Tiago Natal da Luz was born in Torres Novas, Portugal on April 17, 1977, and finished his graduation in Biology by the University of Coimbra in November 2001 with the grade of 14 (out of 20). In July of 2005 he concluded the Master in Ecology by the University of Coimbra entitled "*Avoidance tests with soil organisms as early screening tools for site specific risk assessment of contaminated soils*" with maximum degree of "*Very good*". He has collaborated in projects related with risk assessment of contaminated areas due to industrial activities and use of agrochemicals and wastes in soils, essentially based on toxicity assays with terrestrial organisms (e.g. collembolans and earthworms). Recent projects are mainly focused on waste ecotoxicological characterization. In January of 2007 he benefited of grant supported by the Portuguese Science and Technology Foundation for the PhD entitled "Integrated evaluation of the ecotoxicological risk of using sewage sludges in agriculture and in soil restoration".

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Integrated evaluation of the ecotoxicological risk of using sewage sludges in agriculture and in soil restoration

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Resumo

A análise química exigida por lei para regulamentar o uso de lamas residuais na agricultura é claramente insuficiente para avaliar o risco potencial da utilização deste tipo de resíduos em solo, dado que nem o efeito de contaminantes não incluídos na análise química nem os efeitos que ocorrem como resultado de interacções entre compostos químicos são tidos em linha de conta. Por essa razão a necessidade em complementar as análises químicas com a realização de uma bateria de bioensaios para uma adequada caracterização ecotoxicológica deste tipo de resíduos é amplamente reconhecida. Dos contaminantes frequentemente encontrados em lamas residuais os metais constituem um risco potencial para os organismos edáficos. O grau de contaminação no solo é geralmente avaliado pela concentração total de metais; no entanto, o risco real é determinado pela fracção de metais que está biologicamente disponível para os organismos. Essa fracção está relacionada com a força da ligação do metal à matriz e pode variar ao longo do tempo devido a processos naturais ou antropogénicos. Além de factores abióticos, também processos biológicos, como a actividade das minhocas, são capazes de induzir mudanças na disponibilidade de metais, influenciando a sua distribuição no solo. Além da contaminação por metais, a toxicidade deste tipo de resíduos também pode estar relacionada com a presença de outros produtos químicos perigosos gerados por actividades humanas, como é o caso dos hidrocarbonetos aromáticos policíclicos (PAHs). Tem sido demonstrada a capacidade das minhocas para promover a biodegradação de PAHs no solo. No entanto, até agora, o mecanismo através do qual as minhocas exercem essa influência ainda é desconhecido.

Neste contexto, os principais objectivos deste trabalho foram: i) contribuir para a discussão e definição de estratégias adequadas para a caracterização ecotoxicológica de resíduos; ii) avaliar as alterações na disponibilidade de metais numa lama contaminada com metais ao longo do tempo e o papel de alguns factores como a matriz de contaminação e a actividade de minhocas nessas

alterações; e iii) avaliar a influência da actividade das minhocas em processos microbianos do solo que possam estar de alguma forma relacionados com o aumento da biodegradação de PAHs.

No Capítulo 2, foi testada uma bateria de ensaios ecotoxicológicos com plantas (*Brassica rapa* e *Avena sativa*) e invertebrados do solo (*Eisenia andrei* e *Folsomia candida*) para a caracterização ecotoxicológica de três lamas provenientes de fontes distintas (ETAR urbana e de indústria de processamento de azeite e galvanoplastia), fornecendo informações sobre os seus potenciais riscos e "níveis seguros" de aplicação. Os resultados mostraram que a avaliação ecotoxicológica de resíduos pode ser usada como uma ferramenta de controlo ambiental para a utilização de lamas na agricultura e apoiam a adopção de uma abordagem em diferentes etapas ("tiered approach") para este efeito. No Capítulo 3 foi avaliada a adequabilidade do uso de ensaios de fuga com colêmbolos em etapas preliminares da avaliação de risco ("lower tier"), e a sua capacidade para despoletar ensaios de reprodução numa etapa posterior ("higher tiers") nas mesmas misturas de lama e solo utilizadas no Capítulo 2, após 0, 4 e 12 semanas de incubação. Os resultados comprovaram a eficiência destes ensaios de fuga na caracterização ecotoxicológica preliminar de lamas perigosas e ainda na avaliação das alterações da toxicidade ao longo do tempo. No Capítulo 4, foi avaliada a toxicidade de uma mistura de crómio (Cr), cobre (Cu), níquel (Ni) e zinco (Zn) aplicada no solo directamente ou através de uma matriz orgânica por intermédio de ensaios de reprodução com minhocas (*E. andrei*) e colêmbolos (*F. candida*). Os resultados demonstraram que uma avaliação comparativa deste tipo fornece informação útil sobre o efeito da lama (contaminantes) e da matriz na toxicidade dos metais.

No Capítulo 5, foram avaliadas as alterações na disponibilidade de metais a curto prazo, quando aplicados directamente no solo (solos com solução de metais) ou através de uma matriz orgânica (solos com lama) através de um ensaio de laboratório em microcosmos ao longo de 12 semanas. Os resultados demonstraram que a matriz lama contribuiu, de um modo geral, para reduzir a

mobilidade dos metais no solo. Nos solos contaminados com solução de metais a disponibilidade de metais diminuiu independentemente da concentração e nos solos contaminados com lama a disponibilidade de metais manteve-se estável ao longo do tempo em todas as doses. No Capítulo 6 foi realizada uma experiência complementar à apresentada no Capítulo 5, onde foram adicionadas minhocas da espécie *Dendrobaena veneta* aos microcosmos para avaliar a influência da actividade das minhocas na disponibilidade dos metais a curto prazo. Os resultados mostraram que a actividade das minhocas não alterou a disponibilidade dos metais em nenhum tratamento ao longo do tempo, contudo interferiu no conteúdo em metais dos percolados. As concentrações de Ni, Cu e Cr em *D. veneta* foram maiores nos tratamentos com concentrações de metais mais elevadas; já a concentração interna de Zn não apresentou esta tendência sendo regulada pelas minhocas. Modelos retirados da literatura não foram capazes de prever os níveis de metais medidos em *D. veneta*. No Capítulo 7, foi realizado um ensaio de mesocosmos em campo ao longo de um ano para avaliar as alterações na disponibilidade de metais a longo prazo, comparando também o efeito da matriz (solos contaminados com lama *vs* solução de metais) e a influência da actividade das minhocas (numa densidade realista de 500 minhocas *D. veneta* por m^2). Os resultados não revelaram alterações na concentração de metais totais mas demonstraram uma diminuição na extractabilidade do Ni ao longo do tempo. A actividade das minhocas não interferiu na concentração de metais ao longo do tempo. As concentrações de Cr e Ni nas minhocas foram dependentes das respectivas concentrações no solo em alguns tratamentos e as concentrações internas de Cu e Zn foram reguladas pelas minhocas. Os modelos testados estimaram melhor as concentrações de Cu, Ni e Cr nas minhocas, mas não as de Zn.

No Capítulo 8, foi realizada uma experiência de laboratório em microcosmos para avaliar a influência da colonização de minhocas na actividade microbiana relacionada com a biodegradação de PAHs no solo. Foram efectuadas amostragens destrutivas de colunas de solo com um estrato superficial de

sedimento dragado, contaminado predominantemente com PAHs, ao longo de 18 semanas, para medir parâmetros químicos e microbianos. Os resultados sugerem que não há relação directa entre as alterações da actividade microbiana facilitada pela actividade das minhocas e o aumento da degradação dos PAHs no solo. No entanto, o papel das alterações na estrutura da comunidade microbiana do solo, induzida pelas minhocas, na remoção de PAHs necessita de mais investigação.

Palavras-chave: Ensaios ecotoxicológicos, lamas, *Folsomia candida*, *Eisenia andrei*, *Dendrobaena veneta*, *Brassica rapa*, *Avena sativa*, actividade microbiana, diversidade microbiana, disponibilidade de metais, PAHs

Summary

Chemical analysis required by law to regulate sewage sludge use in agriculture is clearly insufficient to indicate potential risk of sludge amendments in soil because neither the effect of contaminants not screened in chemical analysis nor the effects that occur as a result of multi-chemical interactions are taken into account. Because of that, the need for a test battery of ecotoxicological assays for a proper ecotoxicological characterization of sludges as a complement of chemical analysis is widely recognized. The high level of metals often found in sewage sludges constitutes a potential risk for soil organisms. The degree of soil contamination is generally evaluated by total metal concentrations; however, the real risk of metals is determined by the fraction that is biologically available for the organisms. The available fraction is highly related with the strength of metal binding by the matrix and may change over time due to natural or anthropogenic processes. Besides abiotic factors also biotic processes, like earthworm activity, may induce changes in metal availability by influencing metal partitioning in soil. Besides metal contamination, the toxicity of wastes may also be related to the presence of other hazardous chemicals generated by human activities, including polycyclic aromatic hydrocarbons (PAHs). The ability of earthworms to promote biodegradation of PAHs in soil has been reported. However, until date, the mechanism through which the earthworms exert such influence is still unknown.

Under this context, the main objectives of this thesis were i) to discuss and give a contribute to the definition of suitable strategies for ecotoxicological waste characterization; ii) to evaluate the changes in metal availability of a metal-contaminated sludge over time and the role of factors like soil matrix and earthworm activity in those changes; and iii) to evaluate the influence of earthworm activity on soil microbial processes that may be related with increasing PAH biodegradation.

In Chapter 2 a battery of ecotoxicological assays using plant (*Brassica rapa* and *Avena sativa*) and soil invertebrate species (*Eisenia andrei* and *Folsomia candida*) is proposed for the ecotoxicological characterization of three sludges from distinct sources (urban, olive-processing, and electroplating industries), providing information on their potential hazard and identifying "safe" application levels. Results showed that the ecotoxicological evaluation of wastes can be used as an environmental safety control of sludge use in agriculture and that a tiered approach may be adopted for this purpose. The use of Collembola avoidance tests in a screening level (low tier) acting as a trigger for collembolan reproduction tests (high tier) was assessed in Chapter 3 for the same soil-sludge mixtures used in Chapter 2 after 0, 4, and 12 weeks of incubation. Results demonstrated the ability of collembolan avoidance tests to assess changes in sewage sludge toxicity over time and its potential for hazardous sludge characterization at low tier levels. In Chapter 4, the toxicity of a mixture of chromium (Cr), copper (Cu), nickel (Ni), and zinc (Zn) applied to soil directly or via an organic matrix was evaluated through earthworm (*E. andrei*) and Collembola (*F. candida*) reproduction tests. Results demonstrated that this comparative approach provides useful information on the effect of the sludge matrix on the toxicity of metals.

In Chapter 5 the short-term changes in metal availability when applied to soil directly (metal-spiked soils) or via an organic matrix (sludge-amended soils) were evaluated in a microcosm laboratory experiment over 12 weeks. Results demonstrated that the sludge matrix generally contributed to reduce the mobility of metals in soil. In spiked treatments, metal availability decreased independently of test concentration and in sludge-amended soils the availability of metals remained stable over time in all treatments. In Chapter 6 a complementary experiment was performed with the inclusion of earthworms (*Dendrobaena veneta*) to the microcosms to assess the influence of earthworm activity on metal availability on a short-term basis. Results showed that earthworm activity did not affect metal availability of any treatment over time,

but their burrowing activities did interfere with the metal content of percolates. Nickel, Cu and Cr concentrations in *D. veneta* were higher at the highest treatment levels, whereas Zn internal concentration was regulated. Models taken from the literature were not able to predict the metal levels measured in *D. veneta*. In Chapter 7, an outdoors mesocosm experiment was conducted over one year to evaluate long-term changes of metal availability, also comparing the effect of the matrix (sludge amended *vs* spiked soils) and the activity of earthworms (a realistic density of 500 *D. veneta* per m^2). Results showed no changes in total metal concentrations and a decrease only in Ni extractability over time. Earthworm activity did not affect metal concentrations over time. Earthworm Cr and Ni concentrations were dependent on soil metal concentrations in some treatments and internal Cu and Zn concentrations were regulated by *D. veneta*. Models taken from the literature best estimated Cu, Ni, and Cr but not Zn concentrations in the earthworms.

In Chapter 8, a microcosm laboratory experiment was conducted to evaluate the influence of earthworm colonization and activity in facilitating microbial processes related to the biodegradation of PAHs in soil. Columns containing a layer of dredge sediment contaminated predominantly with PAHs on top of uncontaminated natural soil without and with low and high *E. andrei* densities were destructively sampled over 18 weeks for measurement of chemical and microbial parameters. Results suggest no direct relationship between changes in the microbial activity mediated by earthworms and the increased PAH degradation. However, the role in PAH decrease of shifts in soil microbial community structure induced by earthworms needs further investigation.

Key words: Ecotoxicological assays, sludges, *Folsomia candida*, *Eisenia andrei*, *Dendrobaena veneta*, *Brassica rapa*, *Avena sativa*, microbial activity, microbial diversity, metal availability, PAHs

Chapter 1

General introduction

Legal framework on land application of wastes

Europe produces annually over 250 million tonnes of municipal waste and more than 850 million tonnes of industrial waste, an amount that since 1985 on increases annually by around 3% (SOER 2010). One of the major ways of waste disposal is land application. This practice, however, constitutes a potential source of contamination of soils and ground water due to the presence in wastes of different chemicals that can have adverse effects on the environment (Düring and Gäth 2002). Because of that, since 1986, sewage sludge application to agricultural soils is regulated and monitored following the European Directive 86/278/EEC (European Community 1986). This Directive defines limit values for heavy metal concentrations in sludge and in the soil to which the sludge is applied, and maximum heavy metal loadings to agricultural soils. The sludge must be analyzed at least twice a year for levels of cadmium (Cd), copper (Cu), nickel, lead (Pb), zinc (Zn), mercury, and chromium and for other parameters such as pH and dry matter, organic matter (OM), total nitrogen, nitric and ammoniac nitrogen and total phosphorous contents. The threshold values defined are dependent on pH and metal, nitrogen and phosphorous contents of the soil. Limits for organic pollutants are not considered. However in a draft Working Document on Sludge (EU 2000) the European Union proposed additional limits of concentrations for the following organic contaminants: AOX (sum of halogenated organic compounds), LAS (linear alkylbenzene sulfonates), DEHP (di(2-ethylhexyl)phthalate), NPE (nonylphenol and nonylphenolethoxylate), PAH (sum of 10 polycyclic aromatic hydrocarbons: acenaphthene, phenanthrene, fluorene, fluoranthene, pyrene, benzo(*b*+*j*+*k*)fluoranthene, benzo(*a*)pyrene, benzo(*ghi*)perylene, indeno(1,2,3-*c,d*)pyrene), PCB (polychlorinated biphenyls), and PCDD/F (polychlorinated dibenzodioxins / dibenzofurans). In developed European countries, metal input to soils is regulated by National Directives based on the European Directive 86/278/EEC (European Community 1986). For instance, in Portugal, a recent national Directive (Diário de República 2006)

includes the lower limits for metals proposed by European Directive (European Community 1986) and the limits for organic pollutants proposed by the European Union (EU 2000). Furthermore a proposal for a standard for compost application in agricultural soils is under discussion ("Proposta de Norma Técnica sobre Qualidade e Utilizações de Composto", draft document, 2004, unpublished). In Germany, two Directives regulate sewage sludge (AbfKlärV 1992) and compost application (BioAbfV 1998), which include different limits for heavy metals (some of them lower than those of the European Directive) and limits for some organic contaminants in sewage sludge (AOX, PCB, and PCDD/F; AbfKlärV 1992). In France, a national Directive on the regulation of sewage sludge application (Gavalda et al. 2004) includes limits similar to those of the Portuguese Directive (Diário de República 2006) for the majority of metals, and limits for some PAHs and PCBs are considered (French Decree 1997). Outside Europe, the United States Environmental Protection Agency (USEPA) developed use and disposal regulations for sewage sludge also including pollutant limits, operational requirements and management practices (USEPA 1993). For metals, the USEPA permits the highest limit values among developed nations, including limit concentrations also for arsenic, molybdenum and selenium (USEPA 1999). Limits for PCDD/F are also considered (USEPA 2000).

The determination of specific pollutants in complex mixtures of unknown composition, which usually occur in wastes, does not necessarily allow an accurate estimation of toxicity and does not include possible additive, synergistic and antagonistic effects (Thomas et al. 1986). Moreover, sewage sludge is only a small fraction of the wastes that are annually produced in Europe. Aiming to promote adequate management and regulation, in 1994 the European Union established a list of wastes (European Community 1993) and a list of hazardous wastes (European Community 1994). Implementation of these lists was obligatory for European Member States by January 2002. These lists were amended several times and can be revised when necessary (e.g. European

Community 2001). Actually, the waste lists comprise about 850 different waste types of which 450 are defined as hazardous wastes and more than 200 as so-called "mirror entries". The "mirror entries" include waste types that were previously considered as non-hazardous, but in the revised list can either be hazardous or non-hazardous, depending on the composition of the waste. In Europe, hazardous wastes are classified according to 14 properties, which render them hazardous as defined in Annex III of the Council Directive 91/689/EEC on hazardous waste (Table 1.1; European Community 1991) that is derived from the Council Directive 67/548/EEC (European Community 1967) on dangerous substances. Most of these properties (e.g. toxic, harmful, corrosive, irritant, carcinogenic, teratogenic, mutagenic) are based on the concentration of dangerous substances and, therefore can be attributed on the basis of the criteria laid down by Annex VI of Council Directive 67/548/EEC (European Community 1967) or in subsequent Directives adapting Directive 67/548/EEC to technical progress (European Community 1987; European Community 1992). However, the H14 or "ecotoxic" property, which comprises "substances and preparations that present or may present immediate or delayed risks for one or more sectors of the environment" (European Community 1991), does not refer to specific methods. The classification of ecotoxic or non-ecotoxic for more than 200 mirror entries is left open, which highlights the necessity of developing strategies for assessing the H14 waste property.

Table 1.1 Hazard criteria defined in Annex III of the Council Directive 91/689/EEC (European Community 1991).

Criteria	Definition
H1	«Explosive»: may explode when under effect of flame or sensitive to shocks
H2	«Oxidising»: exhibit highly exothermic reactions in contact with other substances
H3-A	«Highly flammable»: liquids with flash point < 21ºC, catch fire on contact with air, readily ignited, flammable gases, evolve highly flammable gas on contact with water
H3-B	«Flammable»: liquids having flashpoint between < 21ºC and 55ºC
H4	«Irritant»: non corrosive substances which cause inflammation on contact with skin
H5	«Harmful»: if inhaled, ingested or penetrate the skin may involve limited health risks
H6	«Toxic»: may involve serious, acute or chronic health risk and even death
H7	«Carcinogenic»: may induce cancer or increase its incidence
H8	«Corrosive»: may destroy living tissue on contact
H9	«Infectious»: substances containing viable micro-organisms or their toxins which are known or believed to cause disease in man or other living organisms
H10	«Toxic for reproduction»: affect the incidence of non-heritable adverse effects in the progeny and/or male or female reproductive functions or capacity
H11	«Mutagenic»: may induce hereditary genetic defects or increase their incidence
H12	Substances which release toxic gases in contact with water, air or an acid
H13	Substances capable by any means after disposal of yielding another substance which possess any of the characteristics listed above
H14	«Ecotoxic»: may present immediate or delayed risks for one or more sectors of the environment

Assessment of the "Ecotoxic" property of wastes

The ecotoxicity of wastes can be estimated by using chemical- and biologically based approaches. In the first case, chemical analyses are performed and results are compared to threshold values for the substances identified. In the second case, toxicity is directly measured using biological tests. The latter approach is usually considered the best method for assessing potential toxicity due to the integrative character of bioassays, which not only includes possible interactions between chemicals but also integrates the effect of contaminants not considered or detected by chemical analyses (Thomas et al. 1986). The first attempts to use ecotoxicological tests to characterize wastes were based on adaptations of test protocols originally developed for characterization of chemicals and waste waters. These studies largely focused on waste and landfill leachates (Atwater et al. 1983; Assmuth and Penttila 1995; Clement et al. 1997; Wundram et al. 1996) assuming that water constituted the principal carrier of contaminants. More recently, a guidance to standardize the preparation of waste samples for toxicity tests was developed by the European Committee for Standardization (CEN 2003) as EN 14735, which contributed to increase reproducibility and comparability of results between studies. Test batteries have been proposed using test organisms of different trophic levels representing the terrestrial and the aquatic compartments. The Ministry for Environment and transport Baden-Württemberg of Germany sponsored a study to propose a battery of tests to determine the H14 property of 24 waste types (Deventer and Zipperle 2004). Out of the six bioassays tested, a minimum test battery consisting of an algae test (DIN 1991), a higher plant test (OECD 2000) and a bacteria contact test (DIN 2002) was suggested. With the same purpose, the French Agency for Environment and Energy Management and the French Ministry of Environment compiled a database called ECOTOX ANADEME, which includes results of six bioassays on 160 wastes. Due to the fact that some heterogeneity among the data was

obtained related to the variability of procedures adopted (particularly in terms of dilution medium and pH adjustment), there was a need to optimize this battery of bioassays. Therefore, the same battery of six standardized tests was applied to 40 of the 160 wastes and the test results obtained were analyzed using various linear and nonlinear multivariate statistical methods (Pandard et al. 2006). It was shown that the number of tests can be reduced without significantly changing the typology of the wastes. The test battery including tests on *Vibrio fischeri* (AFNOR 1999), *Ceriodaphnia dubia* (AFNOR 2000), and *Lactuca sativa* (ISO 1999c) was considered the best solution for estimating the ecotoxicity of wastes at reduced cost. In a study conducted by Alvarenga et al. (2007), three biodegradable organic residues were subjected to chemical characterization (including total metal quantification) and to toxicity assessment using bioassays. Tests on plant growth (*Lepidium sativum* and *Hordeum vulgare*; ISO 1999a) and earthworm mortality (*Eisenia fetida*; ASTM 1997) were performed and leachates from the residues were tested in bioassays on *V. fischeri* (ISO 1998b), seed germination (Fuentes et al. 2004) and *Daphnia magna* immobilization (ISO 1996). Results demonstrate that the use of a battery of toxicity tests in conjunction with chemical analyses is the most suitable strategy to evaluate the risk of disposal or land application of biodegradable organic residues. A study conducted by Rosa et al. (2007), using a battery of six bioassays to evaluate the short-term ecotoxicity potential of fresh and stabilized textile sludges and their leachates, demonstrated that tests on higher plant growth (ISO 1999c), earthworm mortality (ISO 1993), and algae growth inhibition (ISO 1990) provided good indications of the fertilizer/conditioner potential of the industrial wastes assessed. Wilke et al. (2008) evaluated the ecotoxicity property of four different waste types using a battery of six bioassays including leachate and solid phase tests. According to their findings, the use of chronic or sub-chronic tests instead of acute tests is recommended for toxicity testing of wastes. They concluded that a test battery should include at least a producer and a consumer from the terrestrial and the aquatic environment. Their findings also supported

the inclusion of genotoxicity tests on eluates (ISO 2007) and secondary consumers tests on solid wastes as well. A method for a final classification and ranking of wastes was also proposed. Moreira et al. (2008) evaluated the toxicity of a digested sewage sludge and six derived composts with avoidance and reproduction tests using earthworms (*Eisenia andrei*; ISO 2005, 1998c) and springtails (*Folsomia candida*; ISO 1999b) and higher plant growth tests (*Avena sativa* and *Brassica rapa*; ISO 1999c). They concluded that bioassays constitute important tools for early detection of ecotoxicological risks of soil amendments with organic wastes. More recently, an international ring test was co-organised by the German Federal Environment Agency and ECT Oekotoxikologie GmbH with the participation of 60 laboratories from 15 countries (Moser and Römbke 2009). A basic test battery consisting of algae (ISO 2004), *Daphnia acute* (ISO 1996), Microtox (ISO 1998a, 1998b), earthworm acute (ISO 1993) and plant tests (ISO 1999c) was used and ten additional tests (five aquatic and five terrestrial) were performed. The tests of the basic battery were generally considered suitable for the ecotoxicological characterization of wastes. However, checking alternatives to the earthworm acute test, simplification of existing methods (using microplates or other miniaturized systems) and inclusion of a genotox test in the standard battery were recommended. Gaining further experience with different waste types was also advised. The earthworm reproduction test was the most sensitive terrestrial test and the earthworm avoidance test was considered a promising terrestrial bioassay due to its high sensitivity and short test period. It was suggested that using other organisms in avoidance tests (e.g. collembolans) might be useful for certain waste materials. The inclusion of screening tests (e.g. avoidance tests) for hazardous wastes characterization integrated in a tiered assessment strategy can indeed optimize the waste characterization process in terms of time investment and work effort without compromising the quality of the assessment. With the use of rapid bioassays, the assessment of the toxicity of a waste over time after soil application may also become more practicable. This may provide information

about the waste stabilization state and the development with time of the potential environmental risk associated to sludge disposal. However, further research is needed to confirm these assumptions.

Assessment of soil metal contamination

The environmental risk associated with waste disposal in the field lies in the fact that it often contains significant quantities of metals (Petruzzelli et al. 1994). These potentially toxic elements persist and accumulate in the upper layers of the soil where they can reach levels that can be toxic to terrestrial organisms (Spurgeon et al. 1994; Gzik et al. 2003). Total metal concentrations in soil have been used as criteria to control sewage sludge application (European Community 1986), however, these levels are not necessarily good predictors of waste toxicity. The actual risk of metals is determined by the fraction that is biologically available for organisms. Metal bioavailability is not only related to the particular route of exposure and the matrix in which the organisms are exposed, but depends also on the effective exposure time. Metal availability in soil may change over time due to natural or anthropogenic processes like acidification, salinization or organic matter mineralization, which can influence metal partitioning in the soil (Allen 2002). The concepts of "bioavailability" and "bioaccessibility" were defined to express whether the concentration of a contaminant will have effects on organisms (Meyer 2002). Bioavailability refers "the fraction of the total amount of a chemical present in a specific environmental compartment that, within a given time span, is either available or can be made available for uptake by (micro)organisms from either the direct surrounding of the organism or by ingestion of food". The bioaccessible fraction is defined as "the fraction of the total amount of a chemical present in ingested food, water or ingested soil and sediment that at maximum can be released during digestion" (Peijnenburg and Jager 2003). The present thesis will focus on the bioavailable fraction and, although the bioaccessible fraction is included in

the bioavailable fraction, bioaccessibility will not be further discussed. As a dynamic process, bioavailability of metals should be handled considering a three-step approach including exposure (oral or dermal), which results in metal uptake (bioaccumulation) that may subsequently provoke toxic effects (so-called toxicological bioavailability; Peijnenburg et al. 2007). In coherence with this three-step dynamic process, the management and assessment of metal-contaminated sites can be performed by means of three types of approaches:

- Monitoring strategies;
- Bioaccumulation tests;
- Ecotoxicological bioassays.

Mechanistic models have been developed to allow for an integrated assessment of the effect of metals on organisms based on the concepts of critical body residues (Van Wensem et al. 1994), the free ion activity model (FIAM) (Morel 1983) and the biotic ligand model (BLM) (Di Toro et al. 2001; Gorsuch et al. 2002; Paquin et al. 2002). However, since these approaches are developed to aquatic environments (particularly FIAM and BLMs), their applicability to soil still needs further investigation.

Soil metal contamination - Monitoring strategies

Several methods and sampling strategies have been developed to quantify the reactive metal fraction in soils, aiming to improve the accuracy of ecological risk assessments (Peijnenburg et al. 2007). However, no single analytical method can provide a completely reliable picture of all chemical forms (species) involved in the reactivity and availability of metals (Sigg et al. 2006). The currently available methods for assessing the potential and actual available fractions can be categorized along three main lines of action:

1. Direct measurement and modelling of metal activities;
2. Application of semi-permeable devices;
3. Soil extraction methods.

Point 1 includes techniques to measure free metal ion concentrations or activities in soil pore water, for instance using ion-selective electrodes, which may be the most direct method for determining metal speciation. In addition, other equilibrium and dynamic techniques may be used, like the Donnan membrane technique (Temminghoff et al. 2000), stripping voltametry or adsorption stripping voltametry (Xue and Sigg 2002), stripping chronopotentiometry (Town and Van Leeuwen 2004) and permeation liquid membranes (Salaun and Buffle 2004). This line of action, which requires proper sampling of soil pore water, also includes speciation models that can be used to calculate the chemical speciation of metals in a solution of known composition. The use of these models constitutes a computation of speciation, using for instance the Windermere humic aqueous model (Tipping 1998), MINTEQ or Visual MINTEQ (Allison et al. 1991), the non-ideal competitive adsorption-Donnan model (Kinniburgh et al. 1999), and models to estimate free metal ions in the pore water based on the concentration of metal associated with the sorption phases present in the soil (De Vries et al. 2004).
Point 2 comprises the use of ion exchange membrane technology (Qian and Schoenau 1997), and anion-exchange membranes (e.g. resin membranes; Liang and Schoenau 1995) to mimic bioavailability of metals to organisms.
Point 3 refers to the use of various chemical extractions to describe chemical forms or availability of metals present in soil. Extraction tests are widely used to assess metal content of soils, sludges and sediments. Single (using only one extractant) and sequential extraction methods (usually involving 3 to 8 treatments of the solid phase; e.g. Tessier et al. 1979) can be performed. Initially, single extractions were mainly used for soil analysis, sequential extractions predominantly for sediments. The traditional single extractions are still widely

used and sequential extraction is a standard technique predominantly used for investigation of metal speciation in both soils and sediments (Peijnenburg et al. 2007). For single extractions, the choice of the extracting agent is dependent on the specific metal fraction intended. Commonly used extractants can be divided as follows according to the extraction efficiency:

- Weak extractants: water and diluted salt solution, e.g., calcium chloride ($CaCl_2$), calcium nitrate ($Ca(NO_3)_2$), ammonium acetate ($C_2H_3O_2NH_4$), Mg-salts, barium chloride ($BaCl_2$);
- Reductive extractants: sodium ascorbate ($C_6H_7NaO_6$), hydroxylamine-HCl, sodium dithionite or sodium hydrosulphite ($Na_2S_2O_4$);
- Weak acids: diluted solution of especially acetic or citric acid;
- Chelating agents: e.g., Ethylenediamine tetraacetic acid (EDTA), Diethylene triamine pentaacetic acid (DTPA; sometimes in combination with triethylamine and ascorbic acid), nitrilo triacetic acid (NTA);
- Combined salt-acid extractants: e.g., ammonium oxalate ($C_2H_8N_2O_4$) + oxalic acid ($H_2C_2O_4$), sodium acetate ($C_2H_3NaO_2$) + acetic acid ($C_2H_4O_2$), HNO_3 + NH_4F + $C_2H_4O_2$ + HN_4NO_3 + EDTA (Mehlich III);
- Dilute strong acids: HNO_3, HCl, HCl + H_2SO_4 (Mehlich I);
- Concentrated strong acids: HNO_3, HCl, HNO_3 + HF, *aqua regia* (concentrated HNO_3 + HCl), Fleischmann acid.

Total metal concentration in soil is often determined using *aqua regia* (HCl:HNO_3, 3:1, v:v) digestion. Boiling soil samples in concentrated nitric acid has also been successfully used in some studies (Morgan and Morgan 1988). These extractants only exclude part of the immobile fraction of metals from parent materials which are strongly bound to or incorporated in silicate minerals (Peijnenburg et al. 2007). The use of milder extractants (e.g., 0.01M $CaCl_2$, ammonium acetate, and water) allows extracting more available and reactive metal fractions from soil (Houba et al. 2000; Ma et al. 2006; Sizmur and Hodson

2008). A 0.01M solution of $CaCl_2$ is often preferred as single extractant for the following reasons:

- its ionic strength is more or less the same as the average salt concentration in many soil solutions;
- since Ca^{2+} is a dominant cation on the adsorption complex of soils, the $CaCl_2$ solution is better able to extract other adsorbed cations than solutions containing other cations;
- various elements (important nutrients, heavy metals and soluble organic carbon, nitrogen, phosphorous and sulphur) can be extracted simultaneously;
- it gives better coagulation in suspensions than extractions based on monovalent cations, such as sodium (Na^+) and ammonium (NH_4^+).

In addition, $CaCl_2$ extraction in combination with total metal contents using strong acid digestion allows estimating the distribution coefficient (K_d) under standardized conditions, allowing the comparison of metal availability and mobility across different soils or amendments. These advantages have contributed to an increasing use of 0.01M $CaCl_2$ extraction as an alternative for the many extraction procedures for nutrient or pollutants that are still in use nowadays (Houba et al. 2000).

Soil metal contamination - Bioaccumulation tests

The measurement of concentrations of soil contaminants inside organisms exposed to soil gives an indication of their bioavailability. Based on this assumption, extensive monitoring programs have been conducted, initially for marine environments like the “mussel watch” in the United States (Goldberg et al. 1978) and later also for the terrestrial environment particularly using isopods and earthworms (Dallinger et al. 1992; Spurgeon et al. 1996). Although metal

accumulation does not forcibly imply adverse effects (Vijver et al. 2004), concentrations inside the organism must directly be related with the bioavailable fraction in soil and to the potential risk of adverse effects. Aiming to relate internal concentrations with toxicity, the concept of "lethal body concentration" (LBC) was introduced. This concept assumes that the organism will die when its internal concentration exceeds a certain threshold. Lethal body concentrations have been determined for several compounds (including metals) for different organisms like earthworms (Lanno et al. 1998) and Collembola, mites, isopods and diplopods (Crommentuijn et al. 1994). Several studies have been conducted to investigate the earthworm body burden as a function of soil metal content and other environmental factors (Nahmani et al. 2007). To facilitate a comparison between studies, a standard protocol was developed to standardize procedures to assess bioaccumulation of chemicals in terrestrial oligochaetes (OECD 2010). Other studies have developed equations to estimate metal accumulation in earthworms from soil metal concentrations for cases where direct measurement of metal contents in on-site earthworms may not be feasible (e.g., Neuhauser et al. 1995; Peijnenburg et al. 1999).

The LBC concept, however, does not take into account metal compartmentalization within the organisms' body, which may determine toxic effects since the concentration in the target organ may be responsible for the effect. Metal compartmentalization is related with the different accumulation strategies that organisms from different species follow upon metal exposure (Vijver et al. 2004). These different strategies lead to differences in metal accumulation levels in species from different taxonomic groups of terrestrial invertebrates (Heikens et al. 2001). Some authors have reported that metal accumulation in earthworms is species dependent (Langdon et al. 2005; Spurgeon et al. 1996). In addition, some studies have demonstrated the influence of soil properties on metal uptake by earthworms (Ma et al. 1983; Janssen et al. 1997). A recent review by Nahmani et al. (2007) on studies to assess metal uptake by earthworms concluded that the existing models to estimate body

burden from soil metal contents need to be tested on different soils using independent results. They also highlighted the need for more studies using different earthworm species to allow interspecies comparison. The performance of field or terrestrial model ecosystems is encouraged to reduce experimental constraints that might influence the earthworm's response.

Soil metal contamination - Ecotoxicological bioassays

As mentioned above, ecotoxicological bioassays are useful tools for waste characterization because they integrate the effects of all contaminants including additive, synergistic and antagonistic effects (Thomas et al. 1986). Ecotoxicological test methods have been developed for aquatic and terrestrial organisms based on ecologically relevant sublethal criteria like reproduction (ISO 1998c, 1999b) and growth (ISO 1990, 1999c; OECD 2000; AFNOR 2000) or on lethal criteria (ISO 1993, 1996). Ecotoxicity tests have demonstrated that metals may have hazardous effects on test organism (e.g., Van Gestel et al. 1991; Ribeiro et al. 2000). Several ecotoxicological studies have focused on exposure and effects of single compounds (Yang 1994), and the criteria to control sewage sludge applications in soil are mainly based on single-metal levels (European Community 1986). Since organisms in a polluted environment are simultaneously exposed to many pollutants, the joint effect of chemical mixtures has to be taken into account. Mixture toxicity experiments therefore may provide a more realistic assessment of the potential risk of mixtures of chemicals. Although scattered, the toxicity of metal mixtures has been documented for a wide range of terrestrial invertebrates like beetles (Medici and Taylor 1967), isopods (Odendaal and Reinecke 2004), collembolans (Van Gestel and Hensbergen 1997) and earthworms (Lock and Janssen 2002). The existing methods to assess the joint action of components in a mixture of contaminants are based on the conceptual work developed by Bliss (1939) for individual compounds having the same mode of action. These methods were expanded by

Plackett and Hewlett (1952) with four possible types of interaction that can occur between the chemicals in a mixture (Table 1.2). Because these interaction types may occur at the level of the system or tissue/organ, not having consequences at the level of the individual, an extension of the Plackett and Hewlett (1952) model was proposed by Ashford (1981). From the interactions defined by Plackett and Hewlett (1952) and Ashford (1981), mathematical descriptions are only available for simple similar mode of action (concentration addition model - CA) and independent dissimilar mode of action (response addition model - RA) (Greco et al. 1992). Due to their high complexity, no models are available yet for the description of interactive actions (non-additive).

Table 1.2 The four interaction types between chemical components of mixtures defined by Plackett and Hewlett (1952).

Action	Similar action	Dissimilar action
Non-interactive (additive)	Simple similar action (concentration addition - CA)	Independent dissimilar action (response addition - RA)
Interactive (non-additive)	Complex similar action	Dependent dissimilar action

In the CA model a similar mode of action of the mixture components is assumed and the concentrations are expressed as toxic units (TUs), with TU defined as a fraction of an effective concentration (c), usually the EC_{50} or LC_{50} ($TU = c/EC_{50}$; Sprague 1970). In the CA model, the toxic potential of a mixture is described as the sum of the TUs of the individual chemicals, or:

$$\sum c_i / EC_{50,\,mix} = \sum (c_i / EC_{50,i}) \qquad (1.1)$$

with c_i = concentration of component i in the mixture, $EC_{50,\,mix}$ = concentration of mixture that produces an adverse effect of 50%, $EC_{50,i}$ = concentration of

component *i* of the mixture that produces an adverse effect of 50% when applied alone.

The RA model assumes different modes of action of the mixture components and, therefore, uncorrelated sensitivities to the different toxicants. The RA model is described as:

$$E(c_{mix}) = 1 - (1 - E(c_1)) * (1 - E(c_2)) * \ldots * (1 - E(c_n)) \quad (1.2)$$

with $E(c_{mix})$ = effect of mixture in a concentration c, $E(c_n)$ = effect of component i in a concentration c (i = 1, …., n) when applied alone.

Most studies on metal mixture effects have been focused on aquatic organisms (e.g. Enserink et al. 1991). Consistent results have been obtained in these experiments, with approximately 70% of the mixtures acting in agreement with the CA model (De Zwart and Posthuma 2005). Less consistent data has been obtained in studies on the toxicity of metal mixtures to soil invertebrates. In a study conducted by Van Gestel and Hensbergen (1997) the effects of a mixture of Cd and Zn on the growth of *Folsomia candida* were antagonistic but the effects on reproduction were additive. For the isopod *Porcellio laevis* an antagonistic effect on weight was reported for mixtures of Cd and Zn by Odendaal and Reinecke (2004). A study on the sublethal toxicity (reproduction, growth) of mixtures of Cd, Cu, Pb, and Zn to the earthworms *Eisenia andrei*, *Eisenia fetida*, *Aporrectodea caliginosa* and the potworm *Enchytraeus crypticus*, reported mainly antagonistic effects for total soil concentrations and nearly concentration-additive for 0.01M $CaCl_2$-extractable soil concentrations (Weltje 1998). Other studies investigating effects on the reproduction of *Enchytraeus crypticus* when exposed to mixtures of Cu and Zn (Posthuma et al. 1997) and on growth, cocoon production and survival of *Aporrectodea caliginosa* when exposed to mixtures of Cu, Cd, and Zn (Khalil et al. 1996a, 1996b) reported antagonistic effects. In an experiment on the toxicity of binary mixtures of Cd, Cu, Pb, and Zn to *Enchytraeus albidus* conducted by Lock and Janssen (2002),

predictions based on the CA model resulted in stronger effects than predictions on the basis of the RA model. The authors therefore concluded that the CA model seems to be acceptable as a worst-case scenario for the environmental risk assessment of soils contaminated with metal mixtures. Since the ecotoxicity of wastes may be due to the presence of contaminants that include not only metals, comparative evaluation of the toxicity of sludge-amended and metal-spiked soils (with the same mixture of metals as in the sludge) based on the CA model may provide information on the role of the sludge matrix and other contaminants.

Polycyclic Aromatic Hydrocarbon biodegradation in soil

The waste disposal on soils (e.g. dredge materials) may increase soil concentrations of a wide range of hazardous chemicals generated by human activities, including polycyclic aromatic hydrocarbons (PAHs). The fate of PAHs in nature is of great environment concern due to their toxic, mutagenic and carcinogenic properties (Cerniglia 1992). PAHs are produced by pyrolysis of organic carbon-based materials and are usually associated with residues from combustion (e.g. waste incineration and fossil fuel combustion), coke production, petroleum refining, automobile exhaust, gas works and other high-temperature industrial processes (Sims and Overcash 1983; Weissenfels et al. 1992). PAHs consist of fused aromatic rings that are highly resistant to nucleophilic attacks due to the dense clouds of π-electrons on both sides of the ring structure. In addition, their low aqueous solubility (which decreases with increasing molecular mass) and high solid-water distribution ratios may hinder their ready microbial utilization, contributing to their accumulation in soils. In nature, PAHs ranging in size from naphthalene (two rings, $C_{10}H_8$) to coronene (seven rings, $C_{24}H_{12}$) can be found (Johnsen et al. 2005). PAHs may be subject to chemical oxidation, photolysis, and volatilization, but aerobic biological degradation is the major process affecting the persistence of PAHs in nature. In anaerobic environments PAH-degradation is usually limited (Cerniglia 1992).

Biological degradation may occur by three different processes comprising distinct functions: i) assimilative biodegradation with the mineralization of the compound or part of it, which yields carbon and energy for the degrading organism; ii) intracellular detoxication where the PAHs become water-soluble to allow excretion; iii) co-metabolism as a result of a substrate competing with the structural similar primary substrate for the enzyme's active site (Johnsen et al. 2005), which may not have a direct benefit to the organism but may promote subsequent attack by another organisms (Keck et al. 1989). It is believed that in soil the microbial degradation of PAHs is dependent on the amount dissolved in the water phase that is available to the organisms, contrasting with sorbed, crystalline and non-aqueous phase liquid-dissolved PAHs (the high molecular weight PAHs) which are unavailable to PAH-degrading organisms (e.g., Ogram et al. 1985; Harms and Bosma 1997; Bosma et al. 1997). Consequently, the largest fraction of bacterial PAH degradation occurs in the water phase. When the PAH concentration dissolved in the water phase becomes limiting, bacteria adopt biological mechanisms that allow maximizing transport by diffusion of PAHs to cells to increase degradation rates. These adaptations may occur through different strategies like using mechanisms to manage the optimal PAH-concentration at the cell-surface (Wick et al. 2001), releasing biosurfactants (Volkering et al. 1998), or producing extracellular polymeric substances (Dohse and Lion 1994). On the other hand, the high molecular weight PAHs generally do not serve as growth substrates for any single microbial organism, but are thought to be oxidized in a series of steps by consortia of microbes (Perry 1979). Bouchez et al. (1995) demonstrated that degradation of a PAH mixture constituted a co-operative process involving a consortium of bacterial strains with complementary capacities. In soil, co-metabolic side-reactions act on the PAHs producing a multitude of metabolites that generally have higher polarity than the parent compounds and, therefore, at least part of them may enter the pool of soil dissolved carbon (Richnow et al. 1997). In addition to bacteria, fungi may also contribute considerably to the biodegradation of PAH molecules in soil

(Cerniglia 1992). The results obtained by Kotterman et al. (1998) even suggest that the initial attack of high molecular weight PAHs in soil is more likely by fungal exoenzymes than by bacterial intracellular enzymes. The metabolites resulting from the oxidation by fungi may be more bioavailable to the microbial community. Some studies have given strength to the hypothesis that a catabolic cooperation between fungi and bacteria may promote PAH degradation (e.g. Nurmiaho-Lassila et al. 1997; Boonchan et al. 2000). The ability of plants to stimulate biodegradation of PAHs has also been reported. This ability ranges from negative or negligible to highly stimulatory effects depending on the plant species (Phillips et al. 2006; Mueller and Shann 2006; Aprill and Sims 1990). Possible mechanisms by which the most effective plants enhance removal of PAHs have been proposed. Those mechanisms may involve the stimulation of PAH degrading rhizosphere bacteria or, alternatively, uptake by the plant with subsequent accumulation in the plant tissues, enzymatic degradation, or volatilization (Binet et al. 2000; Miya and Firestone 2001). Soil fauna is also in close contact with PAHs and may take PAHs through the body surface or by feeding (Ma et al. 1995; Van Brummelen et al. 1996). These behaviours trigger detoxification reactions, which have been reported in several invertebrates from soil such as oligochaetes (Achazi et al. 1998) and isopods (Stroomberg et al. 1999) and from bentic environments such as star fish (Den Besten et al. 1992), lobsters (Li and James 1993), and polychaetes (Driscoll and McElroy 1996). These mechanisms are generally based on the hydroxylation and conjugation of PAHs taken up by the soil animals through the activity of cytochrome P450 enzymes followed by excretion of the resulting metabolites to the surroundings where they will be available to bacteria (Stroomberg et al. 1999). Such detoxification mechanisms are, however, believed to be of limited importance for PAH degradation. More important are the interactions between soil invertebrates and microbial communities, which improve PAH degradation. For instance, the ability of earthworms to change soil microbial communities has been widely reported (e.g. Schaefer et al. 2005; Aira et al. 2006; Sen and Chandra 2009). Due

to their bioturbation behaviour they mix organic and mineral constituents with microorganisms, promoting soil aeration (Lavelle et al. 1989). However, the knowledge concerning the mechanisms involved in earthworm activity that stimulate PAH degradation is still sparse. Further research is needed to identify which type of changes in microbial communities provoked by earthworm activity may be related with increased soil PAH degradation.

Outline of the thesis

The major goals of the study presented in this thesis were to provide further ecotoxicological information and to promote the discussion about a suitable evaluation strategy for wastes, and to gain further insights about the role of soil fauna, specifically of soil engineers, in mediating metal availability and in promoting PAH degradation in waste amended soil.

The work presented in this thesis can be grouped into three main topics on waste ecotoxicology.

The first topic is related to ecotoxicological tests to evaluate the toxicity of waste materials. It comprises Chapters 2, 3, and 4, which describe three different approaches essentially based on standard ecotoxicological tests. In Chapter 2, the need for an ecotoxicological assessment as an environmental quality control measure in the process of sewage sludge amendment to agricultural soils is highlighted. Accordingly, a battery of bioassays is suggested, allowing a proper ecotoxicological characterization of sludges (the "ecotoxic" property), and providing information on their potential hazard and on suitable "safe" application levels. The strategy adopted in this Chapter included the evaluation of screening and chronic ecotoxicological tests assuming the suitability of a tiered approach on the ecotoxicity characterization of wastes. Chapter 3 aimed at demonstrating the potential of Collembola avoidance tests as a complementary tool in a monitoring process after sewage sludge application on an agricultural soil. The strategy adopted was based on the assumption that the inclusion of

screening ecotoxicological tests for waste characterization and management integrated in a tier approach (particularly in a lower tier) may contribute to optimize the risk assessment process. Chapter 4 aimed at evaluating the toxicity of a certain metal-contaminated industrial sludge to soil invertebrates, represented by the earthworm *Eisenia andrei* and the collembolan *Folsomia candida*. To attain this purpose a comparative approach between sludge-amended soils and metal-spiked soils was used, allowing for an evaluation of the role of the sludge matrix and of contaminants present in the sludge other than metals.

The second and the third topics refer to the development with time of soil contaminants that originate from sewage sludge application and the influence of the contamination matrix and/or earthworm activity in the process. More specifically, the second topic concerns changes in soil metal availability over time, and is discussed in Chapters 5, 6, and 7. These Chapters describe comparative studies to evaluate changes in a mixture of metals originating from an industrial sludge (the same as used in Chapter 4) or from a metal spiking in soil over short-term and long-term periods. The experiment in Chapter 5 aimed at evaluating metal availability changes in sludge-amended and metal-spiked soils on a short-term basis. Soil metal content was measured over 12 weeks through soil extractions using 69% HNO_3 (total metal concentration) and 0.01M $CaCl_2$ (extractable metal concentration) solutions and by direct measurement in percolates. In Chapter 6 a complementary experiment was performed with the difference that earthworms of the species *Dendrobaena veneta* (at a realistic density) were added to the sludge-amended and metal-spiked soils. This experiment aimed at evaluating the influence of earthworm activity on metal availability on a short-term basis. The influence of matrices on internal metal concentrations and the accuracy of existing models to predict metal body concentrations were also evaluated. To attain these objectives, the development of metal concentrations over time obtained in this experiment was compared with that obtained in the experiment of Chapter 5. In addition to total and extractable metal concentrations in soil and metal content in percolates,

earthworm metal concentrations were measured over 12 weeks. Chapter 7 describes a field assay, which had as main objective to evaluate soil metal availability changes in sludge-amended and metal-spiked soils on a long-term basis. The influence of earthworm activity (the species *D. veneta*) and contamination matrix on the development over time of metal availability and the influence of matrices on earthworm metal concentrations were also addressed. Also in this experiment, the metal concentrations in earthworms were compared to those estimated by existing models to evaluate their accuracy. Total and extractable metal concentrations in soil were measured using 69% HNO_3 and 0.01M $CaCl_2$ extractions, respectively and earthworm metal concentrations were determined over a period of one year. The third topic, which is covered by Chapter 8, focuses on the influence of earthworm activity on microbial processes related with the degradation of persistent organic pollutants. This Chapter describes a laboratory experiment that aimed at identifying which changes in soil microbial communities promoted by earthworm activity can be related with the increased biodegradation of polycyclic aromatic hydrocarbons (PAHs). In this study, PAH-contaminated dredge sediment was used and two densities of the earthworm *Eisenia andrei* were added. Total metal and PAH concentrations in soil were measured and the microbial parameters determined were dehydrogenase activity, microbial biomass, functional diversity (Biolog Ecoplate^{TM}) and structural diversity (PCR-DGGE).

In Chapter 9 an integrated discussion of the results obtained in the Chapters 2-8 is presented.

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Chapter 2

The use of sewage sludge as soil amendment. The need for an ecotoxicological evaluation

Based on the following manuscript:

Natal-da-Luz T, Tidona S, Jesus B, Morais PV, Sousa JP, 2009. The use of sewage sludge as soil amendment. The need for an ecotoxicological evaluation. *Journal of Soils and Sediments* 9: 246 – 260.

Abstract

Sewage sludge use in agriculture should be limited by the presence of metals and other persistent environmental pollutants. The present study aims to contribute for the definition of a test battery of ecotoxicological assays that allows a proper ecotoxicological characterization of sludges, providing information on their potential hazard and identify 'safe' application levels. Three sludges from distinct sources (urban, olive processing and electroplating industries) were tested using avoidance and reproduction tests with earthworms (*Eisenia andrei*) and springtails (*Folsomia candida*) and plant growth tests with turnip (*Brassica rapa*) and oat (*Avena sativa*). Different soil-sludge mixture concentrations mimicking recommended/realistic field dosages were tested. Only the sludge from the electroplating industry induced an avoidance response from the earthworms (EC_{50} = 0.4 t/ha) and collembolans (NOEC = 15 t/ha). This sludge was again the only responsible for an effect in the reproductive output of the earthworms (EC_{50} = 7.74 t/ha). Regarding collembolans none of the sludges tested caused any significant decrease in reproduction. In higher plant tests, the two industrial sludges were toxic, causing a decrease in growth on both species. The EC_{20} values determined for *B. rapa* were 20.3 and 24.2 t/ha and for *A. sativa* 14.7 and 16.2 t/ha for sludges from olive processing and electroplating industries, respectively. The metal loadings of the different test sludges could partially explain the results obtained. The toxicity of the test sludge from electroplating industry observed on the tested invertebrates and plants could be explained by the high amount of total chromium (Cr) from which 22.3% was in the most toxic oxidation state – Cr(VI). However, the toxicity caused by the sludge from the olive processing industry in the test plants could be attributed to the presence of other compounds (not measured in this study) since the metal content was not high enough to induce such an effect. The absence of toxicity showed by the urban test sludge was in agreement with its low levels of metals. The response of the different test organisms and endpoints varied according to

the sludge type. The urban sludge was non-toxic whereas the sludge from the electroplating industry caused a toxic effect on almost all parameters measured (avoidance behaviour of both test organisms, reproduction of earthworms and growth of both plant species). Sludge from the olive processing industry only caused a toxic effect on growth of both plant species. Analysing the sensitivity of the different parameters at the most toxic sludge, avoidance and reproduction were more sensitive than plant growth, whereas plant seed germination was not sensitive at all. The ecotoxicological evaluation of wastes can be used as an environmental safety control of sludge use in agriculture. A tiered approach could be adopted for this purpose incorporating avoidance tests in the first tier (screening level) and reproduction and plant growth tests in a second tier. But more evidence aiming to define the most suitable ecotoxicological test battery for specific sludges with a different contamination profile is still needed.

Key words: Avoidance tests, higher plant growth tests, reproduction tests, sludge characterization, test battery

Background, aim and scope

The increasing industrial activity and demographic pressure in urban areas implied a regulation for the resulting pollutant discharges into water courses in order to preserve the environment. Nowadays, all the wastes resulting from industrial and urban activities have to pass a wastewater treatment plant before their discharge in streams and rivers. The sludge resulting from the treatment process is commonly used as fertilizer or soil amendment in agricultural soils. Besides being a cheap method of disposal, it has the capacity to improve soil aggregation and structural stability which facilitates water infiltration (Al-Assiuty et al. 2000). Furthermore, their high levels of organic matter and microbial activity suggest that the benefits of their use are much higher than the hazards to the environment (Düring and Gäth 2002). However, the metals and the persistent environmental organic pollutants usually present in this type of material may have deleterious effects on soil biota (Düring and Gäth 2002; Petersen et al. 2003). For these reasons, since 1986, the incorporation of wastes in soil has been regulated and monitored following the European Directive 86/278/CEE (European Community 1986). According to this Directive, the amount of sludge allowed to be used per hectare in an agricultural soil depends on metal concentrations in the sludge and in the soil. The sludge must be analyzed at least twice a year for levels of several metals and other chemical parameters. When metal levels are within legal limits, the application threshold values are dependent on the pH, and the metal, nitrogen and phosphorous contents of the soil where the waste is to be applied. Nevertheless, some ecotoxicological assessments evaluating the risks involved in the use of wastes in soil have demonstrated that additional substances and additive effects resulting from certain contaminant mixtures not included in the EU Directive 86/278/CE provide deleterious effects in soil systems (Vikelsøe et al. 2002). Although sludge incorporation in soil usually provides an input of plant available nutrients

and seems to favour invertebrate communities, noxious effects have been found in laboratory studies (e.g. Andrés and Domene 2005).

According to Sørensen et al. (2001) the amendment of agricultural soils with sludge nutrients should be included in the risk assessment due to the accumulation of xenobiotic substances. Notwithstanding these facts, an ecotoxicological assessment is not required by law in most countries. Aiming to take into account all the hazardous factors present in wastes that are not measurable by chemical analysis, the European Union Council Directive 91/689/EEC regulated the waste classification concerning their potential harmful effect according to 14 properties (European Community 1991). It has been subject to several revisions during the last years, the most significant one being in January 2001 (European Community 2001). "Ecotoxic" (H14) is one property that is attributed to the substances and preparations, which present or may present immediate or delayed risks for the environment. For this reason, the definition of a battery of ecotoxicological assays to evaluate and quantify the "Ecotoxic" property of a waste is urgent.

Until now, several strategies have been followed to evaluate the potentially harmful effect of wastes. Many of them have used organisms representative of soil communities as bio-indicators of ecotoxicity: e.g., collembola (e.g. Lubben 1989; Domene et al. 2007a, 2007b), beetles, nematodes, mites (Bruce et al. 1999), Diptera (Redborg et al. 1983), enchytraeids (Gejlsbjerg et al. 2001), earthworms (e.g. Moreira et al. 2008), and plants (e.g., Petersen et al. 2003; Fjällborg and Dave 2004). However, the selection of the most suitable bioassays to characterize wastes is still needed. Aiming to fill this gap, some studies have been conducted during the last years (e.g., Renoux et al. 2001; Robidoux et al. 2001; Pandard et al. 2006; Domene et al. 2008). An inter-laboratory test to evaluate the validity of a test battery for ecotoxicological characterization of wastes was carried out during 2007. Nevertheless, since the suitability of a bioassay can be tightly dependent on the contamination profile of the waste to be assessed, the success of a test battery is related with the type of waste. This

implies more experiments using not only other bioassays and bio-indicators but also several waste types.

The present study, therefore, aims at highlighting the need for an ecotoxicological assessment as an environmental quality control in the process of sewage sludge amendment to agricultural soils. It also aims at suggesting a battery of tests that allows the evaluation of the "Ecotoxic" property of sludges as waste materials. For this purpose, the ecotoxicological potential of three activated sludges from different sources (urban, electroplating and food industries) was assessed. Due to its high sensitivity to evaluate unfavourable conditions over a short period of time, avoidance assays with soil invertebrates were selected as screening tests in this study. Therefore, the test battery consisted of avoidance and reproduction (chronic) tests using organisms representative of the soil community, i.e., collembolans (*Folsomia candida*), earthworms (*Eisenia andrei*). Higher plant growth tests using monocotyledonous (*Avena sativa*) and dicotyledonous (*Brassica rapa*) species were also performed. The assessment was conducted using different sludge doses mixed in with an agricultural soil according to the usual concentrations applied in fertilization assays.

Materials and methods

Sample processing

Reference soil

Field-collected soil from an agricultural area in sub-urban limits of the city of Coimbra, Portugal, was used as a control. This soil was free of pesticides and fertilizer applications for more than 5 years.

The reference soil was sieved (5 mm) and defaunated applying two freeze-thawing cycles (48 h at -20ºC followed by 48 h at 25ºC per cycle) before mixing with the test sludges. The microbial community of the reference soil was re-established by inoculating the bulk soil with an elutriate obtained from a fresh

soil sample (1:10 fresh soil:distilled water (w:w) mixed for 30 minutes). The soil parameters measured were soil pH (1M KCl 1:6 v:v), water holding capacity (WHC; ISO, 1999), cation exchange capacity (CEC; ISO 1994a), organic matter content (OM; loss on ignition at 500ºC for 6 h), soil texture (LNEC 1970) and metals in bulk soil (Table 2.1).

Table 2.1 Physical and chemical characterization of the reference soil (average ± standard deviation) and the upper limit values of metals allowed for an agricultural soil with a pH higher than 7 to allow a sludge incorporation according to Directive 86/278/CEE of the European Community (1986).

		Reference soil	**Limit values (pH >7)**
pH (1M KCl)		7.86 ± 0.08	-
Water-holding capacity (%)		46.3 ± 2.6	-
Cation exchange capacity (meq/kg)		90.4	-
Organic matter (%)		2.9 ± 0.2	-
Metals in bulk soil	Cadmium	< 2.8	4
(mg/kg DW)	Chromium	11	300
	Copper	12	200
	Lead	61	450
	Mercury	*ND*	2.0
	Nickel	< 14	110
	Zinc	96	450
Soil texture (%)	Sand	88.8	-
	Silt	7.00	-
	Clay	4.2	-
Soil type		Loamy Sand	-

ND - not determined parameter.

Sludge samples

Sewage sludges from different sources were characterized by different levels of metals, OM content and pH (Table 2.2). The sludges were chosen to represent different pollutant profiles (metals) and OM contents.

Sludge A was obtained from a municipal urban wastewater treatment plant in Coimbra, Portugal; Sludge B resulted from a biological and secondary treatment of wastewater from an olive industry (Mira, Portugal); Sludge C was obtained after a biological and secondary wastewater treatment of industrial sewage from an electroplating industry (Ceira, Portugal).

Table 2.2 Total metal concentrations, pH and organic matter content of the test sludges (average ± standard deviation) and the upper limit values of metals allowed for a sludge to be incorporated in an agricultural soil according to the Directive 86/278/CEE of the European Community (1986).

Sludge		**A**	**B**	**C**	**Limit values**
pH (1M KCl)		6.57 ± 0.13	7.68 ± 0.08	8.57 ± 0.04	-
Organic matter (%)		74.9 ± 2.1	64.6 ± 4.1	4.4 ± 1.1	-
Metals in	Cadmium	3.2	< 0.5	< *QL*	20
bulk sludge	Chromium	121	74	4790[a]	1000
(mg/kg DW)	Copper	436	66	42	1000
	Lead	145	19	3.5	750
	Mercury	*ND*	0.3	0.07	16
	Nickel	39	33	58	300
	Zinc	1731	350	900	2500

[a] 3720 mg Cr(III) per kilogram and 1070 mg Cr(VI) per kilogram.

ND - not determined parameter; *QL* - not detected or present at a concentration below the quantifying limit; A – Urban sludge; B – Sludge from olive industry; C – Sludge from electroplating industry

Each test sludge was mixed in with the reference soil at five different concentrations (0, 6, 15, 25, and 45 t DW/ha representing 0, 4, 10, 16.7, and 30 g DW/kg, respectively; Table 2.3), according to the usual concentrations applied in fertilization assays and taking into account the allowed legal limits (European Community 1986). The test mixtures were prepared considering sludge concentrations defined taking into account a specific mass of 1.5 g/cm^3 for the reference soil and assuming the test sludges would be incorporated to a depth of 10 cm.

Metal analysis

The metal values for each sludge were obtained directly from analytical reports provided by the companies that supplied the test materials. All the analyses were performed at certified laboratories. On sludges A and B, and on the reference soil, the total content of cadmium (Cd), lead (Pb) and zinc (Zn) was measured by inductively coupled plasma (ICP) – mass spectrometry (USEPA 2005) and chromium (Cr), copper (Cu), and nickel (Ni) by ICP - atomic emission spectrometry (USEPA 2001). In sludge C the Cr, Cu, Ni, and Zn total contents were measured by ICP - atomic emission spectrometry (USEPA 2001) and Cd and Pb by atomic absorption spectrometry using graphite furnace (USEPA 1994). All these analyses were considered valid only when the quality control standard recovery occurred between 95% and 115%. Mercury total content was measured only in the test sludges B and C by AAS using cold vapour atomization (USEPA 1986). In this case, recovery percentage of control standard was always within 94% and 97%. In sludge C, the oxidised state of Cr (Cr(VI)) was measured by the diphenylcarbazide method similarly as was performed by Branco et al. (2005), being the reduced state (Cr(III)) content determined by subtracting the total Cr with the content of its oxidised state (Cr(IV)).

Table 2.3 Characterization of the soil-sludge mixtures.

Sludge	Conc (t/ha)	pH (1M KCl)	WHC (%)	Estimated OM (%)	Estimated metal concentrations in bulk soil (mg/kg DW)						
					Cd	Cr (III/VI)	Cu	Pb	Hg	Ni	Zn
A	0	7.8 ± 0.08	46.2 ± 2.60	2.9 ± 0.21	<2.8	11.0	12.0	61.0	*ND*	<14.0	96.0
	6	7.8 ± 0.01	48.2 ± 0.91	3.2 ± 0.21	<2.8	11.4	13.7	61.3	*ND*	<14.1	103
	15	7.8 ± 0.01	50.9 ± 0.63*	3.6 ± 0.23	<2.8	12.1	16.2	61.8	*ND*	<14.2	112
	25	7.8 ± 0.03	50.5 ± 0.91*	4.1 ± 0.24	<2.8	12.8	19.1	62.4	*ND*	<14.4	123
	45	7.7 ± 0.03	57.7 ± 0.87*	5.0 ± 0.27	<2.8	14.2	24.3	63.4	*ND*	<14.7	144
B	6	7.8 ± 0.03	49.8 ± 2.14	3.1 ± 0.21	<2.8	11.2	12.2	60.8	0.001	<14.1	97.0
	15	7.8 ± 0.02	50.1 ± 0.95	3.5 ± 0.25	<2.8	11.6	12.5	60.6	0.003	<14.2	98.5
	25	7.8 ± 0.02	54.2 ± 2.37*	3.9 ± 0.28	<2.8	12.0	12.9	60.3	0.005	<14.3	100
	45	7.5 ± 0.02*	54.1 ± 1.17*	4.7 ± 0.33	<2.8	12.8	13.6	59.8	0.009	<14.6	103
C	6	7.7 ± 0.03	16.5 ± 2.85*	2.9 ± 0.21	<2.8	30.0 (25.8/4.3)	12.1	60.8	0.000	<14.2	99.2
	15	7.8 ± 0.08	19.8 ± 3.23*	2.9 ± 0.22	<2.8	58.3 (47.7/10.6)	12.3	60.4	0.001	<14.4	104
	25	8.0 ± 0.04*	20.4 ± 2.27*	2.9 ± 0.22	<2.8	90.9 (73.0/17.9)	12.5	60.0	0.001	<14.7	109
	45	8.1 ± 0.06*	22.7 ± 2.31*	2.9 ± 0.24	<2.8	150 (119/31.2)	12.9	59.3	0.002	<15.3	119

The pH and the water-holding capacity (WHC) were measured; the organic matter content (OM) and the metals in bulk soil were estimated for each treatment from the measured values presented in Table 2.2.

See Table 2.2 for sludge codes. *ND* - not determined parameter. * - statistically significant difference ($p \leq 0.05$) comparing with the control (0 t/ha).

Bioassays with invertebrates

Invertebrate species

The avoidance and reproduction assays used the earthworm *Eisenia andrei* (Lumbricidae: Oligochaeta) and the collembola *Folsomia candida* (Collembola: Isotomidae), which are currently used in avoidance and reproduction tests (e.g., Van Gestel et al. 1993; Hund-Rinke and Wiechering 2001; Crouau et al. 2002; Natal-da-Luz et al. 2004), being standardized in ISO guidelines (ISO 1996; 1999; 2007a; 2007b).

Both test species were obtained from laboratory cultures under a photoperiod of 16 h light and 8 h dark at 20 ± 2ºC. The earthworms were kept in plastic culture containers (36 cm length, 22 cm width, and 11 cm height) using horse dung and *Sphagnum* sp. peat as substrate in a ratio of 1:1 (w:w). Cooked oatmeal was given as food twice a month. Worms holder than one month were used in the avoidance tests and adults, with an average individual fresh weight of 402 ± 71.0 mg (average ± SD) and with a well developed clitellum were used in the reproduction assays. The worms were acclimatized during 7 days in the reference soil before being used.

Springtails were cultured in cylindrical transparent plastic boxes (11 cm diameter and 4 cm height) using a mixture of plaster of Paris and activated charcoal in a ratio of 8:1 (w:w) as substrate. Granulated dry yeast was added as food in small amounts to avoid spoilage by fungi. When detected mouldy food was removed from the culture containers. Collembolans of 10 to 12 days old, obtained from synchronized cultures, were used in both reproduction and avoidance assays.

For avoidance and reproduction assays with earthworms and collembolans, moisture content of each soil-sludge mixture was adjusted to 50% of the WHC. Tests were conducted at 20 ± 2ºC under a photoperiod of 16 h light and 8 h dark.

Avoidance tests with earthworms

The test procedure was based on ISO (2007a), with some modifications. Plastic boxes (20 cm length, 12 cm width, and 5 cm height), vertically divided into two equal sections by a plastic card were used. Three hundred grams of reference soil (dry weight equivalent) were placed into one of the two sections (control side); the other section (treatment side) received the same amount of a soil-sludge mixture concentration. The divider was removed and five earthworms (previously washed and wiped dry) were added on the middle line of each test container. All replicates were covered by a perforated and transparent lid to prevent worms from escaping. All concentrations of the three sludges tested were combined with the reference soil (0 t/ha). Four replicates per combination were performed. After seven days of exposure, the two sections of each test container were divided again and the number of worms placed in each section was determined. Individual earthworms found under the midline were recorded as 0.5, independent of the length of the remaining body. Missing worms were considered to have died during the test period. Soil pH was measured at the beginning and at the end of the assays for all combinations tested.

An additional combination was included containing reference soil on both sides of the test container to ensure that no avoidance reaction is detected (dual-control test) (Hund-Rinke and Wiechering 2001; Natal-da-Luz et al. 2004). The avoidance test is considered valid if mortality is lower than 20% in this dual-control test.

Avoidance tests with collembola

In these assays similar procedures to those described for earthworms were adopted. Cylindrical plastic boxes (diameter 7 cm and height 6 cm) were divided into two equal sections by means of a card divider introduced vertically. In one of the sections, 30 g (dry weight equivalent) of a particular soil-sludge mixture concentration was placed, while the other one receive reference soil. Five replicates were tested for each soil-sludge combination. After removing the

plastic divider, 20 individuals (without visible signs of damage) were added to each test vessel. An additional container without individuals was prepared for each soil-sludge combination, for pH and moisture determination at the end of test. To avoid water losses by evaporation and to prevent collembolans from escaping, the containers were closed with a transparent lid. At the end of the 7-day test period the two sections of each test container were divided again and emptied into different vessels. These vessels were filled with water and a few drops of blue ink were added. After gentle stirring, the individuals floating on the water surface were counted. Missing individuals were considered dead. The pH of both sections was determined at the beginning and at the end of the test, for each combination tested. A test with reference soil in both sides was also included (dual-control test). As for the earthworm test, validity criterion assumes mortality lower than 20%.

Reproduction tests with earthworms

The methodology applied was based on Environment Canada (2002). Ten replicates per dose were prepared, each one consisting of a cylindrical plastic box (11 cm diameter and 12 cm height) with approximately 200 g (dry weight equivalent) of a single soil-sludge mixture concentration, properly moistened. Two worms, previously washed and weighted, were placed in each replicate. To prevent worms from escaping, the test vessels were covered with transparent lids, which were overlaid by aluminium foil in order to avoid excessive light on top of the soil. Some holes were punched in these lids to facilitate air circulation. Five grams of cooked oat meal was added per test container as food at the start and after 14, 28, and 42 days of exposure. At day 28, the adult worms were removed and their fresh weight was measured to determine biomass change. At day 56, the number of juveniles hatched per surviving adult was determined in each replicate.

Reproduction tests with collembola

The methodology adopted was in agreement with ISO (1999). Five replicates per dose were prepared, composed of a glass flask (4 cm diameter and 7 cm height) with 30 g (dry weight equivalent) of a particular soil-sludge mixture. In each test container, ten springtails were placed and 2 mg of granulated dry yeast were added as food. To avoid water loss by evaporation and to protect springtails from escaping, all replicates were covered with a transparent lid. At day 14 of exposure, the test containers were opened for few seconds to allow aeration and moisture content was checked by weighing the test containers. When the loss of weight was higher than 2%, water loss was restored. At the end of the 28-day test period each test container was emptied into a small vessel, which subsequently was filled with water. After the addition of a few drops of blue ink and gentle stirring, the animals floating on the water surface were photographed and counted. Missing adult springtails were considered dead. An additional replicate per test concentration, but without organisms, was prepared and submitted to the same conditions in order to allow pH and moisture measurement at the end of the test.

Bioassays with higher plants

Plant species

Two higher plant species were selected from the species list suggested by ISO guideline 11269-2 (ISO 1994b): the oat *Avena sativa* (monocotyledonous) and the turnip *Brassica rapa* (dicotyledonous). Seeds of both species were purchased from commercial sources.

Higher plant growth test

This assay was based on ISO (1994b). Plastic containers (12 cm length, 9 cm width, 6 cm height) were filled with 400 g (dry weight equivalent) of soil from a single treatment. The moisture content of each soil-sludge mixture was adjusted

to 70% WHC. Eight replicates per soil-sludge mixture concentration (4 for *B. rapa* and 4 for *A. sativa*) were prepared. After 24 h, ten uniform undressed seeds of the selected plant species were sown in each pot. The test ran in a plant growth chamber under a photoperiod of 16 h light (8000 ± 2000 lx) and 8 h dark at 23 ± 3ºC. After the emergence of at least 50% of the seedlings in the control soil, the number of plants was determined and in some cases thinned to obtain a total of five evenly spaced representative specimens per test container. Moisture content of each replicate was checked every two days by weighing test vessels and restoring water loss (plant weight was assumed to be negligible compared with soil mass). The test vessels were randomly distributed and re-arranged every two days to prevent effects of unequal lighting, temperature, humidity or ventilation on plant growth. At the end of the 42-day test period the test plants were harvested at the soil surface and the individual dry weight was determined (the fresh samples were placed in an oven at 68ºC during 48 h).

Statistical analyses

Soil properties

The differences between measured pH and WHC values between each treatment and the control (0 t/ha) were evaluated using an one-way ANOVA followed by Dunnett's post-hoc test (Table 2.3).

Avoidance tests with earthworms and collembola

Avoidance assays assume no avoidance behaviour when equal or higher number of organisms is found in the treatment side than in the control side. When a higher number of organisms was found in the control side, the significance of the avoidance behaviour was tested using the Fisher exact test (Zar 1998). This statistical tool is based on a contingency table in which the observed behaviour in a specific treatment is compared with a hypothetical distribution in which no avoidance response is observed (null hypothesis). For the avoidance assays, a

one-tailed test was used. The null hypothesis assumes that 50% of the test organisms stay in the treatment that is being assessed and that no organism leaves that compartment, simulating a situation in which no avoidance behaviour is detected. For analysis of the dual-control test (reference soil in both sides) a two-tailed test was used, assuming an equal distribution of the individuals over both sections of the replicate, which means that the number of organisms that stays in one side is equal to the number that leaves the same compartment. In both cases, the null hypotheses were rejected for a probability equal or lower than 0.05 ($p \leq 0.05$). The number of dead individuals was evenly attributed to both sides of the replicates. For each tested sludge, the no observed effect concentration (NOEC) was determined by the highest soil-sludge concentration which did not provoke any avoidance reaction. The lowest observed effect concentration (LOEC) was the lowest concentration that induced avoidance behaviour by the test organisms. The concentrations inducing 50% (EC_{50}) and 20% (EC_{20}) avoidance behaviour were calculated using probit regression applying the Priprobit software. The model used assumes that in a combination with reference soil on both sides, 50% of the test organisms will be found in each section of the test container (absence of avoidance behaviour). The total number of organisms in each combination used in the Priprobit software did not take into account dead individuals.

Reproduction tests with earthworms and collembola

The data obtained in the reproduction assays was analysed using STATISTICA, version 6.0. The increment in biomass and the reproductive output of earthworms and collembolans was statistically analysed with a one-way ANOVA analysis followed by Dunnett's post-hoc test. Data was Log transformed in order to fulfil the assumptions for the ANOVA. EC_{50} and EC_{20} values were calculated using a logistic model, but only for the data sets characterized by decreasing trends since the EC is defined as a concentration that reduces reproduction rate at a certain percentage. The logistic model used the

following equations to arrive at EC_{50} and EC_{20} values (Environment Canada 2004):

EC_{50}: $Response = b/((1+([Conc]/x)t))$ (2.1)

EC_{20}: $Response = b/((1+(0.2/0.8)\times([Conc]/x)t))$ (2.2)

Where:

b = y-intercept (the control response)

x = EC_x for the data set

$[Conc]$ = exposure concentration

t = scale parameter (estimated between 1 and 4)

Higher plant growth test

The percentage of emergence and plant individual dry weight increment at each treatment after 42 days of exposure were determined. These endpoints for each test concentration of test sludge were compared with those in the control by means of a one-way ANOVA followed by a Dunnett's test. When ANOVA assumptions were not met (after logarithmic transformation of the data) a Kruskall-Wallis test was used followed by the Fisher LSD post-hoc test. The statistical analysis did not include outlier values, which were identified using the Grubbs/Dixon outlier test (Sachs 2002).

Results

Soil and sludge contamination

The metal content of the natural reference soil used in this study was considerably lower than the threshold values above which the addition of sludge would not be allowed according to the European Directive 86/278/CEE (European Community 1986) (Table 2.1). As shown in Table 2.2, sludge A

(urban sludge) contains mainly Zn and Cu, although at concentrations lower than the legal limits. Sludge A had the highest organic matter content. Sludge B (olive industry) had the lowest levels of metals. Test sludge C (electroplating industry) had the lowest organic matter content and high concentration of Cr (22.3% of which is in the most toxic oxidation state - Cr(VI)), which exceeds the limit established by the Directive on sludge by almost a factor of five. This makes the sludge unsuitable for use in agricultural fields as fertilizer. The highest test dose adopted in the present study (45 t/ha), however, corresponds to an increment of total Cr in the soil (144 mg Cr/kg) that is lower than the maximum annual amount allowed (1000 mg Cr/kg) by the same Directive. Taking into account the level of metals present in reference soil (Table 2.1) and in test sludges (Table 2.2), the amount of each metal in each test soil-sludge mixture was calculated (Table 2.3). The same was done for the OM content.

Avoidance tests

Earthworms

No earthworm mortality occurred in the avoidance tests. No significant differences ($p > 0.05$) were detected in the number of animals found in both sections of the dual-control tests. The soil-sludge C mixtures were the only ones that were always significantly avoided by the earthworms ($p \leq 0.05$). The LOEC considered was 6 t/ha, while the EC_{50} was even lower (0.4 t/ha; Table 2.4). Neither sludge A nor sludge B induced any avoidance reaction by the earthworms in the concentrations tested (Figure 2.1).

Table 2.4 No observed effect concentrations (NOEC), lowest observed effect concentrations (LOEC) and 20% (EC_{20}) and 50% (EC_{50}) effect concentrations (with 95% confidence intervals in parentheses) for the toxicity of the test sludges A, B, and C to plants (*Brassica rapa* and *Avena sativa*), earthworms (*Eisenia andrei*) and springtails (*Folsomia candida*).

Test sludges	Test species	Endpoint	Exposure time (days)	NOEC (t/ha)	LOEC (t/ha)	EC_{20} (t/ha)	EC_{50} (t/ha)
A	*A. sativa*	Emergence	5	25	45	-	-
B	*B. rapa*	Individual dry weight	42	<6	6	20.3 (13.3-27.4)	-
	A. sativa	Individual dry weight	42	<6	6	14.7 (4.2-25.2)	36.7 (10.5-62.9)
C	*E. andrei*	Adult growth	28	6	15	4.0 (2.4-5.5)	9.9 (5.9-13.9)
		Reproduction	56	< 6	6	7.7 (2.8-12.7)	11.6 (7.4-15.8)
		Avoidance behaviour	7	<6	6	0.02	0.4
	F. candida	Avoidance behaviour	7	15	25	-	-
	B. rapa	Individual dry weight	42	<6	6	24.2 (16.2-32.2)	-
	A. sativa	Individual dry weight	42	<6	6	16.2 (8.5-23.9)	40.5 (21.4-59.6)

Missing parameters indicate no toxic effects for the specific test organism. See Table 2.2 for sludge codes.

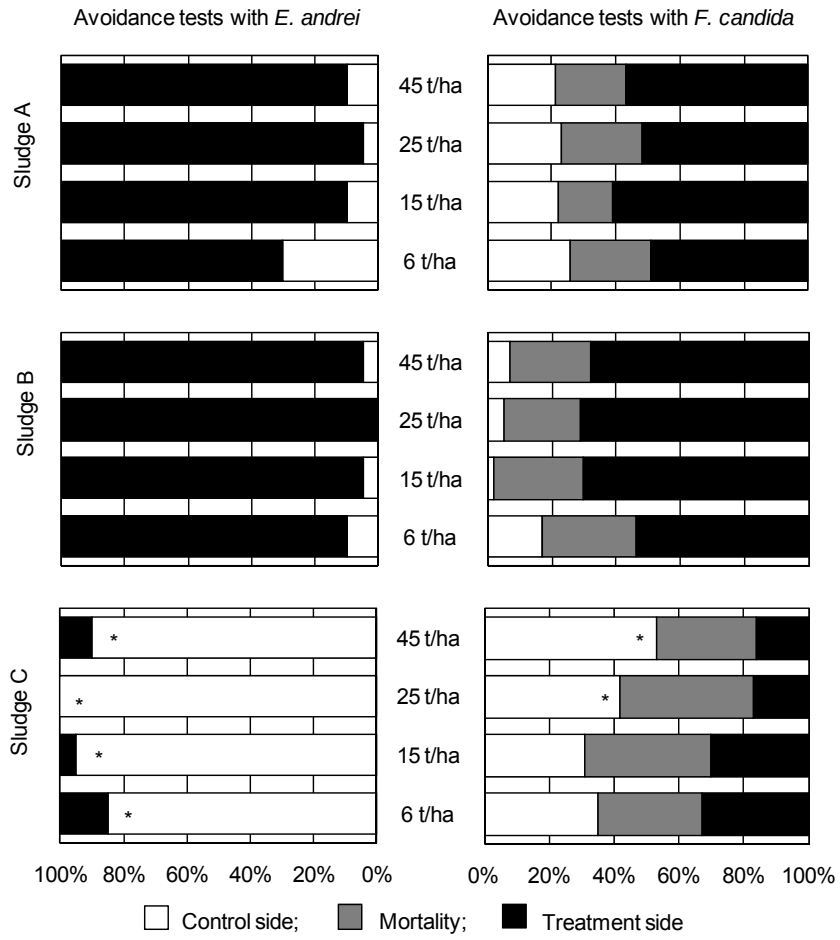

Figure 2.1 Avoidance response of earthworms (*Eisenia andrei* - left graphs) and collembolans (*Folsomia candida* - right graphs) to different sludges mixed at different dosages with a non-polluted control soil. Values are the average percentage of organisms found dead or on control or treatment sides. Each horizontal bar represents a mixture of the sludge concentration with the reference soil (control). * - significantly ($p \leq 0.05$) higher percentage of organisms on the control side than on the treatment side.

Collembola

In the dual-control test no significant differences were observed between both sections of test vessels and on average 9% of the collembola died. The combinations with sludge C were the most lethal inducing a mortality of 37.8%. In the soil-sludge A and B mixtures, average mortality was 22.3% and 26.5%, respectively.

Only the two highest soil-sludge C mixtures (25 and 45 t/ha) induced significant avoidance reaction in the collembola (Figure 2.1). Although the LOEC for sludge C was 15 t/ha, the avoidance behaviour observed did not allow for the calculation of EC_{20} and EC_{50} values (Table 2.4).

Reproduction tests

Earthworms

No earthworm mortality was observed after 28 days of exposure to the tested soil-sludge mixtures. The reproductive output in the reference soil fulfilled the test validity criteria of at least 3 juveniles per adult in controls (Environment Canada 2002). The soil-sludge A mixtures with a concentration of 15 t/ha or higher, induced a significant increase of juvenile production when compared to the control (Figure 2.2). A similar increase was observed in earthworm biomass at concentrations of 25 and 45 t/ha (Figure 2.2) The soil-sludge B mixtures, although to a lower extent, also induced an increment of reproduction (only at 15 t/ha), and significantly stimulated earthworm growth at 15 and 45 t/ha. The soil-sludge C mixtures caused a significant decrease only in juvenile production starting at 6 t/ha (see Figure 2.2). The NOEC for this test sludge therefore was lower than 6 t/ha and the EC_{20} was 7.7 (2.8 - 12.7) t/ha (Table 2.4).

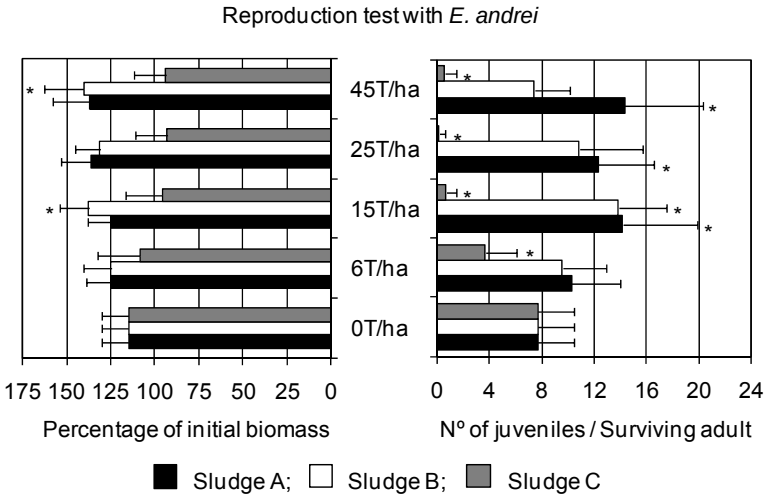

Figure 2.2 Effects of different sludges mixed with a non-polluted control soil on the reproduction of earthworms (*Eisenia andrei*). Percentage of the initial biomass of the adult organisms (average + standard deviation) after 28 days exposure (left graph) and number of juveniles per surviving adult (average + standard deviation) after 56 days exposure (right graph) in the soil-sludge mixtures. * - statistically significant difference ($p \leq 0.05$) comparing with the control (0 t/ha).

Collembola

Collembola mortality in the reproduction test was lower than 20% and the number of juveniles per test container was higher than 100 in the control, fulfilling the test validity criteria (ISO 1999). Survival of the collembolans in the soil-sludge mixtures tested was between 85 and 90% and did not significantly differ between treatments. Only the soil-sludge B mixtures induced a significant increase in the reproduction of *F. candida* at concentrations 15 t/ha and higher. All the other soil-sludge mixtures did not have any influence on the reproductive performance of the collembolans (Figure 2.3).

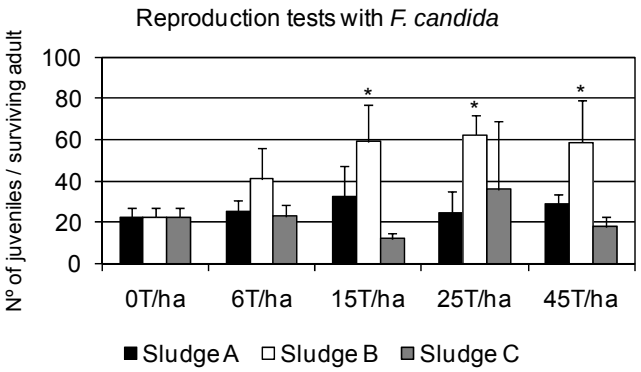

Figure 2.3 Effects of different sludges mixed with a non-polluted control soil on the reproduction of collembolans (*Folsomia candida*). Number of juveniles per surviving adult (average + standard deviation) after 28 days exposure in the soil-sludge mixtures. * - statistically significant difference ($p \leq 0.05$) comparing with the control (0 t/ha).

Higher plant growth test

Turnip

The average emergence in the control pots (0 t/ha concentration) was 65%, fulfilling the test validity criteria, which demand that the emergence should be sufficient to provide five healthy seedlings per control pot (ISO 1994b). The emergence in the soil-sludge mixtures tested was not significantly different from that in the control (Figure 2.4). The soil-sludge A mixtures did not affect the growth of *B. rapa* but both sludges B and C inhibited turnip growth at concentrations of 6 t/ha and higher (see Figure 2.4). LOEC growth for these two sludges was 6 t/ha, and EC_{20} values were 20.3 t/ha for sludge B and 24.2 t/ha for sludge C (see Table 2.4).

Oat

The emergence obtained in the control soil at the beginning of the test was 50%. In soil-sludge A mixtures, at 45 t/ha all seedlings died and a high variability was observed in the emergence detected in the other concentrations. This fact did not allow the calculation of any EC_x values. In soil-sludge B mixtures, a significant decrease in emergence was found at 6 and 45 t/ha. In mixtures with sludge C, the emergence of oat seeds was inhibited at 15 t/ha but not at higher concentrations, where emergence was even significantly higher than that observed in the control at 25 t/ha (see Figure 2.4). Since for the mixtures of sludges B and C no dose-related response was found, no NOEC, LOEC and EC_x values could be determined for emergence.

Concerning plant growth, the soil-sludge A mixtures did not affect *A. sativa*. The soil-sludge B and C mixtures inhibited biomass increment at all concentrations tested. Accordingly, the LOEC for both sludges B and C was 6 t/ha and the EC_{20} values were 14.7 and 16.2 t/ha, respectively (see Figure 2.4 and Table 2.4).

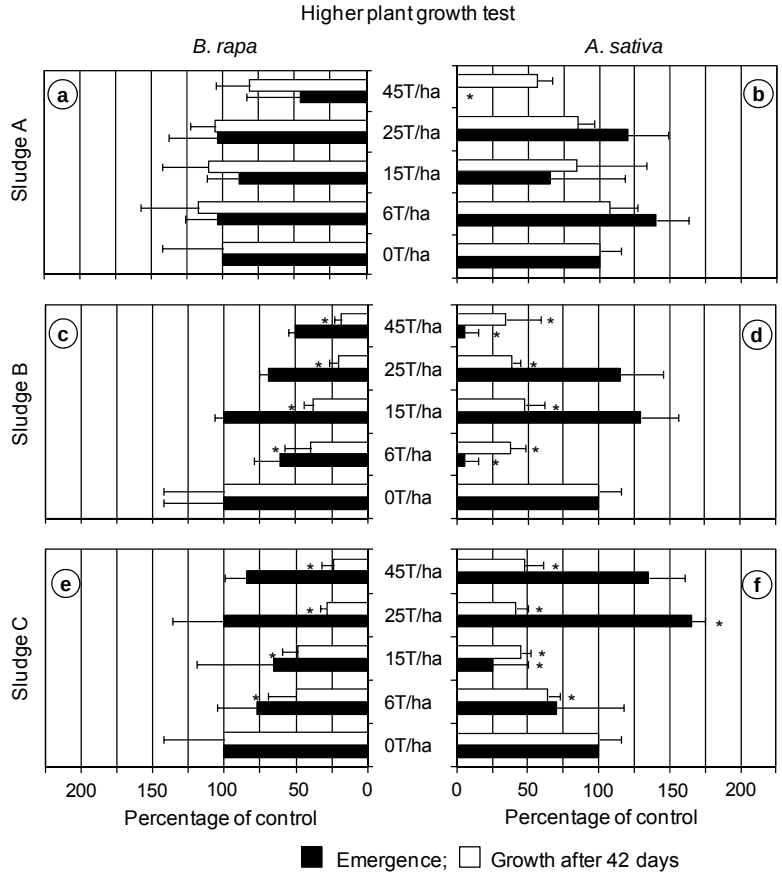

Figure 2.4 Effects of different sludges mixed with a non-polluted control soil on the growth of *Brassica rapa* (left graphs) and *Avena sativa* (right graphs). Values are the percentage of emergence (average + standard deviation) and the percentage of biomass increase (average + standard deviation) after 42 days of exposure, all in relation to the average biomass in the control. First, second, and third line of graphs represent values obtained in soil-sludge A, B, and C concentrations, respectively. * - statistically significant difference ($p \leq 0.05$) comparing with the control (0 t/ha).

Discussion

Avoidance tests

In combinations with soil-sludge A and B mixtures, more organisms were found in the sections with soil-sludge mixture. This attraction, that was more evident with earthworms then with collembolans, is probably related to the higher amount of OM present in the soil-sludge mixtures (correspondent to the sludge fraction of the mixtures) which, in the absence of a sufficient repulsive effect of the contaminants present in the sludges, can favour the movement of the test organisms towards the amended soils. Natal-da-Luz et al. (2008b) observed that, in avoidance tests comparing artificial soils with different organic matter content, both *E. andrei* and *F. candida* are attracted to those soils with higher organic matter content. This response, similarly to the observed in the present study, was more evident in earthworms. These findings indicate that the influence of OM content in the avoidance response of these organisms is relevant and should be taken into account when interpreting the results of avoidance tests. An attraction response for soils (or amended soils with organic matrices) with a higher organic matter content may not exclude the possibility of the occurrence of toxic effects at chronic level (e.g. reproduction). When it occurs at this screening stage, it should be evaluated with chronic tests.

Significant mortality of the collembolans was found in the avoidance tests with all soil-sludge mixtures. This lethal effect could be related to the exposure period of 7 days. According to Natal-da-Luz et al. (2008a) increasing the exposure time from 2 to 14 days increases the mortality of collembolans in avoidance tests. In this study, the lethal effect probably explains for the less pronounced effects of sludge C mixtures on the collembolans. The lower sensitivity of the avoidance behaviour of collembolans, when compared with earthworms, is in agreement with Greenslade and Vaughan (2003). In fact, in sludge C mixtures an avoidance reaction was observed at concentrations as low as 6 t/ha for earthworms and 25

t/ha for springtails. This difference in avoidance behaviour, that was not found for the soil-sludge A and B mixtures, might be due to the Cr content of sludge C. Van Gestel et al. (1992) found an EC_{50} of 155 mg Cr/kg soil DW for the effect on cocoon production by *E. andrei* after 28 days in artificial standard soil spiked with Cr(III). Lock and Janssen (2002) evaluated the effect of Cr(III) on *F. candida* and *E. fetida* reproduction after an exposure of 28 and 21 days, respectively, finding EC_{50} values of 604 and 892 mg Cr/kg soil DW, respectively. Chromium may exist in the environment as Cr(VI) – that is carcinogenic and mobile being able to cross cell membranes – or as Cr(III) – that is less toxic and less mobile since it is not able to penetrate cell membranes. In the present study, 22.3% of the Cr present in test sludge C was chromate (CrO_4^{2-}), which is Cr(VI). This may explain the low EC_{50} for *E. andrei* for avoidance behaviour in sludge C (EC_{50} < 6 t/ha). Therefore, both the results obtained in previous studies with only Cr(III) and the present study with soil-sludge C mixtures are in agreement with the expectations related to the presence of a metal in its different oxidation states. Our results confirm the high toxicity of chromate in the environment.

Reproduction tests

Earthworms

The soil-sludge A mixtures besides having contributed to earthworm growth, they stimulated reproduction, which suggests that the Cu and Zn levels in sludge A were not high enough to negatively affect earthworm reproduction. This assumption is supported by Spurgeon et al. (1994) who determined the effects of Cd, Cu, Pb, and Zn on the growth, reproduction, and survival of *E. fetida* in a standard artificial soil. After 56 days of exposure, no effect on worm growth was observed at any of the metal doses tested, and EC_{50} cocoon production was 53.3 and 276 mg/kg soil DW for Cu and Zn, respectively. Van Gestel et al. (1993) found an EC_{50} for the effect on cocoon production of *E. andrei* of 512 mg Zn/kg

soil DW. These EC_{50} values are significantly higher than the Cu and Zn concentrations in the highest concentration of sludge A (Table 2.3). Apparently contradictory results were obtained by Spurgeon and Hopkin (1996), who, when evaluating the influence of soil OM content on the toxicity of Zn to cocoon production of *E. fetida* in manipulated standard artificial soils, found an EC_{50} value of 136 mg Zn/kg soil DW at 5% OM content and a pH of 6. This is the most similar one to the natural reference soil used in the present study. In agreement with these results, a deleterious effect would be expected at the highest concentration of the soil-sludge mixture A, where the level of Zn was of the same order of magnitude as the reported EC_{50} value. However, the same authors also reported that the availability, and consequently the toxicity of Zn, is usually higher in freshly spiked standard artificial soil than in natural soils. The observed stimulation of earthworm reproduction in soil-sludge A mixtures could be related with the use of sludge as food by these organisms. A similar stimulation in cocoon production in *E. andrei* was observed by Elvira et al. (1999) in a gradient of mixtures of a sludge from a paper-mill factory and cow manure.

The statistically significant increase of the growth rate in the 15 and 45 t/ha concentrations of soil-sludge B mixtures was not totally reflected in the reproductive output of the earthworms, since a significant increase of the reproduction was only observed at 15 t/ha. This growth effect might be related to the fact that this sludge originated from olive-processing industry, which means that significant quantities of oils and fats may be present. Sludges from olive industries already have been shown to induce a biomass increase of *E. andrei* (Benitez et al. 2002).

The absence of toxic effects on the growth of earthworms of soil-sludge mixture C is in agreement with Spurgeon et al. (1994) who, as mentioned above, observed no effect on growth rate of *E. fetida* in a standard artificial soil freshly spiked with 400 mg Zn/kg soil DW. This agrees with Van Gestel et al. (1992) who did not observe any growth reduction of *E. andrei* after 3 weeks exposure in

artificial spiked soil with Cr(III) nitrate at 287 mg Cr/ kg soil DW. The effect on earthworm reproduction may be attributed to the high levels of Cr in an oxidation state which is toxic (Cr(VI)). Considering the EC_{50} values for the effect of Zn on cocoon production determined by Spurgeon et al. (1994) and Van Gestel et al. (1993), the levels of Zn present at the higher concentrations of soil-sludge C mixtures were not high enough to affect the reproductive output of the earthworms. However, considering the EC_{50} for cocoon production determined by Spurgeon and Hopkins (1996), a toxic effect would be expected at the highest concentrations of sludge C. The decrease in earthworm reproduction is in agreement with Van Gestel et al. (1992) who found an EC_{50} of 155 mg Cr/kg soil DW in artificial soil contaminated with Cr(III) nitrate. However, Lock and Janssen (2002) found an EC_{50} for the effect of Cr(III) nitrate on cocoon production of *E. andrei* of 892 mg Cr/kg soil DW, also in artificial soil. Molnár et al. (1989), from a study with *E. fetida* in peat and horse manure, reported an EC_{50} for effects on reproduction of 250 mg Cr/kg soil WW. In these three studies different substrates were used with different organic matter fractions, which in addition to the oxidation state of the Cr salt used had an influence on the availability of Cr. In substrates with high OM content the availability of Cr seems to be lower, since the EC_{50} values tend to be higher. Since the substrate used in the present study had a lower OM content than the substrates used in the three studies mentioned above, and since over 20% of the Cr in sludge C was in the most toxic oxidation state (Cr(VI)), the decrease of the reproduction of *E. andrei* in the soil-sludge C mixtures was expected.

Collembola

The reproductive performance of *F. candida* was considerably different comparing from that of earthworms, emphasizing the need for using both test organisms in waste characterization. From the absence of deleterious effects of soil-sludge A mixtures on the reproduction of collembolans, it may be concluded that the amounts of Cu and Zn were not high enough to affect their normal

behaviour. These data are in agreement with Filser et al. (2000) who found even a preference of two species of the genus *Folsomia* (*F. quadrioculata* and *F. manolachei*) in Cu-contaminated environments. Smit and Van Gestel (1998), reporting EC_{50} values of 261 and 473 mg Zn/kg soil DW for *F. candida* reproduction in Zn spiked natural (2.9% OM) and standard artificial soil respectively, support this conclusion. Lubben (1989) observed a significant increase in the number of juveniles of *F. candida* during 3 months of exposure after the incorporation of a sewage sludge (4 t/ha DW) artificially contaminated with several metals (Zn, Cd, Cu, Ni, Cr, and Pb) in a field with a loamy sand soil. After sludge amendment the soil contained 156 and 38.5 mg/kg soil DW of Zn and Cu, respectively. These levels are in range of those in the highest concentration of sludge A (45 t/ha) of our study. Lubben (1989) also found no effects on the number of juveniles at a three times higher concentration (12 t/ha) of the artificially contaminated sludge. The increased number of juveniles at 4 t/ha was probably related with environmental site factors, which cannot be controlled in a field study. The absence of deleterious effect in the soil-sludge A mixtures is also in agreement with Crouau et al. (2002) who performed a chronic test with *F. candida* in standard artificial soil on a dilution gradient of sewage sludge from a municipal waste water treatment plant. These authors reported a NOEC of 12.5% and an EC_{50} above 50%. Considering that the sludge was incorporated in the soil at a depth of 10 cm, 12.5% and 50% correspond to 187.5 t/ha and 750 t/ha respectively. The NOEC found is four times higher than the highest concentration tested in the present experiment.

Since sludge B had the lowest level of metals, no deleterious effects on the reproduction of the test organisms were expected. The increased reproduction may be related with the high amount of organic compounds present in the sludge mixtures (from the wastes produced in food industry), and the possible use of the sludge as food source (Domene et al. 2007b). These facts may have provided favourable conditions for *F. candida* reproduction.

The absence of deleterious effects in soil-sludge C mixtures contradicts the toxicity seen for the earthworms. The amount of Cr and Zn probably was not high enough to induce toxic effects on the reproduction of *F. candida*. This fact is supported by Lock and Janssen (2001) who evaluated the effects of Zn on the reproduction of *F. candida* in three different spiked substrates (standard artificial, sandy field and loamy field soils) after 28 days of exposure. Toxicity was lowest in the loamy field soil with 1.5% OM (most similar with the soil used in this study) with an EC_{50} of 522 mg Zn/kg soil DW. According to Lock and Janssen (2002), the EC_{50} after 28 days of exposure in standard artificial soil spiked with Cr(III) nitrate is 604 mg Cr/kg soil DW. Therefore, levels of both Zn and Cr, above which the reproductive performance of springtails is affected by more than 50%, are significantly higher (about five times) than the levels present in the higher concentration of sludge C (45 t/ha) tested.

Higher plant growth test

The absence of toxic effects on the emergence of *B. rapa* for all the applied test sludges is in agreement with Wong et al. (2001) who found no significant adverse effect on seed germination of *B. chinensis* when exposed for 144 h to extracts of anaerobically digested sewage sludges from four wastewater treatment plants in Hong Kong. According to these authors, seed germination was less sensitive than root growth. The atypical pattern observed in the emergence of *A. sativa* (especially in soil-sludge B mixtures) seems to be indicative of the low sensitivity of this parameter in evaluating toxicity, when compared to other test parameters. This is in agreement with Gong et al. (2001) who performed seed germination and early seedling growth bioassays with four plant species (*A. sativa*, *Lepidium sativum*, *B. rapa*, and *Phaseolus vulgaris*) in 15 natural soils. After 14 days of exposure, seed germination was less sensitive, which may be related with the high dependence of this process on the energy reserves in cotyledons. This makes this endpoint less sensitive to stressors

present in the surroundings of the seed. Only the soil-sludge A mixtures did not cause any effect on the growth of both test plants. Apparently, the levels of Cu and Zn in sludge A (elevated above background) were not high enough to induce a decrease in the normal growth of the plant species tested. This assumption is in agreement with Fjällborg and Dave (2004), who found no dose-dependent effect on the growth of any test plant used (*Raphanus sativusi*, *Lactuca sativa*, and *A. sativa*) after 14 days of exposure in a Cu-spiked sludge concentration gradient. On the other hand, the results obtained in the present study with the soil-sludge A mixtures differ from the plant growth study performed by Ramiréz et al. (2008), who evaluated the phytotoxicity to *B. rapa*, *Lolium perenne*, and *Trifolium pratense* of a pig slurry treated by a thermal-drying process and with comparable metal loadings as sludge A. The authors reported an EC_{50} value for *B. rapa* growth (using shoot length as endpoint) of 20 g/kg soil DW, which considering a depth of 10 cm and a soil specific mass of 1.5 g/cm^3, corresponds to a dose of 30 t/ha. However, the toxicity induced by the pig slurry used by Ramiréz et al. (2008) could be attributed to the low stability of OM which led to a quick release of ammonium and other decomposition products with known phytotoxic effects. This assumption probably also holds for the soil-sludge B mixtures, which, in spite of the presence of low levels of metals, caused a higher reduction of plant growth.

The decreased plant growth observed in the soil-sludge C mixtures might be due to the high levels of Cr and Zn in this sludge. According to the EC_{20} values for *B. rapa* and *A. sativa* (24.2 and 16.2 t/ha, respectively), oats were slightly more sensitive to sludge C mixtures than turnips, which apparently contradicts the results of Gong et al. (2001) who concluded that turnips are usually more sensitive than oat.

Conclusions

The toxicity of sewage sludge is highly dependent on its source. Sludge A, from an urban waste water treatment plant, revealed no toxicity for any of the test species used. Contrarily, sludge C, from an electroplating industry, was most toxic, causing deleterious effects on both invertebrates and plants. Sludge B, from an olive industry, was not toxic for earthworms and collembolans, but did affect plant growth. Avoidance response, despite being influenced by the OM content (confounding factor), revealed to be a potential endpoint to be included in a low tier screening phase for the risk assessment of these wastes. The observation of a significant avoidance response towards the treatment soil may be a sign of stress that should be further investigated with chronic tests (as occurred in sludge C). On the other hand, the absence of an avoidance response also led to an absence of impairment in reproduction (sludges A and B). However, when an attraction influenced by certain confounding factors like the organic matter content is observed, this should be interpreted as an uncertainty and should be further investigated with more tests. The higher plant growth tests proved to be an essential complement for the evaluation of sludge toxicity. Overall, the results showed that an ecotoxicological assessment is an important tool for sludge characterization (as an environmental safety control), which may also provide important information to control the use of these biosolids in agriculture, e.g., providing the appropriate levels of sludge amendments onto soil that may optimize crop production without harming the soil invertebrate community. All the tests conducted proved to be valid tools for sludge ecotoxicological characterization that, complemented by other assays, can integrate a tiered assessment strategy comprising a screening phase with short tests (e.g. avoidance tests) and a more detailed evaluation including mostly chronic tests (e.g., reproduction tests, plant tests).

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Chapter 3

The use of Collembola avoidance tests to characterize sewage sludges as soil amendments

Based on the following manuscript:

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Abstract

The ecotoxicological characterization of sewage sludge takes into account the additive, antagonistic, and synergistic effects that occur as a result of multi-chemical interactions. Such an evaluation therefore is essential to complement the chemical analysis that, although required by law, is clearly insufficient. Using a tiered approach in the toxic evaluation of sewage sludge allows for characterization of toxicity in a timely manner. According to the literature, reproduction tests with *Folsomia candida* are suitable tools for the toxic assessment of organic sludges. Therefore, the inclusion of Collembola avoidance tests at a screening level (low tier), and acting as a trigger for longer-period tests (high tier; e.g. reproduction test), may provide a successful strategy, and may complement the currently proposed test battery. To evaluate the use of both avoidance and reproduction tests with collembolans in such a tiered approach, three sewage sludges (urban, olive and electroplating industries) were mixed in with a field-collected soil at different concentrations. Avoidance and reproduction tests were performed with the soil-sludge mixtures after 0, 4, and 12 weeks of incubation. The tests detected no toxicity in soil-sludge mixtures of urban and olive sludges at any incubation period. Mixtures with sludge from the electroplating industry induced toxicity only in the avoidance tests with freshly prepared and 4-week incubated samples. These results demonstrate the ability of Collembola avoidance tests to assess sewage sludge toxicity over time and its potential for hazardous sludge characterization at low tier levels.

Key words: *Folsomia candida*, tiered approach, hazardous sludge characterization, avoidance behaviour over time

Introduction

The growth of the World's industry has contributed to the increase of waste production. The European Community generates approximately 2 billion tons of waste per year (Düring and Gäth 2002). A larger part of these wastes consists of industrial sludges that can constitute valuable soil amendments due to their high organic matter and nutrient contents (Phillips et al. 1997). Therefore, the land application of sewage sludge may be an acceptable form of waste disposal. However, the usual presence of contaminants in sludge (e.g. metals) can provoke adverse effects on the environment (Düring and Gäth 2002).

The ability to evaluate the potential eco(toxico)logical risk associated with sludge application is an important tool to prevent the contamination of soil ecosystems. The use of ecotoxicological tests to evaluate the toxicity of waste materials has gained strength with the European Council Directive 91/689/EEC (European Community 1991), which includes the "Ecotoxic" property as one of the 14 criteria that should be considered when characterizing wastes as hazardous to the environment. The information obtained from chemical analysis is often insufficient because several relevant and persistent pollutants are not included and the interaction between chemicals is not considered. The contaminants included in a sewage sludge can cause additive, antagonistic and synergistic effects resulting from multi-contaminant interactions. These phenomena are tightly dependent on the bioavailable fraction and have to be taken into account to obtain a valuable hazardous waste evaluation. Bio-assays have the potential to evaluate the toxicity of a waste as a whole and therefore work as a complement to chemical analysis (Wilke et al. 2008).

There is still no consensus in the scientific community about the battery of ecotoxicological tests that should be adopted for hazardous waste characterization. Recently, studies have been conducted to evaluate the usefulness and suitability of standardized test batteries aimed at waste characterization (Pandard et al. 2006; Alvarenga et al. 2007; Rosa et al. 2007;

Wilke et al. 2008; Natal-da-Luz et al. 2009 – Chapter 2; Moser and Römbke 2009). The use of soil invertebrates as test organisms in ecotoxicological testing has been shown to be suitable for the evaluation of the toxicity of certain contaminants present in sewage sludges, such as linear alkylbenzene sulphonates (Krogh et al. 2007).

Earthworms comprise the bulk of soil invertebrates mostly used in sludge toxicity evaluations (Pandard et al. 2006; Krogh et al. 2007; Alvarenga et al. 2007). However, the inclusion of other invertebrate test species, with different sensitivities and alternate route(s) of exposure, for this type of evaluation would improve the relevance of test results and provide a useful addition to a standardized test battery. Considering the impact on microbial communities following the application of sewage sludge to agricultural soil, it is predictable that microbivorous organisms like collembolans, which are dependent on fungi and bacteria populations, are more sensitive and, consequently more suitable, as a bio-indicator of bio-solid toxicity in soil (Cole et al. 2001). Moreover, earthworms are more influenced by organic matter (OM) content in the soil than Collembola (Natal-da-Luz et al. 2008a). This leads us to hypothesize that the sensitivity of earthworms can be lower than that of collembolans, due to the presence of a higher OM content in sludge-treated soils that can mask the effect of contaminants.

Crouau et al. (2002) and Domene et al. (2007) showed that the collembolan species *Folsomia candida* has potential to be used in the ecotoxicological assessment of organic wastes. *F. candida* reproduction is a more sensitive endpoint than mortality (Krogh and Petersen 1995) but it takes more time to assess. Therefore, in order to reduce the time needed for hazardous waste characterizations, it is critical to use a tiered assessment strategy comprising a screening (lower tier) and a more detailed phase (higher tier; Moser and Römbke 2009). In a tiered approach, reproduction tests are considered as higher tier levels but are only triggered when impairment is detected with lower tier tests. This procedure prevents the use of certain long-term tests (e.g. with sludges that did

not reveal toxicity at a screening level) without compromising the quality of the assessment.

Avoidance tests using soil invertebrates are rapid tests that can be used as lower tier tools in such an assessment scheme. In fact, earthworm avoidance tests are even included in the screening level of a proposed extended limit test design for ecotoxicological waste characterization (Moser and Römbke 2009). Avoidance tests with *F. candida* are emerging and have been optimized using reference chemicals (Natal-da-Luz et al. 2008a, 2008b) and can be used instead of or complementary to earthworms. An ISO draft for avoidance tests with *F. candida* is currently being prepared (ISO 2007b) in addition to the ISO draft for avoidance tests with earthworms (ISO 2007a). These bio-assays, besides being fast and of low cost, already showed the potential to work as an early screening tool in risk assessment (Natal-da-Luz et al. 2004) and, more recently, in sludge characterization (Moreira et al. 2008; Natal-da-Luz et al. 2009 – Chapter 2). Avoidance tests with collembolans were also used to evaluate the microbial degradation of polycyclic aromatic hydrocarbons (PAHs) over time by Lors et al. (2006). However, the usefulness of this test type to evaluate sludge toxicity over time after its incorporation in soil has not been studied.

To fill this knowledge gap, avoidance and reproduction tests with *F. candida* were performed using concentration gradients in soil of three sewage sludges from three distinct sources. The effect of ageing was also investigated over a 12-week incubation period. The objectives of the present study were: i) to assess the use of *F. candida* avoidance behaviour and reproduction as ecotoxicological endpoints; and ii) to evaluate the potential of using *F. candida* avoidance tests to assess the toxicity of sludge over time.

Materials and methods

Control soil

A loamy sand field soil from Central Portugal, collected in the sub-urban limits of the city of Coimbra, was used as a control soil. The soil was free of pesticide and fertilizer application for more than 5 years. The soil was sieved (5 mm) and defaunated through two freeze-thawing cycles (48 h at -20ºC followed by 48 h at 25ºC). The soil microbial community was re-established by inoculating the bulk soil with an elutriate obtained from a fresh soil sample (1:10 fresh soil:distilled water (w:w) mixed for 30 minutes). The parameters measured were soil pH (1M KCl 1:6 v:v), water holding capacity (WHC; ISO 1999), cation exchange capacity (CEC; ISO 1994), organic matter content (OM; loss on ignition at 500ºC for 6h), soil texture (LNEC 1970) and total metal concentration (see below).

Test sludges

Three sewage sludges were obtained from distinct sources, containing different levels of metals, OM and pH (Table 3.1). Sludge A was obtained from a municipal wastewater treatment plant in Coimbra, Portugal; Sludge B from a biological and secondary treatment of wastewater from an olive processing industry (Mira, Portugal); and Sludge C from a biological and secondary wastewater treatment of sewage from an electroplating industry (Ceira, Portugal).

Table 3.1 Total metal concentrations, pH and organic matter content of the test sludges (average ± standard deviation; n = 3) and the upper limit values of metals allowed for a sludge to be incorporated in agricultural soil according to Directive 86/278/CEE of the European Community (1986). *ND* - not determined; *QL* - not detected or present at a concentration below limit of quantification; A – Urban sludge; B – Sludge from olive processing industry; C – Sludge from electroplating industry.

Sludge		**A**	**B**	**C**	**Limit values**
pH (1M KCl)		6.57 ± 0.13	7.68 ± 0.08	8.57 ± 0.04	-
Organic matter (%)		74.9 ± 2.1	64.6 ± 4.1	4.4 ± 1.1	-
Metals in	Cadmium	3.2	< 0.5	< *QL*	20
bulk sludge	Chromium	121	74	4790[a]	1000
(mg/kg DW)	Copper	436	66	42	1000
	Lead	145	19	3.5	750
	Mercury	*ND*	0.3	0.07	16
	Nickel	39	33	58	300
	Zinc	1731	350	900	2500

[a] 3720 mg Cr(III)/kg and 1070 mg Cr(VI)/kg.

Treatments

The control soil was mixed with the test sludges in different proportions to obtain a concentration gradient for each test sludge equivalent to dosages of 0, 6, 15, 25 and 45 t dry weight (DW)/ha (which represent 0, 4, 10, 16.7, and 30 g DW/kg, respectively). These dosages are in agreement with the typical applications used in fertilization assays, taking into account the allowed legal limits (European Community 1986). Sludge mixtures were prepared assuming a density of 1.5 g/cm^3 for the control soil and that the test sludges would be

incorporated to a depth of 10 cm. Once prepared, pH (1M KCl 1:6 v:v), WHC (ISO 1999), and OM content (loss on ignition at 500ºC for 6 h) of each mixture were determined. Each soil-sludge mixture was moistened to 50% of its respective WHC and incubated in a plastic box (36 cm long, 22 cm wide, and 11 cm high) at a temperature of 20 ± 2ºC and a photoperiod of 16:8 h light:dark. Samples were collected from the incubation containers after 0, 4, and 12 weeks of incubation to perform laboratory ecotoxicological tests with *F. candida*. The results obtained with freshly amended soil (collected at t = 0) have been published in Natal-da-Luz et al. (2009 – Chapter 2).

Metal analysis

The metal concentrations for each sludge (Table 3.1) were obtained directly from analytical reports provided by the companies that supplied the test materials. All these analyses were performed by certified laboratories before the preparation of the soil-sludge mixtures. Metal extraction was performed using *aqua regia* ($HCl:HNO_3$, 3:1, v:v) following the German Institute for Standardization (DIN 1986). In sludges A and B and on the control soil, total cadmium (Cd), lead (Pb) and zinc (Zn) concentrations were measured by inductively coupled plasma (ICP) – mass spectrometry as described by USEPA (2005) and total chromium (Cr), copper (Cu), and nickel (Ni) concentrations by ICP - atomic emission spectrometry following USEPA (2001). In sludge C, total Cr, Cu, Ni, and Zn concentrations were measured by ICP - atomic emission spectrometry following USEPA (2001) and Cd and Pb by atomic absorption spectrometry using a graphite furnace according to USEPA (1994). All these analyses were considered valid with quality control recoveries between 95% and 115%. BCR No 145 (Trace Elements in sewage sludges), certified by Community Bureau of Reference (Commission of the European Communities) was used as reference material. Total mercury content was measured only in sludges B and C using atomic absorption spectroscopy using cold vapour atomization (USEPA 1986).

In this case, recovery of the control standard was always between 94% and 97%. In sludge C, the oxidized state of Cr (Cr(VI)) was measured by the diphenylcarbazide method following Branco et al. (2005), with the reduced state (Cr(III)) content determined by the difference between total Cr and oxidized state (Cr(IV)) contents.

Test species

The collembolan *F. candida* (Collembola: Isotomidae) was the soil invertebrate used in the ecotoxicological assays. This species is currently used in reproduction tests (Crouau et al. 2002; Domene et al. 2007), being standardized in an ISO guideline (ISO 1999), and more recently in avoidance tests (Natal-da-Luz et al. 2004, 2008a, 2008b, 2009 – Chapter 2; Moreira et al. 2008). The test organisms were obtained from laboratory cultures reared under a photoperiod of 16:8 h light:dark at 20 ± 2ºC. A mixture of plaster of Paris and activated charcoal in a ratio of 8:1 (w:w) was used as culture substrate in cylindrical transparent plastic boxes (11 cm diameter and 4 cm height). Granulated dry yeast was added as food in small amounts to avoid spoilage by fungi. When detected, mouldy food was removed from the culture containers. Collembolans of 10 to 12 days old, obtained from synchronized cultures, were used in both reproduction and avoidance assays. Tests were conducted at the same temperature and photoperiod conditions used to maintain the cultures. Moisture content of each soil-sludge mixture was adjusted to 50% of the WHC.

Ecotoxicological tests

Avoidance and reproduction tests were performed with soil-sludge mixtures collected after 0, 4, and 12 weeks of incubation.

The procedure adopted in the avoidance tests was based on ISO (2007b) with some modifications. Cylindrical plastic boxes (diameter 7 cm and height 6 cm)

were divided into two equal sections by means of a card divider introduced vertically. Thirty grams (dry weight equivalent) of a particular soil-sludge mixture was placed in one of the sections, while the other one was filled with control soil. Five replicates were tested for each soil-sludge combination. After removing the plastic divider, 20 individuals (10-12 days old and without visible signs of damage) were added to each test vessel. An additional container without individuals was prepared for each soil-sludge combination, for pH and moisture determination at the end of test. To limit water losses by evaporation and to prevent collembolans from escaping, the containers were closed with transparent lids. At the end of the 7-day test period, the two sections of each test container were divided again and emptied into different vessels. These vessels were filled with water and a few drops of blue ink were added. After gently stirring, the individuals floating on the water surface were counted. Missing individuals were considered dead. The pH of the substrate in both sections was determined at the beginning and at the end of the test, for each combination tested. An additional combination was included containing control soil on both sides of the test container to test for absence of avoidance reaction (dual-control test) (Hund-Rinke and Wiechering 2001; Natal-da-Luz et al. 2004). The avoidance test is considered valid if mortality in the dual-control test is lower than 20%.

Reproduction tests were performed as described by ISO (1999). Five replicate glass containers (4 cm diameter and 7 cm height) were filled with 30 g (dry weight equivalent) of a soil-sludge mixture. Ten springtails were placed into each test container, and 2 mg of granulated dry yeast were added as a food source. To limit water loss by evaporation and to protect springtails from escaping, the containers were closed. At day 14 of exposure, the test containers were opened for a few seconds to allow aeration and moisture content was checked by weighing. When weight loss was higher than 2%, water loss was replenished. At the end of the 28-day test period, each test container was emptied into a small vessel that was filled with water. After the addition of a few drops of blue ink and gently stirring, the animals floating on the water surface were

photographed and counted. Missing adult springtails were considered dead. An additional replicate per test concentration, but without organisms, was prepared and subjected to the same conditions to allow for pH and moisture measurements at the end of the test.

Statistical analysis

In the avoidance tests, it was assumed that no avoidance behaviour was detected when the number of organisms in the treatment section was equal to or higher than in the control section. When a higher number of organisms was found in the control section, the significance of the avoidance behaviour was tested using the Fisher exact test (Zar 1998). This statistical tool is based on a contingency table in which the observed behaviour in a specific treatment is compared with a hypothetical distribution in which no avoidance response is observed (null hypothesis). For the avoidance tests, a one-tailed test was used. The null hypothesis assumes that 50% of the test organisms stay in the treatment and that no organism leaves that section, simulating a situation in which no avoidance behaviour occurred. For analysis of the dual-control test (control soil in both sections), a two-tailed test was used, assuming an equal distribution of the individuals over both sections of the replicate, which means that the number of organisms staying in one section is equal to the number that leaves the same compartment. In both cases, the null hypotheses were rejected for a probability equal or lower than 0.05 ($p \leq 0.05$). The number of dead individuals was evenly attributed to both sides of the replicates. For each tested sludge, the no observed effect concentration ($NOEC_{av}$) was determined by the highest soil-sludge concentration that did not provoked any avoidance reaction. The lowest observed effect concentration ($LOEC_{av}$) was the lowest concentration significantly inducing avoidance behaviour of the test organisms.

The reproduction rates of collembolans in the sludge treatments and the control soil were compared by one-way ANOVA analysis followed by Dunnett's post-

hoc test. The reproductive outputs between sampling dates in each specific soil-sludge mixture were compared using the percentage of reproduction of each soil-sludge mixture in relation to that of the control from the same sampling date. The statistical difference between these percentages was analyzed by a one-way ANOVA followed by Duncan's post-hoc multi-stage test. When the assumptions for one-way ANOVA were not fulfilled, a Kruskall-Wallis test was used followed by multiple comparisons of mean ranks for all groups. The statistical analysis did not include outlier values, which were identified using the Grubbs/Dixon outlier test (Sachs 2002). All of the analyses were performed using STATISTICA, version 6.

Results

Soil and sludge contamination

The metal content of the control soil was significantly lower than the threshold values above which the addition of sludge would not be allowed according to the European Directive 86/278/CEE (European Community 1986; Table 3.2). As shown in Table 3.1, sludge A (urban sludge) was mainly contaminated with Zn and Cu, although at concentrations lower than the legal limits. Sludge A had the highest OM content, and sludge B (olive industry) had the lowest metal levels. Test sludge C (electroplating industry) had the lowest OM content and a high concentration of Cr (22.3% of which was in the most toxic oxidation state - Cr(VI)), exceeding the limit established by the Directive on sludge by almost a factor of five. This makes the sludge unsuitable for use in agricultural fields as amendment agent. The highest test dose adopted in the present study (45 t/ha), however, corresponds to an increment of total Cr level in the soil (144 mg Cr/kg) that is lower than the maximum annual amount allowed (1000 mg Cr/kg) by the same Directive.

The control and sludge-treated soils had high pH-KCl values (> 7.5), which were significantly reduced by the highest sludge B concentration and significantly increased by the highest two sludge C dosages. OM content increased with increasing sludge dosages, except for 6 t/ha concentrations of soil-sludge A and C mixtures (Table 3.3).

Table 3.2 Physical and chemical properties of the reference soil (average ± standard deviation; n = 3) and the upper limit values of metals allowed for an agricultural soil with a pH higher than 7 to allow sludge incorporation according to Directive 86/278/CEE of the European Community (1986). *ND* - not determined.

		Reference soil	**Limit values (pH > 7)**
pH (1M KCl)		7.86 ± 0.08	-
Water-holding capacity (%)		46.3 ± 2.6	-
Cation exchange capacity (meq/kg)		90.4	-
Organic matter (%)		2.9 ± 0.2	-
Metals in bulk soil	Cadmium	< 2.8	4
(mg/kg DW)	Chromium	11	300
	Copper	12	200
	Lead	61	450
	Mercury	*ND*	2.0
	Nickel	< 14	110
	Zinc	96	450
Soil texture (%)	Sand	88.8	-
	Silt	7.00	-
	Clay	4.2	-
Soil type		Loamy Sand	-

Table 3.3 Characterization of the soil-sludge mixtures used in the avoidance and reproduction tests with *Folsomia candida* (average ± standard deviation; $n = 3$). See Table 3.1 for sludge codes.

Sludge	Conc (t/ha)	pH (1M KCl)	Water-holding capacity (%)	Organic matter (%)
	0 t/ha	7.8 ± 0.08	46.2 ± 2.60	2.9 ± 0.21
A	6 t/ha	7.8 ± 0.01	48.2 ± 0.91	3.1 ± 0.14
	15 t/ha	7.8 ± 0.01	50.9 ± 0.63*	3.4 ± 0.04*
	25 t/ha	7.8 ± 0.03	50.5 ± 0.91*	3.6 ± 0.10*
	45 t/ha	7.7 ± 0.03	57.7 ± 0.87*	4.9 ± 0.18*
B	6 t/ha	7.8 ± 0.03	49.8 ± 2.14	3.3 ± 0.09*
	15 t/ha	7.8 ± 0.02	50.1 ± 0.95	3.6 ± 0.05*
	25 t/ha	7.8 ± 0.02	54.2 ± 2.37*	3.4 ± 0.03*
	45 t/ha	7.5 ± 0.02*	54.1 ± 1.17*	4.1 ± 0.01*
C	6 t/ha	7.7 ± 0.03	16.5 ± 2.85*	3.1 ± 0.24
	15 t/ha	7.8 ± 0.08	19.8 ± 3.23*	3.3 ± 0.15*
	25 t/ha	8.0 ± 0.04*	20.4 ± 2.27*	3.5 ± 0.16*
	45 t/ha	8.1 ± 0.06*	22.7 ± 2.31*	3.9 ± 0.26*

* - significantly different ($p \leq 0.05$) from the control (0 t/ha).

Avoidance tests

In the dual-control tests no significant avoidance was observed and average mortality was 9, 8, and 16% in samples tested after 0, 4, and 12 weeks of incubation, respectively. In the other combinations (treatment *vs* control soil), mortality was always highest in soil-sludge C mixtures with values of 37.8, 27.8, and 30.5% for 0, 4, and 12 weeks of incubation, respectively. At these sampling times, mortality in combinations with soil-sludge A mixtures was 22.3, 18.3, and 21.0% and with soil-sludge B mixtures 26.5, 17.3, and 27.8%, respectively. Sludge B did not induce avoidance behaviour at any treatment level on any sampling date (Figure 3.1).

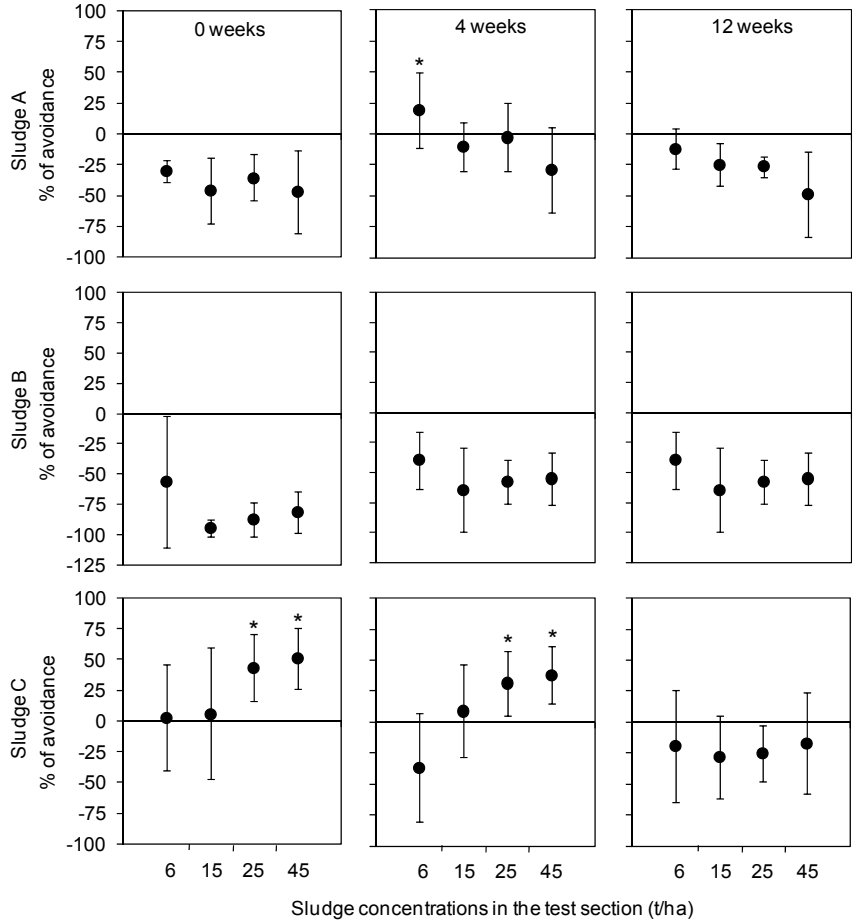

Figure 3.1 Avoidance response of collembolans (*Folsomia candida*) to different sludges mixed at different dosages with a non-polluted control soil and incubated for different periods. Values are the average percentage of avoidance ([No control – No test]/[No control + No tests] x 100) ± standard deviation; $n = 5$. Data for the freshly treated soils ($t = 0$ weeks) are taken from Natal-da-Luz et al. (2009 – Chapter 2). * - significantly ($p \leq 0.05$) higher percentage of organisms in the control section than in the test section; Sludge A: urban; Sludge B: olive processing industry; Sludge C: electroplating industry.

For soil-sludge A mixtures, only the 4-week samples triggered an avoidance behaviour at the 6 t/ha treatment. Since no avoidance reaction was detected in any other combination, no toxicity values ($NOEC_{av}$ and $LOEC_{av}$) could be determined. The mixtures with sludge C were most toxic, with the freshly and 4-week incubated samples of the highest concentrations (25 and 45 t/ha) inducing statistically significant avoidance behaviour of *F. candida*. In the samples collected after 12 weeks incubation, no avoidance behaviour was observed in any combination tested. Therefore, $NOEC_{av}$ and $LOEC_{av}$ values of 15 and 25 t/ha, respectively were determined only for the freshly treated and 4-week incubated samples. The avoidance behaviour towards soil-sludge C mixtures was not strong enough to allow calculation of EC_x values.

Reproduction tests

In the control soil, survival was, on average 88, 90, and 85% in the 0, 4, and 12 weeks incubated samples, respectively and the number of juveniles per test container was always higher than 100, fulfilling the validity criteria (ISO 1999). Survival of collembolans in the test mixtures was not proportional to the sludge concentration applied, nor was it related to the test sludge type. However, in the most aged samples, survival in most treatments was higher, being on average 85.4, 80.2, and 88.3% in the samples incubated for 0, 4, and 12 weeks, respectively.

The reproduction rate of *F. candida* was not affected by soil-sludge A and B mixtures at any sampling date. The soil-sludge B mixtures significantly stimulated reproduction at concentrations above 6 t/ha in fresh spiked samples, at 25 t/ha in 4-week incubated samples and at 15 and 45 t/ha in samples incubated for 12 weeks. With respect to the evolution of reproduction over time at each treatment (evolution of relative values in comparison to the respective control), in the soil-sludge A mixtures, no significant differences in reproduction were found, except for the 6 t/ha treatment where reproduction significantly increased

in the 12-week incubated samples relative to the values obtained from the fresh spiked samples. In soil-sludge B mixtures, a tendency for a higher reproduction in aged samples was observed. Exceptions were the 6 t/ha treatment that presented relative reproduction values not statistically different between freshly and 12 weeks incubated samples and the 25 t/ha treatment where the reproduction did not change significantly between 4 and 12 weeks. The soil-sludge C mixtures were toxic at 15 t/ha in both freshly and 4-week incubated samples. This response profile, however, did not allow for determination of NOEC or LOEC values. The reproduction rates over time did not change significantly, except for the 6 t/ha treatment where a significant increase of reproduction was observed after 12 weeks (Figure 3.2).

Discussion

The survival rate obtained in both reproduction and avoidance tests was not related to the test sludge type. In avoidance tests with sludge C mixtures, mortality was higher than in the other combinations but the response was not dose-related. Mortality in the dual-control tests was always lower than 20%, proving the healthy condition of the test organisms used. According to Natal-da-Luz et al. (2008b) the mortality of collembolans in avoidance tests increases when increasing the exposure time from 2 to 14 days. In that study, the mortality observed in 7-day dual-control and avoidance tests was 24.2 and 21.0%, respectively. In the present study mortality was always significantly lower in the dual-control tests. In the avoidance tests with soil-sludge A mixtures, mortality was similar as reported by Natal-da-Luz et al. (2008b), but was higher in combinations with soil-sludge B and C mixtures. Since mortality was not dose-related, this effect was considered not to be caused by pollutants present in the sludges.

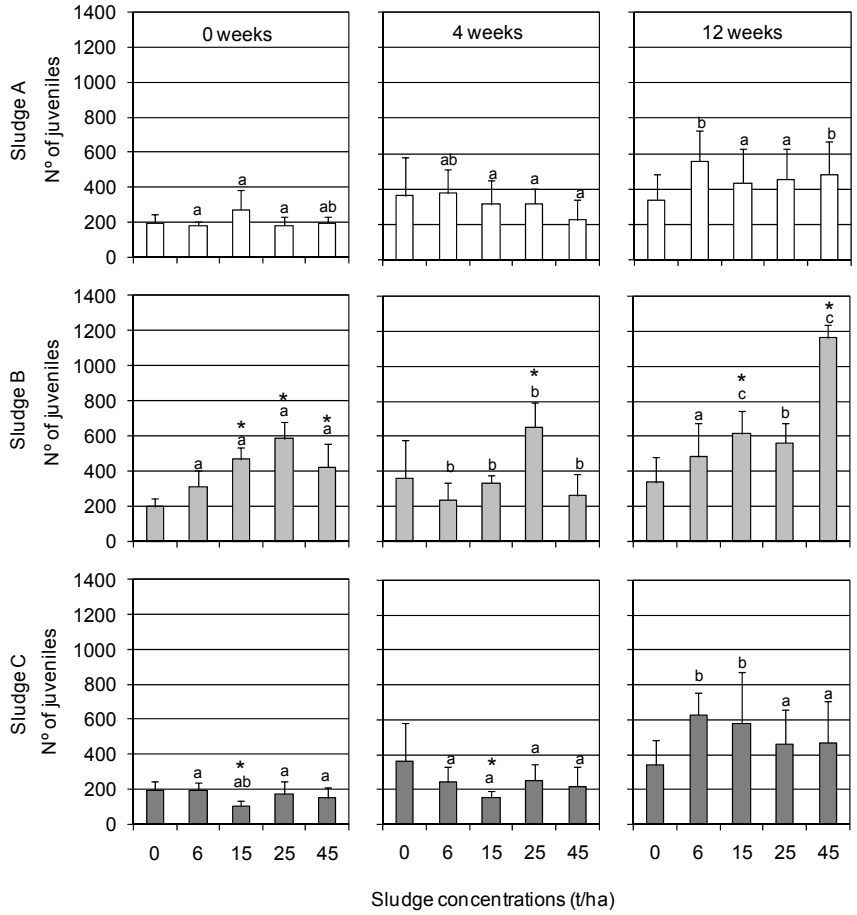

Figure 3.2 Reproduction of collembolans (*Folsomia candida*) in different sludges mixed with a non-polluted control soil and incubated for different periods. Number of juveniles (average + standard deviation; n = 5) produced after 28 days exposure in the soil-sludge mixtures. Data for the freshly treated soils (t = 0 weeks) are taken from Natal-da-Luz et al. (2009 – Chapter 2). * - significantly different ($p \leq 0.05$) compared with the control (0 t/ha) of the same sampling date; bars for the three incubation periods (0, 4, and 12 weeks) of the same soil-sludge mixture concentration with the same letter are not significantly different from each other (see details on the text on how this comparison was done); Sludge A: urban; Sludge B: olive processing industry; Sludge C: electroplating industry.

No toxicity was found in any of the soil-sludge A and B mixtures, in both ecotoxicological tests. In avoidance tests with these treatments, a higher number of organisms were found in the test section, especially for sludge B mixtures (Figure 3.1, negative values). As discussed by Natal-da-Luz et al. (2008a), this behaviour could be induced by the higher OM content of these test mixtures. When this happens it may mask the potential toxicity of the sludge. Therefore, when a significant difference in OM content exists between control soil and test mixtures, and an attraction of the organisms to the treatment section is observed, reproduction tests (less prone to be influenced by OM content in the medium) should be performed in order to evaluate the potential toxicity of the sludge. In this case, the reproduction assays confirmed the results of avoidance tests showing absence of toxicity in soil-sludge A and B mixtures. Although reproduction may also be influenced by soil OM content, the present study showed that this endpoint is less influenced by OM content than avoidance behaviour. Collembolan reproduction was significantly increased in some mixtures of sludge B, however, it was not affected by soil-sludge A mixtures. The absence of toxicity in sludge A mixtures was predictable considering its low level of metals. This is supported by the results of Sandifer and Hopkin (1996) who performed reproduction tests with *F. candida* in OECD artificial soil to evaluate the effect of pH on the toxicity of several metals (including Cu and Zn). The EC_{50} values at pH 6, which is much lower than the pHs of the mixtures tested in the present study, for Cu and Zn (nitrate) were 700 and 900 mg /kg soil DW, respectively. These values are much higher than the values calculated for the highest dose of sludge A (approx. 24.3 and 144 mg Cu and Zn/kg soil DW, respectively). Moreover, Lock and Janssen (2003), using *F. candida* to evaluate the toxicity of zinc chloride, zinc oxide and zinc powder in OECD artificial soil found EC_{50} values of 391, 461, and 393 mg Zn/kg soil DW, respectively. These values are significantly higher than the Zn content of the mixtures with sludge A (ca. 4 times higher). Furthermore, the high pH of our soil/soil-sludge mixtures has resulted in lower availability of the metals (Sandifer and Hopkin 1996).

In the soil-sludge A and B mixtures, no significant changes in the avoidance response over time were observed. The avoidance response detected for the 6 t/ha soil-sludge A mixtures incubated for 4 weeks was not considered relevant, since it was not dose nor time-related. The relative reproduction in soil-sludge A mixtures did not differ significantly over time (except for the 6 t/ha sludge A treatment). On the other hand, reproduction rates in sludge B treatments varied with the incubation period. Between mixtures freshly prepared and incubated for 4 weeks, significant differences in reproduction rates were detected for all concentrations. Those differences were always related with a decrease in the reproduction output. However, there was an overall increase in reproduction at week 12. The relative reproduction of *F. candida* in the 15 and 45 t/ha concentrations even increased significantly, compared to that obtained in freshly and 4 weeks samples in the same concentrations (Figure 3.2). This significant increase can be related to a decreased bioavailability of pollutants (e.g. metals) after the 12-week incubation period. This phenomenon was most pronounced in the 45 t/ha sludge B treatments, probably because sludge B was mainly contaminated with organic pollutants originating from the olive industry that are susceptible to biodegradation (Lors et al. 2006).

Despite the absence of negative effects at the reproductive level, an avoidance response was observed on the 25 and 45 t/ha treatments after 0 and 4 weeks incubation in the soil-sludge C mixtures. Although these mixtures also had significantly higher OM content compared to the control (as in sludge A and B treatments), the toxicity of the sludge was high enough to counteract the effects of the OM content. According to the literature, the observed toxicity cannot be attributed to the levels of Cr(III) (Cr is clearly the most predominant metal present in sludge C). In a study of Lock and Janssen (2002) using *F. candida*, the EC_{50} for the effects of Cr(III) on reproduction in OECD artificial soil was about four times higher (604 mg Cr/kg soil DW) than the Cr content of the most concentrated soil-sludge C mixture (approx. 150 mg Cr/kg soil DW). Therefore it is more likely that effects were due to Cr(VI). According to Francisco et al.

(2002) Cr(VI) is a strong oxidant, more soluble, more bioavailable, and able to penetrate membranes. According to the metal analysis performed before preparation of the soil-sludge mixtures, 22.3% of the Cr in sludge C was in the most toxic oxidation state (Cr(VI)). Sivakumar and Subbhuraam (2005), measuring survival, behaviour and morphological endpoints in the earthworm *E. fetida*, showed that the toxicity of Cr(VI) was seven times higher than that of Cr(III) in soil. This confirms the contribution of Cr(VI) in the effects of the soil-sludge C mixtures on the avoidance response in fresh and 4-week incubated samples. Since no toxicity was detected in reproduction tests with these samples, avoidance behaviour seems to be more sensitive than reproduction, at least in scenarios of Cr(VI) contamination. Using samples collected after 12 weeks incubation, a significant increase of reproduction was found only in 6 t/ha treatment and no avoidance behaviour was detected. According to the available literature, the stability of Cr(III) state is higher than that of Cr(VI) (Costa el al. 1984). Furthermore, it is known that Cr(VI) in soil can be reduced to Cr(III) both by non-humic organic substances (Elovitz and Fish 1994) and by soil humic substances (Wittbrodt and Palmer 1996). In the light of these studies, it is probable that a significant amount of Cr(VI) had been reduced to Cr(III) after 12 weeks of incubation, making the sludge less avoidable as evidenced by the absence of avoidance behaviour. A similar explanation was suggested by Molnár et al. (1989) who evaluated the effects of Cr(III) and Cr(VI) on the growth of the earthworm *E. fetida* over eight weeks. They observed a higher growth reduction of earthworms by Cr(VI) than by Cr(III), however, that toxicity decreased significantly over time, a fact which the authors also related with Cr(VI) disappearance.

Conclusion

The present study confirms that the avoidance behaviour is a useful indicator for samples that might warrant further investigations. When an avoidance response is detected (as observed for fresh and 4-week incubated soil-sludge C mixtures) further tests should be conducted (e.g. performing reproduction tests). On the other hand, the absence of an avoidance reaction indicates that no impairment exists and no further testing is needed. However, when an attraction of the test organisms by the treated side is observed (as seen for some soil-sludge A and B mixtures), it is recommended to perform further tests to ensure that no confounding factors (e.g. OM content) are masking the results of avoidance tests. Avoidance tests showed to be suitable to assess sludge toxicity over time. The results obtained in reproduction tests (especially in sludge B treatments) and in avoidance tests (especially in sludge C treatments) suggest that the bioavailability of contaminants in the test sludges (e.g. metals) can decrease over time. However, further investigations are needed to better understand these phenomena.

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Chapter 4

Toxicity to *Eisenia andrei* and *Folsomia candida* of a metal mixture applied to soil directly or via an organic matrix

Based on the following manuscript:

Natal-da-Luz T, Ojeda G, Pratas J, Van Gestel CAM, Sousa JP, 2011. Toxicity to *Eisenia andrei* and *Folsomia candida* of a metal mixture applied to soil directly or via an organic matrix. *Ecotoxicology and Environmental Safety* (submitted).

Abstract

Regulatory limits for chemicals and ecological risk assessment are usually based on the effects of single compounds, not taking into account mixture effects. The ecotoxicity of metal-contaminated sludge may, however, not only be due to its metal content. Both the sludge matrix and the presence of other toxicants may mitigate or promote metal toxicity. To test this assumption, the toxicity of soils amended with an industrial sludge predominantly contaminated with chromium, copper, nickel, and zinc and soils spiked with the same mixture of metals was evaluated through earthworm (*Eisenia andrei*) and collembolan (*Folsomia candida*) reproduction tests. The sludge was less toxic than the metal mixture for *E. andrei* but more toxic for *F. candida*. Results obtained for the earthworms suggest a decrease in metal bioavailability promoted by the high organic matter content of the sludge. The higher toxicity of the sludge for *F. candida* was probably due to the additive toxic effect of other pollutants.

Key words: Toxic units, joint effect, metal-contaminated sludge, mixture toxicity

Introduction

The land application of industrial sludge has contributed to the enrichment of metals in soils (Düring and Gäth 2002), and the hazard of metals to soil organisms has been widely demonstrated (e.g., Baath 1989; Bruus Pedersen and Van Gestel 2001; Nahmani and Lavelle 2002). However, organisms in a contaminated soil are generally exposed to many pollutants simultaneously. Nevertheless, most regulatory limits for chemicals in soils are still based on single-substance concentrations (e.g. European Community 1986) and ecological risk assessments and soil quality standards are commonly focused on exposure and effects of single compounds (Yang 1994; Van Gestel et al. 2011).

The additive, antagonistic and synergistic effects resulting from multi-contaminant interactions determine the potential ecological risk of a contaminated area. Joint effects to soil organisms may be similar to or stronger or weaker than expected from the effects of single chemical exposure, depending on factors such as the nature of the chemicals in the mixture, variability of exposure routes, and ranges of sensitivities of the receptor organisms (De Zwart and Posthuma 2005). Because of that, the use of toxicity tests in which each chemical is tested separately is inadequate for assessing the potential risk of mixtures of chemicals for soil ecosystems.

The evaluation of the toxicity of biosolids such as industrial metal-contaminated sludges, when applied to soil, should therefore not be restricted to their metal contents. Beyond the presence of other organic and inorganic toxicants in industrial sludges, there are several interactions between metals and the sludge matrix, such as sorption (Posthuma et al. 1997), which influence the availability and consequently the toxicity of metals to soil organisms. The comparison of the amendment with a sludge contaminated predominantly with a mixture of metals and the spiking of a soil directly with the same mixture of metals may complement the ecotoxicological evaluation of a metal-contaminated sludge. This comparison provides information on the effect of sludge contaminants other

than metals and/or metal-matrix interactions in the toxicity of the sludge, which may be useful for the definition of adequate strategies to reduce sludge toxicity before its use on soil (e.g. composting).

Following this assumption, standard toxicity tests were performed with the collembolan *Folsomia candida* and the earthworm *Eisenia andrei* in soils contaminated with a gradient of metals originating from an industrial sludge (sludge-amended soils) predominantly contaminated with chromium (Cr), copper (Cu), nickel (Ni), and zinc (Zn) or from spiking the soil with the same mixture of metals (metal-spiked soils). The objective of the present study was to compare the toxicity of a mixture of metals in soil when applied directly (soil spiking) or via an organic matrix (sludge amendment). It was assumed that the metals act according to the concentration addition model, and toxicity data for the single metals available in the literature were used. Our working hypothesis is that toxicity of the sludge is lower than that of the mixture of metals applied directly to the soil due to sorption to the organic sludge matrix.

Materials and methods

Test soil

A sandy loam soil (Table 4.1) was collected from the 20 cm top layer of an agricultural field in the sub-urban limit of the city of Vila do Conde in the village of Vairão, North of Portugal. The soil was free of pesticide and fertilizer applications for more than 5 years. The soil was sieved (5 mm) and defaunated through two freeze–thawing cycles (48 h at -20ºC followed by 48 h at 25ºC). Soil pH (1M KCl, 1:6, v:v), water holding capacity (WHC; ISO 1999), cation exchange capacity (CEC; Chapman 1965), organic matter content (OM; loss on ignition at 500ºC for 6 h) and soil texture (LNEC 1970) were measured. Total metal concentrations were determined by digestion with *aqua regia* and analysis by flame atomic absorption spectrometry (AAS; AAnalyst 300, Perkin-Elmer).

To check quality of this analysis, VKIAG CMSA, batch VKI-19-2-0595 (Reference Material QC Municipal Sludge A), certified by VKI Water Quality Denmark Institute, was used as reference material and metal concentrations were always between 90 and 110% of certified reference values.

Test sludge

A sludge (Table 4.1) derived from the wastewater treatment plant of Cu, Cr, Ni, and Zn-plating industries in Águeda, Portugal, was used. The sludge originated from chemically induced precipitation of metals of industrial effluents followed by decantation. After air-drying, the precipitate was sieved (5 mm) and analysed for pH, WHC and, OM content, as described above. Total Cr, Cu, Ni, and Zn, and total Cd and Pb concentrations were measured after *aqua regia* digestion using inductively coupled plasma (ICP) – atomic emission spectrometry (OES; Axial 730 ES, Varian Scientific Instruments) following USEPA (2001) and USEPA (1994), respectively. The quality of procedures was checked using BCR No. 145 (Trace Elements in sewage sludges), certified by Community Bureau of Reference (Commission of the European Communities), as reference material. All the analyses were considered valid with quality control recoveries between 95 and 115%. Total mercury content was measured using AAS (AMA-254, ALTEC) with cold vapour atomization (USEPA 1986). In this case, recovery of the control standard was between 94% and 97%. The oxidized state of chromium (Cr(VI)) was extracted using diphenylcarbazide following Branco et al. (2005) and measured by ICP - OES (Axial 730 ES, Varian Scientific Instruments), with the reduced state (Cr(III)) concentration determined as the difference between total Cr and oxidized state (Cr(VI)) concentrations.

Table 4.1 Physical and chemical characteristics of the reference soil and the test sludge used in this study. *ND* - not determined.

Physical and chemical parameters		**Reference soil**	**Test sludge**
pH (1M KCl, $n = 4$; ± SD)		4.4 ± 0.1	8.1 ± 0.1
Water-holding capacity (%, $n = 4$; ± SD)		77.0 ± 1.4	147 ± 0.7
Cation exchange capacity (meq/kg, $n = 1$)		90.0	*ND*
Organic matter (%, $n = 2$; ± SD)		9.3 ± 0.2	28.0 ± 7.6
Total Metals	Cadmium	< 1.8	< 0.5
(mg/kg DW, $n = 1$)	Chromium	< 4.8	5200[a]
	Cobalt	< 16	*ND*
	Copper	22	1000
	Iron	14248	*ND*
	Lead	31	290
	Manganese	145	*ND*
	Mercury	*ND*	0.18
	Nickel	< 10	7100
	Zinc	46	4100
Texture (%, $n = 1$)	Clay	10.3	*ND*
	Silt	35.6	*ND*
	Sand	54.1	*ND*
Soil type		Sandy Loam	-

[a] Only 0.5 mg/kg of the total Cr was in the oxidation state Cr(VI)

Test organisms and culture conditions

Eisenia andrei (Oligochaeta: Lumbricidae) and *Folsomia candida* (Collembola: Isotomidae) originated from laboratory cultures maintained at a constant temperature of 20 ± 2ºC with a photoperiod of 16:8 h light:dark were used. The earthworms were kept in plastic culture containers (36 cm length, 22 cm width, and 11 cm height) using a 1:1 (w/w) mixture of horse dung and *Sphagnum* sp. peat as substrate at a moisture content of 40-60% of the WHC. Cooked oatmeal was given as food twice a month.

Springtails were cultured in plastic containers lined with an 8:1 mixture of plaster of Paris and activated charcoal. Cultures were kept moist and a small amount of granulated dry yeast was added as a food source once a week to avoid spoilage by fungi. Mouldy food was removed when detected.

Toxicity assays

Chronic toxicity tests with *E. andrei* and *F. candida* followed ISO (1996) and ISO (1999), respectively. Test organisms were exposed to a concentration gradient of test sludge mixed with soil at 0, 14.1, 28.2, 56.4, 112.8, and 225.6 g /kg dry soil (DW; E0, E1, E2, E3, E4, and E5, respectively) for earthworms, and 0, 28.2, 56.4, 112.8, 225.6, and 451.2 g /kg DW (F0, F1, F2, F3, F4, and F5, respectively) for springtails. These gradients were prepared separately for each test species. The concentrations were based on chronic metal toxicity data reported in the literature. Metal-spiked soils were prepared simulating the amount of the four metals predominating in the test sludge (Cr, Cu, Ni, and Zn) at dosages of 0, 7.05, 14.1, 28.2, 56.4, and 112.8 g /kg DW (M0, M1, M2, M3, M4, and M5, respectively), using chromium nitrate ($Cr(NO_3)_3 \cdot 9H_2O$, purity 98%; Panreac, Barcelona, Spain), copper chloride ($CuCl_2 \cdot 2H_2O$, purity 99%; Panreac, Barcelona, Spain), nickel chloride ($NiCl_2 \cdot 6H_2O$, purity 98%; Panreac, Barcelona, Spain) and zinc chloride ($ZnCl_2$, purity 97%; Panreac, Barcelona,

Spain). From a stock solution in water, different spiking solutions were prepared to give similar final spiking volumes and mixed in with the test soil to obtain the desired metal concentrations.

Metal-spiked soils were left for a period of 3 weeks to stabilize prior to the start of the tests (introduction of the test organisms). In sludge-amended soils, organisms were added shortly after soil preparation. Before the start of the tests moisture content of all soils was adjusted to 50% of the corresponding WHC.

Tests were carried out at 20 ± 2ºC and under a photoperiod of 16 h light and 8 h dark. Soil pH and moisture were measured at the beginning and at the end of the tests. In the tests with *E. andrei*, four replicates per test concentration were prepared, each consisting of a cylindrical plastic box (11 cm diameter and 12 cm height) containing approximately 500 g (dry weight equivalent) of soil. Ten previously washed earthworms, with a fully developed clitellum, more than one month old and with an average individual weight of 458 ± 84.8 mg (average ± SD, n = 110), were placed in each replicate. To prevent worms from escaping, the test vessels were covered with transparent lids with some holes in it to facilitate aeration. Ten grams fresh weight of granulated horse dung, previously defaunated (48 h at -20ºC followed by 48 h at 68ºC) and moistened, were added per test container as food at the start and after 14 and 28 days of exposure. At day 28, the surviving adult worms were removed, counted and weighted to determine changes in body mass. At day 56, the number of juveniles hatched was determined in each replicate using a water bath at 50 to 60ºC for juveniles recovers from soil.

The reproduction tests with *F. candida* were conducted with five replicates per test concentration, consisting of a glass flask (4 cm diameter and 7 cm height) with 30 g moist soil. In each test container, ten synchronized 10-12 day old springtails were placed with 2 mg of granulated dry yeast for food. The test containers were covered with a lid, and opened weekly for a few seconds to allow aeration. After 14 days of exposure, moisture content was checked by weighing the test containers. When weight loss was higher than 2%, water loss

was restored. After 28 days, each test container was emptied into a small vessel, which subsequently was filled with water. After the addition of a few drops of blue ink and gentle stirring, the animals floating on the water surface were photographed and the number of juveniles and surviving adults was determined. Missing adult springtails were considered dead. An additional replicate per test concentration, but without springtails, was prepared and submitted to the same conditions for pH and moisture measurements at the end of the test.

Metal analysis

To assess total metal concentrations, three soil samples from each treatment were digested using PDS-6 pressure digestion systems (Loftfields analytical solutions, Neu Eichenberg, Germany). Samples of ≤ 100 mg soil, oven-dried (at 105ºC for 12 h) and gently crushed with an acid-washed porcelain pestle and mortar, were mixed with 2 ml HNO_3 69% (PA-ACS-ISO, Panreac, Barcelona, Spain) and left under pressure in the PDS-6 systems at 150ºC during 10 h. The resulting solution was diluted with ultrapure water and transferred to a plastic vial and adjusted to a final volume of 10 ml. The quality of this analysis was checked using SRM 2709 (San Joaquin Soil - Standard Reference Material) certified by the National Institute of Standards and Technology (Department of Commerce, USA) as reference material and replicate blanks were prepared. The average recoveries were 82.9, 95.7, 98.5, and 105.2% for Cr, Cu, Ni, and Zn, respectively.

Concentration addition approach

To enable a comparison of the toxicity of the sludge-amended and metal-spiked soils, the toxic potency of the metal mixture was estimated as the sum of the Toxic Units (ΣTU), assuming that the metals in the mixture had a similar mode of action:

$$\Sigma TU = \Sigma (c_i/EC_{Xi}) \quad (4.1)$$

where c_i is the concentration of metal i in the mixture (i = 1, 2, … , N), and EC_{Xi} is the concentration of metal i producing a certain effect X when applied alone. The quotient c_i/EC_{Xi} is the toxic unit (TU) of metal i. EC_{50} values taken from the literature were used as EC_{Xi} values (Table 4.2). For that purpose, only toxicity data from tests in artificial soil, having properties most similar to the soil type used in this study, were used. The total concentrations of the metals in the mixture measured in the test soils were used as c_i values.

Statistical analysis

The pH, moisture content, earthworm weight change and earthworm and springtail reproduction in metal-spiked and sludge-amended soils were compared to that in the respective controls by one-way ANOVA analysis followed by Dunnett's post hoc test, to detect statistical differences. EC_{50} values for the effect on reproduction were calculated using a logistic, hormetic or exponential model and LC_{50} values were calculated using probit analysis. EC_{50} and LC_{50} values for the effect of the sludge or metal mixture were expressed on the basis of Toxic Units (ΣTU). Statistical differences between EC_{50} values of metal-spiked and sludge-amended soils for the same test species were determined using a generalized likelihood ratio test. The normality and homogeneity of data (using Barlett test) was always checked before statistics. One-way ANOVAs, EC_{50}

determinations and comparisons were performed using STATISTICA, version 6. The determination of LC_{50} values was performed using Priprobit 1.63 software (Sakuma 1998).

Table 4.2 Literature data on the reproduction toxicity for *Eisenia fetida* (cocoon production) and *Folsomia candida* (number of juveniles) of chromium, copper, nickel, and zinc in standard artificial soil. EC_{50} values (in mg/kg dry weight) are based on actual concentrations except for Cu in *E. fetida*, which are based on nominal concentrations.

	Metal	**Exposure time**	**EC_{50}**	**Reference**
E. fetida	Chromium	21d	892	Lock and Janssen 2002a
	Copper	56d	53.3	Spurgeon et al. 1994
	Nickel	21d	362	Lock and Janssen 2002b
	Zinc	21d	705	Lock and Janssen 2003
F. candida	Chromium	28d	604	Lock and Janssen 2002a
	Copper	28d	658	Løkke and Van Gestel 1998
	Nickel	28d	476	Lock and Janssen 2002b
	Zinc	28d	391	Lock and Janssen 2003

Results

Soils and metal concentrations

The moisture content of treatments was not significantly different from that of control both at the beginning and at the end of the chronic tests (data not shown). Table 4.3 shows the pH and total Cr, Cu, Ni, and Zn concentrations of the test soils. Soil pH significantly increased at the highest sludge concentrations. The same proportion of the four metals measured was reached over the range of sludge amendments (Ni > Zn > Cr > Cu). In metal-spiked soils this proportion

was not obtained at the lowest concentrations, but this did not compromise the toxicity evaluation of the metal mixture. Table 4.4 shows the toxic potency (ΣTUs) of metal mixture in each test treatment for *E. andrei* and *F. candida*, based on EC_{50} values taken from the literature (Table 4.2).

Table 4.3 Total Cr, Cu, Ni, and Zn concentrations (average ± SD, $n = 3$) and pH (mean of initial and final values ± SD, $n = 2$) of the soils treated with sludge for the toxicity tests with *Eisenia andrei* (E0 – E5) or *Folsomia candida* (F0 – F5), and of soils spiked with a mixture of metals to which both species were exposed (M0 – M5). * - pH significantly different compared to the control ($p \leq 0.05$).

Treatment	pH (1M KCl)	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
E0/F0/M0	4.8 ± 0.5	6.56 ± 3.33	20.4 ± 5.40	13.2 ± 6.18	38.1 ± 19.2
E1	4.6 ± 0.1	42.4 ± 9.81	28.6 ± 6.67	94.0 ± 23.2	88.8 ± 24.4
E2	5.1 ± 0.3	59.1 ± 11.1	32.5 ± 2.58	150 ± 20.2	125 ± 8.56
E3	5.6 ± 0.1	126 ± 44.9	59.9 ± 18.5	327 ± 117	256 ± 88.5
E4	5.8 ± 0.7*	241 ± 129	73.9 ± 33.1	481 ± 221	338 ± 144
E5	6.7 ± 0.1*	631 ± 156	152 ± 34.0	1302 ± 55.3	688 ± 242
F1	4.8 ± 0.2	54.2 ± 26.9	35.1 ± 13.7	141 ± 70.2	116 ± 58.1
F2	5.3 ± 0.4	139 ± 1.20	61.4 ± 3.45	330 ± 19.1	259 ± 7.38
F3	5.9 ± 0.6*	364 ± 108	107 ± 26.2	653 ± 162	468 ± 74.1
F4	6.6 ± 6.8*	1111 ± 108	242 ± 22.5	1818 ± 114	1009 ± 269
F5	7.0 ± 0.5*	1645 ± 83.0	353 ± 14.1	2555 ± 99.6	1729 ± 98.9
M1	4.5 ± 0.0	21.8 ± 0.890	25.3 ± 2.24	49.2 ± 6.08	61.2 ± 8.20
M2	4.5 ± 0.0	34.1 ± 12.8	25.2 ± 7.40	84.3 ± 33.6	82.8 ± 32.3
M3	4.4 ± 0.0	77.4 ± 9.11	42.4 ± 4.28	191 ± 15.2	190 ± 8.84
M4	4.4 ± 0.1	168 ± 20.9	76.6 ± 11.3	471 ± 51.8	382 ± 46.5
M5	4.2 ± 0.0	296 ± 65.7	93.8 ± 8.97	705 ± 42.1	482 ± 20.0

Table 4.4 Sum of Toxic Units (ΣTUs) indicating the toxic potency of sludge-amended and metal-spiked test soils. TUs are based on measured metal concentrations (see Table 4.3) and on literature data on reproduction toxicity for *Eisenia fetida* and *Folsomia candida* for chromium, copper, nickel, and zinc (see Table 4.2). E0 – E5: Earthworm reproduction tests on sludge-amended soils; F0 – F5: Collembola reproduction tests on sludge-amended soils; M1 – M5: Earthworm and Collembola reproduction tests on metal-spiked soils.

Treatment	***Eisenia andrei*** **(Σ*TU*)**	***Folsomia candida*** **(Σ*TU*)**
E0/F0/M0	0.481	0.167
E1	0.970	-
E2	1.27	-
E3	2.53	-
E4	3.46	-
E5	8.13	-
F1	-	0.736
F2	-	1.68
F3	-	3.33
F4	-	8.61
F5	-	13.0
M1	0.722	0.334
M2	0.861	0.484
M3	1.68	1.08
M4	3.47	2.36
M5	4.72	3.35

Earthworm and springtail survival and earthworm mass change

In control vessels, no earthworm mortality was observed and springtail survival was 92.5 ± 9.5% (average ± SD, n = 5; Figures 4.1 and 4.2 – right axis). The LC_{50} was lower for earthworms and higher for springtails in sludge-amended soils than in metal-spiked soils. However, that difference was not statistically significant for both test organisms (considering the overlap of 95% confidence intervals). In both cases, the highest LC_{50} values were obtained for earthworms (Table 4.5).

The growth of surviving earthworms was not significantly different to that of control animals (Figure 4.1 – second row).

Earthworm and springtail reproduction

The validity criteria for reproduction in control vessels were fulfilled in all chronic tests of both test organisms, with on average ≥ 30 juveniles per test container for the earthworms and ≥ 100 for the Collembola (Figures 4.1 and 4.2). The EC_{50} values show that sludge-amended soils were significantly less toxic (likelihood ratio test: X^2_{1df} = 43.4, $p \leq 0.05$) for earthworms than metal-spiked soils (Table 4.5). The NOEC values for sludge-amended and spiked soils were 1.27 and 0.722 TUs, respectively (corresponding to treatments E2 and M1, respectively; Figure 4.1 – first row and left axis). For the springtails, sludge-amended soils were significantly (X^2_{1df} = 113, $p \leq 0.05$) more toxic than spiked soils (Table 4.5). NOEC values were < 0.736 and 1.08 TUs for sludge-amended and metal-spiked soils, respectively (corresponding to < F1 and M3, respectively; Figure 4.2 - left axis).

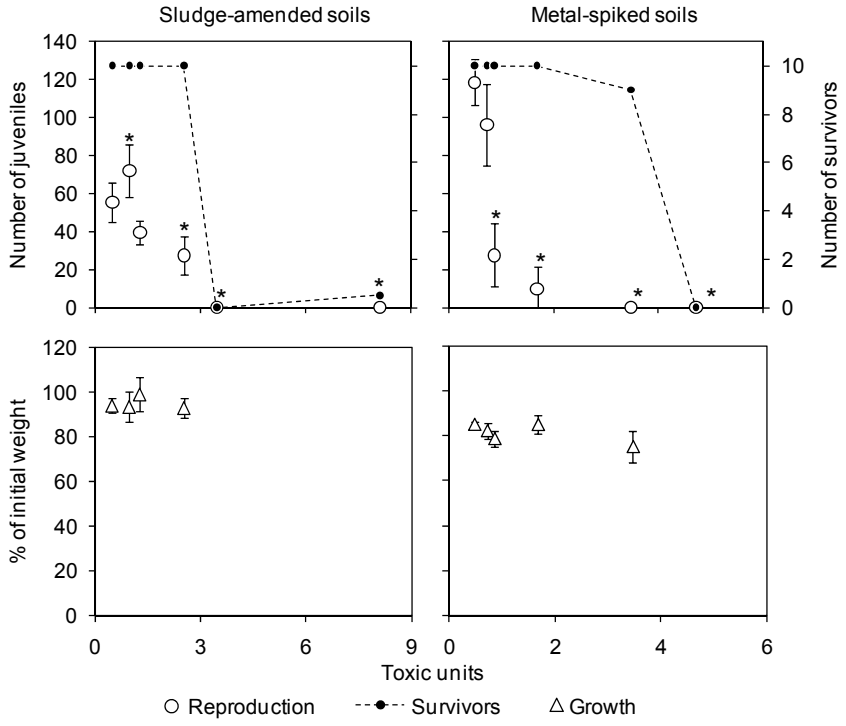

Figure 4.1 Survival, reproduction (first row; right and left axis, respectively; average ± SD, n = 4) and mass change (second row; average ± SD, n = 4) of *Eisenia andrei* when exposed to sludge-amended (left) and metal-spiked soils (right). Metal concentration of treatments is expressed as ΣTUs. * - Number of juveniles significantly different from control ($p \leq 0.05$).

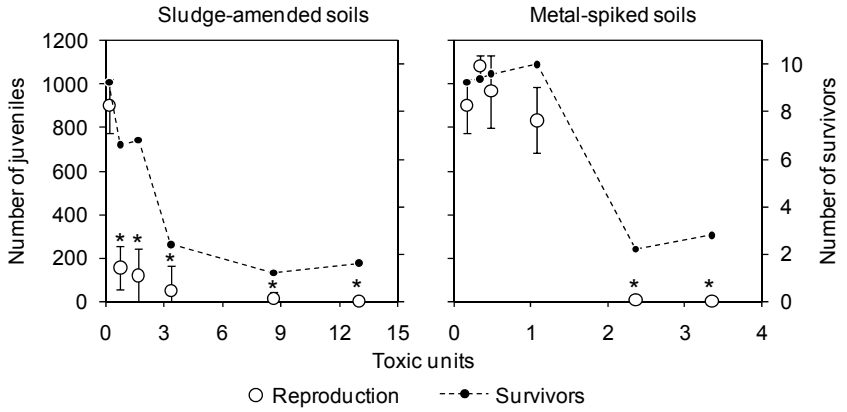

Figure 4.2 Survival (right axis) and reproduction (left axis) of *Folsomia candida* (average ± SD, n = 5) when exposed to sludge-amended (left) and metal-spiked soils (right). Metal concentration of treatments is expressed as ΣTUs. * - Number of juveniles significantly different from control ($p \leq 0.05$).

Table 4.5 LC_{50} and EC_{50} data (with corresponding 95% confidence intervals) for the effects on the survival and reproduction, respectively of *Eisenia andrei* and *Folsomia candida* exposed to a mixture of Cr, Cu, Ni, and Zn in sludge-amended or metal-spiked soils. LC_{50}s and EC_{50}s are expressed on the basis of Toxic Units (TUs) derived from the measured metal concentrations in the test soils and EC_{50} data from the literature (see Table 4.4).

	Sludge-amended soils	**Metal-spiked soils**
E. andrei		
LC_{50}	3.12 (-)*	3.65 (-)*
EC_{50}	2.07 (1.59 – 2.55)	0.796 (0.771 – 0.821)
F. candida		
LC_{50}	1.94 (-)*	1.80 (0.792 – 3.44)
EC_{50}	0.396 (0.256 – 0.536)	1.31 (0.868 – 1.76)

* - data does not allow estimation of a 95% confidence interval.

Discussion

The toxicity data obtained in sludge-amended and metal-spiked soils (LC_{50} and EC_{50} values) were significantly different only for reproduction effects on both test organisms. The mixture of metals was less toxic for *E. andrei* when applied via test sludge, while the sludge-amended soils were more toxic for *F. candida* than the metal-spiked soils. Our working hypothesis, which supposed a decrease in toxicity of the mixture of metals when applied through sludge, was confirmed only for reproduction of *E. andrei*. When comparing the chronic toxicity of sludge-amended and metal-spiked soils, two factors should be taken into account: i) the effect of the sludge matrix on the availability of metals, and ii) the effect of other toxicants included in the sludge, that together with the metal mixture, can influence its toxicity. In addition, the sludge matrix itself may create less optimal conditions for the test organisms, e.g. due to its high organic matter content or pH.

Regarding the effect of the sludge matrix on metal availability, metals were expected to be stronger adsorbed to the sludge and therefore less available than in spiked soils. According to the literature, the adsorption of metals is dependent on physical and chemical properties of the matrix. Several studies have demonstrated that the adsorption of cationic metals like Cu, Ni, and Zn is influenced by soil pH, CEC, $CaCO_3$, iron (Fe), manganese oxides (Mn), clay and OM content (Bibak 1994; Polo et al. 1999; Kabata Pendias and Pendias 2001; Mellis et al. 2004; Adhami et al. 2008). Chromium (III) adsorption in soil is related with the potential to form the Cr organic complex, which is not influenced by soil pH between 5 and 9 (Puzon et al. 2008; Luo et al. 2010). Considering the relatively high OM content of test sludge (28.0 ± 7.6%) and the higher pH of sludge-amended soils (particularly the two highest concentrations) compared to that of spiked soils, it is likely that metals in sludge-amended treatments were less available than in metal-spiked soils. Therefore, a lower toxicity would be expected in sludge-amended than in metal-spiked soils for both

test organisms. Considering the known tolerance of *F. candida* survival and reproduction to a wide range of soil textural classes, OM contents and soil pH (Jänsch et al. 2005), the higher toxicity on reproduction observed for the sludge-amended compared to metal-spiked soils may be due to the presence of other toxicants (not measured) in the test sludge in addition to the metal mixture. Sewage sludge often contains potentially toxic compounds, like anionic surfactants (linear alkylbenzene sulfonates) originating from industrial detergents, which are known to be harmful for collembolans (Holmstrup and Krogh 1996). However, further analytical data would be needed to confirm this assumption.

Conclusions

The toxicity for earthworms of the mixture of metals was mitigated by the test sludge, probably due to the reduction of metal bioavailability by the sludge organic matter. The toxicity of sludge to *F. candida* was not only due to its metal content, but probably enhanced by the presence of other toxicants. The comparison of soils amended with a metal-contaminated sludge or spiked with the same mixture of metals provides useful information on the effect of sludge contamination (not metals) and matrix on its toxicity. Further research is needed to evaluate if the effect of sludge on metal toxicity/availability changes over time.

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Chapter 5

Short-term changes of metal availability in soil I: Comparing sludge-amended and metal-spiked soils

Based on the following manuscript:

Natal-da-Luz T, Ojeda G, Costa M, Pratas J, Lanno RP, Van Gestel CAM, Sousa JP, 2011. Short-term changes of metal availability in soil I: Comparing sludge-amended and metal-spiked soils. *Geochimica et Cosmochimica Acta* (submitted).

Abstract

To control sewage sludge application on soils, total metal content is used to obtain an indication of the potential risk to soil organisms. However, the real risk of metals is determined by the fraction that is biologically available. The available fraction is highly related to the strength of metal binding by the matrix, which is a dynamic process. The evaluation of the fate of metals in time can contribute to improving the accuracy of ecological risk assessment. The objective of the present study was to evaluate the short-term changes in metal availability when metals were applied to soil directly (metal-spiked) or via an organic matrix (sludge-amended). A laboratory experiment was performed using open microcosms filled with agricultural soil. A concentration gradient of industrial sludge from a wastewater treatment plant (11, 15, 55, and 75 t/ha) contaminated predominantly with Cr, Cu, Ni, and Zn, or soil freshly spiked with the same concentrations of these metals were applied on top of the agricultural soil. After 0, 3, 6, and 12 weeks, total (HNO_3 69%) and 0.01M $CaCl_2$ extractable metal concentrations in soil and total metal content in the percolates were measured. Extractable metal concentrations did not change over time in sludge-amended soils, and the extractable fraction was independent of the sludge concentration. In metal-spiked soils, metal extractability decreased over time due to ageing and transport of metals to deeper layers. In general, the sludge matrix increased the adsorption and, consequently, reduced the mobility of metals in soil.

Key words: Extractable metals, total metals, soil matrix, metal mobility

Introduction

Human activities have induced changes in the composition and structure of soils all over the planet. The excessive disposals of waste, the application of various substances and amendments to agricultural soils, and mining activities have contributed to an increased concentration of metals in many soils (Sherameti and Varma 2010). Metals do not degrade but accumulate over time and present a potential risk to soil organisms (e.g., Baath 1989; Bruus Pedersen and Van Gestel 2001; Nahmani and Lavelle 2002). The degree of soil contamination is generally evaluated by total metal concentrations (Allen 2002). However, total metal levels are not necessarily good predictors of adverse effects. The actual risk of metals is determined by the fraction that is biologically available for organisms. Bioavailability is related to the binding strength of metals to various solid phases of the soil matrix (Lanno et al. 2004). Binding strength is dependent on pH, dissolved and total organic matter content, clay content, mineralogical forms of the elements, and other soil properties. In addition, metal partitioning, and consequently metal availability in soil, may change over time due to natural or anthropogenic processes, such as acidification, salinization, or organic matter mineralization (Allen 2002). Contact time may also increase the binding strength, a phenomenon called ageing. Therefore, monitoring the fate of metals in soil over time is the only way to understand partitioning dynamics in terrestrial systems and better predict their potential bioavailability.

The land application of industrial sludges, in particular sewage sludges, as waste disposal has been intensified due to the increased waste production resulting from growing industrial activities. Although sludge application practices are regulated by European Community directive 86/278/EEC (European Community 1986), the enrichment of metal levels in soils has been attributed to long-term sewage sludge applications (Düring and Gäth 2002). Some studies have assessed the fate of metals in long-term plots of sewage sludge-amended soils by extracting total and available metal fractions over time (e.g., McGrath and

Cegarra 1992; McBride et al. 2000). Other researchers have evaluated the influence of soil organism activity (e.g., earthworms) on metal availability (e.g., Sizmur and Hodson 2008). The actual knowledge base regarding the influence of specific combinations of chemical, biological, and environmental parameters on the extractable fraction of metals in soil remains sparse. For instance, changes detected in the availability of metals in a sewage sludge-amended soil over time may be modified by interactions between metals and the type of sludge used. The physical and chemical properties of a specific sludge promote particular changes in metal partitioning that do not occur in soil spiked with a metal salt. Therefore, the comparison of both routes of contamination (metal-spiked *vs* sludge-amended soils) may contribute to understanding the interactions between metals and sludge matrices. Since relevant organic matter transformations can take place during the first weeks following sludge amendments (Tarrasón et al. 2008), the evaluation of changes in metal availability over a short period of time would be desirable. In this context, the present study aims at evaluating the short-term changes in metal availability when applied to soil directly (metal-spiked) or via an organic matrix (sludge-amended). A laboratory experiment was conducted using agricultural soil, treated with an industrial sludge contaminated predominantly with chromium (Cr), copper (Cu), nickel (Ni), and zinc (Zn), or freshly spiked with similar concentrations of these metals. Total and extractable metal concentrations were measured at various intervals over a 12-week period.

More specifically, the objectives of the present study were: i) to evaluate changes in the availability of a specific pool of metals over time in metal-spiked and sludge-amended soils on a short-term basis; and ii) to assess the influence of initial concentration on the development of metal availability over time. Our working hypotheses are that on a short-term basis: i) metal availability is lower in sludge-amended than in metal-spiked soils due to metal adsorption to sludge organic matter (OM); ii) over time metal availability will decrease in spiked soils due to ageing and/or metal transport to deeper layers and remain stable in sludge treatments mainly due to OM stability (assuming that no significant degradation

of sludge OM occurs within 12 weeks); and iii) changes in metal availability in metal-spiked soils will be more pronounced at higher concentrations due to saturation of the metal sorption capacity of the soil while metal availability will not differ among concentrations of sludge-amended soils due to sludge OM stability over time.

Materials and methods

Reference soil

A sandy loam soil collected from the 20-cm top layer of an agricultural field in the suburban limit of Vila do Conde in a small village called Vairão, in northern Portugal, was used as reference soil (Table 5.1). Pesticides and fertilizers were not applied to the soil for more than five years prior to sampling. The soil was sieved (5 mm) and defaunated through two freeze–thaw cycles (48 h at -20ºC followed by 48 h at 25ºC). Soil pH (1M KCl, 1:6, v:v), water-holding capacity (WHC; ISO 1999), cation-exchange capacity (CEC; Chapman 1965), organic matter content (OM; loss on ignition at 500ºC for 6 h), and soil texture (LNEC 1970) were measured. Total metal concentrations were extracted using *aqua regia* and measured by flame atomic absorption spectrometry (AAS; AAnalyst 300, Perkin-Elmer). The quality of this analysis was checked using VKIAG CMSA, batch VKI-19-2-0595 (Reference Material QC Municipal Sludge A), certified by VKI Water Quality Denmark Institute as reference material, and metal concentrations were always between 90 and 110% of certified reference values.

Table 5.1 Physical and chemical characteristics of the reference soil and test sludge used to determine development of metal availability with time. *ND* – not determined.

Physical and chemical parameters		**Reference soil**	**Test sludge**
pH (1M KCl, $n = 4$)		4.4 ± 0.1	8.1 ± 0.1
Water-holding capacity (%, $n = 4$)		77.0 ± 1.4	147 ± 0.7
Cation exchange capacity (meq/kg, $n = 1$)		90.0	*ND*
Organic matter (%, $n = 2$)		9.3 ± 0.2	28.0 ± 7.6
Total Metals	Cadmium	< 1.8	< 0.5
(mg/kg DW, $n = 1$)	Chromium	< 4.8	5200[a]
	Cobalt	< 16	*ND*
	Copper	22	1000
	Iron	14248	*ND*
	Lead	31	290
	Manganese	145	*ND*
	Mercury	*ND*	0.18
	Nickel	< 10	7100
	Zinc	46	4100
Texture (%, $n = 1$)	Clay	10.3	*ND*
	Silt	35.6	*ND*
	Sand	54.1	*ND*
Soil type		Sandy Loam	-

[a] Only 0.5 mg/kg of the total Cr was in the oxidation state Cr(VI)

Test sludge

Sludge (Table 5.1) derived from the wastewater treatment plant of the Cu, Cr, Ni, and Zn-plating industries in Águeda, Portugal, was used. The sludge was produced by chemically induced precipitation of metals of industrial effluents followed by decantation. After air-drying, the precipitate was sieved (5 mm). The parameters measured were pH, WHC and, OM content, as characterized above. After *aqua regia* digestion, total Cr, Cu, Ni, and Zn concentrations were measured using inductively coupled plasma (ICP) – atomic emission spectrometry (OES Axial 730 ES, Varian Scientific Instruments) following USEPA (2001), and cadmium (Cd) and lead (Pb) concentrations were determined by ICP (OES Axial 730 ES, Varian Scientific Instruments) according to USEPA (1994). The accuracy of these procedures was checked using BCR No. 145 (Trace Elements in sewage sludges), certified by Community Bureau of Reference (Commission of the European Communities) as reference material. All the analyses were considered valid with quality control recoveries between 95 and 115%. Total mercury (Hg) content was measured using AAS (AMA-254, ALTEC) using cold vapour atomization (USEPA 1986). In this case, recovery of the control standard was between 94% and 97%. The oxidized state of Cr (Cr(VI)) was extracted using diphenylcarbazide following Branco et al. (2005) and measured by ICP (OES Axial 730 ES, Varian Scientific Instruments), with the reduced state (Cr(III)) concentration determined by the difference between total Cr and oxidized state (Cr(IV)) concentrations.

Treatments

The reference soil was mixed with different proportions of test sludge to obtain a concentration gradient (S0, S1, S2, S3, S4) equivalent to dosages of 0, 11, 15, 55, 75 t dry weight (DW)/ha, respectively (corresponding to 0, 15.4, 20.5, 76.1, 102.5 g DW/kg, respectively, assuming a specific mass of 1.5 g/cm^3 for

reference soil and a sludge incorporation to a depth of 10 cm). These concentrations were selected based upon toxicity data from previous chronic toxicity tests with *Eisenia andrei* and *Folsomia candida* for the same mixture of metals (Natal-da-Luz et al. 2011 – Chapter 4).

Metal-spiked soils were simultaneously prepared to reflect concentrations of the four metals predominating in the test sludge from each sludge treatment. The sources of Cr, Cu, Ni, and Zn were chromium nitrate ($Cr(NO_3)_3 \cdot 9H_2O$, purity 98%; Panreac, Barcelona, Spain), copper chloride ($CuCl_2 \cdot 2H_2O$, purity 99%; Panreac, Barcelona, Spain), nickel chloride ($NiCl_2 \cdot 6H_2O$, purity 98%; Panreac, Barcelona, Spain) and zinc chloride ($ZnCl_2$, purity 97%; Panreac, Barcelona, Spain), respectively. A stock solution with these four chemicals was prepared and different volumes of stock solution were diluted with distilled water to a final volume equal for all spiked-soil treatments. These dilutions were mixed with the reference soil to obtain the desired gradient of metals (M0, M1, M2, M3, M4), mimicking the metal concentration gradient in the sludge-amended soil.

Experimental procedure

A microcosm laboratory experiment was conducted using destructive sampling of replicates. The microcosms consisted of transparent plastic bottles (height 30 cm; diameter 8.5 cm) with the bottom removed and the bottleneck covered with a plastic net (1.9 mm). The bottles were placed upside-down and filled to a depth of 1 cm with Ø2-mm glass beads (to facilitate percolate collection), covered by a 19-cm layer of reference soil and a 10-cm top layer with the test treatments. Each bottle was encased in a plastic beaker (height 15 cm; diameter 8.5 cm) which acted as percolate collector. The water content of treated and reference soils was previously adjusted to 45% of their respective WHCs. The microcosms were filled two weeks before the experiment started and rain simulations were performed twice a week. Each rain simulation consisted of 20 ml of tap water

sprayed on the surface of the microcosm, corresponding to an annual rainfall of approx 370 mm. The upper 10 cm of the soil column and respective percolate (when available) were sampled after 0, 3, 6, and 12 weeks of incubation, using four replicates per treatment. Soil pH (1M KCl, 1:6, v:v) and moisture content (drying at 105ºC for 12 h) were determined on soil samples, and the total percolate volume was recorded. The soil aliquot that was oven-dried to determine moisture content was stored at 4ºC for total metal extractions, while the remainder of the sample was air-dried at room temperature for the assessment of extractable metal concentrations.

Metal analysis over time

Total metal digestions of soil samples were performed using PDS-6 pressure digestion systems (Loftfields Analytical Solutions, Neu Eichenberg, Germany). To 100 mg oven-dried soil (gently crushed with an acid-washed porcelain pestle and mortar), 2 ml HNO_3 69% (PA-ACS-ISO, Panreac, Barcelona, Spain) were added. The mixtures were left under pressure in PDS-6 systems at 150ºC for 10 h. After the digestion period, the resulting solution was diluted with ultrapure water to a final volume of 10 ml and transferred to a plastic vial. The quality of this analysis was checked using SRM 2709 (San Joaquin Soil - Standard Reference Material) certified by the National Institute of Standards and Technology (Department of Commerce, USA) as reference material and replicate blanks were prepared. The average recoveries were 82.9, 95.7, 98.5, and 105.2% for Cr, Cu, Ni, and Zn, respectively.

Extractable metal fractions were determined according to Houba et al. (2000). Twenty grams of air-dried soil were extracted by shaking (2 h, 400 rpm) with 200 ml of 0.01M $CaCl_2$ (21074, Sigma-Aldrich, St Louis, USA) solution. The resulting mixtures were filtered through Whatman No. 1 filter paper discs (Cat. No 1001150, Maidstone, England).

The concentration of Cr, Cu, Ni, and Zn in HNO_3 and $CaCl_2$ extracts and in percolates was determined by flame AAS (2380 Absorption Atomic Spectrometer, Perkin-Elmer). For the measurements in $CaCl_2$ extracts, wash solution, blanks, and standards were prepared using 0.01M $CaCl_2$ solution. Total and extractable metal concentrations were expressed as mg/kg dry soil.

Statistical analysis

For statistical analysis, data from each microcosm from each treatment was expressed as follows: (i) total metal concentrations were expressed as the percentage of the initial concentration, (ii) available metal concentration (extracted with $CaCl_2$) was expressed as percentage of total metal concentration for each replicate, and (iii) the amount of metal detected in the percolates was expressed as percentage of the mean initial total amount of metal in the soil column (both treated and non-treated layer).

Regression analyses were performed to detect significant trends in total metal concentrations over time. Similar regressions were performed for the available concentrations, using all values even if below the detection limit (when positive), and for the predominant metals found in the percolates. For these regressions, when higher R^2 values were obtained, the exponential equation was used instead of the normal linear equation. Regressions were performed for Cr, Cu, Ni, and Zn separately. Normality and homogeneity of variance (using Kolmogorov–Smirnov and Bartlett tests, respectively) of data was always checked before conducting regression analysis. When these assumptions were not fulfilled, values were arcsin transformed. Regressions were performed using STATISTICA version 6. The slopes obtained in regressions comparing the same variables (for total, available, and leached metals) were compared by analysis of covariance (ANCOVAs) after data linearization. The comparisons performed were: slopes obtained for different metals in each treatment (e.g., Ni from M4 *vs* Zn from M4), slopes obtained for different treatments in the same test

concentration for each metal (e.g., Ni from M4 *vs* Ni from S4) and slopes obtained for each metal at different test concentrations of each treatment (e.g., Ni from M4 *vs* Ni from M3 *vs* Ni from M2 *vs* Ni from M1). The level of statistical significance for all analyses was $\alpha = 0.01$.

Results

The soil water content (data not shown) and the pH did not change over time in any of the treatments. The pH was higher in the most concentrated sludge treatments, with mean (± SD; $n = 16$) values of 4.4 ± 0.0, 4.9 ± 0.0, 4.9 ± 0.0, 5.8 ± 0.1, and 6.0 ± 0.0 for the S0, S1, S2, S3, and S4 treatments, respectively. pH of the metal-spiked soils did not differ between concentrations and was on average 4.4 ± 0.1 ($n = 80$).

Total Metal concentrations over time

Total Cr, Cu, Ni, and Zn concentrations in sludge-amended treatments were generally higher than in spiked soils (Table 5.2 for initial total metal concentrations and Table A.1 in the Annex A for total metal concentrations over time – pages 167 to 169). No significant changes in total Cr and Cu concentrations with time were observed in most test treatments, except for Cu in the M2 treatment. No changes with time were observed for total Ni and Zn concentrations in sludge-amended soils. However, in metal-spiked treatments, the concentrations of these two metals decreased with time, as demonstrated by the significant negative slopes of regressions of M2, M3, and M4 treatments relating total concentrations with time (Figure 5.1 and Table A.2 – page 170).
Comparing the slopes, no changes in total concentrations of different metals with time were observed among sludge-amended and spiked soils. Neither different metal sources nor different test concentrations influenced total metal

concentrations over time.

Table 5.2 Initial total Cr, Cu, Ni, and Zn concentrations in the soils treated with sludge (S0 – S4) or spiked with metals (M0 – M4; average ± SD, $n = 4$).

Treatments	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
S0/M0	8.95 ± 3.69	19.6 ± 3.36	13.7 ± 2.97	55.9 ± 35.3
S1	57.2 ± 39.6	36.8 ± 13.0	141 ± 101	163 ± 78.1
S2	200 ± 78.2	67.8 ± 19.5	406 ± 126	335 ± 77.5
S3	265 ± 194	86.7 ± 43.8	511 ± 297	390 ± 170
S4	950 ± 198	215 ± 32.1	1389 ± 281	727 ± 60.9
M1	34.7 ± 2.25	24.8 ± 1.66	87.5 ± 13.9	117 ± 7.36
M2	49.6 ± 12.6	36.0 ± 3.75	134 ± 15.5	131 ± 14.1
M3	216 ± 63.0	85.6 ± 18.1	543 ± 81.6	421 ± 51.7
M4	335 ± 91.9	101 ± 19.7	599 ± 68.1	432 ± 46.6

Extractable metal concentrations over time

Extractable Ni and Zn concentrations were higher in metal-spiked soils than in sludge-amended soils, both initially and over time, while extractable Cr and Cu concentrations were very low in all treatments (Table 5.3 for initial extractable metal concentrations and Table A.3 for extractable metal concentrations over time – pages 171 to 173). The extractable concentrations of Cu were below the detection limit in all sludge treatments and in some of the spiked soils (M1 and M2). The extractability of Cu in M3 was 0.746% ($n = 16$) of total concentration and 2.26% in M4 ($n = 16$; on average of all samplings). The extractable Cr concentrations were below the detection limits in most treatments varying between 0.007 and 0.501% ($n = 4$) of total concentration in all treatments and

samplings. Extractability of Cr was not related to time and that of Cu followed an exponential decrease with time only in M4 treatments (Table A.4 – page 174). Notwithstanding, even in M4 treatments, extractable Cu levels were not biologically meaningful at any of the sampling dates.

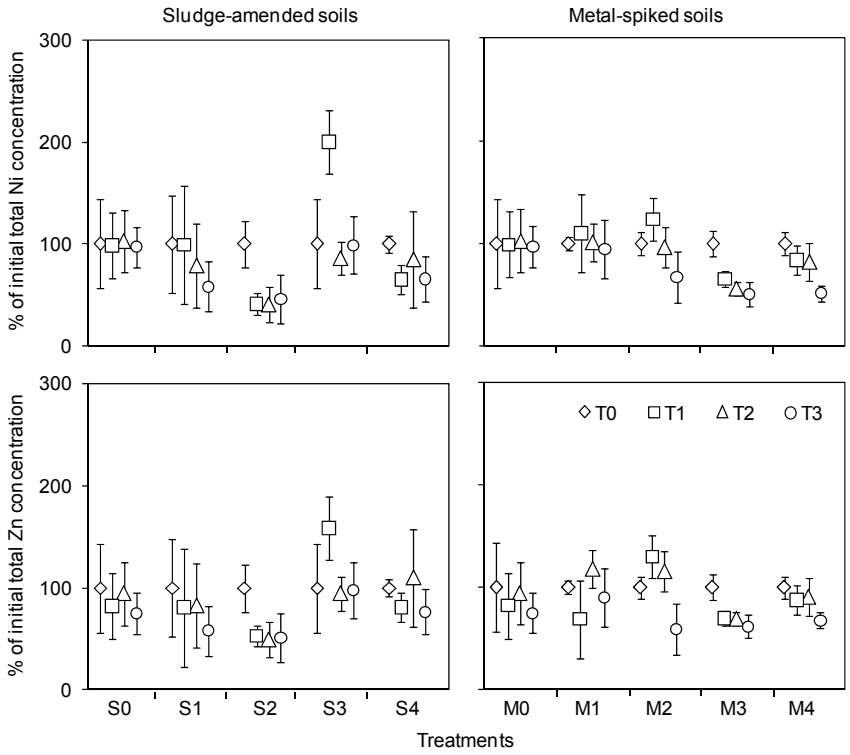

Figure 5.1 Development with time of total Ni (first row) and Zn (second row) concentrations in sludge-amended (S0 – S4; left) and metal-spiked (M0 – M4; right) soils incubated in microcosms on top of a layer of clean soil. Concentrations are expressed as percentage of initial concentrations. S0, S1, S2, S3, S4 – sludge-amended soils with 0, 11, 15, 55, 75 t DW sludge/ha, respectively; M0, M1, M2, M3, M4 – metal-spiked soils simulating the metal content in S0, S1, S2, S3, and S4, respectively. T0, T1, T2, T3 – 0, 3, 6, and 12 weeks sampling.

Table 5.3 Initial 0.01M $CaCl_2$ extractable Cr, Cu, Ni, and Zn concentrations in the soils treated with sludge (S0 – S4) or spiked with metals (M0 – M4; average ± SD, $n = 4$).

Treatments	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
S0/M0	< 0.249	< 0.349	< 0.427	< 0.108
S1	0.252 ± 0.075	< 0.349	1.80 ± 0.770	1.03 ± 0.655
S2	0.292 ± 0.079	< 0.349	3.20 ± 0.226	2.30 ± 0.371
S3	< 0.249	< 0.349	5.86 ± 0.803	1.03 ± 0.445
S4	< 0.249	< 0.349	8.89 ± 0.492	0.514 ± 0.027
M1	< 0.249	< 0.349	28.9 ± 2.79	21.7 ± 2.04
M2	< 0.249	< 0.349	43.6 ± 3.26	34.8 ± 2.94
M3	0.449 ± 0.099	1.09 ± 0.468	327 ± 23.2	244 ± 14.8
M4	1.06 ± 0.111	2.69 ± 0.067	652 ± 24.2	565 ± 19.1

In sludge-amended soils extractable Ni concentrations varied, on average, between 0.573 and 3.94% ($n = 4$) of total concentration, and those of Zn were 0.050 - 1.42% ($n = 4$). For both metals, the highest extractability was found in spiked soils, specifically in the M4 treatment at the two first samplings (0 and 3 months), where extractability was around 100%, sometimes higher (most probably due to analytical biases or variability; Figure 5.2). Nickel and Zn extractability showed a significant exponential decrease in time in the M4 treatment (Figure 5.2 and Table A.4 – page 174).

The comparison of slopes demonstrated that extractability of the different metals over time did not differ significantly in any treatment. The development of Cr extractability was significantly influenced by the source of metal contamination at the highest test concentration (Cr from M4 *vs* Cr from S4). A similar influence was detected for Zn at the lowest test concentration (Zn from M1 *vs* Zn from S1). Along the gradients of sludge-treated and metal-spiked soils, changes in

metal extractability generally did not differ significantly over time. The exception was Zn extractability between the M1 and M2 treatments, which were significantly different. However, neither M1 nor M2 treatments showed significantly different patterns compared to those of the other concentrations of spiked soils.

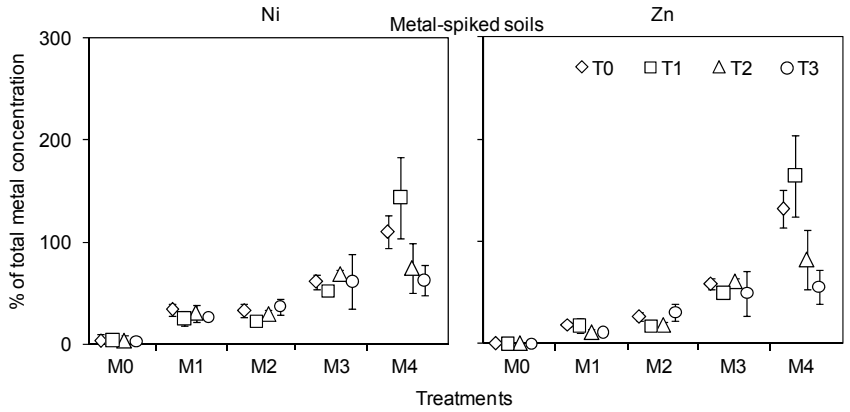

Figure 5.2 Development with time of extractable Ni (left) and Zn (right) concentrations in metal-spiked soils (M0 – M4) incubated in microcosms on top of a layer of clean soil. Concentrations are expressed as percentage of total concentration. See Figure 5.1 for treatment codes and sampling dates.

Metal concentrations in percolates over time

In most treatments, percolates were obtained in at least one replicate from the second sampling date on (3 weeks after the start of the experiment). Exceptions were the S3 microcosms, from which no percolate was collected before 12 weeks and the M3 microcosms from which no percolate was obtained until 6 weeks. Therefore, the collection of percolates from S3 treatments was possible only at the last sampling date, hampering the evaluation of the metal leaching with time for that treatment.

Only Ni and Zn were detected in percolate samples, in general with higher concentrations for metal-spiked soils than for sludge treatments (Table A.5 – pages 175 to 177).

The leaching of Ni and Zn as a percentage of the total amount of metal increased linearly with time in sludge treated and spiked soils, except for the M1 treatment for both metals, and the S1 and M3 treatments for Zn and Ni, respectively (Figure 5.3 and Table A.6 – page 178).

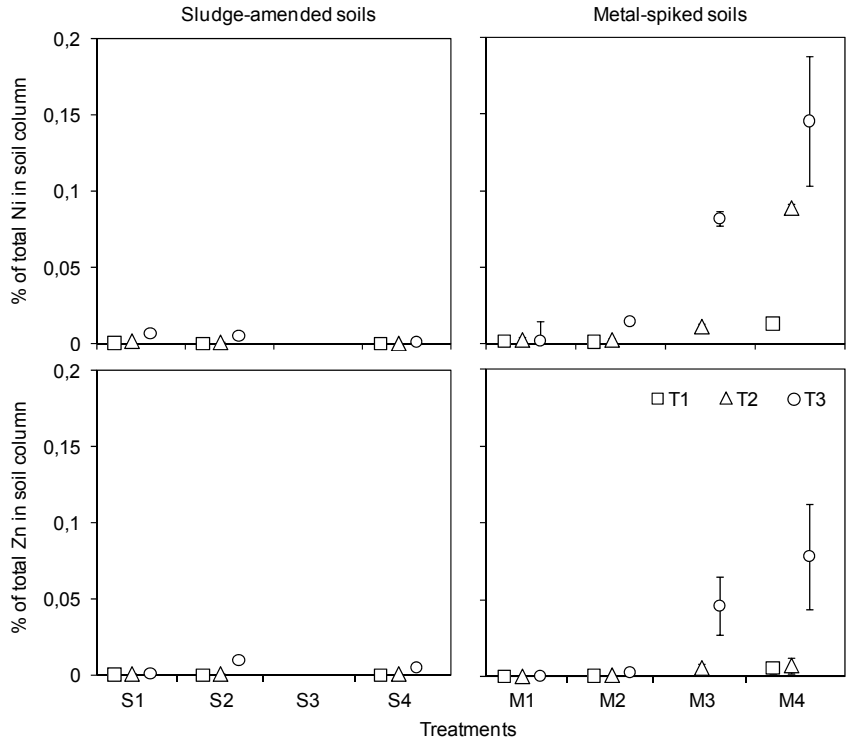

Figure 5.3 Development with time of Ni (first row) and Zn (second row) concentrations in percolates from microcosms with sludge-amended (S1 – S4; left) and metal-spiked (M1 – M4; right) soils on top of a layer of clean soil. Concentrations are expressed as percentage of total amount of metal in soil column. See Figure 5.1 for treatment codes and sampling dates.

The percentage increase in the total amount of Ni in percolates from each sludge-amended soil treatment over time was significantly different from that of Zn in the same treatments. In metal-spiked soils, a significant difference was detected only in the M2 treatment. The contamination source influenced the leaching of Ni and Zn, except for Zn in the lowest concentration (Zn from M1 *vs* Zn from S1). Changes in the percentage of the total amount of Ni and Zn in the percolates were significantly different between sludge concentrations, except for Ni in the S1 compared to the S2 treatment, and for Zn in the S2 compared to the S4 treatment. In metal-spiked soils, leaching changes of these metals in the M1 treatment over time were significantly different from those in the M3 and M4 treatments. In the M2 treatment, Zn leaching changes over time were significantly different from those in the M3 and M4 treatments.

Discussion

Total metal losses over time

In sludge-amended soils no significant losses from the top 10-cm layer were detected for all metals measured. This fact is in agreement with field experiments suggesting that total levels of biosolids-applied metals including Cr, Cu, Ni, and Zn generally tend to remain in the surface soil layer at least for several years (Canet et al. 1997; Sloan et al. 1998). On the other hand, in the M2, M3, and M4 treatments total Ni and Zn losses were significantly related with time. This suggests that the sludge matrix has a crucial role in conditioning the mobility of Ni and Zn. It is likely that these losses were due to transport of the metals to deeper soil layers. The higher mobility of Ni and Zn is demonstrated by the high percentage found in the $CaCl_2$ extracts and by their presence in percolates. Since the first rain simulation was performed one day after soil preparation, the mobility of metals might be overestimated as metals from spiked soils may not have had enough time to interact with soil particles before the first rain

application. No significant losses of total Cr and Cu were detected over time in any treatments (except M2 for Cu), which is consistent with the low $CaCl_2$ extractability and low or negligible leaching. The slopes of total metal concentrations *vs* time suggest that in treatments with spiked soils, Cr and Cu were less mobile than Ni and Zn and that Ni was the most mobile.

Adsorption is likely the major factor that controls the mobility of metals in soil. According to Buchter et al. (1989), *K* values for a soil similar to that used in the present experiment (pH 3.9, 11.6% OM, and 17.6% clay) demonstrate that the adsorption of Cu was highest followed by Ni, Cr, and Zn (*K* values of 221, 50, 30, and 20 dm^3/kg, respectively). It therefore would be expected that Zn and Cr would have the highest total concentration losses over time in spiked treatments. In a study conducted by Posthuma et al. (1997), a stronger sorption of Cu than zinc (using 0.01M $CaCl_2$ extractions) was observed in OECD artificial soil with *K* values differing by a factor of 4. This finding supports the higher mobility of Zn compared to Cu in the metal-spiked soils of our study. Using the Freundlich isotherm ($C_s = K_f * C_w^n$), metal adsorption could also be estimated in our experiment, considering C_s as the total metal concentration and C_w as the concentration of metal in the 0.01M $CaCl_2$ extracts (mg/L). For Cr and Zn in the sludge-treated soils the isotherm did not fit, while for Cu no fit was possible due to the fact that concentrations in the extracts were below the detection limit in most cases. For all other cases, a good fit was obtained. In sludge-amended soils the K_f for Ni was 1399 dm^3/kg with $n = 1.30$. In the metal-spiked soils K_f values were 1076, 49.2, and 81.2 for Cr, Ni, and Zn, respectively with *n* values of 0.45 - 0.64. In case of Ni these values confirm the higher mobility in metal-spiked soils than in sludge-amended soils. The data also confirm the higher mobility of Ni compared to Zn and the low mobility of Cr in the metal-spiked soils.

Other studies have been conducted to evaluate the long-term fate of metals applied to agricultural soils. McBride et al. (2000), evaluating metal changes in a sludge-amended field over the period between 1979 and 1997, using *aqua regia* and nitric-perchloric acid digestions, found that the levels of Cu, Ni, and Zn

tended to decrease in the top 5 cm layer. The decreases were similar for Cu, Ni, and Zn levels (depending on the nature of sewage sludge used) after 18 years of sludge application. They concluded that the decrease may be related not only to leaching of the metals but also to organic matter accumulation at the soil surface (diluting metal concentration in top soil), the activity of soil organisms, and wind and water erosion. Regarding the short-term period and the laboratory conditions under which our study was conducted, leaching is the only mechanism that could be responsible for the losses of metal from the top soil layer observed only in metal-spiked soils. Slightly different results were obtained by Walter et al. (2002), who measured metal contents 1, 5, and 9 years after the last biosolid applications in a soil treated for eight years with high rates of anaerobically-digested biosolids. Using HNO_3-HCl digestions, the authors found significant decreases of total Cu and Zn concentrations in the top 20-cm soil layer and no significant changes of total Cr and Ni levels. Taking into account that in the present study total metal concentration did not change over 12 weeks in sludge-amended soils for any metal, the decrease of total Cu and Zn observed by Walter et al. (2002) probably occurred more than 12 weeks after the last biosolids applications.

Extractability of metals over time

The lower metal extractability in soil samples from the sludge treatments compared to the metal-spiked soils demonstrates the importance of the sludge matrix in modifying the mobility of the metals measured. These findings are in agreement with our first working hypothesis, which assumed a lower metal availability in sludge-amended soils. As confirmed by other studies, the increase of soil pH, the changes in physical properties of the soil-sludge mixtures, and the increase of OM content following the application of the sludge, contributed to a decreased mobility of metals. Brun et al. (1998) evaluated the relationship between extractable Cu (using several extraction methods including 0.01M

$CaCl_2$), soil properties and Cu uptake by wild plants in a study area contaminated with Cu due to excessive use of fungicides. They found that the increase of soil pH and OM content contributed to the decreased extractability of Cu. In agreement with that, Ge et al. (2000), investigating trace metal speciation and bioavailability in three urban soils, found that high pH and organic matter content contribute to immobilizing metals like Cu, Ni, and Zn at the soil particle surface. The extractability of metals in soil is dependent upon their adsorption, which is influenced by several physical and chemical properties of the soil matrix. The adsorption of Cu, Ni, and Zn is influenced by soil pH, CEC, $CaCO_3$, iron (Fe) and manganese (Mn) oxides, clay and OM content (Bibak 1994; Polo et al. 1999; Pendias et al. 2002; Mellis et al. 2004; Adhami et al. 2008). The adsorption of Cr(III) to soil particles is dependent on the solubility of Cr organic complexes that exist in the soil (Puzon et al. 2008). An experiment performed by Luo et al. (2010) to evaluate the sorption of nine Cr(III) organic complexes in soil demonstrated that Cr sorption is not influenced by soil pH between 5 and 9 and that the percentage of Cr sorption can vary between 75 and 2%, depending on the Cr organic complex. Although that study used organic ligands less complex than those found in natural organic matter, adsorption of Cr(III) in soil is highly variable and cannot be predicted without knowing the predominant Cr organic complexes in the soil. Considering all factors that can influence adsorption in soil, it is probable that in our study the metal sorption was mainly determined by the physical and chemical changes induced by the sludge application, which favoured metal adsorption. On the other hand, in the M4 treatment, the extractability of Ni and Zn rounded 100%, but only in the first two samplings. The significant relationship between decreased extractability of Cu, Ni, and Zn with time in the M4 treatment might be related to ageing. Taking into account the significant losses of total Ni and Zn in the M2, M3, and M4 treatments over time, the decrease of Ni and Zn availability in M4 treatment may also be due to leaching. The metal fraction extractable by $CaCl_2$ is supposedly more susceptible to leaching over time. According to that assumption, it is

possible that the extractable fraction of Ni and Zn in the top soil layer at the last sampling date was lower than at the first samplings. These findings are in agreement with our second working hypothesis. Although Cr extractability did not decrease over time, the extractability of the other metals decreased over time in M4. Regarding the third working hypothesis, the results suggest detectable decreases in extractability at higher metal concentrations, with extractability decreasing significantly with time for Cu, Ni, and Zn in the M4 treatment. The slopes of the concentrations below the M4 treatment, however, do not suggest a more pronounced gradual decrease in metal extractability in time with higher metal concentrations.

Metals in percolates over time

The higher total Ni and Zn losses from the top 10-cm soil layers of the most concentrated spiked treatments explain the higher concentrations of Ni and Zn in percolates from these treatments. This suggests that a portion of the losses of Ni and Zn was due to leaching and most of the Ni and Zn were retained in deeper soil layer of the test system. The absence of Cr and Cu in percolates is in agreement with their lower concentrations in $CaCl_2$ extractions. According to Bolt and Bruggenwert (1976), the vertical transport of metals in soil can be described by the equation:

$$X_p = V*t/(Ø + \rho_b*K) \qquad (5.1)$$

where X_p is the distance (depth) to which the chemical is transported (cm), V is the amount of rain (cm/day), t is the time (day), $Ø$ is the water-filled pore volume (cm^3/cm^3), ρ_b is the specific gravity in the soil (kg/dm^3), K is the partition constant (dm^3/kg). This equation assumes transport only takes place through water-filled pores. The transport of metal dissolved in water is retarded by sorption to soil particles.

Estimating the depth at which metals might have been transported in our microcosm experiment using the K value calculated for metals in spiked soils, which corresponded to the most mobile metal (worst case scenario was the K_f value for Ni of 49.2 dm^3/kg), assuming that no evaporation occurred and values for ρ_b and $Ø$ parameters of 1.4 kg/dm^3 and 0.3 cm^3/cm^3, respectively (reasonable values for sandy loam soils), no metal from top layers would be expected in the percolates. Although these estimations predict that the highest vertical transport occurred for Ni (0.122 cm over 12 weeks), that depth was not sufficient to reach the percolate collector. The vertical distance estimated for Cr and Zn was even lower, and also for Ni in the sludge treatment. This suggests that the Ni and Zn found in percolates were due to preferential flow of rain water through cracks in the soil or along the wall of the column.

The comparison of slopes of metal concentrations in percolates against time suggests that the increase of Ni and Zn levels in percolates over time was faster for sludge-amended soils at the lowest test concentrations. However, at the highest concentrations the increase is more pronounced in percolates from metal-spiked treatments. This behaviour is probably related with the fact that in sludge-amended soils the capacity to retain rain water increased with increasing sludge concentration due to the higher OM content of sludge compared to that of soil. Because of that, at the lowest concentrations of sludge-amended soils, more rain water can be collected as percolate (Table A.5 – pages 175 to 177) and, therefore, more metals are transported to percolates allowing a pronounced increase of metal levels in percolates over time. At the highest concentrations of sludge-amended soils, more rain water was retained in top layers and therefore less metal was transported to percolates, resulting only in a slight increase of metal levels in percolates over time. In metal-spiked soils the capacity to retain rain water was assumed to be independent of the metal concentration. In this case it is likely that the increase of metal levels in percolates over time was more pronounced at the treatments with the highest metal concentrations.

Conclusions

Our results demonstrate that metal availability decreased in spiked soils over time due to ageing and leaching of metals, which reduced the most mobile metal fraction in the top soil layers. The decrease of metal availability in spiked treatments was not concentration related. In sludge-amended soils, the availability of metals remained stable over time and was not related with test concentrations. Metals were more mobile in metal-spiked than in sludge-amended soils. These differences in metal behaviour suggest that the particular physical and chemical properties of the test sludge (e.g., high pH and OM content) contributed to a decreased metal mobility, reducing metal availability in sludge treatments.

These experiments demonstrated the behaviour of metals in a particular natural soil under controlled conditions. Some factors that exist in the environment were not taken into account in our study, such as the activity of soil organism (e.g., earthworms) that influences the mobility and fate of metals. Further evaluation of the influence of soil organism activity on metal availability over time in sludge-amended and metal-spiked soils is needed.

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Annex A

Table A.1 Total Cr, Cu, Ni, and Zn concentrations (average ± SD, n = 4) in the top 10-cm soil layer unamended (S0/M0) or amended with sludge (S1 – S4) or spiked with metals (M1 – M4) after 3, 6, and 12 weeks of incubation.

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
3 weeks	S0/M0	6.21 ± 0.75	19.5 ± 2.98	13.4 ± 1.20	41.2 ± 6.26
	S1	55.1 ± 40.5	37.2 ± 18.5	140 ± 94.8	132 ± 94.0
	S2	66.7 ± 24.2	36.1 ± 7.58	170 ± 63.1	178 ± 35.0
	S3	672 ± 225	171 ± 52.8	1021 ± 327	617 ± 120
	S4	600 ± 198	149 ± 48.8	909 ± 235	590 ± 105
	M1	35.2 ± 9.33	26.3 ± 6.49	96.7 ± 29.3	81.2 ± 44.6
	M2	47.0 ± 8.31	37.9 ± 4.58	166 ± 29.5	170 ± 27.9
	M3	160 ± 23.6	69.0 ± 8.74	355 ± 46.1	294 ± 31.5
	M4	309 ± 87.9	96.6 ± 22.7	505 ± 103	381 ± 61.1

Table A.1 (Continued)

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
6 weeks	S0/M0	6.47 ± 1.72	20.8 ± 3.66	13.9 ± 2.20	48.9 ± 9.19
	S1	49.3 ± 32.0	28.3 ± 11.8	111 ± 64.8	136 ± 67.0
	S2	69.4 ± 34.4	37.7 ± 9.50	165 ± 56.0	167 ± 58.4
	S3	240 ± 97.9	72.6 ± 17.3	441 ± 140	368 ± 64.1
	S4	888 ± 383	180 ± 62.3	1179 ± 421	797 ± 342
	M1	36.3 ± 5.63	28.7 ± 4.57	88.5 ± 21.3	139 ± 21.3
	M2	53.9 ± 20.8	35.5 ± 5.61	130 ± 33.4	152 ± 26.0
	M3	170 ± 20.1	69.4 ± 9.14	304 ± 46.6	290 ± 27.8
	M4	344 ± 74.4	113 ± 16.2	489 ± 135	392 ± 79.4

Table A.1 (Continued)

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
12 weeks	S0/M0	5.14 ± 1.08	18.5 ± 3.29	13.2 ± 2.10	39.3 ± 6.50
	S1	35.3 ± 14.9	24.6 ± 5.34	82.5 ± 35.7	94.7 ± 40.0
	S2	82.2 ± 32.4	38.2 ± 10.9	187.9 ± 60.4	172 ± 81.4
	S3	275 ± 218	80.7 ± 28.4	506 ± 253	381 ± 108
	S4	620 ± 323	132 ± 60.3	919 ± 444	556 ± 161
	M1	38.1 ± 2.52	27.0 ± 3.52	82.5 ± 7.64	106 ± 33.4
	M2	37.2 ± 7.11	26.3 ± 4.92	90.5 ± 21.0	77.7 ± 32.4
	M3	179 ± 36.7	72.1 ± 17.7	277 ± 52.4	261 ± 48.8
	M4	279 ± 96.0	93.1 ± 18.3	310 ± 58.0	293 ± 33.2

Table A.2 Regression slopes for total Cr, Cu, Ni, and Zn concentrations as percentage of initial doses (% initial TMC) against time for different metals in unamended (S0/M0), sludge-amended (S1 – S4) and metal-spiked soils (M1 – M4).

		Time (independent variable)							
			Sludge-amended soils				Metal-spiked soils			
	Metal	S0/M0	S1	S2	S3	S4	M1	M2	M3	M4
% initial TMC (depend. variable)	Cr	-0.038[a]*	-3.30	-3.76	- 417	-0.030[a]	0.009[a]	-0.022[a]	-0.937	-0.014[a]
	Cu	-0.005[a]	-3.08	-2.82	-3.52	-0.038[a]	0.007[a]	-0.030[a]*	-0.011[a]	-0.413
	Ni	-0.002[a]	-3.74	-2.14	-3.06	-0.030[a]	-0.007[a]	-0.042[a]*	-0.052[a]*	-0.054[a]*
	Zn	-1.69	-3.20	-2.28	-1.90	-0.019[a]	0.003[a]	-0.056[a]*	-0.035[a]*	-0.031[a]*

* - statistically significant ($p \leq 0.01$); slopes were obtained using normal linear equation except those with [a] that were obtained using the exponential equation.

Table A.3 $CaCl_2$ (0.01M) extractable Cr, Cu, Ni, and Zn concentrations (average ± SD, n = 4) in the top 10-cm soil layer unamended (S0/M0) or amended with sludge (S1 – S4) or spiked with metals (M1 – M4) after 3, 6, and 12 weeks of incubation.

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
3 weeks	S0/M0	< 0.249	< 0.349	0.501 ± 0.461	< 0.108
	S1	< 0.249	< 0.349	2.04 ± 0.256	0.896 ± 0.140
	S2	< 0.249	< 0.349	2.80 ± 0.408	1.36 ± 0.135
	S3	< 0.249	< 0.349	5.85 ± 1.07	0.711 ± 0.239
	S4	< 0.249	< 0.349	7.75 ± 0.666	0.344 ± 0.094
	M1	< 0.249	< 0.349	22.7 ± 2.54	12.1 ± 1.31
	M2	< 0.249	< 0.349	35.5 ± 4.24	28.3 ± 2.23
	M3	0.434 ± 0.118	0.368 ± 0.046	185 ± 27.0	145 ± 10.2
	M4	0.832 ± 0.113	2.97 ± 0.227	697 ± 95.2	611 ± 93.9

Table A.3 (Continued)

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
6 weeks	S0/M0	< 0.249	< 0.349	0.428 ± 0.707	< 0.108
	S1	< 0.249	< 0.349	2.30 ± 0.239	0.799 ± 0.114
	S2	< 0.249	< 0.349	2.99 ± 0.481	1.57 ± 0.491
	S3	< 0.249	< 0.349	5.23 ± 0.750	0.459 ± 0.182
	S4	< 0.249	< 0.349	7.06 ± 1.41	0.396 ± 0.029
	M1	< 0.249	< 0.349	25.6 ± 2.60	16.1 ± 2.45
	M2	< 0.249	< 0.349	36.4 ± 3.94	27.9 ± 3.80
	M3	< 0.249	0.468 ± 0.102	206 ± 18.5	176 ± 24.7
	M4	0.567 ± 0.054	2.22 ± 0.219	354 ± 111	317 ± 103

Table A.3 (Continued)

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
12 weeks	S0/M0	< 0.249	< 0.349	< 0.427	< 0.108
	S1	< 0.249	< 0.349	3.25 ± 1.62	1.13 ± 0.158
	S2	< 0.249	< 0.349	3.35 ± 0.734	2.44 ± 1.10
	S3	< 0.249	< 0.349	5.20 ± 0.516	0.799 ± 0.308
	S4	< 0.249	< 0.349	5.83 ± 0.601	0.354 ± 0.204
	M1	< 0.249	< 0.349	22.2 ± 1.18	12.4 ± 1.04
	M2	< 0.249	< 0.349	32.1 ± 3.76	22.2 ± 2.58
	M3	0.254 ± 0.017	0.363 ± 0.149	163 ± 51.7	122 ± 32.7
	M4	0.684 ± 0.107	1.26 ± 0.132	188 ± 9.88	160 ± 27.4

Table A.4 Regression slopes for 0.01M $CaCl_2$ extractable Cr, Cu, Ni, and Zn concentrations as percentage of total metal concentration (% TMC) against time for different metals in unamended (S0/M0), sludge-amended (S1 – S4), and metal-spiked soils (M1 – M4).

		Time (independent variable)							
			Sludge-amended soils				Metal-spiked soils			
	Metal	S0/M0	S1	S2	S3	S4	M1	M2	M3	M4
% TMC (depend. variable)	Cr	0.015	0.018[a]	0.007[a]	-0.003	0.106[a]	-0.010	0.008	-0.046[a]	-0.023[a]
	Cu	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	-0.030[ab]	-0.066[a]*
	Ni	-0.054	0.088[a]	0.055[a]	0.007[a]	-0.004[a]	-0.437	0.590	0.365	-0.060[a]*
	Zn	-0.115[b]	0.060[a]	0.035[ab]	-0.007[a]	-0.025[a]	-0.706	0.571	-0.015[a]	-0.087[a]*

* - statistically significant ($p \leq 0.01$); *ND* – not determined; slopes were obtained using normal linear equation except those with [a] that were obtained using the exponential equation; [b] – using values arcsin transformed.

Table A.5 Cr, Cu, Ni, and Zn concentrations in the percolates and total volume of percolate collected from soils unamended (S0/M0) or amended with sludge (S1 – S4) or spiked with metals (M1 – M4) after 3, 6, and 12 weeks of incubation (average ± SD, $n = 4$)

Incubation period	Treatment	Percolate volume (ml)	Ni (mg/L)	Zn (mg/L)
3 weeks	S0/M0	20 ± 0.82	0.04 ± 0.02	0.03 ± 0.02
	S1	18 ± 1.0[a]	0.05 ± 0.01[a]	0.05 ± 0.04[a]
	S2	20 ± 0.8	0.03 ± 0.02	0.02 ± 0.01
	S3	0	-	-
	S4	3.0[b]	0.05[b]	0.45[b]
	M1	16 ± 0.82	0.10 ± 0.10	0.03 ± 0.02
	M2	16 ± 4.5	0.13 ± 0.04	0.10 ± 0.02
	M3	0	-	-
	M4	20 ± 0.82	3.6 ± 1.7	1.4 ± 0.68

Table A.5 (Continued)

Incubation period	Treatment	Percolate volume (ml)	Ni (mg/L)	Zn (mg/L)
6 weeks	S0/M0	69 ± 5.6	0.03 ± 0.01	0.05 ± 0.003
	S1	77 ± 1.4[c]	0.04 ± 0.002[c]	0.04 ± 0.03[c]
	S2	85 ± 12	0.05 ± 0.02	0.04 ± 0.01
	S3	0	-	-
	S4	85 ± 7.1	0.07 ± 0.02	0.06 ± 0.04
	M1	67 ± 0.58[a]	0.04 ± 0.02[a]	0.01 ± 0.01[a]
	M2	61 ± 19	0.06 ± 0.03	0.05 ± 0.02
	M3	98 ± 0.82	0.55 ± 0.13	0.25 ± 0.13
	M4	98 ± 11	4.8 ± 2.4	0.43 ± 0.36

Table A.5 (Continued)

Incubation period	Treatment	Percolate volume (ml)	Ni (mg/L)	Zn (mg/L)
12 weeks	S0/M0	294 ± 4.9	0.04 ± 0.02	0.01 ± 0.01
	S1	267 ± 1.0[a]	0.03 ± 0.02[a]	0.02 ± 0.01[a]
	S2	249 ± 19	0.08 ± 0.02	0.15 ± 0.04
	S3	201 ± 0.82	0.06 ± 0.02	0.07 ± 0.06
	S4	174 ± 7.5	0.08 ± 0.02	0.21 ± 0.07
	M1	209 ± 0.82	0.01 ± 0.01	0.01 ± 0.004
	M2	209 ± 12	0.13 ± 0.06	0.04 ± 0.02
	M3	271 ± 0.82	1.5 ± 0.76	0.78 ± 0.31
	M4	271 ± 17	2.9 ± 1.5	1.3 ± 0.53

[a] – $n = 3$; [b] – $n = 2$; [c] – $n = 1$.

Table A.6 Linear regressions slopes for Ni and Zn concentrations in percolates as percentage of total metal concentration (% TMC) against time for different metals in unameded (S0/M0), sludge-amended (S1 – S4), and metal-spiked soils (M1 – M4).

		Time (independent variable)								
			Sludge-amended soils				Metal-spiked soils			
	Metal	S0/M0	S1	S2	S3	S4	M1	M2	M3	M4
% TMC (depend. variable)	Ni	0.229[b]*	0.001*	0.116[b]*	*NP*	0.0001*	0.000	0.002*	0.012	0.480[b]*
	Zn	0.022[b]	0.000	0.182[b]*	*NP*	0.001*	0.000	0.052[b]*	0.007*	0.432[b]*

* - statistically significant ($p \leq 0.01$); *NP* - no percolate available; [b] – using values arcsin transformed.

Chapter 6

Short-term changes of metal availability in soil II: The influence of earthworm activity

Based on the following manuscript:

Natal-da-Luz T, Ojeda G, Costa M, Pratas J, Lanno RP, Van Gestel CAM, Sousa JP, 2011. Short-term changes of metal availability in soil II: The influence of earthworm activity. *Applied Soil Ecology* (submitted).

Abstract

This study aimed at evaluating short-term earthworm-induced changes in the availability of metals applied to soil directly (metal-spiked) or via an organic matrix (sludge-amended). A laboratory experiment was performed using destructive sampling of microcosms filled with agricultural soil. A concentration gradient of industrial sludge contaminated predominantly with Cr, Cu, Ni, and Zn, and a soil freshly spiked with the same metal concentrations were applied on top of the soil columns. Individuals of *Dendrobaena veneta* (mimicking a realistic density of 500 earthworms per m^2) were introduced in half of the replicates of each treatment. Percolates were collected and total and extractable metal concentrations were measured in the soil after 0, 3, 6, and 12 weeks. Metal concentration in earthworms was also determined. Earthworm activity did not affect metal availability of any treatment over time, but Ni and Cu concentrations in *D. veneta* were higher at the highest treatment levels. Earthworm Zn concentrations were similar in all treatments while Cr concentrations increased with increasing soil total metal content only for sludge treatments. Existing relationships of earthworm metal concentrations with total metal content in soil, taken from the literature, were not able to predict the metal levels measured in *D. veneta*. Results demonstrated that although over 12 weeks earthworm activity did not affect metal availability in soil, their burrowing activities did interfere with the metal concentrations of percolates over time.

Key words: Soil matrix, metal mobility, bioaccumulation, *Dendrobaena veneta*

Introduction

The excessive disposal of wastes, the use of fertilizers in agricultural soils, and mining activities have caused metal contamination of soil systems and induced considerable damage to the environment. The risk of metals to terrestrial organisms has been widely demonstrated (e.g., Bengtsson et al. 1986; Spurgeon et al. 1994; Kelly et al. 1999; Bruus Pedersen and Van Gestel 2001). Total metal concentrations are often used as criteria in managing sewage sludge applications in soil (European Community 1986). However, several studies have demonstrated that total metal concentrations in soils are less predictive of biological effects than the amount of metal that is available and mobile. Several methods and sampling strategies have been developed to quantify available metal pools in soil. Chemical extractions are most commonly used to assess the bioaccessibility of metals from soils, sludges, and sediments (Peijnenburg et al. 2007). Body residues may provide a better estimate of the bioavailability of metals in soil, although residues may be highly dependent on the specific route of exposure of the test organism (Lanno et al. 2004). Body burdens of metals may be very relevant for organisms such as earthworms, due to their intimate contact with soil. The bioavailable fraction of metals is dependent upon soil properties, source of contamination, and various physical and biological processes interacting over time.

Earthworm activity is one biological process that is capable of inducing changes in metal availability (Sizmur and Hodson 2009) and influencing metal partitioning in soil. As predominant members of the soil macrofauna, earthworms have an important role in maintaining soil structure by stabilising aggregates and promoting soil porosity (Lavelle and Spain 2001). Earthworms provide important ecosystem services, participating in organic matter recycling and contributing to water and nutrient cycling (Vandewalle et al. 2008). Based on the annual cast production by earthworms of 5 to 250 t/ha (Bohlen 2002), earthworm activities are predicted to affect metal availability even on a short-

term basis. Several studies using metal-spiked, field contaminated, and sewage sludge-amended soils have investigated this influence, using anecic (e.g., *Lumbricus terrestris*; Zorn et al. 2005a) and endogeic species (e.g., *Aporrectodea tuberculata*; Coeurdassier et al. 2007). However, epigeic species (e.g., *Eisenia fetida*; Wen et al. 2004 and *Eisenia veneta*; Sizmur and Hodson 2008) remain the most commonly used. Although most studies have shown an increase in metal availability due to earthworm activity (e.g., Zorn et al. 2005a, Rada et al. 1996), others found an inverse trend in metal-spiked soils (Zorn et al. 2005b) and sewage sludge (Liu et al. 2005). To date, the influence of earthworm activity on metal availability over time has been examined in separate studies with metal-spiked soils and sludge-amended soils. A comparative evaluation of the influence of earthworms on specific pools of metals originating from sludge or from a metal spike in soil is still needed.

Aiming to fill this void, a laboratory experiment was conducted to evaluate the influence of earthworm activity on short-term changes in the availability of metals applied directly to soil (metal-spiked) or via an organic matrix (sludge-amended). Open microcosms filled with agricultural soil were amended with industrial sludge contaminated predominantly with chromium (Cr), copper (Cu), nickel (Ni), and zinc (Zn), or a soil freshly spiked with the same concentration of these metals. *Dendrobaena veneta* were introduced in half of the microcosms of each treatment and sampled at different times during a 12-week period. The present study aimed to answer the following questions: i) Is the activity of a realistic density of *D. veneta* able to influence the availability of metals in sludge-amended and metal-spiked soils over 12 weeks of incubation?; ii) How do different matrices influence the metal concentrations in earthworms?; and, iii) Are the earthworm metal concentrations predictable by existing models relating body concentrations to total metal concentrations in soil? Following these questions, our working hypotheses are that i) earthworm activity will increase metal availability especially in sludge-amended soils due to consumption of sludge organic matter (OM) reducing the number of binding sites for metals to

OM; ii) metal concentrations are higher in earthworms from metal-spiked soils than from sludge-amended soils due to the higher availability of metals in spiked soils, and iii) the earthworm metal concentrations can be predicted by existing models.

Materials and methods

Reference soil and test sludge

A sandy loam soil was collected from an agricultural field in the suburban limit of the city of Vila do Conde in a small village called Vairão, from North Portugal. The soil was collected from the top 20-cm layer by shovel and was free of pesticide and fertilizer applications for more than five years. The soil was sieved (5 mm), and defaunated through two freeze–thawing cycles (48 h at -20ºC followed by 48 h at 25ºC). A sludge derived from a chemical wastewater treatment plant from Cr, Cu, Ni, and Zn-plating industries in Águeda, Portugal, was used. The sludge originated from the chemically induced precipitation of metals in industrial effluents. After air-drying, the precipitate was sieved (5 mm). Physical and chemical parameters measured in both soil and test sludge were pH (1M KCl, 1:6, v:v), water holding capacity (WHC; ISO 1999), and organic matter content (OM; loss on ignition at 500ºC for 6 h). Cation exchange capacity (CEC; Chapman 1965) and soil texture (LNEC 1970) were measured only for the soil. Table 6.1 shows the properties of the soil and test sludge. Total metals were extracted from the soil using *aqua regia* and measured by flame atomic absorption spectrometry (AAS; AAnalyst 300, Perkin-Elmer). To check quality of this analysis, VKIAG CMSA, batch VKI-19-2-0595 (Reference Material QC Municipal Sludge A), certified by the VKI Water Quality Denmark Institute was used as reference material, and metal concentrations were always between 90 and 110% of certified reference values. For test sludge, total Cr, Cu, Ni, and Zn concentrations were measured after *aqua regia* digestion using inductively

coupled plasma (ICP) – atomic emission spectrometry (OES Axial 730 ES, Varian Scientific Instruments) following USEPA (2001). Cadmium (Cd) and lead (Pb) in digests were determined by ICP (OES Axial 730 ES, Varian Scientific Instruments) according to USEPA (1994). The quality of procedures was checked using BCR No. 145 (Trace Elements in sewage sludges), certified by Community Bureau of Reference (Commission of the European Communities) as reference material. All the analyses were considered valid with quality control recoveries between 95 and 115%. Total mercury concentration was measured using AAS (AMA-254, ALTEC) using cold vapour atomization (USEPA 1986). In this case, recovery of the control standard was between 94 and 97%. The oxidized Cr(VI) form was extracted using diphenylcarbazide following Branco et al. (2005) and measured by ICP (OES Axial 730 ES, Varian Scientific Instruments), with the reduced state (Cr(III)) concentration determined by the difference between total Cr and oxidized state (Cr(IV)) concentrations.

Treatments

The test soil was mixed with different proportions of test sludge to obtain the gradient S/Sw0, S/Sw1, S/Sw2, S/Sw3, and S/Sw4 (S – no worms will be added; Sw – worms will be added) equivalent to dosages of 0, 11, 15, 55, 75 t/ha dry weight (DW), respectively (corresponding to 0, 15.4, 20.5, 76.1, 102.5 g/kg DW, respectively, assuming a specific mass of 1.5 g/cm^3 for reference soil and a sludge incorporation depth of 10 cm). These concentrations were defined based on the toxicity of the test sludge and spiked soils using the same pool of the main four metals (Cr, Cu, Ni, Zn) and determined in standard reproduction tests with the earthworm *Eisenia andrei* (Natal-da-Luz et al. 2011a – Chapter 4). Metal-spiked soils were prepared simultaneously to approximate the concentrations of the four predominant metals in each of the sludge treatments. The sources of Cr, Cu, Ni, and Zn were chromium nitrate ($Cr(NO_3)_3 \cdot 9H_2O$, purity 98%; Panreac, Barcelona, Spain), copper chloride ($CuCl_2 \cdot 2H_2O$, purity 99%; Panreac,

Barcelona, Spain), nickel chloride ($NiCl_2 \cdot 6H_2O$, purity 98%; Panreac, Barcelona, Spain) and zinc chloride ($ZnCl_2$, purity 97%; Panreac, Barcelona, Spain), respectively. Different volumes of a stock solution were mixed in with the test soil to obtain the desired gradient of metals; M/Mw0, M/Mw1, M/Mw2, M/Mw3, and M/Mw4 (M – no worms will be added; Mw – worms will be added).

Table 6.1 Physical and chemical characteristics of the soil and test sludge used to assess metal availability over time.

Physical and chemical parameters		**Reference soil**	**Test sludge**
pH (1M KCl, $n = 4$)		4.4 ± 0.1	8.1 ± 0.1
Water-holding capacity (%, $n = 4$)		77.0 ± 1.4	147 ± 0.7
Cation exchange capacity (meq/kg, $n = 1$)		90.0	*ND*
Organic matter (%, $n = 2$)		9.3 ± 0.2	28.0 ± 7.6
Total Metals	Cadmium	< 1.8	< 0.5
(mg/kg DW, $n = 1$)	Chromium	< 4.8	5200[a]
	Cobalt	< 16	*ND*
	Copper	22	1000
	Iron	14248	*ND*
	Lead	31	290
	Manganese	145	*ND*
	Mercury	*ND*	0.18
	Nickel	< 10	7100
	Zinc	46	4100
Texture (%, $n = 1$)	Clay	10.3	*ND*
	Silt	35.6	*ND*
	Sand	54.1	*ND*
Soil type		Sandy Loam	-

[a] - 0.5 mg/kg of the total Cr is in the oxidation state Cr(VI); *ND* - not determined

Experimental procedure

Microcosms consisted of transparent plastic bottles (height 30 cm; diameter 8.5 cm) with the bottom removed and the bottleneck covered with a plastic net (1.9 mm). The bottles were placed upside-down and filled to a depth of 1 cm with Ø2 mm glass beads (to facilitate percolate collection), followed by a 19-cm layer of soil and a 10-cm top layer with the test treatments. Soil and test mixtures were previously moistened to 45% of their WHCs. A plastic beaker (height 15 cm; diameter 8.5 cm) acted as percolate collector at the base of each microcosm. Two hundred and eighty microcosms were filled two weeks before the experiment started (28 microcosms per test treatment). Rain simulations were performed twice a week from the moment the microcosms were filled until the end of the experiment. Each rain simulation consisted of 20 ml of tap water sprayed on the microcosm surface, which corresponds to an annual rainfall of approximately 370 mm. After two weeks of rain simulations (at week 0), four microcosms of each treatment were destructively sampled to record the initial conditions without the influence of earthworms. Simultaneously, four pre-weighed adult *Dendrobaena veneta* (Oligochaeta : Lumbricidae) of 1259 ± 314 mg fresh weight (± SD, n = 480), obtained from a vermiculturist, were introduced into 12 microcosms per treatment. The earthworms were previously acclimated in the test soil for one week. To prevent earthworm escape, the microcosms were covered with a tight tissue net (< 0.5 mm). At 3, 6, and 12 weeks after earthworm introduction, eight replicates per treatment (four replicates with earthworms – the Sw and Mw treatments – and four replicates without earthworms – the S and M treatments) were destructively sampled. In microcosms with earthworms, the surviving animals found in the top 10-cm layer and in the deeper layer were counted separately (to detect possible avoidance behaviour) and collected. The surviving earthworms were washed, weighed individually, and incubated overnight (for 12 to 24 h) in Petri dishes on moist filter paper, in the dark and at room temperature, to purge the gut. After

that, the earthworms were transferred to 20-ml scintillation vials and stored at -20ºC until metal extraction. Prior to digestion, the worms were oven dried at 105ºC for 12 h. The earthworm biomass production was expressed as the percentage of the initial weight. For all microcosms the top 10-cm layers and percolates (when available) were sampled (total volume of percolate was recorded). For each soil sample, pH (1M KCl, 1:6, v:v) was determined. Part of each soil sample was oven-dried at 105ºC to measure moisture content and stored at 4ºC for total metal extraction. The other portion was air-dried at room temperature and used for the determination of extractable metal concentrations.

Metal analysis over time

Soil and earthworm samples were homogenised using an acid-washed porcelain pestle and mortar and digested using PDS-6 pressure digestion systems (Loftfields analytical solutions, Neu Eichenberg, Germany). Samples of ≤ 100 mg were mixed with 2 ml of 69% HNO_3 (PA-ACS-ISO, Panreac, Barcelona, Spain) and left under pressure in PDS-6 systems at 150ºC for 10 h. The resulting solution was diluted with ultrapure water and transferred to a plastic vial to a final volume of 10 ml. The accuracy of metal analysis was checked using SRM 2709 (San Joaquin Soil - Standard Reference Material) certified by National Institute of Standards and Technology (Department of Commerce, USA) and DOLT-3 (Dogfish Liver Certified Reference Material for Trace Metals) certified by National Research Council Canada as reference material for soil and earthworms, respectively. The average recoveries for Cr, Cu, Ni, and Zn in soil were 82.9, 95.7, 98.5, and 105% and for earthworms, 106, 89.9, 117, and 93.4%. Extractable metal fractions were determined according to Houba et al. (2000). Twenty grams of air-dried soil samples (coarsely homogenised with a spoon) were extracted by shaking (2 h, 400 rpm) with 200 ml of 0.01M $CaCl_2$ (21074, Sigma-Aldrich, St Louis, USA) solution followed by filtration through Whatman No. 1 filter paper discs (Cat. No. 1001150, Maidstone, England).

The concentration of Cr, Cu, Ni, and Zn in HNO_3 and $CaCl_2$ extracts and in percolates was determined by flame AAS (2380 Absorption Atomic Spectrometer, Perkin-Elmer). For metal determinations in $CaCl_2$ extracts, wash solution, blanks and standards were prepared in 0.01M $CaCl_2$ solution.

Prediction of earthworm metal concentrations

Metal concentrations in earthworms of each test treatment were estimated with models from the literature using total metal concentrations in soil, and compared with the measured concentrations. This comparison aimed at evaluating the efficiency of existing models to predict metal concentrations in earthworms. Unfortunately, no such relationships were developed for *D. veneta*. We therefore selected the models developed by Neuhauser et al. (1995) for *Allolobophora tuberculata* for Cu, Ni, and Zn and by Peijnenburg et al. (1999) for *E. andrei* for Cr, Cu, and Zn (Table 6.2). For model validation, all measured values from all sampling dates were used, except for the Cr concentrations in control soil, which were not higher or equal to 0.2 mmol/kg, which is a requirement for the model proposed by Peijnenburg et al. (1999).

Table 6.2 Selected models from the literature used to predict earthworm metal concentrations from total metal concentrations in the soil.

Analyte	Model	R^2	Reference
Cu	$\text{Log}M_{ew} = 0.57\ \text{Log}M_s + 0.39$	0.67	Neuhauser et al. (1995)
Ni	$\text{Log}M_{ew} = 0.98\ \text{Log}M_s - 0.67$	0.66	Neuhauser et al. (1995)
Zn	$\text{Log}M_{ew} = 0.27\ \text{Log}M_s + 2.09$	0.35	Neuhauser et al. (1995)
Cr	$\text{Log}M_{ew} = 0.69\ \text{Log}M_s - 1.05$	0.61	Peijnenburg et al. (1999)
Cu	$\text{Log}M_{ew} = 0.25\ \text{Log}M_s - 0.54$	0.69	Peijnenburg et al. (1999)
Zn	$\text{Log}M_{ew} = 1.45\ \text{Log}M_s + 0.42$	0.83	Peijnenburg et al. (1999)

M_{ew} - metal concentration in earthworm (mg/kg dry body weight); M_s – total metal concentration in soil (mg/kg)

Statistical analysis

Regression analyses were performed to examine relationships of total, extractable, and percolate metal concentrations with time. All values were used including those below the detection limit when positive. For regression analysis, total metal concentrations were expressed as percentage of the initial concentration, available metal concentration (extracted with $CaCl_2$) as percentage of total metal concentration, and the amount of metal detected in percolates as percentage of the mean initial total amount of metal in both treated and untreated soil layers of microcosms. Regressions were also performed to find significant relationships between surviving earthworms and total and extractable metal concentrations, for both metal-spiked and sludge-amended soils.

Metal concentrations in earthworms for each sampling date (3, 6, and 12 weeks) were regressed against soil concentrations using the normal linear equation or the linear form of the Langmuir equation. The linear form of the Langmuir equation used reads:

$$C/X = (1/(n*k)) + (1/k)*C \quad (6.1)$$

where C is the metal concentration in earthworms (mg/kg), X is the total or extractable metal concentration in soil (mg/kg), n and k are constants of Langmuir equation.

The normality and homogeneity (using Kolmogorov–Smirnov and Bartlett tests, respectively) of variables was checked before regressions. When these assumptions were not fulfilled, values were arcsin transformed. The earthworm biomass production at different sampling dates was compared between treatments and the controls (Sw0 and Mw0) by one-way ANOVA followed by Dunnett's post hoc test. All analyses were performed using STATISTICA, version 6. The slopes obtained in regressions relating different metals of the

same variables (for total, available, and leached metals) for replicates with and without earthworms were compared. The slopes from regressions of bioaccumulated metals against total and extractable metal concentrations in soil for sludge-amended and metal-spiked treatments were also compared for samples taken at 3, 6, and 12 weeks. All the slope comparisons were performed by analysis of covariance (ANCOVAs) using Microsoft Office Excel version 2007. The level of statistical significance for all analyses was $\alpha = 0.01$.

To validate the prediction of earthworm metal concentrations, the differences between estimated and measured metal concentrations in earthworms were evaluated using Wilcoxon paired-sample test (Zar 1998). Relative accuracy of models was evaluated by calculating the percentage of over- and underestimated values. The slopes from regressions relating earthworm metal concentrations measured and estimated with total metal concentrations in soil were compared by ANCOVAs as described above. These regressions were performed for all sampling dates and separately for metal-spiked and sludge-amended treatments.

Results

Water content and pH of soil samples over the duration of the experiment

Water content of the top 10-cm soil layer did not change considerably over the duration of the experiment and ranged between 45 and 55% of the WHC for both microcosms with and without earthworms. In metal-spiked soils, pH was 4.4 ± 0.18 (mean ± SD, $n = 140$). In sludge-amended soils (S and Sw treatments) pH increased with increasing sludge concentration. The pH values of S/Sw0, S/Sw1, S/Sw2, S/Sw3, and S/Sw4 were 4.4 ± 0.1, 4.9 ± 0.06, 4.9 ± 0.04, 5.8 ± 0.01, 6.0 ± 0.08 (mean ± SD, $n = 28$), respectively.

Effect of earthworm activity on soil metal concentration over time

Total and extractable metal concentrations in soils and metal concentrations in percolates from microcosms without earthworms (the S and M treatments) were taken from Natal-de-Luz et al. (2011b – Chapter 5; data not shown).

Total metal concentrations in microcosms with earthworms (the Sw and Mw treatments) were higher in sludge-amended soils than in metal-spiked soils when comparing the same treatment level (see Table 6.3 for initial total metal concentrations and Table B.1 from Annex B for total metal concentrations over time – pages 215 to 217). In contrast, when considering the $CaCl_2$ extracts of the same microcosms, the inverse trend was observed (Tables 6.4 and B.2 – pages 218 to 220). Comparing the change over time (comparison of slopes) of total (Table B.3 – page 221) or extractable metal concentrations (Table B.4 – page 222), between replicates with and without earthworms, no significantly different patterns for any metal were found.

After three weeks of exposure, percolates were obtained from most microcosms with earthworms, except for Sw3 and Sw4 that did not produce percolates before week six. Only Ni and Zn were measured in percolate samples from microcosms with earthworms (Table B.5 – pages 223 to 225). Earthworm activity only affected the leaching of Ni and Zn in some metal-spiked soils. Leachability of Ni and/or Zn in percolates over time was significantly different between microcosms with and without earthworms (comparison of slopes) in the Mw1 (for both metals) and the Mw2 treatment (for Zn only). In the Mw1 treatment, Ni and Zn concentrations in percolates from microcosms with earthworms increased faster in time (higher slope) than in replicates without earthworms. In the Mw2 treatment, the increase of Zn concentrations in percolates was slower (lower slope; Table B.6 – page 226).

Table 6.3 Total Cr, Cu, Ni, and Zn concentrations (average ± SD, n = 4) in unamended soil (S/Sw0/M/Mw0) and soils amended with sludge (Sw1 – Sw4) or spiked with metals (Mw1 – Mw4) before introduction of the earthworms (*Dendrobaena veneta*).

Treatments	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
S/Sw0/M/Mw0	8.95 ± 3.69	19.6 ± 3.36	13.7 ± 2.97	55.9 ± 35.3
Sw1	57.2 ± 39.6	36.8 ± 13.0	141 ± 101	163 ± 78.1
Sw2	200 ± 78.2	67.8 ± 19.5	406 ± 126	335 ± 77.5
Sw3	265 ± 194	86.7 ± 43.8	511 ± 297	390 ± 170
Sw4	950 ± 198	215 ± 32.1	1389 ± 281	727 ± 60.9
Mw1	34.7 ± 2.25	24.8 ± 1.66	87.5 ± 13.9	117 ± 7.36
Mw2	49.6 ± 12.6	36.0 ± 3.75	134 ± 15.5	131 ± 14.1
Mw3	216 ± 63.0	85.6 ± 18.1	543 ± 81.6	421 ± 51.7
Mw4	335 ± 91.9	101 ± 19.7	599 ± 68.1	432 ± 46.6

Table 6.4 Initial 0.01M $CaCl_2$ extractable Cr, Cu, Ni, and Zn concentrations (average ± SD, n = 4) in unamended soil (S/Sw0/M/Mw0) and soils amended with sludge (Sw1 – Sw4) or spiked with metals (Mw1 – Mw4) before introduction of the earthworms (*Dendrobaena veneta*).

Treatments	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
S/Sw0/M/Mw0	< 0.249	< 0.349	< 0.427	< 0.108
Sw1	0.252 ± 0.075	< 0.349	1.80 ± 0.770	1.03 ± 0.655
Sw2	0.292 ± 0.079	< 0.349	3.20 ± 0.226	2.30 ± 0.371
Sw3	< 0.249	< 0.349	5.86 ± 0.803	1.03 ± 0.445
Sw4	< 0.249	< 0.349	8.89 ± 0.492	0.514 ± 0.027
Mw1	< 0.249	< 0.349	28.9 ± 2.79	21.7 ± 2.04
Mw2	< 0.249	< 0.349	43.6 ± 3.26	34.8 ± 2.94
Mw3	0.449 ± 0.099	1.09 ± 0.468	327 ± 23.2	244 ± 14.8
Mw4	1.06 ± 0.111	2.69 ± 0.067	652 ± 24.2	565 ± 19.1

Earthworm performance over the experiment

In most microcosms, at least two surviving earthworms (50% of the total) were found per sampling time. Exceptions were one replicate of Mw4 after three weeks, two replicates of Mw4 after six weeks and one replicate of Mw3 after 12 weeks, from which only one earthworm was found. In one replicate of Mw2 after six weeks, no surviving worms were found (Table B.7 – pages 227 to 229). Earthworm survival was significantly related to the total metal concentration in metal-spiked soils (Table B.8 – page 230), and also linearly related with the 0.01M $CaCl_2$ extractable Cr, Ni, and Zn concentrations (Table B.9 – page 230).
On average 20 to 60% of the surviving earthworms were found in the top 10-cm soil layer of the test treatments. The exceptions were the microcosms of Mw1, Mw3, and Mw4 treatments at all sampling dates and Sw1 and Sw3 treatments after 12 and three weeks, respectively (Table B.7 – pages 227 to 229).
The earthworm biomass did not significantly change over time in any treatment (data not shown).

Earthworm metal concentrations

In general, the metal concentrations in earthworms from metal-spiked and sludge-amended soils were in the same order of magnitude. Zinc had the highest concentrations in earthworm tissues, with average levels between 115 and 159 mg/kg DW (average values, n = 4), with no effect of treatment or time (Table B.10 – pages 231 to 233). The lowest metal concentrations were found for Cr, varying from lower than 2.80 to 14.8 mg/kg (average values, n = 4; Table B.10 – pages 231 to 233). Chromium concentrations in earthworms from sludge-amended soils increased linearly with total soil Cr concentration after six and 12 weeks (Table B.11 – page 234). The relationship (slope) between Cr concentrations in earthworms and soil was significantly different between metal-spiked and sludge-amended microcosms after six weeks of incubation only for

total Cr concentrations. Copper concentrations in earthworms were linearly related to total Cu concentrations after three weeks exposure in metal-spiked soils, after six and 12 weeks exposure in sludge-amended soils (Figure 6.1 and Table B.11 – page 234), and with extractable Cu concentrations after 12 weeks in metal-spiked soils (Figure 6.2 and Table B.12 – page 235). Earthworm Ni concentrations were linearly related to extractable and total Ni concentrations in soil (Figures 6.2 and 6.3; Tables B.11 and B.12 – pages 234 and 235). The exceptions were sludge-amended soils for total Ni concentrations after three weeks (not significant) and after 12 weeks, where the relationship between earthworm Ni concentration and total Ni concentration in soil was better described by the linear Langmuir equation. The slopes of relations between earthworm Ni concentrations and extractable Ni concentrations in soil were significantly different between sludge-amended and metal-spiked soils for all samplings. After three weeks of exposure significant differences between slopes were also found when using total Ni concentrations.

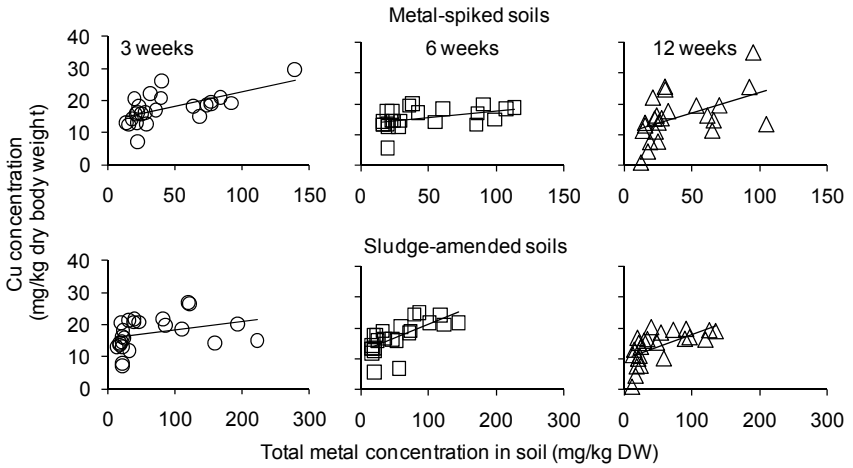

Figure 6.1 Cu concentrations in the earthworm *Dendrobaena veneta* as a function of the total concentration in metal-spiked (first row) and sludge-amended soils (second row) after 3, 6, and 12 weeks exposure (left, middle and right graphs, respectively). Lines show linear regressions.

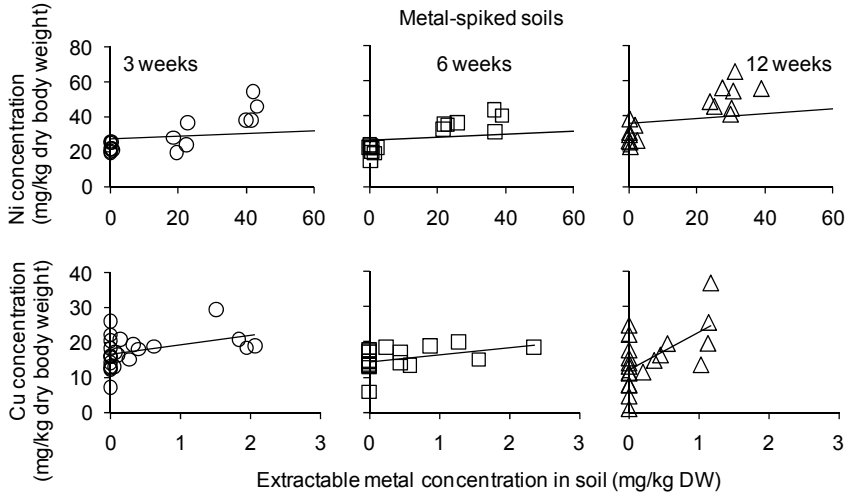

Figure 6.2 Ni and Cu concentrations in the earthworm *Dendrobaena veneta* (first and second row, respectively) as a function of 0.01M $CaCl_2$ extractable concentrations in metal-spiked soils after 3, 6, and 12 weeks exposure (left, middle and right graphs, respectively). Lines show linear regressions.

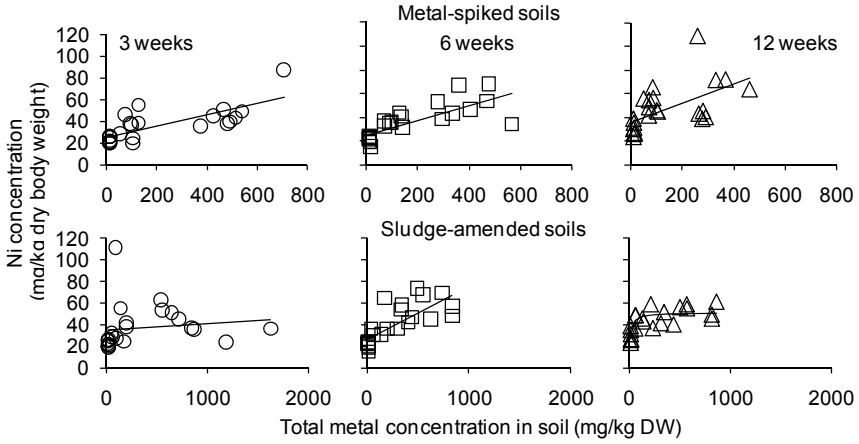

Figure 6.3 Ni concentrations in the earthworm *Dendrobaena veneta* as a function of the total concentration in metal-spiked (first row) and sludge-amended soils (second row) after 3, 6, and 12 weeks exposure (left, middle and right graphs, respectively). Lines show linear regressions. In the graph of Ni concentrations from sludge-amended soils after 12 weeks (right graph from second row), line was fit using the Langmuir equation.

Prediction of earthworm metal concentrations

Metal concentrations in earthworms generally were not accurately estimated by the models of Neuhauser et al. (1995) and Peijnenburg et al. (1999; Table 6.2). When comparing the slopes of regressions of measured and estimated metal concentrations in the earthworms against total soil concentrations, significant differences were found, except for Cr in sludge-amended soils, using the relationship reported by Peijnenburg et al. (1999). Significant differences were found between measured and estimated values when the Wilcoxon paired-sample test was performed for all models tested, except for Ni in both Sw and Mw treatments. Overestimations generated by the models from Peijnenburg et al. (1999) for Zn were, on average, 6761 and 3850% in Sw and Mw treatments, respectively. Chromium and Cu concentrations were underestimated by 39.8 and 94.3% in sludge-amended soils and by 7.28 and 94.5% in spiked soils, respectively. For Cu, Ni, and Zn in sludge-amended soils, the models from Neuhauser et al. (1995) overestimated earthworm Cu, Ni, and Zn concentrations by 57.4, 25.9, and 273%, respectively. In spiked soils, on average, Cu and Zn concentrations in earthworms were overestimated by 42.6 and 249%, respectively, and Ni concentrations were underestimated by 27.3%.

Discussion

Influence of earthworm activity in metal development over time

When compared to a similar study without the presence of earthworms (Natal-da-Luz et al. 2011b – Chapter 5), the present study demonstrates that earthworm activity at realistic densities did not influence total or extractable metal concentrations over time in any treatment. Our first working hypothesis was not confirmed, which suggests that the consumption of sludge OM by the earthworms over 12 weeks of incubation did not significantly reduce its binding

capacity for metals. Several studies have reported the impact of earthworms on metal partitioning in soils. For the present discussion we primarily focused on studies using *D. veneta*, but when such studies were not available in the literature we compared our results with experiments using epigeic earthworm species. Although epigeic species may differ in terms of feeding selectivity, their behaviour is more comparable with that of *D. veneta*. Sizmur and Hodson (2008) evaluated the impact of *E. veneta* (synonym for *Dendrobaena veneta*; Blakemore 2007) on metal mobility in natural soils contaminated with Cu, Pb, and Zn from different sources, using 20 earthworms per 150 g of soil. After 17 days of exposure, they did not find differences between total metal concentrations of soils with and without earthworms. Water-extractable Zn concentrations were variably influenced by earthworm activity depending on the metal source, and extractable Cu concentrations decreased with earthworm activity only in two out of the three soils tested. Wen at al. (2004), investigating the influence of the activity of *E. fetida* on metal availability (including Cr, Cu, Ni, and Zn) on five Chinese cultivated soils found increases in water-extractable metals in soils with earthworms after six weeks with 12 earthworms per kg of soil. These studies suggest a high susceptibility of Cr, Cu, Ni, and Zn behaviour to be influenced by earthworm activity within a short-term period. In both studies however, a much higher earthworm density was used than the four earthworms per 2.5 kg of soil (considering a bulk density of 1.5 g/cm^3) in our study, which corresponds to a density of 500 earthworms per m^2. An impact of earthworm activity on metal availability would therefore be expected, although it might not yet be visible after only 12 weeks of incubation. Epigeic species have been used to determine the effect of earthworm activity on metal extractability using earthworm densities higher than that of the present study. These studies usually report increases in metal availability in the presence of earthworms (Sizmur and Hodson 2009). However, we believe that using a realistic earthworm density increases the ecological relevance of our results.

The only difference in metal behaviour between treatments with and without earthworms was in the Ni and Zn concentrations in percolates from the lowest concentrations of spiked soils over time. These differences might be related to the burrowing activities of the earthworms. As discussed by Natal-da-Luz et al. (2011b – Chapter 5), the depth of the soil column in the microcosms would not be enough to allow the vertical transport by rainwater of metals through the soil matrix and reach the percolate in any treatment. Since increased levels of Ni and Zn were found in percolate samples, it is likely that some rain passed via direct and/or partially direct pathways without passing the soil matrix. It is likely that the burrowing activity of the earthworms, which may enhance soil porosity (Lavelle and Spain 2001), influenced the transport of rain water along the soil columns. Considering this, the same influence of earthworm activity would be expected for Ni and Zn concentrations in percolates from replicates with the highest concentrations of spiked soil over time (Mw3 and Mw4). However, it has to be taken into account that in these treatments, all earthworms avoided the spiked soil layer (top 10 cm) at all sampling dates. The reduced or absent earthworm activity in the top 10-cm layer of these treatments might explain for the absence of an enhanced metal leaching when compared to similar treatments without worms. For sludge-amended soils, the absence of an influence of earthworm activity on the development of Ni and Zn concentrations in percolates over time is probably due to the high stability of metals adsorbed to the sludge matrix.

Earthworm survival and avoidance behaviour

Earthworm survival was higher in sludge-amended than in metal-spiked soils, and linearly related to both total and extractable metal concentrations (except for 0.01M $CaCl_2$-extractable Cu concentrations). Total metal concentrations were highest in sludge-amended soils, but extractable metal concentrations were highest in metal-spiked soils. This suggests that extractable metal concentrations

best explained earthworm survival. Unfortunately, no lethal or sublethal effect concentrations of Cr, Cu, and Zn to the earthworm *D. veneta* are available in the literature. For Ni, a 28-d LC_{50} of 684 mg/kg DW was reported by Scott-Fordsmand et al. (1998) using Lufa 2.2 soil. Bengtsson et al. (1986) found high mortality of *Dendrobaena rubida* (> 80%) after exposure periods longer than one month in a natural soil mixed with decomposed cattle dung (1:2, v:v) spiked with copper nitrate at 100 and 500 mg Cu/kg. Spurgeon et al. (1994) reported 56-d LC_{50} values for *E. fetida* of 555 mg Cu/kg and 745 mg Zn/kg in OECD artificial soil, while 14-day LC_{50} values of 643 mg Cu/kg, 757 mg Ni/kg, and 662 mg Zn/kg DW artificial soil were found by Neuhauser et al. (1985). The 21-d LC_{50} values determined for zinc nitrate by Spurgeon and Hopkin (1996) and zinc chloride by Lock and Janssen (2003) using *E. fetida* in OECD artificial soil were 791 and 1349 mg Zn/kg, respectively. For Cr(III), no mortality of *E. andrei* was found after 3 weeks at 1000 mg/kg (Van Gestel et al. 1992) and for *E. fetida* at 1800 mg/kg after 3 weeks in OECD artificial soil (Lock and Janssen 2002). Taking into account that some of these toxicity tests used soils with higher OM content than our test soil, and assuming additive effects resulting from the pool of metals tested, these LC_{50} values support the lower number of surviving earthworms in the highest metal-spiked treatments. This finding also agrees with the 28-d LC_{50} we found for *E. andrei* in the same metal-spiked test soil, which corresponded to a mixture composed of total Cr, Cu, Ni, and Zn concentrations of 186, 79.3, 505, and 397 mg/kg, respectively (corresponding to 3.65 toxic units based on literature data on reproduction toxicity for *E. fetida*; Natal-da-Luz et al. 2011a – Chapter 4).

The avoidance by the earthworms of the treated top soil layer in the Mw3 and Mw4 microcosms might be caused by the high availability of metals as shown by the 0.01M $CaCl_2$ extractable concentrations. Marinussen et al. (1997a) demonstrated that *D. veneta* in a field experiment was able to avoid the most heavily Cu, Pb, and Zn contaminated areas. Lukkari and Haimi (2005), evaluating the avoidance behaviour of *A. tuberculata*, *Lumbricus rubellus*, and

Dendrobaena octaedra in a concentration gradient of a field soil (13–14% OM) simultaneously spiked with copper and zinc chlorides, found that *D. octaedra* was the most sensitive species after a 48-h exposure. More than 80% of the *D. octaedra* avoided total Cu/Zn concentrations of 44/101 mg/kg, while 100% avoidance was observed at 231/420 mg/kg. More than 80% of the *A. tuberculata* and *L. rubellus* avoided soils with total Cu/Zn concentrations up to 100/203 and 231/420 mg/kg, respectively. These data suggest a high ability of species from the genus *Dendrobaena* to detect and avoid low Cu and Zn concentrations, supporting the avoidance behaviour found in microcosms spiked with the highest metal concentrations.

Earthworm metal concentrations in sludge-amended and metal-spiked soils

Our second working hypothesis was not confirmed since, although metal availability was higher in spiked soils than in sludge-amended soils, the metal concentrations in earthworms from metal-spiked and sludge-amended soils were generally in the same order of magnitude. The regressions obtained demonstrate that for most metals tested, both total and extractable concentrations could explain metal uptake by the earthworms. This fact not only supports findings obtained by Van Gestel (2008) who concluded that in many cases total metal concentrations adequately describe earthworm body concentrations but also agrees with Vijver et al. (2003), who demonstrated that metal accumulation in earthworms is highly dependent on metal in soil solution essentially due to dermal uptake.

The concentration of Zn in the earthworms was not dependent on soil concentration, which demonstrates the ability of *D. veneta* to internally regulate Zn body burdens, at least up to the concentrations tested. The positive correlations of Cu and Ni concentration in earthworms with total and extractable metal concentrations in sludge-amended and metal-spiked soils suggest an inability of *D. veneta* to regulate these two metals over the range of

concentrations tested. In two experiments conducted by Marinussen et al. (1997a, 1997c), Cu concentration in *D. veneta* exposed for two weeks to a gradient of natural contaminated soils was linearly related to total soil concentrations below 150 mg Cu/kg, and a plateau concentration of 57–60 mg Cu/kg was achieved in the earthworms. In the present study, a significant linear relationship between earthworm and soil Cu concentrations was evident in half of the treatments. Although total Cu concentration in metal-spiked soils was always lower than 150 mg Cu/kg, a significant linear relationship was detected only after three weeks. In sludge-amended soils, the total Cu concentration was, on average, higher than 150 mg Cu/kg in Sw3 after three weeks of incubation, but Cu concentrations in the earthworms did not reach a steady state and were always below 57–60 mg Cu/kg. Another experiment performed by Marinussen et al. (1997b) evaluated the influence of Cd and Pb on the bioaccumulation of Cu by *D. veneta*. Earthworms were exposed for 21 days to a natural soil containing about 250 mg Cu/kg and simultaneously spiked with concentration gradients of Cd or Pb. Concentrations above 60 mg Cu/kg were detected in earthworms in all treatments and the steady state observed in the previous studies was never reached. These data do not contradict our results, since although we never measured earthworm Cu concentrations higher than 60 mg/kg in sludge-amended or metal-spiked soils, total Cu concentrations in soil were always lower than 250 mg Cu/kg.

Different Cu levels in *D. veneta* were found by Ireland (1979) who compared, in a field study, the metal concentrations (including Cu and Zn) in three native earthworm species occurring in a sewage sludge-amended area containing 252 ± 5 and 992 ± 53 mg/kg of Cu and Zn, respectively. This author found 134 ± 5 mg Zn/kg in *D. veneta*, which is similar to the levels found in the present study. The Cu concentration measured in that study of 14 ± 2 mg Cu/kg is close to Cu levels measured in earthworms taken from control microcosms after three and six weeks of incubation. These findings also suggest that *D. veneta* is able to regulate Zn. On the other hand, the Cu levels measured in earthworms by Ireland

(1979) suggest a regulation of Cu by *D. veneta*, which is inconsistent with our results and with data from Marinussen et al. (1997a, 1997b, 1997c).

The accumulation of Cr by *D. veneta* was independent of soil Cr concentration in metal-spiked soils. However, in sludge-amended soils, Cr accumulation was linearly and positively related to total Cr concentrations in soil after six and 12 weeks of exposure. These values and the significantly different slopes obtained when regressing Cr concentrations in earthworms against Cr concentrations in soil for sludge-amended and spiked soils, suggest that the source of metal contamination and test matrix affect the uptake of Cr by *D. veneta*. There are no studies reporting the bioaccumulation of Cr in *D. veneta*. Van Gestel et al. (1993) exposed *E. andrei* to artificial soil spiked with chromium nitrate for three weeks and found a dose-related increase of Cr levels in the earthworms up to a total soil concentration of 100 mg Cr/kg. These data are not in agreement with Cr levels found in *D. veneta* from microcosms with metal-spiked soils, which were not linearly related with total Cr concentration in soil. On the other hand, Cr concentrations in *E. andrei* are in agreement with our measurements in *D. veneta* from sludge-amended soils.

Concerning the Ni concentrations measured in *D. veneta*, a comparable bioaccumulation level was described by Laskowski et al. (2010). Uptake of Ni was measured in juvenile *L. terrestris* exposed to a natural soil spiked with nickel chloride at 140 mg Ni/kg over a period of 168 h (1 week). These authors found an initial high Ni concentration in *L. terrestris* (up to > 300 mg/kg) after less than two days of exposure, after which the earthworms switched to constant internal concentration between 51 and 64 mg Ni/kg until the end of exposure. In the present study, earthworms were sampled in the latter stage of the uptake phase in which they are able to maintain a steady internal Ni concentration. This justifies the similar internal Ni levels reported in several treatments of our study, which were similar to those measured by Laskowski et al. (2010; 51 – 64 mg Ni/kg).

Prediction of earthworm metal concentrations

Chromium, Cu, Ni, and Zn concentrations in earthworms estimated by the models developed by Neuhauser et al. (1995) and Peijnenburg et al. (1999) were not validated by our data. Our third working hypothesis was also not confirmed. The closest estimates were generally obtained with the models of Neuhauser et al. (1995). The closest estimated concentration was generated for Cr by the model of Peijnenburg et al. (1999) when applied to sludge-amended soils. Estimations were worst for Zn uptake, which may be related to the ability of *D. veneta* to regulate Zn body concentrations to a fairly constant level.

The validation attempt shows the need for further studies to increase the accuracy of bioaccumulation models to estimate metal uptake in earthworms. We believe that the inclusion of some variables such as soil properties and pH can improve predictions. However, our results suggest that physiological mechanisms specifically used by each earthworm species to regulate internal metal levels are eventually the main factor affecting the accuracy of predictions, suggesting that the development of more species-specific models would be desirable.

Conclusions

The results obtained in the present study demonstrate that the activity of a realistic density of the earthworm *D. veneta* did not influence metal availability in soil over time after an incubation period of 12 weeks, in either sludge-amended or metal-spiked soils. Nickel and Zn concentrations of percolates from metal-spiked microcosms with and without earthworms suggest that the burrowing activity of earthworms may affect metal behaviour in soil. Only Cr and Ni concentrations in the earthworms were influenced by the matrices tested. In earthworms from sludge treatments, internal Cr concentrations were not regulated, which seem to be related to the metal source and matrix. For Ni,

significantly different uptake dynamics were detected in sludge-amended and spiked soils especially considering 0.01M $CaCl_2$-extractable metal levels. The models relating earthworm body concentrations and soil concentrations tested were not able to predict metal concentrations in the earthworms from all treatments, especially for the metals that were regulated by *D. veneta* (e.g. Zn).

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Annex B

Table B.1 Total Cr, Cu, Ni, and Zn concentrations (average ± SD, $n = 4$) in unamended soils (Sw0/Mw0) and in sludge-amended (Sw1 - Sw4) or metal-spiked (Mw1 - Mw4) soils after 3, 6, and 12 weeks of incubation in microcosms with earthworms (*Dendrobaena veneta*).

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
3 weeks	Sw0/Mw0	7.47 ± 0.753	20.8 ± 2.98	12.8 ± 1.20	44.7 ± 6.26
	Sw1	32.5 ± 15.8	23.3 ± 6.01	82.6 ± 41.1	72.0 ± 20.6
	Sw2	70.7 ± 9.82	39.6 ± 5.93	163 ± 48.7	162 ± 45.7
	Sw3	711 ± 342	165 ± 58.6	1057 ± 464	701 ± 301
	Sw4	460 ± 106	108 ± 18.5	689 ± 123	488 ± 67.4
	Mw1	36.9 ± 11.6	27.1 ± 8.54	90.8 ± 24.1	91.1 ± 36.2
	Mw2	48.2 ± 16.7	33.0 ± 9.00	106 ± 25.9	167 ± 98.0
	Mw3	167 ± 15.6	71.5 ± 6.49	459 ± 59.0	365 ± 43.7
	Mw4	318 ± 78.7	97.2 ± 29.0	541 ± 120	402 ± 70.9

Table B.1 (Continued)

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
6 weeks	Sw0/Mw0	5.75 ± 1.72	19.1 ± 3.66	13.1 ± 2.20	53.6 ± 9.19
	Sw1	61.3 ± 56.3	33.2 ± 17.3	146 ± 137	163 ± 105
	Sw2	210 ± 154	70.1 ± 23.5	382 ± 182	328 ± 113
	Sw3	240 ± 148	62.8 ± 24.7	388 ± 169	325 ± 116
	Sw4	510 ± 176	114 ± 30.6	713 ± 185	504 ± 98.6
	Mw1	35.0 ± 4.88	26.0 ± 3.18	82.0 ± 14.8	131 ± 19.6
	Mw2	48.6 ± 6.18	37.5 ± 3.67	129 ± 11.0	160 ± 18.4
	Mw3	176 ± 41.8	71.6 ± 16.4	385 ± 133	335 ± 72.9
	Mw4	304 ± 62.3	102 ± 9.81	409 ± 72.8	349 ± 57.1

Table B.1 (Continued)

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
12 weeks	Sw0/Mw0	6.22 ± 1.08	18.8 ± 3.29	13.0 ± 2.10	38.3 ± 6.50
	Sw1	117 ± 92.0	46.8 ± 23.3	242 ± 179	216 ± 131
	Sw2	83.2 ± 49.9	38.3 ± 16.1	185 ± 111	201 ± 111
	Sw3	184 ± 145	62.2 ± 36.8	336 ± 240	282 ± 171
	Sw4	512 ± 76.9	118 ± 18.7	770 ± 133	531 ± 48.0
	Mw1	38.6 ± 6.44	26.4 ± 4.47	83.0 ± 14.4	97.3 ± 34.6
	Mw2	31.7 ± 10.2	24.0 ± 7.26	77.9 ± 23.2	73.2 ± 38.0
	Mw3	157 ± 12.3	61.2 ± 5.79	274 ± 14.6	245 ± 13.0
	Mw4	251 ± 73.0	90.4 ± 14.7	359 ± 77.2	313 ± 69.8

Table B.2 $CaCl_2$ (0.01M) extractable Cr, Cu, Ni, and Zn concentrations (average ± SD, n = 4) in unamended soil (Sw0/Mw0) and in sludge-amended (Sw1 - Sw4) and metal-spiked (Mw1 - Mw4) soils after 3, 6, and 12 weeks of incubation in microcosms with earthworms (*Dendrobaena veneta*).

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
3 weeks	Sw0/Mw0	< 0.249	< 0.349	0.501 ± 0.461	< 0.108
	Sw1	< 0.249	< 0.349	2.29 ± 0.904	1.04 ± 0.210
	Sw2	< 0.249	< 0.349	3.40 ± 0.362	1.79 ± 0.541
	Sw3	< 0.249	< 0.349	6.00 ± 0.525	0.911 ± 0.252
	Sw4	< 0.249	< 0.349	6.87 ± 0.221	0.456 ± 0.102
	Mw1	< 0.249	< 0.349	20.8 ± 2.03	11.5 ± 1.31
	Mw2	< 0.249	< 0.349	41.6 ± 1.30	31.8 ± 2.47
	Mw3	0.379 ± 0.056	0.405 ± 0.153	202 ± 29.9	155 ± 22.2
	Mw4	0.564 ± 0.033	1.84 ± 0.241	349 ± 35.8	301 ± 32.0

Table B.2 (Continued)

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
6 weeks	Sw0/Mw0	< 0.249	< 0.349	0.428 ± 0.707	< 0.108
	Sw1	< 0.249	< 0.349	2.78 ± 0.475	0.799 ± 0.114
	Sw2	< 0.249	< 0.349	3.31 ± 0.155	1.68 ± 0.108
	Sw3	< 0.249	< 0.349	5.92 ± 0.407	0.764 ± 0.139
	Sw4	< 0.249	< 0.349	8.73 ± 0.633	0.524 ± 0.053
	Mw1	< 0.249	< 0.349	23.1 ± 1.99	15.5 ± 2.24
	Mw2	< 0.249	< 0.349	37.7 ± 1.02	29.3 ± 1.75
	Mw3	0.297 ± 0.076	0.423 ± 0.144	253 ± 24.8	167 ± 27.5
	Mw4	0.582 ± 0.152	1.52 ± 0.625	333 ± 108	248 ± 99.9

Table B.2 (Continued)

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
12 weeks	Sw0/Mw0	< 0.249	< 0.349	< 0.427	< 0.108
	Sw1	< 0.249	< 0.349	2.43 ± 0.164	1.67 ± 0.320
	Sw2	< 0.249	< 0.349	3.89 ± 0.670	2.49 ± 0.215
	Sw3	< 0.249	< 0.349	5.61 ± 0.606	0.774 ± 0.270
	Sw4	< 0.249	< 0.349	6.81 ± 0.806	0.651 ± 0.163
	Mw1	< 0.249	< 0.349	26.9 ± 3.03	16.4 ± 1.32
	Mw2	< 0.249	< 0.349	32.7 ± 4.32	22.9 ± 1.78
	Mw3	< 0.249	0.390 ± 0.149	205 ± 40.1	151 ± 40.7
	Mw4	0.562 ± 0.110	1.12 ± 0.061	198 ± 28.1	163 ± 19.7

Table B.3 Regression slopes for total Cr, Cu, Ni, and Zn concentrations as percentage of initial doses (% initial TMC) against time for different metals in unamended (S0 or M0), sludge-amended (S1 – S4) and metal-spiked soils (M1 – M4) in microcosms without earthworms (data taken from Natal-da-Luz et al. 2011b - Chapter 5) and in unamended (Sw0 or Mw0), sludge-amended (Sw1 – Sw4) and metal-spiked soils (Mw1 – Mw4) in microcosms with the earthworms *Dendrobaena veneta.*

		Time (independent variable)									
		Treatments without earthworms					Treatments with earthworms				
	Metal	S0	S1	S2	S3	S4	Sw0	Sw1	Sw2	Sw3	Sw4
% initial TMC (d.v.)	Cr	-0.038[a]*	-3.30	-3.76	-4.17	-0.030[a]	-0.031[a]	10.3	-0.058[a]	-0.074[a]	-2.92
	Cu	-0.005[a]	-3.08	-2.82	-3.52	-0.038[a]	-0.007[a]	3.29	-0.038[a]	-0.056[a]	-2.89
	Ni	-0.002[a]	-3.74	-2.14	-3.06	-0.030[a]	-0.002[a]	7.33	-0.054[a]	-0.074[a]	-2.85
	Zn	-1.69	-3.20	-2.28	-1.90	-0.019[a]	-2.02	4.04	-0.032[a]	-0.056[a]	-1.67
	Metal	M0	M1	M2	M3	M4	Mw0	Mw1	Mw2	Mw3	Mw4
% initial TMC (d.v.)	Cr	-0.038[a]*	0.009[a]	-0.022[a]	-0.937	-0.014[a]	-0.031[a]	0.008[a]	-0.038[a]	-0.012[a]	-0.025[a]
	Cu	-0.005[a]	0.007[a]	-0.030[a]*	-0.011[a]	-0.413	-0.007[a]	0.004[a]	-0.034[a]	-0.025[a]	-0.757
	Ni	-0.002[a]	-0.007[a]	-0.042[a]*	-0.052[a]*	-0.054[a]*	-0.002[a]	-0.610	-3.03*	-0.057[a]*	-0.045[a]*
	Zn	-1.69	0.003[a]	-0.056[a]*	-0.035[a]*	-0.031[a]*	-2,02	-0.010[a]	-0.053[a]	-0.044[a]*	-0.029[a]*

* - statistically significant ($p \leq 0.01$); slopes were obtained using normal linear equation except those with [a] that were obtained using the exponential equation.

Table B.4 Regression slopes for 0.01M $CaCl_2$ extractable Cr, Cu, Ni, and Zn concentrations as percentage of total metal concentration (% TMC) against time for different metals in unamended (S0 or M0), sludge-amended (S1 – S4) and metal-spiked soils (M1 – M4) in microcosms without earthworms (data taken from Natal-da-Luz et al. 2011b - Chapter 5) and in unamended (Sw0 or Mw0), sludge-amended (Sw1 – Sw4) and metal-spiked soils (Mw1 – Mw4) in microcosms with the earthworms *Dendrobaena veneta.*

		Time (independent variable)									
		Treatments without earthworms					Treatments with earthworms				
	Metal	S0	S1	S2	S3	S4	Sw0	Sw1	Sw2	Sw3	Sw4
% TMC (d.v.)	Cr	0.015	0.018[a]	0.007[a]	-0.003	0.106[a]	0.036	-0.033	0.009	0.010	0.002
	Cu	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*
	Ni	-0.054	0.088[a]	0.055[a]	0.007[a]	-0.004[a]	0.491[b]	-0.008[a]	0.069[a]	0.308[b]	0.021[a]
	Zn	-0.115[b]	0.060[a]	0.035[ab]	-0.007[a]	-0.025[a]	-0.241[b]	0.027	0.535[b]	0.032[a]	0.042[a]*
	Metal	M0	M1	M2	M3	M4	Mw0	Mw1	Mw2	Mw3	Mw4
% TMC (d.v.)	Cr	0.015	-0.010	0.008	-0.046[a]	-0.023[a]	0.036	-0.010	-0.002	-0.036[a]	-0.006
	Cu	*ND*	*ND*	*ND*	-0.030[ab]	-0.066[a]*	*ND*	*ND*	*ND*	-0.041	-0.119*
	Ni	-0.054	-0.437	0.590	0.365	-0.060[a]*	0.491[b]	0.007[a]	0.874	1.79	-0.046[a]
	Zn	-0.115[b]	-0.706	0.571	-0.015[a]	-0.087[a]*	-0.241[b]	0.064	1.01	0.715	-0.068[a]*

* - statistically significant ($p \leq 0.01$); slopes were obtained using normal linear equation except those with [a] that were obtained using the exponential equation; [b] - using values arcsin transformed; *ND* - not determined.

Table B.5 Percolate volumes and Ni and Zn concentrations (average ± SD, $n = 4$) in the percolates collected from unamended soil (Sw0/Mw0) and sludge-amended (Sw1 – Sw4) and metal-spiked (Mw1 – Mw4) soils after 3, 6, and 12 weeks of incubation with earthworms (*Dendrobaena veneta*).

Incubation period	Treatment	Percolate volume (ml)	Ni (mg/L)	Zn (mg/L)
3 weeks	Sw0/Mw0	14.3 ± 4.57	0.026 ± 0.009	0.073 ± 0.021
	Sw1	18.2 ± 1.04[a]	0.063 ± 0.009[a]	0.023 ± 0.016
	Sw2	20.0 ± 0.816	0.069 ± 0.025	0.015 ± 0.010
	Sw3	0	-	-
	Sw4	0	-	-
	Mw1	15.0 ± 0.816	0.060 ± 0.009	0.037 ± 0.038
	Mw2	15.0 ± 3.56	1.45 ± 0.452	0.881 ± 0.308
	Mw3	18.2 ± 1.04[a]	3.11 ± 2.63[a]	1.52 ± 1.28
	Mw4	20.0 ± 0.816	3.74 ± 3.29	1.85 ± 1.89

Table B.5 (Continued)

Incubation period	Treatment	Percolate volume (ml)	Ni (mg/L)	Zn (mg/L)
6 weeks	Sw0/Mw0	126 ± 4.49	0.036 ± 0.016	0.008 ± 0.004
	Sw1	114 ± 0.888	0.031 ± 0.018	0.047 ± 0.072
	Sw2	113 ± 18.2	0.103 ± 0.029	0.031 ± 0.020
	Sw3	56.4 ± 0.566[b]	0.056 ± 0.027[b]	0.011 ± 0.013
	Sw4	29.0 ± 23.2	0.202 ± 0.090	0.182 ± 0.185
	Mw1	103 ± 0.957	0.038 ± 0.031	0.016 ± 0.011
	Mw2	75.0 ± 18.6	0.699 ± 0.264	0.377 ± 0.231
	Mw3	104 ± 0.957	1.40 ± 0.764	0.702 ± 0.445
	Mw4	111 ± 7.74	1.93 ± 1.42	1.07 ± 0.868

Table B.5 (Continued)

Incubation period	Treatment	Percolate volume (ml)	Ni (mg/L)	Zn (mg/L)
12 weeks	Sw0/Mw0	296 ± 9.79	0.029 ± 0.014	0.022 ± 0.015
	Sw1	277 ± 0.830	0.047 ± 0.022	0.017 ± 0.009
	Sw2	274 ± 8.54	0.116 ± 0.044	0.107 ± 0.031
	Sw3	186 ± 0.816	0.167 ± 0.069	0.050 ± 0.025
	Sw4	144 ± 19.7	0.218 ± 0.034	0.334 ± 0.115
	Mw1	280 ± 0.816	0.233 ± 0.133	0.065 ± 0.031
	Mw2	259 ± 14.9	0.299 ± 0.121	0.220 ± 0.073
	Mw3	283 ± 0.816	3.20 ± 0.515	1.54 ± 0.511
	Mw4	289 ± 13.1	5.30 ± 2.92	2.85 ± 1.60

[a] – $n = 3$; [b] – $n = 2$

Table B.6 Linear regression slopes for Ni and Zn concentrations in percolates as percentage of total metal concentration (% TMC) against time for unamended soil (M or Mw0) and metal-spiked soils in microcosms without (M1 – M4; data taken from Natal-da-Luz et al. 2011b - Chapter 5) and with the earthworms *Dendrobaena veneta* (Mw1 – Mw4).

		Time (independent variable)									
		Metal-spiked soils without earthworms					Metal-spiked soils with earthworms				
	Metal	M0	M1	M2	M3	M4	Mw0	Mw1	Mw2	Mw3	Mw4
% TMC (d.v.)	Ni	0.229[a]*	0.000	0.002*	0.012	0.480[a]*	0.002*	0.005*	0.004*	0.021*	0.032*
	Zn	0.022[a]	0.000	0.052[a]*	0.007*	0.432[a]*	0.000*	0.166[a]*	0.002*	0.011*	0.706[a]*

* - statistically significant ($p \leq 0.01$); [a] – using values arcsin transformed.

Table B.7 Survival of the earthworm *Dendrobaena veneta* in microcosms with a 10-cm top layer of either unamended soil (Sw0/Mw0), sludge-amended (Sw1–Sw4), or metal-spiked soils (Mw1–Mw4) after 3, 6, and 12 weeks of exposure. Also indicated is the distribution of the surviving worms between the top 10-cm soil layer (Layer *a*) and deeper soil (Layer *b*), which may indicate avoidance behaviour. All values are mean ± SD ($n = 4$).

Incubation period	Treatment	Survival (%)	Depth distribution of surviving worms (%)	
			Layer *a*	Layer *b*
3 weeks	Sw0/Mw0	93.8 ± 17.7	25.0 ± 23.1	75.0 ± 23.1
	Sw1	75.0 ± 35.4	31.3 ± 47.3	68.8 ± 47.3
	Sw2	87.5 ± 25.0	37.5 ± 30.6	62.5 ± 30.6
	Sw3	93.8 ± 12.5	18.8 ± 23.9	81.3 ± 23.9
	Sw4	100 ± 0.00	71.9 ± 32.9	28.1 ± 32.9
	Mw1	81.3 ± 23.9	9.38 ± 12.0	90.6 ± 12.0
	Mw2	100 ± 0.00	34.4 ± 31.3	65.6 ± 31.2
	Mw3	50.0 ± 0.00	0	100 ± 0.00
	Mw4	75.0 ± 35.4	0	100 ± 0.00

Table B.7 (Continued)

Incubation period	Treatment	Survival (%)	Depth distribution of surviving worms (%)	
			Layer *a*	Layer *b*
6 weeks	Sw0/Mw0	90.6 ± 12.9	40.6 ± 17.1	59.4 ± 17.1
	Sw1	75.0 ± 35.4	33.3 ± 47.1	66.7 ± 47.1
	Sw2	100 ± 0.00	43.8 ± 23.9	56.3 ± 23.9
	Sw3	81.3 ± 37.5	50.0 ± 40.8	50.0 ± 40.8
	Sw4	100 ± 0.00	31.2 ± 23.9	68.8 ± 23.9
	Mw1	75.0 ± 20.4	15.6 ± 23.7	84.4 ± 23.7
	Mw2	75.0 ± 50.0	33.3 ± 14.4[a]	66.7 ± 14.4[a]
	Mw3	75.0 ± 20.4	0	100 ± 0.00
	Mw4	62.5 ± 43.3	0	100 ± 0.00

Table B.7 (Continued)

Incubation period	Treatment	Survival (%)	Depth distribution of surviving worms (%)	
			Layer *a*	Layer *b*
12 weeks	Sw0/Mw0	87.5 ± 18.9	55.2 ± 25.1	44.8 ± 25.1
	Sw1	75.0 ± 28.9	15.6 ± 23.7	84.4 ± 23.7
	Sw2	75.0 ± 35.4	46.9 ± 43.8	53.1 ± 43.8
	Sw3	93.8 ± 12.5	31.2 ± 47.3	68.8 ± 47.3
	Sw4	100 ± 0.00	65.6 ± 27.7	34.4 ± 27.7
	Mw1	68.8 ± 23.9	0	100 ± 0.00
	Mw2	93.8 ± 12.5	38.5 ± 19.7	61.5 ± 19.7
	Mw3	62.5 ± 32.3	0	100 ± 0.00
	Mw4	62.5 ± 14.4	0	100 ± 0.00

[a] - $n = 3$

Table B.8 Linear regression slopes for earthworm (*Dendrobaena veneta*) survival (%) against total concentrations of different metals after 3 to 12 weeks incubation in sludge-amended (Sw) and metal-spiked soils (Mw).

	Treatment	Total metal concentrations (independent variable)			
		Cr	Cu	Ni	Zn
% earthworm survival (dependent variable)	Sw	0.001	0.003	0.000	0.000
	Mw	-0.004*	-0.013*	-0.002*	-0.003*

* - statistically significant

Table B.9 Linear regression slopes for earthworm (*Dendrobaena veneta*) survival (%) against 0.01 M $CaCl_2$-extractable concentrations of different metals after 3 to 12 weeks incubation in sludge-amended (Sw) and metal-spiked soils (Mw).

	Treatment	Extractable metal concentrations (independent variable)			
		Cr	Cu	Ni	Zn
% earthworm survival (dependent variable)	Sw	5.72	*ND*	0.839	-5.92
	Mw	-38.3*	-9.60	-0.068*	-0.083*

* - statistically significant; *ND* - not determined.

Table B.10 Cr, Cu, Ni, and Zn concentrations in the earthworm *Dendrobaena veneta* collected after 3, 6, and 12 weeks of exposure in microcosms with unamended soil (Sw0 – Mw0) and sludge-amended (Sw1 – Sw4) and metal-spiked soils (Mw1 – Mw4) on top of a layer of clean soil. Concentrations are expressed as mg of metal per kg of dry body weight (mean ± SD, $n = 4$).

Incubation period	Treatment	Cr (mg/kg)	Cu (mg/kg)	Ni (mg/kg)	Zn (mg/kg)
3 weeks	Sw0/Mw0	< 2.80	14.8 ± 4.08	22.6 ± 2.59	125 ± 10.9
	Sw1	5.70 ± 4.22	14.4 ± 5.50	56.9 ± 38.4	138 ± 12.6
	Sw2	7.57 ± 4.40	18.8 ± 4.69	32.9 ± 8.37	115 ± 4.44
	Sw3	5.03 ± 6.89	17.3 ± 3.19	39.8 ± 16.6	146 ± 21.0
	Sw4	14.8 ± 6.21	23.5 ± 4.00	46.6 ± 7.25	159 ± 43.7
	Mw1	5.71 ± 2.28	14.6 ± 2.45	27.1 ± 7.23	136 ± 30.6
	Mw2	10.5 ± 3.77	21.3 ± 4.25	44.3 ± 7.90	129 ± 3.28
	Mw3	5.39 ± 1.24	17.9 ± 1.94	41.6 ± 6.85	134 ± 6.27
	Mw4	4.15 ± 3.42	22.1 ± 5.13	55.0 ± 21.7	136 ± 13.0

Table B.10 (Continued)

Incubation period	Treatment	Cr (mg/kg)	Cu (mg/kg)	Ni (mg/kg)	Zn (mg/kg)
6 weeks	Sw0/Mw0	< 2.80	13.6 ± 3.69	20.8 ± 2.90	125 ± 23.1
	Sw1	3.66 ± 3.00	12.8 ± 4.45	38.7 ± 13.1	139 ± 13.5
	Sw2	9.10 ± 4.73	19.5 ± 2.33	39.5 ± 4.36	123 ± 0.660
	Sw3	12.1 ± 4.83	21.1 ± 4.31	64.4 ± 7.99	150 ± 32.0
	Sw4	11.5 ± 2.70	21.4 ± 2.39	54.8 ± 10.3	119 ± 10.9
	Mw1	5.43 ± 2.42	14.1 ± 0.90	34.9 ± 1.66	129 ± 6.99
	Mw2	4.93 ± 3.42[a]	18.9 ± 1.58[a]	38.4 ± 6.51[a]	119 ± 12.0[a]
	Mw3	< 2.80	15.8 ± 2.49	43.1 ± 8.51	140 ± 6.79
	Mw4	< 2.80	18.1 ± 2.16	57.9 ± 11.8	134 ± 8.09

Table B.10 (Continued)

Incubation period	Treatment	Cr (mg/kg)	Cu (mg/kg)	Ni (mg/kg)	Zn (mg/kg)
12 weeks	Sw0/Mw0	< 2.80	8.73 ± 5.34	29.3 ± 7.05	132 ± 11.7
	Sw1	4.52 ± 1.66	14.0 ± 4.67	46.3 ± 4.87	151 ± 19.5
	Sw2	3.36 ± 3.35	16.6 ± 1.43	39.1 ± 3.26	134 ± 4.96
	Sw3	6.21 ± 3.96	17.3 ± 2.18	55.5 ± 4.82	124 ± 12.9
	Sw4	9.88 ± 5.68	18.6 ± 1.59	52.6 ± 6.89	134 ± 7.69
	Mw1	2.89 ± 3.18	16.1 ± 1.12	50.9 ± 4.93	135 ± 6.42
	Mw2	3.36 ± 1.28	21.5 ± 5.34	51.5 ± 11.0	128 ± 9.75
	Mw3	< 2.80	15.4 ± 3.37	59.0 ± 33.2	140 ± 15.4
	Mw4	5.08 ± 3.12	23.7 ± 9.91	61.2 ± 15.8	133 ± 9.56

[a] – $n = 3$

Table B.11 Regression slopes for Cr, Cu, Ni, and Zn concentrations in the earthworm *Dendrobaena veneta* against total concentrations of different metals after 3, 6, and 12 weeks of exposure in sludge-amended (Sw) and metal-spiked soils (Mw).

		Totals metal concentration (independent variable)					
		3 weeks		6 weeks		12 weeks	
	Metal	Sw	Mw	Sw	Mw	Sw	Mw
Metal concentration in earthworm tissue (dependent variable)	Cr	0.005	0.006	0.020*	-0.002	0.016*	0.011
	Cu	0.027	0.092*	0.089*	0.043	0.080*	0.137
	Ni	0.010	0.052*	0.049*	0.063*	-0.063[a]*	0.065*
	Zn	0.042	0.030	0.010	0.037	-0.002	0.025

* - statistically significant ($p \leq 0.01$); slopes were obtained using normal linear regression except for [a] which was obtained using the linear Langmuir equation (slope = *1/k*).

Table B.12 Linear regression slopes for Cr, Cu, Ni, and Zn concentrations in the earthworm *Dendrobaena veneta* against 0.01 M $CaCl_2$ extractable concentrations of different metals after 3, 6, and 12 weeks of exposure in sludge-amended (Sw) and metal-spiked soils (Mw).

		Extractable metal concentration (independent variable)					
		3 weeks		6 weeks		12 weeks	
	Metal	Sw	Mw	Sw	Mw	Sw	Mw
Metal concentration in earthworm tissue (dependent variable)	Cr	1.86	-2.94	-68.4	-2.10	19.1	5.31
	Cu	*ND*	2.81	*ND*	2.04	*ND*	10.6*
	Ni	3.25*	0.077*	4.87*	0.085*	3.57*	0.137*
	Zn	-1.46	0.027	3.00	0.047	4.08	0.045

* - statistically significant ($p \leq 0.01$); *ND* – not determined

Chapter 7

Long-term changes in metal availability of sludge-amended and metal-spiked soils under field conditions. The influence of earthworm activity

Based on the following manuscript under preparation:

Natal-da-Luz T, Ojeda G, Miranda F, Costa M, Lopes Z, Pratas J, Lanno RP, Van Gestel CAM, Sousa JP, 2011. Long-term changes in metal availability of sludge-amended and metal-spiked soils under field conditions. The influence of earthworm activity.

Abstract

The effect of earthworm activity on long-term changes in metal availability in metal-spiked and sludge-amended soils was evaluated in a field experiment. Treatments consisted of control, low and high doses of a sludge mainly contaminated with chromium (Cr), copper (Cu), nickel (Ni), and zinc (Zn) (sludge-amended soils), and similar low and high concentrations of the same metals applied directly to the soil (metal-spiked soils). Half of the treatments were inoculated twice with 500 *Dendrobaena veneta* per m^2. Soil was sampled 0, 3, 6, 10, and 12 months and earthworms after 3, 10, and 12 months. Total and 0.01M $CaCl_2$ extractable metal concentrations were measured in soil samples and internal metal concentrations in the earthworms. Bioaccumulated metal levels were used to validate existing relationship models from the literature. Results showed no changes in total metal contents and a decrease only in Ni extractability over time in the majority of treatments, which was not affected by earthworm activity. Chromium concentration in earthworms increased linearly with increasing total Cr levels in sludge-amended soils after 3 and 12 months. Internal Ni concentrations were related with its extractable contents in metal-spiked soils. Internal Cu and Zn concentrations were regulated by *D. veneta*. Metal concentrations in earthworms generally were higher in metal-spiked soils. Relationship models best estimated Cu, Ni, and Cr but not Zn concentrations in the earthworms.

Key words: Field assay, metal bioaccumulation, *Dendrobaena veneta*, metal extractability

Introduction

Mining activity, manufacturing, the excessive disposal of waste, and the application of various substances and amendments to agricultural soils have resulted in metal contamination of urban and agricultural soils. Because metals tend to accumulate in soil invertebrates and plants, food chain transfer may occur, posing a high risk for human and environmental health. Furthermore, metal effects on terrestrial organisms (e.g., Fritze et al. 2000; Gillet and Ponge 2003; Katanda et al. 2007) may cause disturbances in terrestrial ecosystems compromising soil productivity. For these reasons, applicators of industrial waste or sludge in agricultural soils have to respect regulatory limits based on the total metal concentrations in sludge and in soil (European Community 1986).

The level of soil metal contamination expressed on the basis of total metal concentrations does not necessarily represent the metal fraction to which soil organisms are exposed (Peijnenburg et al. 2007). Metals can be present in soil in several chemical forms, each having a specific mobility and availability to soil organisms. In the environment, metal availability is highly dependent on the binding strength to various phases of the soil matrix (Lanno et al. 2004), and is conditioned by soil pH, dissolved organic matter, calcium, solid-phase metal oxide, and organic matter content, and even by soil fauna activity.

Metal availability may change over time due to natural or anthropogenic processes, such as acidification, salinization, or organic matter mineralization (Allen 2002). Monitoring metal fate in soil over time, using chemical extractions to quantify metal reactivity and available fractions, is the only way to understand long-term changes in metal dynamics in terrestrial systems and better predict their effects. Field assays to evaluate long-term changes in sewage sludge-amended soils have been performed using sequential chemical extractions (Sloan et al. 1998; McGrath and Cegarra 1992) or single mild and strong extractions (Bidwell and Dowdy 1987; McBride et al. 2000). The use of a 0.01M $CaCl_2$ extraction is often adopted to quantify the fraction of metals in soil which is most

available to organisms (Houba et al. 2000). Several other chemical extraction methods have been developed to quantify that fraction; however, no single analytical method is able to directly predict metal uptake by organisms. Body residues may provide a better estimate of the bioavailability of metals in soil, however, metal accumulation is species-related since it is highly dependent on the specific route of exposure of each organism (Nahmani et al. 2007).

Due to their role in soil systems, contributing to the provision of important regulatory and supporting ecosystem services like soil formation and stabilization (Lavelle and Spain 2001), water cycling in soil, and organic matter decomposition and nutrient cycling (Vandewalle et al. 2008), earthworms are widely used as models for studying metal bioaccumulation, providing information on the bioavailability of metals at a contaminated site. Laboratory accumulation studies have been performed mainly using *Eisenia fetida* (e.g., Janssen et al. 1997; Conder and Lanno 2000; Maenpaa et al. 2002), which is the species used in international standard toxicity tests (e.g. ISO 1996). Other species such as *Lumbricus rubellus* (e.g. Langdon et al. 1999), *Lumbricus terrestris* (e.g. Marino and Morgan 1999), *Aporrectodea caliginosa* (e.g. Perämäki et al. 1992), and *Dendrobaena veneta* (e.g. Marinussen at al. 1997a) have been less commonly used. Field studies on metal accumulation in earthworms have also been conducted using predominantly earthworm species from the genus *Lumbricus* and *Aporrectodea* (e.g., Morgan and Morgan 1992; Dai et al. 2004; Ma 2005). Some field studies also used *E. fetida* (Laszczyca et al. 2004), and earthworms from the genus *Dendrobaena* (e.g. *D. veneta* by Ireland 1979).

Since earthworm activity may have a significant influence on metal partitioning in soil, laboratory studies have been conducted to evaluate their impact on metal mobility and availability. Epigeic earthworm species have been the most used (e.g., *E. fetida* by Wen et al. 2004 and *E. veneta* by Sizmur and Hodson 2008), with less attention for anecic (e.g. *L. terrestris* by Zorn et al. 2005a) and endogeic species (e.g. *Aporrectodea tuberculata* by Coeurdassier et al. 2007). In

general, all these studies have evidenced an increase in metal availability favoured by earthworm activity (e.g., Zorn et al. 2005a; Rada et al. 1996). However, other studies found an earthworm-induced decrease in metal availability in chemically spiked (Zorn et al. 2005b) and sludge-amended soils (Liu et al. 2005). The influence of earthworm activity has been studied for metal-spiked soils and for sludge-amended soils separately and only under laboratory conditions. Therefore, a comparative study to evaluate the earthworm activity on these two sources of soil metal contamination under field conditions is still needed.

When the direct measurement of contaminants in earthworm tissues is not possible, estimation of earthworm concentrations is the only alternative. To attain that purpose, studies producing equations relating metal concentrations in the earthworm to total metal content in soil have been conducted (Sample et al. 1999). Because of the strong species dependence of metal accumulation and the influence of soil chemical and physical properties, more studies comparing differences in species response and validating existing models in different soil types are required. Additional experiments evaluating metal bioaccumulation under field conditions are also needed to avoid the possible influence of the experimental approach on the earthworm response (Nahmani et al. 2007). Recently, a comparative evaluation of the short-term changes in the availability of a mixture of metals originating from sludge amendment or a metal spike of soil was conducted under laboratory conditions (Natal-da-Luz et al. 2011a - Chapter 5). The influence of earthworm activity on metal availability was evaluated and metal uptake by earthworms was measured and used to evaluate the accuracy of existing models for estimating earthworm metal concentrations (Natal-da-Luz et al. 2011b - Chapter 6). No influence of *D. veneta* was detected on metal availability over the 12-week experimental period, with metal concentrations in earthworms generally being higher in the most contaminated soils. Except for zinc (Zn) and chromium (Cr), metal uptake by the earthworms was influenced by matrix. The existing models were not able to estimate metal

concentrations in the earthworms. Marinussen and Van der Zee (1997) argue that metal uptake by earthworms in the field can be modified by some factors that are not taken into account in a laboratory study (e.g. temperature variations), influencing the relationship between soil metal content and metal uptake. The influence of earthworm activity on metal availability under field conditions, however, has never been evaluated. But it is likely that 12 weeks was not long enough to detect an influence of earthworm activity on metal availability over time.

To fill this gap, this study aimed at a comparative approach under field conditions and on a long-term basis using amendments with different dosages of a sludge contaminated predominantly with Cr, copper (Cu), nickel (Ni), and Zn and soil spiked with metal solutions to obtain the same dosages of metals. *D. veneta* were introduced in half of the treatments. Total and extractable metal concentrations were measured in soil samples taken over 12 months. Metal bioaccumulation in the earthworms was also measured. The main objectives of the present study were: i) to evaluate changes in the availability of metals over time in metal-spiked and sludge-amended soils on a long-term basis; ii) to assess the influence of earthworm activity on changes in total and extractable metal concentrations; iii) to measure the metal uptake by earthworms from different test matrices; iv) to evaluate the accuracy of existing models to estimate earthworm metal accumulation under field conditions. Our working hypotheses are that i) metal availability will decrease over time especially in spiked soils due to ageing and/or metal transport to deeper layers, which will occur to a more limited extend in sludge-amended soils due to metal adsorption to sludge organic matter (OM); ii) earthworm activity will increase metal availability especially in sludge-amended soils due to consumption of sludge OM by earthworms, which reduces the number of binding places for metals; iii) the metal concentrations will be higher in earthworms from metal-spiked soils than from sludge-amended soils due to the higher bioavailability of metals in spiked soils; and iv) earthworm metal concentrations can be predicted by existing models.

Materials and methods

Experimental area and test sludge

A field assay was conducted in an agricultural field with a sandy loam soil (Table 7.1) at an experimental station of the Ministry of Agriculture, in the village of Vairão, Northern Portugal. The area was free of pesticide and fertilizer applications for more than 5 years. Sludge (Table 7.1) derived from the wastewater treatment plant of Cr, Cu, Ni, and Zn-plating industries in Águeda, Portugal, was used. The sludge was produced by chemically induced precipitation of metals of industrial effluents. After decantation, the sludge was filter pressed to reduce moisture content. After air-drying, the precipitate was 5 mm sieved. The parameters measured in the soil and test sludge were pH (1M KCl, 1:6, v:v), water holding capacity (WHC; ISO 1996) and organic matter content (OM; loss on ignition at 500ºC for 6 h). Cation exchange capacity (CEC; Chapman 1965) and texture (LNEC 1970) were measured only in soil. After *aqua regia* digestion, total metal concentrations in soil were measured by flame atomic absorption spectrometry (AAS; AAnalyst 300, Perkin-Elmer). Digestion was validated using VKIAG CMSA, batch VKI-19-2-0595 (Reference Material QC Municipal Sludge A), certified by VKI Water Quality Denmark Institute as reference material. Metal recoveries were between 90 and 110% of the certified reference values. For the test sludge, total Cr, Cu, Ni, and Zn concentrations were measured after *aqua regia* digestion using inductively coupled plasma (ICP) – atomic emission spectrometry (OES Axial 730 ES, Varian Scientific Instruments) following USEPA (2001) and cadmium (Cd) and lead (Pb) concentrations were determined by ICP (OES Axial 730 ES, Varian Scientific Instruments) according to USEPA (1994). The accuracy of these procedures was checked using BCR No. 145 (Trace Elements in sewage sludges), certified by Community Bureau of Reference (Commission of the European Communities) as reference material. All the analyses were considered valid with quality control

recoveries between 95 and 115%. Total mercury content was measured using AAS (AMA-254, ALTEC) using cold vapour atomization (USEPA 1986). In this case, recovery of the control standard was between 94 and 97%. The oxidized state of chromium (Cr(VI)) was extracted using diphenylcarbazide following Branco et al. (2005) and measured by ICP (OES Axial 730 ES, Varian Scientific Instruments), with the reduced state (Cr(III)) concentration determined by the difference between total Cr and oxidized state (Cr(IV)) concentrations.

Table 7.1 Physical and chemical characterization of soil from the experimental area and test sludge used to assess metal availability over time.

Physical and chemical parameters		**Reference soil**	**Test sludge**
pH (1M KCl, *n* = 4)		4.4 ± 0.1	8.1 ± 0.1
Water-holding capacity (%, *n* = 4)		77.0 ± 1.4	147 ± 0.7
Cation exchange capacity (meq/kg, *n* = 1)		90.0	*ND*
Organic matter (%, *n* = 2)		9.3 ± 0.2	28.0 ± 7.6
Total Metals	Cadmium	< 1.8	< 0.5
(mg/kg DW, *n* = 1)	Chromium	< 4.8	5200[a]
	Cobalt	< 16	*ND*
	Copper	22	1000
	Iron	14248	*ND*
	Lead	31	290
	Manganese	145	*ND*
	Mercury	*ND*	0.18
	Nickel	< 10	7100
	Zinc	46	4100
Texture (%, *n* = 1)	Clay	10.3	*ND*
	Silt	35.6	*ND*
	Sand	54.1	*ND*
Soil type		Sandy Loam	-

[a] Only 0.5 mg/kg of the total Cr is Cr(VI); *ND* – not determined parameter

Test design

The field assay was performed on an agricultural area of about 100 m^2. Each mesocosm consisted of a cylindrical plastic bucket (50 cm deep, Ø 60 cm) with the bottom perforated but lined with a tight net (< 0.5 mm). In November 2008, each mesocosm was buried in the soil forming four lines of 10 units distant from each other by 1 m. Each mesocosm was filled to a depth of 5 cm with cracked stones for road paving (to facilitate leaching), followed by a 35 cm layer of agricultural soil from the same area (collected from a depth below 20 cm) and a 10 cm top layer of the same agricultural soil collected from the 20 cm surface layer. The soil layers were not compacted. After one month, and to control rainfall over the experiment, the area was covered with a plastic semi-cylindrical walk-in tunnel opened at the extremes. Since that day, rain simulations were performed with variable frequencies over the experiment in order to prevent the top 10 cm soil layer from drying out. The irrigations were as homogeneous as possible for all the experimental units. In each line of ten mesocosms, 5 treatments were applied to two units randomly distributed over the line. The treatments consisted of a control (C), a low (SL) and a high (SH) dose application of the test sludge (SL = 15 and SH = 75 t/ha dry weight) and a low and a high spike of a mixture of Cr, Cu, Ni, and Zn (ML and MH, respectively) to obtain the same metal dosages as in the sludge-amended treatments. In the SL and SH treatments, the sludge was mechanically incorporated in the top 10 cm surface layer. In the ML and MH treatments, 5 L of metal solutions were applied to the soil surface using a watering-can. Chromium, Cu, Ni, and Zn were applied as chromium nitrate ($Cr(NO_3)_3 \cdot 9H_2O$, purity 98%; Panreac, Barcelona, Spain), copper chloride ($CuCl_2 \cdot 2H_2O$, purity 99%; Panreac, Barcelona, Spain), nickel chloride ($NiCl_2 \cdot 6H_2O$, purity 98%; Panreac, Barcelona, Spain), and zinc chloride ($ZnCl_2$, purity 97%; Panreac, Barcelona, Spain), respectively. Metal concentrations were based on toxicity data from previous chronic tests with *Eisenia andrei* and *Folsomia candida* as test organisms (Natal-da-Luz et al.

2011c - Chapter 4). A total of 40 mesocosms were prepared (8 per treatment). After two weeks of soil contamination, the experiment started. At that date, the first soil sampling was performed (T0) to characterize the initial conditions of all experimental units. The other soil samplings took place after 3, 6, 10, and 12 months. For each sample from each mesocosm, two soil cores (30 cm deep, Ø 5 cm) were taken using a stainless steel cylinder auger. The empty holes left were filled with Polyvinyl chloride tubes (PVC tubes; 30 cm deep, Ø 5 cm) to reduce the disturbance. Soil samples were taken as far from each other as possible. Soil pH (1M KCl, 1:6, v:v) and moisture content (loss on drying at 105ºC for 12 h) were determined separately for the top 10 cm top layer and the deeper soil layer. From each top 10 cm soil sample, the aliquot that was oven-dried was stored at 4ºC for assessment of total metal concentrations. The other aliquot was air-dried at room temperature and used for determining extractable metal concentration. Total and extractable concentrations of Cr, Cu, Ni, and Zn were measured only in the top 10 cm layer. At T0, 140 adult *Dendrobaena veneta* were introduced in half of the mesocosms from each treatment to obtain an earthworm density of 500 individuals/m^2; these were called the Cw, SLw, SHw, MLw and MHw treatments. The mesocosms in which no worms were introduced, were colonized with earthworms endemic to the experimental area (C, SL, SH, ML and MH treatments), but attaining a lower earthworm density (a homogeneous distribution of endemic earthworms was assumed for the experimental area). Earthworm sampling (up to 4 specimens of *D. veneta* from each earthworm treated mesocosm) was done by hand sorting after 3, 10, and 12 months. One week before the intended 6 month sampling, a failure in the irrigation system caused an excessive watering of the experimental area resulting in high soil moisture content. This may have caused anaerobic conditions killing some earthworms or forcing them to migrate downwards, thus making them inaccessible during our sampling. Because we were not able to find any earthworms at that time, new batches of 140 adult *D. veneta* were introduced in the experimental units. The animals collected at each sampling were washed,

weighted individually and placed together in clean plastic Petri dishes on moist filter paper overnight (for 12 to 24 h), in the dark and at room temperature, to purge the gut. After that period, earthworms from each Petri dish were transferred to 20 ml scintillation vials, frozen at -20ºC and dried at 105ºC for metal analysis. During the first month of the experiment, ryegrass was sown in all units to ensure vegetation cover over the experiment.

Metal analysis over time

Earthworms and soil samples were homogenised using an acid-washed porcelain pestle and mortar and were digested using PDS-6 pressure digestion systems (Loftfields analytical solutions, Neu Eichenberg, Germany). Samples of ≤ 100 mg were mixed with 2 ml HNO_3 69% (PA-ACS-ISO, Panreac, Barcelona, Spain) and left under pressure in PDS-6 devices at 150ºC for 10 h. Next, the resulting solution was diluted with ultrapure water and transferred to a plastic vial to a final volume of 10 ml. The quality of this analysis was checked using SRM 2709 (San Joaquin Soil - Standard Reference Material) certified by the National Institute of Standards and Technology (Department of Commerce, USA) and DOLT-3 (Dogfish Liver Certified Reference Material for Trace Metals) certified by the National Research Council Canada as reference material for soil and earthworm analysis, respectively and replicate blanks were prepared. The average recoveries for soil were 82.9, 95.7, 98.5, and 105% and for earthworms 106, 89.9, 117, and 93.4% for Cr, Cu, Ni, and Zn, respectively.

Extractable metal concentrations in soil were determined according to Houba et al. (2000). Twenty grams of air-dried soil (coarsely homogenised using a spoon) was shaken for 2 h at 400 rpm with 200 ml 0.01M $CaCl_2$ (21074, Sigma-Aldrich, St Louis, USA) solution. The resulting suspensions were filtrated through individual Whatman No 1 filter paper discs (Cat. No 1001150, Maidstone, England). The Cr, Cu, Ni, and Zn in digests and extracts were determined by flame AAS (2380 Absorption Atomic Spectrometer, Perkin-Elmer). For the

measurements in $CaCl_2$ extracts, wash solution, blanks and standards were prepared in 0.01M $CaCl_2$ solution. All metal concentrations in soil were expressed as mg of metal/kg dry soil and in earthworms as mg of metal/kg dry body weight.

Prediction of earthworm metal concentrations

Total metal concentrations were used to validate relationship models from the literature. Since, until date, no models were developed for *D. veneta*, we used models developed by Neuhauser et al. (1995) for Cu, Ni, and Zn uptake and by Peijnenburg et al. (1999) for Cr, Cu, and Zn uptake in *Allolobophora tuberculata* and *E. andrei*, respectively (Table 7.2). For model validation, all measured values from all sampling dates were used, except for the total Cr concentrations in soil samples that were below 0.2 mmol/kg, as required by the model proposed by Peijnenburg et al. (1999).

Table 7.2 Selected models from the literature used to predict earthworm metal concentrations from total metal concentrations in the soil.

Analyte	Model	R^2	Reference
Cu	$\text{Log}M_{ew} = 0.57\ \text{Log}M_s + 0.39$	0.67	Neuhauser et al. (1995)
Ni	$\text{Log}M_{ew} = 0.98\ \text{Log}M_s - 0.67$	0.66	Neuhauser et al. (1995)
Zn	$\text{Log}M_{ew} = 0.27\ \text{Log}M_s + 2.09$	0.35	Neuhauser et al. (1995)
Cr	$\text{Log}M_{ew} = 0.69\ \text{Log}M_s - 1.05$	0.61	Peijnenburg et al. (1999)
Cu	$\text{Log}M_{ew} = 0.25\ \text{Log}M_s - 0.54$	0.69	Peijnenburg et al. (1999)
Zn	$\text{Log}M_{ew} = 1.45\ \text{Log}M_s + 0.42$	0.83	Peijnenburg et al. (1999)

M_{ew} - metal concentration in earthworm (mg/kg dry body weight); M_s - metal concentration in soil (mg/kg dry soil)

Statistical analysis

Regression analyses were performed to detect significant trends in total and extractable metal concentrations over time. For regressions with extractable metal concentrations, all values were used including those below the detection limit when positive. For regressions, total metal concentrations were expressed as percentage of the initial concentration and available metal concentration (extracted with $CaCl_2$) as percentage of total metal concentration. When the normal linear equation did not yield a satisfying fit (low R^2), the exponential equation was used.

Regressions were also performed to find significant relationships between metal concentration in earthworms and soil metal contents (total and extractable metals) separately for metal-spiked and sludge-amended soils, for each sampling date (3, 10, and 12 months). Regression using the linear form of the Langmuir equation was performed instead of linear regression when higher R^2 was obtained. The linear form of the Langmuir equation reads:

$$C/X = (1/(n*k)) + (1/k)*C \quad (7.1)$$

where C is the metal concentration in earthworm tissue (mg/kg), X is the total or extractable metal concentration in soil (mg/kg), n and k are constant of Langmuir equation. Regressions were always performed for Cr, Cu, Ni, and Zn separately. Normality and homocedasticity (using Kolmogorov–Smirnov and Bartlett tests, respectively) was checked before regressions. When these assumptions were not fulfilled, values were arcsin transformed. All analyses were performed using STATISTICA, version 6.

The slopes obtained in regressions comparing the same variables (for total and available metals) were compared. The comparisons performed were: i) slopes obtained for different metals in each treatment (e.g. Ni from MH *vs* Zn from MH); ii) slopes obtained for different metal sources in the same test

concentration (e.g. Ni from MH *vs* Ni from SH); iii) slopes obtained for different test concentrations of each metal source (e.g. Ni from ML *vs* Ni from MH); and iv) slopes obtained for different metals from treatments with and without earthworms (e.g. Ni from MH *vs* Ni from MHw). The slopes from regressions of bioaccumulated metals against metal contents in soil (total and extractable metal concentrations) for sludge-amended and metal-spiked treatments were also compared for 3, 10, and 12 month samplings. All slope comparisons were performed by analysis of covariance (ANCOVAs) using Microsoft Office Excel version 2007. The level of statistical significance for all analyses was $\alpha = 0.01$.

To validate the prediction of earthworm metal concentrations, the differences between estimated and measured metal concentrations in the earthworms were evaluated using Wilcoxon paired-sample test (Zar 1998). Relative accuracy of the models was evaluated by calculating the mean percentage of deviation of estimated values from the measured values. The slopes obtained in regressions comparing tissue metal concentrations with total soil metal concentrations for each metal using estimated and measured uptakes were compared by ANCOVAs. These regressions were performed for all sampling dates and separately for each metal and for sludge-amended and metal-spiked treatments.

Results

Water content and pH of soil samples over the duration of the experiment

The deeper soil layer had a pH-KCl (mean ± SD, $n = 200$) of 4.54 ± 0.09 and a moisture content (mean ± SD, $n = 200$) corresponding to 46.1 ± 6.7% of the WHC over the experiment; these parameters did not differ between treatments. Also in the upper 10 cm soil layer, no effects of earthworm addition were found, with soil moisture content (mean ± SD, $n = 160$) of 43.3 ± 5.2% of the WHC in all samples except for those collected after 6 months which had a moisture content (mean ± SD, $n = 40$) of 54.9 ± 9.2% of the WHC. The pH-KCl of control

and metal-spiked soils (in mesocosms with and without *D. veneta*) was, on average (± SD, n = 120) 4.54 ± 0.06. Sludge amended soils had a higher pH, which increased with increasing sludge dose. On average (± SD, n = 40), at the low sludge dose, soil pH was 5.11 ± 0.29, at the high dose 6.08 ± 0.40.

Metal concentrations over time

Total metal concentrations generally were higher in sludge-amended than in spiked soils at the same test concentrations at all samplings and for all metals measured (Figure 7.1; Table 7.3 and Table C.1 in the Annex C – pages 279 to 282). Changes in soil total metal content over time were not significantly related with time for any metal of mesocosms with or without *D. veneta*. In sludge-amended soils, total metal concentrations slightly decreased over time in mesocosms with earthworms (regression slopes negative) but increased in the absence of earthworms (regression slopes positive; Table C.2 – page 283). In metal-spiked treatments, in general, total metal concentrations decreased over time, independently of earthworm presence (regression slopes negative; Table C.2 – page 283). Both initial total metal concentration (low *vs* high) and matrices (sludge *vs* metal spiking) did not influence the development of total metal content in soils, which did not differ between metals as well.

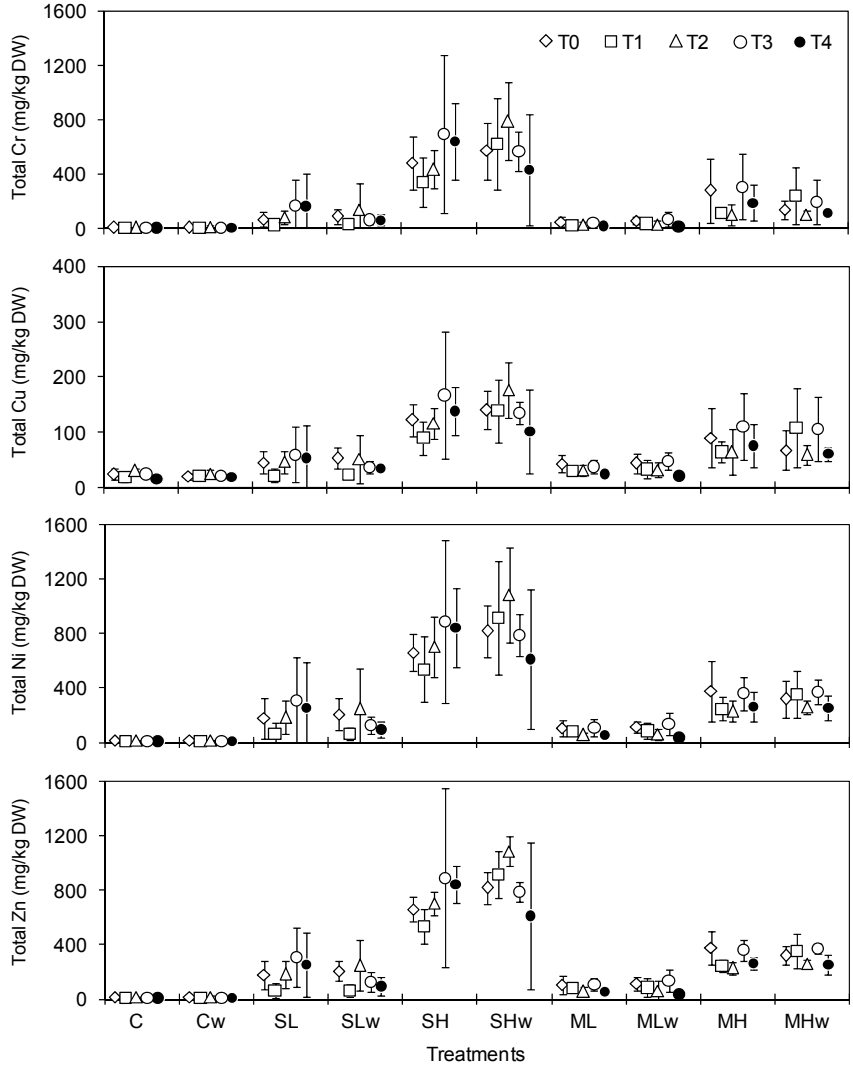

Figure 7.1 Total Cr, Cu, Ni, and Zn concentrations (average ± SD, $n = 4$) in the top 10 cm soil layer from field mesocosms with (treatment codes with w) and without *Dendrobaena veneta*, not treated (C and Cw), treated with a low (SL and SLw) or a high sludge dose (SH and SHw) or spiked with a low (ML and MLw) or a high metal dose (MH and MHw) after 0, 3, 6, 10, and 12 months (T0, T1, T2, T3, and T4, respectively).

Table 7.3 Total Cr, Cu, Ni, and Zn concentrations (average ± SD, $n = 4$) in the top 10 cm soil layer from field mesocosms not treated (C and Cw), treated with a low (SL and SLw) or a high sludge dose (SH and SHw) or spiked with a low (ML and MLw) or a high metal dose (MH and MHw) before the introduction of earthworms (*Dendrobaena veneta*).

Treatments	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
C	6.91 ± 2.87	24.4 ± 10.0	12.3 ± 2.85	46.6 ± 16.1
Cw	7.89 ± 2.16	20.7 ± 4.83	12.5 ± 1.42	46.9 ± 13.6
SL	69.5 ± 56.4	46.0 ± 19.8	176 ± 147	188 ± 104
SLw	90.5 ± 52.9	53.4 ± 19.2	209 ± 115	205 ± 76.4
SH	484 ± 198	122 ± 29.1	663 ± 138	577 ± 91.0
SHw	574 ± 208	140 ± 34.1	819 ± 188	569 ± 116
ML	51.5 ± 32.8	43.0 ± 15.7	104 ± 58.5	140 ± 65.9
MLw	60.6 ± 26.6	44.6 ± 17.7	113 ± 38.6	161 ± 53.8
MH	280 ± 238	90.6 ± 53.2	379 ± 222	287 ± 123
MHw	140 ± 65.8	68.2 ± 35.7	321 ± 136	284 ± 65.8

Extractable Cr, Ni, and Zn concentrations were lower in sludge-amended than in spiked soils (Table 7.4 and Table C.3 – pages 284 to 287). No extractable Cu was detected in any of the soil samples (negative values were always measured) and extractable Cr was higher than the detection limit only in some samplings of the most concentrated spiked soils. Since the measurements below the detection limit of extractable Cr were generally positive, those values could be used for statistical analysis. Metal extractability (as percentage of total metal concentration) generally decreased with time, except for Zn in most of the sludge-amended treatments (Figure 7.2, second row), for Cr in the ML treatments and for Ni in the SLw treatments. Only Ni extractability was significantly related with time, except for the SL, SLw and MLw treatments

(Table C.4 – page 288; Figure 7.2, first row). The development of Ni extractability over time was significantly different from that of Cr and Zn in mesocosms with the highest treatments without *D. veneta* and from that of Zn in the mesocosms with earthworms with the highest sludge dose. Initial concentration (low *vs* high) did not influence the extractability of metals over time in any treatment. Matrix (sludge *vs* metal spiking) only significantly influenced the extractability of Ni in mesocosms with the highest test concentrations. Earthworm activity did not affect development of metal extractability.

Table 7.4 Initial 0.01M $CaCl_2$ extractable Cr, Cu, Ni, and Zn concentrations (average ± SD, n = 4) in the top 10 cm soil layer from field mesocosms not treated (C and Cw), treated with a low (SL and SLw) or a high sludge dose (SH and SHw) or spiked with a low (ML and MLw) or a high metal dose (MH and MHw) before the introduction of earthworms (*Dendrobaena veneta*).

Treatments	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
C	< 0.249	< 0.349	< 0.427	0.154 ± 0.155
Cw	< 0.249	< 0.349	0.449 ± 0.766	0.946 ± 0.781
SL	< 0.249	< 0.349	2.62 ± 1.57	1.60 ± 0.991
SLw	< 0.249	< 0.349	2.81 ± 1.72	0.721 ± 0.651
SH	< 0.249	< 0.349	7.47 ± 1.91	1.36 ± 0.603
SHw	< 0.249	< 0.349	8.73 ± 1.50	0.719 ± 0.277
ML	< 0.249	< 0.349	12.3 ± 10.0	9.56 ± 8.75
MLw	< 0.249	< 0.349	12.7 ± 6.35	12.6 ± 5.42
MH	0.345 ± 0.116	< 0.349	142 ± 80.9	103 ± 65.6
MHw	0.337 ± 0.057	< 0.349	110 ± 79.3	76.3 ± 51.2

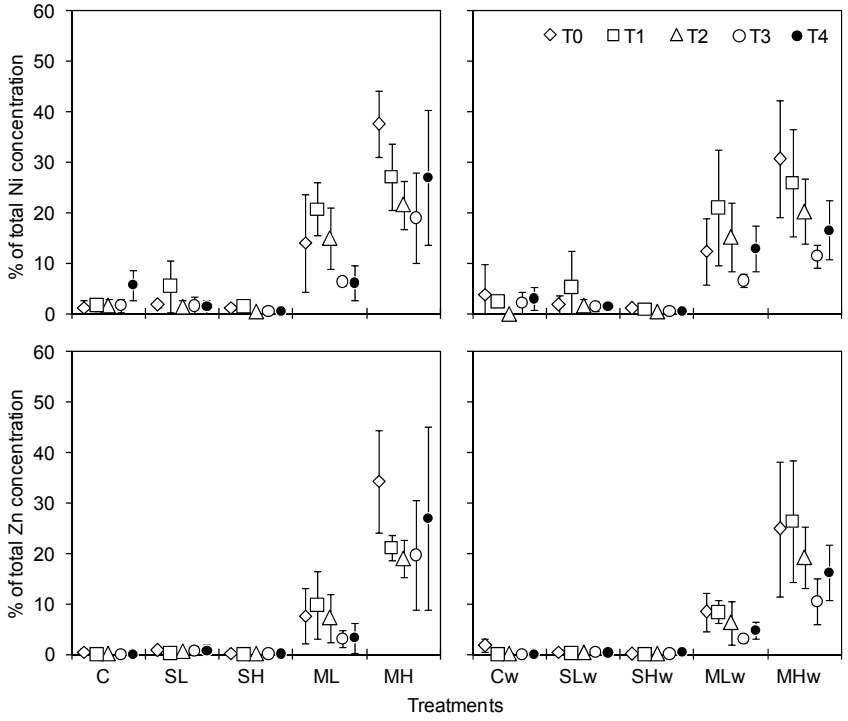

Figure 7.2 $CaCl_2$ (0.01M) extractable concentrations of Ni (first row) and Zn (second row) in field soil samples (average ± SD, n = 4) incubated for different times without (left) and with earthworms (*Dendrobaena veneta*; right). See Figure 7.1 for treatment and sampling codes.

Earthworm metal concentrations

In most cases more than one adult specimen of *D. veneta* was collected at each sampling date from more than one mesocosm of each treatment. Exceptions were the SHw treatment after 3 months and MHw after 10 and 12 months from which adult *D. veneta* could be collected only in one mesocosm. In MHw at 3 months and in SHw at 10 months, no earthworms were found.

Zinc had the highest concentrations in earthworms followed by Ni, with lower internal concentrations found for Cr and Cu. In general, the earthworms from

MHw had the highest internal metal concentrations (Table C.5 – page 289). Earthworm Cr concentrations increased significantly with increasing total Cr concentrations in sludge-amended soils after 3 and 12 months and in spiked soils after 10 months (Figure 7.3).

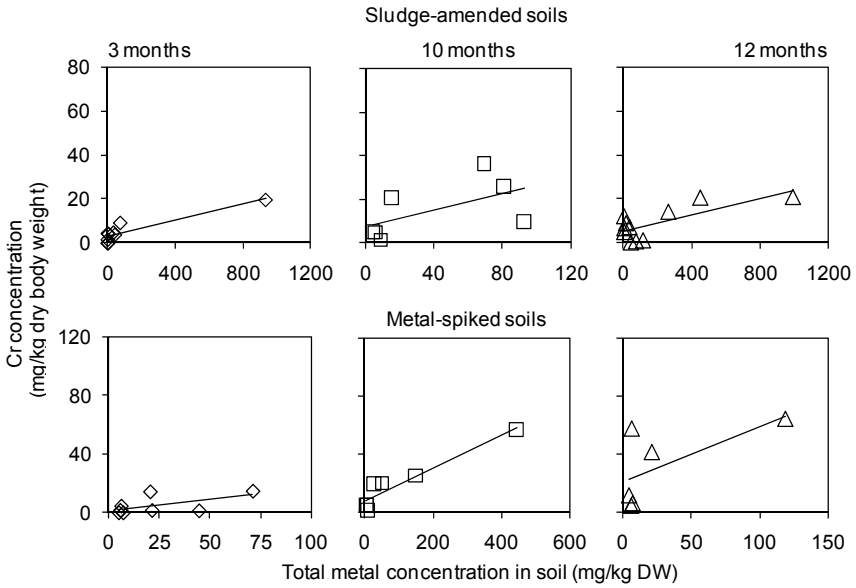

Figure 7.3 Cr concentrations in the earthworm *Dendrobaena veneta* as a function of the total concentration in sludge-amended soils (first row) and metal-spiked soils (second row) after 3, 10, and 12 months of exposure in field mesocosms (left, middle, and right graphs, respectively). Lines show linear regressions.

Internal Cr concentrations, however, were not related with its extractable concentration in any treatment. Copper concentration in earthworms was independent of its total level in soil and Ni concentrations were significantly related with total soil content only in metal-spiked soils after 10 months (Figure 7.4), and with extractable concentration in spiked soils after 3 and 10 months and in sludge-amended soils after 3 months. Internal Zn concentration in earthworms was independent of total and extractable Zn concentrations in soil (Tables C.6 and C.7 – pages 290 and 291).

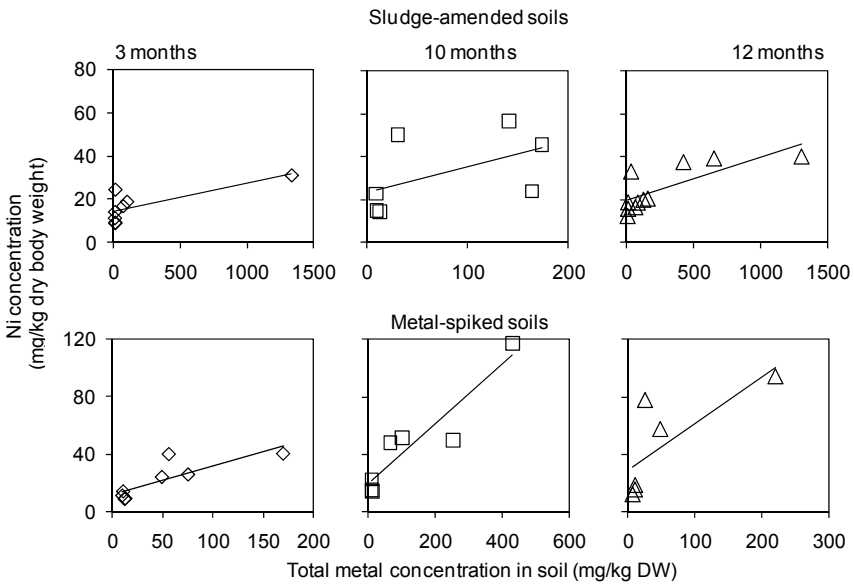

Figure 7.4 Ni concentrations in the earthworm *Dendrobaena veneta* as a function of the total concentration in sludge-amended soils (first row) and metal-spiked soils (second row) after 3, 10, and 12 months of exposure in field mesocosms (left, middle, and right graphs, respectively). Lines show linear regressions.

The test matrices (sludge *vs* metal spiking) influenced the relationship between metal bioaccumulation and total soil metal concentration for both Ni and Zn after 12 months and only for Ni after 3 months. The relationship between earthworm metal concentrations and extractable soil contents were not influenced by the test matrices.

Prediction of earthworm metal concentrations

Metal concentrations in the earthworms estimated with the models of Neuhauser et al. (1995) for Cu and Ni, in sludge-amended soils, were the only ones that agreed with measured values (Wilcoxon paired-sample test: $p > 0.01$). The Cu and Ni concentrations estimated for S treatments and of Zn for S and M treatments were, on average, overestimated by 8.99, 14.6, 189, and 128%, respectively. Estimated values for Cu and Ni in M treatments were, on average, underestimated by 26.7 and 68.1%, respectively. Using the Neuhauser et al. (1995) model, describing relationships between earthworm metal concentrations and total metal concentrations in soil, gave correct predictions for Cu and Ni in metal-spiked soils. The relationship models proposed by Peijnenburg et al. (1999) did not accurately estimate earthworm metal concentrations in any treatment. Zinc concentrations were overestimated in sludge-amended and spiked soils, on average, by 5386 and 1332%, respectively. Chromium and Cu concentrations were underestimated in the greater part of treatments, for Cr by 40.3 and 80.6% and for Cu by 96.3 and 97.0% in sludge-amended and spiked soils, respectively. No significant differences were found between estimated and measured earthworm Cr and Cu concentrations, when related with total concentrations in sludge-amended soils, and for Cr related with total concentrations in metal-spiked soils.

Discussion

Metal behaviour over the experiment

Total metal concentrations showed no significant losses over the 12 months of the experiment. Extractability of Ni showed a significant linear decrease with time, which cannot be attributed to leaching of the most mobile Ni fraction to deeper layers over time as such transport would cause a decrease in total Ni concentrations. This decrease in Ni extractability might be related with ageing (Lock and Janssen 2003; Jalali and Khanlari 2008). The working hypothesis, which assumed a more pronounced decrease in metal extractability over time in metal-spiked soils was confirmed for Ni and Zn. Although the decreases in Zn extractability over time in metal-spiked soils were not significant, the regression slopes of extractable Zn concentrations with time were generally more pronounced in spiked soils than in sludge-amended soils. Slightly different results were obtained by Natal-da-Luz et al (2011a - Chapter 5), when total and extractable metal concentrations were measured in a laboratory microcosm experiment, using the same treatments after 0, 3, 6, and 12 weeks of incubation under weekly rain simulations equivalent to a rainfall of 370 mm per year. Metal extractabilities in the present study were lower than in the laboratory microcosm experiment, which may suggest the influence of environmental factors on the availability of metals, which seems to promote metal adsorption to the solid phase. In the laboratory experiment a decrease of Cu, Ni, and Zn availability over time was observed in the metal-spiked soils at the highest concentration (which was similar to the MH and MHw treatments). On the other hand, total Ni and Zn concentrations decreased significantly with time in treatments equal to ML/MLw and MH/MHw of the present study. As discussed by the authors, these losses might be due to metal transportation to deeper soil layers promoted by the rain simulations. In the present study, the frequency of irrigation was dependent on the weather since the objective was to prevent the 10 cm top layer from

drying out. Because of that, the first irrigation was performed only two weeks after treatment and the second one two weeks later. The rain simulations in the microcosm laboratory experiment were a total of 7 mm/week, while in the field experiment each of the two first irrigations was equivalent to a total of 1.25 mm/week. As a consequence, in the field experiment metals (especially from the metal-spiked treatments) had more time to interact with the soil, explaining the lower mobility. Furthermore, under field conditions, metals are exposed to several factors not taken into account in a laboratory experiment, like the activity of soil microflora, plant roots and edaphic fauna, which may contribute to retaining and capturing metals and preventing their leaching. These facts may explain the absence of total metal concentration decreases over time in the field.

Similarly to the observation by Natal-da-Luz et al. (2011a - Chapter 5), Ni and Zn had the highest extractability and therefore were more mobile than Cr and Cu. The metals were generally more available in metal-spiked than in sludge-amended soils. These data are consistent with McGrath and Cegarra (1992), who evaluated the long-term availability of metals in field plots amended with metal-contaminated sewage sludge. The authors adopted a sequential chemical extraction procedure using in the first step extraction with 0.1M $CaCl_2$. In soil samples collected 6, 11, 19, and 22 years after the last sludge application, extractabilities were always higher for Ni and Zn (approx. 5% of total concentration) than for Cu and Cr (close to 0%). Metal extractabilities did not significantly change over 22 years. Although our data showed a significant decrease of Ni extractability with time in the highest sludge doses over one year, the results of McGrath and Cegarra (1992) are in agreement with the high extractabilities found in the present study for Ni and Zn and the low or negligible ones for Cr and Cu. These differences between metals are highly related with adsorption reactions which are probably the major factor that condition the mobility and extractability of metals in soil. McBride et al. (2000) monitored metal changes in a sludge-amended field from samples collected in 1979 and 1997, using *aqua regia* and nitric-perchloric acid digestions, respectively. Total

concentrations decreased by about 30 to 50% for all metals investigated including Cr, Cu, Ni, and Zn. Slightly different results were obtained by Walter et al. (2002) in a long-term field experiment on a soil treated for eight years at a high rate of anaerobically digested biosolids. One, 5, and 9 years after the last biosolid application, total Cu and Zn concentrations in the top 20 cm soil layer significantly decreased, but total Cr and Ni levels did not change. These studies do not support our results. However, several studies have been performed reporting relatively little movement of sludge-applied metals, which is in agreement with our data. Bidwell and Dowdy (1987) did not find significant changes in total Cd and Zn concentrations in a soil amended with three annual sludge applications sampled 6 years after the last sludge application. In another study, Canet et al. (1997) found no changes in total metal concentrations (including Cr, Cu, Ni, and Zn) in soil layers below 20 cm depth of a sludge-amended soil during 7 years of sludge applications. Barbarik et al. (1998), investigating metal concentrations (including Cu, Ni, and Zn) of a soil amended with several metal-contaminated sludges, found that only Zn levels increased below the plough layer 11 years after sludge applications. Sloan et al. (1998) found no effects of three annual sludge applications on metal concentrations (including Cu, Ni, and Zn) in the soil below 45 cm after 16 years.

Influence of earthworm activity on metal development over time

The absence of significant differences between slopes of regressions relating total and extractable metal concentrations with time for soils with and without *D. veneta* demonstrates that earthworm activity did not change the behaviour of metals over time. The working hypothesis that earthworm activity would promote metal availability therefore was not confirmed. Similar results were obtained by Natal-da-Luz et al. (2011b - Chapter 6), in a 12-week laboratory microcosm experiment to evaluate the influence of *D. veneta* on short-term changes in metal availability using the same treatments and earthworm density

(500 earthworm/m^2) as in this study. However, changes in soil metal concentrations over time promoted by earthworm activity have been reported in other laboratory experiments. Sizmur and Hodson (2008) demonstrated that the earthworm *E. veneta* (a synonym for *D. veneta*; Blakemore 2007) can promote changes in water-extractability of Zn and Cu after a short period of incubation. In a pot experiment, using two natural contaminated soils and a natural metal-spiked soil with 20 earthworms per 150g soil (corresponding to 133.3 worms/kg), after 17 days water-extractable Zn concentrations decreased in spiked soil and increased in natural contaminated soils. Earthworm activity also decreased water-extractable Cu levels in spiked soil and in a natural contaminated soil. Wen at al. (2004) found an increase of water-extractable metals (including Cr, Cu, Ni, and Zn) due to the activity of *E. fetida* in five uncontaminated soils. These findings were obtained after 6 weeks incubation using a ratio of 12 earthworms per kg of soil. Apparently, the results obtained in the latter two studies do not support the absence of an influence of earthworm activity on changes in metal availability over time found in the present experiment. However, it should be taken into account that besides the differences in soil properties, a much lower earthworm density (1.1 worm/kg soil) was used in this study, considering only the top 30 cm soil layer (most used by epigeic earthworm species) and a soil density of 1.5 g/cm^3. Changes in metal availability in earthworm casts have been also reported for epigeic earthworm species (e.g. Ireland 1975; Udovic et al. 2007), showing that changes in metal availability promoted by earthworm activity might result also from changes in casts. The available literature data generally suggest that the activity of epigeic earthworm species may promote changes in soil metal availability over time. We believe that, although the incubation period of the present study was much longer (one year) than usually used in laboratory experiments, it is likely that both environmental factors and the low earthworm density (although realistic) mitigated the possible effect of earthworm activity on metal availability.

Metal concentrations in earthworms from sludge-amended and metal-spiked soils

Metal bioaccumulation, on average, was slightly higher in earthworms from metal-spiked than from sludge-amended soils at the same contamination levels. Such differences confirm the working hypothesis that expected higher metal concentrations in earthworms from metal-spiked than from sludge-amended soils and demonstrates the important role of matrices on metal uptake by *D. veneta*.
Chromium uptake was linearly related with total metal concentrations after 10 months in spiked soils and after 3 and 12 months in sludge-amended soils, which suggest an inability of *D. veneta* to control its Cr internal concentration, at least up to the concentrations tested. Chromium bioaccumulation was not significantly related with Cr extractable concentrations in soil, which suggests that Cr bioaccumulation was better explained by soil total metal contents. This is in agreement with Van Gestel (2008) who concluded that in many cases total metal concentrations give a better description of earthworm body concentrations. Other studies, however, demonstrated that soluble metal pools are best descriptors of metal accumulation in earthworms (Spurgeon and Hopkin 1996; Peijnenburg et al. 1999). In agreement with that, Ni concentration in earthworms was linearly related with extractable Ni concentration in sludge-amended soils after 3 months and in spiked soils after 3 and 10 months. Ni bioaccumulation was generally not significantly related to soil total metal contents. The relationships between earthworm Ni concentrations and extractable Ni levels in soil suggest an inability of *D. veneta* to regulate internal Ni concentrations. The concentrations of Cu and Zn in earthworms were independent of the total and extractable soil concentrations in all treatments. These findings suggest that *D. veneta* is able to regulate its internal Cu and Zn concentrations at least for the soil concentrations tested in this study. Our results are only partially in agreement with the data obtained in the laboratory microcosm experiment performed by Natal-da-Luz et al. (2011b - Chapter 6), who found that Cr bioaccumulation linearly increased

with total Cr content in the soil, but only in sludge-amended soils. In the present study, a linear relationship for Cr was obtained in spiked soils after 10 months. They also found regulation of earthworm Zn concentrations, although in general lower Zn levels were measured in the earthworms than in this study. The authors found that earthworm Cu concentrations significantly increased with total Cu levels in soil, especially in earthworms from sludge-amended soils, while Ni uptake in most cases was significantly correlated with both extractable and total soil Ni concentration. Ireland (1979), collecting native *D. veneta* specimens from a soil containing 992 mg Zn/kg and 252 mg Cu/kg DW, found Zn and Cu levels in the earthworms of 134 mg Zn/kg and 14 mg Cu/kg. These internal concentrations are slightly lower than those obtained in the present experiment, although soil concentrations were higher. The internal Zn and Cu levels found by Ireland (1979) suggest an ability of native *D. veneta* to regulate the internal levels of both metals also at soil concentrations higher than those of our treatments, which supports our findings. In a study conducted by Marinussen et al. (1997b) who introduced *D. veneta* in a metal (Cu, Pb, and Zn) contaminated site to investigate Cu bioaccumulation, earthworm Cu concentrations between 33 and 69 mg/kg were found after periods up to 5 weeks of exposure. In the same study, Marinussen et al. (1997b) also assessed the bioaccumulation of Cu by *D. veneta* in a laboratory experiment. Earthworm Cu levels linearly increased with total Cu content in soil up to a concentration of 150 mg of Cu/kg of soil DW, reaching a plateau of 57 mg of Cu/kg DW in the earthworms. Similar results were obtained by Marinussen et al. (1997a) in a laboratory experiment on metal kinetics in *D. veneta* using contaminated soils with total Cu contents of 242 and 815 mg/kg. The earthworm Cu concentrations reached a plateau of 60 mg/kg after 14 days of exposure for both soils, confirming the ability of *D. veneta* to regulate Cu when exposed above a certain soil Cu content. In our study, internal Cu concentrations measured were between 10.8 and 36.6 mg/kg DW, and the ability of *D. veneta* to regulate its internal Cu concentration at concentrations below 150 mg/kg was demonstrated. Higher Cu concentrations in *D. veneta* were

found in another laboratory experiment of Marinussen et al. (1997c), in which the worms were exposed to natural soil spiked with about 250 mg Cu/kg and a range of Cd or Pb levels. After 21 days of exposure, Cu concentrations in *D. veneta* were between 78 and 110 mg/kg. These high levels might be promoted by physical and chemical properties of the natural soil used, which seem to have favoured the accumulation of Cu by *D. veneta*.

To date Cr and Ni kinetics in *D. veneta* have not been studied. Van Gestel et al. (1993) determined the accumulation of Cr and Zn in *E. andrei* after 3 weeks exposure in artificial soil spiked with Cr nitrate and Zn chloride. They found a dose-related increase of Cr uptake up to a total soil concentration of 100 mg Cr/kg and a regulation of Zn body content to concentrations below 1000 mg Zn/kg in soil. The results on Cr uptake in *E. andrei* support our data, especially for earthworms from sludge-amended soils. The Zn bioaccumulation data, confirm the ability of earthworms to regulate their internal Zn concentration. Laskowski et al. (2010) determining Ni toxicokinetics in juvenile *L. terrestris* found this species to be able to control its internal concentration at 51-64 mg Ni/kg after 2 days of exposure to a natural soil spiked with 140 mg Ni/kg. In the present study the Ni internal concentrations were generally outside this range of 51 – 64 mg/kg, which may be related to *D. veneta* being an epigeic species and *L. terrestris* an anecic one. Also the different physical and chemical properties of the soil used by Laskowski et al. (2010) (clay loam, pH 7.1) may have contributed to the different Ni bioaccumulation patterns.

Earthworm metal concentration prediction with existing models

Our data demonstrate that, for both models used, Zn estimations were less accurate, which may be explained by the ability of *D. veneta* to regulate its internal Zn concentration. Although our results also suggest some regulation ability of earthworms for Cu, the uptake of this metal was more accurately predicted by the model of Neuhauser at al. (1995). In the present study, more

accurate predictions of metal concentrations in *D. veneta* were obtained than by Natal-da-Luz et al. (2011b - Chapter 6) applying the same relationship models to data from a laboratory microcosm experiment. This finding suggests that the accuracy of the models tested is higher for field data than for laboratory data. Further studies are needed to confirm this assumption.

Conclusions

Total metal concentrations in sludge-amended and metal-spiked soils did not change over one year, while only for nickel extractability decreased with time. Changes in metal availability were not influenced by the test matrix. Earthworm activity did not affect changes in metal availability with time. Metal concentrations were slightly higher in earthworms exposed to metal-spiked than to sludge-amended soils. The relationship between earthworm metal concentration and soil metal content was influenced by the matrix only after 12 months for Ni and Zn. Chromium bioaccumulation was linearly related with total soil concentration in sludge-amended soils, and Ni bioaccumulation with extractable concentration in metal-spiked soils. *D. veneta* was able to regulate its body concentration of Cu and Zn. The relationship models tested estimated Cu uptake in the earthworms with high accuracy, followed by Ni and Cr. The present study provides important information on changes in metal availability in soil over time and the effect of earthworm activity. Understanding the way soil fauna (e.g. earthworms) interacts with contaminants may contribute to estimating the ecological risk of a contaminated area.

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Annex C

Table C.1 Total Cr, Cu, Ni, and Zn concentrations (average ± SD, n = 4) in the top 10 cm soil layer of field mesocosms, not treated (C and Cw), treated with a low (SL and SLw) or a high sludge dose (SH and SHw), or spiked with a low (ML and MLw) or a high metal dose (MH and MHw) after 3, 6, 10, and 12 months of incubation, with (treatment codes with w) or without earthworms (*Dendrobaena veneta*).

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
3 months	C	6.48 ± 1.80	19.5 ± 8.02	10.7 ± 1.34	49.8 ± 19.0
	Cw	5.87 ± 0.924	22.4 ± 7.35	11.0 ± 1.35	54.1 ± 14.6
	SL	28.5 ± 36.4	22.4 ± 12.6	63.3 ± 82.3	78.3 ± 58.1
	SLw	32.1 ± 19.8	23.9 ± 9.10	61.8 ± 44.6	97.0 ± 43.7
	SH	342 ± 179	90.1 ± 29.6	538 ± 241	394 ± 127
	SHw	626 ± 334	140 ± 56.7	917 ± 419	527 ± 174
	ML	27.9 ± 11.2	30.7 ± 5.16	79.2 ± 21.2	112 ± 35.7
	MLw	39.4 ± 24.0	33.9 ± 16.8	87.7 ± 56.2	129 ± 65.8
	MH	115 ± 28.9	65.5 ± 19.0	248 ± 83.6	225 ± 45.9
	MHw	242 ± 209	109 ± 70.9	352 ± 174	281 ± 127

Table C.1 (Continued)

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
6 months	C	9.25 ± 1.27	31.2 ± 4.25	14.4 ± 0.930	55.3 ± 4.35
	Cw	12.3 ± 8.53	25.2 ± 8.13	14.0 ± 1.42	57.2 ± 16.7
	SL	84.2 ± 54.2	47.0 ± 20.2	184 ± 123	208 ± 102
	SLw	141 ± 197	52.1 ± 43.1	247 ± 295	240 ± 185
	SH	437 ± 138	117 ± 28.1	708 ± 223	479 ± 86.7
	SHw	790 ± 288	177 ± 50.6	1087 ± 347	642 ± 110
	ML	33.9 ± 20.8	32.1 ± 9.97	57.0 ± 15.2	92.3 ± 37.6
	MLw	33.1 ± 32.1	32.5 ± 13.6	60.3 ± 37.6	119 ± 72.2
	MH	104 ± 76.3	64.5 ± 41.1	229 ± 77.8	209 ± 47.7
	MHw	105 ± 26.6	59.9 ± 17.6	262 ± 48.7	257 ± 33.4

Table C.1 (Continued)

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
10 months	C	7.44 ± 1.58	25.3 ± 4.50	11.2 ± 1.52	46.3 ± 4.90
	Cw	6.46 ± 2.01	22.5 ± 4.30	10.6 ± 1.56	41.9 ± 9.90
	SL	168 ± 192	60.4 ± 50.0	309 ± 322	272 ± 220
	SLw	64.6 ± 34.1	37.4 ± 10.9	127 ± 65.8	162 ± 68.9
	SH	694 ± 582	168 ± 114	892 ± 602	770 ± 659
	SHw	573 ± 145	135 ± 20.6	792 ± 154	534 ± 72.8
	ML	44.6 ± 20.2	38.8 ± 12.5	107 ± 64.9	132 ± 47.3
	MLw	73.3 ± 53.0	48.4 ± 16.1	136 ± 81.6	180 ± 79.0
	MH	309 ± 241	111 ± 59.3	361 ± 124	275 ± 75.8
	MHw	200 ± 164	106 ± 57.4	373 ± 94.3	365 ± 32.6

Table C.1 (Continued)

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
12 months	C	4.67 ± 2.09	16.1 ± 2.86	7.38 ± 0.957	34.1 ± 5.44
	Cw	6.82 ± 1.52	19.4 ± 2.48	10.0 ± 1.74	43.0 ± 7.89
	SL	166 ± 243	54.2 ± 58.0	254 ± 339	224 ± 236
	SLw	63.1 ± 42.5	34.8 ± 5.16	94.9 ± 55.8	155 ± 71.7
	SH	642 ± 279	138 ± 43.2	846 ± 292	568 ± 136
	SHw	434 ± 406	102 ± 75.3	612 ± 512	572 ± 540
	ML	21.5 ± 4.79	24.9 ± 2.46	55.3 ± 13.2	67.8 ± 11.2
	MLw	14.5 ± 7.35	22.3 ± 1.68	37.7 ± 11.4	57.7 ± 2.57
	MH	191 ± 130	76.2 ± 38.9	262 ± 109	217 ± 47.5
	MHw	115 ± 18.8	61.4 ± 12.3	252 ± 91.0	227 ± 72.4

Table C.2 Regression slopes for total Cr, Cu, Ni, and Zn concentrations as percentage of initial doses (% initial TMC) against time for different metals of controls (C and Cw), sludge-amended (SL, SLw, SH and SHw) and metal-spiked soils (ML, MLw, MH, and MHw) incubated in field mesocosms with (treatment codes with w) and without earthworms (*Dendrobaena veneta*).

		Time (independent variable)									
				Sludge-amended soils				Metal-spiked soils			
	Metal	C	Cw	SL	SLw	SH	SHw	ML	MLw	MH	MHw
% initial TMC (depend. variable)	Cr	-1.51	-0.669	1.06[b]	-1.14	4.65	-0.044[a]	-2.42	-0.056[a]	0.007[ab]	-1.68
	Cu	-1.30	-0.36	3.98	-0.261[b]	1.54	-0.033[a]	-0.023[a]	-0.018[a]	0.911	-0.346
	Ni	-0.029[a]	-0.016[a]	7.75	-2.69	3.62	-0.042[a]	-0.012[ab]	-0.041[a]	-0.813	-0.869
	Zn	-1.90	-1.48	4.79	-1.96	0.208	-0.036[a]	-2.61	-0.038[a]	-0.857	-0.001[a]

Slopes were obtained using normal linear regression except for [a] which were obtained using the exponential equation; [b] – using values arcsin transformed.

Table C.3 $CaCl_2$ (0.01M) extractable Cr, Cu, Ni, and Zn concentrations (average ± SD, n = 4) in the top 10 cm soil layer of field mesocosms not treated (C and Cw), treated with a low (SL and SLw) or a high sludge dose (SH and SHw), or spiked with a low (ML and MLw) or a high metal dose (MH and MHw) after 3, 6, 10, and 12 months of incubation with (treatment codes with w) or without earthworms (*Dendrobaena veneta*).

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
3 months	C	< 0.249	< 0.349	< 0.427	< 0.108
	Cw	< 0.249	< 0.349	< 0.427	< 0.108
	SL	< 0.249	< 0.349	1.50 ± 0.667	0.191 ± 0.153
	SLw	< 0.249	< 0.349	2.11 ± 0.396	0.196 ± 0.199
	SH	< 0.249	< 0.349	7.00 ± 0.957	0.336 ± 0.152
	SHw	< 0.249	< 0.349	6.07 ± 1.11	0.261 ± 0.211
	ML	< 0.249	< 0.349	16.4 ± 5.60	9.39 ± 3.37
	MLw	< 0.249	< 0.349	15.1 ± 4.40	9.89 ± 3.46
	MH	< 0.249	< 0.349	63.8 ± 7.61	46.9 ± 6.42
	MHw	0.335 ± 0.203	< 0.349	102 ± 85.0	80.1 ± 61.5

Table C.3 (Continued)

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
6 months	C	< 0.249	< 0.349	< 0.427	< 0.108
	Cw	< 0.249	< 0.349	< 0.427	< 0.108
	SL	< 0.249	< 0.349	1.73 ± 0.414	0.846 ± 0.163
	SLw	< 0.249	< 0.349	1.60 ± 0.247	0.729 ± 0.291
	SH	< 0.249	< 0.349	2.71 ± 0.500	0.531 ± 0.177
	SHw	< 0.249	< 0.349	3.34 ± 0.505	0.704 ± 0.078
	ML	< 0.249	< 0.349	7.81 ± 1.98	5.87 ± 2.16
	MLw	< 0.249	< 0.349	7.81 ± 2.28	5.45 ± 1.39
	MH	0.265 ± 0.110	< 0.349	44.0 ± 14.4	38.1 ± 15.7
	MHw	0.232 ± 0.103	< 0.349	53.7 ± 22.1	49.9 ± 19.4

Table C.3 (Continued)

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
10 months	C	< 0.249	< 0.349	< 0.427	< 0.108
	Cw	< 0.249	< 0.349	< 0.427	< 0.108
	SL	< 0.249	< 0.349	1.60 ± 0.380	0.964 ± 0.469
	SLw	< 0.249	< 0.349	1.49 ± 0.501	0.616 ± 0.088
	SH	< 0.249	< 0.349	3.29 ± 0.688	0.684 ± 0.221
	SHw	< 0.249	< 0.349	3.39 ± 0.401	0.841 ± 0.196
	ML	< 0.249	< 0.349	6.92 ± 4.78	4.51 ± 4.07
	MLw	< 0.249	< 0.349	8.94 ± 5.97	5.89 ± 3.85
	MH	< 0.249	< 0.349	73.1 ± 50.6	57.8 ± 38.1
	MHw	< 0.249	< 0.349	43.6 ± 17.6	38.8 ± 17.7

Table C.3 (Continued)

Incubation period	Treatment	Cr (mg/kg DW)	Cu (mg/kg DW)	Ni (mg/kg DW)	Zn (mg/kg DW)
12 months	C	< 0.249	< 0.349	< 0.427	< 0.108
	Cw	< 0.249	< 0.349	< 0.427	< 0.108
	SL	< 0.249	< 0.349	1.36 ± 0.852	0.741 ± 0.712
	SLw	< 0.249	< 0.349	1.20 ± 0.540	0.589 ± 0.298
	SH	< 0.249	< 0.349	3.51 ± 0.708	0.814 ± 0.388
	SHw	< 0.249	< 0.349	3.53 ± 0.645	0.971 ± 0.542
	ML	< 0.249	< 0.349	3.27 ± 1.81	2.22 ± 1.98
	MLw	< 0.249	< 0.349	7.73 ± 6.41	4.50 ± 3.53
	MH	< 0.249	< 0.349	77.1 ± 67.2	62.7 ± 50.3
	MHw	< 0.249	< 0.349	40.2 ± 13.8	35.9 ± 13.4

Table C.4 Regression slopes for 0.01M $CaCl_2$ extractable Cr, Ni, and Zn concentrations as percentage of total metal concentration (% TMC) against time for different metals of unamended (C and Cw), sludge-amended (SL, SLw, SH, and SHw) and metal-spiked soils (ML, MLw, MH and MHw) incubated in field mesocosms with (treatment codes with w) and without earthworms (*Dendrobaena veneta*).

		Time (independent variable)									
				Sludge-amended soils				Metal-spiked soils			
	Metal	C	Cw	SL	SLw	SH	SHw	ML	MLw	MH	MHw
% TMC (depend. variable)	Cr	0.160[b]	1.10[b]	-0.659[b]	-0.005	-0.001	-0.002	0.016[a]	-0.005	-0.004	-0.009
	Ni	0.166	0.129	-0.046[ab]	0.036[a]	-0.101[a]*	-0.056*	-0.261[b]*	-0.332	-1.44*	-1.43*
	Zn	-0.238[b]	-0.877[b]	0.020	0.066[a]	-0.005	0.004	-0.094[a]	-0.073[a]*	-0.030[a]	-1.12

* - statistically significant ($p \leq 0.01$); slopes were obtained using normal linear regression except for [a] which were obtained using the exponential equation; [b] – using values arcsin transformed.

Table C.5 Cr, Cu, Ni, and Zn concentrations in the earthworm *Dendrobaena veneta* collected after 3, 10, and 12 months of exposure from field mesocosms with unamended (Cw), sludge-amended (SLw and SHw), and metal-spiked soils (MLw and MHw) on top of a layer of clean soil. Concentrations are expressed as mg of metal per kg of dry body weight (mean ± SD, $n = 4$).

Incubation period	Treatment	Cr (mg/kg)	Cu (mg/kg)	Ni (mg/kg)	Zn (mg/kg)
3 months	Cw	< 2.80	15.6 ± 2.45	10.7 ± 2.43	178 ± 44.7
	SLw	5.52 ± 2.53	18.7 ± 1.83	20.9 ± 3.68	188 ± 68.0
	SHw	19.7[a]	10.8[a]	31.2[a]	151[a]
	MLw	7.84 ± 7.38	25.9 ± 5.76	32.7 ± 8.88	222 ± 72.2
	MHw	*ND*	*ND*	*ND*	*ND*
10 months	Cw	3.79 ± 2.16[b]	21.8 ± 7.92[b]	17.5 ± 4.59[b]	147 ± 17.5[b]
	SLw	23.2 ± 11.0	32.7 ± 12.4	44.2 ± 14.1	162 ± 14.8
	SHw	*ND*	*ND*	*ND*	*ND*
	MLw	21.8 ± 3.19[b]	28.5 ± 3.67[b]	49.9 ± 1.71[b]	152 ± 4.74[b]
	MHw	57.3[a]	36.6[a]	117[a]	185[a]
12 months	Cw	7.76 ± 3.76[b]	20.1 ± 5.83[b]	15.9 ± 3.21[b]	162 ± 6.55[b]
	SLw	< 2.80	22.1 ± 3.10	22.8 ± 7.30	153 ± 8.18
	SHw	15.6 ± 6.50	22.1 ± 8.46	34.2 ± 10.2	155 ± 8.17
	MLw	36.0 ± 25.6	33.6 ± 2.68	53.9 ± 26.5	242 ± 167
	MHw	64.9[a]	34.7[a]	94.6[a]	540[a]

[a] – $n = 1$; [b] – $n = 3$; *ND* – not determined

Table C.6 Regession slopes for Cr, Cu, Ni, and Zn concentrations in the earthworm *Dendrobaena veneta* against total concentrations of different metals in soil after 3, 10, and 12 months of exposure in field mesocosms with sludge-amended (Sw) and metal-spiked soils (Mw).

		Total metal concentrations (independent variable)					
		3 months		10 months		12 months	
	Metal	Sw	Mw	Sw	Mw	Sw	Mw
Metal concentration in earthworm tissue (depend. variable)	Cr	0.018*	0.150	0.190	0.114*	0.018*	0.388
	Cu	-0.030	0.333	0.018[a]	0.082	0.025	0.252
	Ni	0.000	0.200	0.117	0.207*	0.020	0.324
	Zn	-0.052	0.234	0.134	0.073	-0.010	2.35

* - statistically significant ($p \leq 0.01$); slopes were obtained using normal linear regression except for [a] which was obtained using the linear Langmuir equation (slope = $1/k$).

Table C.7 Regression slopes for Cr, Ni, and Zn concentrations in the earthworm *Dendrobaena veneta* against 0.01M $CaCl_2$ extractable concentrations of different metals after 3, 10, and 12 months of exposure in field mesocosms with sludge-amended (Sw) and metal-spiked soils (Mw).

		Extractable metal concentrations (independent variable)					
		3 months		10 months		12 months	
	Metal	Sw	Mw	Sw	Mw	Sw	Mw
Metal concentration in earthworm tissue (depend. variable)	Cr	59.3	37.1	-100	50.5	-5.41	6.73[a]
	Ni	4.85*	1.33*	15.2	1.46*	3.91	2.14
	Zn	223	3.79	32.8	0.681	-4.68	17.0

* - statistically significant ($p \leq 0.01$); slopes were obtained using normal linear regression except for [a] which was obtained using the linear Langmuir equation (slope = *1/k*).

Chapter 8

The influence of earthworm activity on microbial processes related with the degradation of persistent pollutants

Based on the following manuscript:

Natal-da-Luz T, Lee I, Verweij RA, Morais PV, Van Velzen MJM, Sousa JP, Van Gestel CAM, 2011. The influence of earthworm activity on microbial processes related with the degradation of persistent pollutants. *Environmental Toxicology and Chemistry* (submitted).

Abstract

Earthworms may promote the biodegradation of polycyclic aromatic hydrocarbons (PAHs) in soil, but the mechanism through which they exert such influence is still unknown. To investigate if the stimulation of PAH degradation by earthworms is related with changes in microbial communities, a microcosm experiment was conducted consisting of columns with natural uncontaminated soil covered with PAH-contaminated dredge sediment. Columns without and with low and high *Eisenia andrei* densities were prepared. Organic matter and PAH content, microbial biomass and dehydrogenase (DHA) activity were measured in soil and sediment over time. Biolog Ecoplate™ and PCR-DGGE gel methods were used to evaluate changes in metabolic and structural diversity of the microbial community, respectively. Earthworm activity promoted PAH degradation in soil, which was significant for biphenyl, benzo(*a*)pyrene and benzo(*e*)pyrene. Microbial biomass and DHA activity generally did not change over the experiment. Earthworm activity did change microbial community structure, but this did not affect its functioning in terms of carbon substrate consumption. Results suggest no relationship between changes in microbial community by earthworm activity and increased PAH disappearance. The role of shifts in soil microbial community structure induced by earthworms in PAH removal needs further investigation.

Key words: Biodegradation, polycyclic aromatic hydrocarbons, *Eisenia andrei*, Biolog Ecoplate™, PCR-DGGE.

Introduction

Earthworms are important members of the soil fauna, contributing to soil porosity and the formation and stabilization of aggregates, influencing pedogenesis and promoting litter breakdown (Lavelle et al. 1989). Moreover, by their burrowing and casting activities, earthworms mediate the provision of ecosystem services like nutrient cycling, organic matter decomposition and water cycling in soils (Vandewalle et al. 2008). Earthworms also have the potential to influence the availability of inorganic contaminants like metals (Sizmur and Hodson 2009) and to promote the biodegradation of organic pollutants such as polycyclic aromatic hydrocarbons (PAHs; Eijsackers et al. 2001).

PAHs are substances potentially genotoxic and carcinogenic (Cerniglia 1992), usually resulting from coke production, petroleum refining, fossil fuel combustion and other high-temperature industrial processes, and accumulate in organic rich soils and sediments. The regular dredging of waterways, which is common practice in The Netherlands, followed by sediment deposition on adjacent areas may cause PAH contamination of bordering grasslands. All PAH are adsorbed by organic matter, and may persist mainly under anaerobic conditions. Especially the high molecular weight PAHs are less volatile and more persistent in soil (Cerniglia 1992).

Earthworm activity in soil may stimulate aerobic PAH biodegradation and by consuming organic matter they may reduce PAH adsorption, increasing bioavailability (Eijsackers et al. 2001). The ability of earthworms to change the structure, biomass, and functioning of soil microbial communities (Sheehan et al. 2008) may indirectly stimulate PAH biodegradation, which is predominantly dependent on microbial activity (Weissenfels et al. 1992; Shuttleworth and Cerniglia 1995). Therefore, the introduction of earthworms constitutes a potential bioremediation tool for soils amended with dredge sediment polluted with persistent organic contaminants. Studies have reported the influence of earthworm activity on decreasing (Devliegher and Verstraete 1995; Zhang et al.

2000) or increasing soil microbial biomass (Aira et al. 2007) and on enzyme activity including dehydrogenase, generally contributing to stimulate soil microbial activity (Businelli et al. 1984; Aira et al. 2006). Other studies reported the ability of earthworms to stimulate the increase of certain fatty acids and respiration activity in soil (Tiunov and Scheu 2000; Schaefer et al. 2005; Sampedro and Whalen 2007).

During the last decade, new methods to characterize soil microbial community structure and functioning have gained importance. The Biolog Ecoplate™ method, based on the ability of microorganisms to degrade a wide range of organic substrates, provides significant information on the functional structure of soil microbial communities (Garland and Mills 1991; Insam et al. 1996). The use of polymerase chain reaction (PCR) to analyse 16S rRNA genes using denatured gradient gel electrophoresis (DGGE) allows to follow shifts in soil bacterial diversity (Ishii and Takii 2003). Although these molecular methods constitute rapid, easy and low cost ways to characterize soil microbial communities, only in recent years Biolog Ecoplate™ (Aira et al. 2006; Sheehan et al. 2008) and PCR-DGGE profiling (Egert et al. 2004; Sen and Chandra 2009; Zhao et al. 2010) have been used to evaluate the influence of earthworm activity on soil microbial function and diversity.

To better understand the role of earthworms in the regulation of the biodegradation of persistent pollutants, this study aimed at evaluating their influence on microbial processes when exposed to PAH-contaminated soils. For that purpose, a laboratory experiment was performed using columns filled with natural uncontaminated soil covered with a layer of PAH contaminated dredge sediment. Columns received no or low and high densities of the earthworm *Eisenia andrei*. More specifically, the objective of the present study was to evaluate shifts in the soil microbial community promoted by earthworm activity and relate them with the biodegradation of PAHs. Our working hypotheses were that: i) microbial communities in the sediment layer and adjacent soil of columns without earthworms tend to be more different from those of columns with

earthworms over time due to the influence of earthworm activity; and, ii) a higher earthworm density increases the similarity between microbial community structures of the sediment and soil layers over time due to the homogenizations of substrate promoted by earthworm activity.

Materials and methods

Natural soil and dredge sediment

A natural sandy soil (4.8% clay) was obtained from a field site in De Kwakel, The Netherlands. Dredge sediment was collected from the Noord-Hollands Kanaal in Alkmaar, The Netherlands. According to previous reports, the sediment was predominantly contaminated with PAHs and some lead (Pb) and zinc (Zn). Both soil and sediment were sieved (4 mm) to remove large particles and analysed for pH (0.01M $CaCl_2$, 1:5, v/v), organic matter (OM) content (loss on ignition at 500ºC for 6 h), PAH concentration (see below) and water holding capacity (WHC; ISO 1997).

Experimental procedure

For the laboratory microcosm test, a test system that was also employed for earlier Terrestrial Model Ecosystem (TME) tests (Knacker et al. 2004) was used. High-density polyethylene tubes (Ø 17.5 cm and 40 cm depth) were filled with uncontaminated soil until 6 cm from the top. The columns were equipped with a bottom plate with a net (± 1 mm) and placed inside a temperature controlled box, with soil temperature maintained at 12-14 ºC. Rain simulations were performed with artificial rain water, using rain-heads, as described by Knacker et al. (2004). Leachate was collected in polyethylene bottles. After filling the columns, three rain simulations were performed over a two week period, each one consisting of 350 ml of rainwater per microcosm (14.6 mm water layer). After these

irrigations, all columns had produced leachate. Next, a 5 cm layer of dredge sediment was placed on top of the soil column. After three days, 175 ml artificial rain was applied to each column and the next day earthworms were introduced and the experiment started. The columns received either 0, 6 or 24 individuals of the earthworm *E. andrei* representing densities of 0 (control, C), 250 (low density, L), and 1000 (high density, H) individuals per m^2, respectively. Earthworm individual mass was on average 415 ± 112 mg fresh weight. The earthworms were obtained from a synchronized laboratory culture at VU University, and were acclimated by incubation in uncontaminated soil for one day. Treatments were randomly distributed over the test system. All columns received 175 ml artificial rainwater twice a week.

The experiment was run at 24 ± 5ºC, at a relative humidity of 45-85% (controlled by air humidifiers), a light intensity of 8,000 to 12,000 lux and a photoperiod of 16:8 h light:dark (using high pressure sodium vapour lamps, Philips SON-T 400 watt).

After 2, 4, 8, and 18 weeks (T1, T2, T3, and T4, respectively), five replicate columns per treatment were destructively sampled. Cores (Ø 10 cm) of the sediment layer and the top 10 cm soil layer were collected. Moisture (upon drying at 60ºC for 48 h) and OM content (loss on ignition at 500ºC for 6 h) were measured and samples were stored for further analyses. An aliquot of each sample was dried at 60ºC for 48 h and stored at room temperature for metal analysis, another was freshly frozen at -20ºC for PAH extraction and functional (Biolog Ecoplate^{TM}) and structural (PCR-DGGE) microbial community analysis, and a third aliquot was freshly stored at 4ºC for measurement of microbial biomass (C_{mic}) and DHA activity. At each sampling, the surviving *E. andrei* and the native earthworms were counted in each test column. The volume of leachate from each column was recorded twice a week and pH of the leachate from three randomly selected replicates of each treatment was measured. Some leachate samples were also analysed for PAH content.

Metal analysis

Dry soil and sediment samples were homogenised using an acid-washed porcelain pestle and mortar. Approx. 130 mg of each sample, together with 2 ml of destruction mixture (HNO_3:HCl, 4:1, v/v) was placed in a Teflon destruction bomb that was tightly closed and heated at 140ºC for 7 h. The resulting extract was diluted with ultrapure water to a final volume of 10 ml, and transferred to a 13 ml plastic tube. Replicate blanks were run simultaneously. Concentrations of Pb and Zn were determined by flame atomic absorption spectrometry (Perkin Elmer AAnalyst 100 AAS). The quality of the analysis was checked using ISE sample 989 (International Soil-Analytical Exchange) certified by Wageningen Evaluating Programs for Analytical Laboratories as reference material. Average recoveries of Pb and Zn were 96.9 and 114%, of the certified concentrations, respectively.

PAH analysis

Soil and sediment samples collected before the experiment and after 4 and 18 weeks were analysed for PAHs. Less than 5 g soil or 1 g sediment, previously freeze dried and homogenised, were extracted in a pressurized liquid extraction system (ASE 350, Dionex, USA) using hexane:acetone, 3:1, v/v as solvent. Prior to extraction, an internal standard solution of deuterated PAHs was added to correct for losses during cleanup (ES-2528, Cambridge Isotope Laboratories, Inc, USA). For quality control CRM 535 freshwater harbour sediment (NIST, USA) and blanks were analyzed simultaneously. Extracts were concentrated by evaporation at 30ºC in a water-bath under a stream of N_2. Extract were cleaned up through a glass column containing 15 g alumina-oxide (Al_2O_3, deactivated with 8% H_2O, w/w) with 1 g of Na_2SO_4 on top. The column was pre-wetted with 20 ml pentane, the extract was added on the column and eluted with 170 ml pentane. The pentane was concentrated by Kuderna-Danish distillation at 45ºC

after adding 0.5 ml of iso-octane as a keeper. After evaporation until 1 ml, activated copper powder was added to remove the sulphur. Extracts were further fractionated on a column containing 1.8 g silica (deactivated with 1.5% H_2O, w/w) and 0.5 g Na_2SO_4 on top. The column was pre-wetted with 4 ml hexane and the extract was applied to the column followed by 14 ml hexane. When all hexane passed the column a clean collection tube was placed and 10 ml of hexane:diethylether (hexane:DEE; 85:15, v/v) was introduced to elute the PAHs. After adding 1 ml of iso-octane all hexane:DEE was evaporated on a water-bath (30°C) under a gentle stream of N_2. The resulting extracts were stored in crimp-top vials at 4ºC until analysis. All the clean-up and fractionation steps were performed protecting extracts from light to avoid photodegradation of the PAHs. The detection of PAHs was performed by a gas chromatograph equipped with mass spectrometer (GC-MS; Agilent 6890 GC with a 5975 MSD). The following PAHs were measured: biphenyl, acenaphthylene, acenaphthene, fluorene, dibenzothiophene, phenanthrene, anthracene, fluoranthene, 4H-cyclopenta(*def*)phenanthrene, pyrene, benz(*a*)anthracene, chrysene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*e*)pyrene, benzo(*a*)pyrene, indeno(*1,2,3-cd*)pyrene, dibenz(*a,h*)anthracene, and benzo(*ghi*)perylene.

Percolates from two replicates of the L and H treatments and from one control replicate of the T4 sampling were also analysed for PAH content. For this analysis, 50 ml samples were hand shaking with 13 ml hexane in a 100 ml separating funnel for 1 minute. The hexane fraction was separated and reduced to 1 ml by evaporation in a water-bath at 30ºC under a stream of N_2 gas and cleaned up on a 1.5 g silica gel 6G column with 13 ml of a mixture of hexane:dichloromethane (85:15, v/v), which was then evaporated until 1 ml, transferred to acetonitrile (ACN) and evaporated until 0.75 ml. The PAHs remained in the ACN and were measured using a HPLC (Dionex Ultimate 3000 pump autosampler and column oven) with a Vydac 25 cm 18 C reversed phase column with a diode array detector (UVD320s Gynkotek, Germering, Germany) combined with a fluorescence detector (model FP-1520; Jasco, Essex, UK). The

PAHs measured were naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz(*a*)anthracene, chrysene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*a*)pyrene, dibenz(*a,h*)anthracene, benzo(*ghi*)perylene, and indeno(*1,2,3-cd*)pyrene.

DHA activity and microbial biomass

Dehydrogenase activity (DHA) in soil was measured using the method of Öhlinger (1996) adapted for a microplate reader. For each sample, 1 g portions of fresh soil were placed in four 15 ml test tubes. One ml of 1% 2,3,5-triphenyltetrazolium chloride (TTC) in 0.1M Tris buffer, pH 7.4, was added to three of the tubes, and the fourth tube was prepared without TTC (control). Test tubes were sealed, shaken and incubated for 24 h at 40ºC. The formed triphenyl formazan (TPF) was extracted using 5 ml acetone by shaking in an orbital shaker for 2 h, in the dark. Next, test mixture was centrifuged (4 min at 3000 rpm) and 200 µl of supernatant from each test tube were placed in microplate cells. TPF was determined photometrically at 546 nm, within 1 h, using a microplate reader (Tecan, Sunrise remote, Austria). Concentration of TPF was calculated using a calibration curve made with standard solution of TPF (0.017, 0.033, 0.1, 0.13, 0.2 mg/ml) after subtracting the absorbance measured in the control tube. DHA activity was expressed as µg TPF/g DW/h.

Microbial biomass (C_{mic}) was determined by the chloroform fumigation-extraction method (ISO 1997) using samples with a moisture content > 30% of the WHC. Three fumigated (with ethanol-free chloroform for 24 h at room temperature) and three unfumigated portions (25 g DW) of each sample were extracted with 100 ml 0.5M K_2SO_4 solution for 30 min on horizontal shakers (approx. 400 rpm). The extracts were filtered through individual Whatman No 1 filter paper discs (Cat. No 1001150, Maidstone, England) and stored at -20ºC until measurements. Extracts from both fumigated and unfumigated portions were analysed for soluble organic carbon using a combustion infrared method

with LiquiTOC (Elementar Analysensysteme GmBH, Germany). C_{mic} was estimated as the difference between the organic carbon contents in the fumigated and unfumigated portions, divided by the fumigation-extraction method efficiency factor K_{ec} = 0.38 (ISO 1997).

Microbial community functional profiling

Microbial functional profiling was assessed using the Biolog Ecoplate™ (Biolog inc., Hayward, CA, USA) system. Soil and sediment samples were collected from all test treatments (C, L, and H) before the experiment and after 2 and 18 weeks. Approx. 2 g FW was weighted twice into two test tubes, which were incubated in the dark at 20 ± 2ºC for one week. After that period, 20 ml of a 0.2% pyrophosphate solution was added in each test tube. After mixing, 1 ml of the sample was transferred into another test tube and 18 ml pyrophosphate solution was added. The tube was mixed and 140 µl aliquots of this solution were placed in wells of the Biolog Ecoplate™. The inoculated plates (one for each sample) were incubated in the dark at 20 ± 2ºC for 15 days and, the absorbance of plates was registered twice a day with a microplate reader (Tecan, Sunrise remote, Austria) at 590 nm.

PCR-DGGE fingerprinting of 16S rRNA gene

Soil and sediment samples taken before the experiment (T0) and after 18 weeks (T4) were used (1 g FW per sample) to assess the diversity of the microbial community. DNA extraction was performed with a soil DNA isolation kit (E.Z.N.A. soil DNA isolation kit, Omega Bio-Tek, Inc., Norcross, Georgia, USA) according to manufacturer's instructions. PCR for the bacterial 16S rRNA gene was performed using Platinum Taq DNA polymerase (Invitrogen, USA) with the forward primer 27F (5'- GAG TTT GAT CCT GGC TCA G -3'; *Escherichia coli* position: 8 to 27) and the reverse primer 1525R (5'- AGA AAG

GAG GTG ATC CAG CC -3'; *E. coli* position: 1495; Rainey et al. 1992). The V3 region of the bacterial 16S rRNA gene was amplified (PCR nested) using Biopro Taq DNA polymerase (Bioline, UK) with the forward primer 341F (5'-CCT ACG GGA GGC AGC AG -3'; *E. coli* position: 341) with a 40 bp GC clamp and the reverse primer 534R (5'- ATT ACC GCG GCT GCT GG -3'; Muyzer et al. 1993). Aliquots of 50 µl of respective GC clamped nested PCR products (with 10% loading buffer) were used for DGGE electrophoresis, which was performed in a D-Code system (BioRad, USA). Polyacrylamide gels were prepared with denaturating gradients ranging from 30 to 70% and were run at 60ºC and 70V for 17 h. Gels were stained for 30 min with ethidium bromide (50 µg/ml). After that period, gels were visualized and photographed on a UV transilluminator table (Gel Doc, BioRad, USA). Photographs were analyzed with BioRad Quantity One software package.

Statistical analysis

The difference in OM content, PAH concentration (as % of initial T0 level), DHA activity, and C_{mic} between treatments with (L and H) and without earthworms (C) was evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's comparison test for each sampling date.

The optical densities measured in Biolog Ecoplates™ were corrected by subtracting the initial colour intensity. Each substrate was then blanked against the control well according to Garland (1996). Because the absorbance of the control well can be slightly higher than that of some substrates, negative values were considered zero. To reduce the impact of inoculum density on well colour development (WCD) when comparing all samples, absorbance values were normalized by dividing by the average well colour development (AWCD) of all 31 carbon sources of the respective plates (Garland 1997). The normalized WCD from a plate reading time between 100 and 119 h were chosen (corresponding to the mid to late exponential phase of colour development). The Bray-Curtis index

of similarity (Faith et al. 1987) was used to evaluate the resemblance of substrate consumption between i) sediment and soil samples from the same treatment; and, ii) between L and H treatments and control separately for soil and sediment samples for each sampling date. The influence of each carbon substrate (in %) for the distance between such samples was determined by Simper analysis (Clarke 1993). Similarities between sediment and soil of L and H treatments were compared with those of control by one-way ANOVA followed by Dunett's post-hoc test. The comparison of similarity between replicates from the control (within control similarity) and similarity between the L and H treatments and that of the control was performed using a one sample *t*-test, separately for sediment and for soil samples. The metabolic diversity of microbial communities was evaluated by determination of substrate richness (the number of used substrates), substrate evenness (the equitability of activities across all used substrates), and substrate diversity (using Shannon's diversity index; Zak et al. 1994) using absorbance values not normalized and higher than 0.25 to eliminate weak false positive responses (Garland 1996). The ecological indices of treatments sampled at different incubation times were compared to those of the controls by means of one-way ANOVA followed by Dunnett´s post-hoc test.

As for substrate consumptions in Biolog Ecoplates^{TM}, in PCR-DGGE fingerprinting, the similarity between i) sediment and soil samples from the same treatment; and, ii) between L and H treatments and control separately for soil and sediment samples for the last sampling date (18 weeks) was evaluated by means of Bray-Curtis index. In this case no Simper analysis was performed. The structural diversity was also calculated using Shannon's diversity index (Zak et al. 1994). All the statistics for the PCR-DGGE were based on the presence/absence matrix obtained from DGGE bands.

In all statistical analyses, prior to one-way ANOVAs and one sample *t*-tests, the data were always checked for normality and homogeneity of variances using the Kolmogorov–Smirnov and Bartlett tests, respectively. When these assumptions were not met, data were arcsin transformed. Both similarities by Bray-Curtis

indices and Simper analysis were determined using Primer 5 for Windows version 5.2.6. All other statistical analyses were performed using Statistica 6.

Results

Organic matter content was higher in the reference soil compared to the test sediment. Total PAH concentration was high in the sediment and low in the soil (Table 8.1).

Table 8.1 Physical and chemical characteristics of the reference soil and the dredge sediment used in this study to construct microcosms (average ± SD, n = 3).

	Reference soil	**Dredge sediment**
pH (0.01M $CaCl_2$)	7.02 ± 0.14	7.67 ± 0.01
Organic matter (%)	12.9 ± 0.90	4.00 ± 0.20
Water-holding capacity (%)	73.2 ± 2.20	68.8 ± 7.90
Biphenyl (µg/kg)	2.26 ± 0.102	29.2 ± 12.3
Benzo(*e*)pyrene (µg/kg)	245 ± 113	1,466 ± 785
Benzo(*a*)pyrene (µg/kg)	239 ± 127	1,448 ± 817
ΣPAHs (mg/kg)	1.33 ± 0.542	18.2 ± 11.0

One week after the dredge sediment was placed on top of the test columns, the surface became hard and cracks appeared. The 5 cm sediment layer gradually dried out, slightly reducing its volume. In spite of that, the deeper sediment remained less dense and accessible for the earthworms. On the sediment of some columns a thin layer of mosses developed during the experiment. Two replicates of C and one replicate of L treatments from the 18-week (T4) sampling were

colonised by plants (*Chenopodium* sp., *Stellaria media*, and *Rumex* sp.), with an average biomass of 14.6 ± 8.10 g (± SD, n = 3).

E. andrei survival was higher than 87% in all treatments at all sampling dates. Earthworm biomass generally increased, except for week 18, when body weight was 82.7% of the initial value (Table 8.2). Autochthonous earthworm species were found only at T4, with an average of 5.2 ± 2.5 individuals per column (± SD, n = 15). No more than 3 juveniles were found in few columns after 8 and 18 weeks. No earthworms were found in the sediment layer after 2, 4, and 8 weeks. After 18 weeks most earthworms were found in the soil, although the distribution over soil and sediment was not quantified.

Table 8.2 Survival and weight change (% of initial) of the earthworm *Eisenia andrei* (average ± SD, n = 15) after incubation in mesocosms containing a natural soil covered with a 5 cm layer of PAH contaminated sediment (Table 8.1), for different periods.

Exposure time (weeks)	Survival (%)	Weight change (% of initial)
2	93.8 ± 10.4	165 ± 21.1
4	87.1 ± 10.7	167 ± 15.5
8	90.3 ± 12.0	167 ± 19.5
18	87.5 ± 10.4	82.7 ± 17.3

Moisture content and pH of sediment and soil layers were similar between treatments over time. The total volume and pH of percolates were not influenced by the presence of earthworms and no PAHs were detected in the percolates.

The total Pb and Zn concentrations in soil and sediment showed some fluctuations over time. Lead concentrations ranged between (mean ± SD, n = 5) 54.8 ± 8.6 and 71.3 ± 28.0 mg Pb/kg dry sediment and between 34.9 ± 2.30 and 45.0 ± 3.00 mg/kg dry soil. In sediment samples from H treatments, after 2

weeks (T1), the Zn content was significantly lower than that in the control with 35.2 ± 10.6 *vs* 151 ± 6.90 mg/kg dry sediment; in all other treatments at the other sampling dates (after 4, 8, and 18 weeks corresponding to T2, T3, and T4, respectively) Zn values did not significantly differ from that of control and ranged between 59.0 ± 56.7 and 141 ± 7.80 mg/kg dry sediment and between 36.2 ± 3.90 and 44.1 ± 3.90 mg/kg dry soil. The OM content was similar between treatments and did not change over time, with values between 4.50 ± 0.30 and 5.60 ± 0.60% in sediment and between 12.3 ± 0.30 and 13.9 ± 1.80% in soil.

Earthworm activity influenced the ΣPAH concentration only in soil after 18 weeks with a decrease observed at both *E. andrei* densities. Considering individual PAHs, a higher degradation compared to control was detected only for biphenyl and benzo(*a*)pyrene in columns with low earthworm density and for biphenyl, benzo(*e*)pyrene and benzo(*a*)pyrene with high earthworm density (Figure 8.1). In the sediment layer, after 18 weeks, PAH concentrations seemed to increase at high earthworm density, but this increase was not significant.

DHA activity in controls ranged on average (± SD, $n = 5$) between 1.90 ± 1.82 and 6.26 ± 0.504 µg TPF/g dry sediment/h, and between 2.79 ± 0.547 and 8.92 ± 0.692 µg TPF/g dry soil/h. In columns with earthworms, DHA activity did not significantly differ from that in the controls, but was significantly higher in soil from the L treatment after 4 weeks (T2; Figure 8.2). Microbial biomass in soil and sediment did not significantly differ between treatments over time and ranged on average (± SD, $n = 6$) between 21.8 ± 11.2 and 75.1 ± 34.3 mg/kg dry soil, and between 12.8 ± 13.3 and 44.1 ± 17.3 mg/kg dry sediment.

Richness, diversity, and evenness of the carbon substrate consumption in Biolog Ecoplates™ by microbial communities from columns with earthworms did not significantly differ from that of the controls over time (Table 8.3). The similarities between substrate consumption of microbial communities in soil and sediment were not influenced by earthworm activity over time (Figure 8.3). However, the influence of the different substrates on the distance between such

samples slightly changed between treatments over time. After 2 weeks (T1), the distances were predominantly influenced by amino acid substrates in the C and L treatments (14.4 and 9.28%, respectively), but in H treatments, the highest influence was from carboxylic acid substrates (16.0%). After 18 weeks (T4), the distance between control columns (C) was mainly influenced by carbohydrate substrates (13.7%) and in the L and H treatments predominantly by carboxylic acids (10.8 and 22.1%, respectively).

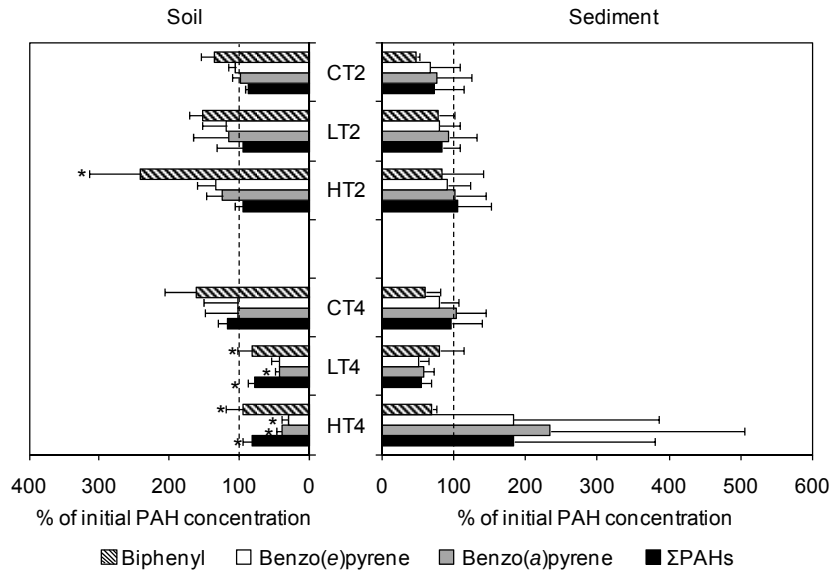

Figure 8.1 Mean levels (± SD, n = 3; % of initial concentration) of polycyclic aromatic hydrocarbons (ΣPAHs) and three individual PAHs (biphenyl, benzo(*a*)pyrene and benzo(*e*)pyrene) in the soil and sediment layers of microcosms containing a natural soil covered with a 5 cm layer of PAH contaminated sediment (Table 8.1), incubated without earthworms (C), and with low (L) and high (H) earthworm densities for 4 (T2) and 18 (T4) weeks. * significantly different ($p \leq 0.05$) compared with the control.

The similarities between carbon substrate consumptions of microbial communities in soil from control and earthworm containing columns, did not show significant changes over time. In sediment samples, this similarity significantly increased for both L and H treatments after 18 weeks (T4; Figure 8.4). For soil, the distances between samples were generally influenced by the consumption of amino acid substrates over time (after 2 weeks 20.4 and 18.5% and after 18 weeks 15.0 and 12.4% for L and H, respectively). In sediment, distances were mainly influenced by amino acids only in L treatments after 2 weeks (T1; 17.5%). In the H treatment after 2 weeks and the L and H treatments after 18 weeks, the main influence was from consumption of carboxylic acids (21.1, 13.5, and 16.2%, respectively).

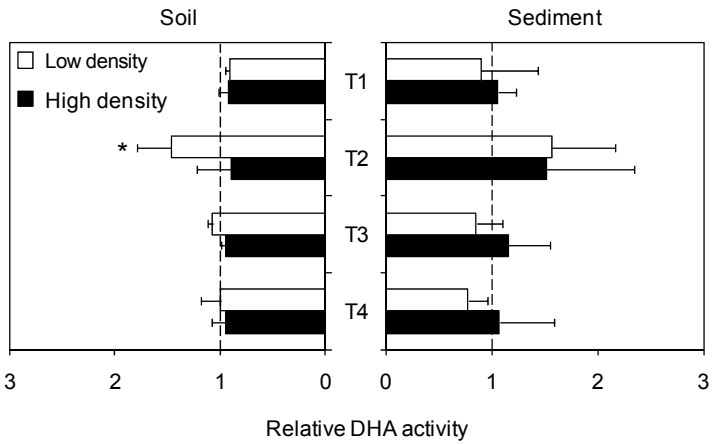

Figure 8.2 Dehydrogenase (DHA) activity (average ± SD, n = 5; relative to control at the same sampling date) in the soil and sediment layers of microcosms containing a natural soil covered with a 5 cm layer of PAH contaminated sediment (Table 8.1), incubated without earthworms (control), and with low and high earthworm densities for 2, 4, 8, and 18 weeks (T1, T2, T3, and T4, respectively). Values > 1 mean average DHA activity higher than control; * - significantly different ($p \leq 0.05$) compared with the control.

Table 8.3 Values (average ± SD, $n = 2$) of different ecological indices (substrate richness, diversity and evenness) calculated from the absorption measured in Biolog Ecoplates™ inoculated with microorganisms from soil and sediment from microcosms containing a natural soil covered with a 5 cm layer of PAH contaminated sediment (Table 8.1), incubated without earthworms (C), and with low (L) and high (L) earthworm densities.

Samplings (weeks)	Substrate richness			Substrate diversity			Substrate evenness		
	C	L	H	C	L	H	C	L	H
Sediment									
0	22.0 ± 2.8	22.0 ± 2.8	22.0 ± 2.8	4.23 ± 0.17	4.23 ± 0.17	4.23 ± 0.17	0.95 ± 0.00	0.95 ± 0.00	0.95 ± 0.00
2	24.5 ± 0.7	24.0 ± 7.1	19.0 ± 11.3	4.37 ± 0.07	4.41 ± 0.45	3.90 ± 0.84	0.95 ± 0.01	0.97 ± 0.01	0.95 ± 0.01
18	16.0 ± 1.4	18.0 ± 2.8	19.0 ± 4.2	3.79 ± 0.20	4.00 ± 0.24	4.02 ± 0.25	0.95 ± 0.02	0.96 ± 0.01	0.95 ± 0.01
Soil									
0	21.0 ± 1.4	21.0 ± 1.4	21.0 ± 1.4	4.17 ± 0.09	4.17 ± 0.09	4.17 ± 0.09	0.95 ± 0.00	0.95 ± 0.00	0.95 ± 0.00
2	20.0 ± 0.0	20.0 ± 2.8	23.5 ± 2.1	4.12 ± 0.07	4.12 ± 0.18	4.40 ± 0.10	0.95 ± 0.02	0.96 ± 0.00	0.97 ± 0.01
18	21.5 ± 2.1	20.5 ± 2.1	22.5 ± 0.7	4.22 ± 0.17	4.16 ± 0.15	4.32 ± 0.10	0.95 ± 0.01	0.96 ± 0.00	0.96 ± 0.01

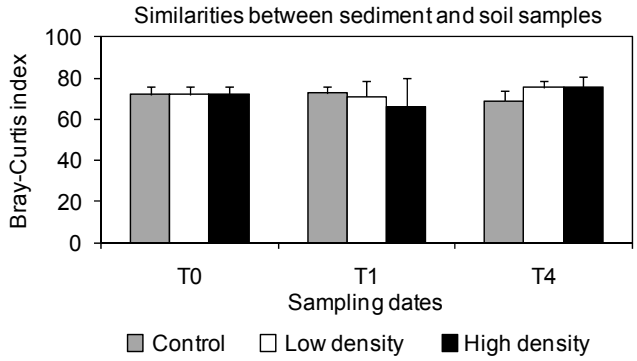

Figure 8.3 Bray-Curtis similarities (average ± SD, n = 5) for the carbon substrate consumption in Biolog Ecoplates™ by microorganisms between soil and sediment layers of microcosms containing a natural soil covered with a 5 cm layer of PAH contaminated sediment (Table 8.1), incubated without earthworms (Control; grey bars), and with low (white bars) and high (black bars) earthworm densities for 0, 2, and 18 weeks (T0, T1, and T4, respectively).

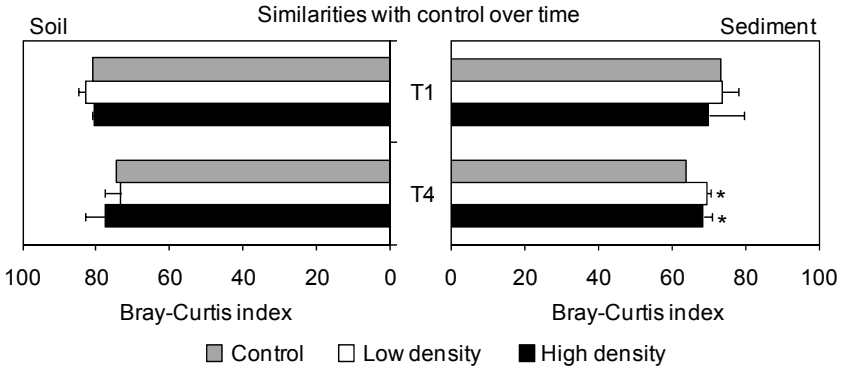

Figure 8.4 Bray-Curtis similarities (average ± SD, n = 5) for the carbon substrate consumption in Biolog Ecoplates™ by microorganisms between soil samples (left) and between sediment samples (right) of control (grey bars) and low (white bars) and high (black bars) earthworm density treatments of microcosms containing a natural soil covered with a 5 cm layer of PAH contaminated sediment (Table 8.1), incubated for 2 and 18 weeks (T1 and T4, respectively). * - significantly different ($p \leq 0.05$) compared with the control.

At T0 and after 18 weeks (T4), the PCR-DGGE gels showed distinct bacterial community profiles between sediment and soil (Figure 8.5).

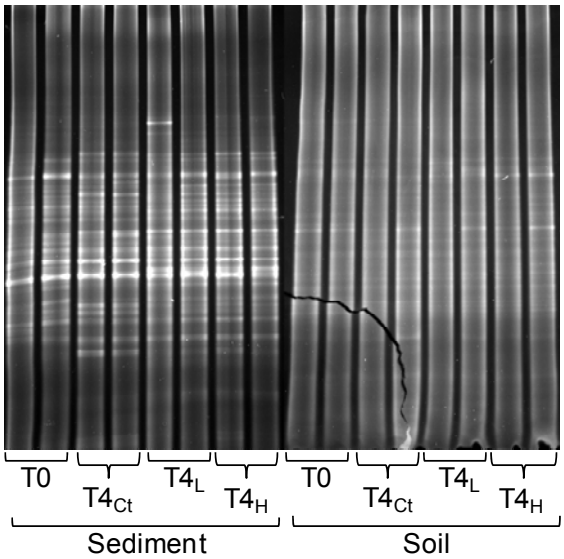

Figure 8.5 PCR-DGGE profiles (16S rRNA genes) of bacterial communities in 16 sediment and soil samples from microcosms containing a natural soil covered with a 5 cm layer of PAH contaminated sediment (Table 8.1), incubated without earthworms (Ct), and with low (L) and high (H) earthworm densities. T0: samples collected before earthworm introduction; T4: samples collected after 18 weeks of experiment.

The similarity between sediment and soil layers significantly increased from an average (± SD, $n = 4$) of 10.3 ± 0.92 to 40.0 ± 9.24 in control columns at T0 and T4, respectively. After 18 weeks (T4) that similarity was higher for treatments with earthworms (L and H) with significant differences compared to that of control only for the H treatment (48.8 ± 4.60 and 55.3 ± 7.24 for L and H, respectively; average ± SD, $n = 4$). Similarities between samples of columns with earthworms and those of control columns were generally significantly lower

than between samples of control columns (71.4 and 94.4 for soil and sediment of control columns, respectively). The exception was for soil samples of the H treatments. These similarities, however, did not decrease with increasing earthworm density (53.4 ± 7.92 and 57.1 ± 11.1 for soil and 65.3 ± 7.49 and 68.3 ± 1.92 for sediment samples in L and H treatments, respectively; average ± SD, n = 4). Samples of sediment at T0 presented higher structural diversity than soil as evidenced by the Shannon indices of 4.16 ± 0.256 and 2.80 ± 0.416, respectively (average ± SD, n = 8). Biodiversity increased in the 18-week test period, with Shannon indices in sediment increasing from 3.85 ± 0.212 (n = 2) to 4.26 ± 0.178 (n = 6) and in soil from 2.32 ± 0.00 (n = 2) to 2.96 ± 0.344 (n = 6). The Shannon indices demonstrated that after 18 weeks the biodiversity on average tended to increase with increasing earthworm density in both sediment (4.17 ± 0.00, 4.20 ± 0.277, and 4.43 ± 0.047 for C, L, and H, respectively, n = 2) and soil (2.81 ± 0.00, 2.95 ± 0.521, and 3.13 ± 0.461 for C, L, and H, respectively, n = 2). Cluster analysis of the DGGE bands distinguished T0 soil samples from the other samples, which were grouped into two main sub-clusters, one including sediment samples and the other soil samples. The cluster of T4 soil samples was separated into two sub-clusters including samples from control and samples from treatments with earthworms. The cluster composed by sediment samples was firstly separated into two sub-clusters, which included T0 and T4 sediment samples, respectively. The T4 samples were separated into samples from control and samples from treatments with earthworms (Figure 8.6).

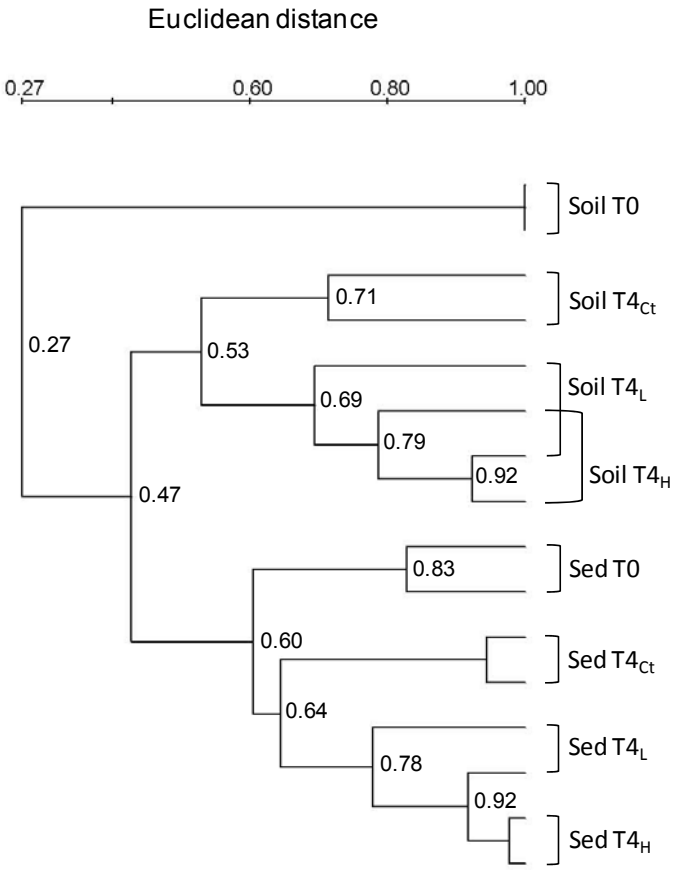

Figure 8.6 Cluster analysis of the DGGE banding patterns in 16 sediment (Sed) and soil samples from microcosms containing a natural soil covered with a 5 cm layer of PAH contaminated sediment (Table 8.1), incubated without earthworms (Ct), and with low (L) and high (H) earthworm densities. Clustering was calculated by UPGMA. The dendogram depicts the hierarchical clustering of the samples based on Euclidean distances (using BioRad Quantity One software). T0: samples collected before earthworm introduction; T4: samples collected after 18 weeks of experiment.

Discussion

E. andrei survival in the L and H treatments (≥ 87.1%) was consistent with the LC_{50} values between 921 and 1949 mg/kg reported for the toxicity of PAH mixtures for *E. fetida* (Potter et al. 1999; Sayles et al. 1999; Eom et al. 2007), which are considerably higher than the ΣPAH concentration in the sediment used in this study (18.2 mg/kg). Literature data available on the toxicity of selected individual PAHs show that the survival of *E. fetida* is not affected by benzo(*a*)pyrene, phenanthrene, anthracene and fluoranthene up to 50-100 mg/kg dry soil (Schaub and Achazi 1996; Contreras-Ramos et al. 2006, 2009). After 10-11 weeks exposure in soils containing phenanthrene, anthracene and benzo(*a*)pyrene up to concentrations of 100, 500-1000, and 150 mg/kg, respectively, weight losses of 23-40% were detected (Contreras-Ramos et al. 2006, 2009). Also these findings support the high survival and absence of effects on body weight changes of *E. andrei* in the present study with sediment and soil containing much lower PAH concentrations.

Earthworm activity promoted PAH degradation only in the soil layer. The fact that earthworms were found in the sediment only after 18 weeks suggests that more time would be needed for earthworm colonisation of sediment layers, and, therefore for a clear effect of earthworm activity on PAH degradation in sediment. Eijsackers et al. (2001) found that earthworm colonisation and their subsequent activities in freshly deposited and aged peat sediments in the field were not affected by a low level of PAH contamination (1.62-2.40 mg ΣPAH/kg). However, in laboratory experiments they found a gradual decrease in burrowing activities of *Lumbricus rubellus* in fresh sediment over a period of four days, which could not be explained from PAH toxicity. In our study, in addition to the higher ΣPAH concentration, the higher compaction of the sediment layer compared to that of soil layer may have delayed earthworm colonisation.

According to Cerniglia (1992), the persistence of PAHs in soil increases with increasing molecular mass (which increases with the number of rings). In spite of that, the increased PAH degradation in the soil from columns with earthworms compared to the control was not related to the structure of the PAHs since concentrations of both 2-ring (biphenyl) and 5-ring PAHs (benzo(*a*)pyrene and benzo(*e*)pyrene) decreased (Figure 8.1). Microorganisms are known to be able to degrade PAHs, including those for which biodegradation rate increased in soil columns with earthworms (e.g., Kanaly et al. 2000; Grishchenkov et al. 2002; Al-Turki 2009). The increased degradation of benzo(*a*)pyrene and benzo(*e*)pyrene was therefore less expected but might be promoted by earthworm activity (Contreras-Ramos et al. 2006). Several authors demonstrated that the disappearance of several PAHs was accelerated by the presence of *E. fetida* and *L. rubellus* (Ma et al. 1995; Eijsackers et al. 2001; Contreras-Ramos et al. 2006, 2008). Nevertheless, the rate of PAH biodegradation is highly variable and dependent not only on PAH structure but also on the intimate association with the soil matrix, determining bioavailability, and on the composition and activity of soil microbial communities (Shuttleworth and Cerniglia 1995).

DHA activity is an indicator of microbial activity related to the enzymes that catalyze metabolic reactions producing ATP through the oxidation of organic matter. C_{mic} is a measure of the total microbial biomass of a microbial community. Together, these two parameters give information on the total activity of the microbial community. DHA activity and C_{mic} levels suggest that earthworm activity did not influence the activity of the microbial community in sediment and soil. Carbon substrate consumption measured in the Biolog Ecoplates™ showed that earthworm activity did have an influence on microbes responsible for the consumption of carboxylic acid substrates. However, the similarity and ecological indices clearly demonstrated that metabolic diversity between treatments was maintained throughout the experiment. These findings do not confirm our working hypotheses. The differences between microbial communities of soil and sediment persist and microbial communities from

treatments with and without earthworms were not divergent over the experiment. Nevertheless, the PCR-DGGE profiles demonstrated that the earthworms promoted changes in microbial community structure including increasing diversity. The proximity of the DGGE fingerprints of microbial communities in the controls at the start and after 18 weeks, evidenced by the dendogram based on Euclidean distances (Figure 8.6), demonstrates a low intra-sample variability in sediment and soil layers in the absence of earthworms. In addition, the Bray-Curtis indices suggest that the earthworms contribute to increasing the distance between samples of columns with earthworms and those without earthworms, which is in agreement with our first working hypothesis. The increasing similarity between microbial community structures of sediment and soil layers with higher earthworm density was also demonstrated by the Bray-Curtis index, which confirms our second working hypothesis. The influence of earthworm activity on microbial community structure in the DGGE analysis does not counteract the maintenance of metabolic diversity between treatments with and without earthworms since carbon substrate consumption in the Biolog Ecoplate^{TM} can be due to the activity of only a part of the microbial community (Bossio and Scow 1998).

Data on the relationship between earthworms and soil microorganisms have been based on studies on vermicomposting and changes in soil after transit through the gut (earthworm casts). Translation of these findings to the real terrestrial environment have to be done with care, since vermicomposting processes or earthworm casts may not represent a microbial community indigenous to a natural soil. Nevertheless, these approaches have demonstrated the capability of earthworms to change soil microbial communities. Maintenance of microbial biomass, similar to that in the present study, was found by Daniel and Anderson (1992) in *L. rubellus* casts. A decrease in microbial biomass however, has been reported more frequently, for instance in *L. terrestris* casts (Devliegher and Verstraete 1995), in a mixed community of *L. terrestris* and *Aporrectodea tuberculata* (Bohlen and Edwards 1995), *Metaphire guillelmi* (Zhang et al.

2000), in earthworm incubated soils and in *Eudrilus eugeniae* casts (Aira et al. 2006). Scheu (1987) and Aira et al. (2003) found an increased microbial biomass in casts of *A. caliginosa*. Similar to our findings, DHA activity was not affected by *E. eugeniae* in composting sugar industry waste over 60 days (Sen and Chandra 2009), but it was decreased in vermicomposting with *E. andrei* and *E. fetida* (Benitez et al. 1999; Aira et al. 2002) or in fresh casts of *E. eugeniae* (Aira et al. 2006). Increased DHA activities were reported by Parthasarathi and Ranganathan (1999) in casts of *E. eugeniae* and by Bansal and Kapoor (2000) and Kaushik and Garg (2003) during vermicomposting of cattle and cow dung with *E. fetida*.

It seems that effects on microbial biomass and DHA activity are dependent on the species of earthworms and on the kind and availability of food sources (Tiunov and Scheu 2000; Flegel and Schrader 2000). According to Aira et al. (2007) *E. fetida* may induce a decrease in the number of different carbon compounds used by microorganisms in the vermicomposting of pig manure. Aira et al. (2006), however, found an increased number of substrates used by microflora in pig slurry after passing the gut of *E. eugeniae*. Sen and Chandra (2009) did not find any effect of *E. eugeniae* on the substrate utilization by microorganisms during composting, but DGGE fingerprints from compost with earthworms were different from those without earthworms. Similar results were observed in our experiment, where structural changes promoted by earthworms in microbial communities did not correspond to changes in microbial functioning. This can be explained by a large soil microbial diversity where substantial overlap in function between microbial species together with a fast growth rate allow the quick filling of empty niches in a changing environment (Müller et al. 2002).

Conclusions

The earthworm *E. andrei* is able to promote the degradation of PAHs in soil, especially biphenyl, benzo(*a*)pyrene and benzo(*e*)pyrene. The faster disappearance of PAHs may be related to increased soil aeration and organic matter consumption by the earthworms. Earthworm activity did not influence the total microbial activity and metabolic diversity. However, changes in the structure of the microbial communities were induced by the presence of earthworms. Further studies are needed to assess the role of earthworms in shifts of soil microbial communities and increasing PAH removal under different soil physicochemical conditions.

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Chapter 9

General discussion

Ecotoxicological characterization of wastes

The results obtained in Chapter 2 demonstrate that the regulation of sewage sludge application in agricultural soil, based uniquely on the individual total concentration of metals in sludge and soil, is insufficient to prevent deleterious effects in terrestrial ecosystems. Although the metal content of sludge from olive-processing industry (sludge B) was below the legal threshold limits (European Community 1986), toxic effects were detected in the higher plant growth tests with *Brassica rapa* and *Avena sativa*. Since it is likely that these deleterious effects were provoked by organic compounds (not measured), performing the analyses advised by the European Union for organic pollutants (EU 2000) the toxicity found in this sludge would probably be confirmed. In spite of that, the possible joint effects of contaminants that may occur in the sludge and the presence of contaminants not detected or measured by chemical analysis make ecotoxicological assays advantageous and adequate tools for the risk assessment of sewage sludges. Furthermore, the low costs of ecotoxicological assays compared to chemical analysis (particularly for the organic compounds AOX, LAS, DEHP, NPE, PAH, PCB, and PCDD/F) allow for a more representative/intensive control of sludge toxicity (e.g. using more samples and/or testing more frequently). The results obtained in Chapter 2 also suggest the usefulness of a tiered approach for the ecotoxicological characterization of sludge. A series of tiers that progressively allow collecting information with increasing levels of complexity, and consequently the perception of the toxicity of a waste under assessment, may optimize and standardize waste characterization. The advantage of such an approach is that the process can stop after a tier where the risk of the waste appears negligible. The tiered approach may comprise a screening phase (low tier) composed by fast assays like avoidance tests, and a more detailed evaluation (high tier) mostly composed by chronic tests like reproduction and plant growth tests.

In Chapter 3 the potential of ecotoxicological tests to assess sludge toxicity after its application in soil, allowing the evaluation of the development of sludge

toxicity over time, was demonstrated. The ability of the screening tests on avoidance behaviour to detect toxicity of sludges (high sensitivity) within a short test period and at low costs makes this type of tests suitable for use in a decision processes. The use of avoidance tests in low tier levels was also shown to trigger more refined assessments (e.g. reproduction tests), which may optimize the process of waste characterization (saving time) without compromising the quality of the assessment. Nevertheless, avoidance and reproduction tests with *F. candida* would not be sufficient for an adequate assessment of waste toxicity over time. The use of several species from different ecological groups, representing distinct routes of exposure to contaminants (e.g., Collembola, earthworms, and plants), is essential for a suitable evaluation of the actual risk of a sludge. This was clearly demonstrated in both Chapters 2 and 4. In Chapter 2 the sludge from olive-processing industry (sludge B) showed toxicity only for plants, whereas the sludge from electroplating industry (sludge C) induced avoidance behaviour for both Collembola and earthworms and affected earthworm reproduction. In Chapter 4, where the toxicity of a mixture of metals was evaluated when applied via test sludge (sludge-amended soils) or directly in soil (metal-spiked soils), metal-contaminated sludge was more toxic for reproduction of Collembola and metal-spiked soil more toxic for earthworms. These findings not only highlighted the importance of the use of different species in ecotoxicological evaluations but also demonstrated that ecotoxicological tests may provide information on the availability of contaminants to the organisms, which cannot be obtained by chemical analysis. The need for the use of more than one test species in biotests for waste characterization was also suggested by the results obtained in the international ring test recently co-organised by the German Federal Environment Agency and ECT Oekotoxikologie GmbH (Moser and Römbke 2009). These results demonstrated that the earthworm avoidance test constitutes a promising soil bioassay due to its high sensitivity and short test period, but the use of other organisms in avoidance tests (e.g. collembolans) was encouraged for certain waste materials.

Changes in metal availability over time in different contamination matrices: laboratory *vs* field

The results obtained in Chapters 5 and 7 suggest that changes in total metal concentrations over time might be different under laboratory or field conditions. In the laboratory experiment (Chapter 5) the total Ni and Zn concentrations showed a significant exponential decrease in most metal-spiked soils. In the field assay (Chapter 7 – considering treatments without *D. veneta*), although in most treatments total metal concentrations decreased with time, this decrease was never significant. However, it has to be taken into account that, while the development of total metal concentrations in the laboratory was analysed over 12 weeks, that of the field assays was followed over one year. It is possible that the losses of Ni and Zn in metal-spiked soils stopped, or were significantly reduced, after 3 months of incubation, resulting in non-significant relationships of total Ni and Zn concentrations with time after that period.

Extractable metal concentrations were generally lower in field soils (Chapter 7) than in the laboratory experiment (Chapter 5). The extractability of Ni and Zn, but not of Cr, in samples from the same test concentrations collected after 3 months were significantly higher in laboratory (treatments S2, S4, M2, and M4 from Chapter 5) than in field experiments (treatments SL, SH, ML, and MH from Chapter 7) only for metal-spiked soils (Figure 9.1). It is likely that soil handling preceding the laboratory experiment, particularly sieving at 5 mm and submitting to freeze–thaw cycles for soil defaunation, caused the higher metal extractability in the laboratory compared to the field assay. In addition, during the first month of the field experiment, ryegrass was sown in all mesocosms to ensure vegetation cover over the experiment, which may have contributed to the decreased metal availability in the field.

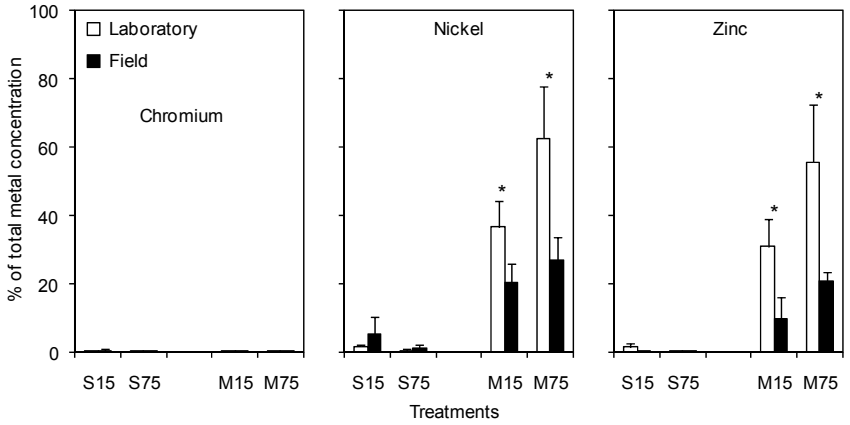

Figure 9.1 Extractability of Cr, Ni, and Zn (average ± SD, n = 4) in soil amended with sludge at concentrations of 15 and 75 t/kg (S15 and S75, respectively) or with metal solution with the same metal concentration of the 15 and 75 t/kg sludge doses (M15 and M75, respectively) in laboratory (the S2 and S4 treatments from Chapter 5; white bars) and field (the SL and SH treatments from Chapter 7; black bars) experiments after 3 months of incubation. Extractability is expressed as the percentage of soil total metal concentration. * - significantly different ($p \leq 0.01$ by t-test).

The development of metal extractability over time also showed some differences between laboratory and field experiments. Whereas in the laboratory, over a 12 week period, Cu, Ni, and Zn extractabilities significantly decreased with time in the most concentrated metal-spiked soils, in the field, over a 12 month period, only the decrease in Ni extractability was significantly related with time in most spiked soils and in the most concentrated sludge-amended soils. In the laboratory experiment the observed decrease for the 3 metals could be attributed to leaching and/or ageing, while in the field experiment, given the constant total metal concentrations over time, the observed decrease of Ni extractability was probably due to ageing that did not seem to occur for the other metals. This suggests that Cu and Zn decreases are not detectable only 3 months after its application to soil because the metals are more adsorbed to the soil organic matter and, consequently, less susceptible to be leached. Given the influence of

field conditions on metal extractability, further research is needed to confirm if during the first 3 months after soil contamination under field conditions, the development of metal extractability is similar to that found in the laboratory experiments.

Comparing the two contamination matrices, metal availability was higher in metal-spiked than in sludge-amended soils both in laboratory and field experiments. Under laboratory conditions, our working hypothesis that assumed a decrease of metal availability over time in spiked soils (due to metal leaching and/or ageing) and a constant metal availability in sludge amended soils due to sludge organic matter stability was confirmed. Under field conditions, this working hypothesis was confirmed for all metals in sludge amended soils, but in spiked soils only for Ni. A second working hypothesis that assumed a more pronounced decrease of metal availability at higher concentration in spiked soils was not confirmed. The decrease in metal availability found over time both in laboratory and field experiments was independent from metal concentration.

Earthworm metal concentrations over time: laboratory *vs* field

With the exception of Ni, the metal concentrations in earthworms were generally higher in animals from the field assay (Chapter 7) than in those found in laboratory samples (Chapter 6). Nevertheless, the concentration factors (CF; ratio between earthworm and total soil concentrations) were generally similar for earthworms from both experiments, except for soils with lower total metal concentrations. The CF for each metal generally tended to decrease with increasing total soil concentrations (Figure 9.2), which is a phenomenon typical for metals (McGeer et al. 2003). Data evidences a higher accumulation of Cr and Cu in *D. veneta* from the field assay than in animals from the laboratory experiment at the lowest metal concentrations in both metal-spiked (soils with concentrations ≤ 21 mg Cr/kg and ≤ 23.8 mg of Cu/kg) and sludge-amended soils (soils with concentrations ≤ 15.5 mg Cr/kg and ≤ 41.9 mg Cu/kg). For Ni,

CFs of earthworms from field and laboratory did not differ in metal-spiked soils. However, in earthworms from sludge-amended soils higher CFs were found under laboratory conditions at the lowest concentrations. The results obtained for Cu are generally in agreement with Marinussen et al. (1997) who measured Cu bioaccumulation in *D. veneta* exposed to metal-contaminated soil under laboratory and field conditions after exposure periods of 2 weeks and 1, 2, and 5 weeks, respectively. They also found lower earthworm Cu concentrations in the laboratory experiment in soils with total Cu concentrations ≤ 30 mg/kg. Given the lower extractability of metals in soils from field assays compared to the laboratory experiment (as discussed above), the differences in metal bioaccumulation by *D. veneta* under field and laboratory conditions may be influenced by environmental factors like soil temperature, which is known to influence the metal uptake of earthworms (Marinussen and Van der Zee 1997), rather than by differences in metal bioavailability.

The results obtained in the laboratory experiment suggest an inability of *D. veneta* to regulate the test metals except for Zn. This fact can be confirmed in Figure 9.2, where CF values for this metal consistently decrease with increasing soil concentration, which is due to the fact that earthworm body concentration of this metal is regulated within a fairly small range and independent of exposure concentration. The same does not seem to occur in the other metals, except maybe for Cu under field conditions where the CFs were the most variable showing a tendency to an internal regulation by the earthworms. It is likely that such variability contributed to the significant correlations found in laboratory between Cu internal levels and soil Cu concentrations.

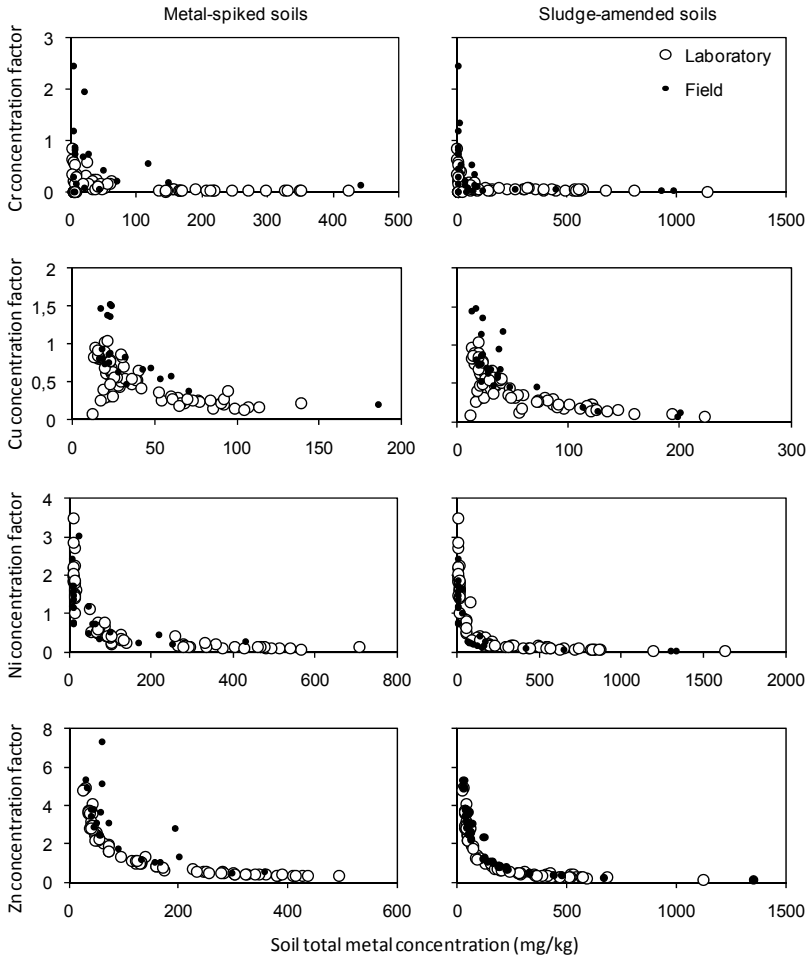

Figure 9.2 Concentration factors (ratio of internal concentration to total soil concentration) for Cr, Cu, Ni, and Zn as a function of soil total concentrations for earthworms (*Dendrobaena veneta*) exposed under laboratory conditions (open circles; data from Chapter 6) or field conditions (solid circles; data from Chapter 7) to metal-spiked (left) and sludge-amended soils (right).

Considering regressions between metal concentrations in earthworms and soil metal concentrations, more significant relationships were found in the laboratory experiment than in the field for both total and extractable metal concentrations. This is probably due to the higher variability of soil metal contents in the field compared to that in the laboratory experiments. It has to be taken into account that in the field the test sludge was incorporated in the top 10 cm surface soil layer using a spoon and the metal solution was simply applied on the soil surface with a watering-can. For the laboratory experiment, soil treatments were mixed, homogenized and prepared separately and then introduced as a top 10 cm layer in the microcosms. The latter procedure provided more homogeneous mixtures of top layers compared to the field assay. In addition, the influence of some environmental factors like soil temperature in metal uptake by the earthworms (Marinussen and Van der Zee 1997) may also have contributed to the limited number of significant relationships between metal body burdens and soil metal contents in the field compared to the laboratory experiment.

The working hypothesis that assumed higher internal metal concentrations in earthworms from spiked treatments compared to those from sludge-amended soils due to the higher metal availability was confirmed only in the field. Under laboratory conditions earthworms from the same concentration of different contamination matrices had metal concentrations within the same magnitude. The accuracy of models tested relating earthworm metal concentrations and soil total metal levels in predicting internal metal concentrations was higher for field data. Zinc estimations were the less accurate both in laboratory and field probably due to the ability of *D. veneta* to regulate this metal.

The influence of earthworm activity on soil pollutants – main findings

The results obtained in Chapters 6 and 7 demonstrated that the activity of 500 *D. veneta* individuals per m^2 did not impact the development of Cr, Cu, Ni, and Zn extractability in metal-spiked or sludge-amended soils over 3 months under laboratory and over one year under field conditions. As discussed in these Chapters, the impact of *D. veneta* (Sizmur and Hodson 2008) and other epigeic earthworm species (e.g. Wen et al. 2004) on metal availability has been shown. However, in those studies earthworm densities higher than 500 per m^2 have been used (10 to 100 times higher). The results obtained in Chapters 6 and 7 therefore suggest that the impact of earthworm activity on soil metal availability in those studies would not have been detected using a realistic earthworm density. The working hypothesis supporting that earthworm activity would increase metal availability especially in sludge-amended soils due to OM consumption was not confirmed neither in laboratory nor field.

On the other hand, in Chapter 8 it was demonstrated that the activity of 250 and 1000 earthworms per m^2 of the epigeic species *E. andrei* increased the degradation of PAHs in soil. The effect of earthworm activity on soil PAH degradation has been reported in other studies (e.g. Eijsackers et al. 2001). According to the results obtained in Chapter 8, such influence is not related with changes in soil microbial activity or metabolic profiling, but rather to shifts in microbial community (structural diversity). In fact data from PCR-DGGE was the only confirming the working hypothesis assuming an higher dissimilarity between treatments with and without earthworms and an increase in the resemblance between soil and PAH-contaminated sediment in treatments with earthworms. The observed shifts in microbial composition could have been induced by soil aeration and/or organic matter consumption by the earthworms. It is likely that the high dependence of soil PAH biodegradation on aerobic

conditions and OM adsorption (Cerniglia 1992) makes PAH-contaminated soils highly susceptible to be impacted by earthworm activity.

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