INFLUENCE OF SOIL PROPERTIES ON THE TOXICITY OF METAL-POLLUTED SOILS:

COMPARISON OF DIFFERENT BIOASSAY METHODS

Ana Romero- Freire

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door

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geboren te Noia, Spanje

promotoren: prof.dr. N.M. van Straalen

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Granada, a 22 de septiembre del 2015.

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Influence of soil properties on the toxicity of metal-polluted soils. Comparison of different bioassay methods.

Promotors: **prof.dr. N.M. van Straalen**
prof.dr. Francisco J. Martín Peinado

Copromotor: **dr.ir. C.A.M. van Gestel**

Memoria de Tesis Doctoral presentada por **Dª Ana Romero Freire** para optar al grado de doctor con mención internacional por la Universidad de Granada.

Septiembre 2015

There is a huge amount to be done if we are to reach a state of sustainability. Do not despair, do not be weighed down by it. All I ask of you is that you go home and do what you can.

Wangari Maathai

CONTENT

CHAPTER 1

General introduction

1.1. Environmental pollution

The current exponential urban growth in space and time comes with environmental changes that lead to an increase in pollution (Nriagu, 1990). The problem of environmental pollution was almost negligible before the beginning of industrialization, but after the nineteenth century rapid demographic development and wide-spread application of industrial products has resulted in an increase in the variety and amount of pollutants released into our environment (Daghan and Ozturk, 2015). The promptness of industrialization and urbanization caused a rise of people's necessities and activities, such as vehicle traffic and industrial production of goods (Grigoratos et al., 2014), which are directly related to atmospheric emissions (air pollution) and their subsequent deposition in water and soil (Rodriguez Martin et al., 2015).

Nowadays, relevant anthropogenic impacts include climate change, biodiversity loss, changes in nitrogen and phosphorous cycles, stratospheric ozone depletion, ocean acidification, global freshwater waste, changes in land use, atmospheric aerosol loading and chemical pollution (Rockström et al., 2009; Diamond et al., 2015). The latter one, chemical pollution, is caused by a wide number of chemicals emitted from different sources and released in a series of steps to the different regions of our planet. It may be questioned whether the system earth is capable of coping with such anthropogenically-released chemicals. In 2006, the First International Conference on Chemicals Management established the Strategic Approach to International Chemicals Management (SAICM), a multi-stakeholder policy framework for achieving a safe management of chemicals worldwide by 2020 (UNEP, 2007). Currently many challenges remain and the lack of prioritization of sound management of chemicals, the limitations of legislation, the lack of information and the lack of adequate financial resources, including funding of activities concerning remediation of contaminated sites, are still major obstacles to achieving the 2020 goal (CSD, 2010; UNEP, 2012). The challenge is to find global conditions that allow continuing human development in a safe world (Rockström et al., 2009).

1.1.1. Soil ecosystem degradation

Soil is one of the most important components of the life-cycle in nature alongside water and air (Daghan and Ozturk, 2015). Soil is an essential part of the ecosystem and normally has a higher buffering capacity against pollution compared to other ecosystem compartments. Nevertheless, the continuous exposure to pollutants may lead to soil degradation over time. In a general sense, soil degradation can be described as the deterioration of soil quality that involves the partial or entire loss of one or more of its functions (Blum, 1998; van Lynden, 2000). Pollution is defined as an introduction of contaminants which harm the abiotic environment and living organisms, and is considered one the most serious soil degradation type. Although soil has a high capacity to cope with pollution, it is not considered a renewable resource. Depending on climate conditions and parent material, it takes 200 to 1000 years on average for the formation of only 1 cm of soil (van Lynden, 2000) but we can lose it within minutes to hours.

Soil-water-air relations in the environment are very complex. When contaminants end up in the environment, they can be stored, transformed, or transferred among these three media, being easily retained in soils. In this way, the presence of contaminants in soils needs a correct environmental assessment in order to minimize their release and spread over the ecosystem and to reduce the risk of exposure to living organisms.

Soils are continuously contaminated by pollutants originating from agriculture (fertilizers, pesticides and herbicides), biological, anthropogenic or nuclear wastes, as well as fossil fuel or mine depositions (Hooda, 2007; Ismail, 2012). One of the most harmful and common contaminants are metals which within Europe are responsible for 37% of the soil pollution (Daghan and Ozturk, 2015).

1.1.2. Environmental distribution of metals and metalloids

Over decades metals have been introduced into our ecosystems by emissions of ash and solid particles (Napa et al., 2015). Several millions of hectares of land all over the world are continuously receiving a wide variety of contaminants (Oldeman et al., 1991). Among them, metals and metalloids are permanent pollutants in our soils, because they cannot be degraded and bind strongly to soil components; representing one of the most harmful problems of our times (Singh et al., 2003). Certain metals are essential for plant growth and for animal health with specific biochemical functions in metabolism and other biochemical processes. Regarding their functions in plants, copper (Cu) and zinc (Zn) can be considered micronutrients (Russell, 1973). However, if nutrient supply increases above certain concentrations they will become toxic, as "*the dose makes the poison*" (Paracelsus, 1567). Metal emissions have decreased in the European Union during the last decades, for example lead emission declined from 23,214 Mg year^{-1} in 1990 to 2,541 Mg year^{-1} in 2011 (Figure 1) (EEA, 2013). Nevertheless, metals can be mobilized in soils over a long time period (Napa et al., 2015) and soils can act as a natural buffer controlling the transport of these chemical elements (Kabata Pendias, 2011). The undue high accumulation of metals in soils with a high buffer capacity leads to a phenomenon called "chemical time bomb" (Batjes, 1997), producing serious environmental disasters once metal storage capacity is exceeded or disrupted due to environmental changes such as acidification (Salomons and Stigliani, 1995).

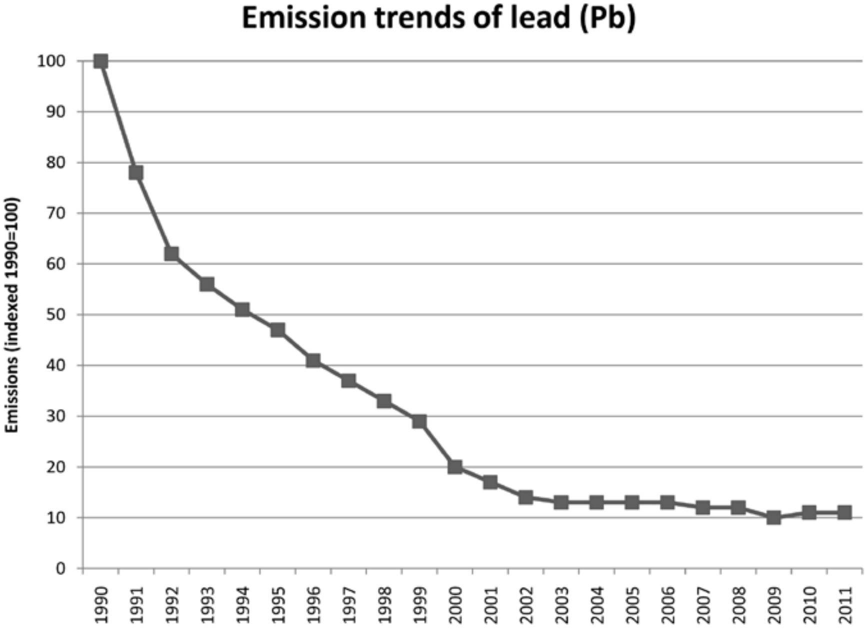

Figure 1. Emission trends of lead (Pb) from 1990 to 2011 in 27 countries, both from EEA member countries and EEA cooperating countries. Data are expressed as a percentage relative to the anthropogenic emission in 1990 (23,214 Mg = 100%). Information provided by the European Environment Agency (EEA).

The occurrence, distribution, origin and environmental mobility of metals have received substantial attention in recent years. Knowledge of natural geochemical and biological processes that govern metal transportation and transformation in the environment is necessary for gaining a better understanding. Figure 2 shows schematically the processes of emission, transformation and distribution of metal and metalloid pollution in the environment (adapted from Wang and Mulligan, 2006). Emissions from sources listed on the left take place to all three environmental compartments (soil, water and atmosphere). Eventually, by far the largest pool is in soils, sediments and rocks, and most transitions are between soil and water. Metals in the atmosphere are mostly bound to aerosols and dust, but some metalloids are present in a gaseous phase.

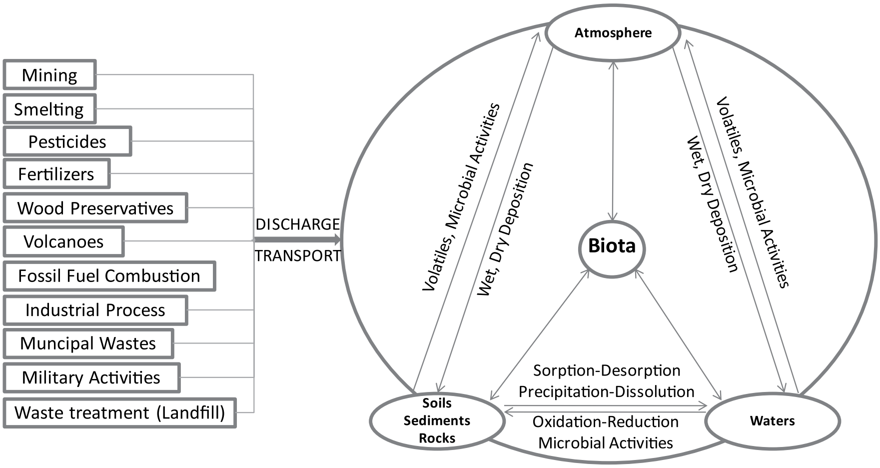

Figure 2. Sources and pathways of the distribution of metal and metalloids in the environment (adapted from Wang and Mulligan, 2006).

Metals and metalloids, as previously mentioned, are released from different anthropogenic activities, with mining activities being one of the most worrying sources (Rodriguez Martin et al., 2015). Mining activities frequently give rise to the release of metals (Zn, Pb and Cu) and metalloids (As) that may be very dangerous when they accumulate in the environment. Due to their potential to seriously affect soil ecosystems (Burgos et al., 2008), and their significant toxicological risks to organisms (Song et al., 2009), these four elements have been chosen for this thesis. The concern of soil pollution by these metal(loid)s needs to be viewed in the context of the continued urban growth and the need to develop methods for sustainable soil protection (Lv et al., 2013).

Many studies have assessed the effects of the individual elements in laboratory conditions but these data are not sufficient for a complete assessment of soil pollution. In an environmentally realistic situation, pollutants act as complex mixtures which may cause significant combined toxic effects, even if all individual chemicals are present at non-toxic concentrations (Diamond et al., 2015). Therefore it is essential to study both the individual and combined toxic effects of metals and metalloids under realistic environmental conditions.

1.1.3. Nanomaterials: the new hazard

The current industrial and societal developments have resulted in the invention of new materials and therefore new forms of pollutants. The concept of nanotechnology is relatively modern and it was introduced by Feynman in 1959. The rapid development of nanotechnology and the wide-spread use of nanoparticle-forms of metals in consumer products has not been followed by an equally rapid growth of risk assessment methodology geared towards nanoparticles. There is a considerable knowledge gap between the environmental release of nanomaterials and the development of data on their potential hazard (Whitley et al., 2013).

Daily consumer products are increasingly using manufactured nanoparticles (NPs). Addressing the risk these substances may pose to both the environment and human health has become one of the main objectives of environmental toxicology (Roco, 2011). There is evidence that the impact of manufactured NPs differs from that of their micro- or macro-particle equivalents (bulk forms) (Batley and McLaughlin, 2008). Therefore it is essential to study the stability and toxicity of NPs in soil over short- and long-term exposure periods and in comparison with the corresponding ionic metal species on which current risk assessment usually is based.

One of the most commonly used types of metal-based nanoparticles is zinc oxide (ZnO). ZnO NPs have the third highest annual production volume (550 tons per year) (Merdzan et al., 2014), and can easily enter the environment ultimately ending up in soils (Hooper et al., 2011). Hence, it is important to have a thorough knowledge of the behaviour and effects of NPs in the environment for a proper risk assessment (Gottschalk and Nowack, 2011). ZnO NPs were chosen in this thesis with the aim to compare their effects with those of their corresponding ionic Zn form.

1.2. Speciation of metals in soils

The transport, bioavailability, fate and toxicity of metal(loid)s depend upon their physical and chemical (speciation) forms, rather than on the total amount that is present (Alloway, 1995; Inaba and Takenaka; 2005). The most important factors influencing bioavailability of metals in soils are soil properties and ageing (Smolders et al., 2009). Figure 3 gives a conceptual scheme of metal partitioning in soils. Metals can be present in the soil solution as free ion but also as complexes with organic or inorganic ligands. The free ion may adsorb to the soil solid phase. The partitioning of the metal ions between the solid phase and the solution depends on the composition of both the solid phase (e.g., clay, oxide and organic matter content) and the solution (pH, concentration of competing cations). The metal sorbed to the solid phase acts as a buffer for the metal in solution. However, not all solid phase metal is in equilibrium with the solution. For instance, precipitation or diffusion into soil particles may result in metal fixation. The fixed metal form will not or only very slowly be exchanged with the solution phase (Buekers et al., 2007), and therefore will be hardly bioavailable for living organisms. In line of what has been exposed, soil properties largely control the mobility, bioavailability and therefore potential deficiency or toxicity of metals for plants and animals (Sheppard and Evenden; 1988). In addition, all these processes will also be influenced by weathering conditions and time. Therefore, metal speciation will be key to assess metal bioavailability in soils.

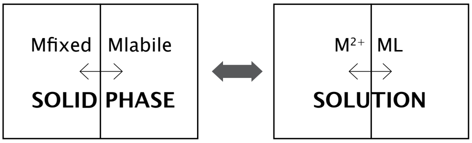

Figure 3. Schematic presentation of metal speciation in soil (M: metal, L: ligand).

One of the main environmental concerns in soil pollution with trace metals is the potential effect of changes in soil conditions, which will lead to a change in metal mobility and availability (Charlatchka and Cambier, 1999). Soils can act as a natural pollution boundary and retain toxic elements from the different processes explained above, however, what will happen when supplies exceed the limit between essential and harmful or the maximum storage capacity of the soils? In response to changes in environmental conditions, sudden transformation in the soil storage reservoir (chemical bomb) may occur leading to harmful effect. When the boundary is exceeded, soils can lose their buffer ability and metals may become labile and more easily bioavailable to living organisms. When metals finally reach the food chain, they also become a major risk to human health.

1.2.1. Influence of physicochemical soil properties

Heavy metals and metalloids can be involved in a series of complex chemical and biological interactions influenced mainly by properties and components of soils. The ubiquity of metals combined with the complexity of soils makes the study of metals one of the most important disciplines of soil chemistry (Roberts et al., 2005). The most relevant soil properties affecting metal speciation can be summarized as follows:

- **pH**, because of competitive proton sorption, precipitation-dissolution reactions, and pH-dependent metal complexation. Soil solution pH controls the electrical charge of soil colloidal fraction components, which is more negative at alkaline pH and therefore cations are removed from solution and bounded to colloids at higher soil pH values (Russell, 1973). In addition, the solubility of metal salts is often pH dependent, causing higher concentrations of cationic metal ions in the soil solution at low pH.

- **Redox potential:** Förstner (1991) identified redox potential as one of the key factors controlling metal biogeochemistry and their uptake by organisms. The redox equilibrium of elements largely determines their chemical and biological behaviour, therefore their mobility and potential toxicity. Soils with low redox potential favour the reduced species of metals, often causing precipitation reactions, e.g. of Fe(II) salts.

- Nature, content and quality of **organic matter**: the amount and type of organic matter present in the soil also affect metal solubility by sorption and complexation processes, e.g. the increase of dissolved organic carbon levels in the soil solution will increase the dissolved fractions of Zn or Cu bound to OM (Degryse et al., 2006). An increase in organic carbon can, however, lead to a decrease of availability (Sherene et al., 2010) since organic carbon associated with clay minerals in the solid phase has a high capacity to bind metals and deplete the soil solution.

- Presence of **Fe/Mn/Al oxides** and hydroxide minerals, which are considered the most important mineral sorbents for trace elements in soils. Some elements show a high affinity to oxide surfaces, e.g. As with iron oxides, which leads to adsorption or co-precipitation of these elements in soils (Simón et al., 2002).
- Soil solution **ionic strength:** increasing ionic strength is known to decrease metal adsorption capacity, and different electrolytes have varying effects on metal adsorption (Fike, 2001).
- Dissolved **calcium**, which significantly can reduce adsorption of other elements to soils, because of competition for adsorption sites between Ca and these elements (Simón et al., 2015). Precipitation reactions largely occur in alkaline and calcareous soils (Hue, 1988); at high pH related to high Ca concentrations, metals can be sorbed by soil colloids leading to decreased bioavailability.
- **Clay minerals:** mineralogical characterization of soils provides much insight for predicting soil properties like shrink and swell capacity, cation exchange capacity, and drainage; and composition of soil solution, i.e. anions, cations, and other complexing agents. Metals in soils mainly complex with clay minerals, oxides, oxyhydroxides and humic substances by covalent or ionic binding interactions (Sparks, 2003).
- **Cation exchange capacity (CEC)**, a measure of the number of negatively-charged sorption sites, is dependent on organic carbon content, clay content as well as the presence of other adsorption phases such as iron and manganese oxyhydroxides and soil pH.

1.2.2. Changes in metal availability with time

After emission of metals to the soil compartment, speciation and sorption processes are usually not in equilibrium. In fact, a remarkably long time, measured in years rather than months, is required to reach equilibrium between pollutants and soils. This process is called ageing (Scheckel et al., 2009). Chemical ageing in soil involves the movement of chemicals from the surface of soil particles into less accessible sites (Linz and Nakles, 1997). Due to ageing processes, metal solubilization, availability and toxicity is greatly modified (Richards et al., 2000; Schreck et al., 2011). Ageing is influenced by external conditions (climate) as well as by soil properties and components (Jalali and Khanlari, 2008). Therefore, it is essential to examine the effects of soils on chemical sequestration and the availability of metal(loid)s with time in order to arrive at a realistic risk assessment.

Metals may associate with many components of soils in various ways and the mechanisms of their ageing could be different from that of other pollutants, such as organic chemicals (Alexander, 1999). Unlike organic contaminants, metal(loid)s do not undergo microbial or chemical degradation and persist for a long time after their introduction in soils (Bolan et al., 2014). It is generally known that the availability of metals in soil decreases with time after contamination (Lock and Janssen, 2003). Generally, metals show their highest bioavailability immediately after application to soil, but can become non-exchangeable in the first hours due to the high sorption capacities of soils (Jalali and Khanlari, 2008). Therefore residence time is a key factor which will directly explain the bioavailability of metals and, hence, their potential toxicity in soils.

Knowing that metal solubility may change with time, studies using artificially spiked soils should be considered with care. In most laboratory experiments large quantities of metals are added as soluble salts during a short period of time and experiments are performed just after the spiked process, which it is at all not indicative of what one may find in field-polluted soils. The potential reactions that may occur between metal ions and soils will be explained from the soil properties mentioned above, the most important ones being: complexation processes, surface adsorption, exchange reactions, chelation processes and precipitation reactions (Hooda and Alloway, 1993). All these processes will require equilibration time and will contribute to explaining metal availability. Therefore, different ageing processes and equilibration times should be compared in view of obtaining the best simulation of the stabilization processes occurring under natural environmental conditions in order to establish generic environmental quality standards.

1.3. Ecotoxicological risk assessment

In the European Union, the evaluation of toxic effects and environmental risk assessment (ERA) is done primarily to protect human health and terrestrial, aquatic and atmospheric ecosystems (Song et al., 2006). There are two distinguished approaches: a) predicting the possible effects of chemicals in the environment with the aim to prevent such effects to occur (predictive ERA), and b) assessing the actual ecological risk or damage under pollution conditions with the aim to remediate or decide on measures to reduce the risk (diagnostic ERA) (van Gestel, 2012). The ERA of contaminated land is carried out through the so-called TRIAD approach, which combines the use of chemical methods, toxicological assays and ecological studies for the characterization of the risk and the associated effect (Jensen and Mesman, 2006).

The use of toxicity tests involving living organisms is essential for both predictive and diagnostic environmental risk assessment. In addition, the use of bioassays with soil organisms is a key element to assess the actual ecological risk and to support legislative regulation of contaminated soils, in order to complement the current regulations when declaring a soil as contaminated (Tarazona, 2005).

1.3.1. Bioassays as a tool for risk assessment

Bioassays are experiments in which selected test organisms are exposed to a soil to be diagnosed, and some biological response is measured. Bioassays differ from classical toxicity tests in that organisms are exposed to a soil taken from the field, rather than a reference soil freshly spiked with a pollutant in the laboratory. Bioassays are used to determine toxicity because they show the direct responses of organisms exposed to potentially polluting elements, and thus are indicative of the actual risk of pollutants, taking into account their bioavailability (Petänen et al., 2003). For terrestrial ecosystems, the most commonly used tests for toxicity assessment include the most important groups of soil organisms: plants, soil microorganisms and mesofauna.

The International Standardization Organization (ISO) and the Organization for Economic Co-operation and Development (OECD) as well as other national and international organizations (EPA, ASTM, …) have developed a number of standardized toxicity tests with plants, invertebrates and microorganisms/microbial communities. In order to find a proper risk assessment, a battery of tests is desirable to establish safe levels of pollutants in soils. Criteria to select the most suitable tests to perform are based on: the practicability and feasibility of the test, the acceptability of the test (standardization), the ecological sensitivity of the test and its reproducibility (van Gestel, 2012).

Bioassays for soil can be divided into two groups: a) those using a liquid phase (soil extract, pore water, leachate, etc.), and b) those using the soil solid phase (Farré and Barceló, 2003; Martin et al., 2010). Lors et al. (2011) conducted a comparison of bioassays performed in soil and in soil extracts. They concluded that there was a good correlation between the two tests ($r^2 = 0.996$), indicating that soil extracts could reflect the toxicity of the soil solid phase. In general, extractions remove contaminants soluble in water; subsequently, they reflect the behaviour of the mobile phases and evaluate the short-term risk of dispersion, solubilization and bioavailability of pollutants in the soil. To assess environmentally relevant soil toxicity, the use of a diverse set of exposure routes is recommended, thus, the set of bioassays selected should jointly take into account exposure to both the solid and the soluble phases of the soil (Rodriguez-Ruíz et al., 2014).

1.3.2. Variability in ecotoxicological data

There is an enormous disparity between the outcomes of laboratory and field studies on metal toxicity (Bååth, 1989). The main questions are: (1) how to establish critical metal concentrations for soil organisms? (2) how to extrapolate from short-term laboratory tests to the field conditions? and (3) how can we ensure that established metal limits protect all the involved populations and processes? (Giller et al., 1998).

In addition to the need to select the metal species to study and its concentration, also the soil type (variability in soil properties) as well as the bioassay or battery of bioassays which produce the best results in terms of applicability need careful consideration. It is essential to select, first of all, the methodological approach for the study. Metal toxicity studies can include laboratory tests with artificially spiked or naturally contaminated field soils or field tests performed under natural conditions. The main differences to take into account are that laboratory tests mainly determine acute metal toxicity, while field conditions determine the chronic effects resulting from long-term exposure. Studies using artificially contaminated soils in the laboratory should be interpreted with caution because the composition of the soil solution may differ from that of contaminated soils *in situ*. The advantages of laboratory studies with freshly spiked soils are that they are fast, simple and reproducible. They however, tend to overestimate the availability of metals in field soils (Smolders et al., 2009), so the toxicity levels defined by these studies may be more conservative and lead to unnecessary large safety margins for environmental risk assessment.

Field polluted soil is often contaminated with more than one metal and may also contain other (organic) pollutants (mixture toxicity). Predicting mixture toxicity has been an enduring challenge but is essential to properly assess soil toxicity. Traditional effect and risk assessment have been routinely focused on exposures to single chemicals and assuming additivity, which may underestimate the risk associated with mixtures (Barata et al., 2006). Recently, an increasing number of studies have been published, dealing with the combined (additive, synergistic or antagonistic) effects of mixtures of contaminants (Chen et al., 2015). More studies are needed to investigate the toxicity of multi-component chemical mixtures in soil with the main objective of finding a correct application in environmental risk assessment and soil remediation both in laboratory and *in situ*. Bioassays may be useful tools for such an assessment of soils contaminated with complex mixtures of pollutants.

1.4. Ecotoxicological insights applied to legislation

The establishment of a legislative instrument to soil protection in Europe has developed in different stages. Assessment methods for chemicals have been implemented in

European legal frameworks such as REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) (Scholz et al., 2013). In the case of soils, the European Commission proposed a framework and common objectives to prevent soil degradation, preserving its functions and rehabilitating degraded soils. This is described in the Thematic Strategy for Soil Protection (COM, 2006), which modifies Directive 2004/35/EC. For a long time, the European Commission proposed the development of a Soil Framework Directive (in analogy to the Water Framework Directive), however, the Soil Framework Directive was withdrawn in May 2014. The Seventh Environmental Action Program, which entered into force on 17 January 2014, recognises that soil degradation is a serious challenge. However, nowadays, there is no international consensus and there are no official criteria to declare a soil as contaminated, although programs have been developed to assess potentially contaminated sites. In general, soil contamination is considered within national environmental regulations without a European framework (Ferguson, 1998).

Reference values or quality criteria for the protection of human health and / or ecosystems have been established in different countries, however, these regulations apply to different processes and show great variability among countries. Examples are, for instance, the United States of America (US), where the Environmental Protection Agency (US EPA) has developed a series of Ecological Soil Screening Levels (Eco-SSLs) to protect terrestrial organisms from effects of soil contamination. In the Netherlands, the Ministry of the Environment developed a set of reference values for contaminated sites based on remediation and health protection criteria (VROM, 2000). In Canada, soil quality guidelines (SQGs) were developed by the Canadian Council of Ministers of the Environment (CCME) in order to assess contaminated soils (CCME, 1999 and 2006). These SQGs are mainly based on data on the toxicity of chemicals in laboratory and field tests. In Germany, the law for the protection of contaminated sites (BBodSchV, 1999) provides preventive values for investigating and intervening to protect ecosystems against negative effects of soil contamination. This also is the case in Spain, where the central government has determined that each region has the responsibility to develop reference levels to decide on declaring a soil as polluted (Law 22/2011). But even today such reference values have not been established in all regions.

1.4.1. Establishment of reference values

The process of ecological risk assessment (ERA) of contaminated soil represents a difficult task given the heterogeneity of the soil as well as the use and management of it. These processes are greatly influenced by differences between scientists, private and administrative interests (Jensen and Pedersen, 2006). So far, this has made the methods to be applied in the analysis of contaminated soils much less advanced than those applied to aquatic systems.

Any ERA process comprises a series of predictive and descriptive phases. Jørgensen (2001) divided the process into nine steps. These steps try to answer questions that may help identifying how the risk should be classified and how it can be treated. Nowadays most ERA methods are implemented distinguishing four main phases (Perrodin et al., 2011). Based on these two approaches, Table 1 shows a current adaptation of this ERA process.

Table 1. Steps to assess the risk of chemical compounds in soils based on Jørgensen (2001) and Perrodin et al. (2011).

PHASE	DESCRIPTION	QUESTION	TOOLS
1	Identification of hazard	Which hazards are associated with the studied chemical?	Characterization and description of contaminants as well as the place (soil type and use)
2	Emission assessment	What are the sources and quantities of emissions?	Production and use of the chemical
3	Effect assessment	What is the relation between dose and responses?	Thresholds: NOEC, LOEC, ECx, LCx
	Effect prediction	Which uncertainties factors to take into account to extrapolate experimental data to real situations?	Predicted no effect concentration (PNEC)
	Modelling concentration	What is the actual exposure concentration?	Predicted Environmental Concentration (PEC)
	Risk quotient (RQ)	What is the ratio PEC/ PNEC?	RQ≥1 indicates a risk to the environment
4	Risk classification	How will we classify the risk?	Reference level (RQ<1) Investigation level (RQ=1) Intervention level (RQ>1)
	Risk-benefit analysis	What is the relation between risk and benefit?	Socioeconomic, political and technical factors
	Risk reduction	How can the risk be reduced to an acceptable level?	Management, restoration and monitoring of soil decontamination activities performed

With the achievement of each of the proposed phases there is a greater approximation to the final risk assessment. The first step (1) involves an approach that should provide an integrated view of the scenario, where the formulation of the problem should be done identifying the pollutant and the subsequent phases of the ERA.

The second phase (2) should be studied with the collaboration of factories and enterprises involved in the possible emission of the pollutant. It includes the analysis of sources of pollutants, transfers and distribution in the environment as well as the possible amounts which can be released.

The third phase (3) will need the use of ecotoxicological studies and models. This phase also involves the selection of suitable bioassays as well as representative endpoints to provide reliable and realistic thresholds values. In this phase, the aim is to obtain endpoints from the organism response to soil toxicity in order to extrapolate these obtained endpoints to soil quality guidelines. One of the endpoints most commonly used in ERA is the no observed effect concentration (NOEC), which is the highest concentration of the studied pollutant which does not produce a significant toxic effect on the studied organism. The lowest observed effect concentration (LOEC) is the lowest concentration tested where a significant toxic effect is observed compared to the control. The effective concentration (ECx) is the concentration where the pollutant is producing a certain effect (x) (i.e. EC50, the parameter studied, e.g. reproduction, is reduced with 50% compared to the control) and the lethal concentration (LCx) is the concentration causing x% death of the test organisms (i.e. LC25, 25% of the studied organisms died). The next steps of this phase involve the use of models to derive safe levels, such as the Predicted No Effect Concentration (PNEC) which is compared with the predicted or measured exposure concentration to estimate the risk.

The last phase (4) will be based on political decisions supported by the information obtained in the previous phases, including the regulatory levels to classify a contaminated soil, the socioeconomic implications, and the restoration activities.

1.4.2. Omissions in current regulation

Most soil quality criteria proposed in different countries do not take into account soil properties and are based on toxicity tests without considering the factor time. As explained earlier, these two factors (soil type and ageing) are key in the bioavailability of metals and other pollutants. Environmental protection policies are based on concentration levels of a certain element or compound, which is usually linked to the soil type or its use. In general three guideline values have been defined, with an increasing degree of requirement: 1) reference level (maximum concentration of an element without producing undesirable effects), 2) investigation level (concentration of an element above the reference level and which may result in potentially harmful effects), and 3) intervention level (contaminated soil where decontamination measures are needed) (Diez, 2006).

The guideline concentrations proposed for soil protection vary enormously. Table 2 shows different guideline values of metal(loid)s in three selected countries. This table shows for instance that for Zn, a soil will be considered polluted with concentrations above 720 mg Zn kg^{-1} soil in The Netherlands, while in Germany this level is set at 3000 mg Zn kg^{-1} soil for residential and industrial use and at 600 mg Zn kg^{-1} soil for agricultural soils, and in Andalusia region (Spain) this level is fixed at 10,000 mg Zn kg^{-1} soil regardless the soil use.

Table 2. Different guideline values for some metal(loid)s in three selected European countries.

	The Netherlands		**Germany**			**Spain (Andalusia region)**		
	VROM (2000, 2009)		(German Federal Government, 2002)			Decree 18/2015		
Soil use classification	Target value (2000)	Intervention value (2009)	Industrial use	Residential use	Agricultural use	Industrial use	Urban use	Other uses
As (mg kg^{-1})	29	76	150	80	50	40	36	36
Pb (mg kg^{-1})	85	530	2000	2000	1000	2750	275	275
Zn (mg kg^{-1})	140	720	3000	3000	600	10000	10000	10000
Cu (mg kg^{-1})	36	190	1000	600	200	10000	3130	595

Defining soil policies is not an easy task because of the high variability of soils, as well as the possible pollutants involved and the complexity of their behaviour in soils. However, the high diversity in soil quality criteria or guideline values is obviously based on the lack of consensus and the applied methodology. Therefore the development of effective soil environmental policies, first of all, is a crucial task for ERA. Joining all policies into an operational way is a joint task of all member states of the European Union. In this regard, soil environmental protection and pollution control legislation has still a long way to go.

1.5. Aim and outline of this thesis

Soil properties are recognised as the main factors that control mobility, (bio) availability and toxicity of metals in terrestrial ecosystems. However, to declare a soil as contaminated, in general, the influence of soil properties is usually not taken into account. Moreover, the use of guideline values to designate a soil as polluted is currently based on laboratory studies without taking into account key processes such as long-term equilibration (ageing), which could change the fate and bioavailability of metals in the soil.

The focus of this thesis is to study the fate and ecotoxicological effects of metals and metalloids as well as metal-based nanoparticles in soils. This includes studying the influence of soil properties and also the effects of ageing (long-term equilibration) on the (bio) availability of metals and metal based nanoparticles in soil. For this purpose, bioassays were used to assess metal bioavailability and toxicity to different organisms. In addition, this study aimed at implementing the obtained results in a framework of risk assessment, for instance by establishing toxicity thresholds for metals in soils that can be used for legislation. The main goal of this thesis is to improve the scientific basis for environmental risk assessment of metals and metalloids in soils.

The work described in this thesis includes application of bioassays with different organisms that jointly represent the major groups of soil organisms (invertebrates, primary producers, microbes, etc.), to assess the bioavailability and toxicity of As, Pb, Zn, and Cu, taking into account ageing processes and soil properties. My study used both laboratory-spiked soils with different properties and field soils affected by residual contamination (Aznalcóllar mine spill, Spain). It also included the assessment of the toxicity of ZnO nanoparticles in relation to soil properties and ageing. Research was carried out in different steps described below.

1. Influence of ageing on metal bioavailability and toxicity in soils

Long-term effects (ageing) on the bioavailability and toxicity (from bioassays with the earthworm *Eisenia andrei*) were studied in three soils with contrasting properties spiked with ZnO nanoparticles (ZnO NP) and ionic Zn ($ZnCl_2$). Metal availability and toxicity were evaluated at different time points during 6 months together with the toxicity effect and bioaccumulation in earthworms (**Chapter 2**).

2. Use of bioassays to derive guideline values

A variety of bioassays were selected as indicator of metal(loid) stress in polluted soils. In order to determine the effect of soil properties, seven different representative soils were chosen and spiked with different elements individually. Soils were incubated during 4 weeks to take into account the effect of time on metal availability. Toxicity of arsenic (As) and its availability in different soils were studied with two bioassays, using the plant *Lactuca sativa* and the bacterium *Vibrio fischeri*, to propose soil guideline values for As (**Chapter 3**). Moreover for the same element, a test with the earthworm *Eisenia andrei* was performed to endorse the importance of bioassay variety as well as the guideline values obtained (**Chapter 4**). The toxicity of lead (Pb) was measured using three bioassays: a seed germination and root elongation test with *Lactuca sativa*, the Microtox® test with *Vibrio fischeri* and a soil basal respiration test (**Chapter 5**). Finally, to justify if studying soil pollution only from a bioassay is effective as well as to assess the effectiveness and reliability of the soil respiration test, we conducted an experiment with four different elements, including As and Pb as well as zinc (Zn) and also soils polluted with copper (Cu) (**Chapter 6**).

3. Relationship with laboratory and *in situ* contaminated soils

The results obtained in the previous steps were applied to a natural polluted area (Aznalcóllar mine) with the aim to test and optimize ecotoxicological studies generally performed in the laboratory under controlled conditions with artificial spiked soils. Long-term effects and the availability of metals in field-contaminated soils were determined 6 and 15 years after the mining spill in areas affected by residual pollution (**Chapter 7**). And to determine the actual risk of contamination to living organisms and ecosystems, two bioassays (*L. sativa* and soil respiration test) were applied to unrecovered areas after 15 years of the Aznalcóllar mine spill (**Chapter 8**).

In the final chapter of this Ph.D. thesis (**Chapter 9**) the results obtained in previous chapters are summarized, discussed and integrated.

CHAPTER **2**

Effects of ageing and soil properties on the bioavailability of zinc nanoparticles to the earthworm *Eisenia andrei*

Abstract

To assess the influence of soil properties and ageing on the availability and toxicity of Zn applied as nanoparticles (ZnO NP) or as Zn^{2+} ions ($ZnCl_2$), three soils were spiked and incubated for up to 6 months. Availability of Zn was determined with pore water and 0.1 M copper nitrate extractions, while exposures of the earthworm *Eisenia andrei* were performed to study Zn bioavailability. Zn availability was lower when applied as nanoparticles, and influenced by soil pH. Zn availability for both Zn forms was affected by ageing, but varied among the tested soils, showing the influence of soil properties. Internal zinc concentration in the earthworms (ZnE) was highest for the soil with high organic carbon content and basic pH spiked with ZnO NP, however for the same soil spiked with $ZnCl_2$ earthworms showed the lowest variation in internal Zn concentration compared to the control treatment. Survival, weight change and reproduction of the earthworms were affected by both Zn forms, but differences in toxicity could not be explained from soil properties or ageing. This shows that ZnO NP and $ZnCl_2$ behave different in soils depending on soil properties and ageing processes, but differences in earthworm toxicity remain unexplained.

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Submitted

2.1. Introduction

The use of nanoparticles (NPs) in a variety of applications has exponentially increased over the last 30 years (Conde et al., 2014). As a consequence, these manufactured NPs are increasingly entering the environment (Nowack and Buschelli, 2007), but only limited data is available on their potential hazard (Nel et al., 2006). The high production of NPs as well as the potential for release into the environment, and subsequent effects on ecosystem health, are becoming a growing concern. A thorough knowledge of the behaviour and effects of NPs in environmental media is essential for a proper risk assessment (Gottschalk et al., 2011).

Zinc oxide (ZnO) is one of the most commonly used types of metal-based nanoparticles. They are the NPs with the third highest annual production volume (550 tons per year) (Merdzan et al., 2014). Nowadays, ZnO NPs are used for electronics applications, solar panel devices, medicine, cosmetics, sunscreens (UV-filters) or applied as antibacterial agents (Ma et al., 2013). ZnO NPs can enter the environment via wastewater at industrial sites or through domestic sewage, and by application of sewage sludge in agriculture they may also end up in soil (Hooper et al., 2011).

The distribution, mobility and bioavailability of Zn in soils are controlled by a range of physico-chemical characteristics, including the nature and heterogeneity of the soil constituents, the surface charge of soil colloids, and variations in soil pH and redox status (Donner et al., 2010). The properties of nanoscale materials may differ substantially from those of the respective bulk materials (Watson et al., 2015). And under the influence of soil properties, such as pH and organic matter content, ZnO NPs show a high variability in bioavailability and therefore toxicity (Tourinho et al., 2013; Waalewijn-Kool et al., 2013; Heggelund et al., 2014). The study of the potential ecotoxicological effects of ZnO NPs is essential to determine under which conditions and how these NPs contribute to toxicity for assessing their hazard and potential risk. Nevertheless, there is a significant lack of characterization of the ZnO NPs and the exposure system in the ecotoxicity studies conducted thus far. Once in the soil or water media, complex processes can affect NPs, which can act as colloids. The NPs may form aggregates or agglomerates, which can lead to sedimentation, or may be prone to dissolution and release free metal ions (Tourinho et al., 2012). Upon contact with water, ZnO NPs dissolves partially to zinc (Zn^{2+}), so their effects may partly be due to the soluble forms of Zn (Brun et al., 2014). This process may also occur in soil, and it therefore is essential to substantiate whether Zn toxicity is produced by the nanoparticle forms as well as the free ions released in soil.

It has been observed that it requires long time periods to reach equilibrium for soil spiked with ionic zinc, a process called ageing (Crout et al., 2006). In soils spiked with ZnO NPs the same process occur (Scheckel et al., 2010), while an additional dissolution to ionic Zn forms may make the time needed to reach an apparent equilibrium even longer. These long-term processes have shown to decrease bulk zinc toxicity in soil over time, while ageing also seems to reduce ZnO NP toxicity (and bioavailability) in soil, as was shown for springtails by Waalewijn-Kool et al. (2014). Hence future ecotoxicity tests with ZnO NPs should focus on their long-term effects in relation to their fate and bioavailability (Ma et al., 2013). Another important fact to consider is that the difference in zinc bioavailability between freshly and aged spiked soils can be explained by major soil properties, like pH and cation exchange capacity (CEC) (Lock and Janssen, 2003).

With the aim of verify the potential ecotoxicological effects of metals, bioaccumulation in soil organisms has been studied extensively (Nahmani et al., 2007; Díez-Ortiz et al., 2010). Earthworms are common in a wide range of soils and have largely been used in bioassays for evaluating hazardous chemicals in soils (Antunes et al., 2008). Earthworms are more susceptible to metal pollution than many other soil invertebrates and have a number of characteristics (large size, behaviour and high biomass), which make them highly suitable for use as bioindicator organisms for determining the toxicity of chemicals in soils (Spurgeon and Hopkin, 1996). As so-called eco-engineers, they play an important role in decomposition and soil-forming processes, but they also can easily accumulate chemicals from soil and in that way, as important prey, introduce then into the food chain (Díez-Ortiz et al., 2010). Consequently, earthworms have been adopted as standard organisms for ecotoxicological testing (EEC, 1984).

The purpose of this study was to determine the influence of ageing on the availability and toxicity of ZnO nanoparticles to earthworms in soils with contrasting properties. Three natural soils with different physical-chemical soil properties were selected to assess the fate and effects of nanoparticulate Zn (ZnO NPs). Ionic Zn ($ZnCl_2$) was included for comparison. Availability of Zn was studied by applying two different chemical extraction methods (pore water and a novel mild copper nitrate extraction) to assess changes in the availability of the two studied Zn forms in the course of a 6 months ageing period. Pore water concentrations may provide an indication of actual Zn availability. Extraction with 0.1M $Cu(NO_3)_2$ was intended as a test of whether it was possible to selectively extract the reactive (labile) pool of ionic zinc in the presence of ZnO NPs. Zinc bioavailability and toxicity was measured by exposing earthworms of the species *Eisenia andrei* to soil incubated for different incubation periods. Internal Zn concentrations in the animals, survival, weight change and reproduction were estimated to evaluate Zn bioavailability and toxicity.

2.2. Materials and methods

2.2.1. Soils

Three uncontaminated soils with contrasting properties were selected from different countries. Two soils were collected from the surface horizon of fields in Spain (SPCA; forestland in Granada) and the Netherlands (NLGA; a garden in Bilthoven), homogenized, sieved through a 5 mm mesh and air dried. The third soil was the LUFA 2.2 natural standard soil (LUFA Speyer, Germany). Before the start of the tests, the following physical and chemical properties were determined: soil pH-KCl (soil:1 M KCl, ratio 1:2.5) and pH in pore water, electric conductivity (EC), calcium carbonate content ($CaCO_3$), organic carbon (OC) content, texture, cation exchange capacity (CEC) and base content according Romero-Freire et al. (2014) and water holding capacity (WHC) (ISO, 1999) (Table 1).

Table 1. Physicochemical properties of the soils used to assess the effects of ageing and the influence of soil properties on the (bio)availability of ZnO nanoparticles and ionic Zn (applied as $ZnCl_2$).

Soil	Country	pH (KCl)	pH (PW)	EC (mS cm^{-1})	$CaCO_3$ (%)	OC (%)	Clay (%)	CEC (cmol+kg^{-1})	P (mg kg^{-1})	WHC (%)	Background Zn (mg kg^{-1})
LUFA2.2	Germany	5.6	6.7	0.05	<1	1.55	8.27	8.19	5.64	45	28.5
NLGA	Netherlands	5.9	6.9	0.03	<1	3.44	4.80	18.8	25.5	51	92.8
SPCA	Spain	7.6	8.0	0.08	37	5.43	23.6	21.4	8.26	62	129

Soils were spiked in the laboratory with ZnO nanoparticles (Nanosun Zinc Oxide P99/30) with a primary particle diameter size of 20-40 nm (Figure SI-1). To study the effect of dissolved Zn, a treatment with the soluble salt $ZnCl_2$ (Merck, zinc chloride pure) was included. Test concentrations were based on toxicity data found in earlier studies (Heggelund et al., 2014). Concentrations used were selected to correspond with EC10 and EC50 for the effects of ZnO NPs and EC50 for effects of $ZnCl_2$ on earthworm reproduction, and values were adjusted to differences in soil properties. Test concentrations of $ZnCl_2$ were 500 mg Zn kg^{-1} in LUFA and NLGA and 1250 mg Zn kg^{-1} in SPCA; ZnO NP concentrations tested were 500 and 1000 mg Zn kg^{-1} in LUFA and NLGA and 1250 and 2500 mg Zn kg^{-1} in SPCA. Uncontaminated controls were also included.

The ZnO NPs were mixed in with the soils as powder, while $ZnCl_2$ was introduced as a solution in water. Soils were intensively mixed to achieve an as homogenous distribution of the Zn as possible. After spiking, soils were moistened to 50% of their WHC. All replicates for all sampling times were dosed together as one batch of soil. Soils were incubated at 20°C and 75% air humidity, with a light/dark cycle of 12/12 hours. Soil moisture content was checked by weighing the test containers and if needed readjusted weekly.

2.2.2. Extraction procedures to assess Zn availability

Two methods were applied to assess the effect of ageing on Zn availability in the ZnO NP and $ZnCl_2$ spiked soils. Extractions with 0.1 M $Cu(NO_3)_2$ $3H_2O$ (Alfa Aesar, copper nitrate 98% pure) were performed at 1, 3, 7, 10, 14, 28, 56, 112, 168 and 196 days after spiking the soils. For that, 1 g (dry weight) soil and 20 ml copper nitrate solution was shaken overnight in falcon tubes (Centrifuge Falcon 6/300 series, CFC Free) at each sampling time (n=4). Sample tubes were centrifuged at 2000 RCF (relative centrifuge force) during 1 hour at 10ºC (MSE Falcon 6/300 centrifuge). The supernatant was sampled using syringes (Terumo 10 ml) with a 0.45 µm filter (Minisart Sartorius hydrophilic) and stored at 5ºC for subsequent analysis.

At 1, 3, 56 and 168 days after spiking the soils, pore water extractions were performed. For that purpose, 50 g of soil samples were placed in Teflon containers and moistened to 100% Water Holding Capacity, mixed and equilibrated for 7 days at room temperature. After that, soils were centrifuged for 45 min at 2000 RCF at 10ºC, over a 0.45 µm membrane filter (Whatman NC45, cellulose nitrate Ø 47 mm) placed in between two round filters (Whatman filter paper cat. no 10001-047, Ø 47 mm) (Hobbelen et al., 2004). Pore water was analysed for pH with a WTW pH 7110 pH meter and EC with a WTW Multiline P4. Samples were acidified (nitric acid 60%) and refrigerated to further analysis. Pore water extraction was performed in duplicate for each treatment, including two controls with only water as a negative control.

2.2.3. Earthworm tests

Earthworm tests were performed using soils incubated for 1, 56 and 140 days after spiking. Earthworms of the species *Eisenia andrei* were obtained from a laboratory culture at the Department of Ecological Science of the VU University in Amsterdam. The earthworms were fed with horse manure free of any pharmaceutical and incubated at 20ºC. The tests used adult earthworms with well-developed clitellum, which were acclimatized for 24 h in the respective control soil before starting the exposures.

The tests with the earthworms followed OECD guideline 222 (2004), including a 28-day exposure period of adult animals and another 28 days incubation of cocoons to enable assessing juvenile production. Four replicate test containers were used for each zinc concentration and control, containing approx. 500 g soil (dry weight equivalent) moistened to 50% WHC. Ten adult earthworms were added to each exposure container after being gently cleaned on moistened paper towels and weighed. Furthermore, 10 g (wet weight) of horse manure:distilled water (1:1 ratio) were added to each container to feed the earthworms. The containers were maintained in an incubator chamber at 20°C, 75% air humidity and with

12 h of light per day. Container weights were monitored to maintain moisture content and additional food was added if required.

After the first 28 days, test containers were emptied into a tray and surviving adults were collected by hand sorting and weighed. Surviving earthworms were incubated on moist filter paper in Petri dishes for approx. 24 h to void their gut contents. After that, they were frozen and later freeze-dried (EFY Modelyo) during two days and stored for analysis. Soil samples, which contained cocoons, were returned to the respective containers and incubated for another 28 days. After this period the number of juveniles was determined by placing the containers in a water bath at 60ºC forcing juveniles to emerge to the surface, where they were counted.

2.2.4. Metal analysis

To check spiked concentrations, approx. 0.1 g oven-dried soil samples were digested in 2 ml of a 4:1 mixture of nitric acid (65% pro analysis; Riedel-de Haen) and hydrochloric acid (37% pro analysis, Baker) in tightly closed Teflon@ bombs which were heated in an oven at 140ºC for 7 h. To determine the Zn concentration in earthworms, one freeze-dried individual earthworm of each replicate sample was digested using the same acid mixture and procedure as described for soil samples (n=4). Total zinc concentrations in soils (ZnT) and earthworms (ZnE) were measured by flame atomic absorption spectrometry (AAS; Perkin Elmer AAnalyst 100). Zn concentrations in copper extracts (ZnCu) and pore water (ZnPW) were also measured by flame AAS. Instrumental drift was monitored by regularly running standard element solutions between samples. All Zn analyses included procedural blanks and a certified reference material (DOLT-4). Procedural blanks for estimating the detection limits (n=20) were <0.003 mg L^{-1} for Zn. Digested blanks contained Zn concentrations below the limit of detection. Recovery of Zn from the DOLT-4 reference material was 97±2.4%.

2.2.5. Data analysis

Normal distribution of the data was verified from a Kolmogorov-Smirnov test. Significant differences were determined by ANOVA and multiple comparisons were performed with Tukey's test ($p < 0.05$). Partition coefficients (Kd) were calculated as the total Zn concentration in soil (ZnT) (mg kg^{-1}) divided by the pore water Zn (ZnPW) (mg L^{-1}) (KdPW) or by Zn concentrations measured in copper extractions (ZnCu) (mg L^{-1}) (KdCu). Bioaccumulation Factors (BAFs) for the accumulation of Zn in the earthworms were calculated by dividing concentrations in the surviving animals (ZnE) by measured total concentrations in the test soils (ZnT) (mg kg^{-1}). To analyze the influence of soil properties and ageing on Zn availability and earthworm responses for the three studied soils, principal component analyses (PCA) were done using the 'CANOCO for Windows' program v4.02. Ordination diagrams were explained with soils samples represented as points and most of studied variables (Kd_{PW}, pH_{PW}, BAF, ZnE and W_L) as arrows, according to González-Alcaraz et al. (2014).

2.3. Results

2.3.1. Available Zn

Measured concentrations in the test soils on average were 97 ± 6% of the nominal (added) Zn concentrations (Table 2). Measured Zn concentrations were used in all data analyses.

Zinc concentration in pore water extractions (soluble Zn) was lower in ZnO NP treatments than in soil spiked with $ZnCl_2$ (Table 2). On average, soluble Zn concentrations from $ZnCl_2$ corresponded with 5.04-11.4% of the total Zn in LUFA, 1.71-2.57% in NLGA and 0.08-0.34% in SPCA soil. In treatments with ZnO NPs, pore water concentrations corresponded with < 2.53 % of total Zn concentration in LUFA soil and < 0.61% in the other two soils, showing the lowest soluble Zn level in SPCA (Table 2). Zn concentrations in the pore water of LUFA and NLGA tended to increase with time for all treatments, while in SPCA they remained constant or showed a slight decline with time.

Table 2. Effect of ageing on the availability of Zn in soil. Shown are the nominal and average (±SD; n=3) measured total Zn concentrations (ZnT) in soils spiked with $ZnCl_2$ or ZnO NPs, and pore water Zn (PW; n=2) and 0.1 M copper nitrate extractable Zn concentrations (ZnCu; n=4), both expressed as mg Zn L^{-1}, obtained at different points in time after spiking the soil. For the complete set of extractions performed with copper nitrate, see Table SI-2 in the Supporting Information). Zn recoveries, in % of total Zn concentration in soil, in pore water (%R_{PW}) and extractions with copper nitrate (%R_{Cu}) are also shown.

SOIL	**TREATMENT**	**Nominal Zn**	**Measured ZnT**	**TIME**	**ZnPW**	**Recovery**	**ZnCu**	**Recovery**
		mg Zn kg^{-1} soil	mg Zn kg^{-1} soil	Days	mg L^{-1}	(%R_{PW})	mg L^{-1}	(%R_{Cu})
LUFA	$ZnCl_2$	500	487 (±87.4)	1	84.1a	7.78	23.4ab	91
				3	60.5a	5.59	21.7a	84
				56	54.4a	5.04	26.4b	82
				168	124b	11.4	24.2ab	52
	ZnO NP	500	497 (±36.7)	1	2.64a	0.24	19.1a	74
				3	3.26a	0.30	20.7ab	80
				56	12.3b	1.12	25.2b	77
				168	25.4c	2.30	21.3ab	41
	ZnO NP	1000	873 (±38.6)	1	5.51a	0.28	40.6b	95
				3	5.46a	0.28	33.4a	77
				56	37.4b	1.93	40.5b	79
				168	49.0c	2.53	36.8ab	61
NLGA	$ZnCl_2$	500	495 (±55.3)	1	16.5a	1.71	31.6b	100
				3	19.1ab	1.98	30.9ab	100
				56	23.6b	2.45	29.2a	68
				168	24.8b	2.57	29.2a	79
	ZnO NP	500	490 (±63.9)	1	2.48a	0.26	30.6ab	100
				3	2.12a	0.22	31.1b	100
				56	2.04a	0.22	29.2ab	68
				168	5.68b	0.60	28.4a	76
	ZnO NP	1000	858 (±49.0)	1	2.95a	0.18	55.0b	100
				3	3.06a	0.18	54.4b	100
				56	2.67a	0.16	50.9a	93
				168	10.2b	0.61	52.9ab	100

SPCA	$ZnCl_2$	1250	1259 (±230)	1	6.84c	0.34	56.2b	93
				3	4.95bc	0.24	50.6b	86
				56	1.57a	0.08	42.5a	66
				168	2.13ab	0.10	40.9a	51
	ZnO NP	1250	1287 (±134)	1	0.91	0.04	51.4b	84
				3	0.73	0.04	47.2b	78
				56	0.81	0.04	39.1a	58
				168	0.83	0.04	38.6a	46
	ZnO NP	2500	2514 (±258)	1	2.05	0.05	118b	97
				3	1.43	0.04	92.4ab	78
				56	1.33	0.03	97.6ab	76
				168	1.46	0.04	77.9a	48

Lowercase letters represent significance difference between sampling days for each treatment (Tukey HSD test. $p < 0.05$).

The 0.1 M $Cu(NO_3)_2$ extractable zinc fraction did not correlate with the soluble zinc concentration for any of the soils or treatment levels. Copper extraction was highly effective, with 100% of the spiked Zn being extracted in the NLGA soil at the first two sampling times and ≥ 74% for the other soils at day 1 (Table 2). The amount of zinc extractable with copper nitrate did not differ among the three studied soils for the two treatments (ZnO NPs or $ZnCl_2$) added at the same concentration and extractability was highest at the highest nominal concentrations. In general, Zn extractability decreased with ageing, this effect was more pronounced in the SPCA soil followed by LUFA. In the NLGA soil spiked with both Zn forms extractability showed small variations, it decreased after 56 days but increased again after 168 days; the decrease was most pronounced at 500 mg Zn kg^{-1} soil (Table 2).

Partition coefficients (Kd) were calculated to facilitate comparison of the chemical availability of Zn for the different treatments. Kd_{PW} values calculated from pore water Zn concentrations were significantly affected by ageing in LUFA for both $ZnCl_2$ and ZnO NPs. NLGA showed only a significant decrease with ageing of Kd_{PW} values for ZnO NPs. SPCA did not show significant changes of Kd_{PW} values for ZnO NPs but Kd_{PW} values for $ZnCl_2$ increased with time. Partition coefficients calculated from $Cu(NO_3)_2$ extractable concentrations did not show significant differences (Figure 1).

2.3.2. Toxicity and bioaccumulation of Zn in earthworms

Earthworm responses to the different test soils and treatments at different times of ageing are shown in Table 3. Survival was higher than 78% for all treatments and sampling times except for SPCA spiked with $ZnCl_2$ after 56 days of ageing where survival was only 25%. Earthworm weight loss, after 28 days of incubation, increased with ageing and was significantly different with the only exception of NLGA spiked with ZnO NPs (500 mg kg^{-1}).

The number of juveniles per earthworm during 28 days of exposition in the controls was 1.8 in NLGA, 3.1 in LUFA and 3.6 in SPCA at time 1 day. Earthworm reproduction compared to the control was most affected by $ZnCl_2$, with complete inhibition of reproduction in LUFA and SPCA at all ageing times and with 82% or more reduction of reproduction in NLGA (Table 3). Reproduction effect also showed some variation depending on the tested soil and ageing period. For ZnO NP treatments, the LUFA soil showed a significant decrease of earthworm reproduction with time. NLGA did not show variation in reproduction; however it showed increased reproduction after 140 d at both ZnO NP treatments. SPCA showed a trend of decreasing reproduction with time in the ZnO NP treatment of 1250 mg Zn kg^{-1} soil while 2500 mg kg^{-1} showed an unusual behaviour with almost no reduction compared to the control after 56 days.

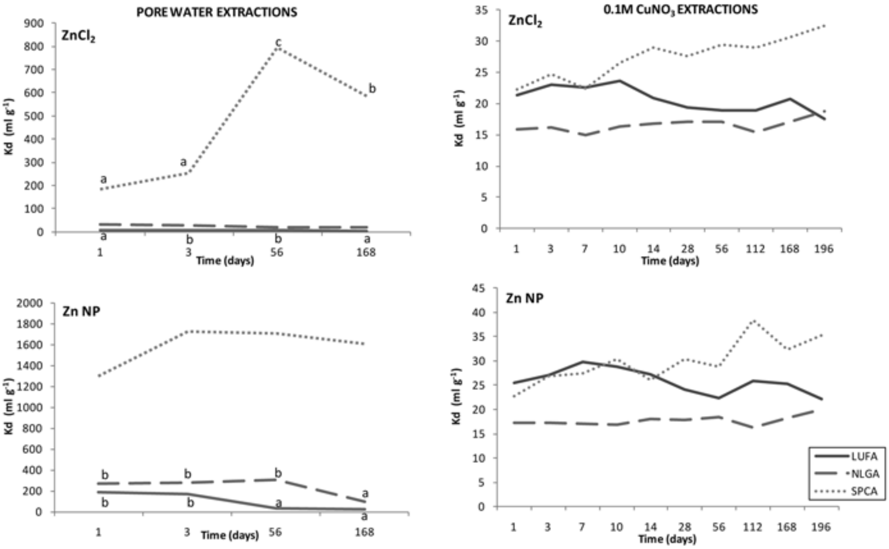


Figure 1. Partition coefficients (Kd, in ml g^{-1}), expressed as the total Zn concentration in soil (ZnT) divided by extractable Zn concentrations, calculated for pore water Zn (ZnPW) (n=2) at 1, 3, 56 and 168 days after spiking and for 0.1 M $CuNO_3$ extractable Zn (ZnCu) (n=4) after 1, 3, 7, 10, 14, 28, 56, 112, 168 and 196 days. Kd values are shown for the three test soils spiked with $ZnCl_2$ or ZnO NP. Letters in pore water extraction show significant difference among times (Tukey p<0.05).

Table 3. Effect of ageing on the toxicity of ZnO NPs and $ZnCl_2$ to the earthworm *Eisenia andrei* exposed to three different natural soils. Shown are average survival (%; n=40) and weight loss (W_L; %) (n=4) after 4 weeks, and reduction of the number of juveniles produced per earthworm after 8 weeks in % compared to the untreated control. Earthworm assays were performed after different periods of ageing of the spiked soils.

Soil	**Treatment**	**Time** (days)	**%Survival**	**% W_L**	**% Reproduction R.**
LUFA	$ZnCl_2$ (500 mg Zn kg^{-1} soil)	1	90	11 a	n.r.
		56	98	12 a	n.r.
		140	95	54 b	n.r.
	ZnO NP (500 mg Zn kg^{-1} soil)	1	98	4 a	31a
		56	100	8 a	89b
		140	93	31 b	100b
	ZnO NP (1000 mg Zn kg^{-1} soil)	1	98	4 a	90a
		56	78	10 a	96ab
		140	85	49 b	100b
NLGA	$ZnCl_2$ (500 mg Zn kg^{-1} soil)	1	100	15 a	98
		56	95	29 b	94
		140	95	30 b	82
	ZnO NP (500 mg Zn kg^{-1} soil)	1	98	26	41
		56	93	26	-5
		140	95	25	22
	ZnO NP (1000 mg Zn kg^{-1} soil)	1	95	18 a	74
		56	98	27 b	79
		140	100	21 ab	61
SPCA	$ZnCl_2$ (1250 mg Zn kg^{-1} soil)	1	98b	21 a	n.r.
		56	25a	32 b	n.r.
		140	83b	41 c	n.r.
	ZnO NP (1250 mg Zn kg^{-1} soil)	1	100b	14 a	45
		56	88a	18 ab	41
		140	100b	26 b	75
	ZnO NP (2500 mg Zn kg^{-1} soil)	1	100	9 a	83c
		56	95	16 b	1a
		140	100	27 c	45b

Lowercase letters represent significance difference between treatments (Tukey HSD test. $p < 0.05$); n.r. no reproduction.

Zn concentrations in earthworms (ZnE) differed within each soil (Table 4). Earthworms exposed to control treatments showed a range of ZnE from 105 to 143 µg Zn g^{-1} earthworm, with an increase in concentration among soils following this trend: SPCA>NLGA>LUFA. For each of the studied soils there were significance differences in ZnE with treatments. Treatments with Zn added differed from the controls (both for ZnCl$_2$ and ZnO NP), however SPCA soil spiked with ZnCl$_2$ showed similar ZnE concentrations that control soils. In general, ZnE in soils spiked with ZnO NP showed a trend to increase with Zn soil concentration added. The highest internal concentrations appeared in the treatments of 1000 mg Zn kg^{-1} soil in LUFA and NLGA (284 and 387 µg Zn g^{-1} earthworm respectively); and SPCA soil spiked with 2500 mg kg^{-1} Zn had 408 µg Zn g^{-1} earthworm, which was the highest earthworm concentration registered. In LUFA aged for 140 days, earthworm concentrations were decreased for all Zn treatments compared to the first sampling. The earthworms exposed to NLGA showed an increase in internal concentrations after 56 days of ageing and a decrease after 140 days which was significant for all treatments. Earthworms kept in soil SPCA did not show remarkable changes upon ageing (Table 4).

Table 4. Average Zn concentrations in earthworms *Eisenia andrei* with corresponding standard deviation(ZnE±SD; n=4) after 4 weeks exposure to ZnCl$_2$ or ZnO NPs in three different soils aged for different periods of time. Also given are bioaccumulation factors (BAF) calculated as Zn concentrations in the earthworms divided by measured total Zn concentrations in the soils.

	TREATMENT	**Nominal Zn mg** Zn kg^{-1} soil	**DAY**	**ZnE±SD µg g^{-1}**		**BAF**
LUFA	Control	0	1	108 ± 3.8	a	7.06
			56	105 ± 10.6	a	6.90
			140	117 ± 7.6	ab	7.66
	ZnCl$_2$	500	1	214 ± 33.3	de	0.44 b
			56	204 ± 24.0	cde	0.42 b
			140	161 ± 7.4	bc	0.33 b
	ZnO NP	500	1	188 ± 21.1	cde	0.38 b
			56	195 ± 32.0	cde	0.39 ab
			140	182 ± 26.1	cd	0.37 b
	ZnO NP	1000	1	229 ± 33.2	e	0.26 a
			56	284 ± 49.1	f	0.33 a
			140	190 ± 36.7	cde	0.22 a
NLGA	Control	0	1	125 ± 8.2	a	1.35
			56	118 ± 5.7	a	1.28
			140	128 ± 10.1	a	1.39

	$ZnCl_2$	500	1	213 ± 19.7	bc	0.43 b
			56	330 ± 59.0	ef	0.67 b
			140	208 ± 26.3	bc	0.42 b
	ZnO NP	500	1	203 ± 33.7	bc	0.42 b
			56	302 ± 91.4	de	0.62 ab
			140	189 ± 20.6	ab	0.39 b
	ZnO NP	1000	1	249 ± 44.4	bcd	0.29 a
			56	387 ± 64.9	f	0.45 a
			140	268 ± 46.3	cde	0.31 a
SPCA	Control	0	1	143 ± 8.1	a	0.93
			56	139 ± 6.8	a	0.90
			140	132 ± 9.9	a	0.86
	$ZnCl_2$	1250	1	192 ± 37.2	ab	0.15 b
			56	175 ± 14.5	ab	0.14 a
			140	168 ± 21.7	ab	0.13 a
	ZnO NP	1250	1	286 ± 38.4	bc	0.22 c
			56	239 ± 38.4	ab	0.19 b
			140	243 ± 50.0	ab	0.19 b
	ZnO NP	2500	1	292 ± 54.8	bc	0.12 a
			56	408 ± 158.4	c	0.15 ab
			140	336 ± 88.9	c	0.13 a

Lowercase letters in ZnE indicate significant differences into each soil sample. Lowercase letters in BAF represent significant differences between treatment for each sampling day (both with Tukey HSD test. $p < 0.05$).

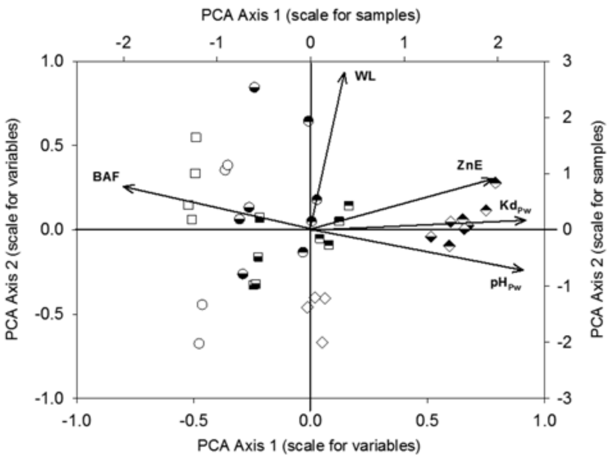
Bioconcentration factors (BAF) calculated for the controls with the zinc background differed among samples with the following pattern: LUFA>NLGA>SPCA. In the treatments with Zn also the SPCA soil showed the lowest BAF but NLGA and LUFA has similar BAF values or they were higher in NLGA. Soil SPCA had the highest BAF in the lowest treatment of Zn added as ZnO NPs (1250 mg Zn kg^{-1} soil), while under $ZnCl_2$ treatments BAF values were similar to the treatment of 2500 mg Zn kg^{-1} soil added as nanoparticles. BAF decreased with ageing in this soil except for the higher treatment with ZnO NP. In LUFA and NLGA, BAF values were higher in the treatment with $ZnCl_2$ and decreased with ageing. Under ZnO NP treatments BAF values showed a dose-related decrease with the increase in soil Zn concentrations.

2.3.3. Influence of soil properties and ageing on Zn bioavailability

Soil pH measured in pore water varied among treatments and incubation times (Table S1). Soil pH was higher in soils spiked with ZnO NPs than in the control soils, while in soils spiked with ionic Zn the pH decreased or remained similar compared to the control. With ageing, in general, soil pH decreased for all treatments; this decrease was most pronounced in LUFA and NLGA soils spiked with ZnO NPs.

In order to study the influence of soil properties, principal component analysis (PCA) with Zn concentration in earthworms (ZnE), bioaccumulation factor (BAF), weight loss (W_L), partition coefficient calculated from pore water (Kd_{PW}) and pH of the pore water for the three studied soils and the different treatments ($ZnCl_2$ and ZnO NPs) as well as for two different ageing periods (day 1 and day 168) were performed. Because reproduction in $ZnCl_2$ treatments was very low in the soil NLGA and null in the other two soils (Tables 3), this earthworm variable was not taken into account in the PCA analysis. On day 1, the main gradient (X-axis) grouped on the positive side the alkaline Spanish soil (SPCA) with the variables pH_{PW}, Kd_{PW} and ZnE (Figure 2a). Treatments of Zn applied as $ZnCl_2$ in this soil appeared more separate from the ZnO NP treatment and variables. NLGA spiked with ZnO NPs appeared in the centre of the gradient and shifted to the negative side of the X-axes when it was spiked with $ZnCl_2$. In this second case, the pH and Kd_{PW} were negatively related and bioaccumulation (BAF) was positively related. LUFA soil showed similar results as NLGA but with less clear differences between treatments. In the secondary gradient (Y-axis), the variable weight loss (W_L) appeared negatively correlated with SPCA soil spiked with $ZnCl_2$ (negative side). The remaining soils were not well segregated in this gradient. The results obtained 168 days after spiking the soils (Figure 2b) showed similarity in the aggregation of variables after 1 day, but soils were better segregated with a higher percentage of the variance explained (85.3%).

a) Time 1 day



b) Time 168 days

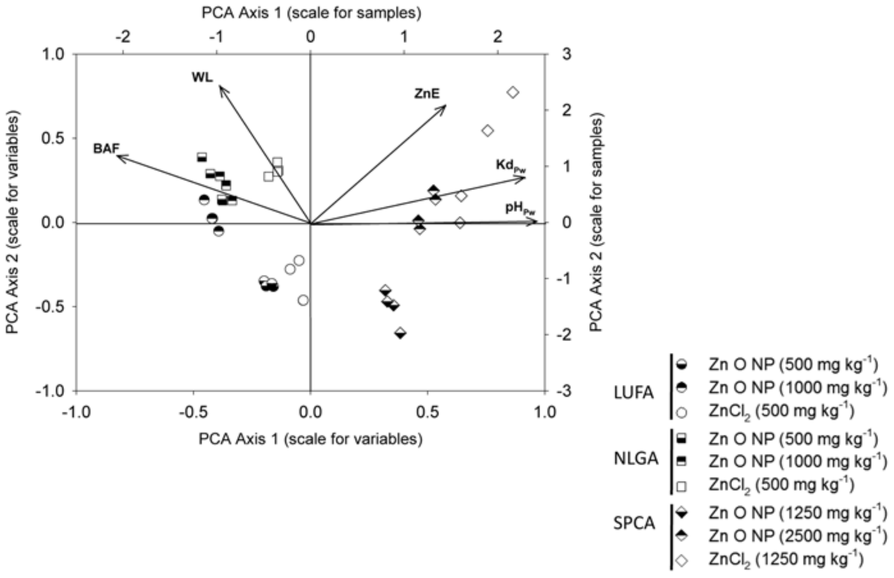


Figure 2. PCAs for the three studied soils with the different treatments (ZnO NP and $ZnCl_2$) and partitioning coefficient calculated in the pore water extractions (Kd_{PW}), pH of the pore water (pH_{PW}), bioaccumulation factor (BAF), Zn concentration in earthworm (ZnE) and earthworm weight loss (W_L) for two incubation periods (day 1 and day 168). Figure 2a) time 1 day, variance explained by the two first components 69.3 % (X-axes: 45.7 %; Y-axes: 23.6 %). Figure 2b) time 168 days, variance explained by the two first components 85.3 % (X-axes: 58 %; Y-axes: 27.3 %).

2.4. Discussion

2.4.1. Zinc solubility and availability

Our results showed that at the same concentration of Zn added, soils spiked with ZnO NPs had lower pore water Zn concentrations than those spiked with $ZnCl_2$. This was also found by Waalewijn-Kool et al. (2013) and indicates that ZnO NPs have a different behaviour compared to ionic Zn. The lower soluble concentration obtained for the ZnO NPs compared with $ZnCl_2$ could suggest that a considerable proportion of the ZnO NPs remained in the particulate form, probably as agglomerates (Hooper et al., 2011). On the other hand, soil pH was affected by the addition of Zn, with an increase in pH in soils spiked with the ZnO NPs and a decrease in pH in soils treated with ionic Zn. Difference in solubility can also be related to soil properties, such as pH. Franklin et al. (2007) found that dissolved Zn concentrations in pore water were higher in soils with lower pH, which matches with the results obtained in our study. Low Zn availability at higher pH has been explained from stronger sorption to the solid phase in basic soils (McLaughlin and Smolders, 2001) as was observed in the carbonate-rich SPCA soil. Moreover in the SPCA soil less Zn was available in the samples spiked with ZnO NPs. It has been demonstrated that water solubility of ZnO is highly pH-dependent (Franklin et al., 2007). In addition was also reported that soils with low pH and low organic matter content have higher availability of Zn (Ma and Bonten, 2011), which is in agreement with our results, where LUFA soil (with the lowest OC content and low pH) showed the highest extractable Zn for both ZnO NP and $ZnCl_2$. NLGA soil presented a similar pH to LUFA, however, higher OC content, and it showed lower pore water Zn compared to LUFA for $ZnCl_2$ treatment but similar pore water Zn in the treatments of ZnO NP. It is known that natural organic matter can modify the surface charge of nanoparticles, affecting their aggregation (Franklin et al., 2007), and Li et al. (2011a) found that Zn ions could have a high affinity for binding to or complexation with dissolved organic carbon. Waalewijn-Kool et al. (2013), in a study with ZnO NPs, found the highest pore water Zn concentrations in the most organic soil (with 15% of organic matter), although under acidic pH ($pH(CaCl_2)=5$). In our study, SPCA soil had the higher OC content but alkaline pH, so it seems that pH could be the factor which mainly controls Zn availability in soils spiked with ZnO NPs, while organic matter content seems to be of less importance in treatments with this Zn form. Partition coefficients (Kd) can be used to express the adsorption of dissolved Zn by soil (USEPA, 1999). Our results showed a decrease of Kd values for the three tested soils in the order: SPCA>NLGA>LUFA, regardless of the applied Zn form. This indicates the highest adsorption and therefore lowest availability of Zn in SPCA soil and the highest Zn availability in LUFA soil. The Kd values diminished with the decrease of the pH in the studied soils, which agrees with literature data (Heemsbergen et al., 2010).

Ageing showed that Zn availability increased in LUFA and NLGA for all studied treatments, while in SPCA soil no significant differences were found when spiked with ZnO NPs and a decrease when spiked with $ZnCl_2$. Our results for days 1 to 3, which is relatively short-term, showed a greater decrease in Zn availability in the LUFA soil (with the lowest CEC), although it increased again after 6 months of incubation. Lock and Janssen (2003) also observed a faster rate of adsorption in soils with low cation exchange capacity. Therefore ageing or equilibration of contaminated soil might provide a more realistic insight into ZnO NP behaviour and therefore its potential toxicity under natural conditions. The equilibrium processes of metals between pore water and soil solid phases are rather complex and nanoparticle solubility could be continuously changing with time (Franklin et al., 2007), so the influence of soil components on Zn solubility cannot be ignored (Waalewijn-Kool et al., 2013).

$Cu(NO_3)_2$ extraction was applied to test whether it could selectively extract the labile Zn from the soils in the presence of ZnO NPs. In the $ZnCl_2$ spiked soils, the extracted Zn followed long-term trends characteristic of the ageing of the Zn added to chemically unreactive forms (Crout et al., 2006). In the ZnO NP spiked soils, % Zn recovered would have been expected to be initially small, if the extraction were selectively removing the chemically reactive Zn while not dissolving the nanoparticles. However, the % recoveries of Zn from the soils spiked with ZnO NP on days 1 and 3 (Table 2) strongly suggest that the extraction dissolves a significant proportion of the ZnO NPs and therefore this extraction method was not suitable for estimating the labile Zn in ZnO NP-spiked soils. Due to the non-suitability of the method previously mentioned, no further explanations are done referring to these extractions.

2.4.2. Zinc toxicity and Zn bioaccumulation by earthworms

Zn concentrations selected for this study were based on effective concentrations (EC50) for effects on earthworm reproduction, therefore no effects on survival were expected. This indeed was the case for almost all soils and treatments (>78% of survival) except for one soil at only one sampling time (Table 3). Nevertheless, survival was high enough to confirm the soils were providing optimal conditions for the earthworms (OECD, 2010).

For the comparison of the earthworm weight loss (W_L) in different soils at different ageing times the % reduction of weight compared to the corresponding control was used. Body weight showed a decrease in all soils and varied between treatments and soil samples, which suggests that obtained differences can be related to soil type. According to Janssen et al. (1997), earthworm weight loss could be caused primarily by soil factors. For the controls, there were significant differences in weight loss for the LUFA soil compared to the other two studied soils (data not shown). Hooper et al. (2011) observed that the earthworm *Eisenia veneta* exposed to 750 mg Zn kg^{-1} soil as ZnO NPs showed greater weight loss compared to treatments with ionic zinc, which disagrees with our results where greater or the same weight loss was observed in the $ZnCl_2$ treatments compared to the ZnO NPs in all studied soils. Heggelund et al. (2014) found a dose-related increase in weight loss of the earthworm *Eisenia fetida* in $ZnCl_2$ treated soils, but not in soils spiked with ZnO NPs at concentrations of 238 to 2500 mg Zn kg^{-1} d.w. soil. In our ZnO NP treatments, earthworms in LUFA and SPCA showed similar results at the two test concentrations while in NLGA there was a small weight decrease with increasing soil Zn concentration. In addition, ageing effects were observed in all soil samples, with an increase in weight loss with time in all studied treatments. The higher W_L was observed for the LUFA soil for both $ZnCl_2$ and ZnO NPs. The observed earthworm weight changes in our studied soils suggest that, upon exposure to ZnO NPs, weight loss is not dose-related and it is probably influenced by soil properties. It also suggests that there is an ageing effect with an increase in earthworm weigh loss with time, influenced also by soil properties.

Reproduction of *Eisenia andrei* is, in general, more sensitive and more ecologically relevant than the other endpoints (Heggelund et al., 2014; Romero-Freire et al., 2015). It is well known that earthworm reproduction is influenced by soil properties (van Gestel et al., 1993), and this indeed was observed from the difference in juvenile numbers in the controls. For that reason, in order to compare earthworm reproduction in different soils at different ageing times, we always used % reduction of reproduction compared to the corresponding control. Earthworm reproduction was more affected by $ZnCl_2$ than by ZnO NPs, with almost total inhibition of reproduction in soils spiked with ionic Zn. A dose-related effect on reproduction was seen for the ZnO NP treatments in our studied soils. It is remarkable that in SPCA soil, spiked with higher Zn concentrations, reproduction was similar to that in the other two soils spiked at lower total Zn concentrations. This could be explained from the low available Zn concentration in the pore water of this soil (Table 2). It however, has to be noted that also in SPCA earthworm reproduction was not always dose-related inhibited, with in some cases stronger inhibition at lower available Zn levels (e.g. after 56 and 140 days).

Results of ecotoxicity tests on Zn with freshly spiked soils could differ from those with field-contaminated soils (Smolders et al., 2009). In our study, an increase of earthworm weight loss was seen in all studied soils and treatments with ageing, while only a decrease in earthworm reproduction with time was observed in the case of LUFA treated with ZnO NPs. In a study with enchytraeids, no effect of ageing on Zn toxicity was detected, which was explained by the high adsorption capacity of soil components (clay content and organic matter content) (Lock and Janssen, 2002). The changes in reproduction toxicity of Zn with ageing in our study could mainly be attributed to the higher Zn availability in LUFA soil that showed an increase with ageing (Table 2). Therefore, further studies on ZnO NP toxicity with ageing are needed using different soil types and exposure levels.

Earthworms are able to sequester and retain as well as autoregulate Zn internally for essential functions, therefore their internal concentration can remain constant regardless of the total and available Zn levels in soil (van Gestel et al., 1993). McGeer et al. (2003) found in polluted soil an internal mean value of 216±121 µg Zn g^{-1} earthworm for *Eisenia fetida*, which is within our mean values for spiked soils (from 161 to 408 µg Zn g^{-1} earthworm). We found somewhat higher earthworm Zn body concentrations in the NLGA and SPCA soil spiked with the highest treatment of ZnO NP (387±65 and 408±158 µg Zn g^{-1} earthworm, respectively). The observed internal earthworm concentrations in our studied soils spiked with different concentrations and forms of Zn highlight the need for further studies on the influence of soil type on Zn bioavailability as well as their potential role in the capability of earthworms to regulate their Zn body concentrations (Ma and Bonten, 2011). In order to enable comparison among soils, treated with different Zn concentrations and different Zn forms, bioaccumulation factors (BAF) were calculated. BAF often does not give enough information because of metal auto-regulation mechanisms in earthworms, however it is a good factor to compare among soils, taking into account the differences in the applied concentrations. In our study BAFs, based on total Zn concentrations in soil, were lowest for SPCA compared to the other studied soils, for both Zn forms. In general, BAFs showed a dose-dependent pattern (opposite to Kd_{PW} in the PCAs, Figure 2) with the lowest values for the highest Zn concentrations. This behaviour had been observed before for other essential elements, such as molybdenum, which internal concentrations may be regulated to fairly constant levels by earthworms (Díez-Ortiz et al., 2010).

BAF was also oppositely related with pH_{PW} which suggests that, along with the available Zn, the pH could have an important role in earthworm bioaccumulation. This agrees with Spurgeon et al. (2006), who indicated that Zn uptake in earthworms can be dependent on pH, so Zn uptake by earthworm is poorly predicted by available Zn due to the pH effect (Ma and Bonten, 2011). However, no such clear trend was found for the SPCA soil where lowest BAFs were found in the $ZnCl_2$ treatment. The BAF or internal Zn concentrations in the earthworms did not explain the endpoint weight loss (W_L) (Figure 2), with the earthworms having the lowest internal zinc concentration having the highest weight loss. The lowest weight loss was found in SPCA soil, which suggests again an effect of soils properties rather than of ZnE. These results suggest that in this soil earthworms may be capable of sequestering zinc, leading to higher body concentrations than expected when Zn was applied as ZnO NPs. Additional studies are needed to unravel the complex mechanism of zinc bioaccumulation in earthworms related with nano-particulate Zn and soil properties.

2.5. Conclusion

In this study we compared the effect of Zn applied as nanoparticles (ZnO NPs) and as Zn^{2+} ions ($ZnCl_2$) on zinc availability and bioavailabilty to the earthworm *Eisenia andrei* at different incubation times after spiking to three natural soils with contrasting properties. Zn concentrations in pore water were lower in soils spiked with ZnO NP in comparison to soils treated with $ZnCl_2$. Zn availability was lowest Zn in the soil with alkaline pH and high organic carbon content. For treatments with ZnO NP only the soil pH explained the differences in Zn availability, while organic carbon content explained Zn availability in soils spiked with $ZnCl_2$. The effect of ageing on the availability of Zn showed differences without regular trends among soils as well between treatments ($ZnCl_2$ and ZnO NPs). Earthworms showed varying internal zinc concentrations among soils, which were highest in the soil with the highest OC content and basic pH, following exposure to Zn applied as ZnO NPs, even though this was the soil which showed the lowest pore water Zn concentrations. Reproduction toxicity of Zn to the earthworms was highest for $ZnCl_2$ treatments, with almost complete reproduction inhibition, but there were no clear differences in survival and weight loss between treatments. An effect of ageing on Zn toxicity to the earthworms was only observed for weight loss, which increased with time. No differences were seen for the other variables, so no significant effects of ageing were detected that could explain differences in earthworm toxicity.

More research is necessary to understand ZnO NP interactions with different soil constituents and how soil properties control Zn availability. It is also essential to deepen the knowledge on the importance of long-term processes for Zn availability for a proper risk assessment of ZnO NPs as well as Zn-polluted soils.

Supplementary data

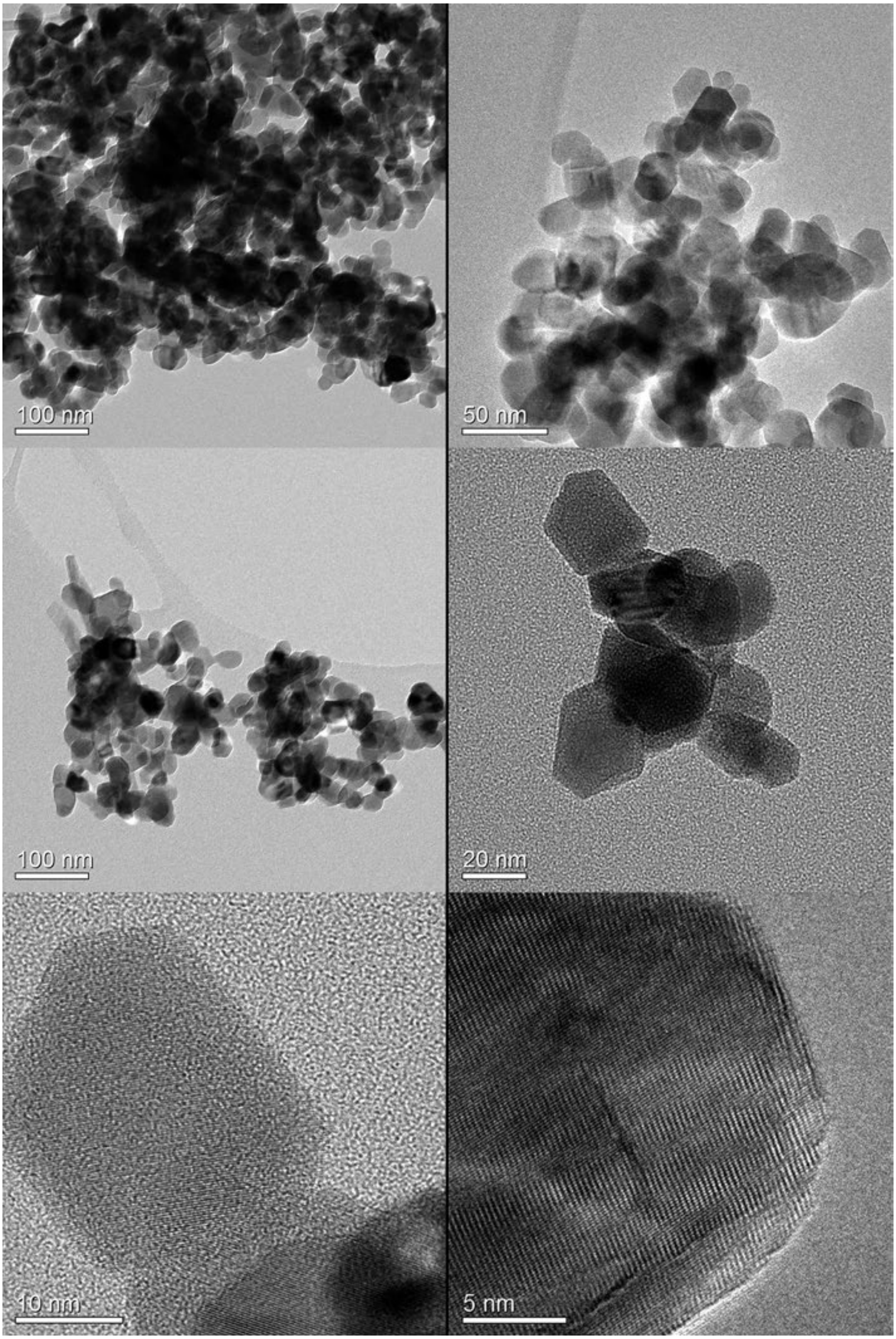


Figure SI-2.1. Transmission Electron Microscopy (TEM) of Nanosun Zinc Oxide P99/30, used in this study to assess effect of soil properties and ageing on Zn bioavailability and toxicity to earthworms.

Table SI-2.1. Average pH$_{PW}$ measured in the soil pore water (n=2) of three soils spiked with ZnCl$_2$ or ZnO NPs and incubated for different times of ageing.

		TREATMENT			
SOIL	**TIME**	**CONTROL**	**ZnCl$_2$**	**ZnO NP**	**ZnO NP**
		0 mg Zn kg^{-1}	500 mg Zn kg^{-1}	500 mg Zn kg^{-1}	1000 mg Zn kg^{-1}
LUFA	1	6.7	6.7	7.5 a	7.5 ab
	3	6.4	6.6	7.5 a	7.6 ab
	56	6.5	6.2	6.8 ab	6.8 ab
	168	6.2	6.1	6.0 b	6.4 b
		0 mg Zn kg^{-1}	500 mg Zn kg^{-1}	500 mg Zn kg^{-1}	1000 mg Zn kg^{-1}
NLGA	1	6.9 a	6.0 a	7.3 a	7.4 a
	3	6.0 b	6.1 a	6.9 ab	7.3 a
	56	5.9 bca	5.5 b	6.4 ab	6.5 b
	168	5.5 c	5.2 c	5.6 b	5.8 c
		0 mg Zn kg^{-1}	1250 mg Zn kg^{-1}	1250 mg Zn kg^{-1}	2500 mg Zn kg^{-1}
SPCA	1	8.0 a	7.6 b	8.2	8.1 ab
	3	7.9 a	7.6 b	8.1	8.1 ab
	56	7.9 a	7.2 a	7.9	7.9 bc
	168	7.6 b	7.4 ab	7.7	7.7 c

Letters show significance difference among times (Tukey p<0.05).

Table SI-2.2. Average 0.1 M $CuNO_3$ extractable Zn concentrations (±SD, n=4) expressed as mg Zn L^{-1} measured in three different soils spiked with ZnO NPs and $ZnCl_2$ and incubated for different periods of ageing.

	mg Zn L^{-1}		
TIME	**LUFA**		
(days)	ZnO NP 500 mg Zn kg^{-1} soil	ZnO NP 1000 mg Zn kg^{-1} soil	$ZnCl_2$ 500 mg Zn kg^{-1} soil
1	19.1 ± 1.5 cde	40.6 ± 0.3 ab	23.3 ± 2.0 bcd
3	20.7 ± 4.4 bcde	33.4 ± 4.3 c	21.7 ± 1.2 cd
7	16.9 ± 0.5 e	33.4 ± 2.2 c	22.2 ± 0.2 bcd
10	17.6 ± 1.0 de	34.2 ± 1.2 c	21.2 ± 0.5 d
14	19.6 ± 0. cde	34.7 ± 0.8 c	23.9 ± 0.8 bcd
28	22.5 ± 0.9 abc	38.2 ± 4.6 abc	25.7 ± 0.7 abc
56	25.2 ± 1.2 abc	40.5 ± 1.7 ab	26.3 ± 1.2 abc
112	21.3 ± 0.8 abcd	35.4 ± 1.7 bc	26.5 ± 1.9 abc
168	21.3 ± 0.5 abcd	36.8 ± 1.0 abc	24.1 ± 1.7 abcd
196	24.8 ± 1.6 ab	41.5 ± 1.9 a	28.5 ± 4.2 a

	mg Zn L^{-1}		
TIME	**NLGA**		
(days)	ZnO NP 500 mg Zn kg^{-1} soil	ZnO NP 1000 mg Zn kg^{-1}soil	$ZnCl_2$ 500 mg Zn kg_{-1} soil
1	30.5 ± 1.0 ab	55.0 ± 1.2 abc	31.6 ± 1.3 a
3	31.1 ± 0.9 ab	54.4 ± 1.3 abcd	30.9 ± 1.0 ab
7	32.2 ± 0.9 a	53.7 ± 1.5 bcd	33.6 ± 2.1 a
10	32.1 ± 2.0 ab	55.6 ± 1.6 ab	30.8 ± 1.8 ab
14	30.2 ± 1.8 ab	51.5 ± 1.4 cde	29.7 ± 1.0 ab
28	29.7 ± 2.3 abc	53.1 ± 2.4 bcd	29.3 ± 4.5 ab
56	29.2 ± 1.5 abc	50.9 ± 1.3 de	29.2 ± 1.0 ab
112	32.7 ± 2.2 a	57.4 ± 1.3 a	32.6 ± 1.8 a
168	28.4 ± 1.2 bc	52.9 ± 1.2 bcd	29.2 ± 0.9 ab
196	26.3 ± 0.8 c	48.1 ± 0.9 e	26.6 ± 0.4 b

	mg Zn L^{-1}		
TIME	**SPCA**		
(days)	ZnO NP 1250 mg Zn kg^{-1} soil	ZnO NP 2500 mg Zn kg^{-1} soil	$ZnCl_2$ 1250 mg Zn kg^{-1} soil
1	51.4 ± 3.4 a	117.7 ± 4.1 a	56.2 ± 6.6 a
3	47.2 ± 3.3 ab	92.4 ± 4.3 abcd	50.6 ± 2.8 ab
7	44.1 ± 4.0 bc	94.0 ± 6.9 abcd	55.7 ± 15.2 a
10	42.6 ± 1.3 bc	79.6 ± 17.7 bcde	47.1 ± 4.7 ab
14	43.1 ± 1.6 bc	107.1 ± 5.0 abcd	43.0 ± 2.0 ab
28	40.4 ± 3.1 bcd	83.7 ± 18.4 bcde	45.3 ± 5.2 ab
56	39.1 ± 4.0 cd	97.5 ± 11.7 abc	42.5 ± 0.9 ab
112	35.3 ± 3.4 d	60.7 ± 0.7 e	43.2 ± 4.8 ab
168	38.6 ± 0.6 cd	77.8 ± 20.5 cde	40.9 ± 1.8 b
196	38.1 ± 2.2 cd	66.3 ± 1.3 de	38.5 ± 0.8 b

Letters show significance difference among times (Tukey $p<0.05$).

CHAPTER 3

Toxicity of arsenic in relation to soil properties: implications to regulatory purposes

Abstract

The present work evaluates the influence of different soil properties and constituents on As solubility in laboratory-contaminated soils, with the aim of assessing the toxicity of this element from the use of bioassays to evaluate the soil leachate toxicity and thereby propose soil guideline values for studies of environmental-risk assessment in soil contamination. Seven soils with contrasting properties were artificially contaminated in laboratory with increasing concentrations of As. Samples were incubated for 4 weeks and afterwards, soil solution (1:1) was obtained after shaking for 24 h. The soil leachate toxicity was assessed with two commonly used bioassays (seed germination test with *Lactuca sativa* and Microtox ® test with *Vibrio fischeri*). The relation between soluble As and soil properties indicated that iron oxides and organic-matter content were the variables most closely related to the reduction of the As solubility, while pH and $CaCO_3$ increased As solubility in the soil solutions. Toxicity bioassays showed significant differences between soils depending on their properties, with a reduction of the toxicity in the iron-rich soil (NOEC = 150 mg kg^{-1}) and a significant increase in the highly carbonate samples (NOEC between 15 – 25 mg kg^{-1}). Soil guideline values for regulatory purposes usually set a single value for large areas (regions or countries) which can produce over- or under-estimation of efforts in soil remediation actions. These values should consider different levels according to the main soil properties controlling arsenic mobility and the soil leachate toxicity.

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3.1. Introduction

Arsenic (As) is an ubiquitous element in the environment which appears in concentrations that vary widely from one area to another. Its presence is associated with both natural and anthropogenic factors, and the high toxicity often threatens human health and even global ecosystems (Nriagu et al., 2007). Arsenic has properties intermediate between metals and non-metals, although its electronegativity and ionization energy are more characteristic of non-metals. Under natural conditions, this element has a complexation chemistry rather similar to phosphorus (CCME 2001), and tends to appear in anionic forms (Moreno-Jiménez et al., 2009).

The mean value of As in the earth's crust is 1.8 mg kg^{-1}, while concentrations in natural soils are generally below 10 mg kg^{-1} (Adriano, 2001; Mench et al., 2009). In soils, As usually accumulates due to its low mobility in this medium (Mench et al., 2009; Beesley et al., 2010), and it is often found as inorganic species because organic compounds have lower solubility (Nriagu et al., 2007; Martínez-Sánchez et al., 2011). In the normal range of pH-Eh soil conditions, arsenates are more common than arsenites (Bowell, 1994), the presence of these species being essential to control toxicity in soil, sediment, or water (Dobran and Zagury, 2006). The As concentration in soil varies widely because it depends on the initial concentration (background) in parent material, natural geochemical cycles, and soil type (Díez et al., 2007), therefore, the mean values of As in natural soils, according to the literature range between 0.1 and 80 mg kg^{-1} worldwide (Alloway, 1995; Adriano, 2001; Bohn et al., 2001; Kabata-Pendias and Mukherjee, 2007).

Arsenic can be taken up by plants together with other micronutrients, but so far no conclusive results are available concerning the possible biological role of As in living organisms, although concentrations between 1 and 1.7 mg kg^{-1} have been reported as normal values in plants (Kabata-Pendias, 2011). Mobility, bioavailability and therefore the potential toxicity or deficiency of most elements for plants and microorganisms in terrestrial system are controlled largely by soil properties (Sheppard and Evenden, 1988). The main soil properties affecting As availability are: Fe oxides, pH, calcium carbonate, clay content, cation-exchange capacity and organic matter content (Lock and Janssen, 2001; Smolders et al., 2003; Smolders et al., 2004; Rooney et al., 2006; Song et al., 2006).

In recent decades, new methods have been developed to determine the impact of As on human health and the risk of accumulation of As in agricultural crops (Bhattacharya et al., 2007). Biological assays for toxicity determination show direct responses of organisms experimentally exposed to potentially polluting elements, and thus are indicative of the potential impact of pollutants on living organisms (Petänen et al., 2003). In the European Union, environmental-risk assessment (ERA) is conducted primarily to assess the effects on human health and on the terrestrial, aquatic and atmospheric resources (Song et al., 2006). The use of bioassays is essential for ERA, and is applied to determine the potential risk of contamination to living organisms and ecosystems. In soils, these bioassays can be divided into two groups: those using the liquid phase (soil-solution extracts, pore water, leachates, etc.), and those using the solid phase of the soil that serves as a substrate for living organisms (Farré and Barceló, 2003; Martín et al., 2010). Lors et al. (2011) comparing bioassays in the solid phase as well as in soil extracts, found a high correlation between the two phases, indicating that soil extracts usually reflect the toxicity of the solid phases. However, the correlation between the total and soluble forms of a pollutant is not always direct, and in the case of As, its solubility and toxicity are usually more tightly controlled by the soil properties than by the total amounts present in the sample (Martín et al., 2011; Martín Peinado et al., 2012). In this sense, bioassays using the liquid phase extract can more readily reflect the behaviour of mobile phases, evaluating the short-term risk of dispersion, solubilization, and bioavailability of pollutants in the environment.

Laboratory studies, using artificially contaminated soil, may differ in the composition of the soil solution against *in situ* contaminated soil. However, laboratory experiments trend to overestimate the solubility and availability of metals in relation to field contaminated soils (Smolders et al., 2009), so that the toxicity level defined by these studies usually overrates the effects, which can raise the safety threshold for the environmental-risk assessment.

The setting of reference levels in regulations of many countries is often controversial, because a single value for large areas (regions or countries) is often set, regardless of spatial variations in soil types, and the extent of contamination or the associated environmental risk in specific areas may be misjudged (Díez et al., 2009). For example, the cleanup guidelines for arsenic in US soils for unrestricted use range from 7 to 40 mg kg^{-1} depending on the State (Teaf et al., 2010); Dutch standards fixed the target value in 29 mg kg^{-1} and the intervention value in 55 mg kg^{-1} (RIVM, 2000); meanwhile Soil Guideline values in UK for residential/allotments is 20 mg kg^{-1} and for commercial/industrial use is 500 mg kg^{-1} (DEFRA 2002). In Spain, some regions have different reference levels for different land-use scenarios (Macías and Calvo de Anta, 2009). Specifically, in Andalusia (southern Spain), a preliminary approximation for establishing these levels fixed the total As thresholds for agricultural soils, natural parks, and industrial areas at 50, 100, and 300 mg kg^{-1}, respectively (Aguilar et al., 1999). Anyway, most guideline values are based on natural background concentrations or in artificial standard soils, preventing about variations according soil type but lacking about additional data.

The present work evaluates the influence of different soil properties and constituents on As solubility in laboratory-contaminated soils, with the aim of assessing the toxicity of this element from the use of bioassays to evaluate the soil leachate toxicity and thereby propose soil guideline values for studies of environmental-risk assessment in soil contamination.

3.2. Materials and methods

3.2.1. Soil samples

Seven soil horizons with different properties, representing most of the main soil groups in Spain, were selected (Table 1). The main parameters analysed were: pH (soil:water ratio 1:2.5), electrical conductivity (EC), texture, organic carbon (OC), water-holding capacity, cation-exchange capacity (CEC), specific surface area (SA), and calcium carbonate content ($CaCO_3$). The main physico-chemical properties were analysed according to the official analysis methods (MAPA, 1994). Moreover, free and amorphous iron, aluminium, and manganese oxides were analysed according to different procedures (Holmgren, 1967; Schwertmann and Taylor ,1977). All analyses were made in triplicate.

The total arsenic concentration (AsT) was determined from acid digestion in strong acids (HNO3 + HF) and water soluble As (AsW) was determined from soil:water extracts (1:1 ratio). In all cases, As was measured by ICP-MS (Inductively Coupled Plasma-Mass Spectrometry) in a spectrometer ICP-MS NEXION 300D. Instrumental drift was monitored by regularly running standard element solutions between samples. For calibration, two sets of standards containing the analyte of interest at five concentrations were prepared using rhodium as an internal standard. Procedural blanks for estimating the detection limits ($3*\sigma$; n = 6) were <0.21 ppb for As. The analytical precision was better than ±5% in all cases.

Table 1. Main properties of selected samples (mean, *standard deviation*).

	SAMPLES													
Soil Type (IUSS, 2006)	Calcaric Cambisol (humic)		Calcaric Cambisol (humic)		Calcaric Kastanozem (antric)		Leptic Cambisol (eutric)		Leptic Regosol (eutric)		Leptic Regosol (distric)		Cutanic Luvisol (chromic)	
Soil horizon	Ah		Bw		Ck		Bw		Ah		C1		Bt	
Name of sample	**H1**		**H2**		**H3**		**H4**		**H5**		**H6**		**H7**	
pH (S)	7.96	*0.06*	8.67	*0.03*	8.79	*0.02*	6.74	*0.19*	7.20	*0.09*	5.87	*0.09*	7.03	*0.44*
EC (dS m^{-1})	0.08	*0.04*	0.07	*0.01*	0.07	*0.01*	0.04	*0.01*	0.09	*0.01*	0.05	*0.01*	0.05	*0.01*
I (mol l^{-1})[a]	7.3	*0.6*	2.0	*0.1*	1.7	*0.1*	0.9	*0.1*	13.0	*3.1*	0.5	*0.1*	1.6	*0.3*
$CaCO_3$ (%)	37.11	*0.44*	72.39	*0.86*	92.32	*1.80*	nd	-	nd	-	nd	-	0.92	*0.16*
OC (%)	5.43	*0.38*	0.42	*0.12*	0.38	*0.17*	0.61	*0.10*	8.22	*0.02*	0.49	*0.02*	0.66	*0.25*
Clay (%)	23.61	*0.90*	11.79	*0.44*	7.70	*0.58*	19.05	*0.38*	23.79	*0.12*	8.31	*0.12*	54.76	*1.13*
CEC ($cmol_{+}$ kg^{-1})	21.43	*2.00*	9.83	*1.00*	2.94	*0.13*	9.91	*0.42*	25.90	*0.37*	3.83	*0.37*	15.53	*1.01*
SA (m^2 g^{-1})	0.047	*0.002*	0.008	*0.001*	0.003	*0.001*	0.015	*0.001*	0.057	*0.002*	0.004	*0.001*	0.083	*0.003*
As T (mg kg^{-1})	15.51	*0.34*	9.07	*1.22*	3.39	*0.15*	16.23	*1.23*	12.27	*0.10*	4.39	*0.10*	25.66	*1.89*
As W (mg kg^{-1})	0.021	*0.001*	0.007	*0.001*	0.010	*0.001*	0.077	*0.015*	0.014	*0.001*	0.010	*0.001*	0.010	*0.001*
Fed (g kg^{-1})	18.97	*0.81*	8.67	*0.71*	3.29	*0.02*	17.90	*1.15*	19.41	*1.21*	7.77	*0.01*	82.58	*7.16*
Mnd (g kg^{-1})	0.54	*0.02*	0.13	*0.01*	0.01	*0.01*	0.32	*0.03*	0.85	*0.01*	0.15	*0.01*	0.13	*0.01*
Ald (g kg^{-1})	2.40	*0.02*	1.10	*0.01*	0.60	*0.02*	1.90	*0.09*	1.90	*0.07*	0.90	*0.10*	5.10	*0.16*
Feo (g kg^{-1})	0.68	*0.03*	0.20	*0.02*	0.01	*0.01*	0.52	*0.09*	0.65	*0.14*	1.00	*0.06*	0.78	*0.07*
Mno (g kg^{-1})	0.06	*0.01*	0.02	*0.01*	bdl	-	0.18	*0.02*	0.41	*0.07*	0.09	*0.01*	0.03	*0.01*
Alo (g kg^{-1})	1.17	*0.03*	0.31	*0.02*	0.15	*0.02*	0.38	*0.07*	0.50	*0.06*	0.27	*0.06*	0.73	*0.06*

nd: non-detected; bdl: below detection limit; EC: electrical conductivity; I: ionic strength; OC: organic carbon; CEC: cation exchange capacity; SA: specific area; AsT: total arsenic concentration; AsW: soluble in water arsenic concentration; $Fe_d/Mn_d/Al_d$: free oxides; $Fe_o/Mn_o/Al_o$: amorphous forms. [a] values x 10^3

3.2.2. Soil spiking

Soil samples were spiked in laboratory with increasing amounts of As from sodium arsenate solution (Na_2HAsO_4 $7H_2O$) with the following concentration range: 50-100-300-600-1200 mg As kg^{-1} soil. These levels were chosen according to the reference values (mg kg^{-1}) proposed for Andalusia (Aguilar et al., 1999), (50 = agricultural soils; 100 = natural areas; 300 = industrial zones, and multiplying the highest concentrations of industrial zones by 2 and 4, respectively). Furthermore, an uncontaminated sample (blank) was left, making a total of 6 treatments. The spiking was performed in triplicate on individual samples of 50 g of soil.

Once spiked, samples were incubated for 4 weeks at 25 ± 1 °C and 60% humidity, with a light cycle of 10 h. The incubation period chosen allowed the stabilization of the contaminant added, optimising the time spent on these tests, this protocol being based on similar studies by other authors (Fendorf et al., 2004; Tang et al., 2006).

After the incubation period, a saturation extract was prepared with a soil:water ratio of 1:1. A soil solution was made after shaking for 24 h, through the extraction system of 10 cm Rhizon MOM. In the resulting extracts, the pH, electrical conductivity, and water-soluble As concentrations by ICP-MS were measured. All assays were performed within 48 h of making extracts.

3.2.3. Bioassays

Two toxicity bioassays were selected for this experiment:

1. Seed germination/Root elongation Toxicity Test, according to OECD (2003) and U.S. EPA (1996) recommendations. This test assesses the phytotoxic effects on seed germination and seedling growth in the first days of growth (Torres, 2003). In Petri dishes, 15 seeds of *Lactuca sativa* and 5 ml of soluble extract from As-contaminated soils were placed in an incubator at 25 ± 1 °C, and the number of seeds germinated and the length of the germinated seed roots were measured after 120 h. Two endpoints were calculated: a) the percentage of germinated seeds (SG) in relation to the control; and b) the root-elongation reduction (LsR) in relation to the control (distilled water).

2. Microtox® test (ASTM 2004), is based on the reduction of the light emitted by a nonpathogenic strain of luminescent marine bacterium *Vibrio fischeri* upon exposure to a toxic sample (Ribó and Kaiser, 1987). The test was performed in a Microtox 500 analyser from Microbics Corporation, according to a modification of Microtox Basic Test for Aqueous Extracts Protocol (Azur Environmental, 1998; Martín et al., 2010). The

luminescence was measured before the mixture with the As-contaminated solutions (0 min). The inhibition of bioluminescence was measured at 15 min after the mixture with the different extracts coming from the As–contaminated soils. The results were expressed as the luminescence reduction in the sample in relation to the control (VfR). The values range from 0, for samples that do not exhibit toxicity, to 100 for samples with maximum toxicity.

3.2.4. Data analysis

After it was verified that the data distribution fit a normal distribution, significant effects were determined with an ANOVA and, for multiple comparisons, Tukey's test ($p < 0.05$) was applied. For the analysis of the influence of soil properties on As solubility and toxicity, Spearman's correlations were performed, whereas a factor analysis was made from a rotated-component analysis using the Varimax with Kaiser normalization method. All these analyses were performed at a confidence level of 95%. Statistical analyses were performed using SPSS v.15.0 (SPSS Inc., Chicago, USA).

The ECx (effective concentration causing a x% -50 and 10%- of reduction in the endpoints) and its 95% confidence interval were established by the fitting to a log-logistic model (Doelman and Haanstra, 1989), using the method of Marquardt (proc NLIN, SAS 9.1, SAS Institute, Cary, NC, USA). No observed effect concentration (NOEC) compared to the uncontaminated sample was calculated by analysis of variance and Tukey's test in multiple-range data from the log-logistic model (Romero-Freire, 2012).

3.3. Results

3.3.1. Arsenic solubility and influence of soil properties

The water extracts taken from the contaminated samples indicated significant differences in the solubility of As between the different treatments and soil samples, these differences being higher with the increase in the As concentration added to the soils (Fig. 1). In all treatments the sample with the lowest As concentration in the solution was H7 (red Mediterranean soil); meanwhile, the highest solubility was found in the highly carbonate samples (H2 and H3). In the highest concentration of spiked soils (1200 mg kg^{-1}) the As solubility also significantly differed among samples, with higher solubility in the slightly acidic and non-carbonate samples (H6 and H4) in relation to the organic horizons (H5 and H1).

The correlation analysis (Spearman) between the water-soluble As concentrations and the soil properties for each sample considered individually indicated that the soluble arsenic was significantly ($p < 0.01$) and positively related with two main variables: the total As added to the soil, and the ionic strength of the soil solution, with correlation coefficients greater than 0.972 and 0.822, respectively, in all cases.

For the different treatments considered individually, the solubility of As in all cases was significantly and inversely related to the free iron (Fed) forms, with an increasing correlation coefficient when the As concentration rose (Table 2). At lower concentrations of added As (≤ 300 mg kg^{-1}), other properties such as soil pH and $CaCO_3$ content significantly influenced the solubility of this element; while for the treatments with high concentrations of As (≥ 600 mg kg^{-1}), an inverse relation with organic carbon (OC) was found.

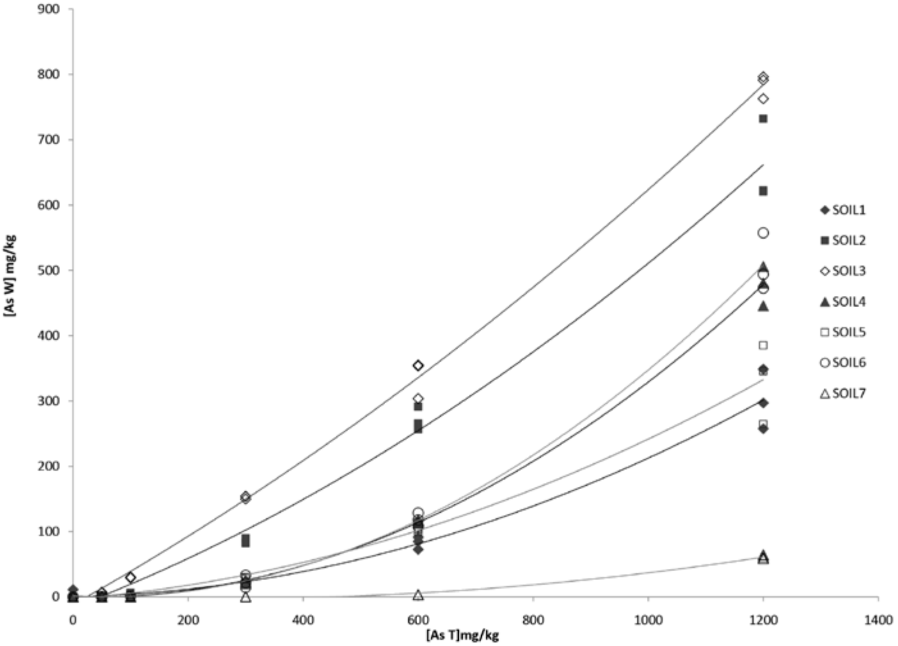

Figure 1. Concentration of soluble arsenic (As W) against total (As T) for the 7 samples studied in all treatments for the three replicates. Mean quadratic fits of trend lines ($R^2 > 0.980$ in all cases).

Table 2. Correlation coefficients (Spearman) between water-soluble As (As W) and some soil properties. Treatment: 50, 100, 300, 600, 1200 mg $kg^{-1}$of total As added to the soils; OC: organic carbon; $CaCO_3$: calcium carbonate content; Fed: free iron oxides; pH S: soil pH. (* p <0.05, ** p <0.01)

Treatment	**OC**	**$CaCO_3$**	**Fe_d**	**pH S**
50	-0.087	0.757**	-0.556**	0.703**
100	-0.206	0.826**	-0.616**	0.770**
300	-0.301	0.687**	-0.719**	0.627**
600	-0.544*	0.416	-0.866**	0.428
1200	-0.621**	0.419	-0.910**	0.399

3.3.2. Toxicity bioassays

3.3.2.1. *Lactuca sativa*

In the seed germination (SG) test, the carbonate soils (H3, H2 and H1) showed stronger inhibition of germination than the other soils, while the sample with the lowest inhibition was the red Mediterranean soil (H7). The germination was inhibited for concentrations ≥ 300 mg kg^{-1} in sample H3, for concentrations ≥ 600 mg kg^{-1} in samples H1 and H2, and for concentrations of ≥ 1200 mg kg^{-1} in samples H4, H5 and H6 (non-carbonate soils); in sample H7, germination was not inhibited in any treatment, with germination values of 87% at the maximum contamination level (1200 mg kg^{-1}).

In the root elongation (RE) test, the reduction of elongation increases with the amount of As added to the samples (Fig. 2). The inhibition of elongation was stronger in carbonated soils in relation to the other samples. For the first level of contamination (50 mg kg^{-1}) the percentage of elongation in relation to the uncontaminated soils for the samples H3, H2, and H1 was 32, 76, and 88%, respectively, whereas for the second level of contamination (100 mg kg^{-1}) it was 4, 29, and 68%, respectively. Samples of non-carbonate soils (H4, H5 and H6) did not significantly differ, with elongation values higher than 50% for the contamination with 100 mg kg^{-1} and higher than 10% for the contamination with 300 mg kg^{-1}. The soil with the lowest phytotoxicity was H7, registering root-elongation values of 49% in relation to the uncontaminated sample for the contamination level of 600 mg kg^{-1}.

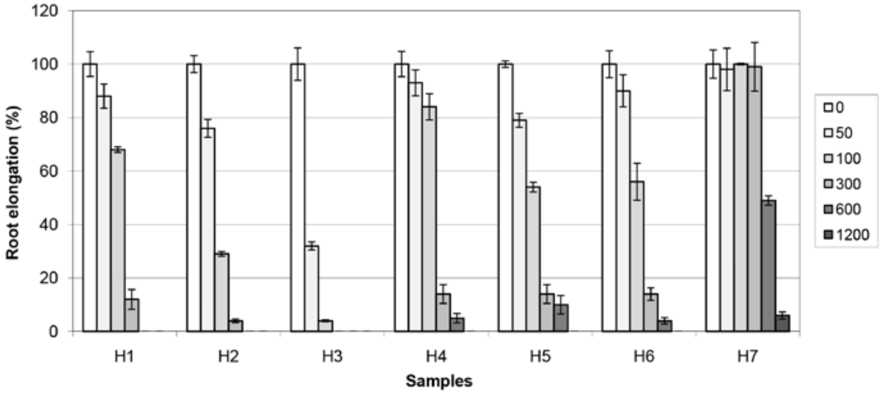

Figure 2. The root-elongation percentage in relation to the control for the different samples and different treatments of total As added (0-50-100-300-600-1200 mg kg^{-1}). Values higher than 100% could indicate hormesis.

3.3.2.2. *Vibrio fischeri*

Reduction values at 15 min of the initial luminescence increased with the amount of As added to the soils (Fig. 3). Highly carbonate soils (samples H2 and H3) showed the greatest reduction, with luminescence values lower than 32% in relation to the uncontaminated sample for the treatment of 100 mg kg^{-1}, followed by the organic horizons (H1 and H5) with luminescence around 60% in relation to the uncontaminated soil, for the same contamination level. The non-carbonate soils (H4 and H6) presented a luminescence higher than 95% for the contamination with 100 mg kg^{-1}, and around 30% for 300 mg kg^{-1} of As added to the soil. The red Mediterranean soil (H7) again showed the least toxicity, no inhibition was detected for the contamination with 300 mg kg^{-1}, and luminescence higher than 55% in relation to the uncontaminated soil for the treatment of 600 mg kg^{-1} of As added to the soil.

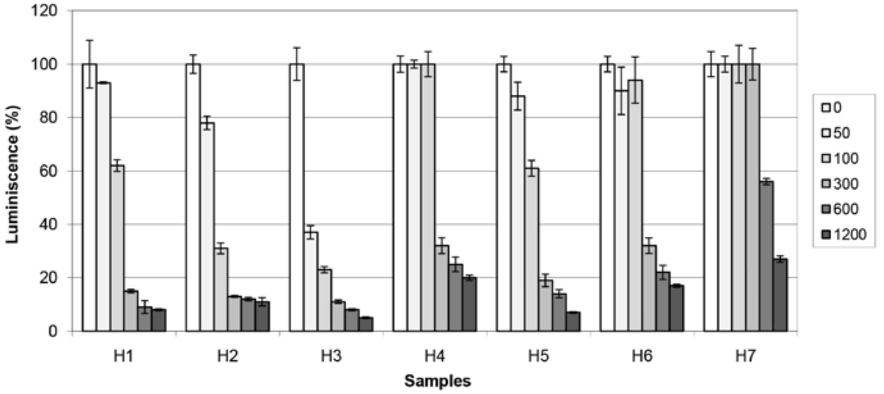

Figure 3. Percentage of luminescence at 15 min in relation to the control for different samples and different treatments of total As added (0-50-100-300-600-1200 mg kg^{-1}). Values higher than 100% could indicate hormesis.

3.3.3. Relation between toxicity and soil properties

The soil properties with highest influence on arsenic toxicity were studied using a correlation analysis (Spearman) between the data obtained in the bioassays and the main variables analysed (Table 3). The root-elongation reduction of *L. sativa* in relation to the control (LsR) was significantly correlated in all treatments with water-soluble As (AsW), and free iron (Fe_d) in direct and inverse relation, respectively. Furthermore, for treatments ≤ 600 mg kg^{-1}, pH (pH S) and calcium carbonate content were directly and significantly related to the increase in phytotoxicity, and for treatments ≥ 600 mg kg^{-1}, toxicity was also significantly related to the pH (pH W) and ionic strength (I) of the soil solution.

Table 3. Correlation coefficients (Spearman) between the percentage of root-elongation reduction of *L. sativa* (LsR) and different variables of soils, for all treatments.

	AsW	Fe_d	$CaCO_3$	pH W	pH S	I
50	0.792**	-0.579**	0.641**	0.300	0.636**	0.330
100	0.941**	-0.763**	0.687**	0.233	0.601**	0.310
300	0.734**	-0.694**	0.626**	0.380	0.653**	0.257
600	0.628**	-0.596**	0.655**	0.797**	0.667**	0.449*
1200	0.605**	-0.608**	0.214	0.509*	0.178	0.606**

Treatment: 50, 100, 300, 600, 1200 mg kg^{-1} of total As added to the soils; AsW: soluble in water As; Fed: free iron oxides; $CaCO_3$: calcium carbonate content; pH W: water extract pH; pH S: soil pH; I: ionic strength. (* $p < 0.05$, ** $p < 0.01$).

According to luminescence reduction of *V. fischeri* in relation to the control (VfR), the variables significantly correlated to this toxicity index in all treatments were the calcium carbonate content and soil pH, in direct relation, and Fe_d in inverse relation to VfR (Table 4). Treatments ≤ 300 mg kg^{-1} had higher correlation coefficients with the water-soluble As (AsW) and the free iron forms (Fe_d) than did treatments with higher As concentrations, with no significant relation with AsW in the treatment of 1200 mg kg^{-1}. Furthermore, for the treatments ≥ 100 mg kg^{-1}, the toxicity was significantly related to the pH and ionic strength (pH W and I) of the soil solution.

Table 4. Correlation coefficients (Spearman) between the percentage reduction in luminescence of *V. fischeri* (VFR) and other soil variables, for all the treatments.

	AsW	Fe_d	$CaCO_3$	pH W	pH S	I
50	0.816**	-0.642**	0.644**	0.338	0.630**	0.279
100	0.936**	-0.606**	0.857**	0.465*	0.837**	0.563**
300	0.857**	-0.561**	0.863**	0.582**	0.833**	0.580**
600	0.495*	-0.447*	0.768**	0.706**	0.731**	0.712**
1200	0.419	-0.447*	0.716**	0.586**	0.662**	0.881**

Treatment: 50, 100, 300, 600, 1200 mg kg^{-1}of total As added to the soils; AsW: water-soluble As; Fed: free iron oxides; CaCO3: calcium carbonate content; pH W: pH of water extract; pH S: soil pH; I: ionic strength. (* p <0.05, ** p <0.01).

The factorial analysis made with all the variables studied showed that 90.96% of the variance was explained by a total of 4 components (Table 5). The responses of the toxicity bioassays (LsR and VfR) were grouped in component 2, explaining 27.16% of the variance, with the total As (AsT), water-soluble As (AsW), and ionic strength (I) of the solution.

Table 5. Factorial analysis of rotated component matrix.

	Component			
	1	2	3	4
AsT		0.953		
AsW		0.852		
pH W				0.492
pH S				0.979
I		0.689		
$CaCO_3$				0.878
OC			0.977	
SA	0.881			
Clay	0.977			
Fed	0.969			
Ald	0.973			
Mnd			0.977	
CEC			0.883	
LsR		0.861		
VfR		0.847		
% ac.ex.var.	27.51	52.78	76.54	90.97

AsT: total As concentration; AsW: water-soluble As concentration; pH W: pH of water extract; pH S: soil pH; I: ionic strength; OC: organic carbon; SA: specific area; Fe_d/Mn_d/Al_d: free oxides; CEC: cation-exchange capacity; LsR: *Lactuca sativa* reduction; VfR: *Vibrio fischeri* reduction.(% ac.ex.var.= % of accumulated explained variance).

3.3.4. Toxicity index

3.3.4.1. *Lactuca sativa*

The effective concentrations of water-soluble As (EC10 and EC50) for root elongation data between the different samples showed no significant differences. The water-soluble As concentrations that inhibited 10% of root elongation ranged between 0.92 and 0.19 mg kg^{-1}, while the 50% reduction of the elongation was caused by values between 4.30 and 1.81 mg kg^{-1}. The no-observed-effect concentrations (NOEC) ranged between 0.10 and 0.02 mg kg^{-1} of soluble As, without significant differences between soil samples.

In relation to the total As (Table 6) the sample that presented the highest toxicity was the highly carbonated soil horizon (H3), wherein concentrations of 23 and 43 mg kg^{-1} of As inhibited the root elongation by 10 and 50%, respectively. The sample with lowest toxicity was the red Mediterranean soil (H7), with an EC10 value of close to 400 mg kg^{-1} and EC50 of more than 620 mg kg^{-1}. The non-carbonate soil H4 also presented low toxicity, in which concentrations of 95 and 185 mg kg^{-1} of total As in soil reduced the root elongation by 10 and 50%, respectively. The other samples did not significantly differ, with EC10 values ranging between 30 and 51 mg kg^{-1}, and EC50 values ranging between 84 and 147 mg kg^{-1} of total As in soil.

For the no-observed-effect concentrations (NOEC), significant differences were found between the different groups of soil. Carbonate soils (H1, H2, and H3), fixed the highest value of this concentration in 25 mg kg^{-1}, while in non-carbonate soils (H4, H5, and H6) this value rose to 40 mg kg^{-1}, and for the red Mediterranean soil (H7) no phytotoxic effects were observed below a concentration of 150 mg kg^{-1}.

Table 6. Effective concentrations (EC50 and EC10) for the *L. sativa* test of total As in soil. (95% CI: confidence interval for the mean 95%).

	Total As (mg kg^{-1})					
	EC50	95% C.I.		EC10	95% C.I.	
H1	147.24	132.65	163.43	62.15	50.69	76.22
H2	83.57	79.62	87.72	42.62	37.96	47.86
H3	43.02	37.89	48.84	23.17	15.39	34.88
H4	185.49	163.49	210.46	94.68	75.41	118.87
H5	123.83	111.73	137.24	37.92	30.19	47.62
H6	126.20	107.68	147.90	47.17	35.39	62.87
H7	621.64	571.38	676.31	394.95	293.71	531.08

3.3.4.2. *Vibrio fischeri*

The effective concentration EC10 of water soluble As showed no significant differences between samples and ranged between 0.06 and 0.61 mg kg^{-1}. For the EC50, samples of carbonate soils (H1, H2 and H3) differed with respect to the other samples; in this case, the 50% reduction in carbonate soils was caused by concentrations between 3.02 and 3.69 mg kg^{-1}, whereas for the other samples ranged between 6.78 and 15.21 mg kg^{-1}. The values of no-observed-effect concentrations (NOEC) for water-soluble As were very similar for all samples with values ranging between 0.002 and 0.003 mg kg^{-1} in all cases.

In relation to the total As (Table 7), the sample with the highest toxicity was again the highly carbonate soil (H3), wherein concentrations of 5.5 and 37.3 mg kg^{-1} inhibited luminescence at 10 and 50%, respectively. The sample with the lowest toxicity was also the red Mediterranean soil (H7), with EC10 concentration higher than 270 mg kg^{-1} and EC50 concentration of 765 mg kg^{-1} of total As. The other samples did not significantly differ, with EC10 values ranging between 39 and 102 mg kg^{-1}, and EC50 values ranging between 88 and 291 mg kg^{-1} of total arsenic in soil.

For the no-observed-effect concentrations (NOEC), no significant differences were detected between samples. In all cases, the NOEC was between 5 and 15 mg kg^{-1} and did not reflect the previously observed differences in solubility or toxicity of As between different samples.

Table 7. Effective concentrations (EC50 and EC10) for *V. fischeri* test of total As. (95% CI: confidence interval for the mean 95%).

	Total As (mg kg^{-1})					
	EC50	95% C.I.		EC10	95% C.I.	
H1	152.65	136.86	170.27	58.21	47.72	71.02
H2	87.84	76.26	101.18	38.82	22.05	68.33
H3	37.30	30.97	44.92	5.51	3.43	8.87
H4	290.64	231.37	365.09	101.88	63.83	162.62
H5	154.87	134.73	178.01	44.38	33.35	59.06
H6	242.20	202.34	289.92	57.54	37.87	87.42
H7	767.54	701.38	839.94	332.14	279.57	394.60

3.4. Discussion

3.4.1. Arsenic solubility and soil properties

Soil properties have a critical influence over the speciation, bioavailability, and solubility of As (Bissen and Frimmel, 2003; Juhasz et al., 2003). From the correlation analysis between the concentrations of water-soluble As and the different properties of the soils studied (Table 2), we determined that the variables having the strongest influence on the solubility of this element were: a) properties related to the soil extract (pH and ionic strength of the solution), b) soil constituents (mainly iron oxides and organic carbon content).

Arsenic is described as one of the few metalloids that can move within the normal pH range (6.5-8.5) under oxidizing as well as reducing conditions (Dzombak and Morel, 1990). In our soils, we detected an increase in As solubility with the rise in pH (also strongly related to the $CaCO_3$ content). The causes of this increased mobility could be associated with desorption of Fe oxides and mobilization of soil carbon under these conditions (Wang and Mulligan, 2006; Klitzke and Lang, 2009). Furthermore, the positive correlation between the solubility of As and the ionic strength found in our samples has been described as a key factor in increasing the mobility of heavy metals in the soil (Acosta et al., 2011), and it has been found that a rise in the ionic strength of aqueous solutions boosts the effectiveness of decontamination in soils affected by As (Deliyanni et al., 2003).

In terms of recuperation of contaminated soils by metal(oid)s, one of the most widely used techniques is the increase of soil pH by liming techniques (Bolan and Duraisamy, 2003). However, according to our results, when As is one of the contaminants, this liming should be done with caution since, under certain conditions, a higher pH may bring on the solublization of certain forms of As. Our results agree with Simón et al. (2005, 2010) who studied the recovery and liming of soils after the accident at the Aznalcóllar mine (Seville, Spain) and recommended not to raise the pH above neutrality so as not to increase As mobility.

Otherwise, the results of correlation between water soluble As and soil constituents indicated a highly significant inverse relation with free iron oxides (Fe_d). In this respect, the Fe oxides and Fe hydroxides of soil have been widely described as the main active constituents in soil-As retention (Fitz and Wenzel, 2002). Many remediation technologies have been developed using iron compounds as the most effective way to immobilize As both from soil as well as in water-contaminated areas (Mohan and Pittman, 2007; Simón et al., 2010).

Also, we found a highly significant inverse relation between soluble As and clay content and specific area, in agreement with the findings of several authors who have suggested that the presence of Fe is closely linked to the large surface areas and small particle sizes (Warren et al., 2003; Yamaguchi et al., 2011). Although the high correlation coefficients between Fe oxides and clay content (0.938) and the specific area (0.929), may be indicative of the effects of collinearity between these variables which have to be carefully analysed to establish adsorption models.

According to Yang et al. (2012) As distribution between the soluble and solid phase is directly related to the total As, pH, and OC. These results agree with those observed in this work, although the correlation with OC appears only for the highest concentrations of added As (1200 mg kg^{-1}). This relationship between As adsorption by humic acid is well known, although the precise retention mechanisms remain controversial (Kumpiene et al., 2008), and some experiments indicate that the presence of organic matter causes the opposite effect, competing with As for adsorption sites on iron oxide surfaces (Redman et al., 2002).

3.4.2. As toxicity

In this work, the toxicity determined by the root elongation of *L. sativa* is considered to have a higher degree of sensitivity than does the luminescence of *V. fischeri* when studying As toxicity in soils, in agreement with other authors (Estepa, 2011). Moreover, values of EC50, EC10, and NOEC both for total as well as for water-soluble As are more uniform (narrow confidence interval) and more differences between samples are detected, indicating higher reliability and sensitivity than with the *V. fischeri* bioassay. In addition, the seed-germination test with *L. sativa* is the least sensitive endpoint to the As concentrations because it requires very high values to inhibit germination, and changes that occur between the different treatments are very abrupt (Bagur González et al., 2011).

Comparisons between toxicity indexes and soil properties indicate that the results for inhibition in both bioassays were similar to those for water-soluble As behaviour. The soil parameters with the greatest influence were: iron oxides, calcium carbonate content, pH and ionic strength of the solution, clay content, and specific area. Of these, iron oxides appear to play the most decisive role in toxicity reduction, coinciding with the extensive use of this constituent as an amendment agent in the recovery of As-contaminated soils (Hartley et al., 2004; Miretzky and Fernández, 2010; Nielsen et al., 2011).

Another variable with great influence is pH of the soil solution, observing in our study that a rise in the pH in turn raises toxicity. Even though increased As availability and toxicity in soils under acidic conditions (pH <5) was generally assumed to be due to the increased solubility of iron and aluminium forms that retain this element (O´Neill, 1995), As mobilization at higher pH values can also be important in certain soil types. Low As adsorption in soils with high pH values is related to a gain in more-negatively charged arsenate species, causing repulsion by the negatively charged soil-exchange sites and increasing the bioavailability of this contaminant (Yang et al., 2002). Moreover, the adsorption capacity of As by Fe oxides diminishes with rising pH, due to the decrease in positive surface charges of these oxides (Klitzke and Lang, 2009), which may augment the solubility of arsenates in the normal pH range of soils (Beesley and Marmiroli, 2011). In this sense, As mobilization over time after remediation actions, implying applications of amendment material, is of great concern, because soil pH is one of the properties most strongly affected. In this sense, we have described remobilization of As in soils 10 years after remediation actions in the soils contaminated by the Aznalcóllar mine-pollution accident (Seville, Spain) (Martín et al., 2011) indicating that the monitoring of recovered areas over the medium and long term was necessary in these cases.

3.4.3. Reference values

Today, for regulatory purposes, reference values for As in soils are based on the establishment of a single value for large regions (countries or communities), regardless of the variations between different soil types, without representing the potential risk of contamination (toxicity) for each scenario. In this way, the most restrictive reference values were established for agricultural soils, with large differences between countries: 10 mg kg^{-1} in UK (Barth and L'Hermite, 1987); 19 mg kg^{-1} in Belgium (BWRHABT GG, 1995); and 29 mg kg^{-1} in Holland (RIVM 2000). Otherwise, guidelines values vary across about a 1000-fold range (0.039 to 40 mg kg^{-1}) in the USA (Teaf et al., 2010). In this study, we confirmed that the different soil types are key in controlling As mobility and thus result in different degrees of toxicity for the same total concentration of contaminant.

In relation to the concentrations of water-soluble As, the results are not conclusive because they have very wide confidence intervals, especially for lower toxicity thresholds, causing an overlap of different values for the different samples. Considering the EC50 value as one of the most common thresholds used in ecotoxicity tests (Isnard et al., 2001), in our study, for the *L. sativa* bioassay and water-soluble arsenic concentrations, we found a range of between 1.8 and 4.3 mg kg^{-1}, similar to the value 2.3 mg kg^{-1} reported by Vaughan and Greensdale (1998) for the same type of bioassay. In the case of *V. fischeri*, our values range between 3.0 and 15.2 mg kg^{-1}, similar to those presented by Fulladosa et al. (2004) of 5.7 (at pH 8) and 20.3 mg kg^{-1} (at pH 7) in As-contaminated solutions.

With respect to the total As concentration, the influence of soil properties and constituents lead to sharp differences between the values of the same endpoint in a given bioassay (Table 8).

The studies cited in the table above indicate that the soil properties having the strongest influence in toxicity are pH and the content of sesquioxides and, as in our study, the lowest toxicity (highest values) occurs in soils with the highest iron oxide content. Soil guideline values can be defined according to the background and NOEC concentrations (Crommentuijn et al., 2000) in natural soils. In the case of arsenic, we can preliminary define three levels: 25 mg kg^{-1} for carbonate soils, 40 for non-carbonate soils, and 150 for iron-rich soils. In any case, the variations between the different soil types are very important, so that more studies are needed to propose generic environmental quality standards defined according to the main soil properties and constituents affecting arsenic mobility and toxicity.

Table 8. Data from the literature on reference values for total As (mg kg^{-1}) concentrations by root-elongation test compared with our results.

Reference	**NOEC**	**EC10**	**EC50**
Song et al. (2006) *(barley)*	15 – 125	4 – 207	27 – 458
Cao et al. (2009) *(lettuce and wheat)*	<40 – 200	20 – 156	59 – 426
Our study (lettuce)			
Carbonate soils	15 – 25	25 – 60	45 – 150
Non-carbonate soils	10 – 40	40 – 95	125 – 185
Iron-rich soil	150	400	620

3.5. Conclusions

The solubility of arsenic in the samples studied is strongly controlled by soil properties and constituents, the iron oxides, the organic carbon content, pH, and ionic strength of the soil solution being the most important variables. Also, we also found that the soil properties strongly alter the As toxicity of the soil leachate, assessed by bioassays, showing a significant reduction of the toxicity in the iron-rich soil and significant increase in the highly carbonate samples. The broad range of responses of arsenic toxicity in relation to soil properties can produce over- or under-estimation of guideline values in soil remediation actions. According to these results, soil guideline values of As should be set at different levels depending on soil properties, which could optimise the efficiency and profitability in the use of generic environmental quality standards.

CHAPTER 4

Influence of soil properties on the bioaccumulation and effects of arsenic in the earthworm *Eisenia andrei*

Abstract

This study aimed at assessing the influence of soil properties on the uptake and toxicity effects of arsenic in the earthworm Eisenia andrei exposed for 4 weeks to seven natural soils spiked with different arsenic concentrations. Water-soluble soil concentrations (AsW) and internal As concentrations in the earthworms (AsE) were greatly different between soils. These two variables were highly correlated and were key factors in earthworm toxicity response. AsW was explained by some soil properties, such as the pH, calcium carbonate content, ionic strength, texture or oxide forms. Toxicity showed a clear variation between soils, in some cases without achieving 50 % adverse effect at the highest As concentration added (600 mg kg^{-1}). Nevertheless, soil properties did not show, in general, a high relation with studied toxicity endpoints, although the high correlation with AsW could greatly reduce indirectly As bioavailability and toxicity risk for earthworms. Obtained results suggest that soil properties should be part of the criteria to establishing thresholds for contaminated soils because they will be key in controlling As availability and thus result in different degrees of toxicity.

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4.1. Introduction

Arsenic is a metalloid which is found at widely varying concentrations in different environments (Kabata-Pendias and Pendias, 2001). Currently, its presence is associated with natural and anthropogenic sources, and in many areas, its environmental levels in water, sediments or soils are of major concern due to its potential adverse health effects (Nriagu et al., 2007).

To determine the reference levels of metals in soils, it is necessary to know their contents under natural conditions (soil background) (Martín et al., 2012). Arsenic background concentration in soil is highly variable as it depends on the initial concentration in the parent material, natural geochemical cycles and soil type (Díez et al., 2007). The bioavailability and therefore the toxicity of metals to organisms in terrestrial ecosystems are largely controlled by soil properties (Sheppard and Evenden, 1988). The solubility of arsenic and therefore also its toxicity are known to be strongly controlled by soil properties and constituents (Martín Peinado et al., 2012). The most important variables affecting As availability in soils seem to be the organic carbon content, pH, ionic strength of the soil solution, iron oxides and cation exchange capacity (Song et al., 2006; Romero-Freire et al., 2014).

Evaluation of the effects of contaminants in soils has become a priority for OECD member countries (Arnaud et al., 2000; Saint-Denis et al., 2001). Most environmental protection policies are based on guidance based on total concentrations of a particular element or compound in soil, referring to land-use type but not to the soil type. This creates a large variety of soil quality criteria for metals in different countries. For example, the intervention levels for As in agricultural soils in Europe range from 10 mg As kg^{-1} soil in the UK (Barth and L'hermite, 1987) to 50 mg As kg^{-1} soil in the Netherlands (NMHPPE, 1994).

The accumulation of trace elements from soil to biota has been studied extensively for many species (Nahmani et al., 2007; Díez-Ortiz et al,. 2010). Earthworms are more susceptible to metal pollution than many other soil invertebrates (Bengtsson et al., 1992; Spurgeon and Hopkin, 1996). Furthermore, earthworms have a number of characteristics (large size, behaviour and high biomass) which make them highly suitable animals for use as bioindicator organisms for determining the toxicity of chemicals in soils (Callahan, 1988; Goats and Edwards, 1988; Bouché, 1992; Arnaud et al., 2000). Consequently, they have been adopted as standard organisms for ecotoxicological testing by the European Union (EEC, 1984), with *Eisenia andrei* commonly used as the test species not only in standardized toxicity tests (OECD, 1984) but also in bioassays to assess the toxicity of field-contaminated soils (Cortet et al., 1999; Fleuren et al., 2003).

To determine toxicity guidance values, usually artificially contaminated soils are used, which may differ in composition from in situ contaminated soil. As a consequence, laboratory experiments tend to overestimate the solubility and availability of metals compared to field-contaminated soils (Smolders et al., 2009; van Gestel et al., 2012). The toxicity level defined by laboratory studies commonly overestimates the effects, which can lead to high strict safety thresholds for the environmental risk assessment (Romero-Freire et al., 2014).

The aim of this study was to determine the effect of soil properties on the bioavailability of arsenic by measuring its uptake and toxicity using the earthworm *Eisenia andrei*. The earthworms were exposed to a wide range of soils with contrasting properties spiked with different arsenic concentrations. This study is part of a broader project that involves assessing the toxicity of arsenic to different organisms for the purpose of establishing guideline values to improve the existing regulation regarding soil pollution in Spain (Romero-Freire et al., 2014).

4.2. Materials and methods

4.2.1. Soils

Seven soils with different properties, representing most of the main soil groups in Spain, were selected (Table 1). The main parameters analysed were pH (soil to water or soil to 0.1 M KCl in a ratio 1:2.5), ionic strength of the soil (I) derived from the electric conductivity according to Simón and García (1999), calcium carbonate content ($CaCO_3$), organic carbon content (OC), available phosphorous content (P), water holding capacity (WHC), available water (AW) calculated from differences in moisture contents at the field capacity (h33) and the wilting point (h1500), texture, cation exchange capacity (CEC). These properties were determined according to official methods of analysis (MAPA, 1994). Moreover, concentrations of free and amorphous iron, aluminium and manganese oxides were analysed according to Holmgren (1967) and Schwertmann and Taylor (1977), respectively.

Table 1. Main properties of the soils used to assess the influence of soil properties on the uptake and effects of As in earthworms.

SOIL	As* (mg kg^{-1})	pH (Water)	pH (KCl)	I (mmol l^{-1})	P (mg kg^{-1})	$CaCO_3$ (%)	OC (%)	Clay (%)
H1	15.5	7.96	7.63	7.3	8.3	37.1	5.43	23.6
H2	9.07	8.67	8.11	2.0	bdl	72.4	0.42	11.8
H3	3.39	8.79	8.24	1.7	bdl	92.3	0.38	7.70
H4	16.2	6.74	5.80	0.9	6.5	bdl	0.61	19.0
H5	12.3	7.20	6.72	13	28.1	bdl	8.22	23.8
H6	4.39	5.87	4.58	0.5	1.1	bdl	0.49	8.31
H7	25.7	7.03	5.86	1.6	bdl	0.92	0.66	54.7

SOIL	Silt (%)	CEC (cmol+ kg^{-1})	Al_d ($^0/_{00}$)	Al_o ($^0/_{00}$)	Fe_d ($^0/_{00}$)	Fe_o ($^0/_{00}$)	Mn_d ($^0/_{00}$)	Mn_o ($^0/_{00}$)
H1	42.3	21.4	2.40	1.17	19.0	0.68	0.54	0.06
H2	46.8	9.83	1.10	0.31	8.67	0.20	0.13	0.02
H3	64.0	2.94	0.60	0.15	3.29	0.01	0.01	0.00
H4	24.3	9.91	1.90	0.38	17.9	0.52	0.32	0.18
H5	33.3	25.9	1.90	0.50	19.4	0.65	0.85	0.41
H6	21.2	3.83	0.90	0.27	7.77	1.00	0.15	0.09
H7	15.3	15.5	5.10	0.73	82.6	0.78	0.13	0.03

* Total As background; I: ionic strength; P: available phosphorus content; OC: organic carbon content; CEC: cation exchange capacity; free forms of Al_d, Fe_d and Mn_d and amorphous forms of Al_o, Fe_o and Mn_o; bdl: below detection limit.

Soils were contaminated in the laboratory with increasing concentrations of sodium arsenate (Na_2HAsO_4 $7H_2O$) according to the reference values proposed by the Junta de Andalusia (Aguilar et al., 1999) for agricultural, natural and industrial use (50–100–300 mg As kg^{-1}, respectively) and adding one more level to create a worst case scenario (600 mg As kg^{-1}). Furthermore, an uncontaminated level (control) was included. Contamination was performed by spiking samples of 500 g of soil (dry weight) with aqueous As solutions ($n = 3$). After spiking, the soils were moistened to 60 % of their WHC and incubated for 4 weeks at 25 ± 1 °C and 60 % air humidity, with a light to dark cycle of 10:14 h. Soil moisture content was checked and, if needed, readjusted weekly. The incubation period chosen allows stabilization of the arsenic added and was based on similar studies by other authors (Fendorf et al., 2004; Tang et al., 2006; Romero-Freire et al., 2014). After the incubation period, a saturated extract was prepared with a soil to water ratio of 1:1 and was stirred for 24 h; then, a soil solution was obtained by extraction with a 10-cm Rhizon MOM and analysed for pH, electrical conductivity, and water-soluble As concentrations (Romero-Freire et al., 2014).

4.2.2. Earthworm toxicity testing

Earthworms of the species *E. andrei* were supplied by Lombricor SCA (Córdoba, Spain). Earthworms were cultured at 20 °C in a substrate of soil with high organic matter content, peat and abundant horse manure free of any pharmaceutical. Before the start of the exposures, adult worms with well-developed clitella were selected with an average weight of 0.50 ± 0.08 g.

The earthworm toxicity tests followed OECD guideline 222 (OECD, 2004), including a 4-week exposure period of adult animals. Three replicate test containers were used for each arsenic concentration and control, containing approximately 500 g soil (dry weight equivalent). Ten adult earthworms were added to each test container after being gently cleaned on moistened paper towels and weighed. To feed the worms, 25 g of horse manure:distilled water (1:4 ratio) was added to each container. The containers were kept in an incubator chamber at 20 °C with 12 h of light per day. Container weights were monitored weekly to maintain moisture content, and additional food was added when required.

After 4 weeks, test containers were emptied into a tray, and surviving adults were collected by hand sorting, cleaned and weighed. Surviving earthworms were placed on moist filter paper for approximately 24 h to void their gut contents, following Arnold and Hodson (2007). After weighing, they were freeze-dried and stored for analysis. Soils, which contained cocoons, were returned to their respective containers and incubated for another 4 weeks controlling the water content weekly. After this period, the containers were placed in a water bath at 60 °C, forcing juveniles to emerge to the surface, where they were counted.

4.2.3. Arsenic analysis

Total arsenic background concentrations in soils and earthworm tissues (AsE) were determined after digestion in a mixture of concentrated HNO_3:HCl (4:1). Water-soluble As concentration in soil (AsW) was determined from soil:water extracts (1:1 ratio) after 24 h equilibrium with shaking (Fotovat and Naidu, 1998; Romero-Freire et al., 2014, 2015). In all cases, As was measured by inductively coupled plasma-mass spectrometry (ICP-MS) in a ICP-MS NexION 300D spectrometer. Instrumental drift was monitored by regularly running standard element solutions between samples. For calibration, two sets of standards containing the analyte of interest at five concentrations were prepared using rhodium as an internal standard. Procedural blanks were included for estimating the detection limit ($3 \times \sigma$; n = 6) which was <0.21 µg L^{-1} for As. The analytical precision was better than ±5 % in all cases. The accuracy of the method was confirmed by analysing standard reference material SRM2711 Montana Soil (US NIST, 2003). Total As concentrations (AsT) in spiked soils were checked using portable X-ray fluorescence (PXRF) (Martín Peinado et al., 2010), and measured concentrations ranged between 80 and 97 % (average ± SD 92 ± 5 %; n = 34) of the nominal values.

4.2.4. Data analysis

Soil-water partition coefficient (Kp) was calculated as the ratio of the total As concentration in soil (AsT in mg kg^{-1} dry soil) and the water-soluble As concentration (AsW in mg L^{-1}) and expressed as litres per kilogramme (Blaser et al., 2000). Biota-soil accumulation factors (BSAF) for the uptake of As in the earthworms after reaching the steady state (OECD 317, 2010) were calculated by dividing concentrations in the surviving animals (AsE; in mg As kg^{-1} earthworm dry weight) by total concentrations in the tested soils (AsT; in mg kg^{-1} dry soil) (Peijnenburg et al., 1999). Biota-water accumulation factors (BWAF) were calculated by dividing AsE by water-soluble As concentrations (AsW; in mg L^{-1}) (Peijnenburg et al., 1999). Lethal concentrations causing 10 % mortality (LC10) and effective concentrations causing 50 and 10 % reduction of juvenile production (EC50, EC10) and their corresponding 95 % confidence intervals were calculated by fitting a log-logistic dose-response model to the data (Doelman and Haanstra, 1989) for soil samples which showed a dose-response relationship with AsT, AsW and/or AsE.

To assess As toxicity for earthworm exposition, mortality (M) was calculated as the percentage of worms that died during the 4-week exposure period for each soil and treatment in relation to the control; weight variation (W) was calculated as the percentage of variation in the surviving earthworms recovered after 4 weeks in relation to the initial weight and recalculated in relation to the control soils; juvenile production (J) was calculated from the number of juveniles produced per worm per week and expressed as the percentage in relation to the control.

Normal distribution of the data was verified with a Kolmogorov-Smirnov test. Significant differences were determined by ANOVA, and multiple comparison analyses were performed with Tukey HSD test ($p < 0.05$). To study the influence of soil properties on accumulation and toxicity of arsenic by *E. andrei*, Spearman's correlation analysis and principal component analysis (PCA) after varimax rotation were applied to discriminated different groups of variables according to statistical similarities of the normalized dataset. All these analyses were performed with a confidence level of 95 % by using SPSS v.20.0 (SPSS Inc. Chicago, USA).

4.3. Results

4.3.1. Water-soluble arsenic concentrations and soil properties

Water-soluble As concentrations showed a dose-related increase and were significantly higher ($p < 0.05$) at the highest As concentrations added (>300 mg kg^{-1}) for all studied soils and also differed between the tested soils (Table 2). The lowest water-soluble As concentration was found in the iron-rich soil H7 (red Mediterranean soil) with 3.34 mg As kg^{-1} soil extracted at the highest concentration tested (600 mg As kg^{-1} soil). Soils rich in organic carbon (H1 and H5) also showed low water-soluble arsenic concentrations, with extractions of 82.9 and 106 mg As kg^{-1} soil, respectively, at the highest exposure concentrations. In soils H4 and H6, which are slightly acidic, non-carbonated and had low ionic strength, 116 and 118 mg kg^{-1} of the As were water-soluble at 600 mg As kg^{-1}, respectively. Soils with the highest $CaCO_3$ content and basic pH (H2 and H3) had the highest water-soluble arsenic concentrations (271 and 337 mg As kg^{-1} soil, respectively). Table 2 also includes the partition coefficient (Kp) for As in the different soils. Kp showed a significance difference among treatments in the different studied soils. Higher Kp values appeared in control soils; however, this was not the case in soils H4 and H7, where high values appeared at the lower As treatment levels.

Table 2. Mean water-extractable arsenic concentrations (AsW; mg As kg^{-1} soil); partition coefficients calculated as the ratio of the total As concentration in soil and the water-soluble As concentration expressed as mg As L^{-1} (Kp; L kg^{-1}); arsenic concentrations in earthworms (*Eisenia andrei*) after 4 weeks exposure (AsE; µg As g^{-1} dry body weight); biota-soil accumulation factors (BSAF; kg soil kg^{-1} earthworm); biota-water accumulation factors (BWAF; L kg^{-1} earthworm).

Soil	**As nominal**	**AsW**	**Kp**	**AsE**	**BSAF**	**BWAF**
	mg kg^{-1}	mg kg^{-1}	L kg^{-1}	µg g^{-1} d.w.	kg soil kg^{-1} worm	L kg^{-1} worm
H1	**0**	0.023a	689b	32.5a	2.06a	1376b
	50	0.49a	134a	113ab	1.73a	228a
	100	1.78a	64.9a	170ab	1.50a	94.9a
	300	22.4b	14.1a	504b	1.60a	22.5a
	600	82.9c	7.5a	972c	1.56a	11.6a
H2	**0**	0.007a	1295d	67.9a	7.49b	9710b
	50	0.96a	61.8c	96.7a	1.63a	103a
	100	6.45a	16.9b	182a	1.66a	28.2a
	300	85.3b	3.63a	326ab	1.07a	3.83a
	600	271c	2.27a	525b	0.87a	1.96a
H3	**0**	0.010a	339d	26.01a	7.67c	2600b
	50	6.56a	8.17c	482b	9.03bc	73.9a
	100	29.5a	3.5b	594bc	5.73b	20.2a
	300	153b	2.00a	762c	2.53a	5.0a
	600	337c	1.80a	ns	ns	ns
H4	**0**	0.077a	218b	18.8a	1.17a	258a
	50	0.12a	579c	278ab	4.20b	2482b
	100	0.57a	205b	516c	4.43b	923a
	300	23.1b	13.8a	667c	2.11a	29.4a
	600	116c	5.33a	718c	1.16a	6.2a

H5	**0**	0.014a	1022b	28.35a	2.33b	2311b
	50	1.05a	59.5a	600b	9.63d	573a
	100	3.44a	32.6a	688bc	6.13c	200a
	300	29.3b	10.6a	768c	2.43b	26.2a
	600	106c	5.83a	609b	0.99a	5.83a
H6	**0**	0.010a	439a	32.2a	7.33a	3219c
	50	0.36a	154.2b	606b	11.1b	1743b
	100	1.08a	97.9c	740b	7.09a	698a
	300	23.1b	14.7d	ns	ns	ns
	600	118c	5.13d	ns	ns	ns
H7	**0**	0.010a	2566a	23.3a	0.93ab	2334ab
	50	0.013a	6305b	64.2a	0.83a	5426c
	100	0.023a	5585b	122a	0.97ab	5411bc
	300	0.28b	1189a	540b	1.67ab	1953b
	600	3.34c	188a	1019c	1.93b	362a

All data were calculated for the different soils tested (H1 - H7) at each level of contamination (As nominal: total arsenic added to the soil in mg kg^{-1}). See Table 1 for soil properties. Lowercase letters show difference among treatments for each soil. Post-hoc Tukey HSD test ($p<0.05$). ns: no survival.

The factorial analysis expressed as a principal component analysis (PCA) between AsT, AsW, Kp and some of the main soil properties showed that 62.3 % of the variance was explained by two components (Fig. 1). The arsenic forms (water-soluble and total) were grouped with pHH_2O and $CaCO_3$ content. In component 1, Kp was strongly related with clay content, ionic strength (I) and free forms of Al and Fe, while in component 2, phosphorous content (P), free forms of Mn, organic carbon (OC) content and cation exchange capacity (CEC) were grouped together.

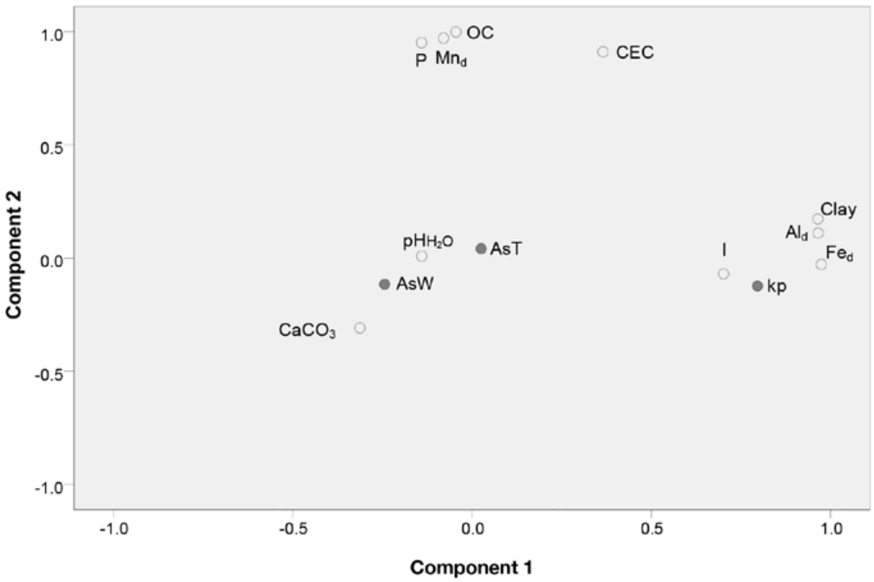

Figure 1. Principal component analysis (PCA) after Varimax rotation including As forms (AsT: total arsenic concentrations; AsW:water-soluble arsenic concentrations) as well as coefficient partitioning (Kp=AsT/AsW) and the main soil properties (I: ionic strenght; Fe_d, Mn_d and Al_d: free forms of iron, manganese and aluminum; CEC: cation exchange capacity; OC: organic carbon content; P: available phosphorous). Accumulate variance explained for Component 1 = 32.8% and for Component 2 = 62.3%.

4.3.2. Arsenic bioaccumulation

Internal As concentrations in earthworms (AsE) increased with increasing soil As concentrations (Table 2). In earthworms from control soils, average arsenic concentration was 32.8 (±16.3) µg As g^{-1} dry weight (n = 21). When exposed to 600 mg As kg^{-1} dry weight, the earthworms showed the highest internal concentration in soils H1, H2, H4 and H7, ranging from 1019 µg As g^{-1} in H7 to 525 µg As g^{-1} in H2, meanwhile soils H3 and H5 presented the highest AsE (762 and 768 µg As g^{-1}, respectively) in the treatment of 300 mg As kg^{-1} dry weight. In soil H6, the maximum value of AsE was 740 µg As g^{-1} in the treatment of 100 mg As kg^{-1}, and there were no survivors at higher exposure concentrations.

Biota-soil accumulation factor (BSAF) ranged between 0.93 and 7.67 kg soil kg^{-1} earthworm in control soils. While for the highest contamination level (600 mg As $kg^{-1)}$, values ranged between 0.87 and 1.93, with the exception of soils H3 and H6 in which there were no survivors for this treatment. In general, BSAF tended to decrease with increasing As concentration in soil (regardless of controls), except for soils H1 and H2, with no significant changes between treatments, and H7 where BSAF increased with increasing AsT (Table 2). Biota-water accumulation factor (BWAF) ranged between 258 and 9710 L kg^{-1} earthworm in the controls, while for the highest As treatment BWAF, values were ranged from 1.96 in H2 to 362 in H7 soils. In general, BWAF decreased with the increase in AsW in relation to the control, except for soils H4 and H7 in which the maximum BWAF values were found at the lowest As concentrations added (treatments of 50 and 100 mg As kg^{-1}) (Table 2).

4.3.3. Arsenic toxicity in earthworms.

According to the OECD 222 guideline, the toxicity test is valid when earthworm mortality in the control is less than 10 % and more than 30 juveniles are produced in each replicate control. Based on this, all tests fulfilled these requirements except soil H1 for juvenile production, in which very low values were reached. Earthworm mortality (M) after 4 weeks was quite differently affected by arsenic contamination according to the different soil types, with less or equal than 7 % mortality in soils H2, less or equal than 20 % mortality in soils H4 and H7, and a dose-related decrease in soils H1, H3, H5 and H6. Mortality was only higher than 50 % in soils H3 and H6 (Table 3) at concentrations added of 300 mg As kg^{-1} soil.

Table 3. Mortality (M) expressed as percentage (%) of worms that died during the 4-week exposure period; weight variation (W) (after four weeks) expressed as % related to the control treatment (0 mg As kg^{-1} soil); and juvenile production (J) of the earthworm Eisenia andrei expressed as % compared to control treatment.

	H1			**H2**			**H3**			**H4**		
As nominal (mg kg^{-1})	**M**	**W**	**J**	**M**	**W**	**J***	**M**	**W**	**J**	**M**	**W**	**J**
0	0a	0.33a	100	3	0	100a	3a	0	100a	7	0b	100a
50	0a	-2.34a	150	0	-2.69	102a	3a	-8.20	70b	3	-15.37ab	91a
100	0a	-2.38a	217	3	3.71	230b	17a	-9.36	1.4c	17	0.91b	71b
300	3ab	12.75ab	0	0	0.36	238b	60b	18.40	0c	0	-28.63a	5.5c
600	10b	22.26b	0	7	14.22	14a	97b	ns	ns	17	-16.02ab	0c

	H5			**H6**			**H7**		
As nominal (mg kg^{-1})	**M**	**W**	**J**	**M**	**W**	**J***	**M**	**W**	**J**
0	3ab	0a	100a	0a	0a	100a	10	0	100a
50	0a	9.32a	42b	33b	50.66ab	0b	3	13.59	74a
100	3ab	3.72a	12c	50b	86.25b	0b	20	16.87	23b
300	23b	11.73a	0.1d	100c	ns	ns	10	10.53	0b
600	47c	41.64b	0d	100c	ns	ns	20	18.11	0b

A negative value in weight variance (W) means weight loss, while a positive value means weight gain. Lowercase letters represent significance difference between treatments (Tukey HSD test. *$p < 0.05$*). ns: not adult survival enough. * anomalous behaviour. See Table 1 for soil properties.

The average individual earthworms weight in the controls at the beginning of the exposures was 501 ± 72 mg (±SD; n = 210), while after 4 weeks of incubation, it was 420 ± 65 mg. In the As-spiked soils, earthworm weights showed variation trends that were not always dose-related. In some cases, there was a trend of increasing earthworm weights in relation to the control (W) at higher As concentrations, which was significant for soils H1, H5 and H6. In the other soils, earthworm weight showed no significant dose-related variation compared to the control (Table 3).

Juvenile production (J) in relation to the control showed a decrease with increasing arsenic concentration for most studied soils. Juvenile reduction was significantly different from the control samples ($p < 0.05$) at the lowest treatment (50 mg As kg^{-1}) for the soils H3, H5 and H6, and at the treatment 100 mg As kg^{-1} added for soils H4 and H7. Soil H2 showed an anomalous behaviour with the highest juvenile number in relation to the control at 100 and 300 mg As kg^{-1} soil (Table 3).

The results of the toxicity endpoints (lethal and effective concentrations) are shown in Table 4. For soils H2, H4 and H7, the arsenic concentrations used in this study in relation to earthworm survival (LC10) did not show toxicity response variation in relation to the control soils. LC10 values calculated from total As concentrations (AsT) ranged from 606 mg As kg^{-1} dry soil for H1 (close to the highest As concentration tested) to 31 mg kg^{-1} dry soil for H6. LC10 based on water-soluble As concentrations (AsW) was also highest for H1 soil (82 mg kg^{-1}) and lowest for soil H6 with a value of 0.089 mg kg^{-1} (Table 4).

Table 4. Toxicity values for the effects of arsenic on the survival and reproduction of the earthworm *Eisenia andrei* after 4 weeks and 8 weeks exposure, respectively, to different soils spiked with As.

		SURVIVAL	
		LC10	
	AsT	**AsW**	**AsE**
SOIL	mg As kg^{-1} soil	mg As kg^{-1} soil	µg As g^{-1} worm
H1	606	82	>793
	(476-736)	(46-118)	
H2	nd	nd	nd
H3	132	77	626
	(15-250)	(0-171)	(597-654)
H4	nd	nd	nd
H5	188	12	734
	(57-318)	(0-25)	(702-766)
H6	31	0.089	356
	(12-49)	(0-0.186)	(105-606)
H7	nd	nd	nd

	JUVENILES					
	EC50			EC10		
	AsT	AsW	AsE	AsT	AsW	AsE
SOIL	mg As kg^{-1} soil	mg As kg^{-1} soil	µg As g^{-1} worm	mg As kg^{-1} soil	mg As kg^{-1} soil	µg As g^{-1} worm
H1	ad	ad	ad	ad	ad	ad
H2	ad	ad	ad	ad	ad	ad
H3	60	8.4	490	45	4.4	425
	(55-65)	(7.5-9.4)	(465-515)	(39-51)	(3.5-5.2)	(378-473)
H4	151	1.2	495	82	0.11	366
	(130-172)	(0.6-1.8)	(421-569)	(63-100)	(0-0.23)	(194-537)
H5	56	0.84	584	26	0.19	497
	(48-63)	(0.66-1.03)	(567-601)	(15-37)	(0.06-0.32)	(454-540)
H6	nd	nd	nd	nd	nd	nd
H7	96	0.013	84	59	0.006	46
	(80-111)	(0.008-0.017)	(70-98)	(38-79)	(0.002-0.009)	(29-63)

LC10 for effects on survival and EC50 and EC10 for effects on juveniles production were calculated using the total (AsT), and the water-soluble arsenic (AsW) in the soils and the internal arsenic concentrations (AsE) in the earthworms. AsT and AsW are expressed in mg As kg^{-1} dry soil and AsE as µg As g^{-1} earthworm. See Table 1 for soil properties and Table 3 for survival and reproduction data. Values in between brackets are 95% confidence intervals. Values > (higher) than the highest treatment (600 mg kg^{-1}) adding soil background; ad: anomalous data; nd: no dose-response observed.

Earthworm reproduction based on juvenile production data was more sensitive than survival data. For soils H1, H2 and H6, no toxicity endpoints were calculated because no dose-related variation was observed. Soils H4 and H7 showed the lowest toxicity according to juvenile production for AsT, with an EC50 of 151 and 96 mg As kg^{-1}, respectively; meanwhile, arsenic toxicity was highest in soil H5 with EC50 and EC10 values of 56 and 26 mg kg^{-1} (AsT), respectively. However, in relation to water-soluble concentrations (AsW), soil H3 was the least toxic (EC50 of 8.4 mg As kg^{-1}), and soil H7 was the most toxic for juvenile production (EC50 of 0.013 mg As kg^{-1}) (Table 4).

Toxicity endpoints were also calculated by the internal As concentrations (AsE) in the surviving earthworms. This variable showed lower differences between soils than the endpoints calculated from AsT and AsW. LC10 based on internal As concentrations was lowest in soil H6 (LC10 356 µg g^{-1} dry earthworm) and highest in H1 (LC10 > 793 µg g^{-1} dry earthworm). For juvenile production, EC50 related to AsE was lowest for soil H7, with values of 84 µg g^{-1} dry earthworm, and the highest value was for H5 soil (584 µg g^{-1} dry earthworm) (Table 4).

4.3.4. As bioavailability and toxicity in relation to soil properties

Spearman correlation between the variables related to As bioavailability and toxicity (Table 5) showed that AsE was directly correlated with AsT and AsW, and inversely with Kp; meanwhile, biota-soil accumulation (BSAF) was only inversely correlated with AsT. Biota-water accumulation factor (BWAF) was inversely correlated with AsT, AsW and AsE, and directly with Kp. In relation to earthworm response, earthworm mortality (M) was directly related to AsT and AsE concentrations, and earthworm weight variation (W) was positively correlated with AsE and mortality. Juvenile production (J) showed a negative correlation with AsT, AsW and AsE and also with mortality and weight variation, while Kp was positively correlated with juvenile production.

Table 5. Correlation coefficients (Spearman) between total arsenic (AsT), water extractable As (AsW), partition coefficient (Kp), internal As concentrations in the earthworms (AsE), biota-soil accumulation factors (BSAF); biota-water accumulation factors (BWAF) and earthworm (*Eisenia andrei*) responses (mortality, weight change and juvenile production).

	Spearman correlations								
	AsT	AsW	Kp	AsE	BSAF	BWAF	M	W	J
AsT (mg As kg^{-1} soil)	1	0.777**	-0.581**	0.747**	-0.459**	-0.633**	0.431*		-0.647**
AsW (mg As kg^{-1} soil)		1	-0.957**	0.759**		-0.953**			-0.527**
Kp (L kg^{-1})			1	-0.645**		0.944**			0.399*
AsE (μg As g^{-1} earthworm)			1		-0.576**	0.533**	0.410*	-0.781**	
BSAF (kg soil kg^{-1} earthworm)				1					
BWAF (L kg^{-1} earthworm)						1			
M (%)							1	0.551**	-0.734**
W (%)								1	-0.436**
J (%)									1

(** p<0.01;*p<0.05)

PCA with the toxicity endpoints and the main soil variables of this study showed that 81.3 % of the variance was explained by a total of five components (Table 6). The responses of the earthworm to As toxicity (mortality, weight variation and juvenile production) were grouped in component 2 with the As forms (AsT, AsW and AsE) with a direct relation in all cases except in juvenile production which showed an inverse relation with all these components. Component 1 grouped some of the main soil properties like organic carbon content (OC), available phosphorous content (P), amorphous Mn forms (Mno) and cation exchange capacity (CEC). Component 3 grouped ionic strength (I) and amorphous iron forms (Fe_o) together and inversely related with pHH_2O; weight variation of the earthworms (W) as well as the AsW was also grouped in this component with positive and negative coefficients, respectively, but with a low load in this component. BSAF was included in component 4 and negatively related with AsT, CEC, clay content and amorphous Al forms (Al_o). Finally, BWAF, Kp and clay content were included in component 5 with a positive relation.

Table 6. Factorial analysis of rotated component matrix (Varimax with Kaiser normalization) for As studied forms, the toxicity earthworm endpoints and the main variables of the studied soils.

	Components				
	1	2	3	4	5
AsT (mg As kg^{-1} soil)		0.711		0.507	
AsW (mg As kg^{-1} soil)		0.618	-0.561		
Kp (L kg^{-1})					0.859
AsE (µg As g^{-1} worm)		0.788			
BSAF (kg soil kg^{-1} earthworm)				-0.855	
BWAF (L kg^{-1} earthworm)					0.825
M (%)		0.781			
W (%)		0.539	0.502		
J (%)		-0.794			
pH (Water)			-0.813		
I (mmol l^{-1})			0.628		
OC (%)	0.931				
CEC (cmol+ kg^{-1})	0.821			0.517	
Clay (%)				0.661	0.515
P (mg kg^{-1})	0.984				
Alo				0.764	
Mno	0.913				
Feo			0.890		
% ac.ex.var	19.9	38.0	54.2	69.1	81.3

Al_o, Fe_o and Mn_o: amorphous forms of Al, Fe and Mn. % ac.ex.var.: percent of accumulated explained variance.

4.4. Discussion

According to the arsenic mobility in relation to soil properties and constituents, iron oxides have been widely described as the main active constituents determining As retention in soil (Fitz and Wenzel, 2002), which coincides with our study where the lowest water-soluble As concentrations were found in the iron-rich soil (H7). Moreover, As distribution between the soluble and the solid phases is related also to organic carbon content (Yang et al., 2002). In our case, sample H1 and H5 (with the highest organic carbon contents) showed low values of AsW for the highest treatment (Table 2). A previous study with the same soils treated with the same As concentrations and conditions (Romero-Freire et al., 2014) confirmed the inverse correlation of AsW concentrations with OC content in the treatments with high concentrations of As added. Furthermore, it is known that the solubility of arsenic decreases when ionic strength increases (Acosta et al., 2011), and in our study, soils H6 and H4 (with the lowest ionic strength) presented low AsW concentrations in relation to the total As content. The highest AsW concentrations were found in the soils with the highest $CaCO_3$ content and basic pH (H2 and H3), which is in accordance with other studies revealing that under certain conditions a higher pH may enhance As solubilization (Simón et al., 2010).

Partition coefficient (Kp) is crucial to estimate the potential for the adsorption of dissolved arsenic in contact with soil (USEPA, 1999) and is strongly influenced by soil parameters. However, there is no consensus in literature to state these parameters due to it is obtained from a wide range of soil to water ratios (Sauvé et al., 2000a), hindering the comparison among studies. Nevertheless, Kp is a key parameter to compare in our study soils under the same testing conditions and arsenic extraction method. Our study showed that Kp was directly correlated with iron and aluminium forms as well as ionic strength and clay content. Other studies (Song et al., 2006; Romero-Freire et al., 2014) showed that some of these properties play an important role in the As solubility which are strongly related to this coefficient. Sauvé et al. (2000a) also reported some other soil properties like pH or OC as essentials to predict Kp values due to their influence on As solubility.

Internal arsenic concentrations in earthworms (AsE) varied between soils and increased with increasing exposure both AsT and AsW concentrations. AsE reported by other authors showed a wide range of values; Janssen et al. (1997) showed for *E. andrei*, in 20 different Dutch soils, a range from 2.99 to 65.2 μg As g^{-1} dry weight; García-Gómez et al. (2014) found for control soils an average of 18 μg As g^{-1} dry weight for *Eisenia fetida*; and Langdon et al. (2003) reported values between 3 μg As g^{-1} (soil total concentration 5 mg As kg^{-1}) and 900 μg As g^{-1} (soil total concentration 87 mg As kg^{-1}) based on a review for different species of earthworms. However, other authors showed lower concentrations. Peijnenburg et al. (1999) found in soils without arsenic pollution a mean tissue concentration of 3.75 μg As g^{-1} dry weight for *E. andrei*, and Beyer et al. (1985) gave an internal arsenic concentration of 5–6 µg As g^{-1} for *E. fetida*. In earthworms assay with different genera of the genus Eisenoides, Beyer and Cromartie (1987) found an internal concentration of 0.17–1.5 µg As g^{-1} dry weight, suggesting that different earthworm species showed great differences in metal accumulation. Fischer and Koszorus (1992) defined the maximal accumulation capacity at 902 µg As g^{-1} dry weight for *E. fetida* at sub-lethal exposure concentrations in long-term studies, which is close to our findings (maximal accumulation capacity obtained 1019 ± 167 µg As g^{-1} dry weight); at higher body concentrations, all earthworms died.

BSAF and BWAF values showed a decrease with increasing exposure levels in most of the studied soils, suggesting that arsenic is mainly autoregulated by *E. andrei* in these soils. Such negative relationship is common for metals (McGeer et al., 2003) and was found for molybdenum (Díez-Ortiz et al., 2010) and also for the bioaccumulation of As in *E. fetida* exposed to a mine soil containing high As concentrations (García-Gómez et al., 2014). As a consequence, the highest BSAF and BWAF values usually occur at low background levels and decrease as pollution levels increase (Williams et al., 2006), which suggests some control over As bioaccumulation. However, in soil H7, BSAF only increased at the highest total As concentration (Table 2), and in soils H4, H5 and H6, BSAF values showed an increase compared to the control at different treatments of As added. These cases suggest the earthworms may be capable of sequestering arsenic, leading to higher body concentrations than expected, but it remains unclear what is the mechanism of arsenic sequestration. Fischer and Koszorus (1992) reported BSAF values for As in *E. fetida* between 18.1 and 10.3 upon exposure to soils polluted with 23–87 mg As kg^{-1} soil, which are higher than the BSAF values found in our study with *E. andrei*.

Otherwise, BWAF values decreased with increasing soil As concentrations, except for soils H4 and H7 (Table 2). In soil H4, BWAF was higher than that in the control at 50 and 100 mg As kg^{-1} dry soil, while in soil H7, this was the case for all treatments except for the highest one (600 mg As kg^{-1}). These findings suggest that As concentrations in earthworms did not change proportionally with changes in water-soluble arsenic induced by increasing total As concentration in soil. US EPA (2007) also reported that for As, and other nonessential metals, accumulation is nonlinear with respect to exposure concentration. In general, BWAF values could be comparable to BCF due to both factors were calculated from the water-soluble arsenic. In this sense, the bioconcentration factor (BCF) proposed by the arsenic ambient water quality criteria (AWQC), calculated from the milligramme of As in a litre of water, is 44 L kg^{-1}, derived from BCF values of 1 for fish and 350 for oysters (Williams et al., 2006); in this paper, the highest BCF values reported by other studies were 1600 and 3091 L kg^{-1} in different fish species. Otherwise, US EPA (2003) suggested a BCF range between 150 and 10,000 L kg^{-1} for different species. Results obtained in our study showed in general also high BWAF values in the range of the reported values. Our results and those obtained by other authors highlight the potential of As for bioaccumulation in food chains (McGeer et al., 2003; Williams et al., 2006), although the variation in BSAF and BWAF values in relation to the contamination levels indicates that these are no good indicators of potential environmental risks (McGeer et al., 2003; García-Gómez et al., 2014).

Earthworm mortality was correlated with AsE in our studied soils, although total or soluble arsenic concentrations in soil should better explain the mortality observed in earthworms than the body accumulation (Table 5). In fact, internal concentrations in earthworms do not necessarily correlate with the concentration at the site or the toxic action (Smith et al., 2012; García-Gómez et al., 2014). However, in some of our studied soils, mortality did not reach 50 % at the highest As concentration added (600 mg As kg^{-1} soil), which reflects the importance of soil properties in reducing As availability and therefore toxicity. Mortality also was related to earthworm weight, with a significant trend to increasing earthworm weights at the highest As concentrations in soils H1, H5 and H6. This suggest that only the biggest earthworms survived arsenic exposure, although it might also be possible that surviving earthworms were more tolerant to As and benefitted from the reduced density and resulting higher food availability to grow bigger. Some authors suggest that earthworm weight loss could be caused primarily by soil factors, especially with soil pH, while body metal concentrations play a minor role (Janssen et al., 1997). In our study, weight variation appeared inversely related with pHH_2O (Table 6), as well as it was correlated with body As concentrations, finding the highest mortality in sample H6, soil with the lowest pH (Table 3). Moreover van Gestel et al. (1992) found that the growth of *E. andrei* was negatively related with reproduction, which also was found in this study (Table 5). Reproduction was more sensitive to As than survival. A large variation in juvenile production was found in the different tested soils, which could be due to the variation in soil properties (van Gestel et al., 1992). According to the OECD guideline 222, the requirements for the control sample need that the earthworm mortality should be less than 10 % and juvenile production higher than 30 juveniles. Control reproduction was good in all samples with the exception of H1 soil, being that all earthworms were taken from the same batch, it must be concluded that the low reproduction in soil H1 was due to the unfavorable properties of the soil rather than to problems with the health of the tested animals. Juvenile production was negatively correlated with total and water-soluble As concentrations in the soils and with internal concentrations in the earthworms. Reproduction was lowest in soil H7, having a high clay content (which could affect water availability) and a high aluminium content, that could have influenced in the reduction of growth and juvenile production (van Gestel and Hoogerwerf, 2001). Optimal environmental conditions for the reproduction of *E. andrei* are high OC content, pH ($CaCl_2$) between 4.5 and 6.5, and a moisture content of 50 % approximately of the maximum soil water holding capacity (van Gestel et al., 1992). Control conditions in soil H5, with a pH-KCl of 6.7 and high organic carbon content, probably were most favourable, explaining the high number of juveniles produced in this soil, with an average of juveniles per worm per week of 6 ± 0.6.

The reproduction of *E. andrei* was more sensitive to arsenic than the other studied parameters; thus, the obtained toxicity endpoints could be considering more accurate. Root elongation test performed with *Lactuca sativa* on the same soils (Romero-Freire et al., 2014) showed similar EC10 values for total As compared with *E. andrei* reproduction in soils H4 and H5 (95 mg kg^{-1} and 38 mg kg^{-1} soil values for lettuce, respectively). Soil H3 showed a more restrictive EC10 for lettuce (23 mg kg^{-1} soils) than for earthworms, with values differing by a factor of two. In soil H7, lettuce was less sensitive (395 mg kg^{-1}), while earthworm reproduction gave an EC10 of 59 mg kg^{-1} in the same soil. These results highlight the influence of soil properties on arsenic bioavailability and therefore toxicity and also the importance of selecting different organisms when defining guideline values for ERA.

The study of the influence of soil properties in As toxicity for earthworm showed controversial results. Other studies with different pollutants showed pH, CEC, Ca content, Mn oxides, OC content, clay and silt content as the most relevant factors affecting earthworm toxicity for metals (Janssen et al., 1997; Peijnenburg et al., 2002; Bradha et al., 2006; van Gestel et al., 2011). However, our results showed that earthworm mortality in different As-treated soils was greatly explained by As soil concentration as well as internal concentration in earthworms and low influence of soil properties was found. Earthworm weight variation, also related with the studied forms of As, was observed that could increase with the rise in I strength of the soils as well as could be indirectly related with pH (highest pH lower weight variation). While juvenile production in our study decreased with higher mortality or earthworm loss body weight, the ionic strength, the pH and the presence of iron may be considered as soil properties which could have a role in earthworm answer to As toxicity. Our study suggested that the influence of soil properties in As solubility and availability indirectly control the influence As toxicity in earthworms. However, related to other bioassays performed with lettuce and a marine bacteria (*V. fischeri*) (Romero-Freire et al., 2014), soil properties had a direct influence in their toxicity answer. In this sense, As behaviour in soils and the complex pathways of incorporation in body oligochaetes should be further studied to propose robust generic environmental quality standards.

Finally, in this study, we worked with soils contaminated with concentrations chosen according to the proposed values for Andalusia representing the criteria to declare a soil as contaminated for different soil uses (Aguilar et al., 1999). The selection of these values was based on data levels proposed by other countries. However, these values do not take into account the difference in soil types. Otherwise, laboratory-spiked soils instead of field-contaminated soil were used in this study because spiked soils tend to overestimate the availability of metals in field soils (Smolders et al., 2009); therefore, the toxicity level defined can provide with more certainty the safety threshold for the environmental risk assessment (ERA). It is therefore necessary to further investigate mobility and bioavailability of As in the ecosystem in relation to soil properties to propose reference values useful in the declaration of contaminated areas.

4.5. Conclusions

Arsenic solubility and therefore availability were largely explained by soil properties and constituents such as pH, calcium carbonate content, oxides forms, clay content and ionic strength of the soil solution and should be considered essential parameters influencing arsenic toxicity in soils. Internal arsenic concentration (AsE) in the studied earthworms (*Eisenia andrei*) increased directly with As solubility in soils which highlight the potential of As for bioaccumulation in food chains. Moreover, AsE varied between soils suggesting that earthworms could be able to sequester arsenic. Further studies to deepen in the relationships between As toxicity of earthworms and soil properties should be performed. The results of the present study in relation to the earthworm answer against As toxicity indicated that earthworm survival, body weight variation and earthworm reproduction are strongly influence by solubility of arsenic as well as AsE; therefore, soil properties could greatly reduce indirectly As bioavailability and toxicity risk for the studied oligochaeta. The comparison of the obtained results with other different organisms against As toxicity showed a high variability and therefore indicated the importance of selecting different organisms when defining guideline values for ERA.

CHAPTER 5

Effect of soil properties on the toxicity of Pb: Assessment of the appropriateness of guideline values

Abstract

Soil contamination with lead is a worldwide problem. Pb can cause adverse effects, but its mobility and availability in the terrestrial environment are strongly controlled by soil properties. The present study investigated the influence of different soil properties on the solubility of lead in laboratory spiked soils, and its toxicity in three bioassays, including *Lactuca sativa* root elongation and *Vibrio fischeri* illumination tests applied to aqueous extracts and basal soil respiration assays. Final aim was to compare soil-dependent toxicity with guideline values. The *L. sativa* bioassay proved to be more sensitive to Pb toxicity than the *V. fischeri* and soil respiration tests. Toxicity was significantly correlated with soil properties, with soil pH, carbonate and organic carbon content being the most important factors. Therefore, these variables should be considered when defining guideline values.

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5.1. Introduction

Lead is a non-essential metal, without metabolic function, that is ubiquitous in the soil. Although it is one of the less mobile heavy metals, lead is easily accumulated in plants when bioavailable (Youn-Joo, 2006). Its average concentration in the Earth's crust is 16 mg kg^{-1} (Nriagu, 1978), whereas natural uncontaminated soils have a mean value of 40 mg kg^{-1}(Davies, 1983). Due to its low mobility, Pb tends to accumulate mainly in the top soil layers (Martín et al., 2014). Finding Pb concentrations in agricultural, urban and industrial soils well defined to different countries (Markus and McBratney, 2001). Around the world, different authors identified a range of Pb concentrations from 0.5 mg kg^{-1}(Dickson et al., 1983) to 16,338 mg kg^{-1} (McGrath and Loveland, 1992). Lead has become widely distributed across the globe as a result of man's actions (Cheng and Hu, 2010), which can cause adverse effects on human health and the environment (Pirkle et al., 1998). The majority of soil Pb comes from anthropogenic sources (USEPA, 2006), including historic ones (e.g. road gasoline emissions). Nowadays, Pb is used widely in buildings, lead-acid batteries, bullets and shot, weights, solder, pewter, and fusible alloys (Cheng and Hu, 2010). These different uses resulted in a registered accumulation of Pb in surface soils exposed to various pollution sources at some sites to levels as high as 135,000 mg kg^{-1}(Adriano, 2001).

Due to its low mobility, properties and constituents of soils are the main factors that explain the different levels of lead toxicity to soil organisms (McLean, 1992). Nevertheless the different risk levels proposed by different countries largely do not take into account soil properties and also cover a wide range of soil concentrations. Moreover these values are based on different national strategies in environmental policies which is reflected in a large variation among soil guideline values (Carlon, 2007). Guideline concentrations for soil Pb vary enormously, with reference values in relation to land use, e.g. residential areas, ranging from 100 mg kg^{-1} in Norway (Ottesen et al., 2008) to 300 mg kg^{-1} in Australia (Laidlaw and Taylor, 2011) and 350 mg kg^{-1} in China (Ren et al., 2006). Other authors propose risk levels that do consider soil properties. Rodrigues et al. (2012), for example, mention soil threshold total concentrations for Pb depending on Alox concentrations from 51-411 mg kg^{-1} at Alox=50 mmol kg^{-1} to 82-789 mg kg^{-1} at Alox=150 mmol kg^{-1}. The Netherlands propose 85 mg kg^{-1} as a target value corresponding with a negligible risk and 530 mg kg^{-1} as the intervention level, independent of soil use but taking into account soil properties (standard soil: 25% clay, 10% OM) (Vegter, 1995). In Andalusia, values proposed for agricultural soils are 350 mg kg^{-1} at $pHH_2O<7$ or 500 mg kg^{-1} at pH>7 (Aguilar et al., 1999).

The chemistry of Pb in soils is affected by three main factors: specific adsorption to different solid phases, precipitation of sparingly soluble compounds, and the formation of relatively stable complexes or chelates with the soil organic matter (Bradl, 2004). Pb accumulation and distribution in soils is dependent on mineral grain sizes (Appel and Ma, 2002). Soil pH is another important factor contributing to the potential retention of Pb by the formation of Pb precipitates at high pH (Harter, 1983; Levonmäki and Hartikainen, 2007). Soil pH is also controlling the solubility of metal hydroxides, metal carbonates and phosphates, hydrolysis, ion-pair formation, organic matter solubility, as well as the surface charge of iron and aluminum oxides and clay edges (McBride, 1994; Sauvé et al., 1997; Shaheen, 2009). Moreover, some authors have reported Pb fixation by soil organic matter as an important factor (Lil and Shuman, 1996; Kabata-Pendias 2011), explaining the accumulation of lead in the upper horizon of soils where organic matter contents is highest (Johnson and Petras, 1998; Sipos et al., 2005).

Since total metal concentration bears little information on the potential risk (Lanno et al., 2004), the use of biological tests is a key tool for determining the bioavailability and toxicity of metal(oid)s. Bioassays and toxicity tests therefore are crucial for assessing the actual ecological risk and to support legislation regarding contaminated soils (Royal Decree 9, 2005). Furthermore, a battery of toxicity tests using a number of representative species of different taxonomic groups is needed to obtain proper insight into the potential hazard of a chemical for the ecosystem (van Gestel, 2012). In addition, it is important to assess how soil properties may affect the toxicity of metals and whether the influence of soil properties is consistent across different species of test organisms. Bioassays may provide direct insight into the exposure of organisms to potentially toxic elements (Petänen et al., 2003), But it is good to mention that, although they are quick and reproducible, standard laboratory bioassays usually overestimate metal bioavailability because of the lack of chemical equilibration (Degryse et al., 2007; Smolders et al., 2009). Moreover toxicity on salt spiked soils may also be aggravated compared to that of the field-contaminated soils because of the associated pH decrease and by the increased salinity (Cheyns et al., 2012). Therefore, the toxicity response in the laboratory spiked soils is usually higher than in the *in situ* contaminated soils. Since laboratory toxicity data generally are used, this may lead to more strict safety levels for environmental risk assessment. However, the application of the safety levels gathered on the legislation could be unsuitable from a diagnostic point of view, because toxicity test usually measure the toxic effects of metals on a limited number of soils and organisms. Moreover, the complexity increases in the field studies because pollution often is caused by a mixture of elements which can mask the problems that could be attributed only to the study metal (Spurgeon and Hopkin, 1995; Li and Thornton, 2001). The aim of this study is to determine the influence of different soil properties on the availability of lead in laboratory-spiked soils and to assess its toxicity in three different toxicity tests, using different organisms and processes. By choosing test concentrations corresponding with threshold values for Pb defined in different Spanish regulations, results from this study could be used to validate risk limits for Pb in soils.

5.2. Materials and methods

5.2.1. Test soils

Seven soils with different properties, representing most of the main soil groups in Spain were selected (Table 1). The main parameters analyzed were: soil pH (pHS, measured in a soil:water ratio 1:2.5), electrical conductivity (EC), texture, organic carbon (OC) content, water holding capacity (WHC), cation exchange capacity (CEC), specific area (SA) and calcium carbonate content ($CaCO_3$). These properties were determined according to the official methods of analysis (MAPA, 1994). Moreover free and amorphous iron, aluminum and manganese oxides were analyzed according to Holmgren (1967) and Schwertmann and Taylor (1977), respectively.

Total soil lead concentration (PbT) was determined after acid digestion of the soils in strong acids (HNO_3 + HF). Water extractable Pb concentration (PbW) was determined in soil:water extracts (1:1 ratio) obtained by shaking for 24 h, followed by centrifuging for 20 min at 3500 rpm and extraction with 10 cm Rhizon MOM (Metal and Organic Matter) soil moisture samplers. In all cases, Pb concentration was measured by Inductively Coupled Plasma-Mass Spectrometry using an ICP-MS NEXION 300D spectrometer. The accuracy of the method was corroborated by analyses of standard reference material SRM 2711; the mean (±SD) experimental value for Pb was 1138±11.0 mg kg^{-1} (n= 6) and the certified value was 1162±31.0 mg kg^{-1}.

5.2.2. Soil spiking

Soils were contaminated in the laboratory with lead (II) nitrate ($Pb(NO_3)_2$: PanReac-ApliChem, 98% purity) in increasing concentrations according to the reference values proposed by the Junta de Andalusia (Aguilar et al., 1999) (500-1000-2000 mg kg^{-1}) and adding two more levels of extreme pollution (4000-8000 mg kg^{-1}). Furthermore an uncontaminated sample (control) was included. The contamination was performed by spiking individual samples of 50 g of soil with aqueous Pb solutions, in triplicate. After spiking, the soils were brought to their 60% of water holding capacity (WHC). The soils were incubated for 4 weeks at 25 ± 1 °C and 60% air humidity, with a light/dark cycle of 10/14 hours. Soil moisture content was checked and if needed readjusted weekly. The incubation period chosen allows stabilization of the Pb added, and duration was based on similar studies by other authors (Fendorf et al., 2004; Romero-Freire et al., 2014).

Total Pb concentrations in the soils after incubation were measured by XRF with a NITON XLt 792 instrument. In all cases, the measured concentrations differed less than 10% from the total estimated concentrations (background value + added Pb). Furthermore a saturation extraction with soil:water ratio 1:1 was performed. Soil solutions were obtained after 24 hours of shaking followed by extraction with a 10 cm Rhizon MOM. In the obtained water extracts, pH (pHW), electrical conductivity, and water-extractable lead concentrations (PbW) were measured. All measurements were performed within 48 hours of obtaining the extracts.

5.2.3. Toxicity tests

Three toxicity tests were selected for this study:

1. Seed germination/Root elongation Toxicity Test, according to US EPA (1996) recommendations. This test assesses the phytotoxic effects on seed germination and initial seedling growth (Torres, 2003). In Petri dishes 15 seeds of *Lactuca sativa* were incubated in 5 ml of aqueous extract from the Pb-spiked soils. The dishes were placed in an incubator at 25 ±1 °C and the number of seeds germinated and the length of the germinated roots seeds were measured after 120h. The endpoint calculated was root elongation reduction (LsR) compared to the control.

2. Microtox® test (ASTM, 2004) based on the reduction of the amount of light emitted by a non- pathogenic strain of luminescent marine bacterium *Vibrio fischeri* upon exposure to a toxic sample (Ribó and Kaiser, 1987). The test was performed in a Microtox 500 analyser from Microbics Corporation, according to a modification of Microtox Basic Test for Aqueous Extracts Protocol (AZUR, 1998; Martín et al., 2010). The luminescence was measured before (0 min) and 15 minutes after mixing the bacteria with aqueous extracts of the Pb-spiked soils. The results were expressed as the luminescence reduction (VfR) in the sample compared to the control.

3. Heterotrophic soil respiration (Rs) was measured by determining the CO_2 flux from treated soils with a microbiological analyzer μ-Trac 4200 SY-LAB model. Soil moisture content was fixed at field capacity and soils were incubated at a constant temperature of 30 °C. CO_2 production was determined by absorption in vials with a solution of potash (KOH 0.2%) during 97 hours, and related to the mass of soil used to obtain a measure of respiration. The results were expressed as the respiration reduction (RsR) compared to the control.

All three toxicity tests were run in triplicate and responses were scaled to range from 0, for samples that did not exhibit toxicity, to 100, for samples with maximum toxicity.

5.2.4. Data analysis

The normal distribution of all variables included in the database was checked by a Kolmogorov-Smirnov test, and significant differences were determined by ANOVA and multiple comparisons were performed with Tukey's test ($p < 0.05$). In order to analyze the influence of soil properties on the extractability and toxicity of lead, Spearman's correlations were performed, whereas factor analysis was applied from a rotated component analysis by the method of Varimax with Kaiser normalization. All these analyses were performed with a confidence level of 95% by using SPSS v.20.0 (SPSS Inc., Chicago, USA).

The effective concentrations causing a 50% and 10% reduction in the endpoints (EC50, EC10) and the corresponding 95% confidence intervals were obtained by the fitting a log-logistic model to the data (Doelman and Haanstra, 1989), using the method of Marquardt (proc NLIN, SAS 9.1, SAS Institute, Cary, NC). The No-Observed Effect Concentration (NOEC) was calculated by analysis of variance and Tukey's test in multiple range data from the log-logistic model (Romero-Freire et al., 2014).

Table 1. Main properties of the soils selected for this study on the toxicity of Pb to different soil organisms and processes.

Soil	**Soil Type** **(WRB, 2006)**	**Soil horizon**	**Background Pb** (mg kg^{-1})	**pH** (H_2O)	**pH** (KCl)	**$CaCO_3$ (%)**	**OC (%)**
H1	Calcaric Cambisol (humic)	Ah	72.6	7.96	7.63	37.1	5.43
H2	Calcaric Cambisol (humic)	Bw	19.3	8.67	8.11	72.4	0.42
H3	Calcaric Kastanozem (antric)	Ck	4.47	8.79	8.24	92.3	0.38
H4	Leptic Cambisol (eutric)	Bw	20.7	6.74	5.80	nd	0.61
H5	Leptic Regosol (eutric)	Ah	120	7.20	6.72	nd	8.22
H6	Leptic Regosol (distric)	C1	5.07	5.87	4.58	nd	0.49
H7	Cutanic Luvisol (chromic)	Bt	31.1	7.03	5.86	0.92	0.66

Soil	Soil Type (WRB, 2006)	SA (m2 g^{-1})	Clay (%)	CEC (cmol+ kg^{-1})	P (mg kg^{-1})	Fe$_o$ (g kg^{-1})	Mno (g kg^{-1})
H1	Calcaric Cambisol (humic)	0.047	23.6	21.4	8.26	0.68	0.06
H2	Calcaric Cambisol (humic)	0.008	11.8	9.83	bdl	0.20	0.02
H3	Calcaric Kastanozem (antric)	0.003	7.7	2.94	bdl	0.01	bdl
H4	Leptic Cambisol (eutric)	0.015	19.1	9.91	6.53	0.52	0.18
H5	Leptic Regosol (eutric)	0.057	23.8	25.9	28.1	0.65	0.41
H6	Leptic Regosol (distric)	0.004	8.3	3.83	1.09	1.00	0.09
H7	Cutanic Luvisol (chromic)	0.083	54.8	15.5	bdl	0.78	0.03

bdl: below detection limit; nd: not detected; $CaCO_3$: Carbonate content; OC: organic carbon; SA: specific area; CEC: cation exchange capacity; P:Phosporus content; Fe_o/Mn_o: amorphous forms

5.3. Results and discussion

5.3.1. Lead availability and influence of soil properties

The water-extractable lead (PbW) concentrations showed significant differences between the different soils especially at the higher added Pb levels (Table 2). The highest PbW concentrations were found in soil H6 (sandy-acidic soil), with values exceeding 30% of the total Pb concentration for the two highest spiking levels. High PbW concentrations were also found in H4, a soil similar to H6 but with higher clay content and lower acidity. In this case water-extractable Pb concentrations reached up to 18% of total Pb at the highest contamination level. Water-extractable Pb concentration in H7 (clayey-neutral-pH) reached a maximum of 2.6% of the total Pb at the highest contamination level. The carbonate-rich soils (H1, H2 and H3) had much lower water-extractable Pb concentrations, below 0.2% of the total Pb content, with the lowest values measured in the high organic carbon soil (H1). Finally, soil H5 (rich in organic carbon) had the lowest water-extractable Pb concentration of all carbonate-poor samples, with < 0.1% of the total Pb being extractable.

Table 2. Mean water-extractable Pb concentrations in soils H1 to H7 at the different contamination levels applied by spiking the soils with $Pb(NO_3)_2$ (PbT: Total Pb added to the soils in mg kg^{-1}). See Table 1 for a description of the soil properties.

	PbW (mg kg^{-1})						
PbT	H1	H2	H3	H4	H5	H6	H7
500	0.04 a	<0.01 a	0.01 a	0.01 a	0.06 a	0.42 a	0.01 a
1000	0.08 ab	0.01 a	0.03 a	0.10 a	0.13 a	13.7 a	0.05 a
2000	0.11 ab	0.05 a	0.08 a	2.43 a	0.22 a	275 b	1.32 a
4000	0.30 b	0.31 b	0.24 a	77.9 a	0.52 b	1405 c	23.3 a
8000	0.88 c	1.02 c	8.95 b	1471 b	1.41 c	2575 d	209 b

Lowercases indicate significant differences between treatments (Tukey, $p<0.05$).

Correlation analysis (Table 3) did not find any significant correlations between PbW and total Pb for most of the contamination levels. The soil properties with the highest influence on Pb extractability were pH (in soil and in soil solution) and calcium carbonate content, and the effect of these parameters increased with increasing contamination level. The role of pH in controlling the Pb concentration in the soil solution is well known (Bur et al., 2012; Ming et al., 2012). Most important in our study is the finding that Pb extractability was below 0.01% of the total Pb content for treatments up to 8000 mg kg^{-1} in the case of the carbonate and organic-rich soil H1. In this case, retention of lead by the soil organic matter can be attributed to the formation of organic complexes (Coppola et al., 2010). Different authors have observed that high levels of organic matter, mainly found in surface soil layers, are an important sink for lead (Sánchez-Camazano et al., 1998; Kabata-Pendias, 2011).

Another significant parameter influencing Pb mobility is related to the soil texture, which relates to the different properties of the soil particles involved like cation exchange capacity and specific surface area that increase with decreasing particle size. This is shown by the positive correlation of water-extractable Pb concentrations with the sand fraction and the negative correlation with the silt fraction (Table 3). These results are consistent with Hooda and Alloway (1998), who found an inverse relationship between Pb retention and the sand fraction, and with the decrease in Pb availability with increasing clay content described by Bur et al. (2012). In our study, the correlation of Pb extractability with clay content was positive but not significant. Maybe the type of clay played a role (Appel and Ma, 2002), with adsorption of Pb to soils being most efficient at high illite contents (Yang et al., 2006). In our case, illite content was highest in soil H7 (>65% of the total clay fraction), which agreed with the low water-extractability of Pb in this carbonate-poor and low organic carbon soil. Available Pb was also found to correlate with the abundance of fine soil fractions, especially silt and very fine sand (Luo et al., 2011). Moreover, Pb extractability was significantly and directly correlated with the Fe and Mn oxide fractions in all treatments except for the highest contamination level. The presence of these oxides and the release of their colloidal forms into the water extraction seems to contribute to lead solubility. This result contradicts the suggestion of several authors that Pb is strongly associated with reducible fractions (Fe and Mn oxides phases) (Li and Thornton, 2001; Sipos et al., 2005; Iavazzo et al., 2012; Boussen et al., 2013).

Table 3. Correlation coefficients (Spearman) relating water-extractable lead concentrations (PbW) with soil properties in seven soils spiked with different concentrations of $Pb(NO_3)_2$ (Total Pb added in mg kg^{-1}). (pHW: pH in the water extract; pHS: soil pHin a soil:water ratio 1:2.5; Feo and Mno: amorphous iron and manganese forms). See Table 1 for soil properties and Table 2 for PbW concentrations.

Treatment	PbT	pHW	pHS	$CaCO_3$	Sand	Coarse Silt	Fe_o	Mno
0	0.375	0.017	-0.645**	-0.562**	0.664**	-0.589**	0.695**	0.808**
500	0.032	-0.075	-0.524*	-0.464*	0.510*	-0.478*	0.623**	0.630**
1000	-0.032	-0.201	-0.787**	-0.738**	0.682**	-0.734**	0.662**	0.788**
2000	-0.174	-0.665**	-0.936**	-0.958**	0.656**	-0.943**	0.721**	0.607**
4000	-0.214	-0.781**	-0.915**	-0.915**	0.668**	-0.910**	0.681**	0.594**
8000	-0.661**	-0.905**	-0.709**	-0,729**	0.436*	-0.717**	0.429	0.267

* $p<0.05$

**$p<0.01$

5.3.2. Lead ecotoxicity

Toxicity of Pb for *Lactuca sativa* (LsR) showed large differences between soil types (Figure 1a). Carbonate-rich soils (H1-H3) showed a very low phytotoxicity, with reductions below 20% at Pb concentrations of 1000 mg kg^{-1} and less than 50% reduction for the highest concentration (8000 mg kg^{-1}). Non-carbonate soils (H4-H7) showed greater toxicity, with lower toxicity in soil H5 (rich in organic carbon) than in the other soils in this group. Soil H6 (sandy-acidic) showed the highest toxicity, with reductions in root elongation higher than 70% at added Pb ≥ 2000 mg kg^{-1}, and 100% reduction at > 4000 mg kg^{-1}. The NOEC, EC10, EC25 and EC50 values and their 95% confidence intervals for the endpoints obtained in this assay are summarized in Table 4. EC50 values could not be calculated for carbonate-rich soils. The non-carbonate soils H6 and H7 showed the highest toxicity when comparing EC50 values. Based on EC10 values, soil H3 (highly carbonated and sandy texture) was most toxic.

Table 4. NOEC, EC10, EC25 and EC50 values with corresponding 95% confidence intervals (C.I.) for the toxicity of Pb in the *Lactuca sativa* root elongation test applied to aqueous extracts of different soils (see Table 1) spiked with $Pb(NO_3)_2$. Values are expressed as Total Pb (mg kg^{-1}) concentrations in the test soils.

				***Lactuca sativa* test**			
Soil	**NOEC**	**EC10**	**C.I.**	**EC25**	**C.I.**	**EC50**	**C.I.**
H1	<500	499	-	>8000		>8000	
H2	500	136	758 - 2451	3360	1651 - 5068	>8000	
H3	<500	254	76 - 845	1564	455 - 2673	>8000	
H4	<500	1097	151 - 7962	1933	781 - 3086	3479	1826 - 6628
H5	<500	3452	2211 - 5391	4643	3394 - 5892	6240	5185 - 7509
H6	<500	498	382 - 649	805	674 - 936	1303	1160 - 1464
H7	500	344	189 - 627	768	451 -1085	1765	1360 - 2291

C.I. 95% confidence intervals;"-" represents wide 95% CI.

The toxicity test with *Vibrio fischeri* (VfR) (Figure 1b) showed similar results as the *L. sativa* test, although with proportionally lower toxicity for all soils. The lowest toxicity was found in the carbonate-rich soils (H1-H3), although >80% reduction was observed in soil H3 at the highest Pb concentration. Non-carbonate soils showed higher toxicity and significant differences were found within this group. Toxicity was lowest in soil H5, with reductions below 20% at all Pb concentrations. The highest reduction of luminescence was found in soil H6 at concentrations ≥ 1000 mg kg^{-1} and soil H7 at concentrations ≥ 2000 mg kg^{-1}.

Toxicity to *V. fischeri* occurred in a narrower range and EC25 and EC50 values were greater than the highest added Pb concentration in many cases. Only for soils H4 and H7 EC50 values could be derived, which amounted 3966 and 2259 mg Pb kg^{-1}, respectively. EC10 values were lowest in non-carbonate and low organic carbon soils (H6, H4 and H7). Due to the scatter in the data, NOEC in some cases was higher than the EC10 (Table 5).

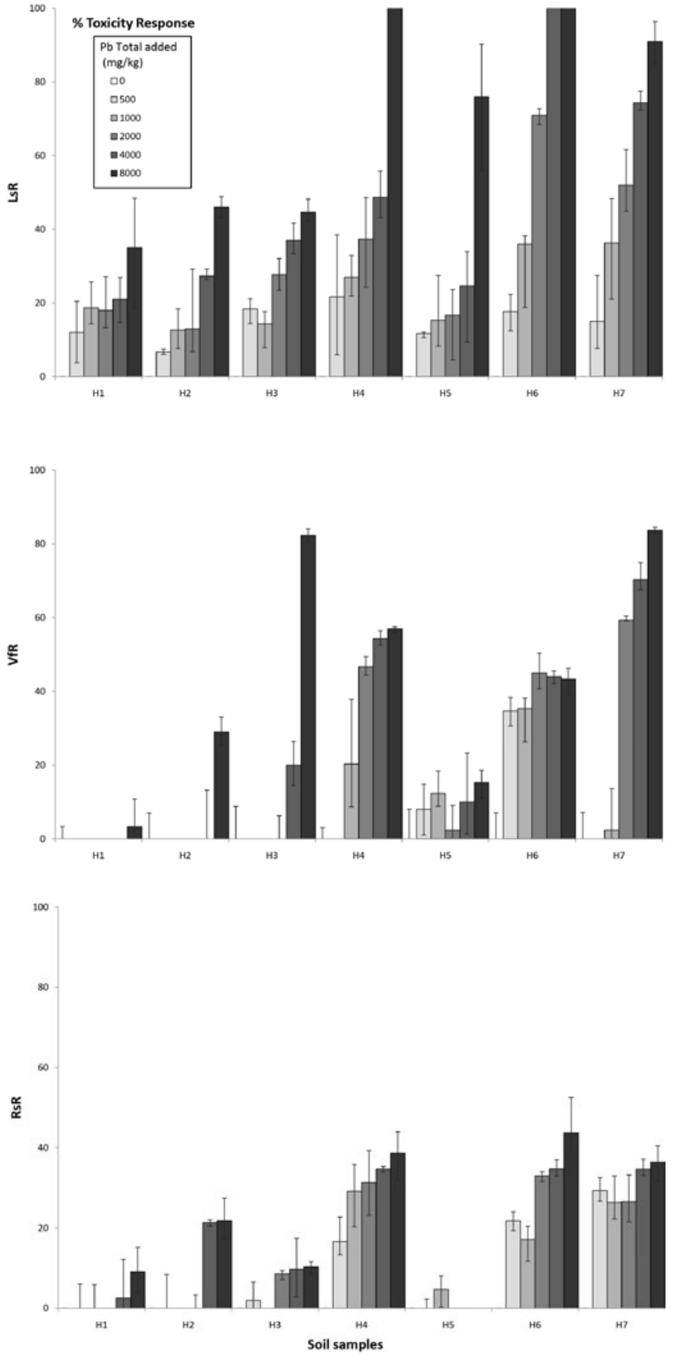

Figure 1. Toxicity of lead (% reduction in relation to the control) in different soils spiked with $Pb(NO_3)_2$ and measured with the *Lactuca sativa* root elongation (LsR) (a) and *Vibrio fischeri* bioluminescence (VfR) (b) tests in aqueous soil extracts or with the Soil respiration (RsR) (c) test performed on the soil solid phase. See Table 1 for a description of the soils and Table 2 for the water-extractable Pb concentrations.

Table 5. NOEC and EC10 values for the toxicity of Pb in the *Vibrio fischeri* bioluminescence test applied to aqueous extracts and in the soil respiration test applied on solid phase of different soils (see Table 1) spiked with $Pb(NO_3)_2$. Values are expressed as Total Pb (mg kg^{-1}) concentrations in the test soils.

	Vibrio fischeri test			**Soil respiration test**		
Soil	**NOEC**	**EC10**	**C.I.**	**NOEC**	**EC10**	**C.I.**
H1	1000	>8000		>8000	>8000	
H2	4000	5337	4357 - 6537	2000	3128	1797 - 5446
H3	4000	2901	1798 - 4682	4000	5951	-
H4	500	386	138 - 1082	<500	90	7 - 1242
H5	≥8000	2473	-	>8000	>8000	
H6	<500	8	1 - 257	<500	122	20 - 758
H7	1000	744	476 - 1164	<500	45	1 - 2477

C.I. 95% confidence intervals;"-" represents wide 95% CI.

The Soil respiration test (RsR) (Figure 1c) showed the lowest Pb toxicity in the organic-rich soils H1 and H5, followed by the carbonate-rich soils H2 and H3. The highest toxicity was found in the non-carbonate soils. This test showed the smallest reduction, therefore no EC50 values could be estimated. Based on estimated EC10 values, Pb was most toxic in the non-carbonate soils (H4, H6, H7). In the organic rich soils (H1, H5), Pb did not cause any toxicity, not even at the highest test concentration (Table 5).

Among the three toxicity tests carried out, the *L. sativa* (vascular plant) test was the most sensitive. Pb toxicity to *L. sativa* spanned a wider concentration range and also showed stronger significant differences between soils obtained the best dose-response curves for this assay. Romero-Freire et al. (2014) and Escoto et al. (2007) recommend the *L. sativa root* elongation test for assessing the toxicity of natural and artificially contaminated soils. Furthermore, tests performed on aqueous extracts are suitable for assessing the short-term potential toxicity because this relates to the easily water-extractable metal forms.

The soil respiration assay (soil microorganisms) presented the lowest sensitivity to lead. The soils with the lowest toxicity in all three tests were rich in carbonate (H2 and H3) and organic carbon (H1 and H5). The non-carbonate soils and low organic matter soils (H4, H6, H7) were the most sensitive in all three tests. These results agree with An et al. (2012) and Kim et al. (2002) who observed that an increase in Ca^{2+} (and Mg^{2+}) content decreases lead toxicity. Other authors (Ming et al., 2012; Cheyns et al., 2012) demonstrated that bioavailability of Pb is pH dependent, with the bioavailable fraction of Pb decreasing with increasing pH. This explains why soils H2 and H3 showed a lower toxicity. The results of the toxicity tests agree with the availability of lead measured in the water extracts, showing that adsorption of Pb onto soil organic matter in high organic matter soils may not only limit its extractability and but also its bioavailability (Sauvé et al., 2000b; Gustafsson et al., 2011; Fleming et al., 2013).

The correlation between the responses of the three toxicity tests in all samples tested was significant (LsR-VfR: 0.688**; LsR-RsR: 0.755**; VfR-RsR: 0.652**). There also was a significant and direct correlation between the test responses (LsR, VfR and RsR) and PbW and PbT (Table 6), while responses were inversely correlated with pHW, pHS and $CaCO_3$. Soil pH (pHS) had a lower influence on toxicity than the soil-solution pH (pHW), highlighting the importance of the measurement of the soil solution pH. Other soil properties like OC and CEC seemed to have little effect on lead toxicity when all soils were grouped, although higher correlation coefficients were found when considering only the non-carbonate soils. This difference could be related to the strong effect of pH (directly related to the presence of calcium carbonate) on Pb availability, which could mask the effect of other variables on Pb toxicity.

Table 6. Correlation between the responses of different toxicity tests (*Lactuca sativa* root elongation (LsR), *Vibrio fischeri* bioluminescence (VfR), soil respiration (RsR)), applied to soils spiked with $Pb(NO_3)_2$ (expressed as % inhibition) and Pb forms (PbW: extractable-in-water Pb; PbT: total Pb) in different test soils and the main soil properties (pHW: pH in the water extract; pHS: soil pH in a soil:water ratio 1:2.5; OC: organic carbon; CEC: cation exchange capacity). See Table 1 for a description of the soils, Table 2 for water-extractable Pb concentrations and Figure 1 for the bioassay responses.

	All soils			**Non-Carbonate soils**		
	LsR	**VfR**	**RsR**	**LsR**	**VfR**	**RsR**
PbW	0.892**	0.761**	0.711**	0.899**	0.837**	0.718**
PbT	0.799**	0.518**	0.478**	0.827**	0.675**	0.489**
pHW	-0.712**	-0.698**	-0.838**	-0.763**	-0.707**	-0.853**
pHS	-0.286*	-0.418*	-0.455**	-0.226	-0.200	-0.345*
OC	-0.099	-0.067	-0.237*	-0.266*	-0.253*	-0.460*
$CaCO_3$	-0.289*	-0.431*	-0.498**	-0.235*	-0.251*	-0.471*
CEC	-0.123	-0.146	-0.256*	-0.248*	-0.254*	-0.448*

Other studies with similar tests confirm our results. In soil respiration tests, for instance, higher soil OC content (and also higher CEC), was related with a lower inhibition of CO_2 emission from soils contaminated with Pb (Doelman and Haanstra, 1979; Nwachukwu and Pulford, 2011). Furthermore, high concentrations of lead were shown to cause an immediate decrease of basal soil respiration (Liao et al., 2007). The effect of soil amendments checked with the *L. sativa* test showed a lower Pb toxicity at higher pH and higher OC content (Ahmad et al., 2012). In tests performed with lettuce and ryegrass on three natural soils artificially spiked and in two natural contaminated soils, Sanderson (2008) found that the plant response was strongly related with pH and clay content of the soils. Kim et al. (2002), using assays with rice, observed that high levels of Ca and Mg decreased Pb content in the plant roots. Similar trends were found for *V. fischeri* where An et al. (2012) also observed that higher concentrations of Ca and Mg in soils caused lower toxicity of Pb.

5.3.3. Comparison with soil guideline values

Guideline values can be defined by using background concentrations added up with safe concentrations derived from laboratory toxicity tests. Usually NOEC values are used for that purpose (Crommentuijn et al., 1994). In many countries guideline values are based on total concentrations estimated from the literature or "imported" from other countries without considering the influence of soil properties (Carlon, 2007). In the case of Spain, each Regional Government has the power to define guideline values for regulatory purposes (Royal Decree 9, 2005) and to set the criteria that declare a soil as contaminated. In Andalusia (Southern Spain), the Regional Government proposed guideline values based on literature data and according to the use of the soil: agriculture, natural and industrial use (Aguilar et al., 1999), discriminating for pH only in the case of agricultural use and only for some elements (Table 7). Taking into account soil properties is needed to further refine the risk limits for Pb in soils in order to optimize decisions on soil remediation. Spanish regulation (Royal Decree 9, 2005) also suggests to estimate generic reference levels (GRL) by using toxicity tests with different organisms, selecting the most sensitive test value. Therefore in our study, the *L. sativa* test was selected to propose indicative guideline values (Table 7). According to the guidelines values proposed by the Regional Government of Andalusia (Spain), and based in the toxicity bioassay with *L. sativa*, we propose the following endpoints as intervention levels: to apply EC10 values for deriving guidelines for agricultural use, EC25 values for natural areas and the EC50 values for industrial areas. In general, these values were consistent with the regulation by the Regional Government in the study area but providing greater accuracy according to different soil types.

Advantage of this approach is that data from the same tests can be used, with slopes of the dose response curve determining the different ECx levels. The slopes of the dose-response curves are dependent on Pb concentrations, but may also be affected by soil properties. Based on our results we therefore propose a methodology to establish future guideline levels taking into account the soil properties (Table 7) that have the greatest influence on the potential toxicity of Pb: calcium carbonate and organic carbon content. Comparison of our proposal and the guidelines from the Andalusia Government indicates some discrepancies. The most important differences occur in the soils with agricultural use, where the guideline values for Andalusia seems to underestimate the potential toxicity of Pb. In other cases, the Andalusian guideline values may overestimate potential toxicity. The differences between soil types should be considered in the declaration of contaminated soils and in the setting of remediation goals for decontamination of polluted areas, which may enable optimised and more profitable land recovery efforts.

In other countries, the establishment of guideline values for metals in soil is not always taking into account soil properties, and if they do, different approaches are applied. In

Belgium and The Netherlands, for instance, guidelines are corrected for organic matter and clay content, while in the UK organic matter content and soil pH are the determining factors (Barth and L'Hermite, 1987). The most restrictive guideline values are for agricultural use and range from 70 mg Pb kg^{-1} dry soil in Canada (CCME, 1999), while a value of 530 mg kg^{-1} is used in the Netherlands as intervention value for a standard soil with 10% organic matter and 25% clay (VROM, 2000). For industrial use the lowest values are set by Canada at 600 mg kg^{-1} (CCME, 1999) or Czech republic at 800 mg kg^{-1} (Czech Regulation 13, 1994), which are much lower than the value of 2500 mg kg^{-1} set by Belgium (Adriano et al., 1997). Our study demonstrates that it is not sufficient to take organic matter content or pH, but that the combination of the two, together with carbonate content should be taken into account to arrive at guideline values that do represent Pb bioavailability. Nevertheless, the values we derived (Table 7) are in agreement with the values established in different countries.

Table 7. Intervention guideline values for Pb concentrations in soils proposed by the Andalusian Regional Government and values proposed based on the results of toxicity tests performed in aqueous extracts of Pb(NO_3)$_2$ spiked soils with *Lactuca sativa*. Values are given as total Pb concentrations in mg kg^{-1} dry soil.

SOIL TYPE	SOIL USE		
	Agriculture	**Natural**	**Industrial**
Andalusia (S Spain)	350-500[1]	1000	2000
Carbonate-rich soils (%$CaCO_3$ ≥2%)[2]	250	1500	3000[4]
Non-Carbonate + Non-Organic-rich[3] soils	350	750	1300
Non-Carbonate + Organic-rich soils	500	3000[4]	3000[4]

[1] Soil pH <7 and >7, respectively.
[2] Based on WRB (2014).
[3] Organic-rich soils %OC>3% (Non-Organic rich soils <3%).
[4] According to Royal Decree 9/2005 to establish contaminated soils from Generic Reference Levels.

5.4. Conclusions

The main soil properties that affected the bioavailability and toxicity of Pb in our test soils were pH, carbonate content, and organic carbon content. Therefore, at least, these variables are essential in defining guideline values for Pb in soils.

The *L. sativa* seed germination/root elongation test was more sensitive to Pb than the microbial tests used. Based on the EC10, EC25 and EC50 values derived from this test applied to seven different soils, we propose preliminary guideline values for Pb in relation to the main soil properties and soil uses. Comparing our proposal with the guideline values of the Andalusian Regional Government indicates that the current regulatory levels could underestimate the potential toxicity of Pb in soils with agricultural use while in other cases an overestimation of the potential toxicity could occur.

More studies are needed to propose robust generic environmental quality standards that take into account a wider range of soil properties affecting lead mobility and toxicity. The differences between soil types observed in our study should be considered in the declaration of contaminated soils and in the setting of remediation goals for polluted areas, because this can optimise and enhance profitability of the remediation efforts.

CHAPTER 6

Is soil basal respiration a good indicator of soil pollution?

Abstract

Metal(loid)s are common pollutants in soils, causing a significant toxicological risk to living organisms and to the ecosystems. Soil basal respiration (SBR) is broadly used as indicator of metal(loid)s stress in polluted soils, although the correlation with toxicity gives in many cases contradictory results. In this paper, we study seven different soils with contrasting properties and with different pollution levels of As, Pb, Zn, and Cu to assess the influence of soil properties and contaminant concentration in the SBR response. In general terms, the SBR showed toxic effects in soils with low organic-matter content and acidic pH values. Low respiration rates were found in soils polluted with As even at very high contamination levels. According to our results, SBR is not a good indicator of pollution by Pb, Zn and Cu in soils rich in organic carbon or in highly carbonate soils. In As-polluted soils, SBR also showed a low sensitivity in all cases. Further studies are needed to assess the role of soil properties and the type of pollutant in the SBR tests.

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6.1. Introduction

Soil pollution can reduce microbial biomass (Shukurov et al., 2014), affect the taxonomic diversity of soil communities (Stefanowicz, et al., 2008), and it may act on a variety of microbial processes in the soil, thereby disturbing the nutrient cycling and the capacity to perform key ecological functions, such as mineralization of organic compounds and synthesis of organic substances (Giller et al., 1998; Moreno et al., 2009). Metals and metalloids do not degrade and may accumulate in soils and sediment (van Gestel, 2008), becoming an environmental concern due to their effects on ecological functions on soils. Metal(loid)s such as As, Pb, Zn or Cu, reach the soil from different anthropogenic activities (industry, mining, smelters, agriculture, etc.), are common pollutants in soil contamination with a serious potential to degrade soil ecosystems (Burgos et al., 2008), and can pose significant toxicological risks to organisms (Song et al., 2009).

Microbial communities constitute one of the most suitable groups to study soil degradation, as they are ubiquitous and respond quickly to changing conditions (Nannipieri et al., 2003). In addition, it has been suggested that they should be included in ecological-risk assessments as key endpoints to follow the toxicity through time (White et al., 1998; Frey et al., 2006). Assessments of metal(loid) effects on SBR require a range of parameters to be measured, related both to chemical properties of the pollutants as well as to soil properties, particularly when the study involves different soils with contrasting properties that can strongly affect the soil-respiration response (Khan and Scullion, 2002). Some authors have observed that the main soil properties which influence soil respiration are clay content (Wang et al., 2013), organic carbon content (Balogh et al., 2011), nitrogen content (Lin et al., 2010; Ramirez et al., 2010), pH and carbonate compounds (Stefanowicz, et al., 2008; Azarbad et al., 2013). Moreover, soil properties also influence the water solubility and bioavailability of metal(loids in soils and therefore, they can modulate the effect of potential pollutants on microbial activity and microbial community composition. In this way, soil respiration has been studied and extensively described as a biochemical process that also depends on physical properties, indicating that soil-water content and temperature are the physical parameters that explain part of the soil-respiration variance (Lloyd and Taylor, 1994; Fang and Moncrieff, 2001; Chen et al., 2010; Balogh et al., 2011; Wang, et al., 2014).

Soil basal respiration (SBR) of microbial biomass is a major attribute related to soil fertility (Niemeyer et al., 2012) and a common indicator of soil quality (ISO, 2002). The presence of pollutant elements in soils can significantly hamper the ability of bacteria to decompose complex substrates (Burkhardt et al., 1993; Nwachukwu and Pulford, 2011), and therefore the amount of CO_2 produced is a reliable index of the effect of metal(loid) contamination on microbial activity (Rost et al., 2001; Nwachukwu and Pulford, 2011; Kaplan et al., 2014). Therefore, respirometry measurements are valuable indicators of soil quality and might signal metal(loid) stress to soil microorganisms (Dai et al., 2004; Azarbad et al., 2013). However, soils are systems with a great complexity and the behaviour of microorganisms to contamination may be very variable, raising doubts on the possibility of use respirometry responses as reliable indicators of soil contamination. Thus, some authors found no evidences in the decrease of soil respiration with the increase of the pollution (Wakelin et al., 2010; Zornoza et al., 2015); moreover, other authors observed that contaminated soils presented higher respiratory activity than unpolluted ones (Scelza et al., 2008) and that respiration rates can augment when the soil contamination level increases (Dinesh et al., 2012). These differences in SBR response in relation to pollution can reflect changes in soil microbial community composition (e.g.: changes in the relative proportions of fungi over bacteria), showing the resistance and resilience of soil microbial communities to certain types of contamination (Scelza et al., 2008; Allison and Martiny, 2008; Hänsh and Emmerling, 2010).

The contradictory results listed above, lead us to provide in this manuscript new information in relation to the comparison between soil toxicity by metal(loid)s and soil basal respiration. We combined soil characterization, chemical extractions and SBR to unravel the link between the effects of contaminants and soil physicochemical properties, with the aim to investigate the effect of pollution by some of the most common trace metal(loid)s in soils (As, Pb, Zn, and Cu) on the soil microorganisms activity, estimated from soil basal respiration (SBR). We focused on studying the influence of soil properties and constituents in the potential toxicity of metal(loid)s and in the role of SBR in the assessment of soil contamination.

6.2. Material and methods

6.2.1. Soil sampling and characterization

Seven soil horizons (H1-H7) with different properties, from the main soil groups in Spain were selected (Table 1). All these uncontaminated soils were collected in the field and cleared of stones and plants. Afterwards, the soils were dried in a thin layer at 25 °C and then sieved through 2 mm. The main parameters analysed were: pH (soil:0.1 M KCl, ratio 1:2.5); texture (Loveland and Whalley, 1991); nutrients, bases, and cation-exchange capacity (CEC) (USDA-SCS, 1972); organic carbon (OC) (Tyurin, 1951); water-holding capacity (WHC) (ISO, 1999); available water (AW) (Richards, 1945) and calcium carbonate content ($CaCO_3$) (Barahona, 1984). Moreover, the amorphous forms of iron, aluminium, and manganese oxides were analysed according to Holmgren (1967) and Schwertmann and Taylor (1977), respectively.

Table 1. Mean values and standard deviation (±SD) of the main properties of selected samples.

Sample	**Soil horizon**	**pH** (KCl)	**AW** (%)	**$CaCO_3$** (%)	**OC** (%)	**N** (%)	**P** ($mg\ kg^{-1}$)	**Clay** (%)	**CEC** ($cmol+\ kg^{-1}$)	**Fe_o** ($g\ kg^{-1}$)	**Mn_o** ($g\ kg^{-1}$)	**Al_o** ($g\ kg^{-1}$)
H1	Ah	7.63	8.08	37.11	5.43	0.35	8.26	23.61	21.43	0.68	0.06	1.17
		(±0.02)	*(±0.23)*	*(±0.44)*	*(±0.38)*	*(±0.03)*	*(±0.81)*	*(±0.90)*	*(±2.00)*	*(±0.03)*	*(±0.01)*	*(±0.03)*
H2	Bw	8.11	5.72	72.39	0.42	0.02	bdl	11.79	9.83	0.20	0.02	0.31
		(±0.09)	(±0.41)	(±0.86)	(±0.12)	(±0.01)	(-)	(±0.44)	(±1.00)	(±0.02)	(±0.01)	(±0.02)
H3	Ck	8.24	5.37	92.32	0.38	0.03	bdl	7.70	2.94	bdl	bdl	0.15
		(±0.09)	(±0.07)	(±1.80)	(±0.17)	(±0.01)	(-)	(±0.58)	(±0.13)	(-)	(-)	(±0.02)
H4	Bw	5.80	6.60	nd	0.61	0.05	6.53	19.05	9.91	0.52	0.18	0.38
		(±0.21)	(±0.18)	(-)	(±0.10)	(±0.02)	(±0.65)	(±0.38)	(±0.42)	(±0.09)	(±0.02)	(±0.07)
H5	Ah	6.72	12.53	nd	8.22	0.44	28.06	23.79	25.90	0.65	0.41	0.50
		(±0.06)	(±2.09)	(-)	(±0.02)	(±0.01)	(±0.69)	(±0.12)	(±0.37)	(±0.14)	(±0.07)	(±0.06)
H6	C	4.58	7.40	nd	0.49	0.44	1.09	8.31	3.83	1.00	0.09	0.27
		(±0.06)	(±0.04)	(-)	(±0.02)	(±0.02)	(±0.11)	(±0.12)	(±0.37)	(±0.06)	(±0.01)	(±0.06)
H7	Bt	5.86	5.49	0.92	0.66	0.04	bdl	54.76	15.53	0.78	0.03	0.73
		(±0.01)	(±0.21)	(±0.16)	(±0.25)	(±0.02)	(-)	(±1.13)	(±1.01)	(±0.07)	(±0.01)	(±0.06)

bdl: below detection limit; AW: available water; $CaCO_3$: calcium carbonate content; OC: organic carbon content; CEC: cation exchange capacity; Fe_o/Mn_o/Al_o: amorphous oxides forms. nd: not detected; bdl: below detection limit.

6.2.2. Soil contamination

Soil samples were individually spiked in the laboratory with the metal(loid)s from solutions of soluble salts of the most abundant chemical species present in the soils: As(V) [Na_2HAsO_4 $7H_2O$], Pb(II) [$Pb(NO_3)_2$], Cu(II) [$Cu(NO_3)_2$ $3H_2O$], and Zn(II) [$ZnCl_2$]. Pollution levels were established by increasing the background concentrations of the soils according to the intervention values proposed by the Regional Government of Andalusia (Aguilar et al., 1999). Five contamination levels were defined: L1 (intervention level for agricultural soil), L2 (intervention level for natural areas), L3 (intervention level for industrial areas), L4 (L3 x 2), and L5 (L3 x 4 in the case of As, Pb, Cu, and L3 x 3 in the case of Zn) (Table 2). In all cases, uncontaminated soil samples were used as a control (L0), making a total of 6 treatments for each studied soil. A total of 126 experimental units (7 soils x 6 treatments x 3 repetitions) were used in this study for each pollutant element.

The contamination was made by spiking 50 g of soil with the individual pollutant, and the moisture was brought to 60% of their water-holding capacity. Soils were incubated for 4 weeks at 25±1°C and 60% air humidity, with a light cycle of 10 hours. The water content of each sample was checked and corrected weekly, maintaining the incubation conditions constant so as not to disturb the microbial activity. The incubation period was chosen to stabilize the contaminant added, optimising the time spent on these tests, and was selected based on similar studies by other authors (Fendorf et al., 2004; Tang et al., 2006; Martín Peinado et al., 2012).

Table 2. Total concentrations (mg metal(loid) kg soil^{-1}) added to samples for the different contamination levels in relation to values from Aguilar et al. (1999). L1 (intervention level for agricultural soil), L2 (intervention level for natural areas), L3 (intervention level for industrial areas), L4 (L3 x 2), and L5 (L3 x 4 in the case of As, Pb, Cu, and L3 x 3 in the case of Zn).

Contamination levels	As	Pb	Zn	Cu
L1	50	500	600	300
L2	100	1000	1000	500
L3	300	2000	3000	1000
L4	600	4000	6000	2000
L5	1200	8000	9000	4000

6.2.3. Metal(loid) analysis

Total trace metal(loid) concentration (mT) in soils was determined from acid digestion in strong acids (HNO_3 + HF). Water-soluble forms (mW) were determined from soil:water extracts (1:1 ratio) obtained by shaking for 24 h and extracted with 10 cm Rhizon MOM (Metal and Organic Matter) soil moisture samplers. In all cases, trace metals were measured by ICP-MS (Inductively Coupled Plasma-Mass Spectrometry) in a spectrometer ICP-MS NEXION 300D. For calibration, two sets of multi-element standards containing all the analytes of interest at five different levels of concentration were prepared using rhodium as the internal standard. All standards were prepared from ICP single-element standard solutions (Merck, Darmstadt, Germany) after dilution with 10% HNO_3. Procedural blanks for estimating the detection limits (3*σ; n = 6) were <0.21 ppb for As, <0.23 ppb for Pb, <2.68 ppb for Zn, and <0.52 ppb for Cu. The analytical precision was better than ±5% in all cases. The accuracy of the method was confirmed by analysing Standard Reference Material SRM2711 Montana Soil (US NIST, 2003), (n=6). For As, Pb, Zn, and Cu the average recovery values ranged between 91% and 105% of the certified reference values.

6.2.4. Soil respiration

Polluted soils were incubated for 4 weeks at 60% of their water-holding capacity. The basal respiration rate (RB), based on ISO 17155 protocol (2002) was measured with SY-LAB equipment, μ-Trac 4200 model. Before starting the test, soil moisture was adjusted at 60% of WHC. The equipment operates with a constant temperature of 30°C and CO_2 production was determined from vials with solution of potash (KOH 0.2%) for 96 hours.

The results were based on the variation of the electrical impedance over time produced by the reaction between the CO_2 emitted (heterotrophic soil respiration) and KOH. Results were calculated as the average rate of CO_2 emitted by each sample and expressed as μg CO_2 day^{-1} g^{-1} soil. For the different pollution levels and for each soil sample, results were calculated as a percentage of basal respiration variation (VR_B, %) compared to the controls using the equation:

$$VR_B = ((R_{B1}-R_{B0}) / R_{B0}) \times 100$$

where R_{B1} is the mean respiration of each sample and R_{B0} is the mean respiration of uncontaminated soil (control) for each studied soil.

6.2.5. Statistics

After the normal distribution of the data was verified by a Kolmogorov-Smirnov test, significant differences were determined by ANOVA and for multiple comparisons Tukey's test was applied ($p < 0.05$). Pearson's correlations and Principal component analysis (PCA) were performed to analyze the influence of soil properties on solubility and toxicity of trace metals and on soil respiration. All these analyses were performed with a confidence level of 95% by using SPSS v.20.0 (SPSS Inc., Chicago, USA).

6.3. Results

6.3.1. Metal(loid) solubility

Water-extractable metal(loid) concentrations varied significantly between the different polluted soils, showing an exponential increase with the rise of total metal concentrations (Figure 1). In all cases, water-extractable forms of As, Pb, Zn, and Cu were directly correlated with total concentrations, with correlation coefficients of 0.819**, 0.438*, 0.619**, and 0.436*, respectively. The highest concentrations of PbW, ZnW, and CuW were found in acidic and non-carbonate soils (H4 and H6), while the highest values of AsW were found in carbonate soils (H2 and H3). Otherwise, the lowest concentrations in the water soluble fraction, at higher pollution levels, were found in carbonate soils (H1, H2 and H3) for Zn and Cu, and in H1, H2 and H5 for Pb polluted samples. Otherwise, the clayey soil (H7) showed the lowest AsW concentration but relatively high concentrations of PbW, ZnW, and CuW were found in relation to carbonate and organic carbon-rich soils.

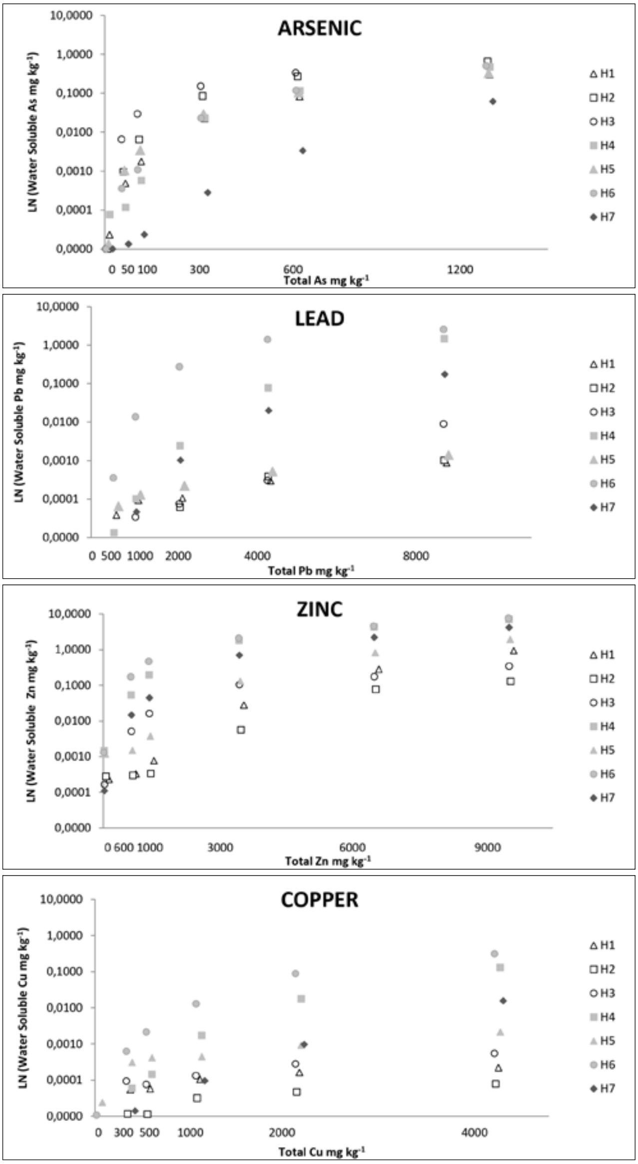

Figure 1. Mean concentration of soluble As, Pb, Zn, and Cu expressed in natural logarithm (y axis) against the total concentration of each metal (x axis) for the seven studied soils in all different treatments.

6.3.2. Soil respiration and soil properties

Basal respiration diverges between unpolluted soil samples (Table 3). The highest soil-respiration rates were found in the soils rich in organic carbon (H1 and H5), with more than 100 µg CO_2 emitted per day per g soil. Otherwise, the clayey soil (H7) showed the lowest basal respiration rate, with 32 µg CO_2 day^{-1} g^{-1} soil. The other soil samples showed intermediate respiration values ranging from 36 to 56 µg CO_2 day^{-1} g^{-1} soil (Table 3). Soil properties with a direct and high significant correlation ($p < 0.01$) in soil respiration in unpolluted samples were the organic carbon content (OC), the available water (AW), the main soil nutrients (nitrogen and potassium content), the cation-exchange capacity (CEC), and the presence of amorphous oxides of Fe, Mn, and Al (Table 4). A multiple stepwise regression equation was performed between the basal soil respiration (R_B) in these unpolluted soils and the main physicochemical soil properties with the highest significant correlation, leading to the following equation:

$$R_B = 70.39 + 10.57\ OC + 109.71\ N - 6.73\ AW \qquad r^2 = 0.725 \qquad (1)$$

This equation indicates that soil respiration was significantly ($p < 0.01$) and directly related to organic carbon and nitrogen content, and inversely related to the capacity of the soil to retain available water.

Table 3. Mean and standard deviation (SD) of Basal Respiration Rate (R_B) (µg CO_2 day^{-1} g $soil^{-1}$) of studied unpolluted soils at 60% of water holding capacity (n=12). Letters represent significant difference between studied samples (Tukey's test (p<0.05).

Sample	**RB**	**SD**
	µg CO_2 day^{-1} g $soil^{-1}$	
H1	120.14	16.70 a
H2	55.83	14.33 b
H3	37.23	2.81 bc
H4	35.86	2.11 bc
H5	116.21	21.49 a
H6	44.52	6.79 bc
H7	31.70	8.28 c

Table 4. Pearson correlation analysis between the basal respiration rate (R_B) of natural soils at 60% of water holding capacity without metal(loid) pollution and the main soil properties (n=84).

Soil Respiration (R_B)	
OC	0.777**
AW	0.692**
N	0.748**
P	0.458*
K	0.747**
CEC	0.617**
Fe_o	0.327*
Mn_o	0.495*
Al_o	0.452*

OC: organic carbon, AW: available water; CEC: cation exchange capacity; Fe_o/Mn_o/Al_o: amorphous oxide forms of Fe, Mn and Al; (*p<0.05; **p>0.01).

6.3.3. Toxicity

6.3.3.1. Soil basal respiration variation

The soil-respiration assay presented a generally low sensitivity to metal pollution without reaching total inhibition in any of our samples and even for metals studied at the highest contamination levels (Figure 2). The one way ANOVA was made for each soil separately to compare statistically significant differences between treatments. Among the metal(loid)s studied, As had the lowest effects on all samples. In most of the soils the microorganism respiration showed no significant variation (H5 and H7) or even stimulation (hormesis) (H2, H4, and H6). The carbonate sample rich in organic carbon (H1) presented a significant reduction in R_B in relation to the control (between 15-20%), where the observed effects started from 50 mg kg^{-1} of arsenic addition, with no differences after the increase in the As concentration. The carbonate sample H3 also showed a slight but significant respiration variation (5%) from the treatment > 600 mg kg^{-1} of As addition (Figure 2a).

Soils spiked with Pb showed different responses depending on the soil sample, although the variation in soil respiration was below 50% in all samples and at all contamination levels. Soil H5 (rich in organic carbon) and H3 (with high carbonate and silt content) did not show a soil-respiration variation in relation to the control with the addition of Pb. Soils which showed the least effect of Pb toxicity were the carbonate soils H1 and H2, where the observed effects started from 4000 mg kg^{-1} of Pb addition (L4), with a reduction in soil respiration of around 25-30% in relation to the control. The highest toxicity was found in the samples without organic matter and with acidic pH values (H4, H6, and H7); in all cases, the toxicity effects started from L1 (500 mg kg^{-1} of Pb addition) and increased with the amount of Pb added to the soil. The maximum inhibition of R_B was achieved in soil H6 (acidic pH and sandy texture) with a reduction of up to 48% in relation to the control (Figure 2b).

In the case of Zn, soils showed greater variations in respiration rates than in the As or Pb treatments (Figure 2c). Basal respiration was 70% lower than in control at treatments higher than 6000 mg Zn kg^{-1} soil (L4) in non-carbonate samples (H4 and H6), while the reduction in RB was above 50% for maximum contamination level in the soil H7. In addition, carbonate samples H1 and H2 showed reductions of less than 30% even at the higher contamination level. Sample H3 (highly carbonated) and H5 (non-carbonated and rich in organic carbon) showed no soil-respiration variation in relation to control with the Zn addition.

Copper showed highest significant variation in RB in more soils than did other studied elements, the sample H3 being the only one that presented no toxicity effects in this bioassay. Soils which showed less effects due to Cu pollution were those rich in organic carbon (H1 and H5), with reductions of less than 30%, followed by clayey soil (H7) with reduction values lower than 50%, even at the highest contamination levels. Soils H4 and H2 showed more sensitivity to Cu pollution, with reductions of more than 70% in relation to the control from the addition of 2000 and 4000 mg Cu kg^{-1} soil (L4 and L5), respectively. The soil with the highest toxicity response in this case was the non-carbonate sandy soil (H6), with a reduction in R_B of more than 70% from the addition of 300 mg Cu kg^{-1} soil (L1) (Figure 2d).

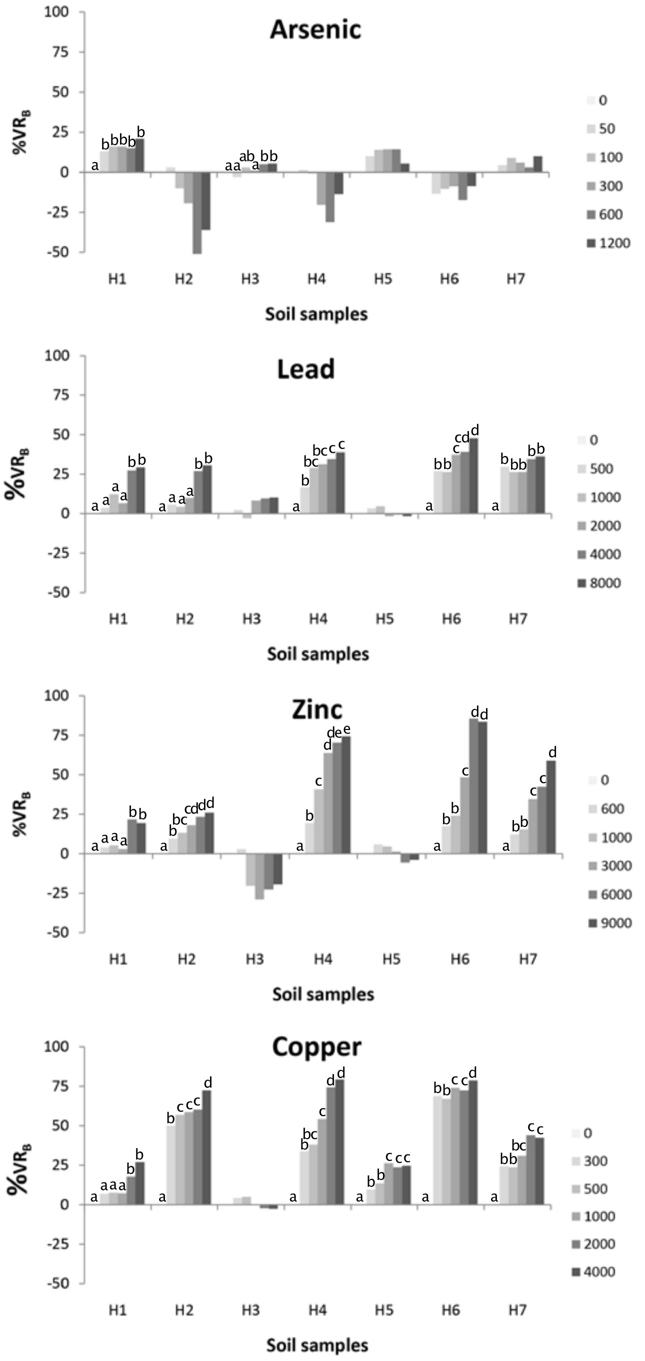

Figure 2. Variation of basal respiration rate (VR_B) in relation to the control for soils spiked with different concentrations of As, Pb, Zn, and Cu (mg metal kg^{-1} soil). Letters indicate statistically significant differences according to ANOVA post hoc Tukey's test ($p<0.05$).

6.3.3.2. Influence of soil properties on basal respiration response

Soils spiked with As did not show high levels of variation (significant differences appearing only in two of the seven soils studied), and the influence of these soil properties were not studied. However, in the two soils (H1 and H3) that presented significant reductions in R_B a direct correlation with the total concentration of As in soil (0.424*) was found.

Principal component analysis was performed between the respiration response and the main soil properties of the samples that showed respiration variation (Table 5). In the case of the other pollutants (Pb, Zn and Cu) more than 84% of the variance is explained by 3 components, and the soil basal respiration is included in C1 and C2. In soils polluted with Pb and Zn, component 1 includes the VR_B directly related to soluble forms of contaminants and sand content, and inversely related to the other soil properties; component 2 includes the VR_B directly related to the pollutant concentration, both total as soluble in water. Soils polluted with Cu presented similar behaviour to Pb and Zn pollution, although soluble in water forms are only included in component 2.

Table 5. Principal component analysis between soil basal respiration variation (VR_B) and the corresponding total metal concentration (mT), water extractable forms (mW) and significant soil properties of the samples that showed significant reduction in R_B.

	LEAD			ZINC			COPPER		
	C1	C2	C3	C1	C2	C3	C1	C2	C3
VR_B	-0.564	0.664		-0.734	0.594		-0.700	0.515	
mT		0.793			0.802			0.876	
mW	-0.565	0.540			-0.661	0.673		0.561	
pH_{KCl}	0.821		0.540	0.810		0.559	0.757		-0.541
OC	0.768			0.752			0.806		
$CaCO_3$	0.570		0.733	0.583		0.747			-0.748
Sand	-0.800			-0.781			-0.812		
K	0.786			0.758			0.835		
CEC	0.881			0.850			0.924		
% Explained variance	36.0	60.7	84.5	36.2	63.4	88.7	38.8	63.6	84.5

6.4. Discussion

6.4.1. Metal(loid) solubility

According to our results, metal(loid) solubility is strongly controlled by soil properties, showing significant differences in relation to soil type. Arsenic had the highest water-extractable concentrations in carbonate soils with low organic carbon content (samples H2 and H3), this increase in solubilization is related to the rise in pH, in accordance with previous studies (Jones et al., 1997; Simón et al., 2010). Otherwise, soluble As concentrations in non-carbonate soils with low organic carbon content (samples H4 and H6) were lower than in carbonate ones, but higher than in soils rich in organic carbon (samples H1 and H5), highlighting the strong influence of acidic conditions and organic matter on the solubility of this element (O'Neill, 1995; Romero Freire et al., 2014). The soil that showed the lowest soluble As concentrations was the clayey and iron-rich sample (H7), in accord with the retention ability of As by iron oxides in soils (Fitz and Wenzel, 2002; Aguilar et al., 2007).

In the case of Pb, the highest PbW concentrations were found in the acidic soils with low organic carbon content (H4 and H6), indicating the important role of pH in controlling Pb solubilization (Bur et al., 2012; Ming et al., 2012). The clayey soil (H7) also showed high concentrations in PbW; in this case, Fe and Mn oxides could favour Pb desorption from soil (Romero-Freire et al., 2015), although this process should be studied in more detail. Otherwise, carbonate soils and/or with high content in organic carbon (H1, H2, H3, and H5) presented significantly lower water-extractable Pb concentrations than did the other soils. The role of organic matter as an important sink for Pb has been described by many authors (Sánchez-Camazano et al., 1998; Kabata-Pendias, 2011); in this way, soil H1 (with slightly alkaline pH and high organic matter content) showed the lowest water-extractable Pb of all the soils studied.

Soluble forms of zinc (ZnW) and copper (CuW) presented the highest concentrations in the acidic soils (H4 and H6) and the lowest solubility was attained in calcareous soils (H1, H2, and H3). According to Ivezic et al. (2012), pH and OC were the parameters that showed the most significant influence on metal solubility; these authors found that low water-extractable Zn came mainly from calcareous soils with pH>7. Moreover, in acidic soils, Zn could be adsorbed predominantly by cation exchange, while the rise in soil pH made the specific Zn adsorption to soil organic matter, clay minerals, oxides, and carbonates more relevant (Jacquat et al., 2008). These results are consistent with those found for soils H7 and H5. On the other hand, Cu presented results similar to those of Zn, showing a strong affinity for OC, as well as for carbonates in alkaline soils and iron oxides in acidic soils (Ivezic et al., 2012). In this way, sample H7 (soil rich in clay and iron oxides) showed high Cu solubility, followed by soil H3 (highest pH and high content in carbonates) and by soil H1 (similar to H3 but with high organic carbon content).

6.4.2. Basal respiration in control soils

Soil-respiration data strongly depend on the measurement method, environmental conditions and soil properties. Accordingly, diverse values have been reported by other authors from different studies; thus, Dinesh and Ghoshal Chaudhuri (2013), studying mangrove areas, registered a value of 25 μg CO_2 g^{-1} day^{-1}, but under plantations they measured a value of 13 μg CO_2 g^{-1} day^{-1}. Meanwhile, Cheng et al. (2013) recorded a maximum basal respiration in forest soils of 15.5 μg CO_2 g^{-1} day^{-1}, and Azarbad et al. (2013) reported values from 0.7 to 1.52 μg CO_2 g^{-1} day^{-1} in polluted soils. However, Fernández et al. (2004) in an experiment performed with the same equipment but with glucose-induced soil respiration, measured an average of 384 μg CO_2 g^{-1} day^{-1} under an artificial control soil. The two main factors influencing soil respiration in the field are temperature and water content (Wang et al., 2014; Bujalský et al., 2014), these two factors also being essential in laboratory experiments, including the method used in the rewetting of dry soil (Meisner et al., 2013). For these reasons, one of the advantages of laboratory methods in relation to field ones is that the main variables affecting soil respiration can be maintained constant throughout the experiment, finding more comparative and reliable results than in field studies. In our case, samples were measured under the same conditions of temperature and moisture, so that the differences between samples can be attributed to the different soil types and contamination treatments. In our samples, soil respiration showed a wide range of values, with the soils rich in organic carbon having the highest CO_2 efflux emitted. Nevertheless, according to the ISO 17155 protocol (2002), the results of an interlaboratory test using different contaminated soils (Annex A) showed that basal respiration rates varied between 49.92 and 126.48 μg CO_2 g^{-1} day^{-1}, which is in the range found in our study.

According to the relationship between soil basal respiration and soil properties, Xu and Qui (2001) reported that soil CO_2 efflux increases with greater OC, N, and P content in the soils, which is in line with our results. The strong influence of organic carbon content to *soil respiration* (Eq. 1) is well known (Smith, 2003, Bahn et al., 2008, Chen et al., 2014), with the organic topsoil as the most active part of soil-carbon pool which potentially influence soil respiration (Vesterdal et al., 2012). In the case of N content, the higher soil-respiration rate is related to the source of proteins that promote microbial growth (Xu and Qui, 2001). The presence of available P is also described as a key factor for the development of microbial biomass in mineral soils (Xu and Qui, 2001). Moreover, Peigné et al. (2009), studying areas with low levels of soil microbial biomass, reported that samples with higher CEC presented higher levels of respiration activity, in agreement with the direct correlation found between soil respiration and CEC in our study. In addition, soil basal respiration strongly depends on soil moisture with the soil-water availability (AW) as an important variable when soils are relatively dry (Howard and Howard, 1993; Reichstein et al., 2003). Furthermore, soil-respiration variability could be influenced by other soil constituents such as the oxide content (Aciego and Brookes, 2008).

6.4.3. Basal respiration in polluted soils

Diversity and microbial biomass variation due to metal(loid) stress in polluted soils has been widely reported (Bååth et al., 2005; Gough et al., 2008), although no correlation between the contamination level and the reduction in soil respiration has been also found (Wakelin et al., 2010; Zornoza at al., 2015). Moreover, hardly any studies examine the impact of the soil properties on basal respiration in polluted soils. In our study, As contamination showed little impact on soil respiration, with five of the seven soils studied presenting no significant toxicity effect even at the highest contamination level of 1200 mg As kg^{-1} soil. This finding agrees with results reported by Speir et al. (1999), which indicated no inhibition of microbial respiration by As(V). Also Turpeinen et al. (2004), reported no significant differences in the toxicity response of microbial community in As-contaminated soils up to 2500 mg As kg^{-1} soil. Otherwise, a slight (< 25% in relation to the control) but significant variation in R_B was found in carbonate samples (H3 and H1) and a positive correlation with the total As added to the soils was found. According to our results, the mechanisms affecting soil basal respiration in As-polluted samples should be further explored.

Variation in soil respiration showed similar results in the case of Pb, Zn, and Cu between the different soils, with soil properties such as pH_{KCl}, calcium carbonate content, organic carbon, and sand content being the main significant factors. Different authors have observed that high levels of organic matter constitute a major sink for metals (Kabata-Pendias, 2011; Minkita et al., 2013; Steinnes, 2013) and their negative effects can be counteracted by reducing their bioavailability, allowing more microbial growth, and boosting soil basal respiration (Balabane, 1999; Nwachukwu and Pulford, 2011). Moreover, higher organic matter content usually implies greater availability of nutrients coming from the mineralization of organic compounds for coping with the stress related to metal pollution, allowing a higher microbial growth rate. In this way, samples H1 and H5 (rich in organic carbon) showed significantly lower soil-respiration variation under Pb, Zn, and Cu contamination.

Soil pH regulates sorption competition between elements, negative charge of the exchangeable complex, dissolution of soil components, and ultimately determine the metal bioavailability and toxicity in relation to soil-respiration response (Azarbad et al., 2013). In our study, the soils with higher pH values showed less respiration-variation rates, being less sensitive to toxicity. The pH influence related to this variation of metal solubility with the alkalinity has been studied in the case of Pb (Shaheen, 2009), Zn (Li et al., 2011b), and Cu (Shaheen et al., 2009). According to the foregoing data on OC and pH, soils H2, H3, H4, and H6, with low organic matter content, showed the greatest respiration-variation rate for Pb, Zn, and Cu contamination as a function of the pH gradient. Soil H3, the most alkaline sample, did not register significant soil respiration variation for the three metals studied, while soil H6 with the most acid pH showed the greatest reduction in respiration rates. The $CaCO_3$

content has an indirect effect on metal solubility, conditioning soil pH, but also has a direct effect through surface interactions. In this way, solubility and bioavailability of Pb, Zn, and Cu in relation to carbonate content, can also justify the lower reduction in soil basal respiration in carbonate soils (H2 and H3) with respect to non-carbonate soils (H4 and H6).

Soil texture and cation-exchange capacity are also two soil properties that govern metal sorption in soils. Fine textures, with high clay content, have a higher cation-exchange capacity and thus a higher tendency for cation sorption than soils having coarse textures (Ma and Uren, 1998; Papini et al., 2004). Our results showed that CEC was directly correlated with the rate of respiration decrease and inversely to the sand fraction in the soils contaminated with Pb, Zn, and Cu. These relations occurred in all the soils studied but were more evident in the H4, H6, and H7 (non-carbonate and low in organic matter content), which had significantly higher differences in CEC and texture than in the other soils. These findings agree with those reported by Cerqueira et al. (2011) and Jalali and Moharrami (2007), who indicated that the retention and sorption of Pb, Zn, and Cu is significantly correlated to CEC and also, but less markedly, with the clay content. Moreover, organic matter and clay content are the main factors determining CEC, whereas the organic matter content has a more predominant role than clays in soils (Sierra et al., 2007). As a result, high OC content and high CEC in soils are two of the main factors that inhibit the toxicity effect on soil respiration for Zn, Cu, and Pb in contaminated soils.

Anyway, the respiration rates found in our study of soils polluted with As, Zn, Cu and Pb indicate that the R_B variation has different results depending on the soil type and the pollutant involved. This has been previously reported (Scelza et al., 2008; Dinesh et al., 2012) and could be related to relative changes in the structure and diversity of the soil microbial community composition (Hänsh and Emmerling, 2010). According to our results, R_B variation is not a good indicator of pollution by Pb, Zn and Cu in soils rich in organic carbon or in highly carbonate soils. Moreover, in As-polluted soils, R_B variation also showed a low sensitivity even at the highest level of pollution (1200 mg kg^{-1}). This work notes the need for further studies in key role of the soil properties and the type of contaminant for the assessment of pollution using S_BR test.

6.5. Conclusions

Soil basal respiration (SBR) assay is frequently used as a sensitive test to determine microbial stress in relation to metal(loid) contamination. According to our results, SBR is not a good indicator of pollution when some soil types or pollutant elements are considered. Our study indicates that SBR showed no toxic effects in highly carbonated and low developed horizon (H3) for Pb, Zn and Cu, even at the highest pollution levels tested in this study. No toxic response was also observed for Pb and Zn in non-carbonated soils rich in organic carbon (H5) at any level of contamination. Low respiration rates were found in soils polluted with As even at very high contamination levels, indicating low sensitivity for As pollution in all soils tested in this study. In any case, the test is useful to define soil properties affecting metal(loid) mobility and toxicity in relation to soil basal respiration variations. The differences between the soils examined in our study should be further analysed and extended to other soil types, in order to test the ability of this assay in the assessment of environmental quality standards.

CHAPTER 7

Long-term contamination in a recovered area

Abstract

Soil pollution from the spill of Aznalcóllar mine (S Spain) was monitored by analysing polluted soils in 1998, 1999, and 2004. Following the methodology used in previous studies, in 2013 we conducted a new sampling and analysis of the soils affected by the spill and the data were compared with those of 2004. The results confirm that the pH tended to rise and concentration of pollutants tended to diminish over time. In 2013, the total concentration of pollutants was within the normal range for uncontaminated soils and close to the background concentration of the soils prior to the spill; while the soluble concentration of pollutants was clearly below the toxic level. These results indicate that remediation measures implemented have been effective. However, the removal of tailings (first remediation measure applied) was deficient and in many places the tailings were mixed with the soil. The high concentration of sulphides and metal(loid)s in the tailings gave rise to spots with very acidic and highly polluted soils devoid of vegetation. In 2013, fifteen years after the spill, these spots of bare soils remain a major source of pollution from which pollutants are scattered through the solid and liquid phases of runoff water, requiring action to immobilize pollutants and encourage the restoration of vegetation on these soils. In this type of pollution in a Mediterranean environment, the complete removal of tailings is more important that the speed at which they are removed.

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7.1. Introduction

On 25 April 1998 a breach in the dam of the holding pond of tailings from a pyrite mine in Aznalcóllar (S Spain) spilled around 4.5 x 10^6 m^3 of acidic waters and toxic tailings containing high concentrations of zinc (Zn), copper (Cu), cadmium (Cd), arsenic (As), lead (Pb), and thallium (Tl) into the Agrio and Guadiamar river basins, affecting some 43 km^2 (Grimalt et al, 1999). The mean concentrations of the more relevant trace elements in the tailing were: 7096 mg Zn kg^{-1}, 1552 mg Cu kg^{-1}, 25.1 mg Cd kg^{-1}, 2878 mg As kg^{-1}, 7888 mg Pb kg^{-1}, and 51.6 mg Tl kg^{-1} (Cabrera et al., 1999). This accident was the second largest of the 59 major mine spills in the world and the largest reported in Europe (Nikolic et al., 2011). One of the most important soil-remediation programmes in Europe was applied to restore the affected area (Domínguez et al., 2008).

During the first months after the accident, soils were polluted in two stages (Simón et al., 2001). First, the toxic water (liquid phase) and tailings (solid phase) covered the soil and infiltrated in depth through macro- and micropores. This penetration was quite heterogeneous depending on the soil properties, mainly texture and structure. In the second stage, drying and oxidation of pyrite tailings resulted in oxidation of sulphides, with a sharp drop in pH and solubilization of contaminants that infiltrated the soil, raising the pollution level. To reduce the concentration, mobility, and bioavailability of the pollutants, as well as to halt the transfer to other components of the ecosystem (mainly water and living organisms), the remediation measurements that were urgently started included mainly: a) removal of the tailings and upper part of the highly polluted soils by heavy machinery and b) addition of sugar-refinery scum (4 kg m^{-2}), organic matter (2 kg m^{-2}) and iron-rich clayey materials (2 kg m^{-2}), followed by tilling of the upper 20 cm of the soils. Furthermore, bioremediation techniques were tested. After three years of intense rehabilitation, soils continued presenting a relatively high concentration in Cu, Zn, As, and Pb, leading the Regional Government to convert the affected area into a natural protected area called Guadiamar Green Corridor, where the afforestation of ca. 4500 ha with autochthonous species was performed (CMA, 2003).

Reports describing the evolution of contamination from the Aznalcóllar spill have examined leaching across the soil profile (Kraus and Wiegand, 2006; Aguilar et al., 2007; Álvarez-Ayuso et al., 2008), the temporal and spatial variations in the pollutant concentrations (Burgos et al., 2006; Vanderlinden et al., 2006; Ordóñez et al., 2007; Simón et al., 2008), and the effects of soil amendments (Walker et al., 2004; Aguilar et al., 2004b; Querol et al., 2006), as well as analysing the effectiveness of phytoremediation techniques (Clemente et al., 2005; Vázquez et al., 2006; Bernal et al., 2007). Although the remediation measures gradually decreased the concentration of pollutants in the affected soils, six years after the accident, especially in the upper part of the affected area (first 18 km downstream from the source of the contamination), an irregular distribution pattern of contamination in the affected soils was easily discernible, with highly polluted spots alternating with other much less polluted areas (Simón et al., 2008). Fifteen years after the accident, the most polluted spots of soils are easily distinguished in the landscape because no vegetation has developed, representing a major source of pollution that should be monitored.

The aim of the present work is to analyse the time course of the pollution in the recovered area affected by the Aznalcóllar mine spill and characterize the main issues concerning the spots where the residual pollution still generates problems in the ecosystem recovery, by the assessment of the soil pollution in the Ágrio and Guadiamar river basins in 2013 (15 years after of the accident). The results were compared with those of 2004. Simultaneously, the pollution of the soil in the spots lacking vegetation was analysed and the results compared with those corresponding to the entire area (2013 sampling).

7.2. Materials and Methods

7.2.1. Soil-sampling method

The soil-sampling method for the soil pollution in the Ágrio and Guadiamar river basins in 2013 was the same as used in previous studies (Simón et al., 2008). In the same 100 georeferenced plots of 10 x 10 m previously selected, 5 soil sub-samples were taken from the four corners and centre at depths of 0-10 cm and intensively homogenized to provide a single sample for each plot. During the 2013 sampling, many spots with failed regeneration of the vegetation (bare soil) were detected. Spots of bare soil (BS) were randomly distributed and had a highly variable surface area, ranging mainly from 1 to 200 m^2. The BS surface area was quantified using satellite images from 2014 Google Earth and the image processing program ImageJ v.1.48 (Fig. 1). In the first 18 km from the tailing pond (Sector 1, predominantly acidic soils), the BS surface area represented about 7% of the total area affected by the spill, while beyond the first 18 km (Sector 2, predominantly neutral or alkaline soils) the BS surface area decreased to 0.4%. Twenty of these spots of BS were selected and the soil between 0 and 10 cm was sampled with the above-mentioned methodology.

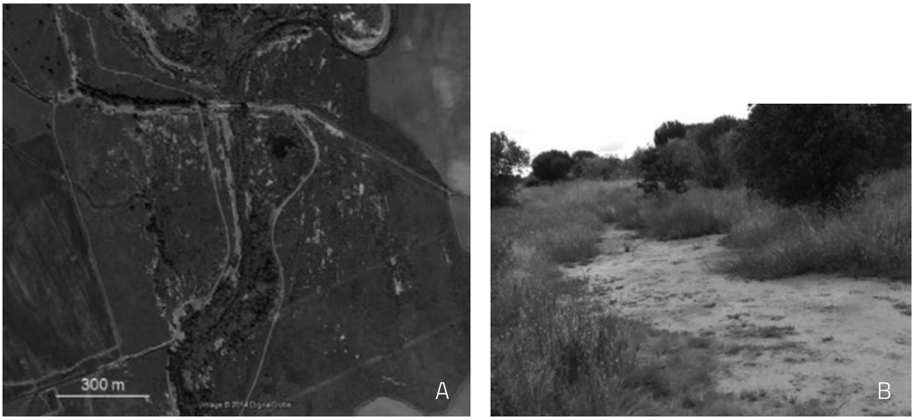

Figure 1. (A) Aerial image of the Sector 1 in the Green Corridor of the Guadiamar river, the light brown spots correspond to bare soils. (B) Detailed image of a spot of bare soil.

7.2.2. Analytical methods

The methodology used in the soil-sample analysis was the same used in the previous studies (Simón et al., 2008). The soil samples were air-dried at 25°C, sieved (2-mm mesh) and analysed following standard soil methods (MAPA, 1994). The total concentration of pollutants was analysed in the laboratory, after fine grinding and acid digestion (HNO_3+HF+HCl), by ICP-MS in a PE SCIEX ELAN-5000A spectrometer. The ICP-MS operating conditions included three replicates in each measurement. For calibration, two sets of multi-element standards containing all the analytes of interest at five different levels of concentration were prepared using rhodium as the internal standard. All standards were prepared from ICP single-element standard solutions (Merck, Darmstadt, Germany) after dilution with 10% HNO_3. Procedural blanks for estimating the detection limits ($3^*\sigma$; n = 6) were <0.21 ppb for As, <2.68 ppb for Zn, <0.52 ppb for Cu, and <0.23 ppb for Pb. The analytical precision was better than ± 5% in all cases.

The accuracy of the method was confirmed by analysing Standard Reference Material SRM2711 Montana Soil (US NIST, 2003), six replicates. For As, Zn, Cu, and Pb the average recoveries ranged between 91% and 105% of the certified reference values. Water-soluble concentrations of pollutant elements were measured in the pore water from the saturated paste of the soils, and analysed by ICP-MS after acidification with HNO_3. The enrichment factor of each pollutant (EFp) was estimated from the equation: $EFp = Ps/Pul$ (Lacatusu, 1998), where Ps is the concentration of each pollutant in the sample and P*ul* is the upper limit of the concentration of each pollutant in uncontaminated soils of the Guadiamar River (38.5 mg As kg^{-1}, 271.0 mg Zn kg^{-1}, 85.0 mg Cu kg^{-1}, and 86.3 mg Pb kg^{-1}; Cabrera et al., 1999).

Table 1. Mean and standard deviation (SD) of the total concentration of pollutants (TP) in soils (mg kg-1) sampled in 2004 and 2013 and bare soil (BS).

		Sector 1					Sector 2				
Sampling		pH	TCu	TZn	TAs	TPb	pH	TCu	TZn	TAs	TPb
2004	Mean	6.2^{a}	88^{a}	328^{a}	105^{a}	175^{a}	7.6^{a}	116^{a}	508^{a}	78^{a}	169^{a}
	SD	1.6	45	191	81	109	0.7	49	259	45	87
2013	Mean	7.7^{b}	81^{a}	248^{a}	61^{a}	131^{a}	8.1^{a}	84^{a}	317^{a}	37^{a}	88^{a}
	SD	0.8	43	239	50	72	0.8	49	199	28	57
BS	Mean	3.3^{c}	125^{b}	162^{b}	298^{b}	757^{b}	3.8^{b}	130^{a}	241^{a}	257^{b}	324^{b}
	SD	0.4	39	67	103	388	1.3	38	120	260	141

In each column, values of the mean followed by the same letter do not significantly differ (Tukey's test, *p*<0.05).

7.2.3. Statistical methods

Data distributions were established by calculating the mean values and the standard deviations by cumulative frequency-distribution curves. The differences between the individual means of the study periods and the soil types were compared using Tukey's test ($p<0.05$). Spearman correlation analysis involving pH and total and soluble pollutant concentration were also performed. The SPSS (PASW Statistics 20) software package was used for all statistical analyses.

7.3. Results

7.3.1. pH values and total concentration of the pollutants

In 2013, the mean pH value increased significantly in both sectors in relation to 2004, to reach a neutral to slightly alkaline pH (between 7.7 and 8.1). However, the differences were significant only in Sector 1, where the mean pH was slightly acidic (6.2) in 2004 (Table 1). In the bare soil (BS), the mean pH strongly fell to clearly acidic values, lower than 4.0 in both sectors. The mean of the total concentration of the pollutants (TP) declined in 2013 in both sectors compared to 2004, although as a result of the heterogeneous pollution of the study area the standard deviations were very high and the differences between mean values were not significant. Regarding the 2013 sampling, the mean of the TP increased sharply in the BS, especially in the case of TAs (5 to 7 times higher) and TPb (3 to 4 times higher); while the increase of the mean values of TCu was lower (1.5 times higher) and, in the case of TZn, especially in the Sector 1, there was a significant reduction in the BS in relation to the previous samplings.

The ratio of the concentration of each pollutant between 2013 and 2004 ($TP_{13/04}$) was less than 1 in approximately 80% of the soils and higher in the remaining 20% (Figure 2). Except in the case of Zn, the $TP_{13/04}$ ratios were significantly ($p<0.001$) and inversely related to the total concentration of the pollutants in 2004 (TP_{04}) by the following linear equations:

$TCu_{13/04} = 33.4\ TCu_{04}^{-1} + 0.46$ $\quad r = 0.429$ $\quad$ (1)

$TAs_{13/04} = 21.7\ TAs_{04}^{-1} + 0.26$ $\quad r = 0.585$ $\quad$ (2)

$TPb_{13/04} = 44.6\ TPb_{04}^{-1} + 0.34$ $\quad r = 0.407$ $\quad$ (3)

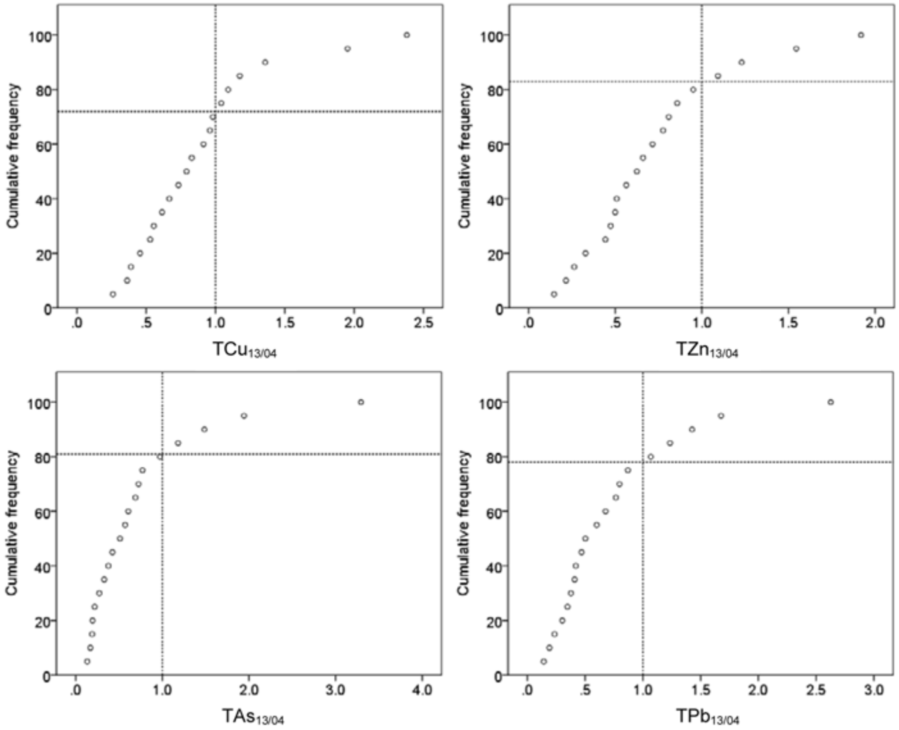

Figure 2. Cumulative frequency distribution for the ratio of the total concentration of each pollutant in 0-10 cm in depth between 2013 and 2004 ($TP_{13/04}$).

The decrease in the TP lowered the mean value of the EF for each pollutant in 2013 in relation to 2004 (Figure 3), reaching in 2013 mean values around 1 (between 0.98 for Cu and 1.13 for Pb). Compared to the sampling of 2013, the mean EF in the BS sharply increased in the case of As and Pb, reaching 8-fold higher values, while the enrichment of Cu was much lower and Zn was depleted (EF <1).

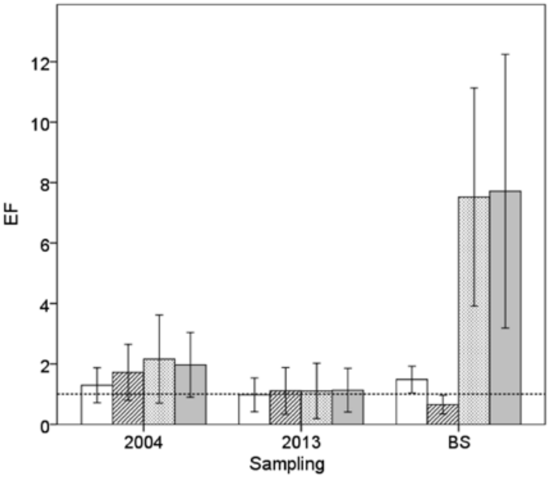

Figure 3. Mean and standard deviation (error bars) of the enrichment factor (EF) of Cu (open bars), Zn (stripped bars), As (dotted bars) and Pb (grey bars) in the sampling of 2004, 2013 and in the bare soil (BS). The dotted line indicates the value of EF = 1.

7.3.2. Soluble concentration of the pollutants

Compared to 2004 values, the mean soluble concentration of the pollutants (SP) in both sectors decreased in 2013 in the case of SCu, SZn, and SPb, and increased in the case of SAs (Table 2). For the same reasons as the TP, the standard deviation was very high and the differences between mean values were not significant, except in the case of SPb in Sector 2. In the BS, as in the TP, the SP increased sharply, especially in the case of SCu and SZn.

Table 2. Mean and standard deviation (SD) of the soluble concentration of pollutants (SP) in soils (μg kg^{-1}) sampled in 2004 and 2013 and bare soil (BS).

		Sector 1				Sector 2			
Sampling		SCu	SZn	SAs	SPb	SCu	SZn	SAs	SPb
2004	Mean	128a	779 a	12a	13a	146a	305a	6a	62a
	SD	59	883	5	5	85	446	2	40
2013	Mean	45a	160a	17a	5a	75a	138a	10a	10b
	SD	17	88	8	2	98	143	5	4
BS	Mean	8206b	65770b	188b	49c	5387b	104805b	334b	96.1c
	SD	3640	29522	60	35	2478	71516	427	26.5

In each column, values of the mean followed by the same letter do not significantly differ (Tukey's test, $p<0.05$).

With all soils sampled in 2013 considered (including the BS), SCu and SZn were inversely and significantly ($p< 0.001$) related to the pH by the following power equations:

$$SCu\ (mg\ kg^{-1}) = 2.84 \times 10^{3} \times pH^{-5.212} \qquad r = 0.961 \qquad (4)$$

$$SZn\ (mg\ kg^{-1}) = 127.4 \times 10^{3} \times pH^{-6.661} \qquad r = 0.941 \qquad (5)$$

While in the case of As and Pb, soluble concentrations were significantly ($p<0.001$) related only to the respective total concentrations by the following linear equations:

$$SAs\ (mg\ kg^{-1}) = 8.34 \times 10^{-4} \times TAs - 24.38 \times 10^{-3} \qquad r = 0.858 \qquad (6)$$

$$SPb\ (mg\ kg^{-1}) = 0.66 \times 10^{-4} \times TPb + 4.71 \times 10^{-3} \qquad r = 0.858 \qquad (7)$$

The difference between As and Pb was that the mean value of the SA:TA ratio tended to increase in 2013 compared to 2004 and increased further in the BS (Figure 4), while the mean value of the SPb:TPb ratio tended to decrease in 2013 compared to 2004 values, but remained constant in the BS in relation to soils sampled in 2013.

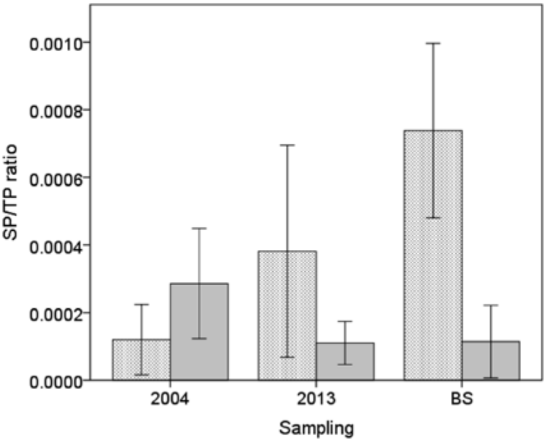

Figure 4. Mean and standard deviation of the ratio between the soluble and total concentration (SP/TP) of As (dotted bars) and Pb (grey bars).

7.4. Discussion

The results for 2013 confirm that the mean pH values tended to rise and the mean of the total concentration of pollutants (TP) tended to diminish over time. In any case, there was an increase of the TP in 2013 for some 20% of the soils (Fig. 2), mainly in the less contaminated soils in 2004; [Eqs. (1)-(3)], suggesting the existence of a spread of pollutants from the most polluted soils to the least polluted ones. This process was presumably related to surface runoff water previously described in the area due to occurrence of frequent rainfall intensities higher than the final infiltration rate of the soils (Simón et al., 2005a). This spreading process, together with the pollutant uptake by plant roots (Dominguez et al., 2008) and the infiltration in depth of the most soluble pollutants such as Zn and Cu (Kraus and Wiegand, 2006), could justify the decrease of the TP in the first 10 cm of the soils over time. Therefore, in 2013 the mean of the TP (Table 1) was close to or lower than the upper limit (250 mg Cu kg^{-1}, 900 mg Zn kg^{-1}, 40 mg As kg^{-1}, and 300 mg Pb kg^{-1}) of the normal range in soils (Bowen, 1979). In addition, the EF (Fig. 2) indicates that in 2013 the pollutant concentration in the soils was similar to the pollutant concentration prior to the spill (mean EF values around 1).

The rise in pH values would be primarily responsible for the decline of the SCu and SZn [Eqs. (4) and (5)], while the decrease of the TAs and TPb caused the reduction of the SAs and SPb over time [Eqs. (6)-(7)]. However, compared to the 2004 sampling, the soluble As in relation to the total As increased in 2013 (Fig. 4), which could be attributed to ageing and higher crystallinity of iron oxy-hydroxides that led to the partial desorption of the As immobilized by these iron compounds (Pedersen et al., 2006). In any case, mean values of the SCu, SZn, SAs, and SPb (Table 2) in 2013 were clearly lower than the toxic levels (700 µg Cu kg^{-1}, 500 µg Zn kg^{-1}, 40 µg As kg^{-1}, and 100 µg Pb kg^{-1}) estimated by Ewers (1991) and Bohn et al. (1985).

In accordance with the above, the remediation measures could be considered effective, to the extent that they have decreased the total and soluble pollutant concentration to a level at which the soil can be considered to have little or no pollution. However, the first remediation measure, consisting in the removal of the tailings and the upper part of the soils using heavy machinery, was very fast (in eight months, 45 km^2 were cleaned) but resulted in a deficient cleanup, leaving spots (BS) in which part of the tailings remained mixed with the soil (Simón et al., 2005a). The heavy concentration of pollutants in the tailings significantly increased the total concentration of pollutants in the soils of the spots, especially As and Pb, where the mean concentrations in the BS were between 3- and 7-fold higher than those of 2013 sampling soils (Table 1). Thus, the As and Pb concentrations exceeded the upper limit of the normal range in soils (Bowen, 1979) and even As exceeded more than 2.5-fold the intervention level (100 mg As kg^{-1}) established by the Environmental Agency of the Regional Government of Andalusia for natural parks (Llamas et al., 2000). In addition, oxidation over time of the sulphides contained in the tailings (Simón et al., 2001) significantly decreased the pH of the BS to clearly acidic values (between 3 and 4), resulting in a significant increase of the soluble concentration of pollutants (Table 2) that widely surpassed the toxic level mentioned above. The higher solubility of Cu and Zn compared to As and Pb, would encourage their spread by the liquid phase of the runoff and their infiltration below the top 10 cm of the soils. Both processes can explain why the EF of Cu and Zn in the BS were clearly lower than EF of As and Pb, especially in the case of Zn (more soluble than Cu) with values lower than 1.

These results indicate the high total and soluble pollutant concentration, which greatly limited the vegetation development in the unrecovered soils where the residues of tailings remained, resulting in spots of bare soil that clearly stand out in the landscape 15 years after the spill. These spots are a major source of pollution from which pollutants are scattered through the solid and liquid phases of runoff water, so that it would be necessary take action to immobilize pollutants and encourage the restoration of vegetation in these BS. These problems, together with the fact that the spill pollutants were still concentrated in the top 10 cm of soil after completion of all the remediation measures (Simón et al., 2007), lead us to consider that, in this type of pollution in a Mediterranean environment, the speed of cleaning would be less important than ensuring the complete removal of the tailings.

7.5. Conclusions

The remediation measures implemented in soils affected by the spill of Aznalcóllar mine were effective, to the extent that 15 years after the accident, the total and soluble concentration of pollutants is within the normal range in soils and close to concentrations that the soils registered prior to the spill. However, the removal of tailings with heavy machinery, although fast, was inadequate, leaving spots where the tailings remained mixed with the soil. In these spots the pH sharply declined, while the total and soluble concentration of pollutants spiked, and the development of the vegetation was drastically limited. Thus, the spots have become major sources of pollution, requiring intervention in order to raise the pH, decrease the solubility of pollutants, and encourage colonization by vegetation. Slower and meticulous removal of tailings, without leaving residues of tailings mixed with soil, would have resulted in a more efficient recovery of the affected area.

CHAPTER 8

Long-term toxicity assessment of soils in a recovered area affected by a mining spill

Abstract

Residual pollution in the Guadiamar Green Corridor still remains after Aználcollar mine spill in 1998. The polluted areas are identified by the absence of vegetation, soil acidic pH and high concentrations of As, Pb, Zn and Cu. Soil toxicity was assessed by lettuce root elongation and induced soil respiration bioassays. In bare soils, total As and Pb concentrations and water-extractable levels for As, Zn and Cu exceeded the toxicity guidelines. Pollutants responsible for toxicity were different depending on the tested organism, with arsenic being most toxic for lettuce and the metal mixture to soil respiration. Soil properties, such as pH or organic carbon content, are key factors to control metal availability and toxicity in the area. According to our results, there is a risk of pollution to living organisms and the soil quality criteria established in the area should be revised to reduce the risk of toxicity.

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Submitted

8.1. Introduction

In 1998 the breaking of the waste dump at the Aznalcóllar pyrite mine (Seville, Spain) spilled around 4.5 x 10^6 m^3 of acidic waters and toxic tailings into the Agrio and Guadiamar river basins (Grimalt et al., 1999). This accident was considered the second largest of the 59 major mine spills in the world and the largest reported in Europe (Nikolic et al., 2011), with a total affected area of 4630 ha of soils with an agricultural and grazing use. Soils were highly polluted with high concentrations of Zn, Cu, As and Pb (Simón et al., 2001) and few months later one of the most important soil remediation programmes in Europe was applied to restore the affected area, concluding with the establishment of the Guadiamar Green Corridor (CMA, 2003). The main remediation actions included: i) removal of the tailings and heavily polluted topsoil; ii) amendment applications to reduce mobility and bioavailability of pollutants (using liming, iron-rich clayey materials, and organic matter); iii) soil phytostabilization with autochthonous species (CMA, 2003; Aguilar et al., 2004a). The remediation measures gradually decreased the concentration of pollutants in the affected soils over time, but an irregular distribution pattern of contamination in the affected soils was easily discernible, with highly polluted areas alternating with other much less polluted areas (Simón et al., 2008). Fifteen years after the accident, most of the affected soils are successfully recovered, however highly polluted spots of soils with variable area (from 1 to 200 m^2) are easily distinguished in the landscape because no vegetation has developed. These spots cover around 20 ha of the total affected area which represent a local source of pollution with high risk of dispersion that should be monitored (Martín Peinado et al., 2015).

The evolution over time of total and available concentration of pollutants in soils is well studied both *in situ* and in the laboratory, but the long-term changes in toxicity are less studied, especially under natural conditions. Ageing studies mainly assessed the development of toxicity in spiked soils (Scheckel et al., 2009). These studies show that long-term incubations are required, at least under laboratory conditions, to obtain results that may help estimating the potential risk in natural soils. In general ageing processes have shown to decrease toxicity in soil over time (Lock and Janssen, 2002). Ecotoxicity tests are key tools in the study of the fate and bioavailability and to assess the potential ecotoxicological effects of metals as well as their possible transfer to the other compartments such as groundwater, or even their transfer to the food chain. The use of biological tests is essential for determining the potential ecological risk of soil contamination to organisms and ecosystems, although the influence of long-term equilibration processes usually is not considered in such tests.

The residual polluted areas in the Guadiamar Green Corridor do not only have an immediate need of study for their pollutant load, but also they are contaminated natural areas that have gone through a long process of stabilization in natural conditions. Our main objective was to assess the residual contamination and potential toxicity using bioassays in soil affected by the mine spill, focusing on the effect of soil properties on the availability of pollutants and their ecotoxicological implications. Natural residual polluted areas could be a key to test and optimize ecotoxicological tests usually performed under controlled laboratory conditions. This work was driven by the hypothesis that the total metal concentration estimated from laboratory ecotoxicological studies (guideline values) are a simplistic approach not representative of field polluted soils, where metal bioavailability and toxicity will be defined by soil properties, ageing processes and presence of multi-element pollution as well as studied organisms. This study will help to understand in a more reliable way both the effects of soil properties and the processes of ageing involving field multi-element pollution in soils.

8.2. Materials and Methods

8.2.1. Soil samples

In the Guadiamar Green Corridor (Seville, Spain), unrecovered soils were detected using satellite images from areas without regeneration of vegetation (bare soils, BS) but surrounded by regenerated areas (recovered soils, RS) (Figure 1). According to the first soil classification of the area (Aguilar et al., 2004b), samples were selected in two different sectors based on the calcium carbonate content in the soil parent material. Sector 1 included soils with high carbonate content, Sector 2 soils with low/null carbonate content in the parent material. The methodology used in the soil sampling was the same as used in previous studies of the area (Simón et al., 2008; Martín Peinado et al., 2015). Samples were collected in the two Sectors in 200 x 200 m plots discriminated in two types: i) plots with evidence of pollution (with spots of bare soils) and ii) plots without evidence of pollution (soils completely covered by vegetation) (Figure 1). In the plots with evidence of pollution, samples were taken both in bare soils (BS) and the adjacent recovered soils (RS). For each soil type, 5 sub-samples were randomly taken at 0-10 cm depth and intensely homogenized to provide a representative sample of BS and RS at each plot. In the plots without evidence of pollution, 5 sub-samples were randomly taken at 0-10 cm depth and intensely homogenized to provide a representative sample of the recovered areas (RA). A total of 28 samples were selected for this study, 14 in Sector 1 (4 BS, 4 adjacent RS and 6 representing RA; and 14 in Sector 2 (4 BS, 4 RS and 6 RA) (Figure 1). Two unpolluted natural control soils with similar properties and constituents to those of the studied soils were selected: CS1: unpolluted control soil similar to soils of sector 1, and CS2: unpolluted control soil similar to soils of sector 2 (Table 1).

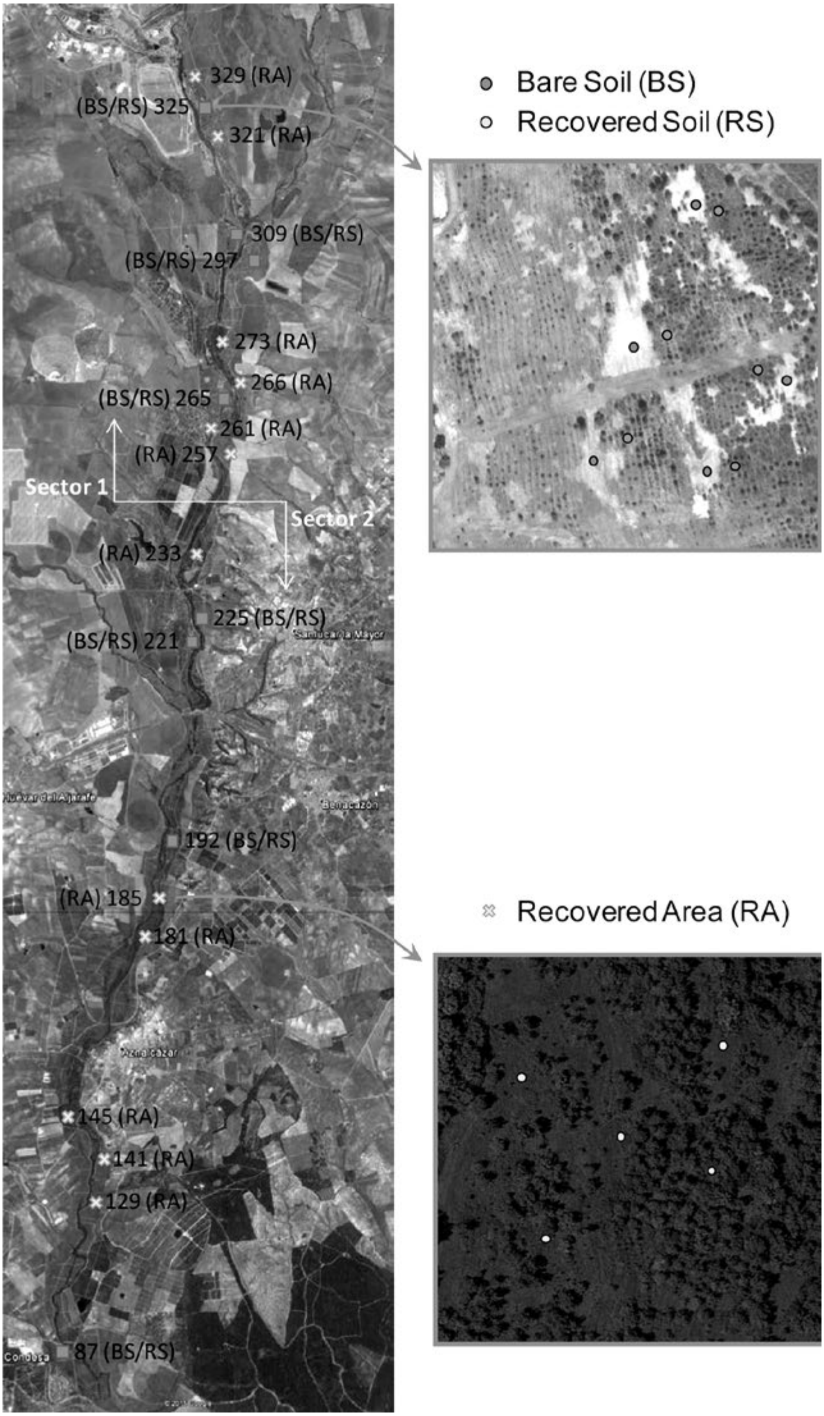

Figure 1. Localization of the studied plots into the Guadiamar Green Corridor (Seville, Spain). Sector 1 included soils with high calcium carbonate content, Sector 2 soils with low/null carbonate content in the parent material. Expected polluted areas were selected according to the absence of vegetation (BS: bare soils) with the neighboring soil with vegetation (RS: recovered soils). Representative recovered areas (RA) were selected to avoid possible errors in understanding pollution due to dispersion.

8.2.2. Analytical methods

The main soil properties and constituents including calcium carbonate content, organic carbon content, electric conductivity, cation exchange capacity and pH were analyzed following standard methods (MAPA, 1994) (Table 1). According to Martín Peinado et al. (2015) the main pollutants in the area were As, Pb, Zn and Cu. Total concentrations of these pollutants (Tx) were determined by PXRF (Martín Peinado et al., 2010). Water extractable (Wx; from soil:water extract 1:2.5; Blakemore, 1987) and exchangeable (Ex; extracted with ammonium acetate 1N; Beckett, 1989) metal concentrations were measured by ICP-MS (PE SCIEX ELAN-5000A spectrometer). The ICP-MS analyses included 3 replicates and for calibration two sets of multi-element standards were used with the studied analytes at five different concentrations (Martín Perinado et al., 2015). Analytical precision was better than ±5% in all cases.

Table 1. Main soil properties of soils sampled for this study. Sector 1 included soils with high carbonate content, Sector 2 soils with low/null carbonate content in the parent material. Expected polluted areas were selected according to the absence of vegetation. BS: bare soils, RS paired recovered soil and RA representative recovered areas. CS1: unpolluted control soil similar to soils of Sector 1; CS2: unpolluted control soil similar to soils of Sector 2.

Sector	Soil type		Vegetation	pH	$CaCO_3$ (%)	OC (%)	EC (dS m^{-1})	CEC (cmol+ kg^{-1})
1	RA	(n=8)	present	8.08	11.33	1.39	0.27	21.5
		SD		(±0.13)	(±7.78)	(±0.66)	(±0.12)	(±7.51)
	RS	(n=4)	present	6.12	6.69	1.47	0.37	17.9
		SD		(±2.29)	(±7.25)	(±0.33)	(±0.24)	(±6.25)
	BS	(n=4)	absent	4.30	1.99	0.62	3.88	9.97
		SD		(±2.24)	(±2.34)	(±0.20)	(±1.13)	(±0.70)
2	RA	(n=8)	present	7.55	3.96	1.35	0.29	18.4
		SD		(±1.01)	(±4.41)	(±0.32)	(±0.23)	(±2.95)
	RS	(n=4)	present	7.57	3.24	1.40	0.27	17.9
		SD		(±0.82)	(±3.69)	(±0.30)	(±0.17)	(±5.29)
	BS	(n=4)	absent	3.19	0.90	0.73	2.20	12.2
		SD		(±0.37)	(±0.19)	(±0.13)	(±0.24)	(±0.63)
CS1				8.67	72.39	0.42	0.14	9.83
				(±0.03)	(±0.86)	(±0.12)	(±0.02)	(±1.00)
CS2				6.74	nd	0.61	0.08	9.91
				(±0.04)	-	(±0.10)	(±0.02)	(±0.42)

The unpolluted control samples (CS1 and CS2) were artificially and individually spiked in the laboratory with the metals most abundantly present in the studied soils: As(V), Pb(II), Zn(II) and Cu(II), using solutions of the following soluble salts: Na_2HAsO_4 $7H_2O$, $Pb(NO_3)_2$, $ZnCl_2$, and $Cu(NO_3)_2$ $3H_2O$, respectively. Pollution levels were established according to the intervention values proposed by the Regional Government of Andalusia (Aguilar et al., 1999) with five concentrations and based on a previous study by Romero-Freire et al. (in press, chapter 6) (Table 2).

Table 2. Contamination levels of different metals (mg kg dry soil^{-1}) added to the unpolluted control soils (CS) according to Aguilar et al. (1999). L1 (intervention level for agricultural soil), L2 (intervention level for natural areas), L3 (intervention level for industrial areas), L4 (L3 x 2), and L5 (L3 x 4 in the case of As, Pb, Cu, and L3 x 3 in the case of Zn).

Contamination levels	As	Pb	Zn	Cu
L1	50	500	600	300
L2	100	1000	1000	500
L3	300	2000	3000	1000
L4	600	4000	6000	2000
L5	1200	8000	9000	4000

8.2.3. Bioassays

Two biological tests were selected for this experiment:

1. Root elongation test, according to OECD (2003) and U.S. EPA (1996) recommendations. This test assesses the phytotoxic effects on seedling growth in the first days of development (Escoto et al., 2007). For this test the water extract of the soils (1:2.5) was used, obtained after 24 hours of shaking, by extraction with 10 cm Rhizon MOM. In Petri dishes 10 seeds of *Lactuca sativa* and 5 ml of water extract were placed, and incubated at 25 ±1°C and the length of the germinated roots were measured after 120 h. Samples were tested in triplicate. The endpoint calculated was root elongation reduction in relation to water controls. For further analyses, the values obtained were scaled between 0 and 1 indicating no reduction and maximal reduction, respectively (*Lactuca sativa* reduction: LsR).

2. Soil respiration test (ISO 17155, 2002). This test measures the mass of CO_2 released per unit mass of soil and per unit of time after addition of glucose as carbon substrate (substrate induced respiration). Samples were measured in a microbiological analyzer µ-Trac 4200 SY-LAB model in triplicate, with soil moisture fixed at field capacity and at a constant temperature of 30ºC. CO_2 produced was trapped in vials with a solution

of potash (KOH 0.2%) during 6 hours, and related to the weight of the soil used for the respiration measurement. The results were expressed as the average respiration in µg CO_2 g^{-1} soil per day (Substrate induced respiration: R_S).

8.2.4. Multi-element pollution study

From the unpolluted control soils (CS1 and CS2) spiked in laboratory, the 50 percent effective concentrations (EC50) for the studied bioassays were calculated by fitting a log-logistic dose-response model to the data for the 4 studied elements (As, Pb, Zn and Cu) according to Doelman and Haanstra (1989). EC50s were based on total soil concentrations. To predict multi-element toxicity in the studied soils, it was assumed that the metals would act in an additive way. Toxic potency of metals in the soils from the area was estimated using the toxic units (TU) approach. A toxic unit was derived by dividing the total concentration of each element by the EC50 obtained in the control soils, selecting the EC50 from CS1 for the soils in Sector 1 and from CS2 for the soils in Sector 2. Total toxic units (TU_T) were the sum of all individual TU for each element and were used to estimate mixture toxicity (Weltje, 1998). The relative contribution of each metal to the total toxic potency of the mixture was expressed as the percentage of the individual TU (%TUx) in relation to the total toxic unit.

8.2.5. Statistical analysis

Data distributions were established by calculating the mean values and the standard deviations by cumulative frequency-distribution curves. The differences between the individual means of the study samples were compared using Tukey’s test ($p<0.05$). Spearman correlation and rotated component analysis using the Varimax with Kaiser normalization method were performed to determine the effects of soil properties, metal concentration and bioassays response. All the analyses were performed using SPSS v.20.0 (SPSS Inc. Chicago, USA).

8.3. Results and discussion

8.3.1. Metal concentration and availability

Total metal concentrations showed significant differences depending on the sector, soil groups (BS, RS and RA) and the element considered (Table 3). The less mobile elements (As and Pb) had significantly higher concentrations in bare soils (BS) than in recovered soils

(RS or RA), with values exceeding the intervention levels (L2 in Table 2, Aguilar et al., 1999) for natural soils. As concentrations were about 4-fold higher in BS than in the other soil groups, with mean values of 257 mg kg^{-1} and 375 mg kg^{-1} in Sector 1 and 2, respectively. For Pb, the mean concentrations in Sector 1 (325 mg kg^{-1}) were twice as high in BS than in recovered soils, while in Sector 2 they were up to 6-fold higher with mean values > 1000 mg kg^{-1}. The more mobile elements (Zn and Cu) showed a different distribution than the other metals, with values below the intervention levels for natural soils in the area. In general, the concentrations of these elements in BS were not significantly higher than those in recovered soils with the exception of Cu in Sector 2, were the concentrations in BS where 1.8-fold higher than those in RS soils.

Table 3. Mean and *standard deviation* (SD) of the total (T), water extractable (W) and exchangeable (E) metal concentration in soils of the two sectors (Sector 1: soils with high carbonate content in the parent material, Sector 2 soils with low/null carbonate content in the parent material) (all values expressed in mg kg dry soil^{-1}).

	Sector 1			**Sector 2**		
	RA	**RS**	**BS**	**RA**	**RS**	**BS**
TAs	63.0	66.0	257	87.8	61.1	375
	(30.0)	*(31.2)*	*(235)*	*(73.7)*	*(11.9)*	*(133)*
TPb	151	139	324	162	136	1008
	(73.6)	*(39.9)*	*(128)*	*(103)*	*(9.8)*	*(268)*
TZn	386	232	241	325	259	154
	(100)	*(118)*	*(108)*	*(372)*	*(90.0)*	*(65.8)*
TCu	97.5	116	130	89.0	69.3	127
	(31.7)	*(63.7)*	*(34.0)*	*(57.8)*	*(10.6)*	*(22.6)*
WAs	0.03	0.04	0.08	0.07	0.05	0.18
	(0.01)	*(0.02)*	*(0.35)*	*(0.04)*	*(0.03)*	*(0.13)*
WPb	0.01	0.04	0.05	0.04	0.10	0.10
	(0.01)	*(0.04)*	*(0.04)*	*(0.07)*	*(0.15)*	*(0.05)*
WZn	0.07	2.39	109	0.12	0.08	59.4
	(0.03)	*(3.65)*	*(126)*	*(0.20)*	*(0.08)*	*(24.3)*
WCu	0.11	0.51	9.89	0.07	0.05	9.74
	(0.02)	*(0.62)*	*(8.14)*	*(0.05)*	*(0.01)*	*(6.49)*
EAs	0.21	0.15	1.13	0.16	0.48	0.81
	(0.08)	*(0.12)*	*(1.96)*	*(0.08)*	*(0.56)*	*(1.02)*
EPb	0.05	0.03	0.12	0.16	0.31	0.23
	(0.05)	*(0.02)*	*(0.11)*	*(0.24)*	*(0.27)*	*(0.25)*
EZn	9.69	8.29	128	6.02	5.53	63.8
	(6.51)	*(1.85)*	*(169)*	*(6.29)*	*(2.15)*	*(49.7)*
ECu	0.59	1.77	5.31	0.34	0.35	4.02
	(0.32)	*(1.66)*	*(4.26)*	*(0.22)*	*(0.23)*	*(3.36)*

The guideline values proposed for soil protection vary enormously among countries and legislations. Recently, the Regional government of Andalusia region, where the affected area is located, has published soil quality criteria with guideline values based on total concentrations (Decree 18/2015). Compared with the established values of the Decree, arsenic concentrations exceeded the guideline level (36 mg As kg^{-1} soil) in all samples (recovered and bare soil), while for Pb this was the case only in bare soils (guideline level = 275 mg Pb kg^{-1} soil). The guideline levels to declare a soil polluted with Zn (10,000 mg Zn kg^{-1} soil) or Cu (from 595 mg Cu kg^{-1} soil, depending soil use) were not exceeded in the studied soils.

Water extractable and exchangeable concentrations were higher in bare soils than in recovered soils (Table 3) in the case of As, Zn and Cu. For these three elements, the concentrations in Sector 2 were much higher than in Sector 1. In the case of Pb, water extractable and exchangeable concentrations did not differ between BS and the other soil groups neither in Sector 1 and 2.Toxic levels based on concentrations in the soil solution were reported for As (0.04 mg kg^{-1}; Bohn et al., 1985) and for Pb, Zn and Cu (1, 0.5 and 0.7 mg kg^{-1}, respectively; Ewers, 1991). The water extractable concentrations of As, Zn and Cu in BS exceeded these values in both Sectors, while in RS and RA soils these levels were not exceeded.

8.3.2. Effect of soil properties in pollutant availability

The differences in total, soluble and exchangeable metal concentrations between BS and the recovered soils could be directly explained by soil properties. Spearman correlation analysis (Table 4) between the total, water extractable and exchangeable metal concentrations in BS indicate different behaviour according to the considered element. Total concentrations of As and Pb were negatively correlated with pH, because in this type of pollution involving sulphide tailings, the oxidation processes generates a strong acidity and a release of metals (Simón et al., 2001). The As and Pb released could co-precipitate even at acidic pH due to the presence of iron in the soil (Simón et al., 2005b), which can explain the great variation in the concentrations of these two elements in our studied soils. The other two metals, Zn and Cu, showed a different distribution, with no significant differences between BS and RS or RA. These two elements were also released after tailing oxidation, but because of their high solubility under acidic conditions (Aguilar et al., 2004b) these element could lixiviate both in depth as in the surrounding recovered soils. This behaviour can also be explained by the significant correlation of the concentrations of these metals with EC, indicating that Zn and Cu are strongly related to the soluble salts (mainly sulphates) formed from sulphide oxidation (Dorronsoro et al., 2002) and can be responsible of the dispersion of these elements from BS to the adjacent soils. Cu concentrations also significantly correlated with organic carbon content. This relation has previously been described and is related to the specific binding of Cu by adsorption to organic matter (McLaren et al., 1981).

Table 4. Spearman correlation between total (Tx), water extractable (Wx) and exchangeable (Ex) concentrations of As, Pb, Zn and Cu and soil properties for the bare soils (BS) of the two studied sectors in the Guadiamar Green Corridor (Seville, Spain) (see figure 1).

	TAs	TPb	TZn	TCu	WAs	WPb	WZn	Wcu	EAs	EPb	EZn	ECu
	mg kg^{-1}				mg kg^{-1}				mg kg^{-1}			
$CaCO_3$					-0.524**	-0.452*	-0.738**	-0.405*			-0.690**	-0.692**
pH	-0.667**	-0.429*			-0.548**		-0.595**	-0.929**	-0.690**		-0.689**	-0.690**
OC				0.762**		0.548**			-0.524**			
EC			0.479*	0.515*						0.405*		
CEC	0.500*	0.857**	-0.429*									

Mostly water extractable and exchangeable metal concentrations were strongly and negatively correlated with soil pH and $CaCO_3$ content (Table 4). Therefore, the highest available metal concentrations were found in BS, where the soil pH was strongly acidic. The reduction of the mobility of metals in these soils were previously studied in the affected area (Simón et al., 2005b; Aguilar et al., 2004b), where liming showed to be one of the most efficient measures to reduce metal mobility and bioavailability in the affected soils (Aguilar et al., 2004a). The presence of $CaCO_3$ under acidic conditions in BS is related to previous liming and its interaction with the high concentrations of iron and sulphur present, where the carbonate particles become coated with amorphous iron forms limiting their buffer capacity (Al et al., 2000; Simón et al., 2005b). The highest concentrations of water extractable and exchangeable As were positively and significantly ($p<0.05$) correlated with the high total concentrations of this element in BS (Spearman's coefficient 0.536 and 0.893, respectively). For Zn and Cu, there was no significant correlation of extractable and total concentrations ($p>0.05$ in all cases), with the acidic conditions being responsible for the high available concentrations of these two elements in BS compared to the recovered areas.

8.3.3. Ecotoxicological effects in natural polluted soils

The results obtained (Figure 2) showed stress effects in bare soils (BS) of both sectors and for both bioassays. In general, RS and RA showed similar mean responses in the lettuce root elongation and soil respiration tests for each sector. However some evidences of toxicity were detected in these areas in soils considered recovered. In Sector 1 maximum reduction reached in the lettuce root elongation (LsR) was 58%, while in Sector 2, with low carbonate content, it was around 65% respect to controls. In the second bioassay, RS from Sector 1 reached really low values of induced soil respiration (Rs) with a minimum of 12.6 µg CO_2 g^{-1} soil day^{-1}. This broad variation in the bioassay responses suggests that soil properties, such as pH or CaCO3 content, which mainly differed between the studied sectors, are essential factors when comparing the toxicity of soils (Romero-Freire et al., 2014; 2015).

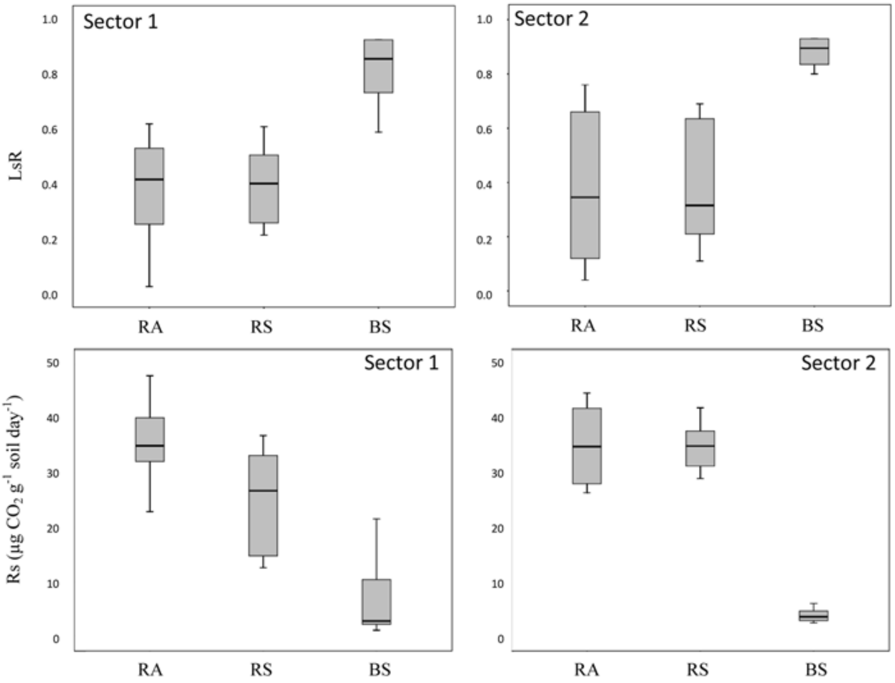

Figure 2. Toxicity of polluted soils in the two studied sectors of the Guadiamar Green Corridor. On top results for the *Lactuca sativa* root elongation test (LsR: root reduction scaled between no reduction (0) and maximal reduction (1)) and on the bottom the soil respiration test (Rs: induced soil respiration expressed as µg CO_2 g^{-1} soil per day). (RA: recovered areas, RS: recovered soils and BS: bare soils).

These findings show that bare soils were most toxic in the two bioassays and it also suggests that in the case of high pollution, the outcome of only one bioassay would be sufficient identify soil toxicity regardless the studied phase (total or water extractable metal) and organism (bacteria or plants). However it was previously reported that exposure pathways could lead to different responses of exposed organisms (García-Gómez et al., 2014). In addition, organisms have their own sensitivity to pollutants and will also show different responses under different exposure conditions (Matejczyk et al., 2011; Romero-Freire et al., *in press*). Therefore the use of only one bioassay may be useful for early identifying pollution problems, but it will not be sufficient as an indicator of toxicity pathways in order to select remediation methods.

Table 5. Effective concentrations (EC50) calculated for the toxicity of metals in the unpolluted control soils (CS) spiked in the laboratory, in toxicity tests focusing on *Lactuca sativa* root elongation or soil respiration. See table 2 for the total metal concentration added into the control soils.

		EC50 **As**	**EC50** **Pb**	**EC50** **Zn**	**EC50** **Cu**
Bioassay	**Control soil**	Based on total concentrations (mg kg dry soil^{-1})			
Lactuca sativa	CS1	84 *(80-88)*	8000*	9000*	4000*
	CS2	191 *(165-222)*	3460 *(1801-6647)*	4155 *(2956-5841)*	1387 *(989-1844)*
Soil respiraton	CS1	1200*	8000*	9000*	283 *(115-695)*
	CS2	1200*	8000*	1842 *(1234-2749)*	742 *(430-1280)*

*: EC50 higher than the highest used concentration. Data in brackets represent the confidence interval for the mean 95%.

To study the effects of multi-element pollution in the two bioassays, effective concentrations inhibiting the response with 50% (EC50) were calculated from the toxicity tests in the control soils (CS) individually polluted under artificial conditions for each element. Selection of thresholds levels is necessary to provide a safe environment, however it is a complex process (Peña-Fernández et al., 2014). There is a wide variation in literature data and robust toxicity thresholds have not yet been defined (Marques et al., 2014). From the toxicity data shown in Table 5, toxic units (TU) were calculated by dividing the total concentration of the element by the calculated EC50 (Weltje, 1998). In order to arrive at a precautionary decision, when the toxicity did not reach 50% effect, even at the highest concentrations applied, we selected the maximum concentration for that metal to estimate toxic units (Table 5). This was the case in the *L. sativa* test for Pb, Zn and Cu in soil with high carbonate content (CS1) and in the soil respiration test for As and Pb in both control soils and for Zn in soil CS1. Table 6 shows the calculated toxic units and the contribution of each metal to the total toxic potency of the field soil. It was observed that metals responsible for the toxicity of the test soils were different depending on the test. In the case of the lettuce bioassay, toxicity in the two sectors was mainly determined by the concentration of As. The response of the soil respiration bioassay was mainly explained by Cu in the soils of Sector 1, whereas in Sector 2 it was mainly explained by Zn and Cu in the case of recovered soils (RS) and by As and Cu in the bare soils (BS). This suggests that the established guideline values for Zn and Cu in the Decree 18/2015 could overestimate the potential pollution in these areas. These results highlight that different responses of different organisms cannot be predicted on the basis of the effects of individual pollutants, which is in accordance of the results obtained by Baderna et al. (2015) who performed tests with plants and 8 pollutants. From these results we may also concluded that a metal which causes toxicity in some organisms does not do so in others and that the complexity of natural polluted soils due to the presence of mixture of elements can mask the effects attributed to the different metals involved (Spurgeon and Hopkin, 1995). Therefore a battery of tests involving more organisms is essential for a proper assessment of the toxicity of multi-element polluted field soils, including the synergistic or antagonistic effects of involved metals as well as their bioavailability.

Table 6. Range of toxic units (TU) for the two bioassays and in the two studied sector to paired soils (recovered soils and bare soils). TUs were calculated from the total concentration of pollutants in soils and the corresponding effective concentration (EC50) of the reference soils (table 5). The weight of each pollutant to the calculated toxic unit is expressed as the percentage (%) of the total toxic unit.

Sector	Sample	TU range	%TUAs	%TUPb	%TUZn	%TUCu
				Lactuca sativa bioassay		
1	**RS**	(0.46-1.34)	90.3	2.2	4.2	3.3
	BS	(1.40-7.79)	95.2	1.8	1.3	1.6
2	**RS**	(0.37-0.56)	67.5	8.4	13.3	10.7
	BS	(1.64-3.27)	81.6	12.8	1.6	4.0
				Soil respiration bioassay		
1	**RS**	(0.28-0.77)	11.0	3.8	7.7	77.6
	BS	(0.44-1.06)	25.7	5.8	3.9	64.6
2	**RS**	(0.26-0.40)	17.3	5.8	45.6	31.4
	BS	(0.50-0.88)	44.2	18.5	12.0	25.3

Bioassays performed with samples taken from the polluted site allow assessing the real risk of pollution under natural conditions (Fernández et al., 2006). Concentration-effect curves for the effects in the *Lactuca sativa* and the soil respiration bioassays were calculated and are shown in Figures 3 and 4, respectively. Lettuce root elongation in the field soils showed a strong relationship with the estimated toxic units (Figure 3), with an EC50 of 1.48 TU, suggesting antagonistic effects in bare soils (Weltje, 1998). This effect supports the higher contribution obtained for As to the total TU (Table 6), which confirms that the lettuce bioassay in the bare soils is mainly attributed to this metalloid, regardless the other involved metals. Therefore we could conclude that remediation is needed for arsenic toxicity. On the other hand, the dose-response curve for induced soil respiration showed that the effect produced by soil pollution was mainly synergistic (EC50 0.31 TU), which suggests that although it was observed different contribution of the different pollutants to TU, any metal involved can have effect in soil respiration reduction (Figure 4). These differences in sensitivity of different toxicity tests have been previously reported by other authors depending on metals occurring in the same soil samples (Baran et al., 2014).

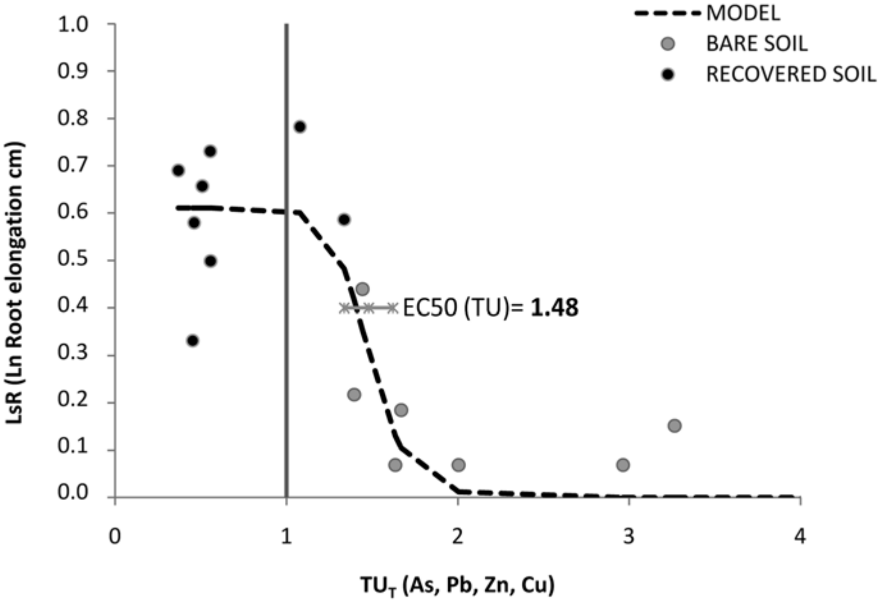

Figure 3. Concentration-effect curve for the toxicity of field-contaminated soils in the lettuce rot elongation test, using the total toxic units of As, Pb, Zn and Cu in recovered soils (RS) (black points) and in bare soils (BS) (grey points) of the two studied sectors as a measure of exposure. Vertical line represents the shift of the curve towards a synergistic or antagonistic effect. EC50 of the calculated toxic unit and its confidence intervals for the mean 95% are expressed in the curve.

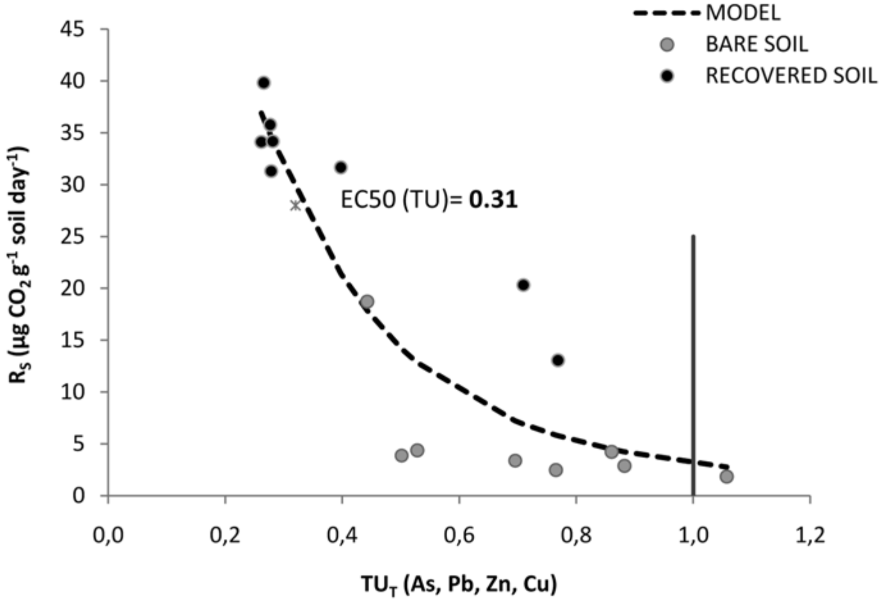

Figure 4. Concentration-effect curve for the toxicity of field-contaminated soils in the substrate induced respiration test, using the total toxic units of As, Pb, Zn and Cu in recovered soils (RS) (black points) and in bare soils (BS) (grey points) of the two studied sectors as a measure of exposure. Vertical line represents the shift of the curve towards a synergistic or antagonistic effect. EC50 of the calculated toxic unit is expressed in the curve; confidence intervals are wider than the mean 95% and therefore are not shown.

8.3.4. Effect of soil properties in metal toxicity

Principal component analysis (PCA) with the toxicity measured in the two bioassays and the main soil properties as well as total metal concentrations and the calculated toxic units showed that 72.3% of the variance was explained by two components (Table 7). Related with bioassays response, soil respiration was grouped in component 1 and negatively correlated with toxic units and specifically with total concentrations of As and Pb in the soils. This means that higher TAs, TPb or TU led to higher toxicity of the soils. Rs was negative correlated with EC and positive with OC content. Root elongation reduction (LsR) appeared in the same component but with an opposite sign, and was positively correlated with toxic unit, i.e., greater toxic unit, greater elongation reduction. Component 2 grouped together and showed positive correlations of pH, $CaCO_3$ content and CEC, and it was related with zinc and copper. These properties appeared to be positive correlated with TZn and negatively with TCu.

Table 7. Principal component analysis (PCA) after Varimax with Kaiser normalization to paired soils (BS and RS) including total metal concentrations, bioassay response (substrate induced respiration -R_S- and reduction of lettuce root elongation -LsR-), toxic units calculated for each bioassay from the total effective metal concentration and the main soil properties.

	Comp. 1	**Comp. 2**
pH (1:2.5)	-0.610	0.730
$CaCO_3$ (%)		0.829
OC (%)	-0.718	
EC (dS m^{-1})	0.892	
CEC (cmol+ kg^{-1})	-0.516	0.602
As_T (mg kg^{-1})	0.870	
Pb_T (mg $kg^{-1)}$	0.644	
Zn_T (mg kg^{-1})		0.730
Cu_T (mg kg^{-1})		-0.807
$TU_{(LsR)}$	0.856	
LsR	0.841	
$TU_{(Rs)}$	0.692	-0.552
R_S	-0.729	0.576
% ac. ex. var.	43.9	72.3

The bioassay responses, as expected, were explained by the toxic units, but appeared mainly influenced by TAs and TPb in soil. Positive correlation between organisms' responses and concentration of metals in soils from areas of mining activities has been reported before (García-Lorenzo et al., 2009). The total metal concentration in bare soils (BS) was much higher than in the paired ones (RS) (Martín-Peinado et al., 2015). The influence of As to toxicity is in accordance with the previous results in the lettuce bioassay, however total Pb explained toxicity only in Sector 2 in both bioassays. Soil pollution with metals increased electric conductivity and therefore correlation with toxicity should be studied in more detail to discriminate the effect of metals from the effect of salinity. Metal toxicity is also largely dependent on their specific chemical form and on their binding state, for instance their complexation with organic ligands (Rodríguez-Ruíz et al., 2014). Therefore a higher OC content will lead to a decreased toxicity for both bioassays. In the PCA component 2, Zn and Cu clustered together with other soil properties. Zn and Cu are the most soluble metals (Kraus and Wiegand, 2006) and in general a rise in soil pH leads to a decline of their concentrations. These elements also are essential for plant nutrition and therefore deficiency effects could be mistaken for toxic responses (García-Gómez et al., 2014). $CaCO_3$ content, which is directly related with soil pH, plays an important role in decreasing the solubility of these elements by precipitation, adsorption or co-precipitation processes (González et al., 2013). However, our results showed contrasting responses for Zn and Cu that should be related with the different behaviour of these two elements under acidic conditions. Simón et al. (2005b) reported that the percentage of both elements precipitated after liming is significantly different in relation to pH. At pH 3.8 the percentage of Zn and Cu precipitated was 5% and 30%, respectively, while at pH 5 this was 5% for Zn and increased to 80% for Cu. These differences can explain that under acidic conditions the amount of total Cu precipitated in soil was much higher than total Zn. More studies are needed to investigate the toxicity of multi-element pollution in soils to advance knowledge for environmental risk assessment and soil remediation.

8.4. Conclusions

Residual polluted areas are still present in the Guadiamar Green Corridor affected by the spill of Aznalcóllar mine, several years after the application of remediation actions and easily identified by the absence of vegetation. Total As and Pb concentrations were significantly higher in polluted soils than in unpolluted areas and toxic levels were exceeded for As, Zn and Cu. The long-term equilibration under natural conditions has changed pollution conditions and therefore metal bioavailability. These *in situ* polluted areas are key to optimize ecotoxicological studies as well as to check if safe environmental thresholds calculated in laboratories are effective for ecotoxicological risk assessment.

Using two bioassays (lettuce root elongation and soil respiration) it was observed that metals mainly responsible for the toxicity of the soils are different depending on the test. Some soil properties, such as pH or organic carbon content, reduce metal availability and therefore soil toxicity and they should be key components to declare pollution levels. According to the existing regulation in the affected area, there is a clear risk of contamination to living organisms but the established soil quality criteria overestimate toxicity. Intervention in the polluted areas is required to control bioavailability as well as to protect metal release to the surrounding recovered areas.

CHAPTER 9

General Discussion

There is a worldwide concern on soil pollution, specifically concerning metals and metalloids, because these elements do not degrade and are retained in soils. Moreover, due to the urban growth new materials involving metals, such as nanoparticles, are released and end up in soils. Soil pollution has received relatively little attention compared to other environmental compartments, because this medium usually has a very high capacity to neutralize contamination. But, to what extent are soils capable of coping with non-degradable chemicals without leading to hazards to man and the environment? Establishing methods for sustainable soil protection is essential. Step-by-step, different international approaches have been developed in order to define guidelines and methodologies to assess metal pollution in soils. However, there is a lack of consensus on soil protection among a wide range of countries, and, even in Europe, official criteria to declare a soil as contaminated have not been established yet in the current legislation of many countries.

This thesis studied the fate and effects of soil pollution by some common metals and metalloids as well as metal-based nanoparticles to improve the scientific basis for their environmental risk assessment (ERA). But, how to establish critical metal concentration in soils for protecting soil organisms? One of the major concerns is the current increase of metal nanoparticle release to the environment, giving these metal forms a different potential hazard compared to their ionic forms. It is known that metal solubility, bioavailability and toxicity are dependent on soil properties and also can vary with time (ageing). The common use of freshly-spiked artificial soils in soil toxicity tests, however, does not take these factors into account, and therefore does not really represent natural conditions. Can we extrapolate from short-term laboratory assays to the real field conditions? In addition, the effects of pollution are usually studied under controlled conditions with soils artificially spiked with single metals or simple combinations of metals. Natural soils however, usually contain combinations of several metals that are present already for long periods of time. As a consequence, toxicity of field-contaminated soils may be hard to predict from the results obtained in laboratory studies.

Referring to the issues raised above, the main objective of this thesis was to verify the effectiveness of existing soil quality criteria and to improve the scientific basis for the environmental risk assessment of metals and metalloids in soils, by taking into account external factors such as soil properties and ageing processes. The discussion below aims at integrating the results presented in the seven research Chapters **(2-8)** and to contribute to generic environmental quality standards for soils polluted with metals.

9.1. Metal-pollution in a changing world: nanoparticles

Although metal emissions have decreased in the European Union during the last decades (EEA, 2013), the production of nanomaterials involving metals has rapidly increased. These nanomaterials exhibit chemical, physical and biological properties that are quite different from those of the corresponding bulk materials, and therefore their potential impact may differ from their equivalent bulk forms (UNEP, 2012). In **Chapter 2** of this thesis we studied one of the most commonly used metal-based nanoparticles, zinc oxide (ZnO NPs), and compared their toxic effects with those of the corresponding ionic (bulk) form (Zn^{2+}). Zn availability was determined in pore water and in 0.1 M copper nitrate extractions and Zn bioavailability and toxicity were measured using the earthworm *Eisenia andrei*, at different incubation times over 6 months after spiking the soils.

Our results show that Zn availability was lower in soils spiked with ZnO NPs in comparison to $ZnCl_2$. Zn availability was influenced by soil type, and was lowest in the soil with alkaline pH and high organic carbon content. Under treatments of Zn added as ZnO NPs, soil pH best explained the observed differences, which suggests that pH mainly controls Zn solubility in soils spiked with ZnO NP. The organic carbon content best explained Zn solubility in soils spiked with $ZnCl_2$. Zn availability for both Zn forms was affected by ageing, but varied among the test soils, highlighting the influence of soil properties on metal equilibration in soils. Toxic effects on earthworm reproduction at the same concentration of Zn in the soils were higher under treatments of $ZnCl_2$, with almost complete reproduction inhibition. But there was no clear difference in the other two endpoints studied (earthworm survival and weight loss) for the two studied forms of Zn added. Zinc-induced weight loss aggravated with ageing, but there were no differences in the other variables. So, not enough significant effects of ageing were detected that could explain differences in earthworm toxicity. Our findings suggest that ZnO NP and $ZnCl_2$ behave different in soils depending on soil properties and ageing processes, but that differences in Zn availability do not translate into differences in Zn bioavailability.

Waalewijn-Kool et al. (2013) found also that Zn availability is lower under treatments with ZnO as nanoparticle. Soil properties, such as pH and organic matter content, caused a high variability in the bioavailability of ZnO NPs, with consequent effects on their toxicity (Tourinho et al., 2013; Heggelund et al., 2014; Waalewijn-Kool et al., 2014). Soil properties also may influence the behaviour of Zn availability over a period of ageing; this underlines the complexity of metal stabilization in soils, which is dependent on processes of agglomeration and aggregation of these small particles. Although our study only revealed significant effects in Zn availability and not in Zn bioavailability, new paradigms for the safety evaluation of NPs are needed to further unravel the mechanisms of NP behaviour in soil (Savolainen, 2014). Further ecotoxicological testing of compounds with nanoparticles is needed for environmental risk assessment and legislative purposes. Such studies should involve the stability and toxicity of nanoparticles in soil over short- and long-term exposure periods, in comparison with the corresponding ionic metal species,. In this manner, a comprehensive understanding of nanomaterial fate and effects can be obtained to establish generic models of NP toxicity (Galera, 2015). This may also help governments in facing the challenge to adapt existing regulations to these new materials in order to protect environmental and human health.

9.2. Influence of soil properties on metal(loid) availability

One of the most important questions in this thesis concerned the effects of soil properties on metal availability and toxicity. It is widely known that soil properties control metal speciation and therefore their bioavailability in the environment (Sheppard and Evenden; 1988). Generally, metal toxicity is correlated with availability. The obtained results for the four main elements studied in this thesis showed that:

- For arsenic (As), the availability in soils appeared to be directly correlated with soil pH, calcium carbonate content and ionic strength of the soil solution. An increase in these parameters leads to greater As availability. On the other hand, the iron and clay content, and the specific surface area of soils can decrease As availability in the soil solution. Organic matter content, which is normally a key soil constituent, appeared negatively related with As solubility but only at high As concentrations (**Chapter 3 and 4**).

- For lead (Pb), the main soil parameters that significantly reduced its availability in the test soils were pH, carbonate content, silt content and iron and manganese content, while soils with sandy textures and low organic carbon content presented higher concentrations of available Pb (**Chapter 5**).

- For zinc (Zn), the availability was higher in soils with acid pH and low or null carbonate content, followed by soils rich in iron and organic carbon and therefore with higher cation exchange capacity. Alkaline soils with high pH and high carbonate content showed the lowest available Zn (**Chapter 6**). Also a recovered area affected by a mining spill (Guadiamar Green Corridor, Spain), soil pH was the main factor affecting Zn solubility, with increasing pH leading to a decline of soluble Zn levels (**Chapter 7**). **Chapter 2** studied ionic and nanoparticles forms of Zn, and showed that soil pH was the main factor controlling the availability of Zn added as nanoparticles, while organic carbon content also had an important role in soils spiked with ionic Zn.

- For copper (Cu), although studied only to a limited extent in this thesis, availability showed some similarities with that of Zn. The highest Cu availability was in soil with null or low carbonate content (acid pH), followed by soils with great iron content. From the soils without carbonates, the organic one showed the lowest available Cu levels. The lowest Cu availability was found in alkaline soils with high carbonate content independent of the organic carbon content (**Chapter 6**). Copper availability in the soils of the Guadiamar Green Corridor was also inversely related to soil pH (**Chapter 7**).

Our findings emphasize that soil properties have a critical influence on metal (bio)availability; moreover, these effects cannot be generalized but depend on the metal. The overriding effects of soil properties make it hardly possible to derive generic standards valid for all soils. Uisng generic standards or one-dimensional guideline values can lead to over- or underestimation of the risks of soil pollution, In conclusion, when soil guideline values for metals are based on laboratory toxicity data, they must take into account soil properties, to optimize their efficiency and profitability in the use of environmental quality standards.

9.3. Metal long-term equilibration

Time is a key factor to reach equilibrium between pollutants and soil systems, both in artificially spiked soils and in natural polluted soils (Lock and Janssen, 2003; Scheckel et al., 2009; Smolders et al., 2009; Martín Peinado et al., 2012). Metal availability may change with time and can be influenced by external conditions, such as climate and precipitation. Therefore tests performed with freshly spiked soils should be considered with caution in order to extrapolate the obtained results to cases of long-term pollution.

In **Chapter 2** of this thesis, the effect of ageing was studied in soils spiked with different forms of Zn in the laboratory over a period of 6 months, while in **Chapter 7** the effects of ageing was evaluated in natural polluted soils 6 and 15 years after a mining spill. Figure 1 shows two cases of the ageing effect in metal availability both to artificial (a) and to natural (b) polluted soils. In general, metals show their highest bioavailability immediately after the pollution, but it can quickly change due to the high sorption capacities of soils leading to conversion into non-exchangeable forms (Jalali and Khanlari, 2008).

Metals may associate with various components of soils in different ways, and these associations may change with ageing. This is clearly shown in Figure 1a where soils that differed widely in their soil properties, after spiking with the same As concentration (100 mg As kg^{-1} soil) showed great diversity of As availability and variable trends in time. The incubation time for the soils used in this thesis was selected based on this research, which was previously done in our department. In order to both optimize the time to perform soil toxicity tests and allow for stabilization of the metal added, the incubation period chosen was 4 weeks. This incubation period was used in **Chapters 3-6**, while in the remaining chapters the effect of ageing was evaluated by determining changes in metal toxicity.

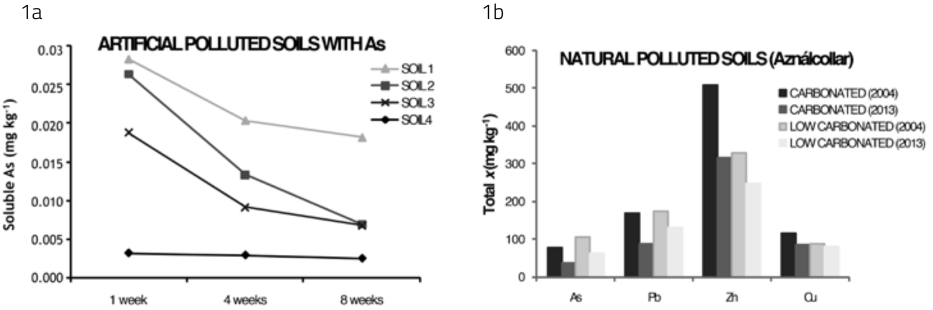

Figure 1. 1a: Soluble As concentrations in four soils with contrasting properties during three incubation periods (Martín Peinado et al., 2012). 1b: Mean total concentration of metals in Guadiamar Green Corridor (Aznalcóllar) soil with low and high carbonate content, sampled in 2004 and 2013 (Chapter 7).

Figure 1b compares the total concentrations of four elements after a metal pollution accident in two areas, one rich in carbonate and the other with low/null carbonate content in the soil. From measurements spanning across nine years (2004-2013), a decrease in the total metal concentration in soil was observed. This decrease over time in total concentration could be related with external parameters such as rainfall and therefore leaching of the metals or their uptake by plants (**Chapter 7**). It was also observed that long-time equilibration of the studied elements was most effective in the soils of the carbonate-rich area, leading to a greater decrease in the concentration with time of all studied elements except for arsenic.

Our findings demonstrate that the availability of metals changes with time, and also that the equilibration time should be measured in years, even decades. Studying ageing and equilibration of contaminated soil over time will provide a more realistic insight into metal behaviour and hence, its potential toxicity under natural conditions. Different ageing processes and equilibration times should be compared in view of obtaining the best simulation of the stabilization processes occurring under natural environmental conditions in order to establish generic soil quality standards for metals.

9.4. Biological assessment of metal availability and toxicity

Many national and international efforts are undertaken to develop, improve and standardize methods for assessing soil quality, particularly for classifying the potential hazards of both contaminated soils and the materials (pollutants) they may contain (van Straalen and Løkke, 1997; OEHHA, 2008). Soil toxicity tests and bioassays are important tools for decision makers, both in defining general soil quality criteria and in assessing contaminated sites and planning remedial actions. Basically, toxicity test are used to determine the potential hazard of a chemical for an organism in standard laboratory test expressed in terms of dose-response. Results of toxicity tests are used to derive risk limits for chemicals in soil. Bioassays are used to assess toxicity of soil samples from a natural contaminated site (van Gestel, 2012), and are the tools for assessing the actual risk of contaminated soils. Bioassays with soil organisms can also be used for long-term monitoring programs or to assess the success of clean-up projects. A variety of standardized testing methods are available commonly looking at the responses of soil microorganisms, terrestrial plants and terrestrial invertebrates and vertebrates. The toxicity of a chemical is often measured from different endpoints such as decrease in reproduction or in survival. The differences in soil type and therefore in soil components and properties will influence the response of the studied organism. Hence, toxicity tests and bioassays are crucial for assessing the actual ecological risk and to support legislation regarding contaminated soils (Royal Decree 9/2005). This should take into account the variation in soil type as well as the differences in sensitivity of different test organisms (Chapter 6; Matejczyk et al., 2011). Therefore, a battery of tests using a number of species representative of different taxonomic groups is needed to obtain proper insight into the potential hazard of a chemical for the ecosystem (van Gestel, 2012). The number of tests and organisms selected should be optimized with respect to the cost and the time, and the required level of detail of information needed for a proper assessment.

This thesis applied biological tests to determine the toxicity of different metals in soils under control conditions as well as to diagnose the actual risk in metal-polluted field soils.

-Biological testing

In this thesis we selected four standard tests with commonly used organisms (**Chapters 2-6**). In one assay we used a soil invertebrate, the earthworm *Eisenia andrei* (Chapters 2 and 4), considering four different endpoints: survival, weight change, reproduction and metal bioaccumulation. Tests with a terrestrial plant, *Lactuca sativa*, were performed in Chapters 3 and 5, focusing on two endpoints (seed germination and root elongation). The Gram-negative bacterium *Vibrio fischeri* (Chapters 3 and 5) was used with luminescence after 5 and 15 min of exposure being the endpoint. The response of soil microbial communities was measured as soil respiration (Chapters 5 and 6), which included basal respiration and substrate-induced respiration.

Earthworm reproduction and lettuce root elongation were most sensitive to metal pollution and showed more reproducible results with narrower confidence intervals than other variables. So, we could classify the four toxicity tests depending on their reliability and sensitivity shown in our studies from greater to lower as: *E. andrei* (reproduction) test ≥ *L. sativa* (root elongation) test > *V. fischeri* luminescence test > soil respiration test.

Guideline values for assessing the environmental risk of contaminated soils should be based on the results of a large number of assays, and could for instance take into account the most sensitive responses among all selected tests (Fernandez and Tarazona, 2008). Our results, which were obtained from four complementary tests with different organisms and different endpoints, may be considered a preliminary assessment of the toxicity and suitable to the check and/or establish current soil quality criteria or guideline values.

-Predictive risk assessment

In a predictive (also called forward) risk assessment soil quality criteria are derived from toxicity tests. These criteria are usually assumed to hold in general and are applied to large areas (regions or countries) without taking into account the spatial variation in soil types. This can lead to over- or under-estimation of the actual environmental risk. It was previously demonstrated that metal availability is highly determined by soil properties or ageing (Chapter 2-7), therefore generic soil quality criteria for large areas, due to the great variability in soil types, may be misleading.

We propose to use the toxicity data obtained with the biological tests applied in this thesis to assess the potential risk of pollution according to soil type or in relation to soil properties. So, the endpoints proposed in our study are:

- The most restrictive value for any use is set at the level of EC10. Depending on the regulations, the EC10 can be considered as Intervention level for agricultural soils.
- The most restrictive value for ecosystem protection is set at the level of EC25. Depending on the regulations, the EC25 can be considered as an Intervention level for urban soils.
- The most restrictive value to protect industrial activities without extractive land use is set at the level of EC50. Depending on the regulations, the EC50 can be considered as Intervention level for industrial soils.

Concentrations exceeding these values should trigger restoration or remediation initiatives or changes in land use. Based on these assumptions, Tables 1 and 2 show the soil quality criteria obtained for arsenic and lead, respectively, the two elements thoroughly studied in this thesis. The obtained values were grouped according to correlation with soil properties and similarity in the toxicity among soils. All values are expressed as total concentration in mg kg^{-1} dry soil. Our values were compared with the recently published regulatory levels for trace elements set by the Regional Government of Andalusia (Decree 18/2015 of 27th January, Annex IV), which are applicable to the study area assess in Chapter 7.

The endpoints obtained for arsenic (Table 1) differ enormously depending on soil properties. Only for carbonate-rich soils, the values proposed by the Andalusian government are in the range of the values obtained in our study (except for the soil respiration test that was not sensitive enough), but for other soil types the regulatory values are sufficiently protective. According to the *L. sativa* data (the most sensitive test in our study), the highest differences are for soils with high iron content, where the proposed value underestimates the role of the iron compounds in the soil in the reduction of As availability and toxicity. Therefore, our data indicate that, in the case of As pollution, regulatory levels proposed by the Regional government of Andalusia may be revised according to at least two main parameters: calcium carbonate and iron content.

Table 1. Comparison of the soil quality guidelines proposed by the Regional government of Andalusia (Decree 18/2015 of 27th January, Annex IV) for arsenic differentiated to soil use and the results obtained in the toxicity tests performed in this thesis.

ARSENIC	**Spain (Andalusia region)**		
	Decree 18/2015		
Soil use classification	**Industrial use**	**Urban use**	**Other uses**
As(mg kg^{-1})	40	36	36
	Results obtained in this thesis		
***Lactuca sativa* test (Chapter 3)**	**EC50**	**EC25**	**EC10**
carbonated soil	45-150	32-101	25-60
low/null carbonated soil	125-185	69-136	40-95
iron rich soil	620	499	400
***Vibrio fischeri* test (Chapter 3)**	**EC50**	**EC25**	**EC10**
carbonated soil	37-153	14-94	6-58
low/null carbonated soil	155-291	83-170	44-102
iron rich soil	768	487	332
***Eisenia andrei* test (Chapter 4)**	**EC50**	**EC25**	**EC10**
carbonated soil	60	52	45
low/null carbonated soil	56-151	38-111	26-82
iron rich soil	96	75	59
Soil respiration test (Chapter 6)			**EC10**
organic carbon rich soil			>50
iron content rich soil			>1200

For lead, toxicity to *V. fischeri* occurred over a narrower range while in the soil respiration test Pb caused only a small reduction, making it impossible to calculate EC25 and EC50 values for all cases (Table 2). The obtained values varied widely depending on soil properties as well as the studied organism. Data for *L. sativa* were within the range of regulatory values in the case of other uses in carbonated soils and to industrial use in low/null carbonated soils, but the proposed values could overestimate Pb toxicity especially in carbonate-rich soils and soils rich in organic carbon. According to the *V. fischeri* test, the values proposed for urban use (EC25) seemed to overestimate the risk of lead pollution in soils with neutral or alkaline pH, underrating the soil's buffer capacity in these cases. In acidic soils (pH<6), the regulatory values are in the range of the values obtained in our tests. But due to the high range of this class, more studies are needed to assess Pb toxicity under strongly acidic conditions, where according to our data, Pb toxicity could be detected at concentrations as low as 8 mg kg^{-1} (EC10). The results of the soil respiration test showed the same pattern: carbonate-rich soils and soils rich in organic carbon had a low Pb toxicity, so the regulatory levels seem too restrictive in comparison to our data. On the other hand, for soils with low/null carbonate content the regulatory levels underestimate Pb toxicity. Hence, our data indicate that, in the case of Pb pollution, regulatory levels proposed by the Regional Government of Andalusia should be qualified according to at least three main soil factors: calcium carbonate, organic carbon content and pH.

Table 2. Comparison of the soil quality guidelines proposed by the Regional government of Andalusia (Decree 18/2015 of 27th January, Annex IV) for lead pollution differentiated to soil use and the results obtained in the toxicity tests performed in this thesis.

LEAD	**Spain (Andalusia region)**		
	Decree 18/2015		
Soil use classification	**Industrial use**	**Urban use**	**Other uses**
Pb (mg kg^{-1})	2750	275	275
	Results obtained in this thesis		
***Lactuca sativa* test (Chapter 5)**	**EC50**	**EC25**	**EC10**
carbonated soil	>8000	1564-3360	254-1363
low/null carbonated soil	1303-3479	768-1933	344-1097
organic carbon rich soil	>6240	>4643	499-3452
***Vibrio fischeri* test (Chapter 5)**	**EC50**	**EC25**	**EC10**
pH<6		29-888	8-386
pH between 6-8	2259-3966	>1300	744-2473
pH>8		>4005	2901-5337
Soil respiration test (Chapter 5, 6)		**EC25**	**EC10**
carbonated soil		>2924	3128-5951
low/null carbonated soil		504-867	45-122
organic carbon rich soil			>8000

In the light of these comparisons the existing guideline values in Andalusia are not sufficiently accurate to classify soil pollution and therefore the risk of pollution could be over- or underestimated. The large differences in obtained values are due to the high diversity in soil types. In addition, the response of organisms exposed to the same pollutant under the same conditions showed considerable variation, which highlights the need to perform a battery of biological tests to properly assess the risk of metals. Therefore a new approach taking into account soil properties and using different organisms is needed for a proper predictive risk assessment of contaminated soils.

-Diagnostic risk assessment

Assessment of field-contaminated soils requires a diagnostic risk assessment. Such an approach starts from the (field-polluted) soil itself and analyses the possible effects on soil organisms that are exposed to it. The experimental approach is usually called bioassay, which may apply the same procedure as the toxicity test used for predictive risk assessment except that exposure is to field-contaminated soil instead of soil spiked with a single chemical or well-defined mixture and that it is more difficult to find a proper control (van Gestel, 2012). In the Guadiamar Green Corridor "hot" spots of metal pollution were indicated by the absence of vegetation (**Chapter 7**). These in situ polluted areas can be key to validate or optimize bioassays as well as to check if soil quality criteria derived from laboratory toxicity data are effective for ecotoxicological risk assessment.

In the **Chapter 8** two biological tests were selected: lettuce root elongation and soil respiration, to assess the actual risk of the non-recovered Guadiamar Green Corridor soils. These tests were also applied to check the appropriateness of the legislative criteria derived from toxicity tests performed in the laboratory. Field observations (presence or absence of vegetation) as well as chemical analysis (total, available and extractable concentrations of metals) were combined with these bioassays to evaluate the actual ecological risk of the residual metal contamination in this area.

Bioassays can tell whether pollutants at toxic levels are present in the studied area and can identify grades of pollution, but they will not be able to identify the pollutants that are responsible of the toxicity (Weeks et al., 2004). In natural polluted areas the main concern is how to deal with mixture toxicity. The more relevant trace metals previously identified in the Guadiamar Green Corridor after the mining spill were As, Pb, Zn and Cu (the four elements studied in this thesis), but the soil also contained cadmium (Cd) and thallium (Tl) (Grimalt et al., 1999). Extrapolation of results from laboratory toxicity tests, usually performed with individual chemicals, to field soils commonly polluted with toxicant mixtures is an enduring challenge. In Chapter 8 mixture toxicity was studied from a simple model (Weltje, 1998), calculating the toxicity of the mixture of metals present in the Guadiamar soils on the basis of the toxicity of the individual metals estimated in laboratory tests and assuming concentration addition. For that purpose, the toxic unit approach was used. From our results, the following conclusions can be drawn:

- The use of bioassays in natural polluted areas is better able to identify soil pollution than only from determining if metal concentration exceeds guideline values. Bioassays performed in natural polluted soils take into account interactions between pollutants (antagonistic, additive or synergistic effects) and their bioavailability.

- Metal toxicity will differ depending on the test organism. The bioassays performed showed toxic effects but their responses did not correlate, which suggests that for the same polluted soil, organisms will be affected by different pollutants.

- Calculation of toxic units to estimate mixture toxicity assuming concentration addition is a simple and easy method. However it depends on the availability of toxicity data for the individual pollutants, which are not always available. It may take into account any other pollutants present in the soils (e.g. in our soils possible presence of cadmium and thallium), but it also depends on the completeness of the set of chemicals analysed.

To sum up, laboratory ecotoxicological tests are useful to define soil quality guideline values based on added metal concentrations, but they are directly influenced by soil type and the choice of the set of test organisms. Therefore laboratory studies have limitations and hence may not be fully representative of field conditions, where metals and other pollutant can act together. The results obtained suggest that the use of data from laboratory tests can be a key to predict thresholds for metal toxicity, but that under field conditions, bioassays performed to diagnose the soils will give more realistic and comprehensive results.

9.5. Extrapolation from laboratory to field

After studying the toxicity of metals in soils spiked in the laboratory and in field-polluted soils, we can deal with the following question: How can we estimate safe levels for the ecological risk assessment (ERA) from the obtained endpoints? One of the last phases of the Screening Level Ecological Risk Assessment (SLERA) is the estimation of the ecological risk, which can be done by calculating the risk quotient (RQ) (Table 1, chapter 1). This RQ can be obtained by dividing the highest measured on-site contaminant concentration by a sensitive screening value (NOEC, EC10, LC5...). If the concentration is higher than the screening value selected (RQ > 1), harmful effects may occur, and the larger the RQ value, the greater the effects may be. The US EPA, however, does not distinguish a more refined scaling and only takes into consideration if the RQ value exceeds 1 to identify contamination (US EPA, 2007). This obviously is something that should be taken into consideration in order to prioritize soil remediation activities.

Based on the results obtained in the toxicity tests performed in the laboratory (**Chapter 2-6**), RQ values were estimated for natural polluted areas in the Guadiamar Green Corridor (**Chapter 8**). To determine the potential risk we selected as screening value the EC10. This approach was performed only for As and Pb pollution based on the maximum and minimum EC10 values obtained for the different test organisms; see tables 1 and 2 (Risk calculated in field from laboratory results). In order to check the reliability of the values proposed by the Regional government, RQ was also calculated dividing the total soil concentrations by the intervention level for other uses (Decree 18/2015) (Risk calculated in field from regulation guidelines values). Finally, assuming that the soil background metal concentration of the recovered adjacent areas, which showed vegetation cover, causes no ecological risk, we also calculated the RQ using the concentrations measured in these areas (risk calculated in the field from adjacent unpolluted areas) (Table 3).

Table 3. Estimation of risk quotients (RQ) obtained by 1. dividing the total concentration of selected metals in soil by the EC10 values derived from toxicity tests (see Tables 1 and 2), 2. dividing total soil concentrations by the most restrictive soil quality criteria (SQC) proposed by the Andalusian government (other uses, see Table 2 in the Introduction), and 3. dividing total soil concentrations by the background concentrations in recovered adjacent areas. RQ with values higher than one indicate pollution risk.

	RQ calculated from EC10 values (Table 1, 2)		**RQ calculated from Andalusian SQC for other uses (See Table 2 in Chapter 1)**		**RQ calculated from adjacent total background concentrations**	
	SECTOR 1	**SECTOR 2**	**SECTOR 1**	**SECTOR 2**	**SECTOR 1**	**SECTOR 2**
	Carbonated soils	Low carbonated	Carbonated soils	Low carbonated	Carbonated soils	Low carbonated
TAs	4.3-42.9	3.7-14.4	7.1	10.4	4.1	4.3
TPb	0.1-1.3	0.3-7.2	1.2	3.7	2.1	6.2
TZn			<0.1	<0.1	0.6	0.5
TCu			0.2	0.2	1.3	1.4

Risk quotients obtained using EC10 values showed greater variability depending on soil type, which highlights the importance of taking into consideration the soil type when assessing the risk of soil pollution. In spite of these widely varying values, the As concentrations showed a high ecological risk in the area. The other two estimated RQ values, based on Andalusian soil quality criteria and background concentrations, showed ecological risks for both As and Pb.

These data suggest that, despite having studied the toxicity risk in three different ways, all of them showed toxicity, so there are possible ecological threats in the Guadiamar Green Corridor. Hence a more detailed ecological risk assessment or intervention actions are needed in these areas. In addition, our results show that soil toxicity, as we observed before, will be controlled by soil properties and studied organisms (wide ranges in the RQ obtained from laboratory studies). RQ calculated from Andalusia guideline values, despite the range obtained depending on soil and organisms in laboratory, are also within our proposed data. Finally, from the results shown, we can recommend the use of the background metal concentration in adjacent soils to check pollution, because it can provide a realistic way to predict toxicity with low efforts.

The ecological risk of a metal in soil is usually determined from its total concentration. However, it is known that the potential ecological risk of metals is strongly related to soluble and available concentrations in the soil. To demonstrate the importance of the available forms of metals in soil and their possible harmful effects, we calculated RQ values assuming that the metal concentration in recovered adjacent areas had no ecological risk. The water extractable (Wx) and exchangeable (Ex) metal concentrations were used to calculate the corresponding RQ (data from chapter 8) (Table 4).

Table 4. Estimation of risk quotients (RQ) for metal pollution in soils of the Guadiamar Green Corridor, obtained by dividing water extractable (Wx) and exchangeable metal concentrations (Ex) in soil by the corresponding concentrations in recovered adjacent areas.

	SECTOR 1	**SECTOR 2**
RQ	Carbonated soils	Low carbonated
WAs	2.7	2.6
WPb	5.0	2.5
WZn	1554	495
WCu	90	139
EAs	5.4	5.1
EPb	2.4	1.4
EZn	13	11
ECu	9.0	12

In this case, all RQs were higher than 1, which suggests that there are possible harmful effects according to the soluble and exchangeable concentrations of the studied metals (bioavailability), even in cases were no effects were expected based on total concentrations (see table 3), such as the cases for Zn and Cu which showed really high RQs in the water extractable forms. This approach highlights that we must not neglect the available concentrations of metals when assessing the ecological risk of metals in soils.

In conclusion

In this thesis it is shown that at least five factors are indispensable to study metal toxicity in soil: the element and its form (single, mixture, bulk or nano-particulate form), total and available concentrations (which implies metal speciation, not studied in this thesis), variability in soil properties and constituents, long-term effects (ageing) and what organisms will be exposed or what we want to protect (soil use). Current soil protection policies show a wide variation in the guideline values or quality criteria proposed for metal pollution in soil among countries, probably because not all these factors were taken into account. It is an undisputed fact that soil type differs from place to place and each country should select its own regulation levels to classify a soil as polluted and which possible use or restoration measurements are needed. But, due to the strong effect of soil properties on the availability and bioavailability of elements studied in this thesis (As, Pb, Zn and Cu), we strongly recommend legislative frameworks to take this factor into account. Deriving soil quality criteria for individual metals is not a good indicator of soil pollution, because under real conditions metals are rarely found in isolation and soils usually contain (complex) mixtures. It is essential to determine the toxic effects and the interaction among involved elements (antagonistic, additive or synergistic effects) to properly assess contaminated soils. Biological tests are indispensable for such an assessment.

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SUMMARY

Influence of soil properties on the toxicity of metal-polluted soils. Comparison of different bioassay methods.

Soil is an essential part of the ecosystem that acts as a sink of contamination since it has a high buffer capacity compared with other ecosystem compartments like water and air. When contaminants end up in soil, they can be stored, transformed, or transferred to other media. The presence of contaminants in soils needs a proper environmental assessment to prevent pollution or to determine measures to cope with contaminated soils. Several millions of hectares of land all over the globe are polluted with a wide variety of contaminants, being metals and metalloids among the substances that generate major problems due to the fact that they do not degrade and can accumulate in the environment. Although metal emissions have decreased in the European Union over the last few years, the development of new hazards involving metals has increased. This especially concerns the widely deployed production of materials involving metal-based nanoparticles, exhibiting chemical and biological properties that are quite different from those of the corresponding bulk materials.

In the problems concerning environmental contamination, soil properties are the main factors that control the mobility, (bio) availability and toxicity of metals in terrestrial ecosystems. However, guideline values to declare a soil as polluted are currently based on laboratory studies without taking into account key factors such as soil properties or long-term equilibration processes (ageing), which could change the fate and bioavailability of metals.

The aim of this thesis was to study the fate and ecotoxicological effects of metals (Pb, Zn and Cu) and metalloids (As) as well as metal-based nanoparticles in soils (ZnO NP). This included determining the influence of soil properties and also the effects of ageing (long-term equilibration) on the (bio)availability and toxicity of these elements. For this purpose, four bioassays representing the major groups of soil organisms were applied, following standard test guidelines: i) Seed germination/ Root elongation Toxicity Test (U.S. EPA 850:4200) with the plant *Lactuca sativa*; ii) Microtox®Test (ASTM, 2004) with the bacterium *Vibrio fischeri*; iii) Soil Respiration Test (ISO 17155, 2002) with soil microorganisms; and iv) Earthworm Survival and Reproduction Test (OECD 207 and 222) with *Eisenia andrei*. These tests were performed both in laboratory-spiked soils with different properties and in soils affected by residual contamination (Aznalcóllar mine spill, Spain). The main goal of this thesis was to improve the scientific basis for the environmental risk assessment of metals and metalloids in soils, with the following specific objectives:

- To study the influence of ageing on metal bioavailability and toxicity in soils,
- to assess the effect of soil properties on metal availability and toxicity,

- to evaluate the use of bioassays to define guideline values,
- to relate the data obtained in soils spiked in laboratory with soils polluted *in situ*,
- and, to implement the obtained results in a framework of risk assessment.

In **chapter 2** we compared the effect of Zn applied as ZnO nanoparticles (ZnO NPs) and as Zn^{2+} ion ($ZnCl_2$) on the earthworm *Eisenia andrei* at different periods after spiking three natural soils with different soil properties. The results showed lower Zn availability in soils when applied as ZnO NP compared to the Zn ion form. Zn availability was different according to the soil type, indicating the high influence of soil properties in the behavior of this element, and highlighting the pH as a main property controlling the potential toxicity of Zn. Internal zinc concentration in earthworms (ZnE) was highest for the soil with high organic carbon content and basic pH spiked with ZnO NP, indicating the key role of soil type in the availability and toxicity of this element. Further scientific research and ecotoxicological testing of compounds with nanoparticles must be performed to support reliable environmental risk assessment for legislative purposes.

The study of ageing and equilibration of contaminated soil over time may provide a more realistic insight into metal behaviour and its potential toxicity under natural conditions. In **chapter 2**, it was shown that Zn availability from ZnO NPs or ionic Zn was differently influenced by ageing depending on the soil type. Although ageing did not show clear effects on Zn toxicity in this study, knowledge on the importance of long-term processes for Zn availability is crucial for a proper risk assessment of ZnO NPs as well as Zn-polluted soils. Long-term effects and the availability of metals and metalloids in field-contaminated soils were determined 6 and 15 years after the mining spill in an area affected by residual pollution (Aznalcóllar mine) in **chapter 7**. In this case, under natural conditions, soil pH tended to increase with time and concentrations of pollutants in soil tended to decrease. Poorly recovered areas were still present 15 years after the spill, indicated by the absence of vegetation and high pollutant concentrations. This study showed that there are possible ecological threats in the studied area, so more detailed ecological risk assessment or intervention actions are needed.

A battery of toxicity tests using a number of representative species belonging to different taxonomic groups is needed to obtain proper insight into the potential hazard of a chemical for the ecosystem. In this thesis different toxicity tests were performed to assess the toxicity of individual metals, while different bioassays were used to determine toxicity of field-contaminated soils (**chapters 2-6**; **chapter 8**). Our findings show that the response of the different tests to the same metal greatly differed. Results indicated that earthworm reproduction and lettuce root elongation tests were the most sensitive tests to metal pollution and gave more uniform toxicity values with smaller confidence intervals. Therefore, for identifying actual soil toxicity, different organisms will give different results, so the use of a battery of tests is essential for performing a more complete and reliable assessment of the toxicity of metals and/or contaminated soils. The number of tests and organisms should be optimized depending on the circumstances and required level of detail of the information needed.

In order to determine the effect of soil properties on metal pollution, seven different representative soils were spiked with different elements individually (As, Pb, Zn and Cu). Soils were incubated for 4 weeks to take into account the effect of time on metal availability. Soil properties effects were studied in **all chapters** of this thesis. Our findings highlight that soil properties have a critical influence on metal (bio)availability and they have to be included in the ecological risk assessment of contaminated soils as well as in the setting of soil quality criteria for metals. The broad range of responses in the test soils in relation to soil properties can lead to over- or under-protective guideline values, which will also influence the efficacy of remediation goals and actions. Consequently, soil guideline values must be differentiated depending on soil properties, in order to optimize their efficiency and profitability in environmental risk assessment.

The guideline values derived in chapters 3-6 were compared with the current legislation as well as with effects observed in natural soils polluted with a range of elements (Aznalcóllar spill). The results (**chapter 8**) showed that the existing guideline values in Andalusia (Decree 18/2015) (where the Aznalcóllar soils are located) are not sufficiently accurate to declare contaminated soils and therefore the risk of pollution could be over- or underestimated depending on the considered element. The large differences in values obtained are due to the high diversity of soil types. Therefore a new approach taking into account soil properties to determine limits to soil toxicity is essential for a proper Ecological Risk Assessment. Current regulation should be revised in order to apply these findings to develop more efficient and effective policy regulation.

SAMENVATTING

De invloed van bodemeigenschappen op de toxiciteit van metaal-vervuilde gronden. Een vergelijking van verschillende bioassays.

De bodem in een essentieel onderdeel van het ecosysteem met een grote buffercapaciteit in vergelijking tot andere milieucompartimenten zoals water en lucht. Daardoor fungeert de bodem vaak als een vergaarbak van verontreinigende stoffen. Wanneer verontreinigingen in de bodem terecht komen, kunnen ze worden opgeslagen, omgezet of doorgevoerd naar andere compartimenten. De aanwezigheid van vervuilende stoffen in de bodem vereist een zorgvuldige beoordeling, om vervuiling te voorkomen of om te bepalen welke maatregelen nodig zijn om de risico's van de vervuiling te beperken. Verspreid over de aarde zijn vele miljoenen hectares vervuild met een grote varieteit aan stoffen. Hierbij vormt de verontreiniging met metalen en metalloïden het grootste probleem, aangezien deze elementen niet afbreken en daardoor kunnen accumuleren in het milieu. Hoewel de emissie van metalen in de Europese Unie de laatste jaren aan het afnemen is, duiken nieuwe risico's op waarin metalen een rol spelen. Dit betreft met name de exponentieel groeiende productie van materialen waarin metaal-houdende nanodeeltjes (NPs) zitten. Deze nanodeeltjes hebben chemische en biologische eigenschappen die sterk afwijken van die van de corresponderende niet-nanomaterialen.

Bodemeigenschappen hebben een grote invloed op de mobiliteit, (biologische) beschikbaarheid en toxiciteit van metalen in de bodem. Criteria voor het classificeren van een bodem als verontreinigd zijn vaak gebaseerd op resultaten van laboratorium-onderzoek, zonder dat daarbij rekening wordt gehouden met de invloed van factoren als bodemeigenschappen of lange-termijn evenwichtsprocessen, die het gedrag en de biologische beschikbaarheid van metalen kunnen veranderen.

Doel van dit proefschrift was het bestuderen van het gedrag en de ecotoxicologische effecten van metalen (Pb, Zn, Cu), metalloïden (As) en metaal-houdende nanodeeltjes (ZnO NPs) in de bodem. Dit omvatte het bepalen van de invloed van bodemeigenschappen en van veroudering (lange-termijn evenwichtsinstelling) op de biologische beschikbaarheid van deze elementen. Voor dit doel zijn biologische testen uitgevoerd met vertegenwoordigers van vier groepen van belangrijke bodemorganismen. Deze testen gebruikten internationaal gestandaardiseerde testmethoden en betroffen: i) Zaadkieming / wortelgroei toxiciteitstest (U.S. EPA 850:4200) met de plant *Lactuca sativa*; ii) Microtox®Test (ASTM, 2004) met de bacterie *Vibrio fischeri*; iii) Bodemademhalingstest (ISO 17155, 2002) met bodem-microorganismen; en iv) Regenwormen overleving- en reproductietesten (OECD 207 en 222) met *Eisenia andrei*. Deze testen werden uitgevoerd op in het laboratorium met metalen behandelde gronden en op gronden die in het veld vervuild waren met metalen door de Aznalcóllar mijnramp (Andalusië, Spanje). Hoofddoelstelling van dit proefschrift was om de wetenschappelijke basis voor de risicobeoordeling van metalen en metalloïden in de bodem te vergroten, en had daarbij de volgende doelen:

- Het bepalen van de invloed van veroudering op de biologische beschikbaarheid en toxiciteit van metalen in de bodem,
- Het bepalen van de invloed van bodemeigenschappen op de biologische beschikbaarheid en toxiciteit van metalen,
- Het evalueren van het gebruik van biologische testen voor het afleiden van normen voor metalen in de bodem,
- Om gegevens verkregen voor in het laboratorium met metalen behandelde gronden te relateren aan die voor in situ vervuilde gronden,
- De verkregen resultaten verwerken in een raamwerk voor risicobeoordeling.

In **hoofdstuk 2** is het effect van Zn, toegediend als ZnO nanodeeltjes (ZnO NPs) en als het Zn^{2+} ion ($ZnCl_2$), op de regenworm *Eisenia andrei* bepaald op verschillende tijden na het behandelen van drie natuurlijke gronden met verschillende bodemeigenschappen. De beschikbaarheid van Zn in de gronden was lager voor ZnO NPs dan voor het Zn ion, en hing af van het bodemtype. Dit toont aan dat bodemeigenschappen een grote invloed kunnen hebben op het gedrag van Zn. De pH van de grond bleek de voornaamste factor te zijn die de mogelijke giftigheid van Zn in de bodem controleert. Zn-gehalten in de regenwormen waren het hoogst op de grond met het hoogste gehalte aan organische stof en een neutrale pH. Dit onderstreept de sleutelrol van bodemtype in het bepalen van de biologische beschikbaarheid en toxiciteit van dit element. Verder onderzoek is nodig om te komen tot een betrouwbare risicobeoordeling van materialen die metaal-houdende nanodeeltjes bevatten.

Studie van veroudering en het process van evenwichtsinstelling van metalen in de tijd in verontreinigde gronden kan een meer realistisch inzicht geven in het gedrag en mogelijke effecten van metalen onder natuurlijke omstandigheden. In **hoofdstuk 2** is aangetoond dat de beschikbaarheid van Zn afkomstig van ZnO NPs of van ionisch Zn op een verschillende manier werd beïnvloed door veroudering, en dat dit proces mede afhankelijk was van het bodemtype. Hoewel veroudering geen duidelijk effect had op de toxiciteit van Zn, is kennis van het belang van lange-termijn processen wel essentieel voor een goede risicobeoordeling van zowel ZnO NPs als Zn-vervuilde gronden. In **hoofdstuk 7** zijn de lange-termijn effecten en de beschikbaarheid van metalen en metalloïden bepaald in veld-vervuilde gronden. Dit gebeurde 6 en 15 jaar na het ongeluk met mijnafval bij de Aznalcóllar mijn (Spanje). In dit geval, onder natuurlijke omstandigheden, nam de pH van de bodem toe in de tijd terwijl de gehalten aan metalen afnamen. Toch was ook 15 jaar na het ongeluk nog nauwelijks herstel opgetreden in een aantal gebieden, hetgeen bleek uit het ontbreken van vegetatie en de aanwezigheid van zeer hoge metaalgehalten. Dit onderzoek laat zien dat er mogelijk sprake is van ecologische risico's en dat een meer gedetailleerd onderzoek nodig is om deze risico's in kaart te brengen of om te bepalen welke saneringsmaatregelen noodzakelijk zijn.

Om goed inzicht te krijgen in het mogelijke ecologische risico van een stof is het van essentieel belang de toxiciteit te bepalen in testen met soorten die representatief zijn voor verschillende taxonomische groepen. In dit proefschrift zijn verschillende toxiciteitstesten uitgevoerd om de toxiciteit van de individuele metalen te bepalen (**hoofdstukken 2-6**). Daarnaast zijn bioassays uitgevoerd om de giftigheid van verontreinigde gronden uit het veld te meten (**hoofdstuk 8**). De resultaten laten zien dat de reactie van de verschillende testen op hetzelfde metaal sterk kan verschillen. De reproductie van regenwormen en de wortellengtegroei van planten waren het meest gevoelig voor metalen en gaven ook de meest consistente waarden met de kleinste betrouwbaarheidsintervallen. Het feit dat verschillende soorten verschillend kunnen reageren op verontreiniging is van groot belang voor het aantonen van het actuele risico van vervuilde gronden. Er zal daarom altijd een verzameling van toxiciteitstesten, met verschillende organismen, moeten worden uitgevoerd om een zo nauwkeurig en volledige mogelijke risicobeoordeling van metalen of metaal-vervuilde gronden mogelijk te maken. Het optimum aantal testen en testorganismen zal afhangen van de situatie en de vereiste mate van detail van informatie die nodig is voor de risicobeoordeling.

Om het effect van bodemeigenschappen op de metaalvervuiling te bepalen werden zeven gronden in het laboratorium behandeld met verschillende metalen apart (As, Pb, Zn en Cu). De gronden werden gedurende 4 weken geïncubeerd om rekening te houden met het effect van de tijd op de beschikbaarheid van de metalen. Het effect van bodemeigenschappen werd bestudeerd in **alle hoofdstukken** van dit proefschrift. De resultaten laten zien dat bodemeigenschappen een grote invloed hebben op de (biologische) beschikbaarheid van metalen en dus moeten worden meegenomen bij de beoordeling van het ecologische risico van verontreinigde gronden en bij het afleiden van normen voor metalen in de bodem. De grote variatie in de respons van de testen in gronden in relatie tot de bodemeigenschappen kan leiden tot te hoge of te lage normen, en dit kan ook invloed hebben op de effectiviteit van saneringsmaatregelen. Normen voor metalen in de bodem moeten dus rekening houden met bodemeigenschappen; alleen op die manier kan worden gekomen tot een efficiënte en kosten-effectieve risicobeoordeling.

De normen die in de hoofdstukken 3-6 zijn afgeleid zijn vergeleken met de huidige wetgeving en ook met de toxiciteit die werd gemeten in gronden die waren vervuild met een heel scala aan metalen ten gevolge van het ongeluk met mijnafval bij de Aznalcóllar mijn (Andalusië, Spanje). De resultaten van deze vergelijking (**hoofdstuk 8**) laten zien dat de huidige normen in Andalusië (Decree 18/2015) niet voldoende nauwkeurig zijn om verontreinigde gronden goed te kunnen classificeren. De risico's van deze gronden kunnen derhalve over- of onderschat worden, afhankelijk van het metaal waarop wordt beoordeeld. De grote verschillen in de verkregen waarden zijn het gevolg van de grote variatie in bodemtypen. Voor een verbetering van de ecologische risicobeoordeling is een nieuwe benadering nodig, die rekening houdt met bodemeigenschappen bij het afleiden van risicogrenzen op basis van de toxiciteit van gronden. De huidige regelgeving moet worden aangepast om deze aanbeveling mee te nemen en te komen tot een efficiënter en effectiever bodembeschermingsbeleid.

RESUMEN

Influencia de las propiedades del suelo en la toxicidad de suelos contaminados por metales. Comparación de diferentes métodos de bioensayos.

El suelo es una parte esencial del ecosistema que actúa como sumidero para la contaminación debido a su gran capacidad amortiguadora en comparación con los otros dos grandes compartimentos de los ecosistemas: el agua y el aire. Cuando algún tipo de elemento potencialmente contaminante llega al sistema suelo, éste puede ser almacenado, transformado o transferido dentro de los ciclos del agua o aire, así como a la cadena trófica. Por lo tanto, la presencia de sustancias contaminantes en los suelos requiere una evaluación ambiental adecuada para evitar la dispersión de la contaminación o para decidir qué medidas se deben tomar a la hora de realizar actuaciones de recuperación. En todo el mundo se ha constatado la existencia de millones de hectáreas de suelos contaminados, estando los metales y metaloides entre las sustancias que generan mayores problemas debido a que se trata de elementos que no se degradan y se acumulan en el medioambiente. Aunque a lo largo de los últimos años las emisiones de metales han disminuido en la Unión Europea, la producción de nuevas sustancias compuestas por metales ha aumentado drásticamente. En concreto, se ha desarrollado un amplio número de productos de uso diario con componentes de nanopartículas con base metálica, los cuales poseen propiedades químicas y biológicas muy diferentes a las formas metálicas más tradicionales.

Dentro de la problemática de la contaminación ambiental, las propiedades del suelo son los principales factores que controlan la movilidad, (bio) disponibilidad y toxicidad de los metales en los ecosistemas terrestres. Sin embargo, los niveles de referencia orientativos para declarar un suelo como contaminado se basan actualmente en estudios realizados en laboratorio, sin tener en cuenta muchos de los factores clave como son el efecto de las propiedades del suelo o los procesos de equilibrio que se producen a largo plazo (envejecimiento). Estos factores podrían cambiar el comportamiento y por lo tanto la biodisponibilidad de los metales en los suelos y deben ser estudiados en profundidad.

A la vista de lo expuesto anteriormente, la finalidad de esta tesis es estudiar el comportamiento y los efectos ecotoxicológicos de metales (Pb, Zn y Cu) y metaloides (As), así como las emergentes formas metálicas de tamaño nanopartícula en los suelos (en concreto se estudia la tercera forma más abundante en la actualidad: ZnO NP). Para ello se evalúa la influencia de las propiedades del suelo así como los efectos del envejecimiento (equilibrio de los metales en suelo a largo plazo) en la (bio) disponibilidad y toxicidad de estos elementos. Estos estudios se realizan a partir de cuatro bioensayos con representación de los principales grupos de organismos del suelo, siguiendo directrices establecidas en pruebas estándar: i) estudio del efecto tóxico en la germinación de semillas y en la elongación de raíz de la planta *Lactuca sativa* (US EPA 850: 4200); ii) efecto de la toxicidad en la reducción de la luminiscencia de la bacteria marina *Vibrio fischeri* a partir de un test realizado con un

Microtox® (ASTM, 2004); iii) estudio de la respiración heterotrófica de los organismos de suelo (ISO 17155, 2002); y iv) estudio de la supervivencia, reproducción y bioacumulación de la lombriz de tierra *Eisenia andrei* (OECD 207 y 222).

Los estudios desarrollados en esta tesis se han realizado tanto en suelos con distintas propiedades contaminados artificialmente en laboratorio, así como en los suelos afectados por el vertido minero de 1998 de la mina de Aznalcóllar (Sevilla, SO España). El objetivo principal de esta tesis es mejorar la base científica para la evaluación del riesgo ambiental de metales y metaloides en suelos, a partir de los siguientes objetivos específicos:

- estudiar la influencia del envejecimiento sobre la biodisponibilidad y toxicidad de metales en suelos;
- evaluar el efecto de las propiedades del suelo en la disponibilidad de los metales y en la toxicidad que producen;
- evaluar el uso de bioensayos como herramientas para definir niveles genéricos de referencia para la contaminación;
- relacionar los datos obtenidos en suelos contaminados artificialmente en el laboratorio con suelos contaminados in situ;
- poner en práctica los resultados obtenidos dentro del marco de evaluación del riesgo ecológico (ERE).

En el **capítulo 2** de esta tesis se compara el efecto de Zn aplicado como nanopartículas (ZnO NP) y como forma iónica ($ZnCl_2$) sobre la lombriz de tierra *Eisenia andrei*, después de diferentes periodos de incubación en tres suelos con propiedades distintas. Los resultados muestran una menor disponibilidad de Zn en suelos cuando son contaminados con ZnO NP en comparación con la forma iónica. La disponibilidad de Zn varía según el suelo estudiado, lo que claramente enfatiza la importancia que las propiedades del suelo tienen en el comportamiento de este elemento, destacando el pH como una propiedad fundamental en el control de la toxicidad potencial de Zn. La concentración interna de Zn en las lombrices de tierra (ZnE) es más alta para el suelo contaminado con Zn aplicado en forma de nanopartícula con alto contenido en carbono orgánico y pH básico, lo cual refuerza la idea de que no sólo la disponibilidad del elemento afecta a su biodisponibilidad y toxicidad sino también el tipo de suelo. A la vista de estos resultados es indiscutible que se deben realizar más pruebas ecotoxicológicas a los compuestos con nanopartículas con objeto de fortalecer la evaluación del riesgo ambiental dentro de la legislación existente y tratar los compuestos que las contengan de forma distinta a las formas metálicas comunes.

El estudio del equilibrio con el tiempo de un suelo contaminado (envejecimiento) puede proporcionar una visión más realista del comportamiento de metal y su toxicidad potencial en condiciones naturales. En el **capítulo 2** también se observa que la disponibilidad de Zn tanto en suelos contaminados con ZnO NPs como con $ZnCl_2$, está influenciada por el proceso de envejecimiento con diferentes resultados dependiendo del tipo de suelo. Aunque el envejecimiento no muestra efectos claros sobre la toxicidad de Zn en este estudio, el conocimiento de los procesos que rigen a largo plazo la disponibilidad de este elemento es clave para una evaluación adecuada del riesgo de los suelos contaminados por Zn en cualquiera de las dos formas estudiadas. Además, los efectos a largo plazo y la disponibilidad de metales y metaloides en suelos contaminados *in situ* se determinan 6 y 15 años después del accidente minero de Aznalcóllar, tal y como se describe en el **capítulo 7**. En este caso, bajo condiciones naturales, el pH del suelo en general tiende a aumentar con el tiempo mientras que las concentraciones de contaminantes disminuyen. Sin embargo, después de 15 años de que se produjera el accidente y se realizaran actividades de recuperación, siguen existiendo áreas mal recuperadas que a simple vista presentan ausencia de vegetación y una vez analizadas muestran tener altas concentraciones de metales. Este estudio demuestra que aún existen posibles amenazas ecológicas en la zona y que son necesarias acciones para la evaluación de riesgos e intervenciones de recuperación de los suelos.

Para obtener una visión adecuada del peligro potencial de una sustancia química para el ecosistema es necesario realizar una batería de ensayos de toxicidad utilizando un número de especies representativas de los distintos grupos taxonómicos (bioensayos). En esta tesis se realizan diferentes pruebas para evaluar la toxicidad de metales de forma individual en suelos contaminados en laboratorio (**capítulos 2-6**), así como la evaluación de la toxicidad por multi-elementos en suelos contaminados de la zona de Aznalcóllar (**capítulo 8**). Los resultados obtenidos muestran que las respuestas obtenidas para los diferentes bioensayos difieren ampliamente para un mismo metal. En concreto, las pruebas de elongación de raíz para lechugas (*L. sativa*) y las de reproducción para lombrices (*E. andrei*) son los tests más sensibles a la contaminación por metales y dan valores de toxicidad más uniformes con intervalos de confianza más pequeños. Por lo tanto, para una evaluación más completa y fiable de la toxicidad de metales o de la contaminación de suelos se debe emplear más de un único bioensayo. El número de pruebas y organismos a estudiar debe ser optimizado en función de las circunstancias y el nivel de detalle requerido según la información que se persigue alcanzar con dichas pruebas.

Con el fin de determinar el efecto de las propiedades del suelo sobre la contaminación por metales, siete suelos con propiedades representativas de los principales grupos de suelos se han contaminado con formas metálicas de forma individual (As, Pb, Zn y Cu). Además, los suelos se incuban durante 4 semanas para evaluar el efecto del tiempo sobre la disponibilidad de metal. Los efectos de las propiedades del suelo se estudian en **todos los capítulos** de esta tesis. Nuestros resultados ponen de relieve que las propiedades del suelo son claves en la (bio)disponibilidad y tienen que ser incluidas en la evaluación del riesgo ecológico de suelos contaminados, así como en el establecimiento de criterios de calidad del suelo para la contaminación por metales. La amplia gama de respuestas que se obtiene en estos suelos demuestra claramente que no tener en cuenta las propiedades edáficas puede llevar a sobreestimar o subestimar la toxicidad y, por lo tanto, invalidar los valores de referencia establecidos generalmente a partir de valores únicos, lo que influirá en la eficacia de los objetivos y acciones de recuperación. En consecuencia, los niveles de referencia del suelo deben diferenciarse en función de las propiedades del suelo, con el fin de optimizar la eficiencia y rentabilidad en la evaluación del riesgo ambiental.

Los datos obtenidos de los bioensayos en los **capítulos 3-6** se comparan con la legislación vigente, así como con los efectos observados en los suelos naturales contaminados de Aznalcóllar. Los resultados obtenidos (**capítulo 8**) muestran que los valores de referencia existentes en Andalucía (Decreto 18/2015), donde se encuentra localizada la zona de estudio, no son suficientemente precisos para declarar un suelo como contaminado y, por lo tanto, el riesgo de contaminación podría estar sobrestimado o subestimado en función del elemento considerado. Las grandes diferencias en los valores obtenidos se deben a la gran diversidad de tipos de suelo.

Por lo tanto, y a la vista de todo lo expuesto, para una adecuada evaluación de riesgos ecológicos, concretamente por contaminación metálica en suelos, es esencial un nuevo enfoque que tenga en cuenta las propiedades del suelo. En consecuencia, para desarrollar una legislación realmente eficiente y útil, la regulación actual debería ser revisada aplicando los resultados obtenidos en esta tesis.

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> que faltan para morir,
> y las contase, y las volviese a contar, para evitar
> errores, hasta la última,
> hasta aquella que tiene la estatura de un niño
> y le besa y le cubre la frente,
> así he vivido yo con una vaga prudencia de caballo de
> cartón en el baño,
> sabiendo que jamás me he equivocado en nada,
> sino en las cosas que yo más quería.
>
> Luis Rosales

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CURRICULUM VITAE

Ana Romero Freire was born in Noia, Galicia, Spain, on the 11st of January, 1984. She has a bachelor degree in Forestry Technical Engineering by the University of Santiago de Compostela (USC, 2007) and a degree in Environmental Science by the University of Granada (UGR, 2011), both in Spain. From September 2009 to June 2010, she held an Erasmus scholarship at the KU Leuven (Belgium) where she did her final bachelor project in relation to the toxicity of manganese for soil living organisms and the effect of waterlogging, under the supervision of Prof. Dr. Erik Smolders. She also has two Master degrees, the first in Management of Environmental Projects and Corporate Social Responsibility (USC, 2009) and the other in Conservation, Management and Restoration of Biodiversity (UGR, 2012). From September 2011 until 2015, she held a PhD grant from the Spanish Ministry of Science and Innovation to perform her PhD thesis in the Department of Soil Science and Agricultural Chemistry of the University of Granada supervised by Dr. Francisco José Martín Peinado. Her research mainly focused on investigating how soil properties can affect the toxicity of metal-polluted soils to living organisms. To achieve a double Doctorate Degree in collaboration with the VU Amsterdam (The Netherlands), she spent eight months at the Department of Ecological Science supervised by Prof. Dr. Nico van Straalen and Dr. Ir. Cornelis AM van Gestel. As part of her PhD training, she also worked for three months in the Centre for Ecology and Hydrology of Oxford (UK) supervised by Dr. Claus Svendsen. As a qualified soil scientist, she has taken part in several research projects: "Toxicity of heavy metals in soils as tool for environmental management" (CGL2010-19902), "Re-use of agricultural waste for optimizing resources in subtropical crops: towards a holistic approach" (CLG2013-46655-R), and "Geohistorical reconstruction of three vegetation on Mediterranean ultramafic soils"(CS02013-47713-P). After completing her PhD thesis, she would like to continue her scientific career in an university or research center.