Collembolans from the species *Folsomia candida* (Isotomidae) were obtained from synchronized cultures maintained at the Center for Research and Innovation in Toxicology of the Technical University of Catalonia (Spain). Animals were cultured at 20 ± 2°C in 145/20 mm Petri dishes filled with a substrate of plaster of Paris and charcoal (8:1, w/w) to a height of approximately 10 mm. Individuals were fed twice a week with granulated dry yeast added in small amounts (approximately 2 mg of yeast per organism and week) to avoid spoilage by fungi. Adult organisms (12–20 days old) were selected for avoidance tests.

A soil from a known natural uncontaminated area near the laboratory (Pereira Miranda et al. 2011) was selected as control soil. Samples from the topsoil (0–20 cm depth) were air-dried and sieved through a 2 mm mesh. Several soil parameters were analyzed: texture (Pipette method), pH (KCl, 1 mol/L) (ISO 2005a), Water Holding Capacity (WHC) (ISO 2011), organic matter (Walkley and Black 1934), moisture (ISO 1993), and cation exchange capacity (CEC) (Schollenberger and Simon 1945) (Table 1).

In this study, avoidance tests with collembolans were carried out following two different experimental procedures: (a) using the ISO standard protocol (ISO 2011) and (b) using 55 mm Petri dishes as test containers. The selected exposure times were 48 h with the ISO procedure and 2, 24 and 48 h for the Petri dish methodology. Median effective concentration (EC_{50}) values were determined three times for each test substance and exposure time in independent experimental runs. Five replicates per test concentration were prepared. Additionally, dual-control tests (ten replicates) with control soil at both sides of the test container were performed with each experimental run in order to validate the tests by checking the homogeneity in the distribution of collembolans. Tests were performed in an environmental chamber at 20 ± 2°C under a 16:8 h light:dark cycle.

The control soil was spiked with the reference chemicals boric acid (Scharlab, 99.8 % pure) and phenmedipham (Sigma-Aldrich, 99.7 % pure) (ISO 2011). A stock solution of each substance was prepared with the proper solvent (deionized water for boric acid and methanol (Labkem, 99.5 % pure) for phenmedipham). Spiking solutions providing the desired concentration of test substance in soil and a moisture content between 40 % and 60 % of the Water Holding Capacity of the soil were obtained by diluting the stock solutions. Batches of control soil were homogeneously contaminated with the corresponding solution and divided into two sub-batches (one for the application of each methodology). The control soil was treated with five concentrations of boric acid corresponding to 350.0, 700.0, 1400.0, 2800.0 and 5600.0 mg/kg dry soil and was left for equilibration before starting the tests. In the case of phenmedipham, the control soil was spiked with the corresponding solution, thoroughly mixed and left overnight until methanol was evaporated. Final concentrations of phenmedipham in soils were 35.0, 70.0, 140.0 and 280.0 mg/kg soil dry weight (dw). Additionally, a soil from a site (hereinafter field soil) contaminated with petroleum hydrocarbons was selected to ensure the transferability of the proposed test design to a more realistic scenario. Sampling and pre-treatment of the field soil were carried out as described for the control soil. Physical–chemical properties of the field soil can be seen in Table 1. Hydrocarbons in soil (C10–C40) were determined through gas chromatography and flame ionization detector (GC–FID) (Table 1). Final test concentrations were 25 %, 35 %, 50 %, 70 % and 100 % of field soil mixed with control soil, corresponding to 1312, 1838, 2625, 3675 and 5250 mg of petroleum hydrocarbons per kg (dw). When dilution of the field soil was needed, it was achieved by mixing it with the control soil (w/w). Prior to the start of the tests, soils were hydrated with deionized water until the desired moisture content was reached.

According to the procedure described in the ISO standard 17512:2 (ISO 2011), cylindrical plastic containers (diameter 8 cm; depth 8 cm) were divided into two equal sections. Approximately 30 g (wet weight) of control and contaminated soils were placed into the corresponding

Table 1 Physical–chemical characteristics of the control and field soils

	Texture	pH_{KCl}	WHC (%)	Organic matter (%)	Moisture (%)	CEC (meq/100 g)	Petroleum hydrocarbons (C10–C40) (mg/kg)
Control Soil	Clay loam	7.6	41.4	10.7	18.6	22.8	–
Field soil	Silty loam	7.9	24.9	8.3	7.5	23.4	5250

section and the divider was removed. Twenty organisms were carefully placed on top of the soils. After 48 h of exposure, the two soils were separated and the soil from each section was carefully emptied. Each subsample was flooded with water and after gentle stirring the animals floating on the water surface were counted. Missing individuals were considered as dead and discarded for the subsequent calculations. The alternative method used plastic Petri dishes (55 mm diameter, 14 mm height) as test vessels. Petri dishes were divided into two sections filled with 6 g (wet weight) of the corresponding soil. Wet soils were pressed by hand in order to obtain a suitable texture that prevented collembolans from hiding into soil. Ten collembolans were carefully placed on top of the line dividing the two sections. The distribution of individuals was recorded after 2, 24 and 48 h of incubation.

Data from dual-control tests were analyzed using the two-tailed Fisher Exact test (Zar 1998) to check the homogeneous distribution of the organisms. Following the recommendations of the ISO 17512:2 standard (ISO 2011), the percentage of avoidance in the avoidance tests was calculated in each replicate by the equation $x = [(n_c - n_t)/N] \times 100$, where x = percent avoidance, n_c = number of individuals in the control soil, n_t = number of individuals in the test soil, and N = total number of individuals. Negative avoidance values (lack of avoidance) were transformed to zero. The avoidance median effective concentration values (EC_{50}) and their 95 % confidence limits were calculated by Probit regression with maximum likelihood estimation. A normal or logistic distribution was assumed depending on the results from the Kolomogorov–Smirnov normality test. EC_{50} values were compared between experimental procedures and exposure times within the same procedure using the confidence interval ratio test recommended by Wheeler et al. (2006). Statistical analysis was performed using SPSS software (SPSS 15.0 for Windows; SPSS Inc., Chicago, IL, USA) and Minitab Statistical Software (Minitab 15.0; Minitab Inc., State College, PA, USA).

Results and Discussion

Dual-control tests with both methodologies showed an even distribution of collembolans, with a number of organisms per section between 40 % and 60 % of the total. Additionally, the number of dead or missing organisms never reached values higher than 20 % per treatment, thus meeting the requirements of the ISO standard (ISO 2011) (Table 2). Results from avoidance tests revealed the high variability inherent in the procedures, with estimated EC_{50} values that markedly varied with the trial within each test substance and experimental procedure. In some cases effective median concentration values could not be reported. In order to improve the results of the avoidance tests, data from the three available trials were combined. To do so, the mean avoidance percentage of all replicates per treatment (N = 15) was used for the calculation of the probit regressions. After combining the results, EC_{50} values were successfully calculated for both experimental procedures (Table 2).

Effective median concentration values estimated after the exposure to the reference substances boric acid and phenmedipham were in some cases higher than those found in literature. For boric acid, previous studies reported EC_{50} of 1440 mg/kg (Becker et al. 2011) after applying the ISO standard 17512:2 in OECD artificial soil and questioned the suitability of boric acid as reference substance in avoidance tests with collembolans due to the low sensitivity of the organisms (Amorim et al. 2012). Our results agreed with those studies, reporting an EC_{50} value for the ISO test of 3397.58 mg/kg (Table 2). Differences in the EC_{50} values between studies can be explained by the soil typology since the percentage of organic matter and clay, soil constituents related with the binding of boron (Goldberg 1997), were higher in our soil (10.7 % and 29.1 % respectively) than in the OECD artificial soil (approximately 8 % and 20 % respectively). Regarding the exposure to phenmedipham, EC_{50} values from both methodologies presented in this study were two orders of magnitude higher than those calculated by Diogo et al. (2007) (4.14–8.01 mg phenmedipham/kg) after applying Betosip® (active ingredient phenmedipham) to OECD artificial soil following the ISO standard. In this case, differences in the results between studies were attributed to soil typology and to the form in which the test substance was applied. The contents of organic matter and silt, soil constituents known to reduce the bioavailability of phenmedipham (Domene et al. 2012), were again higher in our soil (32,4 % of silt) than in the OECD artificial soil (approximately 10 % silt content). More importantly, since the

Table 2 EC_{50} avoidance values, confidence limits and percentage of mortality per replicate (mean ± SD) estimated with the data combined from the available trials (N = 15 replicates per treatment)

Test substance	Procedure	X^2	p	EC_{50}	Confidence limits (95 %)	Mortality (%)
Boric acid (mg/kg)	ISO 48 h	3.45	0.179	3397.58a	2521.10–4578.68	4.8 ± 1.9
	Pd. 2 h	1.30	0.730	1124.63b	893.26–1415.92	0.5 ± 0.9
	Pd. 24 h	1.04	0.792	1034.24b	836.78–1290.21	1.3 ± 1.6
	Pd. 48 h	4.51	0.105	1729.90b	1017.15–2692.90	2.3 ± 2
Phenmedipham (mg/kg)	ISO 48 h	4.67	0.097	289.76a	225.14–372.92	7.9 ± 4.3
	Pd. 2 h	5.08	0.079	127.93b	97.51–167.85	1.7 ± 0.7
	Pd. 24 h	2.79	0.248	155.14ab	83.28–289	4.3 ± 2.2
	Pd. 48 h	1.25	0.263	201.49ab	121.31–334.66	7.3 ± 4.1
Petroleum hydrocarbons (mg/kg)	ISO 48 h	0.42	0.810	2744.70a	2276.93–3308.55	11 ± 1.8
	Pd. 2 h	1.45	0.485	1392.30b	1195.43–1621.73	1.9 ± 2
	Pd. 24 h	3.08	0.214	1487.85b	1326.15–1669.50	2.5 ± 1.6
	Pd. 48 h	5.44	0.066	1615.95b	1463.70–1780–43	4 ± 1.6

EC_{50} values within the same test substance followed by the same letter are not significantly different ($p > 0.05$). Pd: Petri dish

ISO standard 17512:2 only requires a reference substance that has phenmedipham as the unique active ingredient, several products that fulfill this requirement are usually applied. While we used pure phenmedipham as test substance, the study by Diogo et al. (2007) applied the commercial formulation Betosip®, complicating the comparison of results due to the presence of co-formulants with unknown effect on the test organisms. No previous studies were found where avoidance EC_{50} values were estimated after exposing collembolans to pure phenmedipham. Nonetheless, results from the present study suggest that the pure compound is not the best choice as reference substance due to the low sensitivity shown by collembolans. Regarding the exposures to petroleum hydrocarbons, the detected avoidance responses were similar to those documented by Hentati et al. (2013) and Aldaya et al. (2006) after assessing hydrocarbon-contaminated field soils with the ISO standard and a procedure involving Petri dishes respectively, thus confirming the sensitivity of the test organisms towards the presence of hydrocarbons.

For all tested substances, results from the Petri dish procedure presented similar or lower variability and EC_{50} values (i.e. higher sensitivity) than the ISO method. In the exposure to boric acid and the hydrocarbon-contaminated field soil, EC_{50} estimates from the Petri dish procedure after all exposure times were significantly lower than those from the ISO methodology (Table 2). In the case of phenmedipham, a statistically lower EC_{50} value was only found after 2 h of exposure due to the higher variability observed at longer exposure times. The higher sensitivity of avoidance tests with collembolans performed in Petri dishes was also reported by Boiteau et al. (2011) after applying modified versions of the plastic cup test (ISO 2005b) and of the Petri dish avoidance test (Aldaya et al. 2006) in the assessment of the avoidance response of *F. candida* to copper. No clear explanation for the higher sensitivity of the Petri dish procedure was found although we hypothesized that it might be related to the disposal of soil in the test chambers. Due to the much lower volume of soil available for test organisms in the Petri dishes, they had fewer chances to find a suitable spot in the contaminated section and therefore they migrate more likely to the non-contaminated soil.

The application of the Petri dish procedure allowed the observation of temporal trends in the avoidance responses. EC_{50} values for all tested substances tended to increase (i.e. lower sensitivity) throughout time although no statistically significant differences were found between exposure times. Therefore, for the tested substances, an exposure of 2 h may be sufficient when an early screening of soil contamination is required. A shortening of the exposure time was already suggested by Da-Luz et al. (2008) after finding consistent avoidance responses after 24 h. Aldaya et al. (2006) and Lors et al. (2006) also established shorter exposure times of 20–100 min in avoidance tests with collembolans. Even so, caution must be taken since the absence of significant differences between exposure times might be explained by the high variability of the results, especially in the case of phenmedipham.

Findings of our study suggest that the presented procedure could become a valuable tool for an initial screening of soil contamination supplying rapid information for future decision-taking. Despite the suboptimal sensitivity of the test organisms to some of the tested substances, the Petri dish method provided information equivalent or even

more sensitive than the ISO standard and represented an improvement in terms of time and resources needed for the performance of the test. Additionally, data recorded in this study pointed out that an exposure time of 2 h with the Petri dish avoidance test may be enough for an early warning tool. Despite the potential benefits of the presented test, further research is required in order to reduce the high variation of results inherent in avoidance tests. At the same time, the performance of the test and the reduction of the exposure time from 48–2 h should be validated with other soils and chemical substances. Finally, a revision of the reference substances is suggested due to the low sensitivity of *F. candida* to boric acid and pure phenmedipham.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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