Methodologies using soil organisms for the ecotoxicological assessment of organic wastes

Xavier Domene Casadesús

Departament de Biologia Animal, Vegetal i Ecologia
Universitat Autònoma de Barcelona
Centre de Recerca Ecològica i Aplicacions Forestals

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Xavier Domene

Centre de Recerca Ecològica i Aplicacions Forestals (CREAF). Universitat Autònoma de Barcelona. Edifici C. 08193 Bellaterra (Barcelona). Spain

Phone: +34 935 811 312

Fax: +34 935 814 151

La DRA. PILAR ANDRÉS PASTOR, investigadora del Centre de Recerca i Aplicacions Forestals (CREAF) de la Universitat Autònoma de Barcelona,

CERTIFICA:

Que la present memòria, presentada per XAVIER DOMENE CASADESÚS, i titulada "*METHODOLOGIES USING SOIL ORGANISMS FOR THE ECOTOXICOLOGICAL ASSESSMENT OF ORGANIC WASTES*", ha estat realitzada sota la seva direcció i constitueix la seva tesi per optar al grau de Doctor dins del programa de Sòls, Aigua i Medi Ambient.

I perquè així consti, signa el present certificat a Bellaterra el 8 de gener de 2008

CREAF
CENTRE DE RECERCA ECOLÒGICA
I APLICACIONS FORESTALS
UNIVERSITAT AUTÒNOMA DE
BARCELONA - EDIFICI C
E-08193 BELLATERRA

Dra. Pilar Andrés Pastor

Investigadora del Centre de Recerca Ecològica i Aplicacions Forestals (CREAF)

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"*El més curiós és que tots aquells qui estudien seriosament aquesta ciència cauen en una espècie de passió. Verdaderament, el que més plaer proporciona no és el saber, sinó l'estudiar; no la possessió, sinó la conquesta; no l'estar aquí, sinó l'arribar allà.*"

Karl Friedrich Gauss (1777-1855), matemàtic i físic

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Agraïments

Es fa difícil fer uns agraïments en moments com aquest, que assenyalen la fi d'un temps i obren la porta a una nova etapa, quan tanta gent ha contribuït en que aquesta tesi fos una realitat. Probablement és veritat que som com som per la gent que ens hem trobat en el camí, i en el meu cas, ho crec fermament. Per tant, per començar, gràcies a la meva família i amistats.

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Abbreviations

DEPH	Di(2-ethylhexyl)phthalate.
DM	Dry matter.
ECx	Concentration of a test substance estimated to reduce the outcome in a sublethal endpoint rate at the end of the test by x% compared to the control.
Eluate	Extract obtained from the solid-phase.
HC5	Maximum permissible environmental concentration of a substance to affect no more than 5% of the species, giving a 95% protection level.
HRGC-ECD	High resolution gas chromatography with electron capture.
HRGC-HRMS	High resolution gas chromatography/high resolution mass spectrometry.
HRGC-MS	High resolution gas chromatography/mass spectrometry.
LAS	Linear alkylbenzene sulphonates.
LCx	Concentration of a substance estimated to prove lethal at the end of the test by x% compared to the control.
LOEC	Lowest observed effect concentration. It is the lowest concentration of the test substance which is observed to have a significant effect when compared with the control.
NOEC	No observed effect concentration. It is the highest tested concentration of a test substance at which no lethal or other effect is observed.
NPE	Nonylphenol polyethoxylates.
PAH	Polycyclic aromatic hydrocarbons.
PCB	Polychlorinated biphenyls.
PCDD/F	Polychlorinated dioxins and furans.
PEC	Predicted environmental concentration.
PNEC	Predicted no effect concentration.
RQ	Risk Quotient equals PEC divided by PNEC.
Solid-phase	Waste solid matter.
WWTP	Waste water treatment plant.

Resum

La correcta gestió dels residus orgànics és un dels principals reptes actuals a la Unió Europea, en resposta al fort increment en la seva producció en les darreres dècades. Les creixents limitacions en la incineració i el dipòsit en abocador de residus orgànics ha conduit cap a un increment en el reciclatge en el sòl d'aquests residus en forma d'esmena orgànica, perfilant-se aquesta com la principal via de gestió actual i futura. Malgrat tot, els residus orgànics són també el destí final de contaminants originats amb les activitats humanes. Per tant, el reciclatge de residus orgànics en sòls pot també representar un risc, ja que podria malmetre de manera irreparable els organismes edàfics i les funcions que duen a terme, bàsiques per al funcionament dels ecosistemes terrestres i les societats humanes.

Actualment, a la Unió Europea no existeixen requeriments de qualitat mínima per a permetre el reciclatge de residus en el sòl, amb l'excepció dels fangs de depuradora, pels quals existeix una legislació específica. A més, en els escassos casos en què existeix aquesta avaluació de qualitat, el criteri utilitzat és exclusivament basat en anàlisis químiques, i fins ara, els bioassaigs no han estat incorporats en la legislació malgrat els seus avantatges en termes de rellevància per a situacions reals. Malgrat tot, un pas necessari per a aquesta incorporació és la definició de quins bioassaigs són adients per a aquesta finalitat.

En aquesta tesi, diversos bioassaigs amb organismes terrestres i aquàtics (Microtox, cladòcers, microorganismes del sòl, plantes, lumbrícids, enquitreids i col·lèmbols), paràmetres biològics (letals i subletals), i procediments (fase sòlida o extractes dels residus) són comparats per al seu ús el l'avaluació de risc ecotoxicològic de diversos residus orgànics per tal de seleccionar-ne els més adients. Posteriorment, per cada residu, i a partir d'un conjunt de dades de toxicitat per a diversos organismes habitants del sòl, s'estimen dosis segures d'esmena, les quals es comparen amb les dosis habituals i amb treballs de camp sobre els efectes d'esmenes orgàniques en la biota del sòl. Finalment, s'estudia la validesa dels resultats de toxicitat d'un residu orgànic obtingut en el sòl artificial OECD per a fer extrapolacions de resultats en sòls naturals. A més, s'avalua la influència de les propietats tant en els organismes test com en la pròpia toxicitat d'un mateix residu.

Les principals conclusions d'aquesta tesi són:

1. Els tests amb la fase sòlida dels residus són els més adients per a l'avaluació del risc ecotoxicològic de les esmenes amb residus orgànics.
2. La mortalitat de lumbrícids, enquitreids i col·lèmbols és un paràmetre sensible i adient per als bioassaigs amb residus. Els paràmetres subletals com la germinació i creixement vegetals, la reproducció d'enquitreids i col·lèmbols, la inhibició de les activitats d'alimentació en col·lèmbols, i el pes fresc en lumbrícids són també sensibles i adients per a aquesta finalitat.
3. Els mètodes d'anàlisi química no permeten predir el risc ecotoxicològic dels residus, ja que en general la càrrega contaminant no es troba correlacionada amb la toxicitat observada. Per contra, en la major part dels bioassaigs estudiats, l'estabilitat dels residus estava fortament correlacionada amb la toxicitat.
4. Les dosis segures d'esmena per a sòls agrícoles, deduïdes amb el mètode de distribució de la sensibilitat de les espècies i a partir de dades de toxicitat de laboratori, concorden amb les dosis sense efectes per a la biota del sòl descrites en diversos treballs de camp publicats. Malgrat tot, la validesa de les estimacions resta pendent de ser comprovada en condicions de camp.
5. Entre els diversos posttractaments potencialment aplicables als residus orgànics, el compostatge es va comprovar com a millor que l'assecat tèrmic, ja que a més de permetre la higienització i la reducció dels costos de transport, permet disminuir la ecotoxicitat dels residus, maximitzant la seva reutilització segura en sòls.
6. L'ús de fangs deshidratats i assecats tèrmicament, i de purins assecats tèrmicament no es preveu que suposi un risc per als sòls si els aquests residus s'apliquen en base a criteris de demanda de nutrients dels cultius, generalment per sota de 3 t ha^{-1}. Malgrat tot, dosis lleugerament superiors, al voltant de 5 t ha^{-1}, s'espera que puguin malmetre els ecosistemes terrestres.
7. En bioassaigs utilitzant sòl artificial OECD, col·lèmbols, i un mateix residu orgànic, la mortalitat era similar que l'observada en sòls agrícoles naturals. Per contra, els efectes sobre la reproducció eren sobrestimats en el sòl artificial OECD.
8. Els sòls amb textura fina i/o baixos continguts en matèria orgànic són els més vulnerables davant esmenes amb residus orgànics quan s'utilitzen col·lèmbols com a indicadors.

Summary

Correct organic wastes management is one of the main challenges in the European Union given its production increase in the last decades. Increasing limitations to organic waste incineration and landfilling, together with its fertilizing potential, has lead to their recycling as soil amendment. In fact, waste use in soil is currently the main waste management option, and it is predicted to increase in the next years. However, organic wastes also are the final sink of pollutants released by human activities. Disposal in soil might lead to irreparable damage for soil organisms and their functions, which play a central role in terrestrial ecosystems and human societies.

No requirements of a minimum organic wastes quality exist in the European Union for their recycling in soils, with the exception of sewage sludge, for which specific legislation exist. In this particular case, only chemical assays are taken into account and, to date, bioassays are not considered for this purpose, despite their advantages in terms of relevance for real situations. However, a first step for the inclusion of bioassays into legislation for the quality assessment of wastes is to define suitable methods for this purpose.

In this thesis, a wide range of bioassays (Microtox, daphnids, soil microorganisms, plants, earthworms, enchytraeids, and collembolans), endpoints (lethal and sublethal), and procedures (waste's solid-phase and eluates), are evaluated to be used for the ecotoxicological risk assessment of different organic wastes (dewatered, composted and thermally-dried sewage sludges, and thermally-dried pig slurry) in order to select the most suitable methods for this purpose. Furthermore, safe amendment rates for each waste are derived from laboratory data and compared with usual amendment rates and with published field works on waste effects on soil-dwelling organisms. Finally, the validity of OECD artificial soil for waste testing is also assessed by comparison of toxicity results in different natural soils using the same waste. In addition, the biasing influence of soil properties on waste toxicity results is also evaluated, through their effects on the test organisms as well as through their influence on the toxicity of wastes.

The main conclusions of this work are:

1. Waste solid-phase assays on soil organisms are the most suitable approach for organic waste ecotoxicological risk assessment on soils.
2. Earthworms, enchytraeids, and collembolans mortality is a suitable endpoint for waste testing. Sublethal endpoints such as plant germination and growth, enchytraeid and collembolan reproduction, collembolan feeding inhibition and earthworm body weight were also sensitive and suitable for waste testing.
3. Chemical assays are unsuitable to predict the wastes ecotoxicological risk, as toxicity is generally uncorrelated with pollutant burden. In contrast, toxicity and waste stability are highly correlated in most bioassays.
4. Safe amendment rates for agricultural soils derived with the "species sensitivity distribution" method from laboratory toxicity data agree with results from published field studies, but their relevance for actual field amendments has not been verified.
5. Among wastes post-treatments, composting is better than thermally-drying, because in addition to facilitating their hygienization of the wastes and reducing transport costs, composting decreases the ecotoxicity of the wastes, and allows their safe reuse as agricultural soil amendment.
6. Dewatered and thermally-dried sludges or pig slurry are not predicted to be a risk for soils if applied according to crop demands (usually below 3 t ha^{-1} DM). However, slightly higher dosages (around 5 t ha^{-1} DM) are expected to have noxious effects on soil ecosystems.
7. Mortality of collembolans when exposed to an organic waste is the same when using OECD artificial soil or natural agricultural soils, but effects on reproduction are overestimated when the OECD soil is used.
8. Soils with fine texture and/or low organic matter content are the most vulnerable for collembolans to organic wastes amendments.

Chapter 1

Introduction

1.1. Soil protection in the European Union

Soil is a non-renewable resource at the human time scale, given the extreme slowness of its formation and regeneration. At the same time, it is a very dynamic system which performs many functions and delivers services vital to human activities and to the ecosystems functioning. However, no specific legislation on soil protection exists in the EU (Commission of the European Communities 2006b). This is why soil protection is one of the main aims of the Sixth Environment Action Program of the European Community (2002-2012) with sustainable soil use being one of the main goals (European Commission 2006b).

In 2002, the European Commission published the communication "*Towards a Thematic Strategy for Soil Protection*", with the aim of building on the political commitment to soil protection in the coming years. In this document, soil is defined as a key element with the following environmental, social and economic functions (European Commission 2002b):

a) *Food and other biomass production*. Soil supplies water, nutrients and root fixation in agriculture and forestry and is hence essential for human survival.

b) *Storing, filtering and transformation*. Soil performs storage, filtering, buffering and transformation functions. It plays a central role in water protection (acts as natural filter for groundwater) and exchange of gases with the atmosphere (releases CO_2, methane and other gases to the athmosphere).

c) *Habitat and gene pool*. Soil is the habitat of a huge variety of organisms, all with unique gene patterns, which perform essential functions in terrestrial ecosystems.

d) *Physical and cultural environment for mankind*. Soil is the platform for human activity and is also an element of the landscape and cultural heritage.

e) *Source of raw materials*. Soils provide raw materials such as clay, sand, minerals and peat.

In a first attempt to incorporate soil protection to the European legislation, in 2004, the Directive 2004/35/EC on environmental liability regarding prevention and remediation of environmental damage was approved. Its main aim was to establish a common framework for prevention and remediation of any environmental damage as far as they cause damage to water, land, protected species or natural habitats. The legislation forces the operators responsible for the environmental harm to inform and to bear the preventive and remediation costs.

More recently, an amendment of this Directive has been proposed to specifically protect soils (Commission of the European Communities 2006b). Its main aims are "to establish a common European framework for soil protection; to obligate the land users to take precautionary measures when soil use is expected to significantly hamper soil functions; to ensure the maintenance of soil functions and to identify risk areas within the member states; to limit the inputs of dangerous substances to soil that would hamper soil functions and which imply a risk to human health and environment; and to create an inventory of contaminated sites as a previous step to fund remediation programs".

Since 2005, a specific legislation has existed in Spain for contaminated soils, but it only considers soils polluted by industrial activities (Royal Decree 9/2005). However, for the first time in Europe, this legislation has made it possible to determine a soil is polluted based on the results of biological toxicity tests (Tarazona et al. 2006).

1.2. Wastes in the European Union

As a consequence of the economic development, waste production has significantly augmented in Europe. Annual waste production in Europe is around 1.3×10^9 tonnes, with 40×10^6 being hazardous wastes. This is equivalent to an annual release of 3.5 tonnes per European. Furthermore, an additional 700×10^6 of tonnes of agricultural

wastes are released in Europe, increasing the magnitude of the problem. A large percent of the produced wastes are incinerated or landfilled, even though the current legislation tends to discourage such management options, given their environmental consequences in terms of pollution risk for ecosystems and human health, and their greenhouse gases release (European Commission 2006a).

Nowadays, the European legislation on wastes is grouped in six Directives: Directive 75/442/EEC on waste, Directive 91/689/EEC on hazardous waste, Directive 75/439/EEC on oils, Directive 86/278/EEC on sewage sludge, Directive 94/62/EC on packaging waste and Directive 1999/31/EC on waste landfilling (Commission of the European Communities 2006a).

However, most of these rules do not apply to the organic wastes which are used as soil amendment. In particular, animal manure is not covered by any of the above directives, and Directive 75/442/EEC specifically excludes sewage sludge, which is governed by its own Directive (86/278/EEC). Directive 91/689/EEC does not apply to treated sewage sludge suitable for soil use (according to Directive 86/278/EEC) nor to other organic wastes not classifiable as harmful. Finally, Directive 1999/31/EC does not apply to the spreading of organic wastes to soils for fertilization and improvement purposes, but this legislation might apply to organic wastes when they are considered environmentally harmful.

Therefore, no specific regulatory controls exist in the EU for application of organic wastes other than sewage sludge (European Commission 2001a). For sewage sludge, amendments are regulated by Directive 86/278/EEC, which requires raw sludge stabilization and sets maximum heavy metals concentrations for applying the sludge to soil. However, soil amendments with other organic wastes commercialized as fertilisers might be indirectly regulated by the Regulations (EC) No 2003/2003 and No 1774/2002, if their pathogenic and heavy metal content exceed the limit values. Furthermore, Directive 91/676/EEC concerning pollution of water by nitrates from

agricultural sources, might also indirectly limit the use of organic waste on soil in vulnerable regions.

In the Directive 91/689/EEC on hazardous waste, the term "ecotoxicity" was introduced in the European legislation for the first time. Ecotoxicity is one of the criteria used to label hazardous wastes, which are defined as "substances and preparations which present or may imply immediate or delayed risks for one or more sectors of the environment". Nevertheless, no procedure has been validated to assess the ecotoxicological characterization of wastes (Pandard et al. 2006), despite the existence of several standardized soil ecotoxicity protocols. Sewage sludge and other organic wastes currently used on soils are not considered hazardous. Furthermore, the quality of sewage sludge and other organic wastes is assessed exclusively on the basis of chemical methods, and there is no standardized ecotoxicological metodology for this purpose.

1.3. Organic waste production and composition

Production of organic waste increased in the European Union during the last decades. In the case of sewage sludge, it is due to the transposition to the member states of the Directive 91/271/EEC on wastewaters treatment and, in the case of animal manures, to the increase of intensive cattle raising. The most recently published data estimate that 180 x 10^6 tonnes (dry weight) of animal manures are annually produced in the UE (European Commission 2001a). In addition, 5.5 x 10^6 tonnes (dry weight) of sewage sludge were produced in 1992 and, the latest report predicted that 8.3 x 10^6 tonnes were expected for 2005 (European Commission 2000).

Sewage sludge is mainly composed of organic matter, which represents around 50% of its dry weight. Furthermore, it contains significant concentrations of nutrients essential for crops, such as nitrogen, phosphorus, calcium, potassium, sulphur, magnesium, sodium, boron, cobalt, selenium and iodine (European Commission 2001b). In the case of animal manures, dry matter and nutrient content is highly variable among farms and depends on

factors such as type of livestock (species, breed and age), diet, type of production and waste handling system (European Commission 2001a).

Sewage sludge may contain significant concentrations of potentially toxic elements (PTE), a term including metallic (Cd, Cr, Cu, Hg, Ni, Pb, Zn, As, Al, Ti) or non-metallic (e. g. Cl) elements harmful for living organisms. PTEs mainly accumulate in sludges in non bioavailable forms. It has been estimated that 70 to 75% of Zn, Cu, Cd, Cr, Hg, Se, As and Mo, 80% of Pb, and 40% of Ni are transferred during this process from wastewater to sludge (Thornton et al. 2001). Their concentrations in sludge depend on treatments and post-treatments, and in some sludges, they may be high enough to prevent the sludge application to soil according to Directive 86/278/EEC.

Manures and slurries can also contain high levels of PTEs, particularly zinc and copper, due to the use of mineral supplements in animal feed, and veterinary products. This fact is more problematic in the case of pig slurry, which can contain up to 600 mg Kg^{-1} (dry weight) of copper and up to 900 mg Kg^{-1} of zinc, in comparison to the 2500 mg Kg^{-1} for zinc and 1000 mg Kg^{-1} for copper, the current limit values for sewage sludge used in agriculture (European Commission 2001a).

In the case of the organic pollutants, more than 6000 anthropogenic organic compounds have been detected in wastewaters. Some of them can be partly biodegraded during the wastewater treatment, while others accumulate in sludge, given their high affinity for mineral particles and organic matter (Thornton et al. 2001). Among them, the most relevant in terms of concentration in sludge and toxicity are polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), polychlorinated dibenzodioxins/dibenzofuranes (PCDD/F), di(2-ethylhexyl)phthalate (DEHP), nonylphenols (NPE), and linear alkylbenzene sulphonates (LAS).

PAH, PCB and PCDD/F are persistent, given their hydrophobic nature and the inability of most living organisms to metabolize these compounds. Only PAH are known to be partly degraded by composting. In contrast, DEHP, LAS, and NPE compounds are non-

persistent and may be biodegraded during the wastewater treatment. However, they can accumulate significantly in sludge given their high concentrations in wastewaters and the presence of hydrophobic regions in their structures (Thornton et al. 2001). Once in soil, they biodegraded quickly (Petersen et al. 2003). They are also mineralised by composting (Jensen 1999). Organic pollutants in animal manures are generally low or undetectable (European Commission 2001a).

1.4. Use of organic wastes in soil

Application of organic wastes agricultural soils is as old as agriculture. From ancient times, materials composted toa greater or lesser degree from households and husbandry have been applied to the surrounding fields (Jensen 2004). In the last century, such practices have undergone important changes in modern societies, where organic manures have been partly replaced by chemical fertilizers. However, during recent decades, organic wastes have recovered their important role as fertilizers, given their increasing production. Intensive livestock units are segregated from agricultural land, where manure and slurries could be recycled. Furthermore, in recent decades, the implementation of wastewater treatments has lead to a huge increase in sewage sludge production (European Commission 2000) whose disposal has been mainly managed by application to soil (European Commission 2000).

In the specific case of sewage sludge, the most recent available data (Commission of the European Communities 2006a) indicate that Belgium (Wallonia), Denmark, Spain, France, Ireland, UK and Hungary apply to land 50% or more of the sludge they generate to land, while Finland, Sweden and Slovenia apply less than 17% of the produced sludge. Greece, the Netherlands, Belgium (Flanders), Slovakia and the Czech Republic apply lower sludge percentages or do not use sludge for fertilization. Sewage application to soil and incineration are expected to increase, given the policy against biodegradable wastes landfilling (Directive 1999/31/EC) (Figure 1.1).

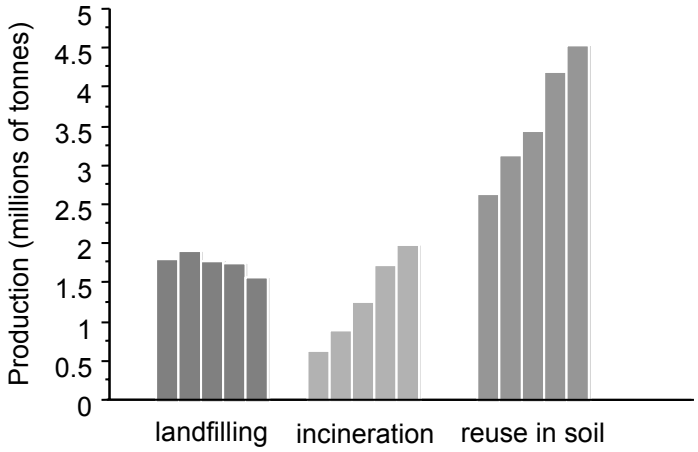

Figure 1.1. Estimated sludge use in 1992, 1995, 1998, 2000 and 2005 (European Commission 2002a).

There are no available data about the percentages of reuse in soil of other organic wastes in Europe (European Commission 2000a), but 50% of the pig slurry produced in Spain is recycled in agricultural soils (MAPA 1997). In Europe, around 90% of the waste spread on land is composed of farm waste and predominantly animal manure, followed by food production wastes, waterways dredgings and paper waste sludge. Industrial organic wastes only account for 1% of the amendments (European Commission 2001a).

The amount of organic waste applied to an agricultural land is generally dictated by its nutrient content (nitrogen and phosphorus) and the crop demands. However, sewage sludges and organic wastes contain a wide variety of substances potentially hazardous for the environment and the human health because their composition reflects how modern societies use chemicals in industry and households (Jensen 2004). The European legislation already described regulates the use of some organic wastes in soil in order to prevent unacceptable environmental effects, to avoid excessive fertilization and to ensure long-term sustainable fertilization. However, waste quality is exclusively assessed on the basis of maximum pollutant concentrations, even though the disadvantages of the

chemical methods when compared with the biological methods are well known (Crouau et al. 2002).

1.5. Approaches to ecological risk assessment of wastes

The goal of using external agricultural inputs (mineral fertilizers, organic amendments, microbial inoculants and pesticides) is to maximize productivity and economic returns, but their side effects on soil organisms are often neglected (Bünemann et al. 2006). Soil has high buffer capacity to pollutants, but also limited resistance. This may result in irreparable damage to the soil system, which might be counteracted only with high monetary expenses. Hence, limiting soil inputs should protect the soil function as a central component of the ecosystem and as a basic necessity for human, animal and plant life (Kördel and Römbke 2001).

Waste hazard estimation may be performed through a chemical-specific approach or through a toxicity-based approach (Pandard et al. 2006). In the former case, chemical analyses are carried out and results are compared with threshold values. In the latter case, toxicity is directly measured by biological tests. Chemical methods are often less suitable than bioassays, particularly for assessing the toxicity of wastes which present a complex and unknown pollutant burden.

Chemical methods require a previous knowledge of the substances to be analyzed and hence do not take into account all the substances present. Neither are they sensitive to pollutant bioavailability or to synergisms and antagonisms between pollutants (Crouau et al. 2002, Pandard et al. 2006).

Despite the wide agreement about the suitability of bioassays for ecotoxicity assessment, only few attempts have been carried out to define waste sample processing for ecotoxicological characterisation (ISO 14735:2005) and to define a suitable test battery (Pandard et al. 2006). This highlights the need for selecting and assessing

ecotoxicological methods for organic wastes, which could be incorporated into future legislation.

1.6. Ecological relevancy of laboratory bioassays

Ecotoxicology has evolved more from toxicology than from ecology, so there are those who argue that is too simplistic (Calow and Forbes 2003). The main criticism is that extrapolation from single-species ecotoxicological laboratory test results to the ecosystem level involves a number of uncertainties which preclude robust predictions (Kammenga and Laskowsky 2000, Calow and Forbes 2003). By contrast with ecotoxicologists, ecologists do not simplify their study objects, nor analyze an idealized part of the reality (Van Straalen 2003).

However, since the 1990s, many attempts to integrate ecological issues into ecotoxicology have been made, but tests with single species are still common and hence environmental standards for toxicants are still based on individual organism response (Van Straalen 2003). However, there have been some successes with the ecological approach, such as the acceptance of multispecies tests (community, enclosure, field) as valid regulatory instruments, and the use of functional endpoints (primary production, decomposition) in addition to survival, growth and reproduction of single species (Van Straalen 2003).

Despite the likely limitations in terms of ecological relevancy of most laboratory single-species ecotoxicological bioassays, their main aim is to obtain data on the effects of pollutants on a given species in order to indicate risks for similar species and ecosystems, and also to obtain cause-effect relationships of events observed in field conditions. This approach should be considered as the best option available, since there are no alternative approaches balancing feasibility and realism. Because there are no practical difficulties in carrying out studies at higher levels of biological organisation, it is necessary to extrapolate individual toxicity data to the population, community and

ecosystem levels (Hommen and Strauss 2003) and several methodologies have been proposed to carry out such extrapolations (Forbes and Calow 2002, Calow and Forbes 2003, Maltby 2006). Some experimental studies have field-validated the ecological relevance of predictions carried out with extrapolation methodologies (Sloof et al. 1986, Versteeg et al. 1999, Smit et al. 2002, Hose & van den Brink 2004, Schroer et al. 2004). These methodologies can be used both for the estimation of soil limit concentration values for pollutants or for carrying out ecological risk assessments of polluted sites.

1.7. Ecological role of soil-dwelling organisms

Soil organisms are major components of terrestrial ecosystems. Even though their biomass in soil is low compared with the mineral or humus fraction, their activity is absolutely crucial for soil and terrestrial ecosystems functioning. Soil inhabiting organisms decompose litter, create humus and transform the parent mineral material into a suitable habitat for biological activity and plant growth, with effects on soil aeration, moisture and nutrient retention. Their main functions are (Van-Camp et al. 2004, Mulder 2006):

- Decomposition of organic material and production of soil organic matter.
- Nutrient cycling and nutrient mineralisation.
- Pollutants degradation and supply of clean water.
- Biological control of agricultural and forestry pests.
- Soil structure formation.
- Regulation, fixation and release of CO_2 and other greenhouse gases (e.g. CH_4, and N_2O).
- Resilience and resistance of ecosystems.

Therefore, sustainable soil use is of major importance for the sustained provision of goods and services that contribute to the livelihoods of land users (Van-Camp et al. 2004).

Soil protection is mainly focused on maintaining soil ecological functions, but maintaining soil functions might also be achieved by protecting the soil ecological structure. The concept of soil ecological structure includes species richness, biomass, feeding groups, etc., while functional aspects include substrates degradation, organic matter processing rate, respiration, nitrification, etc. (Van Stralen 2002).

However, the relationship between ecosystems structure and functioning is not clear (Giller and O'Donovan 2002), and several hypotheses have been proposed (Van Stralen 2002, van-Camp et al. 2004, Figure 1.2):

- The decrease of biodiversity is not dramatic for ecosystem functions because many species are "redundant" in the sense that they perform similar functions (*redundant species hypothesis*). Hence, when a species disappear, it is replaced by its functionally equivalent species.
- A decrease of biodiversity promotes a proportional decrease in the ecosystem functions, since each species carries out slightly different functions. Since the whole community functions better than some subset of species, the loss of one species subtracts their contribution to one or more ecological processes (*rivet hypotheses*).
- The structure-function relationship is not universal but ecosystem-specific (*idiosyncratic hypotheses*).

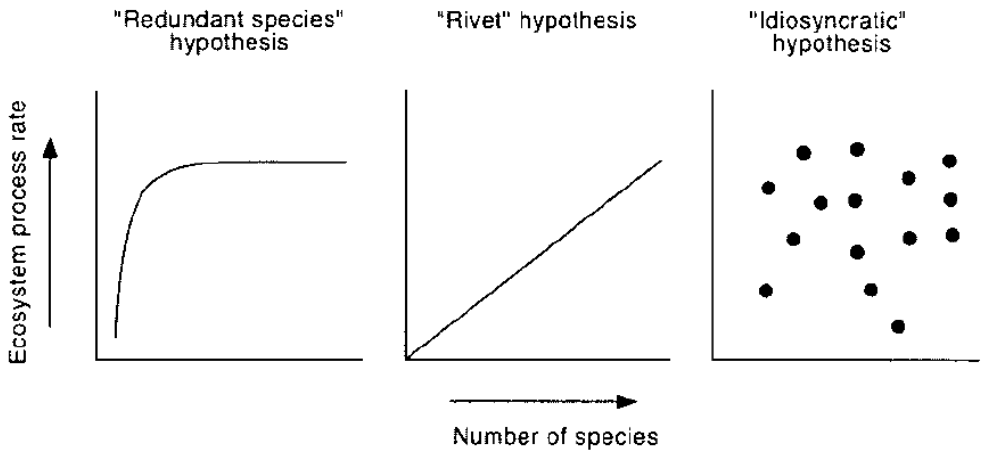

Figure 1.2. Three main hypotheses on the ecosystems function-structure relationship (reproduced from Lawton 1993).

To date, there is no clear empirical evidence to support these hypotheses, but there is a report of experiments with terrestrial mesocosms which show that ecosystem processes are affected by biodiversity losses (Naeem et al. 1995). However, most of the experiments carried out using soil fauna suggest that species composition is likely to be more important than species number (Jones and Bradford 2001). More precisely, it has been showed that removal of entire functional groups in soil is more influential for ecosystem functions than loss in species diversity within these groups (Mikola and Setälä 1998). On the other hand, other studies have supported the idiosyncratic hypotheses (Verhoef 1996, Emmerson et al. 2001).

Whatever the community properties are which determine the association between structure and function in a given ecosystem, and whatever hypotheses are proposed for explaining the mechanisms of association, soil biodiversity protection should ensure the maintenance of its ecological functions.

1.8. Ecotoxicological bioassays

A number of terrestrial ecotoxicological tests have been developed since the early 1990s, but few of them have been standardized and intercalibrated, and none have

been included as alternatives to chemical methods in the European legislation on soil or wastes (Tarazona et al. 2006). Soil ecotoxicological bioassays are performed on soil organisms, and can be grouped into four main categories: laboratory single-species tests, laboratory or semi-field multispecies tests, field multispecies tests, and soil processes tests (CSTEE 2000).

1.8.1. Laboratory single-species tests

These tests are carried out in the laboratory under controlled environmental conditions. Some of them have been standardized, including tests with microorganisms (Microtox), plants (ISO 11269-1:1993, ISO 11269-2:1995) and invertebrates. Most tests belong to the latter group (using protozoa, nematodes, crustaceans, coleopterans, isopods, millipedes, mites, collembolans, enchytraeids, earthworms, and molluscs) but few have been standardized. Among those standardized are the acute toxicity, chronic toxicity, and the avoidance tests protocols standardized for earthworms (*E. fetida* and *E. andrei*) (ISO 11268-1:1993, ISO 11268-2:1998 and ISO 17512 respectively), and also the chronic toxicity tests for collembolans (*Folsomia candida*) and enchytraeids (*Enchytraeus sp.*) (ISO 11267:1999 and ISO 16387:2004 respectively). Pollutants effects on any of these test organisms might indicate effects on similar species in soils, and hence might indicate direct effects on the functions these species carry out in soil as well as indirect effects through their interactions with other groups (Figure 1.3).

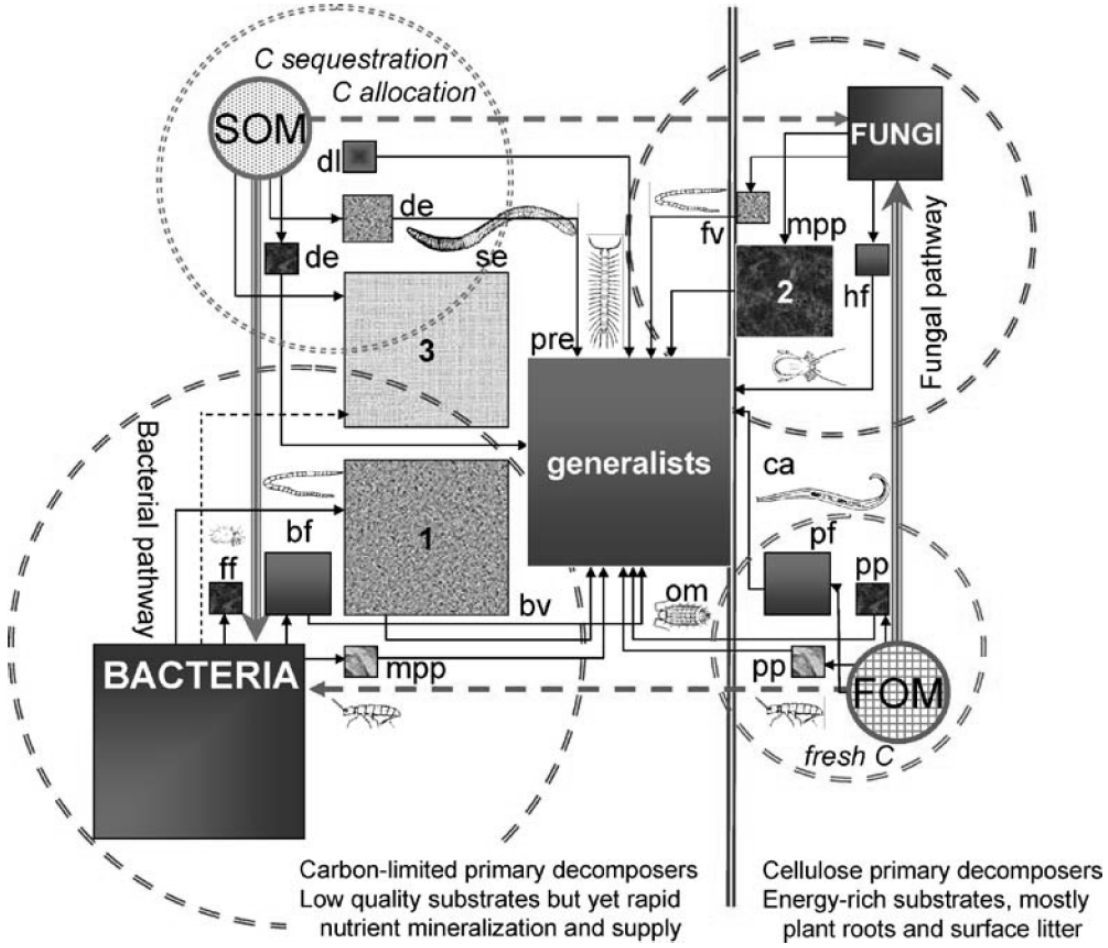

Figure 1.3. Principal feedbacks and trophic links in a typical agroecosystem [bottom right: fresh organic matter (***FOM***); top left: soil organic matter (***SOM***)]. Bacterial pathway on the left, fungal pathway on the right. ***1***, enchytraeids; ***2***, mites; ***3***, earthworms, no numbers indicate either collembolans (paper-textured boxes) or nematodes (blue-greyish boxes). Trophic categories and life strategies: ***bf*** (bacterial-feeding nematodes), ***bv*** (bacterivore enchytraeids), ***ca*** (carnivore nematodes), ***de*** (soil detritivore mites and enchytraeids), ***dl*** (Dauerlarvae, the only nematode resting stage), ***ff*** (filter-feeding histiostomatid mites), ***fv*** (fungivore enchytraeids), ***hf*** (hyphal-feeding nematodes), ***mpp*** [microphytophagous collembolans (mostly high grazing rates) and mycophagous oribatid and prostigmatid mites (low grazing rates)], ***om*** [omnivore (micro)arthropods and non-parasitic nematodes], ***pf*** (plantroot-feeding nematodes), ***pp*** (phytophagous microarthropods, mostly engulfers and fluid feeders), ***pre*** (fluid feeders and nematophagous predating mites), ***se*** (all endogeic soil engineers, earthworms). Reproduced from **Mulder (2006)**.

Roles of macrofauna include direct organic matter processing (by snails, earthworms, enchytraeids, millipedes, ants and termites), predation (spiders, ants) and soil structure formation (earthworms, termites). Micro- and mesofauna (protozoa, nematodes, collembolans and mites) regulate bacterial and fungal activities, hence indirectly influencing organic matter decomposition. Soil nematodes, which are detritus consumers, influence both decomposition and nutrient mineralisation. Earthworms improve the organic matter incorporation under the soil surface, increase the number of water stable soil aggregates, and enhance water infiltration, aeration and root penetration and microbial activity (Pankhurst 1997).

The single-species approach has been used to assess the ecotoxicity of animal manures, sewage sludges, and pig slurries in laboratory conditions by using plants, collembolans and earthworms (Krogh et al. 1997, Robidoux et al. 1998, Renoux et al. 2001, Diez et al. 2001, Crouau et al. 2002, Pandard et al. 2006).

1.8.2. Laboratory and semi-field multispecies tests: microcosms and mesocosms

Multispecies tests, commonly called microcosms or mesocosms tests, have been proposed as a tool to obtain more ecologically relevant ecotoxicological data (Figure 1.4). According to Kampichler et al. (2001), "microcosms" can be defined as ecosystem models completely confined (without materials or individuals exchange with surrounding environment), kept in laboratory or field conditions and consisting of a simplified or whole soil community. "Mesocosms" are microcosms kept in field conditions allowing material exchange (gases, nutrients, water) but with limitations to the individuals exchange by a physical barrier. The term mesocosm was proposed by Odum (1984) to fill the gap between the experiments carried out in confined systems and those not limiting the materials and individuals' movement, "macrocosm" (i.e. the real world).

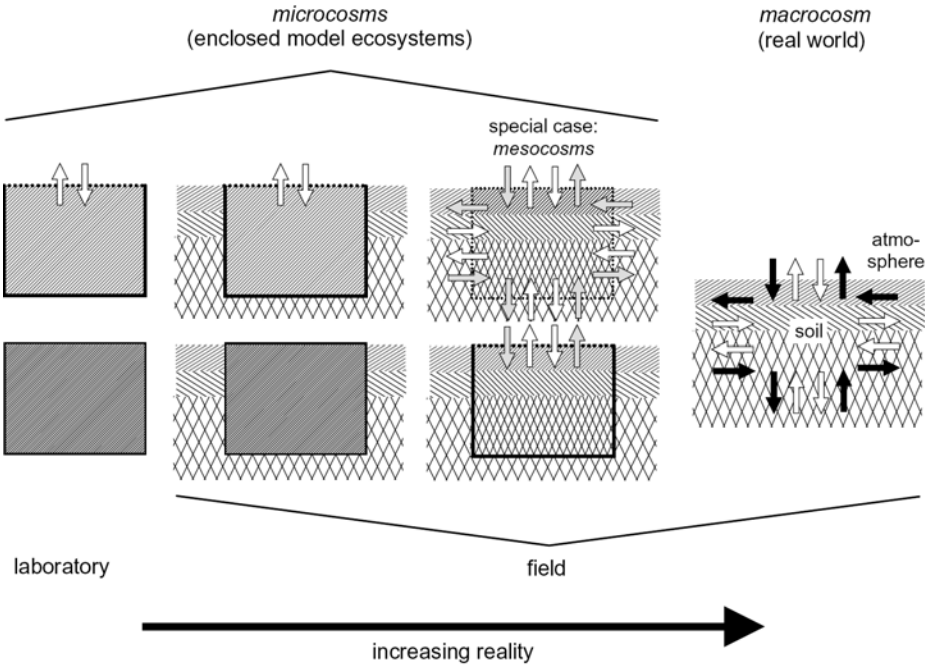

Figure 1.4. Microcosms, mesocosms and macrocosm. White arrows indicate matter exchange (gas, water, nutrients). Movement of soil fauna between the mesocosm and the environment is indicated with shaded arrows (controlled movement) or black arrows (unrestricted movement). Upper and lower microcosm rows represent different degrees of permeability. Reproduced from Kampichler et al. (2001).

Even though microcosms/mesocosms provide ecologically relevant data, the low reproducibility is their main limitation (Edwards et al. 1996), something that might explain why no standardized protocols have been published to date. However, recently an attempt has been carried out to develop a standardized multispecies test for environmental risk assessment, the so-called Terrestrial Model Ecoystems (TME) (Knacker et al. 2004), whose main elements are represented in Figure 1.5.

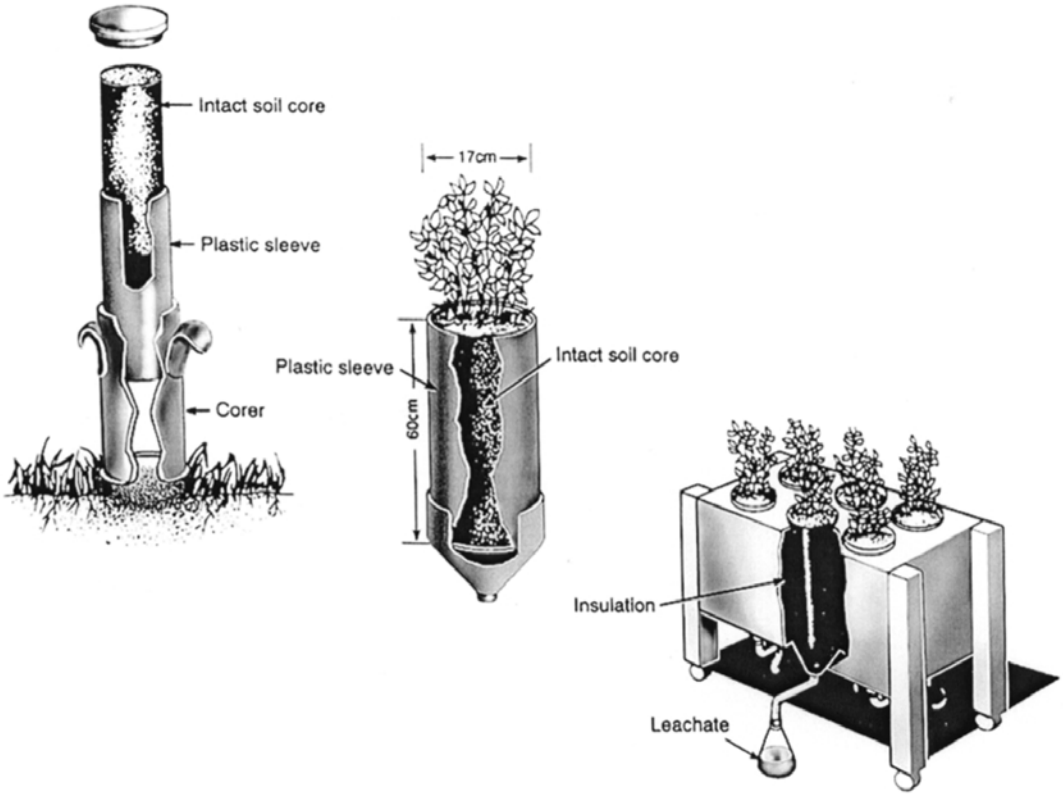

Figure 1.5. Terrestrial Model Ecosystem. Reproduced from Van Straalen (2002).

Experiments using laboratory multispecies tests to assess organic waste ecotoxicity are still scarce. Andrés and Domene (2005) compared three sewage sludges for toxicity through their effects on field-collected soil fauna communities in laboratory microcosms. Pernin et al. (2006) assessed the toxicity of sludge enriched with copper on an artificial soil community of six collembolans two Oribatida mites, one Gamasida mite and one enchytraeid species. In both studies, some species were sensitive while others were tolerant at similar dosages, resulting in changes in the community structure.

1.8.3. Field multispecies tests: macrocosms

No standardized methods have been published for this type of test, with the exception of ISO 11268-3:1999, a protocol for the assessment of pollution effects on earthworm field communities.

Field studies dealing with effects of organic waste amendments are common, but they generally focus on the effects on crops. More scarce are studies centered on the effects of agricultural or degraded land amendment with organic waste organic waste on the remainder of soil-dwelling organisms (Krogh et al. 1997, Andrés 1999, Barrera et al. 2001, Matscheko et al. 2002, Petersen et al. 2003).

1.8.4. Soil processes tests

Tests using soil processes are carried out relatively easily (Kula and Römbke 1998) and can be used in laboratory, semi-field or field conditions. The "cotton strip assay" (Harrison et al. 1988, Kratz 1996) is based on the loss of tensile strength of a cotton strip placed in the soil for a given period. It is used to measure the decomposition activity in soil, but it has been criticized because cotton and natural litter degradation are not entirely comparable (CSTEE 2000).

A similar test is the "litter bag method" (Crossley and Hoglund 1962, Paulus et al. 1999), which measures the natural litter decomposition and hence is more ecologically relevant. This method has also been criticized on the basis of the unnatural conditions provided by the litter bag, which can impede contact between the litter and the polluted, perhaps creating an attractive microclimate for decomposers.

The "bait-lamina test" (Von Törne 1990, Paulus et al. 1999) measures the soil fauna feeding rate. It consists of a plastic strip with holes filled with a food substrate which allow numerically determining feeding rate.

1.9. General objectives

The main objectives of this thesis are:

a. To select suitable methodologies for organic waste ecotoxicity assessment in terms of sample preparation procedures, test organisms and endpoints.

b. To identify the main waste parameters explaining the toxicity of waste to soil-dwelling organisms, and also the most suitable waste treatments to decrease toxicity.

c. To assess the aptitude of laboratory waste bioassays for the ecological risk assessment of organic waste field amendments.

d. To evaluate the suitability of OECD artificial soil as a natural soil surrogate for waste bioassays, and to investigate the influence of different soil properties on test results.

e. To assess the effect of different soils (including OECD) and different soil properties on test organisms.

1.10. Outline of the thesis

In order to approach the main objectives and to provide relevant results for the variety of organic wastes currently applied to agricultural soils, seven types of waste are studied in this thesis: two dewatered sewage sludges, two composted sewage sludges, two thermally-dried sewage sludges, and a thermally-dried pig slurry.

Most work has been carried out using OECD artificial soil, but some work with different natural soils is also included in order to assess the relevancy of results. Some tests have been carried out both with waste solid-phase and others with different waste eluates.

Furthermore, work has been performed with a variety of test organisms and endpoints, ranging from terrestrial to aquatic, from microorganisms to plants and soil invertebrates and from lethal to sublethal endpoints.

In **Chapter 2**, the suitability of the *Folsomia candida* mortality and reproduction test as a tool for the ecotoxicological assessment of organic wastes is assessed. Special attention

is paid to the particular characteristics of waste testing, including variations in the physico-chemical properties of the soil-waste mixtures as waste concentration increases. In **Chapter 3**, an innovative test method, based on the *F. candida* feeding inhibition is evaluated and compared with the former test described. Both methods are compared for sensitivity, correlation of responses and workload. In chapters 2 and 3, the main waste properties contributing to the toxicity are identified.

In **Chapter 4**, waste toxicity using terrestrial assays (*F. candida*) is compared with that obtained with aquatic assays (*Vibrio fischeri* and *Daphnia magna*). Furthermore, representativity of different waste eluates for estimating solid-phase toxicity is assessed, as well as the main parameters contributing to toxicity both in solid-phase and eluate assays.

In **Chapter 5**, data from a battery of different laboratory solid-phase waste bioassays (microorganisms, plants, earthworms, enchytraeids and collembolans) are used to assess the suitability of the species sensitivity distribution method for estimating safe organic waste amendment rates. Risk is assessed by comparison between the estimated safe amendment rates and the plausible amendment rates in agricultural land, according to different realistic scenarios based on crop demands and usual amendment rates in the European Union. Main waste properties determining risks for soil-dwelling species are also verified.

In the two last chapters, the suitability of using OECD artificial soil as a test substrate for extrapolating effects to natural soils is assessed. Furthermore, the influence of soil properties on the toxicity results of these assays waste bioassays is also evaluated assessed. More precisely, in **Chapter 6**, the suitability of OECD artificial soil and natural soils are used in avoidance and reproduction tests with different soil organisms (earthworms, enchytraeids and collembolans). The influence of different soil properties on test results is examined. The suitability of OECD soil as a natural soil surrogate is evaluated. Then, in **Chapter 7**, the toxicity of an organic waste on *F. candida* was

assessed in the OECD artificial soil and in several natural soils, in order to evaluate the relevancy of this substrate for real situations. Main soil properties modulating the toxicity of organic wastes were also determined in order to identify the soils most vulnerable to organic amendments.

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Chapter 2

Ecotoxicological assessment of organic wastes using the soil collembolan *Folsomia candida*[1]

ABSTRACT

The reproduction test with the collembolan *Folsomia candida* is used as a tool to evaluate the ecotoxicological potential of organic wastes currently applied to soil. Seven organic wastes (dewatered sewage sludges, thermally-dried sewage sludges, composted sewage sludges, and a thermally-dried pig slurry) were tested. These wastes had different origins, treatments, and pollutant burdens, and were selected as a representative sample of the wide variety of wastes currently generated. *F. candida* showed varied sensitivity depending on the waste, but also depending on the endpoint assessed. Reproduction was more sensitive than survival, although no correlations between reproduction and physico-chemical parameters and pollutant burden could be found. On the other hand, mortality was directly related to the lack of stability of wastes, probably reflecting the toxicity of end-products such as ammonium. Body length was not shown to be a sensitive endpoint for waste testing, as it was neither affected nor even stimulated by waste concentrations.

Organic matter, pH, and electrical conductivity varied with waste concentration in soil-waste mixtures, although their effect on collembolan performance was expected to be low and part of the complex effect exerted by wastes when applied to real soils. Selection of the water content is the most problematic aspect in waste testing, as it may

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affect the performance of test organisms. In this study a qualitative approach for water content selection in waste testing was considered to be the most suitable.

Treatment of wastes affected composition and toxicity. Composting of sewage sludge increased its stability, compared to the initial sludge, but decreased its non-persistent organic pollutant burden and toxicity. On the other hand, thermally-dried wastes from sludge and pig slurry displayed high toxicity, mainly attributable to their low stability. The results from the study indicate the inability of chemical methods to predict the effects of complex mixtures on living organisms with respect to ecotoxicity bioassays, but also the need for stabilization treatments of organic wastes prior to their reuse in soils.

2.1. INTRODUCTION

The amount of sewage sludge produced in the European Union has increased dramatically in recent years due to the implementation of Directive 91/271/EC. This increase will mainly be managed through its reuse in agricultural soil, despite our poor understanding of the impact of this management option. There is a large amount of experimental evidence which suggests that this practice may enhance soil fertility, but there are also well-known associated environmental risks, including pathogens, nitrate pollution of ground waters, and inputs of heavy metals and organic pollutants (Düring and Gath, 2002).

To date, experimental results of sludge application to agricultural soils indicate a low level of risk for crops, but little is known about its effects on soil biota, a critical element in soil functioning (Giller et al., 1997). Harmful effects on soil invertebrates have been found in laboratory experiments (Krogh et al., 1997; Andrés and Domene, 2005), but some field experiments have shown that soil biota are stimulated when sludge is added to soil at agronomic rates (Krogh and Pedersen, 1997; Petersen et al., 2003).

Measuring pollutant concentration by chemical methods is the most common way to estimate the toxicity of pollutants and wastes, despite the development of biological methods in recent decades and their advantages over chemical methods. For example, the European Union regulation restricts the reuse of sewage sludge in soil taking into account limit values for six heavy metals (Directive 86/278/EC), but no biological tests are mentioned, even in the third draft of the Working Document on Sludge (European Communities, 2000). Furthermore, methods to assess the direct toxicity of solid wastes are not available despite the existence of standardized protocols for single chemicals using terrestrial organisms.

Crouau et al. (2002) concluded that the standardized Collembola reproduction test ISO 11267 (1999) was suitable for this purpose. They also pointed out that reproduction in this species may be affected not only by pollutant content but also by physico-chemical characteristics of waste such as pH, moisture and organic matter content. As a result, bioassays applied to organic wastes were not easy to interpret as two contradictory effects occurred at the same time. On the one hand, the organic matter in residues may have a stimulatory effect on soil organisms, while on the other hand the pollutant burden may exert inhibitory effects (Krogh et al., 1997; Andrés and Domene, 2005). Furthermore, parameters such as water availability or pH may also contribute to the biological effects observed.

The main aim of this study was to assess the suitability of the *F. candida* reproduction test as a tool for the ecotoxicological assessment of organic wastes which are to be applied to soils. Special attention was devoted to the special characteristics of waste testing, which involves variation in the physico-chemical properties of the soil-waste mixtures as the waste concentration increases. In addition, the influence of the origin, treatment, and composition of organic wastes on the ecotoxicological response of *F. candida* were be studied.

2.2. METHODS

2.2.1. Test species

The strain of *F. candida* used in our experiments was provided by the Institute of Ecological Science of the Free University of Amsterdam. Cultures were raised in polyethylene containers 17.5 x 12.5 x 7.5 cm. The substrate consisted of a 1 cm layer of a wet mixture of plaster of Paris and charcoal (9:1 v/v). Cultures were raised in darkness in a climatic chamber at a constant temperature of 21±1°C. The substrate was renewed and the density of individuals was reduced every two months to avoid overcrowding.

2.2.2. Organic wastes

In order to represent a variety of organic wastes currently applied to agricultural soils, we selected seven types of waste: two dewatered sewage sludges, two composted sewage sludges, two thermally-dried sewage sludges, and a thermally-dried pig slurry. Treatments and post-treatments of the wastes differed as summarized in Table 2.1.

Physico-chemical properties, heavy metal and organic pollutant contents of the wastes are recorded in Table 2.2. Dry matter, water holding capacity, water pH, electrical conductivity, total nitrogen, and organic matter were measured according to EN 12880 (2000), ISO 11267 (1999), EN 13037 (1999), EN 13038 (1999), EN 13342 (2000) and EN 12879 (2000), respectively.

Non-hydrolyzable (stable) organic matter and non-hydrolyzable nitrogen were measured as a percentage of organic matter and nitrogen remaining in the sample residue after acid hydrolysis, as described in Rovira and Vallejo (2002). This method removes the more labile fraction of an organic substrate, mainly consisting of polysaccharides and proteins. Hydrolyzable nitrogen was calculated by subtracting the

content of non-hydrolyzable nitrogen from total nitrogen content. N-NH_4 was measured on the distillates obtained from fresh samples.

Table 2.1. Treatments and post-treatments of the organic wastes.

Waste	Origin	Treatment	Post-treatment
AED	Banyoles WWTP	Aerobic digestion, dewatering	None
AEC	Banyoles WWTP	Aerobic digestion, dewatering	Composting in vessel
AET	Banyoles WWTP	Aerobic digestion, dewatering	Thermal drying
AND	Blanes WWTP	Anaerobic digestion, dewatering	None
ANC	Blanes WWTP	Anaerobic digestion, dewatering	Composting in heap
ANT	Blanes WWTP	Anaerobic digestion, dewatering	Thermal drying
SLT	Juneda WTP	Anaerobic digestion, dewatering	Thermal drying

Elemental analysis of P, K, Cd, Cr, Cu, Hg, Ni, Pb and Zn was carried out by ICP-MS according to ISO 11885 (1996). Polychlorinated dibenzodioxins and dibenzofuranes (PCDD/F) were measured with HRGC-HRMS, polychlorinated biphenyls (PCB) by HRGC-ECD, di (2-ethylhexyl) phthalate (DEHP) and nonylphenols (NPE) by HRGC-MS. Polycyclic aromatic hydrocarbons (PAH) and linear alkylbenzene sulphonates (LAS) were determined by HPLC with fluorescence and UV detectors, respectively. Values for each pollutant group were expressed as indicated in the third draft of the Working Document on Sludge (European Communities, 2000). Hence, DEHP, LAS, PCDD/F values represent total values. NPE include nonylphenol and nonylphenol ethoxylates with 1 or 2 ethoxy groups. PAH are the sum of acenapthene, phenanthrene, fluorene, fluoranthene, pyrene, benzo(b+j+k)fluoranthene, benzo(a)pyrene, benzo(ghi)perylene, and indeno(1, 2, 3-c, d)pyrene. PCB is the sum of the polychlorinated biphenyl congeners number 28, 52, 101, 118, 138, 153 and 180.

Table 2.2. Physico-chemical properties, heavy metal and organic pollutant contents of the organic wastes (* = over the limit value in the 3rd draft of Working Document on sludge (European Communities 2000), ** = over the limit value of the current Directive on sludge (86/278/EEC); *w.w.* = wet weight; *d.w.* = dry weight). See Table 2.1 for waste abbreviations.

Parameter	Units	AEC	AED	AET	ANC	AND	ANT	SLT
Dry matter	g kg^{-1} (*w.w.*)	449	150	945	470	199	844	865
WHC	% (*w.w.*)	74.4	63.9	74.7	64.9	64.8	67.9	55.9
pH	water, 1:5 (v/v)	7.8	8.1	6.9	7.2	8.4	7.2	6.4
Electrical conductivity	dS/m, 25°C	1.2	1.5	3.57	4.2	2.25	6.22	64.65
Organic matter	g kg^{-1} (*d.w.*)	622	684	687	551	566	668	612
Stable organic matter	%	50.1	37.8	40.4	54.2	47.7	46.7	36.6
N	g kg^{-1} (*d.w.*)	39.5	62.4	60.6	23.7	38.8	53.3	62.5
Non-hydrolyzable N	g kg^{-1} (*d.w.*)	17.0	16.4	19.1	16.1	12.4	18.4	10.9
Hydrolyzable N	g kg^{-1} (*d.w.*)	22.5	46.0	41.5	7.6	26.4	34.9	51.6
NH_4-N	g kg^{-1} (*w.w.*)	2.7	14.0	8.0	3.4	15.1	11.6	52.9
P	g kg^{-1} (*d.w.*)	22.0	20.4	20.5	28.6	33.6	29.2	20.4
K	g kg^{-1} (*d.w.*)	3.6	1.9	2.2	4.4	2.3	2.5	55
Cd	mg kg^{-1} (*d.w.*)	1.0	1.3	1.3	3.5	3.2	3.1	<0.7
Cr	mg kg^{-1} (*d.w.*)	345	55	30	53	54	127	15
Cu	mg kg^{-1} (*d.w.*)	294	624	645	798	933	833	780
Hg	mg kg^{-1} (*d.w.*)	0.67	1.33	0.95	2.13	2.51	2.25	0.12
Ni	mg kg^{-1} (*d.w.*)	59	80	53	76	64	45	29
Pb	mg kg^{-1} (*d.w.*)	1196**	3940**	3747**	92	78	85	<20
Zn	mg kg^{-1} (*d.w.*)	843	956	952	1028	988	890	2060

DEHP	mg kg^{-1} (*d.w.*)	10	61	27	22	143	71	1
LAS	mg kg^{-1} (*d.w.*)	298	816	331	214	3240*	5572*	60
NPE	mg kg^{-1} (*d.w.*)	86*	153*	76*	158*	513*	573*	54*
PAH	mg kg^{-1} (*d.w.*)	0.1	0.4	0.3	1.6	1.1	1.4	0.05
PCB	mg kg^{-1} (*d.w.*)	0.015	0.034	0.029	0.041	0.023	0.029	<0.007
PCDD/F	ngTEQ kg^{-1} (*d.w.*)	16	15.6	13.7	12.4	7.7	13.2	0.3

It should be noted that each type of waste came from a different batch, and hence, besides differences resulting from contrasting treatments and post-treatments, values for individual pollutants may also be different in wastes from the same plant, given temporal changes in wastewater composition. Despite that, some of the physico-chemical characteristics of wastes changed too dramatically with post-treatments for this to be attributed exclusively to batch differences.

The current final product of wastewater treatment is dewatered sludge, obtained from aerobic or anaerobic digestion followed by centrifugation. Sludge stabilization and dewatering is compulsory prior to its application to the soil, as this process reduces pathogen content and volume. Some wastewater plants perform additional sludge post-treatments, the most common of which are composting and thermal drying. Sludge composts of this work were produced by mixing dewatered sludge with pine wood chips (1:4.5, v/v). For the original anaerobic sludge, composting was carried out in a tunnel with air injection for fifteen days at the wastewater plant itself. For the aerobic sludge, composting was performed in a heap. Components of the heap were well mixed every two days by tumbling the first four weeks, and then every week until the end of the composting period (50 days). At the end of this period, both composts were sieved to 1 cm. Composting decreased total, hydrolyzable and ammonium nitrogen content, and increased organic matter stability compared to dewatered sludge. Composting also

resulted in a reduced concentration of non-persistent organic pollutants (DEHP, NPE and LAS)(Table 2.2).

Thermal drying was carried out by placing dewatered sludge in a heated rotary cylinder and injecting hot air, which provided a temperature of around 130-150ºC for 45 minutes. This treatment reduced the $N\text{-}NH_4$ content of dewatered sludge, but increased its electrical conductivity, and did not decrease pollutant levels with respect to dewatered sludge, with the exception of DEHP.

Pig slurry was obtained from an anaerobic digestion of raw slurry followed by thermal drying at 130ºC, a treatment that provided a waste characterized by high electrical conductivity, high hydrolyzable nitrogen and $N\text{-}NH_4$ content, and low organic matter stability (easily mineralizable).

For the analysis of the wastes, and for the preparation of soil-waste mixtures for the bioassays, each waste was dried at 60ºC for 48-72 hours depending on its initial water content, and then ground. These steps were unavoidable in order to ensure the homogeneity and accuracy of the lower test concentrations.

2.2.3. Test preparation

The experiment was performed as indicated in the standard test ISO 11267 (1999), although several modifications were performed to the protocol in order to adapt it to the experimental aims and waste properties. These changes were based on an unpublished preliminary work that showed that effects on different individual parameters (survival, reproduction, and body length) may occur at quite different concentrations. First, the range-finding assay was not performed and testing was reduced to a single assay. This allowed simultaneous observations of the endpoints studied in each waste. Twelve test concentrations were used: 0, 1, 2, 4, 7.9, 15.8, 31.6, 63.1, 125.9, 251.2, 501.2 and 1000 g kg^{-1} (w/w) of waste in a mixture with OECD artificial soil. Second, given that water holding capacity (WHC) of wastes was higher

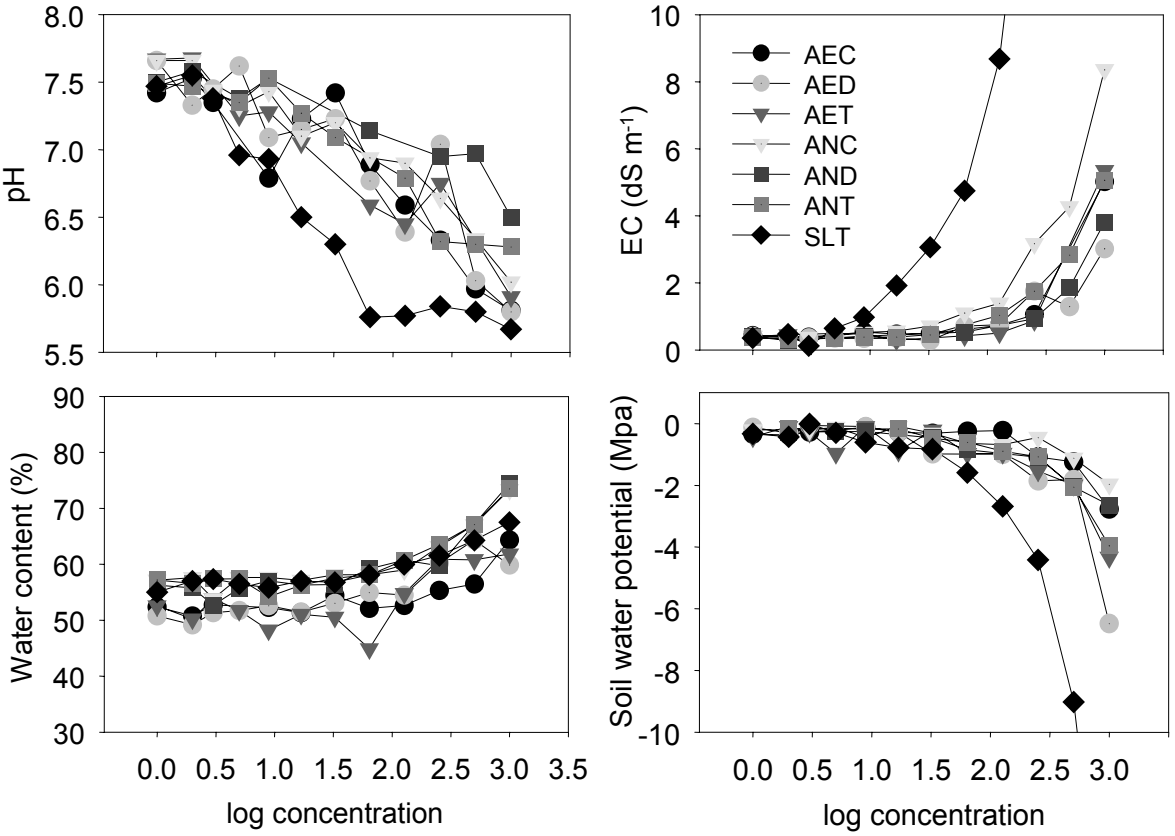
than the artificial soil, water content should be increased with increasing concentrations in soil-waste mixtures in order not to affect the performance of test organisms. A possible approach to this problem might be to provide the same % of the WHC in all the test concentrations, but that might require, before any waste bioassay, a prior assessment of WHC for every test concentration. In this study we took an alternative approach in order to provide a more workable method, which makes any previous work unnecessary. Hence, the suitable moisture for each concentration was determined by the addition of small amounts of water in order to provide the optimum moisture for soil-waste mixtures. As indicated in ISO 17512 (2005), such optimum water content is defined as that when no standing water or free water appears when the soil is compressed. In controls, that point was achieved with humidity around 50-60% (w/dw), and around 55-75% in the highest waste concentration. Using these criteria, all test concentrations had a similarly wet appearance and a crumbly structure.

Each replicate consisted of a 125 ml polyethylene container filled with 30 g of wet test substrate, with a lid that allowed sealing. For each concentration 5 replicates were prepared, as well as an additional replicate to determine changes in pH, electrical conductivity, water content, and water availability (measured as soil water potential) at the end of the test period. The pH and electrical conductivity were measured in a 1:5 soil-water extract obtained according to ISO 10390 (2005) using a Crison MicropH 2000 pHmeter, and a Crison Conductimeter 522 (Crison, Barcelona, Spain), respectively. The soil water potential was measured with a WP4 dew point potentiometer (Decagon Devices, Pullman, USA).

The physico-chemical characteristics of the soil-waste mixtures used in the bioassays are summarized in Figure 2.1. As waste concentration increased, the pH values decreased by 1.5 units at most from controls to the highest concentration, while the electrical conductivity markedly increased from intermediate concentrations. The moisture content was similar at most of the concentrations, but was slightly higher at the higher waste

concentrations, as more water was added in order to provide the optimum moisture content. Water potential values also remained nearly constant at lower waste concentrations, but decreased markedly at intermediate concentrations. This was mainly explained by the high solute concentration provided by wastes, as a significant correlation between log transformed values of electrical conductivity and water potential was detected (Pearson, r = 0.814, P<0.001).

Figure 2.1. Changes in physico-chemical parameters in soil-waste mixtures with increasing waste concentration. Concentrations are expressed as log [1+concentration], in g Kg-1. See Table 2.1 for waste abbreviations.



2.2.4. Test performance

Ten 10-12 day-old individuals of *F. candida* were placed in each container together with 3 mg of granulated dry yeast. Yeast was added again on the 14th day. Containers were kept in the dark at 21±1°C for 28 days, and were opened twice a week. During this period, individuals reached sexual maturity and produced offspring.

At the end of this period, the containers were flooded with water to float the adults and juveniles. A dark dye was added to facilitate counting and a photograph was taken. Adults and juveniles were counted using the image treatment software ImageTool 3.0, and they were distinguished by their clearly different sizes. The mean body length of adults per replicate was measured from the anterior end of the head, between the antennae, to the posterior end of the abdomen, as described by Folker-Hansen et al. (1996).

Relative survival (100*number of adults in replicates/ mean number of adults in controls), relative reproduction (100*number of juveniles in replicates/ mean number of juveniles in controls), and relative body length (100*mean body length of adults in replicates/ adults mean body length in controls) were calculated.

2.2.5. Data treatment

LC50, LC20, EC50, and EC20 were calculated for each type of waste using Statistica 6.0. These values and their 95% confidence intervals were calculated from suitable regression models (exponential, Gompertz, hormesis, linear or logistic). The choice of the model was based on the best fit to the data, based on Stephenson et al. (2000).

In order to find out which individual pollutant, pollutant group, or physico-chemical parameter might be responsible for the observed effects in the whole set of wastes, we calculated Pearson correlation coefficients for the toxicity values (LCx, ECx) with respect to the individual pollutant concentrations in wastes, the sum of heavy metal

concentrations, the sum of organic pollutant concentrations, the sum of persistent organics (PAH, PCB, and PCDD/F), the sum of non-persistent organics (DEHP, LAS, and NPE), the sum of all pollutant concentrations, and individual values for physico-chemical parameters. Pearson correlation coefficients were calculated using SPSS 11.0.

Additionally, we estimated the individual pollutant concentrations at the LC50 and EC50 and we compared them with LC/EC50 values collected from the literature, in order to check if any pollutant was on a range above that expected to exert harmful effects on collembolans.

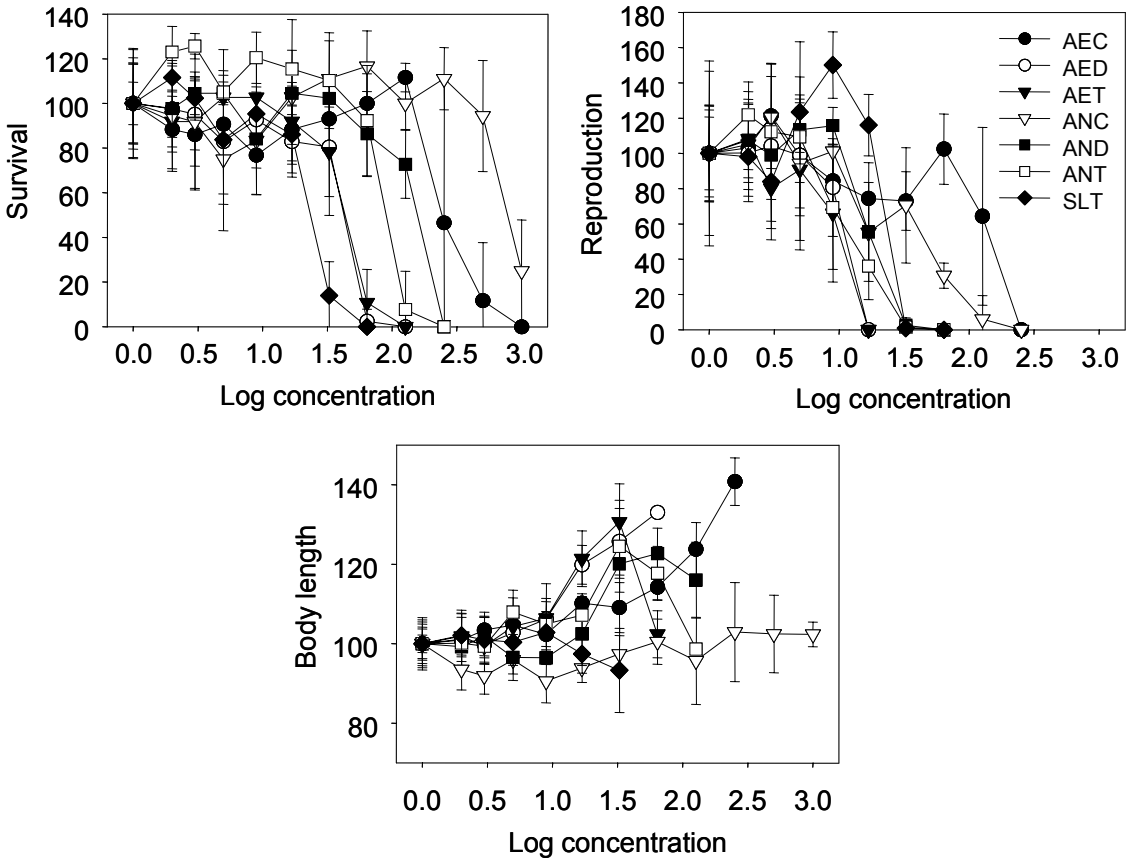
2.3. RESULTS

2.3.1. Toxicity test results

Test results complied with the validity requirements of ISO 11267 (1999), as in controls the number of surviving individuals was over 8 (8.6±0.2), and more than 100 juveniles (348±197) were present. Mean body length of individuals in controls at the end of the assays was 1.59±0.04 mm.

The results of the toxicity tests are shown in Figure 2.2. Survival and reproduction decreased with increasing waste concentrations, survival being the least sensitive. It is also noteworthy that reproduction usually showed a higher variability between replicates than survival. On the other hand, body growth was either not affected by waste concentration or was even slightly stimulated. For most of the studied wastes, body growth increased in parallel with the decrease in reproduction.

Figure 2.2. Mean values for survival, reproduction, and body length of *Folsomia candida* with increasing concentrations of wastes in soil-waste mixtures. Effects on endpoints are expressed as a percentage with respect to controls. Concentrations are expressed as log [1+concentration], in g Kg-1. Bars indicate standard deviation. See Table 2.1 for waste abbreviations.



LCx and ECx values are shown in Table 2.3. No values were calculated for body length, since it was not affected. Survival was strongly inhibited by pig slurry (LC50=24 g kg^{-1}), but to a lesser extent in both composted sludges (LC50=252 and 834 g kg^{-1}) indicating their lower toxicity. Reproduction was hardly inhibited by aerobic thermally-dried sludge (EC50=5.3 g kg^{-1}), followed by aerobic and anaerobic dewatered sludge (EC50=10.0 and 10.4 g kg^{-1}). The lowest inhibition in reproduction was shown by anaerobic composted sludge (207 g kg^{-1}).

Table 2.3. Toxicity values for survival (LCx) and reproduction (ECx) of *F. candida* expressed in g kg^{-1}, with 95% confidence intervals enclosed in parentheses. See Table 2.1 for waste abbreviations.

Waste	LC50	LC20	EC50	EC20
AEC	252 (222, 287)	210 (81, 546)	207 (37, 1142)	26 (4.51, 134)
AED	44 (34, 57)	35 (26, 46)	10 (3.8, 24)	7.9 (5.8, 11)
AET	44 (37, 52)	32 (25, 40)	5.3 (2.8, 9.4)	1.1 (0.7, 1.5)
ANC	834 (626, 1110)	665 (384, 1152)	29 (18, 46)	12 (5.6, 25)
AND	154 (134, 178)	114 (97, 133)	16 (15, 18)	14 (11, 18)
ANT	86 (72, 101)	63 (50, 79)	10.4 (7.5, 14)	6.7 (4.5, 9.9)
SLT	24 (20, 28)	18 (14, 23)	19 (3.8, 86)	18 (6.4, 48)

2.3.2. Waste composition and toxic effects

The comparison of LC50 and EC50 for individual pollutants obtained from the literature with their concentrations in test containers at LC50 and EC50 showed that none of those pollutants were present in concentrations which might be expected to affect survival (Table 2.4). However, some pollutants might affect reproduction (Table 2.5). More precisely, nonylphenol ethoxylates (including 4-nonylphenol) in some wastes (AEC, AND, ANT and AND) showed values above 2.9 mg kg^{-1}, which was reported to be EC50 for 4-nonylphenol on *F. candida* (Greenslade and Vaughan, 2003). Also Zn was present in a range that could affect reproduction in AEC according to Fountain and Hopkin (2005). Finally, LAS concentrations were close to those expected to affect reproduction in AEC, ANT, and AND, according to Jensen et al. (2001a).

Table 2.4. Published LC50 values for the effect of single pollutants on *F. candida*, and equivalent concentrations of these products in studied wastes at the LC50 level. All values reported were from *F. candida* with the exception of LC50 for PAH, DEHP, NPE, and LAS, obtained from *Folsomia fimetaria*, and LC50 for PCDD/F, obtained from Collembola as a group. PCDD/F are expressed as ng TEQ kg^{-1}. See Table 2.1 for waste abbreviations.

Pollutant	LC50	Reference	Pollutant equivalent concentration (mg kg^{-1})						
	(mg kg^{-1})		AEC	AED	AET	ANC	AND	ANT	SLT
Cd	850	Crommentuijn et al. 1993	0.2	0.1	0.1	2.9	0.5	0.3	0.02
Cr	-	-	87.0	2.4	1.3	44.2	8.3	11.2	0.4
Cu	1810	Greenslade and Vaughan 2003	74.2	27.4	28.4	665.4	143.7	73.8	18.5
Hg	-	-	0.17	0.06	0.04	1.78	0.39	0.20	0.00
Ni	-	-	14.9	3.5	2.3	63.4	9.9	4.0	0.7
Pb	980-2900	Bongers et al. 2004	301.7	173.0	164.8	76.7	12.0	7.5	0.5
Zn	5150	Lock and Janssen 2001b	212.7	42.0	41.9	857.1	152.1	78.8	48.8
PCB	>204 (PCB153)	Aldrich and Daniel 2003	0.004	0.001	0.001	0.034	0.003	0.003	<0.001
PAH	67-1025	Sverdrup et al. 2002	0.02	0.02	0.01	1.33	0.17	0.12	0.001
DEHP	>5000	Jensen et al. 2001b	2.5	2.7	1.2	18.3	22.0	6.3	0.02
NPE	99-140 (NP)	Scott-Fordsmand and Krogh 2004	21.7	6.7	3.3	131.7	79.0	50.7	1.23
LAS	>793	Holmstrup and Krogh 2001	75.2	35.8	14.6	178.4	499.0	493.4	1.4
PCDD/F	>10 (OCDD)	Van Straalen et al. 1995	4.04	0.68	0.60	10.34	1.19	1.17	0.01

Table 2.5. Published EC50 values for the effect of single pollutants on *F. candida*, and equivalent concentrations of these products in studied wastes at the EC50 level. Values for NP from *Folsomia fimetaria*. PCDD/F are expressed as ng TEQ kg^{-1}. See Table 2.1 for waste abbreviations.

Pollutant	EC50	Reference	Pollutant equivalent concentration (mg kg^{-1})						
	(mg kg^{-1})		AEC	AED	AET	ANC	AND	ANT	SLT
Cd	51-780	Fountain and Hopkin 2005	0.21	0.01	0.01	0.10	0.05	0.03	0.01
Cr	604	Lock and Janssen 2002a	71.4	0.5	0.2	1.5	0.9	1.3	0.3
Cu	250-1480	Fountain and Hopkin 2005	60.83	6.2	3.4	22.8	15.23	8.7	15.1
Hg	3.26	Lock and Janssen 2001a	0.14	0.01	0.01	0.06	0.04	0.02	0.00
Ni	476	Lock and Janssen 2002b	12.2	0.8	0.3	2.2	1.0	0.5	0.6
Pb	580-3160	Fountain and Hopkin 2005	247.4	39.3	19.9	2.6	1.3	0.9	0.4
Zn	50-2088	Fountain and Hopkin 2005	174.4	9.5	5.0	29.4	16.2	9.3	40.0
PCB	-	-	0.003	<0.001	<0.001	0.001	<0.001	<0.001	<0.001
PAH	-	-	0.021	0.004	0.002	0.046	0.018	0.015	0.001
DEHP	>5000	Jensen et al. 2001b	2.1	0.6	0.1	0.6	2.3	0.7	0.02
NPE	2.9 (NP)	Greenslade and Vaughan 2003	17.8	1.5	0.4	4.5	8.4	6.0	1.0
LAS	91	Jensen et al. 2001a	61.7	8.1	1.7	6.1	53.0	58.00	1.2
PCDD/F	-	-	3.3	0.2	0.1	0.3	0.1	0.1	0.01

Pearson coefficients of toxicity values (LCx, ECx) with concentration of individual pollutants and the sum of concentrations of pollutant groups showed a general lack of association. On the other hand, toxicity values showed significant correlation with physico-chemical properties, such as the positive correlation between survival and stable organic matter (LC50 r = 0.953, LC20 r = 0.947), and negative correlations were found for survival with total nitrogen (LC50 r = -0.971, LC20 r = -0.968), hydrolyzable nitrogen (LC50 r = -0.966, LC20 r = -0.963), and N-NH_4 content (LC50 r = -0.794, LC20 r = -0.801). In contrast, no significant correlations were found between reproduction values and waste physico-chemical properties.

2.4. DISCUSSION

2.4.1. Effects of treatment on waste properties

The changes in waste properties resulting from composting observed in this study coincide with similar published papers. Composted sludge shows a high degree of organic matter stability, since during this post-treatment there is a loss of the more labile fractions through microbial degradation (Grube et al., 2006). In this aerobic process, part of the less persistent organic pollutants (DEHP, LAS, and NPE) are also degraded. This fact has already been reported for DEHP (Bagó et al. 2005), LAS (Sanz et al., 2006), and NPE (Déportes et al., 1995). Despite this, NPE levels in the dewatered sludges studied were so high that composting was not able to reduce their concentration in composted sludges below the limit value of 50 mg kg-1 laid down in the draft of the Working Document on Sludge (European Communities, 2000). This agrees with the opinion that NPE is the most harmful group for the environment of all the non-persistent organic pollutants. On the other hand, heavy metals and more persistent organic pollutants maintained or increased their concentrations with composting, as has already been pointed out by Déportes et al. (1995).

As far as we know, no comparative studies on the effect of thermal drying on the pollutant burden of organic wastes have been published. We did not observe great differences in pollutant contents with respect to dewatered sludge, even for PCDD/F, which has been observed to increase during the thermal dewatering process (Blazer and Pluschke, 1994). Furthermore, it is worth pointing out the lower DEHP level in thermally-dried sludge, already mentioned by Bagó et al. (2005) and attributed to steam distillation during the drying process.

Treatments applied to pig slurry produce a waste product with high values of hydrolyzable nitrogen, ammonia, K and Zn, an extremely high electrical conductivity, and a low proportion of stable organic matter.

2.4.2. Influence of physico-chemical variation in soil-waste mixtures

Crouau et al. (2002) have already pointed that the actual toxicity of wastes is not easy to assess since pH, organic matter and water content may also affect the test organisms as well as the pollutant burden. The effects of pH and organic matter are waste-characteristic but, on the other hand, the water content provided to soil-waste mixtures may exert some influence on the observed results by its direct effects on organisms and indirect effects on pollutant bioavailability.

2.4.2.1. Organic matter, pH, and electrical conductivity.

Increased waste concentrations exerted contradictory effects on *F. candida*. On the one hand, waste inhibited survival and reproduction at higher concentrations, but stimulated reproduction at lower concentrations. Such behavior shows the contradictory effects of polluted organic wastes, the organic matrix of which acts simultaneously as a nutritional resource and as a source of toxicity (Krogh et al., 1997; Andrés and Domene, 2005). The presumed nourishing effect of organic matter from wastes has been confirmed in an unpublished study that showed that *F. candida* ingested sludge from the test substrate, as

has already been shown by Krogh and Pedersen (1997) and by Scott-Fordsmand and Krogh (2004) for *F. fimetaria*. Nevertheless, sludge consumption rates were lower when yeast was available as an additional food source. Since yeast was quickly consumed after its addition to the test replicates, it is likely that individuals use organic wastes as an alternative food resource. This observation indicates that for this species, when organic wastes are tested, exposure could be mediated both by cuticle contact and consumption, as suggested by Krogh and Pedersen (1997).

The observed decrease in pH with waste concentration was unlikely to influence the results, as has already been pointed out by Crouau et al. (2002). The variation in pH between controls and concentrations with effects on survival or reproduction was always less than 1 pH unit (Figure 2.1), too low a variation to affect survival, and reproduction according to Crommentuijn et al. (1997), and Crouau et al. (1999). However, pH variation may influence pollutant bioavailability, although its variation in this study was within a range not expected to affect its uptake, according to Sandifer and Hopkin (1996) and Crommentuijn et al. (1997), but also given the nature of most of the pollutants contained in wastes, sorbed to waste particles.

Electrical conductivity also showed very large increases as waste concentration increased, although this in itself did not explain the observed effects on collembolans, as there was a lack of association between conductivity and toxicity.

In summary, organic matter content, electrical conductivity, and to a lesser extent pH, may in themselves affect survival and reproduction of *F. candida* as will slight differences in pollutant bioavailability. Nevertheless, these influences should be considered as part of the complex effects of wastes on soil biota when applied to real soils rather than as a disturbing factor for the interpretation of ecotoxicological results.

2.4.2.2. Water content and water availability

Water content is the most problematic parameter in waste testing given that the water holding properties of wastes are usually higher than that of soil. This makes it difficult to select a suitable water content without a previous case-by-case knowledge of the maximum water retention properties of soil-waste mixture concentrations, which would make any waste bioassay largely unworkable for current use. In this study, an alternative approach was used, as water content was qualitatively provided to soil-waste mixtures in order to provide a similarly wet and crumbly structure to all test mixtures. This approach is similar to that suggested to provide an optimum humidity of test substrate in a recent standardized protocol for earthworms (ISO 17512-1, 2005). To verify the suitability of such an approach we measured the soil water potential of test mixtures, which is the most realistic and most relevant measure of water availability for collembolans (Holmstrup et al., 2001). According to several authors (Holmstrup, 1997) *F. candida*'s survival is not significantly affected at relative air humidities over 98.5%, which is equivalent to a soil water potential of -2.04 MPa, below the permanent wilting point for plants (-1.5 MPa). In our test concentrations, such values were only attained for most wastes at concentrations over 750 g Kg^{-1}, much higher than the concentrations affecting survival, and especially reproduction (Table 2.3), which in turn is mainly due to the high solute content provided by the wastes rather than water deficiency. Toxic effects generally appeared at a range of concentrations with water potentials below -1 MPa, very close to those of the controls, and not expected in themselves to affect the performance of individuals. These findings support the idea that the soil environment is highly buffered with respect to desiccation, since air in soil pores is always near to saturation whenever soil has a moist appearance (Hillel, 1971). On the other hand, water availability differences may influence pollutant bioavailability. We lack a direct measure of pollutant bioavailability and hence any remarks about this would be premature. Nevertheless, we considered this possibility to be very limited given the little

variation in water availability in the range of concentrations with effects and the already detected low influence of water content variation in pollutant toxicity in this species (Van Gestel and Van Diepen, 1997).

2.4.3. Sensitivity of *F. candida* endpoints to wastes

F. candida is a suitable species for waste testing due to its easy culture and manipulability, and sensitivity to pollutants (Greenslade and Vaughan, 2003), but also because it is a representative species of soil collembolans, a group which performs key functions in soil ecosystems (Fountain and Hopkin, 2005).

All *F. candida* biological endpoints reacted to organic waste, although with different patterns. Survival showed a continuous decrease with waste concentration increase, usually at much higher concentrations than those affecting reproduction. Reproduction increased over the controls at the lowest concentrations, indicating hormetic and/or trophic effects of wastes, followed by an inhibition at higher doses of waste. Furthermore, reproduction showed higher variability between replicates than survival. This has already been noticed for this species (Crouau and Cazes, 2003). On the other hand, body length was not sensitive to waste concentration, as it was unaffected, or only slightly affected, at the highest concentrations with survivors. This lack of sensitivity to pollution agrees with the work of Folker-Hansen et al. (1996) for two collembolan species, although other studies support the sensitivity of this endpoint for collembolans (Scott-Fordsmand and Krogh, 2004). In the present study, stimulation of body length was coupled with inhibition in reproduction. This may be explained by the previously demonstrated negative trade-off between reproduction and growth in other insects (Ernsting et al., 1993).

2.4.4. Waste composition and toxic effects

Despite the fact that wastes showed concentrations for one or more pollutants above the limit values of the Working Document on Sludge (European Communities, 2000), NPE was the only pollutant group with overall high concentrations in all tested wastes, with levels over the 50 mg kg^{-1} mentioned in the draft (European Communities, 2000) (Table 2.2). Surfactants can affect soil microorganisms and invertebrates by dissolving biomembranes (Jensen, 1999), but NPE are also thought to have estrogenic effects and hence to affect the reproduction of invertebrates (Oehlmann and Schulte-Oehlmann, 2003). Toxic effects of NP on reproduction of *F. candida* (EC50 = 2.9 mg kg^{-1}) (Greenslade and Vaughan, 2003), and also on the reproduction and survival of *F. fimetaria* around 40 and 99-140 mg kg^{-1}, respectively (Scott-Fordsmand and Krogh, 2004) have been reported. According to the estimated NPE concentrations shown in Table 2.5, only some of the wastes showed concentrations above 2.9 mg kg^{-1}. Furthermore, no correlation was found between survival or reproduction and NPE levels. Likewise, none of the remaining pollutants or pollutant groups could be directly related to the observed effects, and hence none of them in themselves were able to account for toxic response. This agrees with the extended consideration of chemical methods, compared with bioassays, as unsuitable for the prediction of ecological risk to soil organisms of the complex pollutant burden of wastes, as already pointed out by Crouau et al. (2002).

On the other hand, some physico-chemical properties of the wastes showed a correlation with the observed effects. More precisely, LC50 and LC20 values were positively correlated with stable organic matter (ease of decomposition) and negatively correlated with total nitrogen, hydrolyzable (labile) nitrogen, and ammonium, although no correlation appeared with EC50 reproduction values. The more stabilized a waste is, the higher the proportion of recalcitrant organic matter, and the lower the amount of total, hydrolyzable nitrogen and ammonium released (Martins and Dewes, 1992). This is the

reason why survival is correlated with all these parameters, as all of them reflect the stability of wastes.

The negative correlation between the stability of wastes and their toxicity has been widely reported for plants (Pascual et al., 1997). It has been suggested that phytotoxicity was mediated by the release of ammonium, phenols, and organic acids during waste degradation, but also by competition between plants and microorganisms for available nitrogen, and by the decrease in soil oxygen levels (Déportes et al., 1995). Ammonia was directly related in this work to the observed negative effects on the survival of collembolans, as has already been shown for plants in soils amended with non-stabilized composts (Katayama et al., 1985), but also for short-term reductions of soil fauna density after the application of nitrogenated fertilizers (Seniczak et al., 1994) or organic wastes (Neher, 1999). On the other hand, reproduction inhibition was not significantly associated with waste stability, despite the fact that the more stabilized wastes, composted sludges, had a lower impact on reproduction. This pattern suggests that this endpoint, besides being more sensitive than survival, is affected in a different way by the waste that was tested. Hence, reproduction probably reflects the combined effect of waste physico-chemical properties and pollutant burden of wastes, providing more integrative information.

CONCLUSIONS

F. candida shows differential sensitivity depending on the type of waste, but also depending on the endpoint assessed. Reproduction is far more sensitive than survival, as it is affected at lower waste concentrations, while body length is a non- sensitive endpoint for waste testing. Pollutant burden alone is not able to predict the ecological risk of organic wastes to soil organisms, since neither the concentrations of single pollutants nor the sum of concentrations of pollutant groups can be related with the

observed toxic effects. On the other hand, collembolan mortality is clearly explained by the stability of wastes, which is probably related to releases of secondary metabolites with decomposition, mainly ammonium. In contrast to survival, none of the physico-chemical parameters explains the effects on reproduction, as this endpoint is likely to reflect the combined effects of the physico-chemical parameters and pollutant burden of wastes.

Soil-waste mixtures vary in their organic matter, pH, and electrical conductivity with increasing concentrations, but it would be better to consider them as contributors to the observed effects rather than disturbing factors, as these factors also act in real situations. On the other hand, selection of water content is a problematic step in waste testing, as it needs to be adjusted in order to ensure that water content does not affect test organism performance. In this study, a qualitative approach for the choice of optimum water content is validated as suitable for water content selection.

Treatment of sewage sludge changes its composition and toxicity, especially with composting, which increases its stability, decreases the non-persistent organic pollutant burden, and decreases toxicity. Thermal drying increases toxicity, which is attributable to a decrease in waste stability promoted by high temperatures. It is also worth pointing out the high toxicity of thermally-dried pig slurry, which is mainly due to its low stability.

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Chapter 3

Feeding inhibition in the soil collembolan *Folsomia candida* as an endpoint for the estimation of organic waste ecotoxicity[2]

ABSTRACT

Despite the increasing quantities of organic wastes that are being reused in soils, there are few studies that focus on the selection of bioassays for the ecotoxicological risk assessment of organic wastes to soils. In the present study, differences in feeding inhibition in the soil collembolan *Folsomia candida* were evaluated as an ecotoxicological endpoint for the assessment of risk to soils amended with polluted organic wastes. Seven organic wastes (dewatered sewage sludges, thermally dried sewage sludges, composted sewage sludges, and a thermally dried pig slurry) were tested. These wastes had different origins, treatments, and pollutant burdens, and were selected as a representative sample of the wide variety of wastes currently generated. A clear dose response was observed for this parameter, with an increase in percentage of individual feeding inhibition with increased doses of organic wastes. More significantly, feeding inhibition correlated highly with mortality and reproduction inhibition in the different wastes. Composted sludges displayed the lowest toxicity, followed by thermally-dried sludge and dewatered sludge. Thermally-dried pig slurry showed the highest toxicity for feeding, with lower EC50 values than the lowest dose tested. Among waste physicochemical parameters and pollutants, low organic matter stability appeared to be the main predictor of potential adverse effects on soil fauna, as it correlated

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significantly with feeding inhibition and mortality. Furthermore, feeding inhibition tests were run over a short exposure time (less than 7 d), which, together with the results obtained, makes this bioassay a good screening tool for organic waste toxicity.

3.1. INTRODUCTION

In recent decades, increase in the production of sewage sludge in the European Union, as a result of the implementation of Directive 91/271/EEC, has mainly been recycled to land, over other options such as landfill or incineration ([1]; http://ec.europa.eu/environment/waste/sludge/problems.htm). Despite the undeniable benefits of organic waste amendments in terms of soil fertility, concerns may arise regarding waste with a higher pollutant content, which may have deleterious effects on soil ecosystems and hence, counteract their contribution to soil fertility. Despite the importance of soil fauna for soil ecosystems [2], only a few studies have focused on the harmful effects of sludge amendments to soil invertebrates in the field. None of them have reported noxious effects of sludge at agronomical dosages ([3]; http://www2.mst.dk/Udgiv/publications/1997/87-7810-865-9/pdf/87-7810-865-9.pdf, [4,5]). On the other hand, several laboratory studies have shown significant effects on soil fauna [3,6]. Despite the extent of soil reuse and the existence of limit values for some pollutants in sludge for this practice, there is still a paucity of studies which focus on the selection of laboratory assays to determine the ecotoxicological risk of wastes for soil inhabiting organisms. Among the few attempts that have been conducted are those involving plants and earthworms [7-9], and collembolans [3,10-12]. Reproduction and growth are the most common sublethal endpoints in soil ecotoxicity testing due to their ecological relevancy. However, they require a great effort in terms of time and handling, which in recent decades has encouraged research aimed at finding alternative endpoints which provide similar information but require less experimental

effort [13-15]. Reproduction and growth are the ultimate objectives in a long line of processes that start with biochemical and physiological events [16], and endpoints at the biochemical, physiological or behavioral level might then be used as a complement to these traditional endpoints as they are early-warning indicators which generally provide results faster. This is the main reason for the attempts to relate reproduction or growth with biomarkers [17,18] or avoidance responses [19]. However, it has been indicated that behavioral responses, together with growth and reproduction, only show a weak correlation with effects at higher biological levels. Furthermore, it has also been indicated that biomarkers rarely provide useful predictions of effects at higher biological levels [20,21]. This is why endpoints at the individual level such as reproduction, growth or behavioral responses are preferred to those at lower biological levels and considered as more ecologically relevant. An alternative to conventional individual endpoints might be feeding inhibition, a parameter that has been recognized as a general stress response to toxicants and demonstrated on a variety of aquatic species [15,22,23], although not much is known about its importance in soil organisms. Reduced feeding from artificially-polluted food has been reported in *F. candida* by several authors [24-26], although this pattern has never been tested for polluted organic wastes, despite the evidence in this species of active feeding on these materials [10,27]. Feeding inhibition, either during or after exposure to contaminants, has been used as an ecotoxicological endpoint in a variety of organisms, mainly aquatic but also in terrestrial species. Feeding inhibition during exposure has been especially demonstrated in the freshwater cladoceran, *Daphnia magna*, for a variety of pollutants [14,28-31], and other aquatic invertebrates [13,32-35]. Other authors have detected feeding inhibition behavior in several crustaceans [15,23,36], and fish [37] when relocated to uncontaminated environments, after previous exposure to contaminants (so-called post-feeding inhibition).

On the other hand, only a few studies have addressed feeding inhibition in terrestrial organisms, and with contradictory results. Several authors have shown feeding inhibition with exposure to pollutants in nematodes [38,39] and collembolans [24-26]. However, no feeding inhibition was observed in isopods when leaves contaminated with the herbicide trifluralin were offered as food [40].

Despite the relative abundance of literature using this endpoint, the exact mechanisms involved in feeding inhibition as a response to pollution are still unknown. More precisely, it is not clear whether it is due to direct avoidance of pollutants in food or an indirect physiological or biochemical effect of toxicants that finally impact on feeding activities, or both.

It has been suggested that direct avoidance of contaminated food occurs in collembolans [24-26], soil nematodes [38], and D. magna [41]. The importance of preingestive inhibition may be high in collembolans as it has been demonstrated that their food preferences are linked to odor [42]. Furthermore, regurgitation of polluted food in D. magna has also been reported [30]. Feeding behavior may also be indirectly affected through physiological and biochemical effects. The persistence of feeding inhibition after exposure to contaminants is the main evidence of this mechanism. The explanation is that pollutants are able to affect the organism's biochemistry and physiology, disturbing its sensorial reception, enzymatic activities, and metabolism, with unavoidable influences on feeding behavior and other parameters. Several studies have demonstrated this mechanism in daphnids [15,28] and polychaetes [43]. Other studies have even specifically linked feeding inhibition with a decrease in acetyl cholinesterase (AChE) levels due to pollutant levels, as demonstrated in crustaceans [32], fishes [44], and birds [45].

In summary, feeding inhibition seems to be a complex response involving components which are not easy to separate. On the one hand, pollutants may directly reduce

consumption through active avoidance of polluted food, but conversely, they can disrupt feeding mechanisms through effects on biochemical and physiological processes.

The aim of this work was to evaluate the suitability of feeding inhibition in *F. candida* as an ecotoxicological endpoint to assess potential risks of soil amendments with polluted organic wastes. To achieve this goal, different wastes and exposure times were tested, and feeding inhibition values were compared with effects of survival and reproduction inhibition in the same species in terms of sensitivity, correlation of responses and workload.

3.2. METHODS

3.2.1. Test organisms

The *F. candida* culture used in the tests was raised in our laboratory, and was initiated four years ago from cultures of the Institute of Ecological Science of the Vrije Universiteit (Amsterdam, The Netherlands). Breeding of individuals was performed in polyethylene containers of 17.5 x 12.5 x 7.5 cm, with a 1 cm layer of wet substrate made of a mixture of plaster of Paris and charcoal (9:1 v/v). Cultures were kept in darkness in a climatic chamber at a constant temperature of 21±1°C. Every two months the substrate was renewed and the density of individuals was reduced to avoid overcrowding. Synchronized cultures were used in the tests, prepared as described in the International Organization for Standardization (ISO) Guideline 11267 [46].

3.2.2. Test substrate

Artificial soil prepared as documented by the ISO Guideline 11267 [46] but removing peat from the mixture. By doing this it was ensured that reported consumptions in artificial soil-waste mixtures corresponded only to waste ingestion, as in a preliminary test with conventional artificial soil it was shown that individuals consumed peat from the

substrate, a fact that would disturb the assessment of feeding behavior. The test substrate was composed of 78% quartz sand and 22% kaolin. A suitable amount of calcium carbonate was added to provide a pH of approximately 6±0.5. In all tested doses of the organic wastes, the water content of the mixture was adjusted to 50% of the water-holding capacity of the artificial soil.

3.2.3. Organic wastes

One dried pig slurry and six sewage sludges, with different origins, and subjected to different treatments and post-treatments, were selected in order to provide a wide range of waste properties to evaluate the sensitivity of the bioassay described in this work (Table 3.1). All wastes were dried at 60°C for 48 to 72 h, depending on the initial humidity, and then ground and sieved (<2mm). This step was unavoidable in order to ensure the accuracy of the low doses tested in most of the wastes. The resulting samples were used both for the preparation of soil-waste mixtures and for waste characterization. Details of physicochemical properties and concentrations of metals and organic pollutants in the wastes together with methods for their characterization are described in Domene et al. [12] (Chapter 2, Table 2.2).

Table 3.1. Origin, treatments and post-treatments of the organic wastes.

Waste	Origin	Treatment	Post-treatment
AED	Banyoles WWTP	Aerobic digestion, dewatering	None
AEC	Banyoles WWTP	Aerobic digestion, dewatering	Composting in vessel
AET	Banyoles WWTP	Aerobic digestion, dewatering	Thermal drying
AND	Blanes WWTP	Anaerobic digestion, dewatering	None
ANC	Blanes WWTP	Anaerobic digestion, dewatering	Composting in heap
ANT	Blanes WWTP	Anaerobic digestion, dewatering	Thermal drying
SLT	Juneda WTP	Anaerobic digestion, dewatering	Thermal drying

Sludge composts used in the present study were produced from a 1:4.5 (v/v) mixture of dewatered sludge and pinewood chips. For the anaerobic sludge, composting was carried out with pine splinters in a tunnel with air injection for 15 d, while aerobic sludge was composted in a heap for 50 d using chips from recycled wood and furniture as a bulking agent. At the end of the process, both composts were sieved to 1 cm to remove approximately 90% of the chips. This post-treatment decreased the total, hydrolysable and ammonium nitrogen content of the initial sludge, while increasing organic matter stability.

Thermal drying was carried out by placing dewatered sludge in a heated rotary cylinder and injecting hot air, which subjected the sludge to a temperature of approximately 130 to 150ºC for 45 min. With this post-treatment, pollutant levels did not change significantly, but N-NH4 levels decreased and electrical conductivity increased.

Pig slurry was obtained from a treatment plant where the raw slurry is subjected to anaerobic digestion followed by thermal drying at 130ºC, producing a final product with a dusty appearance. The final product showed high electrical conductivity, high levels of hydrolysable nitrogen and ammonium, and low organic matter stability.

3.2.4. Experimental setup

A 4-d preliminary assay was performed in order to define the range of doses for each organic waste to be tested in the definitive assay. The doses used in this preliminary assay were 1, 10, 50, 100, 300, 500, 700, 1000 g Kg^{-1}. Based on these results, a definitive concentration range was defined for each waste, taking as definitive the range of concentrations with 20% to 80% of individuals showing feeding inhibition. In the definitive test, five doses in an arithmetic progression were defined for each waste, but always within the 1 to 500 g Kg^{-1} range as maximum. Each dose tested was

composed of 5 replicates and an additional replicate for the determination of water content, pH and electric conductivity. The test was carried out using 50 ml polyethylene containers filled with only 5 g of wet substrate mixture in order to maximize retrieving of the individuals and their observation. 15 individuals (10 – 12 d old) were placed in each test container. Three exposure times (2, 4, and 7 d) were defined for each waste and concentration. The number of individuals was determined in a preliminary experiment that showed that feeding patterns were similar regardless of the number of individuals tested (10, 15, and 20). The number of individuals selected (15) is considered high enough to minimize the impact of possible individual mortality or inability to refloat some of the animals, but low enough to allow direct and simultaneous counting of all the individuals in the test container.

Figure 3.1. Group of *Folsomia candida* removed from a test chamber by flotation. Four individuals have the ingested waste clearly visible in their gut.

3.2.5. Observation of feeding behavior

It has been suggested that *F. candida* ingests sludge from test substrate when this is available [10,27]. Since gut content in *F. candida* is easily observed in vivo, given the lack of pigment in its cuticle [26], individuals feeding on the organic wastes could be easily assessed at the end of the test by the presence of dark content in the gut. Direct

observation of the individuals was possible after flooding the test container, allowing the animals to float on the surface of the water (Fig. 3.1).

3.2.6. Data treatment

The 50% effective concentration (EC50) values and their associated 95% confidence intervals were estimated by probit analysis using Minitab 13.2 software (Minitab, State College, PA, USA). A logistic distribution was assumed to carry out probit analysis as it displayed the best adjustment to the data.

After calculations, EC50 values for feeding in each waste after 2, 4, and 7 d of exposure were compared by Pearson correlation with the 50% lethal concentration values (LC50) and EC50 reproduction values for *F. candida*, obtained from the same wastes in a previous study [12]. These values were obtained in accordance with ISO Guideline 11267 [46] with some modifications to adapt it to waste testing, and were derived using linear and non-linear regression models according to Stephenson et al. [47]. EC50 feeding values were also compared by Pearson correlation with all the physicochemical parameters and pollutants assessed for each waste in order to detect any significant relationship. All correlations in the present study were carried out with log-transformed values.

3.3. RESULTS

3.3.1. Feeding behavior

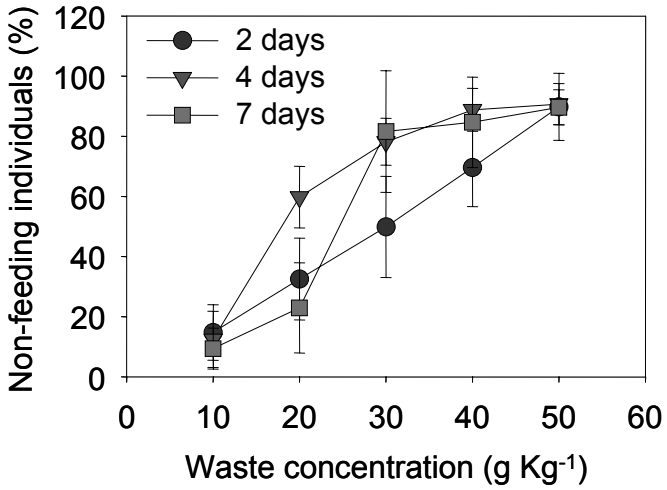
Retrieving of individuals with the substrate used averaged 75%. Losses should be attributed mainly to an inability to flotate all the individuals in this type of substrate rather than to mortality, since losses of individuals in replicates were similar with increasing waste doses and also because these were not expected to affect survival according to the LC50 values found by Domene et al. [12] (Table 3.2).

Table 3.2. Feeding inhibition values (EC50, 2, 4, and 7 d), plus survival (LC50) and reproduction (EC50) (28 d) inhibition, after exposure to contaminated waste. Values expressed as g Kg^{-1} (dry wt). * = from Domene et al. [15]. See Table 3.1 for waste abbreviations; EC50 = concentration of waste estimated to reduce the outcome in a sublethal endpoint rate by 50% compared to the control; LC50 = concentration of waste estimated to prove lethal at the end of the test by 50% compared to the control.

Waste	***Feeding EC50, 2 d***	***Feeding EC50, 4 d***	***Feeding EC50, 7 d***	***LC50, 28 d****	***Reproduction EC50, 28 d****
AEC	445.7 (435.3, 456.2)	455.9 (445.7, 466.0)	423.6 (411.1, 436.0)	252.3 (221.7, 287.2)	206.9 (36.8, 1141.8)
AED	3.9 (3.7, 4.2)	3.6 (3.4, 3.9)	4.4 (4.2, 4.6)	43.9 (34.1, 56.6)	10.0 (3.8, 23.8)
AET	1.7 (1.1, 2.2)	2.9 (2.7, 3.2)	3.9 (3.7, 4.1)	44.0 (37.4, 51.7)	5.3 (2.8, 9.4)
ANC	299.1 (290.3, 308.2)	281.5 (264.2, 304.0)	164.6 (155.2, 173.6)	833.8 (626.4, 1109.7)	28.7 (17.7, 46.0)
AND	18.1 (16.9, 19.3)	26.1 (25.2, 27.1)	20.5 (18.8, 22.0)	154.4 (133.7, 178.3)	16.4 (14.7, 18.2)
ANT	27.3 (26.2, 28.5)	20.0 (19.2, 20.8)	24.7 (24.0, 25.5)	85.6 (72.3, 101.3)	10.4 (7.5, 14.2)
SLT	-	-	-	23.7 (20.2, 27.8)	19.4 (3.8, 86.4)

The main reason may be a lack of peat in the substrate, which gave it a less crumbly structure. This made flotation of individuals more difficult in comparison with conventional artificial soil. For the reasons already indicated, we assumed that this phenomenon did not affect the test results.

Figure 3.2. Feeding inhibition values in *Folsomia candida* exposed to increasing concentrations of the anaerobic thermally-dried sewage sludge (ANT) after 2, 4, and 7 d of exposure. Bars indicate standard deviation. n = 5.



Feeding inhibition increased with increasing doses of the different wastes, presenting a linear or linear-like form depending on waste and exposure time tested (data not shown, see an example in Fig. 2). Mean feeding inhibition rates in the lower test doses varied between 12 and 34% depending on the waste, while at the highest tested doses mean rates were between 68 and 95%. The exception was pig slurry (SLT), which showed an inhibition of approximately 72% at the lowest tested dose (1 g Kg^{-1}). This inhibition continued to increase towards the highest dose (data not shown). An additional experiment (results not presented) showed that the feeding inhibition of this waste remained very high even at concentrations as low as 0.1 g Kg^{-1}. As no feeding EC50

could be calculated for this waste, we excluded this waste for comparisons with reproduction and survival inhibition values.

The EC50 feeding values of the different wastes generally did not overlap in their 95% confidence intervals, suggesting a satisfactory precision of the method (Table 3.2), and were below 5 g Kg^{-1} in the aerobic dewatered (AED) and thermally-dried (AET) sludges, while the anaerobic dewatered (AND) and thermally-dried (ANT) sludges showed values below 30 g Kg^{-1}. On the other hand, composted sludges showed the highest EC50 values, approximately 165 to 300 g Kg^{-1} for ANC, and 423 to 456 g Kg^{-1} for AEC.

Feeding rates for each waste and concentration showed no significant differences between times tested (analysis of variance [ANOVA], $p > 0.05$), indicating that the response of this endpoint remains nearly constant within one week of exposure.

3.3.2. Comparison of endpoints

Feeding inhibition in *F. candida* correlated highly with effects on survival and reproduction (Table 3.3). This correlation was maintained throughout the different exposure times, both with survival and reproduction. Maximum correlation was found with feeding inhibition after 7 d of exposure and reproduction. This relationship is interesting given the lack of correlation between survival and reproduction in the present study ($r = 0.636$, $p = 0.174$). Furthermore, it is also worth noting that feeding behaviour was usually inhibited at lower waste concentrations than those affecting mortality, with the exception of AEC. Conversely, feeding was usually inhibited at concentrations above those affecting reproduction (except AED and AET). It is also noteworthy that, despite being less sensitive than reproduction, feeding inhibition was more reliable, as it displayed narrower confidence intervals.

Table 3.3. Pearson's correlations of values of feeding inhibition (EC50) Folsomia candida after 2, 4, and 7 d of exposure with survival (LC50), reproduction EC50, and some physicochemical parameters. Correlation analyses were carried out with log-transformed values. n = 6.

	Feeding EC50, 2 d	**Feeding EC50, 4 d**	**Feeding EC50, 7 d**
LC50, 28 d	r=0.890, p=0.017	r=0.908, p=0.012	r=0.858, p=0.029
Reproduction EC50, 28 d	r = 0.865, p=0.026	r = 0.880, p = 0.021	r = 0.905, p=0.013
Organic matter stability	r=0.897, p=0.015	r=0.913, p=0.011	r=0.884, p=0.019
NH4-N	r=- 0.773, p=0.071	r=- 0. 801, p=0.056	r=-0.817, p=0.047
Total nitrogen	r=-0.802, p=0.055	r=-0.829, p=0.041	r=-0.764, p=0.077

3.3.3. Influence of waste properties on feeding behavior

The EC50 feeding values correlated positively and significantly with organic matter stability of wastes throughout the experimental period. Furthermore, significant negative correlation was found with ammonium at 7 d, and marginally significant at 4 d. Finally, a significant negative relationship was found with total nitrogen after 4 d of exposure, although the relationship also appeared marginally significant at 2 d (Table 3.3). These correlations agree with a previous study, where LC50 values in *F. candida* were correlated with the same properties [12].

3.4. DISCUSSION

3.4.1. Relevance and sensitivity of feeding inhibition

Feeding inhibition is a suitable endpoint for ecotoxicity testing because it is a general stress response to exposure to toxicants in a variety of species [15,22,23]. Whether feeding inhibition is an avoidance response to polluted food or a result of physiological or biochemical processes finally impacting on feeding behavior, the reduction of food intake has an impact at least at the individual level. Acquisition and allocation of energy

determines developmental rate, growth rate, fecundity, and survival. Hence, any disturbance in the energy allocation to any of these processes, is expected to be also translated into effects at the population level [14,15,34,48,49]. For example, Maltby [50] showed that feeding inhibition in the amphipod, *Gammarus pulex*, correlated both with lethality and reproduction. This pattern has been confirmed in the freshwater rotifer, *Brachionus calcyflorus* [13]. Barata and Baird [14] also concluded for *Daphnia magna* that chemicals affecting endpoints like feeding rate or viability of eggs were predictive of effects in traits like reproduction and survival, given the influence of the former endpoints on the latter. However, Lopes et al. [51] found no correlation between feeding inhibition and survival in *Daphnia longispina* when exposed to copper. Results from our study suggest a link between feeding inhibition in the first week of exposure and reproduction and survival after a month.

Furthermore, some studies have suggested that feeding inhibition might be an ecologically relevant parameter, since impacts of pollutants on this endpoint have correlated with changes at the community and ecosystem levels. Maltby et al. [33] found that in situ feeding rates of the aquatic crustacean, *Gammarus pulex*, correlated significantly with stream macroinvertebrate diversity and detritus processing. On the other hand, McWilliam and Baird [15] found no correlation between postexposure feeding depression of *Daphnia magna* and macroinvertebrate community structure. No studies are available for terrestrial ecosystems, but given the key role of soil fauna in facilitating microbial decomposition and nutrient turnover [52], effects at the individual level may provoke effects at higher biological levels [34].

In the present study, feeding inhibition of *F. candida* in the set of wastes studied correlated highly with mortality and reproduction inhibition, indicating the relevance of this endpoint for estimating effects on other more commonly used endpoints which require a longer experimental period. Furthermore, EC50 values for feeding also remained significantly constant during the first week of exposure. This may permit a reduction of

the exposure time to only two days while still providing relevant information on waste toxicity. In addition, feeding inhibition values displayed narrower confidence intervals than survival and reproduction inhibition values, showing the high reliability of this endpoint. An explanation might be the lower chance of mortality (due to the feeding test running for a shorter period), and/or the best adjustment of the model used for feeding data with respect to those used for survival and reproduction.

When inhibition values were compared, it was apparent that feeding behavior was generally inhibited at lower waste concentrations than mortality, while feeding was usually inhibited at concentrations above those affecting reproduction. This trend is usual for lethality in studies with aquatic organisms exposed to pollutants [13,14,23,29]. However, there is no agreement on what is typical with respect to reproduction. Some published studies have shown that reproduction is generally affected at lower concentrations than feeding [13,5], while others have reported inhibition at similar concentrations [14,39] or the opposite relationship [53].

3.4.2. Suitability of *F. candida* for feeding inhibition bioassays

Among the most commonly used soil test species, *F. candida* is especially recommended for the assessment of feeding inhibition responses, as it is possible to observe the consumption of some food sources, in this case organic wastes, as a dark gut content due to the lack of pigment in its cuticle [26]. Like other collembolans, *F. candida* molts throughout its entire life cycle. During molting the whole cuticle and gut epithelium is completely regenerated, and at this stage individuals stop feeding. This may interfere with the observation of feeding activities [54]. Despite this fact, we assumed that this phenomenon is negligible for the purposes of this study as a synchronized culture was used for the experiments, which should reduce any variability in results caused by these molting events. In addition, the rate of non-feeding individuals in the lower waste concentrations is similar, independent of the waste type and exposure time

(approximately 25%, data not reported), suggesting a low bias on the results of moulting stages. These rates are in accordance with the 20% reported by Thimm et al. [54] in mixed-age cultures, which were assigned by the authors to moulting individuals. Moreover, the feeding rates observed offer an accurate assessment of the instantaneous situation of individuals, as the period between ingestion and excretion of food boluses in this species lasts only 35 minutes in similar experimental conditions to those used in our study [54].

The consumption of sewage sludge has already been suggested for *F. candida* [12] and *F. fimetaria* [27]. However, this consumption seems to be important only when an alternative clean food source is not available. In a previous unpublished work we observed that individuals of *F. candida* showed lower consumption rates of sewage sludge when yeast was available, in accordance with its near relative *F. fimetaria* [3]. This behavior agrees with findings from other authors who reported avoidance of contaminated food in this species [24-26]. Filser and Hölscher [24] also showed that the appeal of potato bait decreased with copper concentration. Pedersen et al. [25] found that both *F. candida* and *F. fimetaria* consumed less contaminated yeast with increasing concentrations of copper. Fountain and Hopkin [26] reported a lower feeding rate for *F. candida* with increasing concentrations of heavy metals in yeast offered as food. These authors also demonstrated an active avoidance of polluted food, as a higher percentage of individuals fed on culture substrate with increasing concentrations of metals in yeast. The authors attributed this behavior to an attempt to use the substrate as an alternative food source, demonstrating the existence of mechanisms of contaminant recognition in this species.

The use of feeding inhibition as an endpoint has the main advantage of providing results more quickly than other endpoints and generally requires simpler experimental setups [13-15]. However, no endpoint could be generally considered to be better than others, as each chemical has its main mode of action and each species may present a different

sensitivity to a given parameter. Despite this, feeding inhibition seems to be a general response to a variety of pollutants with different modes of action and toxicities, making this parameter a sensitive and robust response for use as an ecotoxicological endpoint [15].

3.4.3. Parameters involved in feeding inhibition behavior

Results from this study indicate the suitability of feeding inhibition in *F. candida* as a sensitive endpoint for organic waste ecotoxicity testing, as it was clearly affected at different doses depending on the waste. We have no evidence about the main mechanism involved in the feeding inhibition observed in the present study. However, by not adding an alternative food source to test containers we forced individuals to interact with the waste offered. By doing this we obtained an integrative response, regardless of whether direct avoidance of polluted food and/or biochemical and physiological disruption of feeding behavior were the main cause.

Composted sewage sludges inhibited feeding at much higher doses when compared to the effects obtained with dewatered and thermally-dried sludges. On the other hand, it was not possible to assess the EC50 feeding values of the thermally-dried pig slurry as they were below 1 g Kg^{-1}, given the higher toxicity of this sludge with respect to the other wastes [12].

The main factor influencing feeding patterns in this species was the organic matter stability of wastes, a parameter which reflects ease of decomposition. This relationship has already been pointed out in a previous study [12], where a strong correlation was found between this waste parameter and mortality in this species, although no significant relationship was found with reproduction. During decomposition, the breakdown of the more labile fraction of organic matter decreases the relative quantities of total nitrogen, mainly through ammonia losses [55]. Soil amendments with organic wastes with a low degree of stability may cause problems for soil biota, as during their decomposition

there is a release of secondary metabolites such as ammonium, phenols, and organic acids, among other adverse effects [56]. Correlation between degree of waste stability and toxicity has been widely reported for plants [57], and also for soil fauna [2]. Furthermore, a correlation with the degree of stability may also reflect the already mentioned degradation of the less persistent organic pollutants in wastes through composting [58,59]. Whatever the reason, according to results from this study, organic matter stability appears as the best predictor for anticipating potential adverse effects for soil fauna derived from soil amendments, at least in the short term.

CONCLUSION

Feeding inhibition of *F. candida* is a suitable endpoint to assess the ecotoxicological risk of organic waste amendments in soils. It showed different responses to different wastes, and presented a clear dose-response relationship with increasing waste doses. More significantly, feeding inhibition was correlated highly with mortality and reproduction inhibition in the different wastes. Besides providing equivalent information, results involving feeding inhibition could be obtained over a shorter exposure time (less than 7 d) in comparison with other more conventional endpoints like reproduction. Results from this study indicate the value of this bioassay as a screening tool for organic waste toxicity.

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Chapter 4

Comparison of solid-phase and eluate assays to gauge the ecotoxicological risk of organic wastes on soil organisms

ABSTRACT

Development of methodologies to assess the safety of reusing polluted organic wastes in soil is a priority in Europe. In this study, and coupled with chemical analysis, seven organic wastes were subjected to different bioassays (collembola reproduction test, daphnid immobilization test, and Microtox luminescence test). Tests were carried out with solid-phase waste and three different waste eluates (water, methanol, and dichloromethane).

Bioassays were sensitive to the different wastes, but toxicity patterns were not coherent among them. Solid-phase assays were indicated as the most suitable for waste testing in terms of relevance for real situations, but also because toxicity in eluates was generally not representative of the chronic effects in solid-phase. On the contrary, the use of aqueous eluates in some bioassays was representative for the acute solid-phase toxicity in soils.

No general correlations were found between toxicity and waste pollutant burden, neither in solid-phase nor in eluate assays, showing the inability of chemical methods to predict the ecotoxicological risks of wastes. On the contrary, several physico-chemical parameters reflecting the degree of low organic matter stability in wastes were the main contributor to the acute toxicity seen in collembolans and daphnids.

4.1. INTRODUCTION

There is increasing interest in the development of bioassays to evaluate the suitability of polluted organic wastes for safe application to soils excluding any ecotoxicological risk. The complex nature of such wastes, especially in the case of sewage sludge, containing a huge number of potentially noxious chemicals (Thornton et al. 2001), and the limitations of chemical methods to assess their risk to soils (Crouau et al. 2002) favours using a bioassay approach.

A wide variety of literature dealing with potential effects on crops of polluted organic wastes is available, mainly centred on sewage sludge, while less is known about effects on soil fauna, a key group in soil agroecosystems (Giller et al. 1997, Neher 1999). No harmful effects on soil fauna have been found in field studies using agronomic dosages (Krogh et al. 1997, Kielhorn et al. 1999, Cole et al. 2001, Petersen et al. 2003), although some laboratory studies have indicated risk for soil fauna if such wastes are applied to soils (Krogh et al. 1997, Krogh and Pedersen 1997, Andrés and Domene 2005). This scarcity of studies shows how incomplete the knowledge on this subject is and is a sign of the current need for the selection of bioassays to assess the ecotoxicological risk of wastes to soils.

Among the laboratory studies centered on organic waste ecotoxicity, most have been carried out using waste eluates or leachates and aquatic ecotoxicity tests. However, others have focused on the suitability of solid-phase bioassays for organic wastes using terrestrial organisms. The latter approach is the most relevant as it provides results closer to those expected in field conditions. However, solid-phase assays have several drawbacks associated to the organic matter matrix. Organic matter may mask toxicity through its nutritive effect on soil organisms, but also may modify physico-chemical properties and the water holding capacity of the soil-waste mixtures at increasing

concentrations, therefore affecting to a certain extent the performance of the test organism.

Assays on the aqueous eluate of waste is the most commonly employed method for waste ecotoxicity assessment (Vaajasaari 2005). For organic wastes, this approach has been taken using microorganisms, plants, and daphnids as test organisms. However, to date, correlation of results with those obtained from solid-phase tests has not been reported to our knowledge, indeed the extrapolation of results from aqueous eluates and aquatic bioassays to soil organisms has been criticized for its low ecological relevance (Alexander et al. 2003, McMillen et al. 2003). Despite these criticisms, the use of eluates may compensate for some of the main limitations of the solid-phase tests, as long as results of these eluate assays correlate with those of the solid-phase assays. Furthermore, combined testing of different eluate solvents (water and organic solvents) may give information about the pollutant fraction mainly contributing to the harmful effects of wastes.

The aims of this work are (a) to compare sensitivities of terrestrial and aquatic assays for the ecotoxicity assessment of organic wastes; (b) to examine the representativeness of different waste eluates for the estimation of solid-phase toxicity; and (c) to detect the main contributors to the organic waste toxicity by correlating the biological response with waste composition both in solid-phase and eluate assays. For this purpose, organic waste in solid-phase and its corresponding three eluates (water, methanol and dichloromethane), obtained with a method incurring low experimental and economic costs, were compared by chemical methods and bioassays (survival and reproduction of the soil collembolan *Folsomia candida*, luminescence of the marine bacteria *Vibrio fischeri*, and mobility of the freshwater copepod *Daphnia magna*).

4.2. METHODS

4.2.1. Sample origin and preparation

Six different sewage sludges and one thermally dried pig slurry were selected. Given their contrasting origins and treatments, these samples are representative of a broad range of organic wastes generated in Europe (Table 4.1).

Each waste was dried at 60ºC for 48-72 hours depending on its initial moisture, and then ground and sieved (<2 mm), in order to ensure the homogeneity and accuracy of the lowest test concentrations and for the preparation of eluates.

Table 4.1. Origin, treatments and post-treatments of the organic wastes (AE = aerobically digested sewage, AN = anaerobically digested sewage, SL = pig slurry, D = dewatered, C = composted, T = thermally dried).

Waste	Origin	Treatment	Post-treatment
AED	Banyoles WWTP	Aerobic digestion, dewatering	None
AEC	Banyoles WWTP	Aerobic digestion, dewatering	Composting in vessel
AET	Banyoles WWTP	Aerobic digestion, dewatering	Thermal drying
AND	Blanes WWTP	Anaerobic digestion, dewatering	None
ANC	Blanes WWTP	Anaerobic digestion, dewatering	Composting in heap
ANT	Blanes WWTP	Anaerobic digestion, dewatering	Thermal drying
SLT	Juneda WTP	Anaerobic digestion, dewatering	Thermal drying

4.2.2. Eluate preparation

Three different solvents (water, methanol and dichloromethane) were used to isolate three pollutant fractions, differing in their solubility. Eluates were prepared with deionised water, methanol (99.5% purity), and dichloromethane (99.8% purity) (SDS Chemicals, Peypin, France). Aqueous elution allows dissolution of hydrophylic (polar)

substances and methanol elutes amphyphylic substances (semipolar) while dichlorometane solubilizes hydrophobic (non-polar) substances.

Eluates were prepared by sonication of the dried and ground wastes in contact with the extraction solvent. More precisely, 200 g of dried and ground waste were added to 500 ml of solvent in an Erlenmeyer flask and placed in an ultrasound extraction system for 90 minutes at room temperature. Methanol and dichloromethane eluates were then vacuum-filtered with cellulose filter (0.45-µm poresize), and concentrated in a rotavapour. Aqueous eluates were centrifuged, decanted, vacuum-filtered and concentrated by lyophilization. Final concentrations of each eluate were adjusted to 2500 g L^{-1} by addition of pure solvent. Solid matter in eluates was low (5.6, 4.2, and 3% mean values in water, methanol, and dichloromethane eluates, respectively).

For the Microtox acute toxicity assay, based on seawater bacteria, the eluates have to be applied to an aqueous environment. For this, 2 ml of each eluate were dissolved in Microtox saline solution (2 % NaCl) as indicated in ISO 11348 (1998), to a final volume of 50 ml. For methanol and dichloromethane, previous to this dissolution, 2 ml aliquots were subjected to a continuous flow of nitrogen until complete evaporation of solvent. Then, 50 ml of saline solution was added to the solid remnant and the mixture was sonicated for 1 hour. Finally, the resultant solution was vacuum-filtered in a 0.45 µm pore size filter. Hence, we obtained a final eluate concentration of 100 g L^{-1} for the aqueous, methanol, and dichloromethane eluate assays. For this assay, an additional eluate was prepared, herein called solid-phase eluate, consisting of an aqueous eluate from wastes. 5 g of dry and ground waste were mixed with 50 ml of saline solution. Then, the mixture was sonicated for 1 hour, and finally vacuum-filtered in a 0.45 µm filter. Hence, a final eluate concentration of 100 g L^{-1} was also obtained and used for preparation of dilutions.

For *Daphnia magna* acute toxicity assays, eluates were obtained as indicated in EN 12457-2 (CEN 2002), a procedure used to assess the risk of pollutants leaching from

granular wastes or sludge. Extraction was performed by adding 900 ml of deionised water to 90 g of dry and ground waste, and shaking for 24 hours at room temperature. After 15 minutes of sedimentation, liquid was decanted and the eluate was centrifuged for 30 minutes at 3500 g, decanted again, and vacuum-filtered in a 0.45 µm pore size filter. Only solid-phase aqueous eluates were prepared for this assay, since according to this method, extraction has to be carried out with deionised water, and given the practical limitations of the high volume of waste and extraction solvent that would be required for the preparation of methanol and dichloromethane eluates.

4.2.3. Characterization of wastes and eluates

The most relevant physico-chemical properties of the original wastes are recorded in Table 4.2, and were determined on the same sample allotment used for bioassays. Dry matter and ammonium were determined from fresh samples, while the remaining parameters were measured from the dry and ground samples. Dry matter, water holding capacity, pH, electric conductivity, total nitrogen, and organic matter were measured according to EN 12880, ISO 11267, EN 13037, EN 13038, EN 13342 and EN 12879 (CEN 1999a, 1999b, 2000a, 2000b, 2000c). Non-hydrolysable (stable) organic matter and non-hydrolysable nitrogen were measured as the percentage of organic matter and nitrogen remaining in the sample residue after acid hydrolysis, as described in Rovira and Vallejo (2002). Hydrolysable nitrogen was the difference between total nitrogen and non-hydrolysable nitrogen. Ammonium was measured by distillation of the fresh sample.

Table 4.2. Physico-chemical properties of the wastes studied (from Domene et al. 2007).

Parameter	Units	AEC	AED	AET	ANC	AND	ANT	SLT
Dry matter	g Kg^{-1} (w/w.w.)	449	150	945	470	199	844	865
WHC	% (w/w.w.)	74.4	63.9	74.7	64.9	64.8	67.9	55.9
pH	water, 1:5 (v/v)	7.8	8.1	6.9	7.2	8.4	7.2	6.4
Electrical conductivity	dS/m, 25ºC	1.2	1.5	3.57	4.2	2.25	6.22	64.65
Organic matter	g Kg^{-1} (d.w.)	622	684	687	551	566	668	612
Stable organic matter	%	50.1	37.8	40.4	54.2	47.7	46.7	36.6
N	g Kg^{-1} (d.w.)	39.5	62.4	60.6	23.7	38.8	53.3	62.5
Non-hydrolysable N	g Kg^{-1} (d.w.)	17.0	16.4	19.1	16.1	12.4	18.4	10.9
Hydrolysable N	g Kg^{-1} (d.w.)	22.5	46.0	41.5	7.6	26.4	34.9	51.6
NH_4-N	g Kg^{-1} (d.w.)	2.7	14.0	8.0	3.4	15.1	11.6	52.9
P	g Kg^{-1} (d.w.)	22.0	20.4	20.5	28.6	33.6	29.2	20.4
K	g Kg^{-1} (d.w.)	3.6	1.9	2.2	4.4	2.3	2.5	55.1

Pollutant burden of wastes and eluates used in *F. candida* and Microtox assays are recorded in Table 4.3. No chemical data were available from the aqueous eluates specifically prepared for the *D. magna* assay and those for the Microtox assays. However, we assumed that relative differences in chemical burden between wastes were equivalent to that of the aqueous eluates prepared used in assays of *F. candida*, for which direct measures were available. Heavy metals in wastes were determined by Applus Agroambiental Inc., while heavy metals in eluates were determined by the Chemical Analysis Service of the Autonomous University of Barcelona. Organic pollutants were determined by the Sarrià Chemical Institute of Barcelona. Cd, Cr, Cu, Hg, Ni, and Pb were determined by ICP-MS according to ISO 11885 (ISO 1996a). Polychlorinated dibenzodioxins/dibenzofuranes (PCDD/F) were measured with HRGC-HRMS, polychlorinated biphenyls (PCB) with HRGC-ECD, di(2-ethylhexyl)phthalate (DEHP) and

NPE with HRGC-MS. Polycyclic aromatic hydrocarbons (PAH) and linear alkylbenzene sulphonates (LAS) were measured with HPLC with fluorescence and UV detectors respectively. NPE include nonylphenol and nonylphenol ethoxylates with 1 or 2 ethoxy groups. PAH were the sum of acenapthene, phenanthrene, fluorene, flouranthene, pyrene, benzo(b+j+k)fluoranthene, benzo(a)pyrene, benzo(ghi)perylene, and indeno(1, 2, 3-c, d)pyrene. PCB was the sum of the polychlorinated biphenyl congener number 28, 52, 101, 118, 138, 153 and 180.

Table 4.3. Solid-phase and eluates pollutant burden. Values for the solid-phase are expressed in mg Kg^{-1} except for of PCB (in ng g^{-1}), and for PCDD/F (in ng TE Kg^{-1}). For eluates, concentrations are expressed in the same units, but are in reference to the total mass of dry sludge used for the extraction. *nd* = non-detectable levels.

Assay	Pollutant	AEC	AED	AET	ANC	AND	ANT	SLT
Solid-phase	Cd	1	1.3	1.3	3.5	3.2	3.1	<0.7
	Cr	345	55	30	53	54	127	15
	Cu	294	624	645	798	933	833	780
	Hg	0.67	1.33	0.95	2.13	2.51	2.25	0.12
	Ni	59	80	53	76	64	45	29
	Pb	1196	3940	3747	92	78	85	<20
	Zn	843	956	952	1028	988	890	2060
	DEHP	10	61	27	22	143	71	1
	LAS	298	816	331	214	3240	5572	60
	NPE	86	153	76	158	513	573	54
	PAH	0.1	0.4	0.3	1.6	1.1	1.4	0.05
	PCB	15	34	29	41	23	29	<7
	PCDD/F	16	15.6	13.7	12.4	7.7	13.2	0.3
Water eluate	Cd	0.33	1.17	1.36	2.13	nd	0.72	nd
	Cr	0.63	10.07	0.78	3.96	nd	1.75	nd
	Cu	0.13	3.46	0.63	1.49	nd	1.56	nd
	Hg	0.01	0.18	0.77	0.47	nd	0.01	nd
	Ni	0.05	0.73	10.33	2.57	nd	0.1	nd
	Pb	0.24	2.57	24.3	3.46	0.02	0.04	nd
	Zn	0.11	1.43	28.7	22.6	0.02	0.2	nd
	DEHP	nd	nd	nd	nd	nd	nd	nd
	LAS	nd	nd	nd	nd	nd	nd	nd
	NPE	nd	nd	nd	nd	nd	nd	nd
	PAH	nd	nd	nd	nd	nd	nd	nd
	PCB	nd	nd	nd	nd	nd	nd	nd
	PCDD/F	nd	nd	nd	nd	nd	nd	nd

Methanol eluate	Cd	nd	nd	nd	nd	nd	nd	nd
	Cr	0.07	1.41	0.09	0.09	0.09	0.19	0.06
	Cu	0.23	3.27	1.64	0.25	0.17	2.4	16.6
	Hg	nd	nd	nd	nd	nd	nd	nd
	Ni	0.13	4.5	0.55	nd	nd	0.08	0.65
	Pb	nd	2.4	0.23	nd	nd	nd	nd
	Zn	0.24	8.55	0.33	0.38	0.06	0.38	16.28
	DEHP	8.76	27.2	39.6	24.1	115.9	54.2	0.49
	LAS	0.19	0.15	0.03	0.09	4.25	4.66	0
	NPE	36	62	30	53	237	296	77
	PAH	0.05	0.09	0.12	0.86	0.27	0.43	0.01
	PCB	nd	nd	nd	nd	nd	nd	nd
	PCDD/F	nd	nd	nd	nd	nd	nd	nd
Dichloromethane eluate	Cd	nd	nd	nd	nd	nd	nd	nd
	Cr	0.54	1.36	0.1	0.05	0.65	0.23	nd
	Cu	0.25	0.99	0.29	0.26	1.2	0.91	0.81
	Hg	nd	nd	nd	nd	nd	nd	nd
	Ni	0.25	0.24	0.05	nd	0.06	0.04	nd
	Pb	0.38	9.39	1.13	nd	0.18	0.1	nd
	Zn	0.55	1.58	0.27	0.05	1.17	0.73	0.26
	DEHP	10.4	55.0	27.8	175.3	183.5	368.8	6.13
	LAS	nd	0.03	nd	nd	0.09	0.06	nd
	NPE	36	62	32	59	154	215	25
	PAH	0.09	0.04	0.09	0.44	0.25	0.32	0.01
	PCB	nd	nd	nd	nd	nd	nd	nd
	PCDD/F	nd	nd	nd	nd	nd	nd	nd

4.2.4. *Folsomia candida* reproduction assay

F. candida is a soil collembolan commonly used in soil ecotoxicity tests. The assay was performed in artificial soil according to ISO 11267 (1999). Effects on survival and reproduction at increasing concentrations of waste were measured after 28 days of exposure, the time needed to obtain a first generation from the parent individuals. For the solid-phase assays, toxicity results of *F. candida* were obtained from Domene et al. (2007). In solid-phase assays, twelve test concentrations were prepared by mixing dry waste with artificial soil at 0, 1, 2, 4, 7.9, 15.8, 31.6, 63.1, 125.9, 251.2, 501.2 and 1000 g Kg^{-1}. In the eluate assays, the suitable volume of extract was added to the soil to obtain the same test concentrations used in the solid-phase assays, calculated according to the total waste weight initially used to prepare the extracts. In order to ensure the similitude of treatments in the different concentrations, this suitable volume of extract was raised to a constant volume by the addition of pure solvent. The same

volume of pure solvent was also added to controls. Then, methanol and dichloromethane soil-eluate mixtures were left to evaporate completely for 48 hours in a fume hood at room temperature. For soil-aqueous eluate mixtures, drying was carried out in a stove at 60ºC for 48 hours. Once dry, soil-eluate mixtures were homogenized and deionised water was added to obtain a water content around 55% (w/dw).

Five replicates per concentration were prepared, consisting of 125 ml polyethylene sealed containers with 30 g of wet mixture. In each replicate, 10 individuals 10 to 12 days old were added together with 3 mg of granulated dry yeast. Containers were kept in the dark at 21±1ºC for 28 days, and were aerated twice a week. Yeast was also added the 14th day of the test.

At the end of this period, containers were flooded with water to float the adults and juveniles on the surface. A dark dye was used to facilitate the counting of the individuals, and a picture was taken to assess effects on several endpoints using the image treatment software ImageTool 3.0. From pictures, the number of adults and juveniles per replicate was determined, which were distinguishable by their size, and also the mean body length of adults per replicate. Length was measured from the anterior end of the head between the antennae to the posterior end of the abdomen, as described by Folker-Hansen et al. (1996) and Fountain and Hopkin (2001). From these data, relative survival (100*number of adults in the replicate/mean number of adults in controls) and relative reproduction (100*number of juveniles in the replicate/mean number of juveniles in controls) were calculated.

4.2.5. Microtox acute toxicity assay

This assay was selected due to its common use in aquatic ecotoxicity and to infer potential adverse effects on soil organisms from soil eluates. Microtox acute toxicity test was carried out by the Sarrià Chemical Institute of Barcelona, according to ISO 11348 (1998), and using a Microtox Model 500 Analyzer (Azur Environmental Inc.). The assay is

based on the luminescence inhibition of the strain NRRL B-11177 of the marine bacteria *Vibrio fischeri*. Luminescence is a by-product of its cellular respiration, so any adverse effect on its metabolism is reflected by a loss in bioluminescence. Luminescence inhibition of cultures at 15ºC after 15 minutes of exposure was used as the endpoint. Assays were performed by duplicate with the eluates already described. Four test concentrations (0, 5.6, 11.25, 22.5, and 45% of eluate) were prepared from dilutions of the primary eluates. When these concentrations did not inhibit luminescence, dilutions of the primary eluates (1:5, 1:10, or 1:20) were used.

4.2.6. *Daphnia magna* acute toxicity assay

D. magna is a freshwater copepod widely used for aquatic ecotoxicological purposes and also for soil ecotoxicity using soil eluates. Assays were performed in Environmental Toxicology Laboratory of the Technical University of Catalonia according to ISO 6341 (1996b). The endpoint for toxicity assessment is inhibition of the individuals' mobility after 48 hours of exposure.

From the solid-phase aqueous eluate obtained according to EN 12457-2 (CEN 2002), a dilution range was prepared to provide eight test concentrations (0, 1.5, 3, 6, 12.5, 25, 50, and 100% of eluate). One replicate per concentration was prepared, consisting of a glass flask with 20 individuals. Individuals were exposed to the test concentrations at 20ºC±2ºC in a constant light-dark photoperiod (16:8). After 48 hours, the number of immobilized individuals was determined.

4.2.7. Data treatment

In the *F. candida* assay, LC50 and EC50 values, together with 95% confidence intervals, were calculated by suitable regression models (exponential, Gompertz, hormesis, linear and logistic) using the Statistica 6.0 software package (StatSoft Inc.). The choice of the model was based on best fit to data according to Stephenson et al. (2000).

In the Microtox assay, luminescence inhibition values (EC50) and 95% confidence intervals were determined by means of the MicrotoxOmni software (Azur Environmental Inc.).

In the *D. magna* assay, the individual's mobility EC50 and 95% confidence intervals were determined by means of probit analysis using the statistical software package Minitab 13.2 (Minitab Inc. 2000).

In order to compare toxicity results of the different assays, Pearson correlations of their log-transformed toxicity values (LC50, EC50) were determined, using the statistical package SPSS 13.0. Furthermore, within each bioassay, correlations of toxicity in the solid-phase with that in eluates were sought by the same method.

Pearson correlations were also used to reveal which waste parameters were primarily responsible for the observed toxicity in each bioassay. More precisely, significant correlations of toxicity with the log-transformed values of individual pollutant concentration, the sum of concentrations of pollutant groups, and physico-chemical parameters were sought. The assessed pollutant groups were heavy metals, organic pollutants, persistent organics (PAH, PCB and PCDD/F), non-persistent (DEHP, LAS and NPE), and total pollutant burden. When a concentration value was below the detection limit, zero was used in the correlations.

4.3. RESULTS

4.3.1. Sample and eluate characterization

The studied wastes showed contrasting physico-chemical properties and pollutant burden (Table 4.2 and 4.3). Sludge compost showed higher organic matter stability and lower total nitrogen content, with hydrolysable nitrogen and ammonium. Furthermore, composting reduced concentrations of non-persistent organic pollutants (DEHP, NPE and LAS). Thermal drying reduced the $N\text{-}NH_4$ levels and increased its electrical conductivity

with respect to dewatered sludge. No significant reduction in the pollutant levels was observed, except for DEHP, which showed a slightly lower concentration in the thermally dried than in the dewatered sludges. Thermally dried pig slurry presented low moisture content, extremely high electric conductivity, was highly hydrolysable and had a high total nitrogen content and a low organic matter stability.

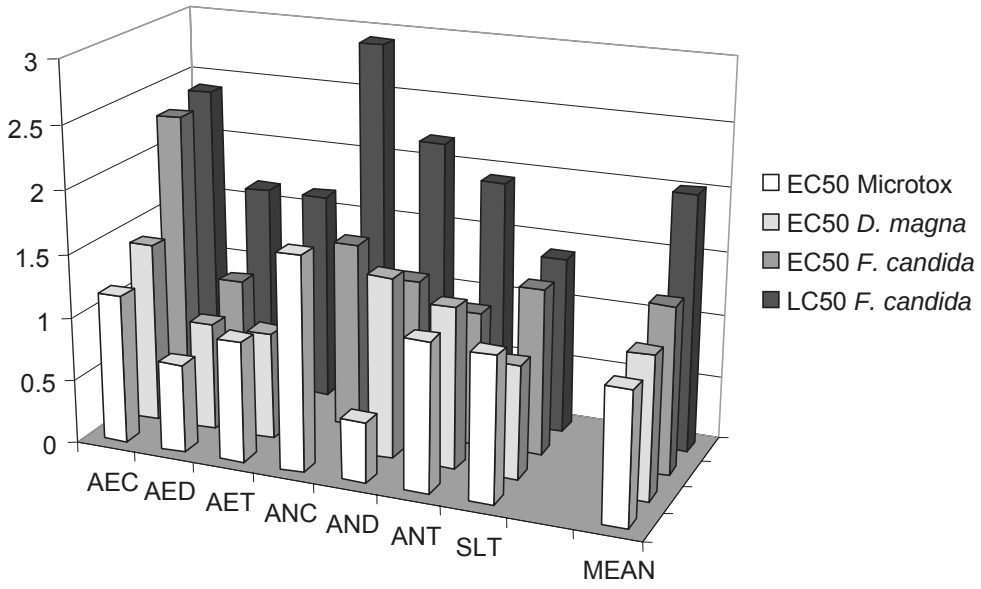
Comparison between the concentration of a particular pollutant in the three eluates and the measured "total" concentration in the solid-phase (Table 4.3), gives an estimation of the extraction efficiencies of each solvent. Heavy metals were mainly in non-extractable form, as mean extraction efficiency for them was 0 to 4% for any solvent, except for Cd (43%) and Hg (15%) in the aqueous eluates. LAS, PCB, and PCDD/Fs were not detectable in eluates. NPE and PAH were not recovered in aqueous eluates, but were present in the methanol (48 and 37% respectively) and dichloromethane (37 and 24% respectively) eluates. DEHP showed high relative concentrations in methanol (80%), but was particularly high in dichloromethane eluates (246%), in which higher concentrations were found in eluates than in the solid-phase in some of the wastes. This unexpected result could be attributed to a higher extraction efficiency of dichloromethane with respect to the method used for "total" DEHP determination, or to an accidental loss from solvent previous to DEHP determination, given the high volatility of dichloromethane. Given that DEHP concentrations in methanol and dichloromethane eluates were correlated with their concentrations in solid-phase, we assumed this result did not invalidate data for correlations with bioassays.

As a general trend, individual heavy metal concentrations in solid-phase were uncorrelated with their concentrations in eluates. On the contrary, solid-phase organic pollutant concentrations were generally correlated with their concentrations in methanol eluates ($p<0.05$ for DEHP, LAS, NPE, and PAH, respectively), and in dichloromethane eluates ($p<0.05$ for DEHP, NPE, and PAH, respectively), but not with aqueous eluates as no organic pollutants were detectable.

4.3.2. Bioassays comparison

Results from the battery of tests generally concord that sludge composts are the least toxic of the wastes studied, both when solid-phase or eluates are tested (Table 4.4, Figure 4.1). The exception was the Microtox assay using aqueous and dichloromethane eluates, which failed to identify composts as the least polluted wastes. On the contrary, there was no consistent pattern for the most toxic wastes, which depended on the bioassay and the pollutant fraction. This is why no correlations between bioassays could be found, with the exception of *F. candida* mortality in solid-phase tests which correlated highly with inhibition of mobility in *D. magna* ($r = 0.922$, $p=0.009$).

Figure 4.1. Bioassays comparison of inhibition values in solid-phase assays of the different wastes. Values were log-transformed and expressed in g Kg^{-1} in *F. candida* assay, and g L^{-1} in *D. magna* and Microtox assays.



Different relative sensitivities of the various bioassays appeared when the different pollutant fractions were compared for their mean toxicity results. In the solid-phase assays, the overall order of sensitivity to wastes was Microtox > *D. magna* > *F. candida* reproduction > *F. candida* survival (Figure 4.1). In aqueous eluate assays, sensitivity was

rated as *F. candida* reproduction > Microtox > *F. candida* survival. In methanol eluate assays, bioassay sensitivity was rated as Microtox > *F. candida* reproduction > *F. candida* survival, with a close range from both the *F. candida* endpoints. In the dichloromethane eluate assays, the same ranking was observed, but with more definite differences between *F. candida* reproduction and survival.

4.3.3. Solid-phase and eluate toxicity comparison

4.3.3.1. F. candida *reproduction assay*

Survival and reproduction were sensitive to solid-phase wastes and eluates, reproduction being affected at lower concentrations than survival (Table 4.4). Concerning toxicity differences between wastes, the only general trend was the lower toxicity of the sludge composts for both endpoints and for all pollutant fractions. It is noteworthy that in composts survival was not inhibited, or inhibition was below 50% in all the eluate assays. Furthermore, the aerobic thermally dried sludge did not inhibit neither survival nor reproduction when applied as a dichloromethane eluate, despite its high toxicity in the solid-phase assays.

Survival in the solid-phase was positively correlated with that in the aqueous eluate tests ($r = 0.927$, $p=0.023$), and also with reproduction in the aqueous and methanol eluate tests ($r =0.831$ $p = 0.041$, and $r = 0.770$ $p = 0.043$, respectively). On the contrary, there was no correlation between effects on survival and reproduction in the solid-phase tests. Effects on reproduction in the solid-phase and in the eluate tests were not correlated, except for aqueous and methanol eluates, for whom a strong positive correlation ($p=0.006$) was found.

Table 4.4. Toxicity values for each waste in the different bioassays. Values of solid-phase assays are expressed as g Kg^{-1} for *F. candida* assay, as $g \cdot L^{-1}$ for *D. magna* assay, and as $mg \cdot L^{-1}$ for Microtox. Toxicity values show their 95% confidence intervals enclosed in parentheses. In the eluate assays, concentration values are expressed based on the mass of waste initially extracted.

Assay	Waste	*F. candida* LC50	*F. candida* EC50	*D. magna* EC50	Microtox EC50
Solid-phase	AEC	252.3 (221.7, 287.2)	207 (36.8, 1142)	25.4 (21.4, 30.6)	13.9 (9.2, 21.7)
	AED	43.9 (34.1, 56.6)	10.0 (3.8, 23.8)	6.1 (4.8, 7.7)	4.4 (2.46, 7.91)
	AET	44.0 (37.4, 51.7)	5.3 (2.8, 9.4)	6.1 (4.9, 7.5)	7.9 (6.5, 9.5)
	ANC	834 (626, 1110)	28.7 (17.7, 46.0)	-	47.9 (29.8, 88.9)
	AND	154 (134, 178)	16.4 (14.7, 18.2)	25.8 (21.7, 31.1)	1.61 (1.09, 2.4)
	ANT	85.6 (72.3, 101)	10.4 (7.5, 14.2)	17.6 (14.2, 21.7)	14.2 (12.3, 16.5)
	SLT	23.7 (20.2, 27.8)	19.4 (3.8, 86.4)	6.8 (5.9, 8.6)	12.5 (9.5, 16.6)
Water eluate	AEC	-	28.6 (11.8, 67.5)		75.4 (56.4, 100.8)
	AED	138 (111, 172)	4.0 (2.9, 5.6)		21.7 (16.4, 28.8)
	AET	245 (207, 290)	7.4 (3.6, 14.1)		65.0 (29.2, 281.5)
	ANC	-	-		-
	AND	628 (527, 750)	8.5 (3.5, 19.1)		60.3 (7.7, 486.3)
	ANT	279 (233, 334)	2.5 (1.6, 3.8)		97.3 (47.9, 204.7)
	SLT	129 (106, 129)	0.7 (0.3, 1.2)		54.6 (32.4,97.6)
Methanol eluate	AEC	-	656 (390, 1105)		23.7 (21.2, 26.4)
	AED	80.7 (68.5, 95.1)	24.6 (19.8, 30.4)		1.9 (0.9, 3.7)
	AET	254 (312, 206)	230 (184, 288)		3.6 (3.1, 4.2)
	ANC	-	548 (283, 1061)		16.2 (13.7, 19.1)
	AND	100 (85.1, 118)	65.5 (56.0, 76.6)		1.0 (0.2, 4.7)
	ANT	87.9 (70.8, 109)	31.3 (20.4, 47.6)		1.57 (1.0, 2.5)
	SLT	311 (234, 413)	8.2 (5.4, 12.0)		10.8 (5.8, 16.2)
Dichloromethane eluate	AEC	-	-		4.3 (3.6, 5.3)
	AED	257 (225, 292)	72.0 (24.0, 212.7)		7.9 (3.0, 20.5)
	AET	-	-		2.1 (1.3, 3,4)
	ANC	-	93.5 (44.6, 194.8)		14.4 (11.3, 18.5)

AND	133 (110, 161)	85.4 (31.0, 232.0)	0.8 (0.6, 1.5)
ANT	248 (210, 291)	58.9 (39.1, 88.3)	2.4 (1.8, 3.3)
SLT	254 (204, 316)	166 (108, 256)	5.4 (3.8, 7.7)

4.3.3.2. Microtox acute toxicity assay

V. fischeri was sensitive to solid-phase and eluates of the different wastes, as it provided significant toxicity values for each waste (Table 4.4). However, the relative toxicities of wastes were not coherent among the different pollutant fractions. Sludge compost toxicity was markedly lower than the remaining wastes in solid-phase eluates and methanol eluates, although this trend disappeared in the other eluates. On the contrary, in the aqueous eluates toxicity was similar for different wastes.

4.3.3.3. D. magna *acute toxicity assay*

Mobility inhibition in *D. magna* was also sensitive to the different wastes (Table 4.4), sludge composts being the least toxic. Anaerobic dewatered sludge exerted moderate toxicity and anaerobic thermally dried and dewatered sludge was the most toxic.

4.3.6. Pollutant concentrations and biological response

No correlation between toxicity and individual pollutant concentrations or pollutant groups could be detected, neither in solid-phase nor in aqueous and dichloromethane eluates. In contrast, some correlations appeared in the methanol eluate assays. More precisely, reproduction of *F. candida* was inversely correlated with copper levels ($r = -0.818$, $p = 0.024$), and the sum of heavy metals ($r = -0.788$, $p = 0.035$). Also luminescence in the Microtox assay was inversely correlated with the sum of organic pollutants ($r = -0.781$, $p = 0.038$), and the sum of non persistent organics ($r = -0.782$, $p = 0.038$).

4.3.7. Physico-chemical properties of wastes and biological response

For *F. candida* assays, some physico-chemical parameters of the original wastes were correlated with toxicity. In the solid-phase assays, survival was highly and positively correlated with the degree of waste stabilization ($r = 0.952$, $p = 0.001$), and negatively correlated with their total nitrogen ($r = -0.972$, $p<0.001$), hydrolysable nitrogen ($r = -0.97$, $p<0.001$), and ammonium content ($r = -0.786$, $p = 0.036$). This pattern was also observed for survival in the aqueous eluate assays, as there was a positive correlation between survival and degree of stabilization ($r = 0.906$, $p = 0.034$), as well as a negative relationship between survival and total nitrogen ($r = -0.941$, $p = 0.017$), and hydrolysable nitrogen ($r = -0.978$, $p = 0.004$). A negative correlation was also found between survival in the methanol eluate assays and the total nitrogen ($r = -0.939$, $p = 0.002$).

No correlations were found between reproduction and waste physico-chemical properties in the solid-phase assays. Nevertheless, negative correlations were observed between reproduction in both aqueous and methanol eluate assays and the ammonium concentration in the original wastes ($r = -0.904$ $p = 0.013$, and $r = -0.013$ $p = 0.002$ respectively), as well as with hydrolysable nitrogen in the aqueous eluate tests ($r = -0.835$, $p = 0.039$).

Similar correlations were also found in the *D. magna* assay, as mobility was positively correlated with waste stability ($r = 0.950$, $p = 0.004$), and negatively correlated with the total nitrogen ($r = -0.944$, $p = 0.005$) and hydrolysable nitrogen ($r = -0.927$, $p = 0.008$). On the contrary, no significant correlations were found between the Microtox assays and the physico-chemical properties of the wastes.

4.4. DISCUSSION

4.4.1. Comparison of waste bioassays

Developing bioassays to assess the safety of polluted organic wastes application to soil is a priority in Europe, given the increased production of these materials and concern about this practice. Selection of such bioassays should primarily be based on their ecological relevance, but also on low experimental and economic costs.

The interest in bioassays has arisen due to the complex pollutant burden of wastes, but also because of the limitations of chemical methods to estimate the risk for ecosystems (Crouau et al. 2002). First, chemical methods require a previous knowledge of the substance groups to be analyzed and, therefore, not all potentially noxious chemicals are monitored. Second, screening the most potentially noxious chemicals is too expensive, given that thousands of them can be present in a polluted substrate. Third, chemical methods give information about the total pollutant burden but not about its bioavailability or release of end products. Finally, chemical methods do not detect synergisms and antagonisms between chemicals. Bioassays overcome these limitations, providing more realistic information on the potential effects of pollutants on living organisms, however they do not provide information on the identity of chemicals causing the effects measured (Brack 2003).

The growing concern for the prediction of the environmental hazard of wastes has led to the development of standardized protocols which measure the harmful properties of wastes from their leachates by using a combination of chemical analyses and aquatic ecotoxicology assays. Nevertheless, most of this work has been limited to characterizing inorganic wastes (Vaajasaari 2005). An example is the European standard EN 14735 (CEN 2005) for preparation of waste samples for ecotoxicity tests, applicable both to inorganic and organic wastes. In this protocol, steps from sampling waste to the performance of the biological tests, either using its solid-phase or its water eluates are

described. Furthermore, a list of potential terrestrial and aquatic organisms to be used is proposed. Results of the present study demonstrate the sensitivity to wastes of some of these bioassays, and despite the generalized lack of coherence among them, their differential sensitivity is shown. Microtox toxicity was not correlated with any of the remaining bioassays and neither was collembolan reproduction. However, mortality of *F. candida* in solid-phase was highly correlated with inhibition in the mobility of *D. magna*.

4.4.2. Solid-phase versus eluate assays in waste testing

Direct measure of waste toxicity for terrestrial soils and sediment organisms (solid-phase tests) should be the most relevant way to estimate its ecotoxicological potential, as it is close to real situations. However, only some works have taken this approach for wastes (Krogh et al. 1997, Krogh and Pedersen 1997, Robidoux et al. 1998, Renoux et al. 2001, Crouau et al 2002, Pandard et al. 2006, Domene et al. 2007), and most of the studies have been performed with aqueous eluates or leachates using as test organisms: microorganisms (Robidoux et al. 1998, Selivanovskaya and Latypova 2004, Mantis et al. 2005, Park et al. 2005, Fuentes et al. 2006), plants (Wong et al. 1981, Garcia et al. 1991, Robidoux et al. 1998, Tiquia and Tam 1998, Renoux et al. 2001, Wong et al. 2001, Selivanovskaya and Latypova 2004, Fuentes et al. 2006, Walter et al. 2006) or daphnids (Selivanovskaya and Latypova 2004, Fjällborg and Dave 2003, Fjällborg et al. 2005, Molina-Barahona et al. 2005).

Some authors have criticized the use of eluates to extrapolate waste effects on soils because eluates only give information on the pollutants instantaneous bioavailability, not reflecting their longer-term bioavailability (McMillen et al. 2003). Furthermore, several confounding phenomena usually hinder finding correlations between pollutant concentrations in eluates and biological responses, making the interpretation of results difficult (Alexander et al. 2003, McMillen et al. 2003). More precisely, when eluates are used in aquatic tests or terrestrial soils after being mixed with a clean soil, synergic or

antagonistic effects between pollutants or changes in their bioavailability may strongly modify the eluates' toxicity with respect to that of the original soils or wastes.

Solid-phase waste assays are, without question, more relevant than eluate assays mainly because they are the closest to real situations. However, solid-phase assays incur several problems in their practical application (Domene et al. 2007). First, organic matter represents a significant percentage of organic wastes, and can mask and underestimate their potential toxicity in short-term bioassays through pollutant sorption and nutritious effects on soil fauna. Second, wastes' physico-chemical properties and water holding retention capacity usually are very different to those in the test substrate, a fact that may generate wide variations in these parameters depending on test concentrations. Indeed test concentrations may influence the chemicals' bioavailability or the response of organisms to their toxic effects. Third, solid-phase bioassays do not allow the identification of the pollutant fraction mainly explaining the observed toxic effects, while a combined testing of different eluates (aqueous and organic solvents) may give indications on the pollutant fraction mainly contributing to the harmful effects exerted by organic wastes. Finally, eluate assays may reduce the experimental effort needed for the bioassays, since some steps of sample preparation for solid-phase testing could be omitted (namely drying and grounding, but especially all work associated with the monitoring of wastes' physico-chemical properties and their water holding capacities).

Therefore, the choice of method is not an easy one, since both solid-phase and eluate approaches imply significant limitations. Furthermore, the number of works comparing toxicities obtained through solid-phase assays with terrestrial organisms and eluate assays with aquatic organisms are still very scarce, although they concord in not finding significant correlations (Sheehan et al. 2003, Loureiro et al. 2005).

Results from this study indicated aqueous eluates were representative of the solid-phase acute toxicity to *F. candida*, but also to that of *D. magna*. Despite this, the lack of correlation between *F. candida* reproduction in the solid-phase and in the aqueous eluate

tests suggest that even aqueous eluates are not suitable for estimating chronic effects. This is consistent with several authors who claim that toxicity results obtained from aquatic tests using soil eluates can only be extrapolated to soils for short-term assays focused on lethal endpoints (McMillen et al. 2005), and that a proper assessment of the long-term ecological risk should be based on chronic assays using soil organisms and sublethal endpoints (van Gestel et al. 2001). Incoherencies between the chronic toxicity results obtained for *F. candida* and those from the Microtox assay, stress the lack of representativeness of the aquatic tests with waste eluates to predict effects on soils. This finding agrees with the work of Sheehan et al. (2003), who failed to find any correlation between toxicity of several polluted soils, their leachates and their corresponding groundwaters, using terrestrial tests (*Eisenia fetida* survival) and aquatic tests (Microtox test, and daphnids immobilization test). Loureiro et al. (2005), comparing results of different bioassays with two polluted soils and a control soil, also did not find any correlation of results between the *F. candida* reproduction test and aquatic test results (Microtox luminescence test, and *Daphnia magna* immobilization and reproduction test). On the contrary, Microtox results have been correlated with fish lethality in assays with wastes from a petroleum refinery (Park et al. 2005).

4.4.3. Main contributors to organic waste toxicity

Both in the solid-phase and eluate assays, composted sewage sludges presented lower toxicity than the other wastes (with the exception of aqueous and dichloromethane eluates in Microtox). However, the battery of tests did not determine the most toxic wastes, as it depended both on test species and pollutant fraction. These results demonstrate the influence of the species' intrinsic sensitivity to a given chemical burden on the observed toxicities, and also the compulsory requirement of test batteries including several organisms for a proper ecotoxicological risk assessment of wastes.

Results from this study did not show correlation of toxicities with pollutant burden neither in the solid-phase, aqueous or dichloromethane eluate assays, in agreement with reports by other authors regarding polluted soils and their corresponding eluates (Sheehan et al. 2003). However, some correlations were ostensible in methanol eluate assays, since the sum of heavy metals explained the reproduction inhibition in *F. candida*, while the sum of all the organic pollutants and the sum of non persistent organics explained the inhibition of luminescence in the Microtox assay. The relationship found for heavy metals in *F. candida* is not surprising, as most of the heavy metal burden in sludge is not water soluble but is adsorbed to the organic matrix (Alonso et al. 2006), which can be partly solubilized by methanol.

On the contrary, several physico-chemical parameters of the original wastes related to organic matter stability were highly explanatory for the acute toxicity observed in *F. candida* survival and *D. magna* mobility in the solid-phase and aqueous eluate assays. Furthermore, non-hydrolysable nitrogen and ammonium concentration in the original wastes also accounted for inhibition in reproduction of *F. candida* in water eluates, while ammonium was the inhibiting factor in methanol eluate assays. Non stabilized wastes are easily decomposed, as an important percentage of their organic matter is labile, mainly polysaccharides and proteins (Rovira and Vallejo 2002). Along the decomposition process, the percentage of stable organic matter increases, while there is a loss of total nitrogen by depletion of its more hydrolysable (labile) fraction, mainly as ammonia releases (Witter and Lopez-Real 1988, Martins and Dewes 1992, Grube et al. 2006). In addition to ammonium, other noxious secondary metabolites of decomposition are released, like phenols and organic acids (Garcia et al. 1991, Mathur et al. 1993, Déportes et al. 1995, Fang et al. 1999, Huang et al. 2004). Hence, the more stabilized an organic waste, the higher is the percentage of stable organic matter, and the lower the levels of total nitrogen, hydrolysable nitrogen, and ammonium. Furthermore, with decomposition, waste toxicity may decrease because non-persistent organic pollutants

can be degraded by microorganisms (Déportes et al. 1995, Marttinen et al. 2004, Bagó et al. 2005, Abad et al. 2005, Sanz et al. 2006), and because pollutant bioavailability is the lowest in the most stabilized wastes (Fuentes et al. 2006). This relationship between waste stability and toxicity has already been reported for plants (Zucconi et al. 1981, Katayama et al. 1985, Pascual et al. 1997, Huang et al. 2004, Zmora-Nahum et al. 2005), but is scarcely documented for soil fauna (Neher 1999). Garcia et al. (1991) already stressed the relevance of organic wastes' aqueous eluates to indicate the wastes' maturity and phytotoxicity (Fuentes et al. 2006). Other authors have also found this association using the Microtox assay (Fuentes et al. 2006, Walter et al. 2006). Particularly, Tiquia and Tam (1998) showed that increasing the stability of pig manure composts decreases ammonium and metal concentrations in eluates, and reduces phytotoxicity.

CONCLUSIONS

The general lack of coherence of results between bioassays, shows the varied sensitivity of different test organisms, and the need to use test batteries for a proper ecotoxicological risk assessment.

Waste eluates were not representative of the chronic toxicity exerted by solid-phase waste. However, aqueous eluates exerted equivalent acute toxicity to that of solid-phase waste in collembolans and daphnids. Therefore, extrapolation to soils of waste toxicity results obtained from aquatic tests using aqueous soil eluates should be only acceptable for short-term assays focused on lethal endpoints.

Chemical methods are not suitable for a proper risk assessment of wastes, as no correlation of toxicities with pollutant burden were found neither in the solid-phase, nor in aqueous or dichloromethane eluate assays. The only direct relationships were found in methanol eluate assays between total heavy metal burden and collembolan chronic

toxicity, and between the total organic and non-persistent organic burden with the Microtox assay.

Physico-chemical parameters related to organic matter stability were highly explanatory of the acute toxicity observed in collembolans and daphnids in the solid-phase and aqueous eluate assays, but also of the chronic toxicity for collembolans in aqueous and methanol eluates. Ammonium and other water-soluble decomposition end products might be the main explanation for this association.

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Chapter 5

Ecological risk assessment of organic wastes amendments using the species sensitivity distribution from a soil organisms test battery

ABSTRACT

An ecological risk assessment of seven different organic wastes was carried out using a battery of soil biota tests: earthworm fresh weight, collembola, enchytraeid and earthworm reproduction, plant emergence and growth, and microbial respiration. For each waste, a safe amendment rate was estimated from the species sensitivity distribution and compared with different realistic amendment scenarios. Assuming that the safe amendment values estimated in the laboratory were representative of actual field situations, none of the wastes was expected to exert noxious effects on soil biota if applied according either to the N or P crop demands or to the usual amendment rates in Europe. However, the ecological risk assessment indicated that some of the wastes might be problematic if applied over 5 t DM ha^{-1}. The main contributors to the toxicity were low organic matter stability and high ammonia contents in wastes, but not pollutant burden. This finding indicates the need to stabilize wastes prior to their reuse in soils in order to avoid an impact on soil communities.

5.1. INTRODUCTION

The use of organic wastes in soils is an increasing management option in the European Union. For sewage, such increase is mainly due to the rising amount of sludge produced, the increasingly stringent controls on landfilling, the public opposition to incineration, and the ban on disposal at sea (Schowanek et al. 2004, Thornton et al. 2001). Preference for the reuse of organic wastes in agricultural land with respect to other management options is also due to the benefits of this practice. Organic amendments enhance soil fertility by adding nutrients, it is a cheaper option that allows reduction in the use of fertilisers, improves the soil structure, the water retention and the resilience to erosion, and it is an inexpensive solution of management of organic wastes (Schowaneck et al. 2004). However, the side effects on the soil-dwelling organisms exposed to wastes' pollutant burden are often neglected (Bünemann et al. 2006), despite their central role in soil agroecosystems functioning (Giller et al. 1997, Neher 1999).

The amount of organic wastes applied to an agricultural land is generally dictated by their nutrient content (nitrogen and phosphorus) and by the crop demands. Furthermore, several criteria for environmental protection exist in Europe to ensure a minimum waste quality before waste application, in order to ensure its long-term sustainable use on land. However, quality relies exclusively on the waste total pollutant content. Sewage sludge amendments are regulated by Directive 86/278/EEC, which compels the raw sludge stabilization and sets heavy metals limit values for sludge reuse on soil. There is no specific legislation for the reuse on agricultural land for non-sewage organic wastes, although it might be limited by their pathogen and heavy metal content when placed on the market as fertilisers (according to each member state transpositions of the Regulation (EC) No 2003/2003 and No 1774/2002). Directive 91/676/EEC, which concerns the protection of waters against pollution caused by nitrates from agricultural sources, can also limit the use of wastes in soil. Hence, only chemical assays are considered to limit the

use of organic wastes in soil, despite their disadvantages compared to biological assays (Crouau et al. 2002).

A great variety of wastes are currently produced in the European Union as a result of the spread of wastes treatment technologies which minimize their volume, and increase their hygienization and ease of handling. Composting of raw wastes or aerobic/anaerobic digestion of raw sludges, followed by composting or thermal drying of the resultant products, are the most common treatments to achieve such goals. It has been shown that such treatments have consequences on the wastes' physical, chemical, and biological parameters (Schowanek et al. 2004), but not much is known about the impact on soil organisms of these different products. The published studies on the effects of organic wastes on soil biota range from laboratory studies to field effects. No harmful effects on the croplands soil fauna have been reported when wastes are applied at agronomic rates, but ecological risk can not be excluded for all wastes or for higher application rates.

Ecological risk assessment has been defined as the process of estimating the likelihood that a particular event will occur under a given set of circumstances (Maltby 2006), which can be used both as a tool for taking decisions in current situations and for predicting future risks. In any risk assessment, the first step is to derive, the "predicted no-effect concentration" (PNEC). This is the concentration at which no harmful effects on the environment are expected. PNEC values are then compared with the "predicted environmental concentrations" (PEC) in the studied soil in order to calculate the "risk quotient" (RQ= PEC/PNEC), which is used to determine the ecological risk (when RQ>1).

In this study the safe amendment rates of different wastes are estimated from a set of laboratory toxicity data obtained from a soil bioassay battery (microorganisms, plants, collembolans, enchytraeids, and earthworms), are compared with several realistic amendment scenarios in order to determine the ecological risk amendments with such wastes.

The aims of the present study were (a) to assess the suitability of the species sensitivity distribution method for estimating organic wastes safe amendment rates, (b) to compare the estimated rates with plausible amendment rates in agricultural soils according to different scenarios based on crop demands and usual amendment rates in the European Union, and (c) to determine the relationship between waste composition and ecotoxicity, and also the influence of waste treatments and post-treatments on their ecotoxicity.

5.2. METHODS

5.2.1. Organic wastes

Seven materials were selected in order to represent the variety of organic wastes currently generated in Europe and used as amendment: two dewatered sewage sludges (AED and AND, obtained from an aerobic and anaerobic digestion of raw sludge respectively), two composted sewage sludges (AEC and ANC, from composting of the aerobic and anaerobic sludge respectively), two thermally-dried sewage sludges (AET and ANT, obtained from the drying at temperatures around 140°C of the aerobic and anaerobic sludge respectively), and a thermally-dried pig slurry (SLT). Wastes treatments and post-treatments are summarized in Table 5.1.

Dewatered sludge is the final product of wastewater treatment, by aerobic or anaerobic digestion, followed by dewatering. This process reduces the sludge volume and pathogen content, and increases its stability. Some treatment plants carry out additional sludge post-treatments in order to enhance hygienization and to further reduce water content, being composting and thermal drying the most common technologies. In the dewatered sludges used in this study (AED, AND), dewatering was carried out by centrifugation.

Table 5.1. Origin, treatments and post-treatments of the organic wastes.

Waste	Origin	Treatment	Post-treatment
AED	Banyoles WWTP	Aerobic digestion, dewatering	None
AEC	Banyoles WWTP	Aerobic digestion, dewatering	Composting in tunnel
AET	Banyoles WWTP	Aerobic digestion, dewatering	Thermal drying
AND	Blanes WWTP	Anaerobic digestion, dewatering	None
ANC	Blanes WWTP	Anaerobic digestion, dewatering	Composting in heap
ANT	Blanes WWTP	Anaerobic digestion, dewatering	Thermal drying
SLT	Juneda WTP	Anaerobic digestion, dewatering	Thermal drying

Both sludge composts (AEC, ANC) were produced with a sludge-pine wood chips mixture (1:4.5 v/v). AEC was composted in a heap for 50 days, with continuous tumbling of the heap during the first month, followed by weekly turning. ANC was composted for 15 days in a rotatory tunnel with air injection. At the end of the composting period, composts were sieved to 1 cm.

Thermally-dried sludges (AET, ANT) were prepared by placing dewatered sludge for 45 minutes in a heated rotatory cylinder and by injecting hot air, which provided a temperature between 130 to 150°C. Thermally dried pig slurry (SLT) was obtained by anaerobic digestion of raw slurry followed by thermal drying in a rotatory tunnel at 130°C.

In the laboratory, each waste was dried at 60°C for 48-72 hours, depending on its initial water content, and then ground and homogenized to ensure the preparation accuracy of the lower waste concentrations. These materials were used for characterization and for the preparation of the soil-waste mixtures used in the bioassays. The physico-chemical properties and pollutant burden of each waste are recorded in Table 5.2.

Dry matter, water holding capacity, water pH, electrical conductivity, total nitrogen and organic matter were measured according to the standards EN 12880 (2000), ISO 11267 (1999), EN 13037 (1999), EN 13038 (1999), EN 13342 (2000) and EN 12879 (2000), respectively. Non-hydrolyzable organic matter (stable), and non-hydrolyzable nitrogen were measured by the percentage of organic matter and nitrogen remaining in the sample residue after acid hydrolysis, mainly consisting of polysaccharides and proteins, as described in Rovira & Vallejo (2002). Hydrolyzable nitrogen was calculated by subtracting the non-hydrolyzable nitrogen from total nitrogen content. N-NH_4 was measured using the distillates obtained from fresh samples.

Table 5.2. Organic wastes physico-chemical properties and heavy metal and organic pollutant contents (*=exceeding the the 3rd draft of Working Document on Sludge (European Commission 2000) limit value; **= exceeding the current Directive on sludge (86/278/EEC) limit value; *w.w* = wet weight; *d.w.* = dry weight). See Table 1 for waste abbreviations. Data obtained from Domene et al. (2007).

Parameter	Units	AEC	AED	AET	ANC	AND	ANT	SLT
Dry matter	g kg^{-1} (*w.w.*)	449	150	945	470	199	844	865
WHC	% (*w.w.*)	74.4	63.9	74.7	64.9	64.8	67.9	55.9
pH	water, 1:5 (v/v)	7.8	8.1	6.9	7.2	8.4	7.2	6.4
Electrical conductivity	dS/m, 25ºC	1.2	1.5	3.57	4.2	2.25	6.22	64.65
Organic matter	g kg^{-1} (*d.w.*)	622	684	687	551	566	668	612
Stable organic matter	%	50.1	37.8	40.4	54.2	47.7	46.7	36.6
N	g kg^{-1} (*d.w.*)	39.5	62.4	60.6	23.7	38.8	53.3	62.5
Non-hydrolyzable N	g kg^{-1} (*d.w.*)	17.0	16.4	19.1	16.1	12.4	18.4	10.9
Hydrolyzable N	g kg^{-1} (*d.w.*)	22.5	46.0	41.5	7.6	26.4	34.9	51.6
NH_4-N	g kg^{-1} (w.w.)	2.7	14.0	8.0	3.4	15.1	11.6	52.9

P	g kg^{-1} (d.w.)	22.0	20.4	20.5	28.6	33.6	29.2	20.4
K	g kg^{-1} (d.w.)	3.6	1.9	2.2	4.4	2.3	2.5	55
Cd	mg kg^{-1} (d.w.)	1.0	1.3	1.3	3.5	3.2	3.1	<0.7
Cr	mg kg^{-1} (d.w.)	345	55	30	53	54	127	15
Cu	mg kg^{-1} (d.w.)	294	624	645	798	933	833	780
Hg	mg kg^{-1} (d.w.)	0.67	1.33	0.95	2.13	2.51	2.25	0.12
Ni	mg kg^{-1} (d.w.)	59	80	53	76	64	45	29
Pb	mg kg^{-1} (d.w.)	1196**	3940**	3747**	92	78	85	<20
Zn	mg kg^{-1} (d.w.)	843	956	952	1028	988	890	2060
DEHP	mg kg^{-1} (d.w.)	10	61	27	22	143	71	1
LAS	mg kg^{-1} (d.w.)	298	816	331	214	3240*	5572*	60
NPE	mg kg^{-1} (d.w.)	86*	153*	76*	158*	513*	573*	54*
PAH	mg kg^{-1} (d.w.)	0.1	0.4	0.3	1.6	1.1	1.4	0.05
PCB	mg kg^{-1} (d.w.)	0.015	0.034	0.029	0.041	0.023	0.029	<0.007
PCDD/F	ngTEQ kg^{-1} (d.w.)	16	15.6	13.7	12.4	7.7	13.2	0.3

Elemental analysis of P, K, Cd, Cr, Cu, Hg, Ni, Pb, and Zn was carried out by ICP-MS according to ISO 11885 (1996). We also measured the organic pollutants and pollutant groups indicated in the third draft of the Working Document on Sludge (European Commission 2000). Polychlorinated dibenzodioxins/dibenzofuranes (PCDD/F) were measured with HRGC-HRMS, polychlorinated biphenyls (PCB) by HRGC-ECD, di(2-ethylhexyl)phthalate (DEHP) and nonylphenols (NPE) by HRGC-MS. Polycyclic aromatic hydrocarbons (PAH) and linear alkylbenzene sulphonates (LAS) were determined by HPLC with fluorescence and UV detectors, respectively. Values of DEHP, LAS and PCDD/F represent total values. NPE includes nonylphenol and nonylphenol ethoxylates with 1 or 2 ethoxy groups. PAH is the sum of acenapthene, phenanthrene, fluorene, fluoranthene, pyrene, benzo(b+j+k)fluoranthene, benzo(a)pyrene, benzo(ghi)perylene,

and indeno(1, 2, 3-c, d)pyrene. PCB is the sum of the polychlorinated biphenyl congeners number 28, 52, 101, 118, 138, 153 and 180.

5.2.2. Effects on soil microorganisms

Toxicity values were calculated from the raw data of (Matana et al., unpublished data), where substrate-induced respiration (SIR) was used as endpoint, measured according to OECD 217 (2000) using glucose as substrate. The top-layer of a sandy natural grassland soil, sieved to 2 mm, was used for soil-waste mixtures preparation. In this study, eight waste concentrations were tested (0, 10, 21.2, 44.8, 94.9, 200.8, 425.1, and 900 g kg^{-1}). Moisture of mixtures was adjusted to 50% of their maximum water holding capacity. Three replicates consisting in a sealed container were prepared for each concentration. As indicated in the protocol, SIR was measured at day 1 (acute toxicity) and day 28 (chronic toxicity).

5.2.3. Effects on plants

Waste toxicity values were calculated from the raw data of Ramírez et al., unpublished data). Effects on seedling emergence and growth of three plant species (*Brassica rapa*, *Lolium perenne*, and *Trifolium pratense*) were assessed according to OECD 208 (1984). Experiments were performed in artificial soil, prepared as indicated in OECD 207 (1984). A preliminary assay using germination as endpoint (0, 1, 10, 100, 1000 g kg^{-1}) was used to determine a range of concentrations for the definitive assay, consisting of six concentrations in a geometric progression. Soil-waste mixtures were adjusted and maintained at 60% of their water-holding capacity during the assay. Each replicate consisted of a 250 ml plastic cup filled with 100 g of soil-waste mixture (dry weight), and five replicates were prepared for each concentration. Then, ten seeds were introduced in each replicate, which were maintained in a 16:8 h light/dark and 15/21°C period at 70% relative humidity. Seedling emergence percentage was determined when

50% of the seeds in controls germinated. At this point, 5 seedlings per replicate were retained, and the remaining plants were removed. After 28 days, seedling growth was measured as shoot length.

5.2.4. Effects on collembolans

Waste toxicity values were calculated from the raw data of Domene et al. (2007). Effects on the survival and reproduction of the soil collembolan *Folsomia candida* were determined based on ISO 11267 (1999). The assay was performed in artificial soil-waste mixtures. Twelve concentrations were tested (0, 1, 2, 3.9, 7.9, 15.8, 31.6, 63.1, 125.9, 251.2, 501.2, and 1000 g kg^{-1}). Soil-waste mixtures were adjusted at around 60% of their water-holding capacity. Five replicates per concentration were prepared in sealed 100 ml-flasks. Ten individuals 10 to12 days-aged were introduced in each flask. Replicates were aerated twice a week and maintained in the dark at 21°C. The animals were fed with 3 mg of yeast at the start of the assay and after 14 days. The number of surviving adults and juveniles was determined at day 28.

5.2.5. Effects on enchytraeids

Effects on the survival and reproduction of the soil enchytraeid *Enchytraeus crypticus* were determined according to ISO 16387 (2004). The assay was also performed in artificial soil-waste mixtures. Ten concentrations were evaluated (0, 2.5, 5, 10, 25.1, 63.1, 158.4, 398.1, 700, and 1000 g kg^{-1}). Water content of soil-waste mixtures was adjusted to 60% of their water-holding capacity. Four replicates per concentration were prepared, each in 150 ml-flasks filled with 30 g of the test substrate. Ten adults (clearly identified by the clitella) were introduced in each flask. The animals were fed with 25 mg of ground oat at the start of the assay, and weekly thereafter. Replicates were aerated twice a week and maintained in the dark at 21°C. The number of surviving adults and juveniles was determined after 28 days.

5.2.6. Effects on earthworms

The earthworm *Eisenia andrei* was used, as indicated in ISO 11268-2 (1996), to determine effects on survival, reproduction, and fresh weight of the individuals. The assay was carried out in artificial soil, using eight test concentrations (0, 10, 25.1, 63.1, 158.4, 398.1, 700, and 1000 g kg^{-1}). The soil-waste mixtures water content was adjusted to 60% of their water holding capacity. Each replicate consisted of a 1000 ml-container covered with a perforated lid that allowed aeration. Ten clitellated individuals of synchronized age (4 weeks difference at most), and 250 to 600 mg weight, were placed in each container. Animals were fed with 5 g cooked oat flakes at the start and weekly thereafter. Replicates were maintained in a 16:8 light:dark photoperiod, at 70% of relative humidity and a constant temperature of 21°C for 28 days. Fresh weight of adults was measured at after 14 and 28 days of exposure. At day 28, adults were counted and removed from the test substrate, and the replicates were incubated 28 more days in order to allow juveniles to emerge and grow. After this period, juveniles were counted.

5.2.7. Data treatment

For all bioassays and chronic endpoints, the "effective concentration" for 20% inhibition (EC20) was calculated using Statistica 6.0, together with its 95% confidence intervals. Values were calculated from suitable regression models (exponential, Gompertz, hormesis, linear or logistic), chosen on the basis of the best fit to the data, and according to the criteria indicated in Stephenson et al. (2000). For the *E. andrei*, the "no observed effect concentration" (NOEC) was calculated instead of EC20 by means of the Bonferroni test, since for most wastes, inhibition with respect to the controls always was lower than 20%.

5.2.8. Wastes ecological risk assessment

5.2.8.1. PNEC estimation

PNEC is the concentration below which an ecosystem is not expected to suffer an unacceptable damage, according to a predefined acceptable effect level (LCx, NOEC, ECx) on different organisms. In the present study, selection of species, endpoint, and acceptable effect level for the risk assessment of organic wastes were based on the recommendations of European Commission (2003) and Traas et al. (2001). Chronic toxicity data for each waste were obtained from different taxonomic groups: primary producers (three plant species), consumers (collembolans, enchytraeids, and earthworms), and decomposers (microorganisms). The acceptable effect level in this study was defined as the EC20 rather than NOEC for each endpoint for two main reasons. First, ECx is more reliable than NOEC, given the higher statistical robustness of the first approach (Moore & Caux 1997, Jager et al. 2006). Second, a 20% reduction of an endpoint may be considered a realistic value of maximum tolerable inhibition, given that several authors have reported that NOEC values are equivalent to a response inhibition of 5 to 30% with respect to the control (Hoeckstra & van Ewijk 1993, Pack 1993, Moore & Caux 1997).

Among the available approaches to the PNEC estimation, we selected the species sensitivity distributions method according to Aldenberg & Jaworska (2000). This method assumes that the acceptable effect level (sensitivity) of the different species in an ecosystem follows a probability function called "species sensitivity distribution". Then, from a limited number of species, and assuming that they are a random sample of the whole ecosystem, an acceptable effect level for all the ecosystem's species can be estimated (Van der Hoeven 2004). PNEC values for each waste were estimated from the set of chronic toxicity values (EC20) of the different bioassays obtained for each waste by means of the software ETX 2.0 (Van Vlardingen et al. 2004). After checking the data

normality, the program calculates a normal distribution of the entered toxicity data and provides the species sensitivity distribution (SSD). From this distribution, the program calculates the hazardous concentration (HC5) and its two-sided 90% confidence interval, which is selected in this study to represent the PNEC. The HC5 is the estimated 5th percentile of the distribution, which represents the concentration expected to be protective of the 95% of the species of an ecosystem. PNEC values obtained in the laboratory (g kg^{-1}) were converted to amendment rates in agricultural soils (t ha^{-1}) assuming an ideal agricultural soil with a 20 cm plough layer, and a density of 1.25 g cm^{-3}.

5.2.8.2. PEC estimation and risk characterization

The predicted environmental concentrations (PEC) were also estimated assuming an ideal soil with a 20 cm plough layer, and a density of 1.25 g cm^{-3}. Different PEC values were determined according to several scenarios. A first group of scenarios was based on different agronomical demands of N and P for different crops obtained from Johansson et al. (1999). More precisely, we estimated for each waste the amendment rate that supply 100 kg N ha^{-1} (oat and spring barley), 150 kg N ha^{-1} (wheat), 10 kg P ha^{-1} (oats, spring wheat, spring barley, and winter wheat), and 20 kg P ha^{-1} (peas, and sugar beet). Finally, the last scenario was based on the median amendment rate in the European Union (3 t ha^{-1}) according to European Commission (2001). Then, for each waste and scenario, the ecological risk quotient was calculated (RQ=PEC/PNEC). Risk was considered as acceptable if RQ was below 1.

5.2.9. Relationship between waste parameters and PNEC values

The contribution of the waste composition (Table 5.2) to the estimated PNEC in the different wastes was assessed by means of Pearson correlation of the PNEC values with the concentration of each individual pollutant, the sum of heavy metal concentrations, the

sum of organic pollutant concentrations, the sum of persistent organics (PAH, PCB, and PCDD/F), the sum of non-persistent organics (DEHP, LAS, and NPE), the sum of all pollutant concentrations, and each physico-chemical parameter. The correlations were calculated with the log-transformed values using SPSS 13.0.

5.3. RESULTS

5.3.1. Wastes toxicity

Plant, enchytraeid, and collembola tests and the assessed endpoints were sensitive to wastes, as they were inhibited with increasing waste concentration (Table 5.3). On the contrary, no inhibition of the soil substrate-induced respiration (SIR) was observed for any waste, with the exception of pig slurry (SLT) at day 1. For most wastes, respiration was enhanced as waste concentration increased then preventing the PNEC calculation. The reproduction of the earthworm *E. andrei* was not used either for PNEC derivation, since the number of juveniles in controls was below 30 in most wastes, which is not acceptable according to the ISO 11268-2 (1996). Effects on fresh weight after 28 days were also discarded since no significant inhibition was found, and due to the high variability between replicates. On the contrary, effects on fresh weight after 14 days of exposure were suitable for PNEC derivation for most of wastes.

All the bioassays and endpoints (lethal or sublethal) were significantly correlated among them (Pearson $p<0.05$). The exception was EC20 for reproduction in *E. crypticus*, not correlated with *F. candida* mortality (LC20) and reproduction (EC20) neither with EC20 for germination in *B. rapa* and *L. perenne*. Even more remarkable is the lack of significant correlation of EC20 for reproduction in *F. candida* with the results in the remainder bioassays. Finally, LC20 in *F. candida* was uncorrelated with its EC20 for reproduction, and also with the *E. crypticus* EC20 for reproduction.

Table 5.3. Lethal concentrations (LC20) and effective concentrations (EC20) expressed as g kg^{-1}, with their 95% confidence intervals, for each waste. Values in *Eisenia andrei* for fresh weight corresponded to NOEC. Blank cells indicate a lack of inhibition. For waste abbreviations see Table 5.1.

Species	Endpoint	AEC	AED	AET	ANC	AND	ANT	SLT
Microorganisms	Respiration	-	-	-	-	-	-	1.6 (1.5, 1.7)
Eisenia andrei	Fresh weight	158	-	-	158	63.1	25.1	10
Eisenia andrei	Survival	11.2 (7.2, 17)	0.48 (0.45, 0.52)	0.49 (0.49, 0.5)	12.3 (6.0, 24.6)	1.0 (0.5, 1.7)	0.5 (0.2, 0.9)	0.5 (0.4, 0.6)
Enchytraeus crypticus	Reproduction	551 (248, 1221)	1.4 (0.9, 2.0)	5.8 (0.8, 25.2)	98.2 (44.5, 215)	1.6 (1.2, 2.2)	17.5 (12, 25.2)	2.3 (1.5, 3.5)
Enchytraeus crypticus	Survival	551 (248, 1221)	23.8 (10.2, 54.0)	10.7 (4.0, 26.7)	320 (137, 746)	23.8 (8.8, 62.0)	73.5 (36.1, 148)	11.32 (8.3, 15.2)
Folsomia candida	Reproduction	26.3 (4.5, 134)	7.9 (5.8, 10.8)	1.1 (0.7, 1.5)	12.1 (5.6, 25.2)	14 (10.8, 18.0)	6.7 (4.5, 9.9)	18.1 (6.4, 48.4)
Folsomia candida	Survival	210 (80.6, 546)	34.7 (26.2, 46.0)	31.6 (24.7, 40.3)	665 (384, 1152)	114 (97, 133)	63.3 (50.3, 79.5)	17.7 (13.6, 23)
Brassica rapa	Emergence	193 (161, 230)	11.6 (5.8, 19.5)	16.4 (13.0, 20.1)	585 (535, 639)	58.9 (27.4, 117)	13.4 (7.6, 21.2)	7.7 (6.1, 9.5)
Brassica rapa	Growth	206 (91.1, 453)	8.1 (6.5, 9.8)	18.5 (6.4, 39.3)	586 (535, 643)	74.5 (55.5, 98.8)	39.5 (25.9, 58.3)	18.6 (14.8, 22.8)
Lolium perenne	Emergence	203 (197, 209)	26.8 (21.4, 33.1)	28.4 (21.4, 33.1)	612 (541, 693)	75.8 (35,5, 129.6)	35.1 (29.4, 62.3)	8.7 (6.5, 11.23)
Lolium perenne	Growth	223 (217, 228)	13.6 (10.9, 16.6)	19.0 (15.9, 22.4)	594 (445, 791)	72.4 (54.8, 108)	33.3 (28.6, 38.6)	17.3 (14.44, 20.59)
Trifolium pratense	Emergence	161 (128, 203)	16.7 (14.1, 19.6)	15.5 (11.3, 20.5)	367 (313, 431)	16.3 (10.3, 24.0)	17.7 (14.5, 21.4)	4.8 (1.69, 4.14)
Trifolium pratense	Growth	183 (173, 192)	13.1 (9.8, 17.0)	15.6 (13.1, 204)	177 (154, 204)	14.1 (10.2, 18.7)	17.1 (13.3, 21.5)	4.5 (0.9, 11.2)

5.3.2. Risk characterization

Derived PNEC (HC5) for each waste and PEC values in the different scenarios are presented in Table 5.4. Highest PNEC values were found for composted sludges. Aerobic dewatered sludge and aerobic thermally dried sludge were the most toxic.

The data for the different fertilization scenarios, show that if the amendment rates of the studied wastes were based on N and P crop demands, rates of all wastes would usually be below 3 t ha^{-1}, which is the median amendment rate in Europe (Table 5.4).

Table 5.4. Estimated PNEC (HC5, the hazardous concentration protecting 95% of the species) and PEC values for each waste, expressed as tonnes of waste (dry weight) per hectare of soil. Low and high crop demands of N and P from Johansson et al. (1999). Median amendment rates from European Commission (2001). For waste abbreviations see Table 5.1.

		PEC				
Waste	**PNEC**	Low N demand (100 kg N ha^{-1})	High N demand (150 kg N ha^{-1})	Low P demand (10 kg P ha^{-1})	High P demand (20 kg P ha^{-1})	Median in EU
AEC	109.6	2.53	3.80	0.68	0.91	3
AED	5.4	1.60	2.40	0.74	0.98	3
AET	4.7	1.65	2.48	0.73	0.98	3
ANC	67.9	4.22	6.33	0.52	0.70	3
AND	6.9	2.58	3.87	0.45	0.60	3
ANT	17.6	1.88	2.81	0.51	0.68	3
SLT	5.8	1.60	2.40	0.74	0.98	3

It is also of worth noticing that the derived PNEC values were significantly correlated with the toxicity results obtained in most of the bioassays (Pearson $p<0.05$), showing that any of them is "ecologically" relevant of the relative ecotoxicity differences among

wastes. The only exception was reproduction in *F. candida*, which was not correlated with PNEC values.

Risk quotients for the different scenarios indicated that no harmful effects on soil ecosystems were likely to occur if wastes were applied according to crop demands of N or P (Table 5.5). Furthermore, using the median amendment rate in Europe (3 t ha^{-1}), no risk for soil ecosystems should be expected for the studied wastes.

Table 5.5. Risk quotient (RQ=PEC/PNEC) for each waste and scenario, expressed as tonnes of waste in soil-waste mixture per hectare of soil (dry weight). Risk is acceptable when RQ is below 1. For waste abbreviations see Table 5.1.

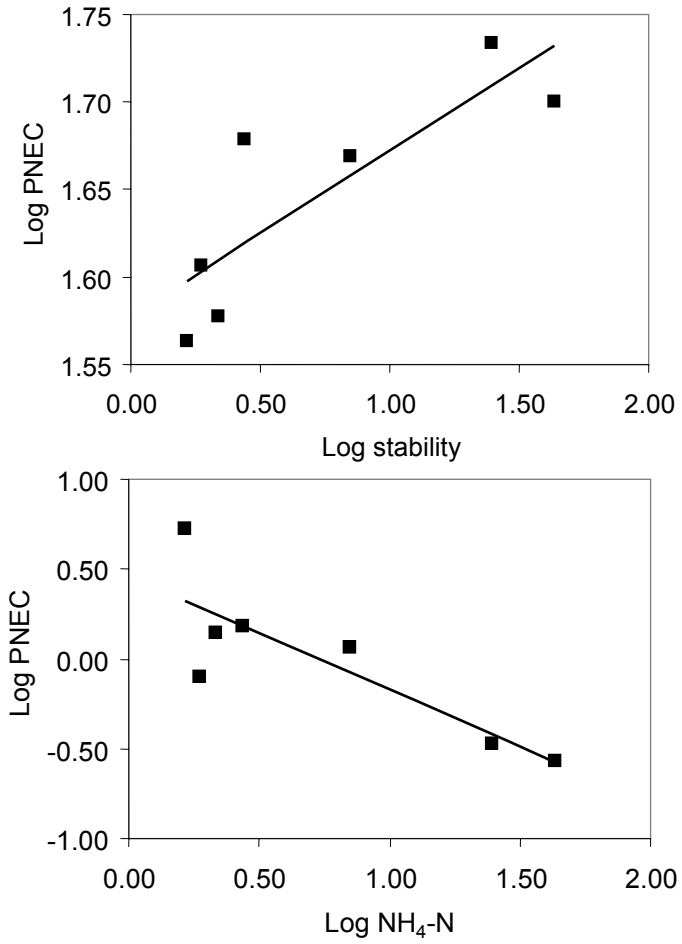
Waste	Low N demand (100 kg ha^{-1})	High N demand (150 kg ha^{-1})	Low P demand (10 kg ha^{-1})	High P demand (20 kg ha^{-1})	Median in EU
AEC	0.02	0.03	0.01	0.01	0.03
AED	0.30	0.44	0.14	0.18	0.55
AET	0.35	0.53	0.16	0.21	0.64
ANC	0.06	0.09	0.01	0.01	0.04
AND	0.38	0.56	0.07	0.09	0.44
ANT	0.11	0.16	0.03	0.04	0.17
SLT	0.27	0.41	0.13	0.17	0.52

5.3.3. Relationship between waste parameters and PNEC values

No significant relationships were found between concentrations of single pollutant or pollutant group and PNEC. On the contrary, there was a significant positive correlation between a waste's stability and PNEC ($r = 0.838$, $p = 0.019$), a significant negative correlation between ammonium content and PNEC ($r = -0.837$, $p = 0.019$), and a marginal negative correlation between PNEC and hydrolysable nitrogen ($r = -0.754$, $p = 0.05$) (Figure 5.1).

The lack of correlation with pollutant burden and the general correlation of toxicity with parameters related to waste's stability has also been found for most of the bioassays and endpoints (Pearson $p<0.05$). The only exception was the reproduction inhibition in *F. candida*, not correlated with any of these properties, indicating that properties might be also influencing the toxicity in these species.

Figure 5.1. Correlation of log-transformed values of stability and ammonium content in wastes with PNEC, the last expressed as g kg^{-1} of waste in soil.



5.4. DISCUSSION

5.4.1. Quality assessment of organic amendments in Europe

Several external inputs (mineral fertilisers, organic amendments, microbial inoculants, and pesticides) are applied to agricultural soils to maximise productivity and economic returns. However, side effects of such amendments on soil organisms are not usually taken into account (Bünemann et al. 2006).

The current transposition to some member states of the European Union legislation concerning sewage sludge and organic wastes which are considered as fertilizers, allows and encourages the reuse in agricultural land of organic wastes with low pollutant content. More precisely, the heavy metals content is taken into account both for sewage sludges (Directive 86/278/EEC), and organic wastes considered as commercial fertilizers (depending on the exact transpositions to the member states of the Regulation (EC) No 2003/2003). The nitrogen content of wastes may also limit these amendments in vulnerable zones (Directive 91/676/EEC). However, it is widely accepted that chemical methods have important limitations to predict waste effects on soil organisms (Crouau et al. 2002). They do not account for all current potential pollutants in wastes, and give no indications about bioavailability, interactions between pollutants, secondary products or final effects on the soil dwelling organisms and soil ecosystem.

Hence, the main problem is not the use of wastes in soil per se, but the lack of ecologically relevant methodologies to monitor the quality and environmental safety of wastes when used as amendments, given the soil limited resistance to pollution (Kördel and Römbke 2001). For this reason, ecotoxicological criteria should be included, in addition to chemical methods, for monitoring organic waste quality.

5.4.2. Use of test batteries in risk assessment

Contaminants, or mixtures of contaminants, have markedly differential effects on the populations of different soil-dwelling species. This highlights the importance of including different species in a battery of bioassays for any ecological risk assessment (Van Gestel et al. 2001).

Test batteries have been widely used in aquatic ecotoxicology (Davoren et al. 2005, Mariani et al. 2006), but are still scarce in soil ecotoxicity evaluation. This approach has been used to evaluate polluted sites (Achazi 2002), to assess the effectiveness of remediation treatments (Mendonça & Picado 2002, Molina-Barahona et al. 2005), to provide data for derivation of non harmful chemical concentrations in soil (Lock & Janssen 2001a, Lock & Janssen 2001b, Lock & Janssen 2002a, Lock & Janssen 2002b, Sverdrup et al. 2002, Lock & Janssen 2003, Kuperman et al. 2004, Kuperman et al. 2006, Römbke et al. 2006) and to assess sewage sludge quality (Renoux et al. 2001, Robidoux et al. 2001, Selivanovskaya & Latypova 2003).

There are no universal rules for suitable species selection, and furthermore, there is no agreement about the most suitable endpoints to predict effects on ecosystems. For some ecologists, maintenance of the ecosystem structure is the main aim, and any loss in population size, species diversity or genetic diversity is detrimental. For others, changes in the ecosystem structure are not critical if functions are preserved. Both approaches have been experimentally evaluated, and also the linkage between them (Wentsel et al. 2003). Hence, the use of a set of endpoints reflecting both structural and functional effects of pollutants on ecosystems would be the best choice. McMillen et al. (2003) recommend a minimum test battery including tests with plants, soft-bodied invertebrates and soil arthropods together with tests designed to assess ecosystem functions such as decomposition or nitrification. Based on this set of species and endpoints, a coarse vision of potential effects on the ecosystem structure and function is possible. Achazi (2002), concluded that a proper assessment of soil pollution was achieved using a test battery

using both aquatic and soil tests (microbial activity and ammonium oxidation, earthworm and collembolan reproduction, and plant germination and growth). In the present study, different bioassays were selected in order to take into account both functional (microbial respiration) and structural endpoints (the performance of several soil organism). Plant emergence and growth, collembolans and enchytraeids reproduction, and earthworm fresh weight showed different responses to wastes. Microbial respiration was not inhibited, so was not useful as an endpoint (Table 5.3).

5.4.3. Species sensitivity distribution in risk assessment of organic wastes

The species sensitivity distribution (SSD) is increasingly used to complement or replace arbitrary assessment factors in chemicals risk assessment (Grist et al. 2002). Its application is recommended by public organisations in Denmark, The Netherlands, and Canada (Jensen et al. 2001) and also, recently, by the European Union (European Commission 2003). This methodology was initially developed by Kooijman (1987), and has been modified and improved by several authors (Aldenberg & Slob 1993, Jagoe & Newman 1997, Wagner & Løkke 1991, Aldenberg & Jaworska 2000, Newman et al. 2000, van der Hoeven 2001, Grist et al. 2002, Posthuma et al. 2002). The method uses acceptable pollutant effect levels (LCx, NOEC, ECx) for a chemical for a limited number of species to determine an exposure level or concentration below which the ecosystem species will not suffer unacceptable damages. The method assumes that the available set of toxicity data for different species is randomly drawn from all species potentially present in the ecosystem (Van der Hoeven 2004).

There are several limitations of the SSD methods for deriving soil quality criteria or assessing ecological risk. The input data are obtained in the laboratory and from a limited number of species and not randomly selected from an ecosystem (van der Hoeven 2004), and only representative of those easily bred in the laboratory (Duboudin et al. 2004). The data sets generally do not reflect the species proportion among trophic

groups (Forbes et al. 2001, Forbes & Calow 2002). The selected exposure time of organisms is arbitrary (Jager et al. 2006), and the endpoints used in different organisms and taxonomic distances varied according to the study (Wagner & Løkke 1991, Newman et al. 2000, Jager et al. 2006). In addition, different HC5 values can be derived depending on the SSD methodology used and on the sample size (Wagner & Løkke 1991, Newman et al. 2000, Duboudin et al. 2004). With respect to the ecological relevancy of estimations carried out with this method, it has been indicated that measures of acceptable effect level (LCx, NOEC, ECx) can not be extrapolated to population and community effects (Newman et al. 2000), and that protecting 95% of the species is an arbitrary decision (Aldenberg & Jaworska 2000). The resulting HC5 value may not be suitable in ecosystems containing a pollution-sensitive dominant or keystone species (Newman et al. 2000), and furthermore, SSD methods do not take into account the modifying influence on the observed toxicity of biotic and abiotic interactions acting in real ecosystems (Wagner & Løkke 1991, van Straalen & Bergema 1995).

Some experimental studies have indicated a lack of ecological relevancy of SSD methodologies (Roessink et al. 2006), but despite all their potential drawbacks, several authors have pointed out that statistical extrapolation methods may produce reliable results if sufficient data are available and if methods are validated at the ecosystem level (Wagner & Løkke 1991, Forbes et al. 2001). Furthermore, most of the comparative studies have showed that the hazardous concentration values calculated using SSD for single-species in the laboratory lead to harmful concentrations similar to that observed in field studies at the community and ecosystem levels (Sloof et al. 1986, Versteeg et al. 1999, Smit et al. 2002, Hose & van den Brink 2004, Schroer et al. 2004).

In the present study, the safe amendment rates derived from SSD according to methodology of Aldenberg & Jaworska (2000), showed contrasting values for the different studied wastes, indicating its suitability for comparative purposes (Table 4).

However, its use for the prediction of harmful effects in real situations can not be confirmed as we lack a field validation of the predictions of this study.

5.4.4. Relevancy of the estimated safe amendment rates

According to the risk quotient determined in our study for different scenarios (Table 5.5), none of the wastes is expected to exert noxious effects on the soil biota if applied at realistic amendment rates. If amendment is based on N or P crop demands or in the 3 t ha^{-1} amendment rate for agriculture in the EU, risk quotients are generally below 0.5, that is, the amendment rate are usually 50% lower than the safe amendment rate.

Despite this, safe amendment rates (PNEC) of the most toxic wastes (AET, AED, and SLT), around 5 t ha^{-1}, are not very different from the usual European amendment rates. AET and AED should not be currently used, according to Directive 86/278/EEC, given their high Pb concentration, but SLT could be used on soils despite its predicted toxicity. The high risk of pig slurry amendments with respect to other wastes agrees with the results of Diez et al. (2001), who indicate negative effects of pig slurry amendment at a 3.6 t ha^{-1} (dry weight) on *F. candida* reproduction in laboratory. On the contrary, they did not found significant inhibition in plants and enchytraeids.

A non exhaustive selection of studies on the ecotoxicological effects of organic waste amendments on soil biota is presented in Table 5.6. As a general pattern, no harmful effects on crops and soil biota have been reported when wastes are applied below or around the usual EU amendment rates (3 t ha^{-1}). On the contrary, noxious effects of sewage sludge to soil biota have been reported above 20 t ha^{-1} (Andrés 1999, Barrera et al. 2001, Andrés & Domene 2005). Other studies have reported effects at lower amendment rates (8.6 t ha^{-1} in Krogh & Pedersen 1997), and also bioaccumulation in earthworms at even lower concentrations (Matscheko et al. 2002). The magnitudes of amendments causing harm to soil biota (Table 5.6) are in accordance with results from the present study. On the other hand, it is noticeable safe amendment rates of the

composted sludges in this study (with predicted safe amendment rates between 68 and 110 t ha^{-1}) are much greater than the amendment rates based on the crop demands. Despite this, these conclusions are only valid for the short-term, as they are based on one-month studies at most with a limited number of species. Furthermore, the toxicity data used for deriving the safe amendment rates were obtained in laboratory using OECD artificial soil and require a field validation.

5.4.5. Relationship between waste parameters and safe amendment rates

In our study, no significant correlations were found between pollutant concentration and safe amendment rates. This finding indicates the failure of chemical methods in predicting effects on organisms and the need for including ecotoxicological criteria in legislation. It is worthy of notice that waste stability and ammonium content are the most influential determinants of the maximum amendment rates derived in this study (Figure 5.1). The more stabilized a waste, the lower is its ammonium content, the lower is its toxicity and the higher the safe amendment rate. The coupled behaviour of both parameters (stability and ammonium content) is not casual, since waste stabilization implies a higher recalcitrant organic matter content and a lower amount of hydrolyzable nitrogen and of ammonium release (Witter & Lopez-Real 1988, Martins & Dewes 1992). During decomposition of wastes in soil, nitrogen losses are initially mainly as ammonium and ammonia. This also explains the marginal correlation between hydrolysable nitrogen and safe amendment rates. These results agree with published reports phytoxicity of amendments with non stabilized organic wastes (Zucconi et al. 1981, Pascual et al. 1997, Atiyeh et al. 2000, Huang et al. 2004, Zmora-Nahum et al. 2005) and specifically attributed to ammonium (Katayama et al. 1985). This pattern has also been indicated for soil fauna after organic amendments (Neher 1999) or application of nitrogen fertilizers (Seniczak et al. 1994).

Table 5.6. Reported effects of organic wastes on soil biota obtained from laboratory and field studies. When explicit information was not available in the reference, waste concentrations were converted to an equivalent field amendment rate assuming an ideal soil with a 20 cm plough layer, and a density of 1.25 g cm^{-3}.

Reference	Site	Waste	T/ha DM	Effect on soil biota
Renoux et al. (2001)	Laboratory	Sewage sludge	≥20	Noxious effects on plants (*Hordeum vulgare, Lactuca sativa*) and earthworms (*Eisenia andrei*).
Andrés & Domene 2005	Laboratory	Sewage sludge	9-23	Decrease in faunal density and disturbance of trophic structure.
Andrés (1999)	Restored land	Sewage sludge	375	Impoverishment of the community structure and decrease in the soil oribatid diversity.
Barrera et al. (2001)	Restored land	Sewage sludge	187.5-375	Increase in the adult and juvenile density of two earthworm species (*Allobophora chlorotica*, *Nicodrilus caliginosus*).
Diez et al. (2001)	Laboratory	Pig slurry	>3.6	Significant decrease in the reproduction of the collembolan *Folsomia candida*.
Krogh & Petersen (1997)	Agricultural land	Cattle manure, sewage sludge	3.5-21	No harm to crops, microarthropods, or earthworms.
Krogh & Petersen (1997)	Laboratory	Cattle manure, sewage sludges	8.6-25.2	Effects on reproduction of *Folsomia fimetaria*.
Matscheko et al. (2002)	Agricultural land	Sewage sludge	≥1	PBDE and PCB bioaccumulation in earthworms.
Pernin et al (2006)	Laboratory	Sewage sludge spiked with copper	150	Noxious effects on the mesofauna community structure.
Petersen et al. (2003)	Agricultural land	Sewage sludge, household compost	3.6-14.9	No harmful effects on crops.

Results from this study point out the importance of stabilization treatments like composting prior to the use of organic wastes in soils, as decomposition of low stabilized wastes generate noxious substances like ammonia, phenols, and organic acids (Déportes et al. 1995). The relationship between these parameters and the level of toxicity may be strong enough in the short-term to exceed and mask the differences in pollutant burden existing between of different wastes.

CONCLUSIONS

The species sensitivity distribution method, as used in the present work, is suitable for comparative purposes of the risk assessment of organic wastes, as demonstrated by clearly differentiated results for different wastes. Predictions for real field situations should to be validated by empirical validation. If the predicted safe amendment rates of the studied wastes are realistic, the usual amendment rate in Europe (3 t DM ha^{-1}) and amendments based on the N and P crop demands are safe. Despite this, some wastes of this study may produce harmful effects if applied only slightly above 5 t DM ha^{-1}.

The toxicity of waste, and therefore the safe amendment rate, is not related mainly to its pollutant burden, at least in the short-term, but primarily to its lack of stability and to noxious compounds such as ammonium, which is released during decomposition of waste in soil. Waste stabilization appears in this study as a suitable treatment to decrease the short-term impact of organic waste on soil biota, therefore to allow its safe reuse application to soil.

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Chapter 6

Soil properties influence on the avoidance behaviour and reproduction of *Eisenia andrei*, *Enchytraeus crypticus*, and *Folsomia candida*

ABSTRACT

Twelve natural soils from three Mediterranean countries of contrasting properties and OECD artificial soil were tested for their influence in ecotoxicological avoidance and reproduction tests of three soil organisms: the earthworm *Eisenia andrei*, the enchytraeid *Enchytraeus crypticus*, and the collembolan *Folsomia candida*.

When used in avoidance tests with natural soils as control, the OECD artificial soil was problematic, as it may highly influence the test species behaviour. On the contrary, reproduction was similar using OECD artificial soil and natural soils.

Generally, avoidance behaviour and reproduction were influenced by the same soil properties, although the properties affecting these endpoints were different depending on the species. *E. andrei* avoided soils with low organic matter levels and fine texture and showed low reproduction in the less organic soils. *E. crypticus* evaded soils with high levels of silt and clay, which also caused the lowest reproduction, together with the soils presenting high pH and CEC values. Finally, *F. candida* preferred fine textured soils and showed the lowest reproduction in sandy soils and in soils with high nitrogen levels.

6.1. INTRODUCTION

Reproduction is one of the most commonly used endpoints in addition to mortality. This arises from the fact that this endpoint is more sensitive than mortality, as chemical concentrations causing slight disturbance at the biochemical and physiological level are able to be transferred through a cascade of processes to effects on reproduction outcome (Gerhardt 1996). Since any change in the energy allocation at the individual level might be translated to effects at the population level, this endpoint it is considered as ecologically relevant (McCauley et al. 1990, Barata and Baird 2000, Kooijman 2000, Muller and Nisbet 2001, Maltby et al. 2001, McWilliam and Baird 2002a, Irving et al. 2003). However, the use of reproduction as endpoint requires considerable effort in terms of time and handling, and this is why, in recent decades, alternative endpoints which provide similar information with less experimental effort have been proposed. Among them, avoidance tests are one of the most usual, although they have been mainly applied to aquatic environments. However recently they have been used for soil ecotoxicity (Natal-da-Luz et al. 2004), most of the attempts being carried out with earthworms (Yeardley et al. 1996, Slimak 1997, Stephenson et al. 1998, Hund-Rinke and Wiechering 2001, Reinecke et al. 2002, Hund-Rinke et al. 2002, Hund-Rinke et al. 2005, Loureiro et al. 2005, Lukkari and Haimi 2005). This has led to the development of a standardized protocol for earthworms (ISO 17512-1:2005). On the contrary, such methods have been only scarcely used with other soil organisms like enchytreids (Rüther and Greven 1990, Sjögren et al. 1995, Salminen and Sulkava 1996, Salminen and Haimi 2001, Achazi et al. 1999, Amorim et al, 2005d), collembolans (Natal-da-Luz et al. 2004, Natal-da-Luz 2005), and isopods (Loureiro et al. 2005, Zidar et al. 2005), despite knowledge about the existence of avoidance responses to pollution in these groups. Avoidance tests are at least as sensitive as reproduction, have a shorter duration, and are easier to perform than the existing acute or reproduction tests

(Yeardley et al. 1996, Stephenson et al. 1998, Natal-da-Luz et al. 2004, Amorim et al. 2005d, Hund-Rinke et al. 2005).

Most of the soil ecotoxicological tests are carried out using OECD artificial soil (OECD 1984), and natural German soil LUFA 2.2 (Schinkel 1985) which allows standardization between laboratories and comparison of the test results from different species (Smit et al. 1998). However, the use of standardized soils is not representative of realistic situations, since it is known that soil properties have an important influence on pollutants bioavailability and toxicity. Hence, the use of natural soils in risk assessments of pollutants or wastes is always advisable if the aim is to generate representative data for real situations.

Soil properties not only influence the fate and bioavailability of pollutants, but also the behaviour of the test species (Römbke et al. 2006). The influence of soil parameters in reproduction has been widely reported in the literature (Van Gestel et al. 1992, Sandifer and Hopkin 1996, Gestel and van Diepen 1997, Crouau et al. 1999, Pedersen et al. 1997, Greenslade and Vaughan 2003, Jänsch et al. 2005a, Amorim et al. 2005a, Amorim et al. 2005b, Amorim et al. 2005c, Römbke et al. 2006), but less is known about their effects in avoidance tests. However, in both reproduction and avoidance tests, the use of controls should correct the impact of such influences. Nevertheless, , in certain circumstances, soil properties might influence the test organisms to such an extent to distort the observed toxicity: 1) In acute or reproduction tests with natural soils, when the soil is far from the requirements of the test species, toxic stress might be magnified by the unsuitable soil properties, overestimating the toxicity (Højer et al. 2001); 2) In the risk assessment of polluted sites, where there is a lack of control, the properties of the soil selected as control, usually an adjacent soil or a standard soil, might provide biased conclusions when its pedological properties are different from those in the test soil (Hund-Rinke and Wiechering 2001, Crouau and Cazes 2005); 3) In the avoidance tests with chemicals, the lack of homogeneity in the test soil can produce small differences in soil

properties that might distort or overcome the differences in toxic burden, producing biased results.

The use of natural soils is advisable to provide more relevant results for real situations, but the control soil has to be accurately chosen both to approach the properties of the soil that has to be assessed and to satisfy the biological requirements of the test species.

The main aim of this study is to assess the influence of a set of natural soils on the avoidance behaviour and reproduction of three commonly used test species (*Eisenia andrei, Enchytraeus crypticus*, and *Folsomia candida*).

6.2. METHODS

6.2.1. Soil selection and characterization

Soils came from three Mediterranean regions: 4 from Alentejo (Portugal), 5 from Catalonia (Spain), and 3 from Liguria (Italia). Furthermore, OECD artificial soil was also used in order to compare it with outcomes in natural soils.

The available pedological information in each region (Cardoso 1965, Cardoso et al. 1973, DARP 2005, Scarin 1971), was used to select the soils, using as main criteria their contrasting properties, and coming from agricultural, shrubland or forest ecosystems. In agricultural soils, those without or with low agrochemical treatments were preferred.

Topsoil samples (0-20 cm depth) were collected, 5-mm sieved, and air-dried. Then soils were defaunated alternating two freezing-thawing cycles consisting of placing soils in at -20ºC for 4 days followed by 4 days at 20ºC. Soils were analysed by current agronomic methods, and heavy metals were also measured to ensure that their concentrations were below those expected to affect the test species. Main properties of the studied soils are presented in Table 6.1.

6.2.2. Test organisms

The earthworm *Eisenia andrei* (Oligochaeta:Lumbricidae), the enchytraeid *Enchytraeus crypticus* (Oligochaeta:Enchytraeidae) and the collembolan *Folsomia candida* (Isotomidae:Collembola), were used as test species. All these species have been widely used in soil ecotoxicity studies given the existence of standardized protocols.

The individuals of *E. andrei* came from the cultures of the Laboratory of Soils of the Instituto do Ambiente e Vida of the University of Coimbra (Portugal). They were bred in a 1:1 (v/v) mixture of peat and horse dung, with pH adjusted around 6.5 by addition of $CaCO_3$, and at 70% of the mixture maximum water holding capacity (WHC). Cooked oat flakes were provided as food to cultures every two weeks. All adults were transferred to a new culture substrate every two months to leave a synchronized aged culture in the old substrate.

The initial strain of *E. crypticus* was provided by ECT GmbH (Flörsheim, Germany). The individuals were cultured in Lufa 2.2 soil, with water content around 70% of the WHC and pH around 6.0. Ground oat was added as food twice a week. Culture substrate was renewed periodically when overcrowding was observed, transferring a few individuals to new substrate.

The individuals of *F. candida* were obtained from cultures of the Instituto do Ambiente e Vida. Cultures were raised in a wet plaster of Paris and charcoal mixture (11:1, w/w). Individuals were fed weekly with granulated dry yeast.

The cultures of all the species were raised in a climatic chamber at constant temperatures 20±2ºC and a 16:8 h (light:dark) photoperiod.

Table 6.1. Properties of the soils. Texture = coarse sand (2 - 0.2 mm) / fine sand (0.2 - 0.02 mm) / silt (0.02 – 0.002 mm) / clay (< 2 µm); Corg = organic carbon; Ntot = total nitrogen; Nmin = mineral nitrogen; C/N = carbon/nitrogen ratio; CEC = cationic exchange capacity; WHC = maximum water holding capacity. The values are expressed as percentage and are referred to the dry matter. Soils came from three mediterranean regions: Alentejo (BR, LIT, LUV, PZ), Catalonia (GAN, GRA, POR, PRA, RIU), and Liguria (IT2, IT3, IT4).

Soil	pH (H_20)	pH (KCl)	Texture	Corg	Ntot	Nmin	C/N	CEC	WHC
			%	%	%	ppm		Cmol/Kg	%
BR	7.6	6.9	10 / 17.9 / 23.6 / 48.5	1.5	0.11	7	13.2	26.8	61.1
GAN	8.3	7.7	1.5 / 74.5 / 12 / 12	0.4	0.03	28	8.8	6.0	37.6
GRA	8.2	7.6	2.1 / 25.7 / 48.5 / 23.7	1.0	0.04	42	9.0	14.2	49.8
IT2	7.7	7.4	23.6 / 15.7 / 44.4 / 16.3	2.8	0.13	67	11.3	18.6	39.8
IT3	7.7	7.3	23.1 / 28.1 / 36.4 / 12.4	1.6	0.25	56	10.1	18.4	43.3
IT4	7.9	7.3	20.2 / 29.1 / 34.7 / 16	1.6	0.16	42	12.5	18.8	47.4
LIT	5.2	4.6	41.9 / 24.8 / 21.6 / 11.7	2.4	0.08	49	15.3	8.6	42.4
LUV	5.5	4.4	29.8 / 38.2 / 20.3 / 11.3	1.2	0.16	18	14.5	9.9	32.1
OECD	7.0	6.1	9.7 / 76.9 / 2.7 / 10.7	3.4	0.07	28	112.1	7.0	75.0
POR	6.9	6.6	46.2 / 21.4 / 20.5 / 11.9	2.5	0.11	56	11.3	18.6	38.8
PRA	5.1	4.5	42.4 / 35 / 12.1 / 10.6	1.3	0.22	112	10.7	11.2	39.4
PZ	5.3	4.2	69.8 / 21.3 / 5.8 / 3.2	1.3	0.13	7	18.3	4.0	30.7
RIU	7.3	6.7	23.7 / 34.9 / 13.8 / 27.6	1.1	0.12	42	8.5	14.9	45.0

6.2.3. Soil preparation

Suitable water content was provided to each soil in order to give a moist and crumbly substrate. For most soils, this content equals between 40 to 60% of WHC. In the clayey and silty soils (BR, GRA, RIU) the water content was adjusted to around 35-45% of the WHC, since higher water content made a doughy soil structure.

6.2.4. Avoidance tests

The general principle of this assay is to prepare 5 replicates consisting of a container filled with two adjacent soil portions, each occupying half the container (Figure 6.1). Then a fixed number of individuals of the test species is placed in the centre of the container, and left under controlled climatic conditions for 48 h (20±2ºC and 16:8 h light:dark photoperiod). After this period, the number of individuals is determined in each soil portion.

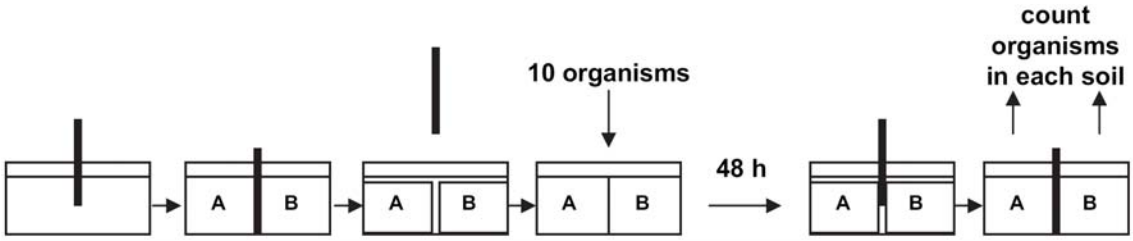


Figure 6.1. General framework of the avoidance tests (reproduced from Loureiro et al. 2005).

In the present study, two different assays were carried out: a) control-dual tests, where both portions were composed of the same soil, with the aim of determining if the individuals' distribution is affected by factors other than soil properties (Yeardley et al. 1996, Hund-Rinke and Wiechering 2001); b) avoidance tests, where pairs of different soils within each region were compared, also including the OECD artificial soil. The interest in these assays was to determine the influence of soil properties on the avoidance/preference behaviour of each species.

The avoidance tests for *E. andrei* were based on ISO 17512 (2005). Two portions consisting of 250 g of wet soil were placed in translucent plastic containers (20x12x5 cm). In order to ensure a clear separation between soils, a thin plastic sheet was put in the middle of the container and was removed after depositing the soils. Ten adult worms were placed at the top surface. The container was covered with a perforated lid (to allow aeration and avoid individuals escaping) which was left under constant climatic conditions for 48 h. After this time, each soil portion was removed, and the number of worms was determined. When one individual was found in the center, 0.5 individuals were assigned to each of the sides.

For *E. crypticus*, avoidance tests were based on Amorim et al (2005d). Two portions of 20g of wet soil were placed in translucent cylindrical containers (6x4 cm). Ten adult individuals were placed in the middle of the container, which was closed with a perforated lid and incubated for 48h under constant climatic conditions. After this period, counting was carried out as described for earthworms.

Avoidance tests with *F. candida* were based on Natal-da-Luz et al. (2004). In this case, two 30 g portions of wet soil were placed in translucent cylindrical containers (7x6 cm). 20 individuals were transferred to each container (10 to 12-days old) and incubated for 48 h. At the end of the period, each soil portion was taken separately, poured into a 200-mL container and flooded with water. Soil was gently stirred in order to force the individuals to float on the water surface to enable counting.

6.2.5. Reproduction tests

The reproduction rate of all the species was determined in the natural soils and in the OECD artificial soil according to standardized protocols. For *E. andrei*, the soils that were generally rejected in avoidance tests were not used for reproduction tests (BR, GAN, GRA), as they were clearly unsuitable for the requirements of this species. All the assays

were carried out under controlled climatic conditions (20±2°C and a 16:8 h light:dark photoperiod).

In *E. andrei*, reproduction was based on ISO 11268-2 (1998). For each soil, four replicates were prepared, each consisting of a 100-mL plastic container with perforated lid filled with 500 g (dry weight) of wet soil. In each replicate, 10 clitellated individuals (with maximum age difference of 2 months) were added and left at constant climatic conditions for 28 days. 5 g (dry weight) of cooked oat flakes were added weekly as food source. At the end of this period, adults were removed from soil, and containers were additionally incubated for 28 days. At the end of this period, the number of juveniles was determined by submerging the containers in a water bath at 60°C, which forces the juveniles to appear at the soil surface.

In *E. crypticus*, reproduction was determined according to ISO 16387 (2004), but shortening the test period to only 28 days, according to the modifications for this species suggested by Kuperman et al. (2006), since the former protocol is designed for *E. albidus,* which has a longer life cycle. Furthermore, adults were maintained in the test container until the end of the test, given the fragility and small size of this species. For each soil, 4 replicates were prepared, consisting of 20 g (dry weight) of wet soil in a 150-mL glass sealed flask. Ten clitellated individuals were added to each replicate. Along the experimental period, 0.5 g of ground oat was added as food. At the end of the test period, the number of juveniles was determined according to ISO 16387:2004. Individuals within each replicate were fixed in ethanol 75% with a few drops of Bengal red (1% solution in ethanol). After 12 hours, the individuals showed a reddish colour which enabled their counting. The number of juveniles was determined after soil washing in a 0.25 mm-mesh sieve to remove the smaller fractions of soil and facilitate counting in a Petri dish.

In *F. candida*, reproduction was determined following the ISO 11267(1999). Five replicates were prepared for each soil, consisting of 30 g (dry weight) of wet soil in a

sealed 150-mL glass flask. The test lasted 28 days under constant climatic conditions. 5 mg of granulated yeast were added at the start and after two weeks. At the end of the test period, the soil was poured into a 200-mL container, flooded with water and stirred in order to float the individuals on the water surface. Then, a picture was taken and the number of juveniles was calculated by means of the image treatment software ImageTool 3.0.

6.2.6. Data treatment

All statistical procedures of this study were carried out using SPSS for Windows 13.0 (SPSS Inc., Chicago, USA).

6.2.6.1. Soils comparison

The comparison between soils of this study was performed by means of a principal components analysis (PCA). To carry out the analysis, certain soil variables in Table 6.1 were not considered. More precisely, we excluded from the PCA variables calculated from other variables or those which showed high correlations with other variables (Pearson $r<0.8$). In the latter case we retained the variable with the highest biological sense. Hence C/N ratio and pH (KCl) were not used

In the PCA, we calculated the factor scores of each soil for each principal component, in order to interpret the similitude of composition of the different soils and the main gradients of properties present in the set of soils.

6.2.6.2. Avoidance tests

For all the species tested, significant differences in the distribution of individuals between both sides of the containers were determined by means of the Fisher's exact test (Zar 1999). This procedure allows comparing the observed distribution of individuals with an expected distribution assuming no avoidance as the null hypothesis, as described in

Natal-da-Luz et al. 2004. For the dual-control tests, a two-tailed test was used, which assumes as the null hypothesis an equal distribution of individuals in both sides (no avoidance). For the avoidance tests with pairs of different soils, a one-tailed test was used, assuming as null hypothesis the lack of avoidance, that is to say, that half of the total individuals tested remain in the soil being assessed (test soil). The null hypothesis was rejected for a probability equal or lower than 0.05. Since statistics were carried out only taking into account surviving individuals, no correction of the mortality of individuals in control-dual tests was carried out.

In order to detect which soil properties are mainly responsible for the avoidance patterns, the avoidance rate was calculated for each pair of different soils, calculated by the equation $A = [(C-T)/N]*100$, where A = % avoidance, C = number of individuals in the control soil, T = number of individuals in the tested soil, and N = total number of individuals, as described in ISO 17512 (2005). A positive value indicates avoidance of the test soil with respect to the control, while a negative value indicates that individuals are attracted by the test soil. Then, for each soil combination and soil parameter, the quotient between the parameter value in the test soil and in the control soil was calculated. Next, we constructed a matrix with soil combinations as rows and quotient values as columns. Some columns were eliminated according to the criteria already described. Hence, pH (KCl), C/N ratio and CEC were not used for the analysis. Afterwards, a PCA was performed with the selected variables, and factor scores were determined for each soil combination. To relate avoidance and soil parameters, the avoidance values for each soil combination were correlated with their factor scores in the principal axes, looking for significant correlations.

6.2.6.3. Reproduction tests

Given the high number of comparisons that could be carried out and given the main interest of this study, we only compared reproduction in natural soils versus that in OECD artificial soil. Significant differences were verified by means of the Bonferroni test.

In order to relate the soil properties to the reproduction outcome, a PCA was carried out with the properties of Table 6.1 (excluding pH (KCl) and C/N ratio as previously indicated), and the factor scores were determined for each soil. To relate reproduction with soil properties, the number of juveniles in each soil was correlated with their corresponding factor scores in each principal axis to find any significant correlations.

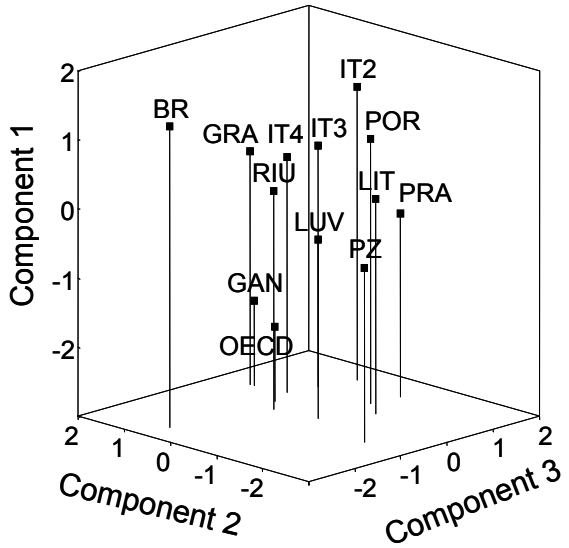
6.3. RESULTS

6.3.1. Soil characterization

PCA discriminated soils according to three main axes which explained 33.6, 28.62 and, 14.7 the variance, respectively. The first axis was associated with the CEC (0.904), silt content (0.767), clay content (0.742), and pH (0.713). The second axis reflected the total nitrogen content (0.778), the fine sand content (-0.774), and the coarse sand content (0.724). The third axis summarised the organic carbon levels (0.936).

The relative positions of the different soils in the three main axes (Figure 6.2) shows a homogeneous cloud of points, with no clearly separated groups, indicating the equilibrated distribution of soil property values among the soils studied. The peripheral position of the BR soil is noteworthy mainly explained by its high WHC, and of the GAN and OECD soils, mainly due to their high fine sand content.

Figure 6.2. Distribution of soils according to PCA factor scores for the three main axes summarizing their soil properties.



6.3.2. Avoidance tests

Mortality during the assay was below 10% in most soil combinations, fulfilling the validity criteria of ISO 17512 (2005). There was no mortality in earthworm tests nor in most of the enchytraeid and collembola tests. However, mortality exceeded the validity criteria in *E. crypticus* (12% in GRA/PRA) and in *F. candida* (GRA/GRA 12%, GAN/PRA 12%, GRA/POR 12%, GRA/PRA 18%, PRA/RIU 20% and PRA/PRA 22%).

Results from dual-control tests indicated that the individuals were distributed at random among the two half-sides of the test containers (Fischer's test $p>0.05$) (Figure 6.3). Hence, no other influence than soil properties may explain any eventual avoidance behaviour in this study.

In most of the avoidance tests using two different soils, the animals' distribution was not random in all the species, showing their sensitivity to soil properties. More precisely, avoidance was observed in 81% of the soil combinations for earthworms (Figure 6.4), in 74% for collembolans (Figure 6.5) and in 71% for enchytraeids (Figure 6.6). It is noteworthy that several soils (BR, GAN, and GRA) were always avoided by *E. andrei*,

who occasionally escaped from the soil and remained in the lid. For this reason these soils were excluded from reproduction assays with earthworms.

When compared with natural soils, the OECD artificial soil was avoided by earthworms in 25% of the combinations, in 33% by enchytraeids, and in 75% by collembolans.

Figure 6.3. Dual-control test results for *E. andrei*. Results are expressed as percent of individuals. (Fisher's exact test, $p<0.05$).

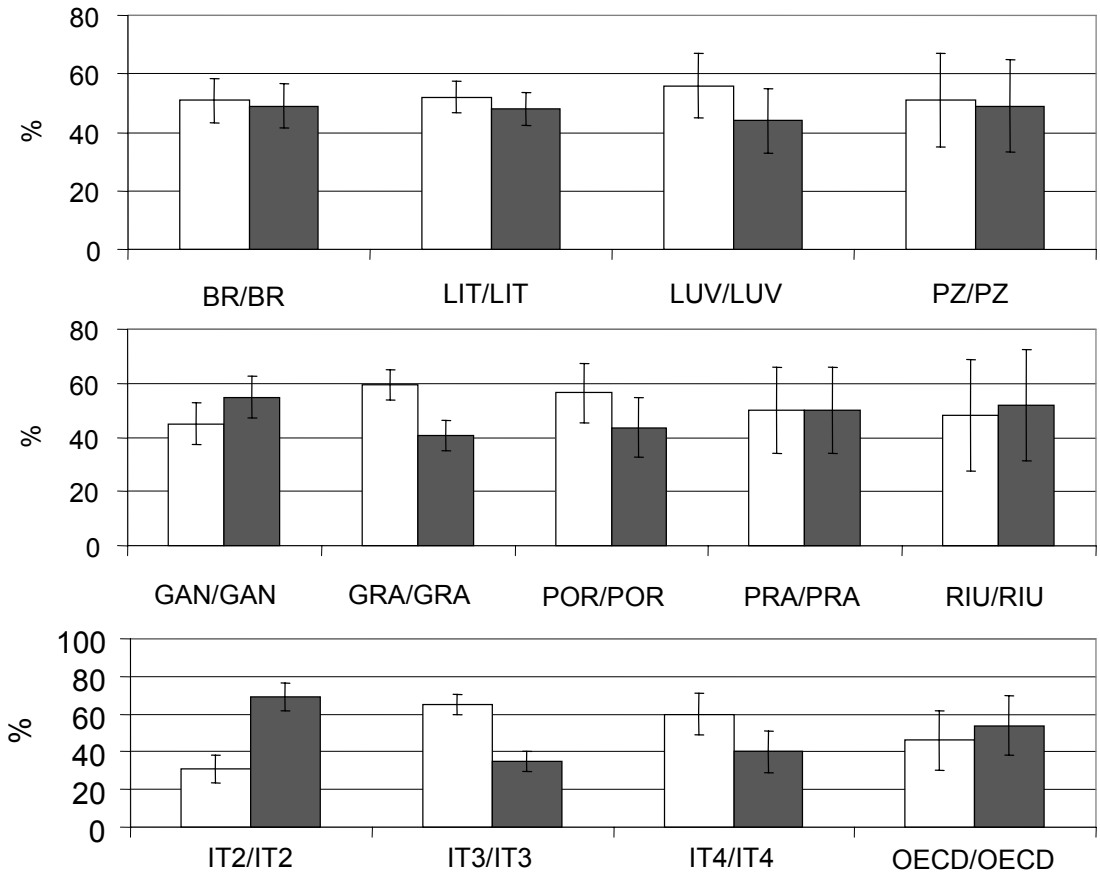


Figure 6.4. Results of the avoidance tests of *E. andrei* exposed to different soil combinations. Results are expressed in percent of the individuals present in each side. Asterisks indicate significant differences in the distribution of the individuals (Fisher's exact test, $p<0.05$).

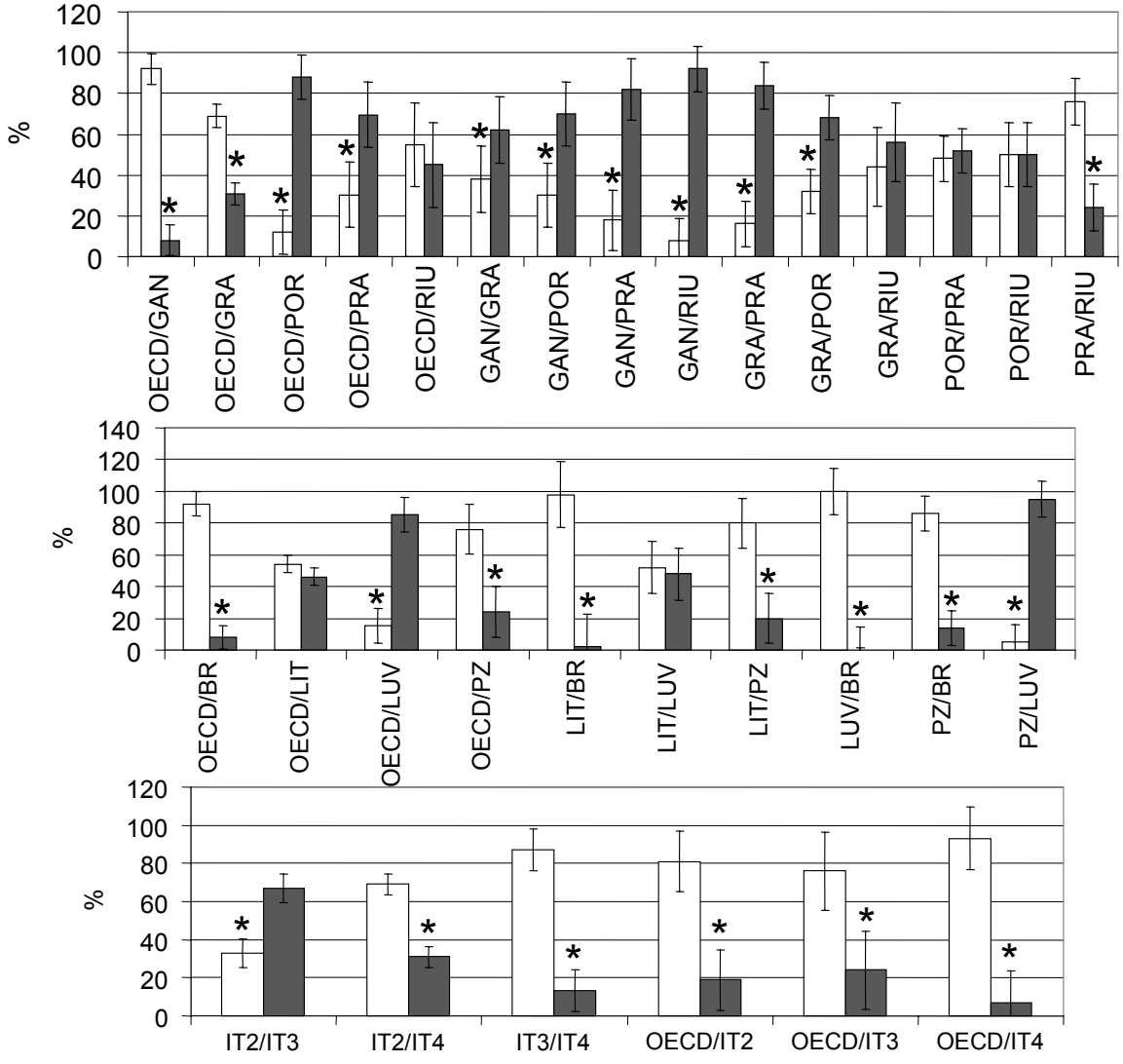


Figure 6.5. Results of the avoidance tests of *E. crypticus* exposed to different soil combinations. Results are expressed in percent of the individuals present in each side. , Asterisks indicate significant differences in the distribution of the individuals (Fisher's exact test, $p<0.05$).

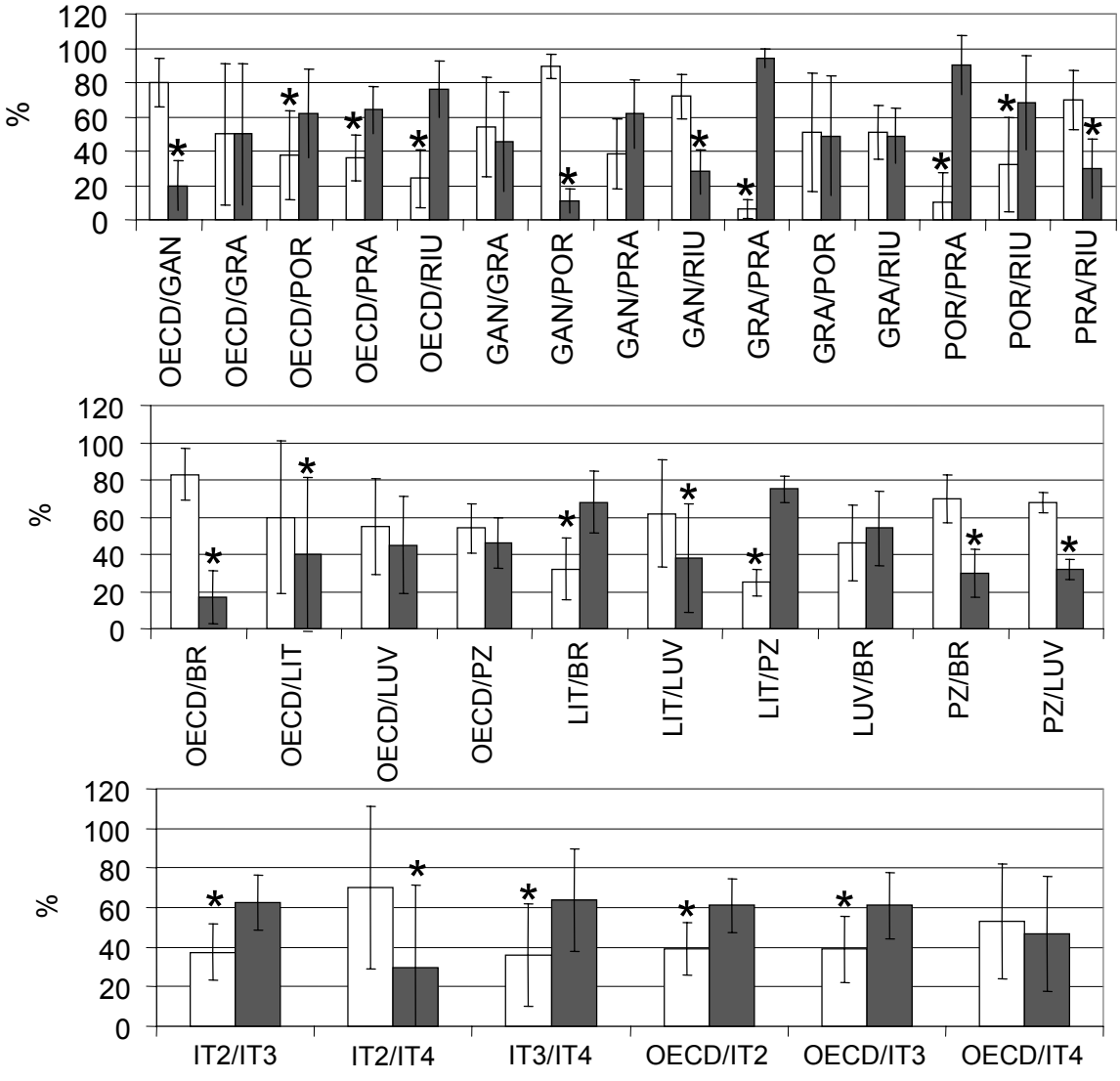
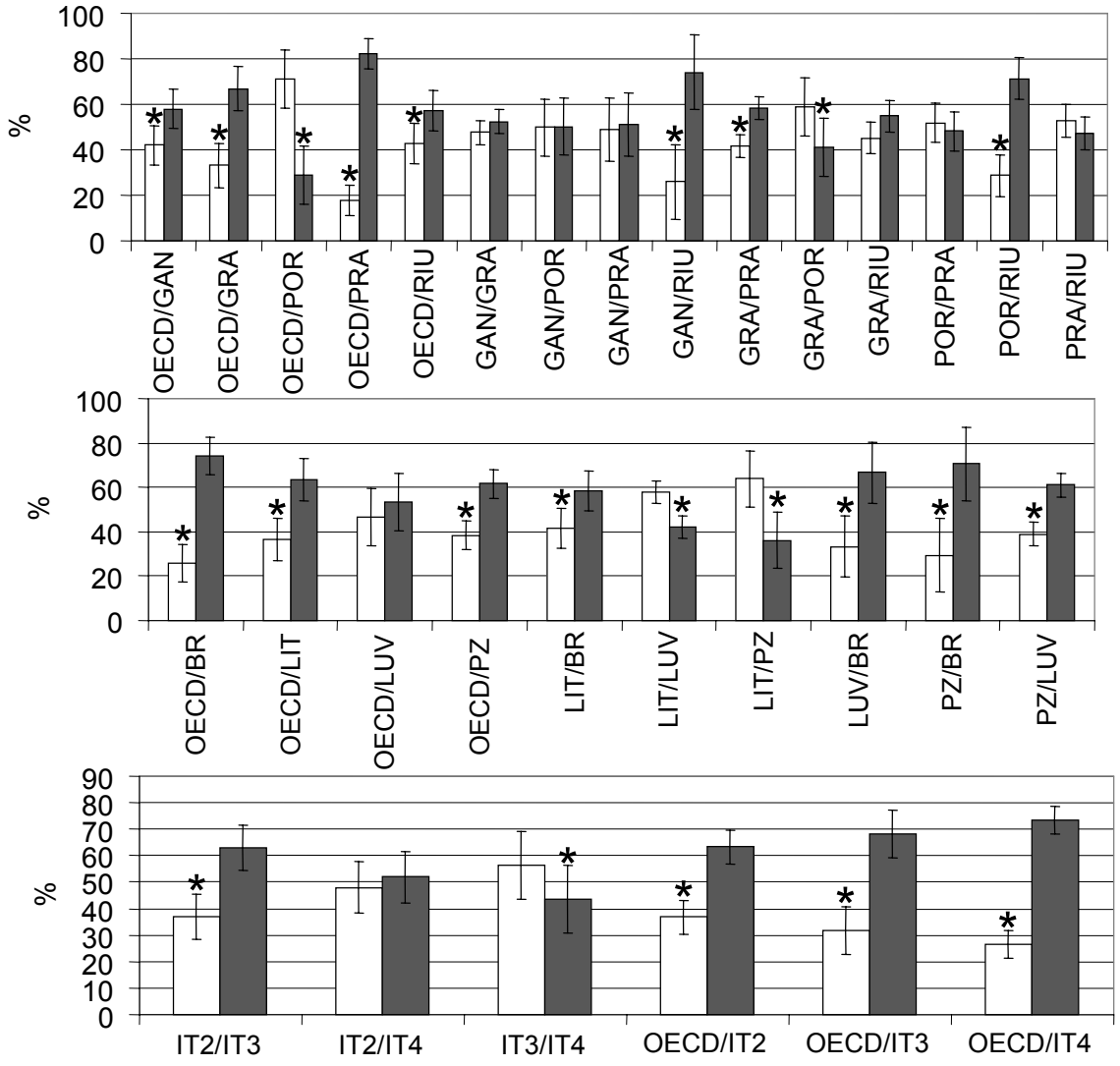


Figure 6.6. Results of the avoidance tests of *F. candida* exposed to different soil combinations. Results are expressed in percent of the individuals present in each side. Asterisks indicate significant differences in the distribution of the individuals (Fisher's exact test, $p<0.05$).



In the PCA analysis of the soil properties quotients, three principal axes explained 35.3, 26.2, and 16.5% of the variance. For *E. andrei*, avoidance was positively correlated with the second axis (Pearson r = 0.4, p = 0.026), mainly associated to the clay (0.891) and silt content (0.855), and was negatively correlated with the third axis (r = -0.61, p<0.001), mainly associated with organic carbon (0.818). For *E. crypticus,* avoidance was positively correlated with the second axis (r = 0.373, p = 0.039), explained by the clay and silt content. Avoidance in *F. candida* was positively correlated with the first axis (r = 0.364, p = 0.044), explained by WHC (0.884) and fine sand content (0.766), but also negatively correlated with the second axis (r = -0.446, p = 0.012), associated to the clay and silt content.

6.3.3. Reproduction tests

In the reproduction tests, mortality was below 10% in *E. andrei* and 20% in the two other species. However, mortality could not be accurately assessed in *E. crypticus*, as adults and juveniles were not clearly distinctively identified in all soils, given the short life cycle of the species.

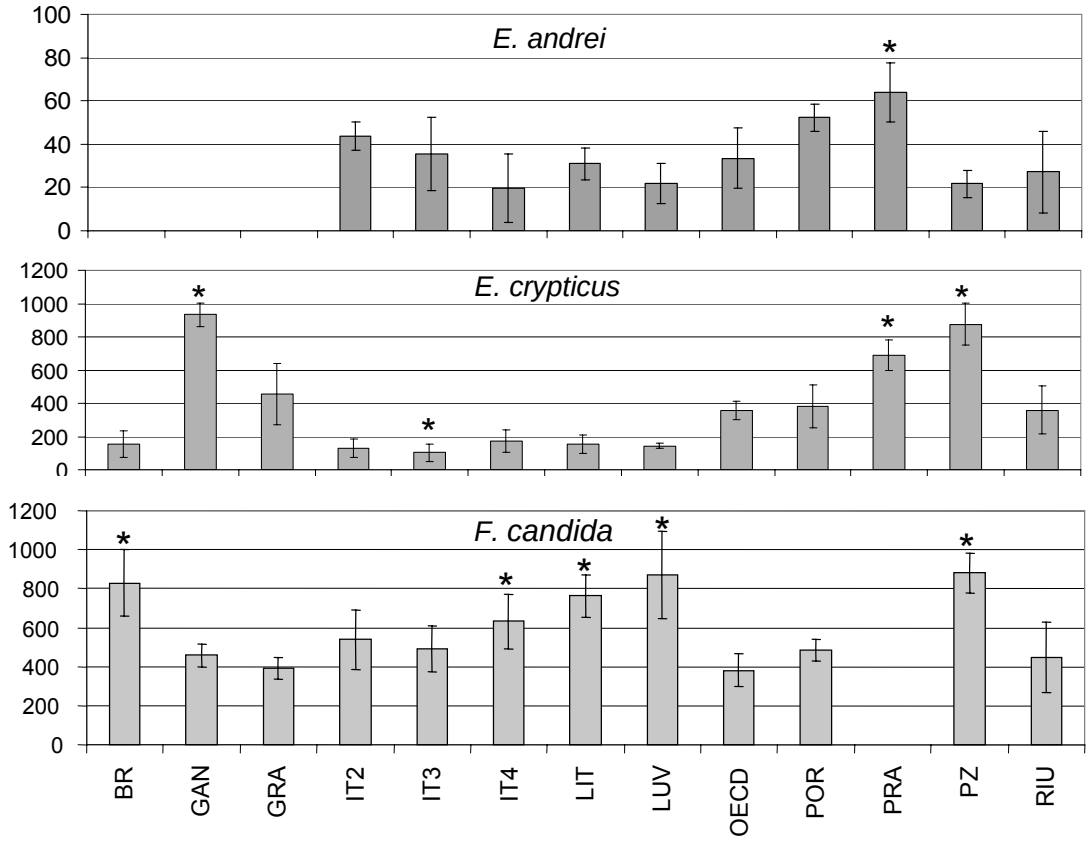
The reproduction of *E. andrei* in BR, GAN, and GRA was not assessed because the species showed a high avoidance. For this species, reproduction in OECD soil showed intermediate values and was not significantly different from natural soils, with the exception of the PRA soil, where the number of juveniles was higher (Figure 6.7). Almost half of soils (IT4, LIT, LUV, PZ, RIU) did not accomplish the reproduction validity criteria (>30 juveniles and variation coefficient below 30%) of the ISO 11268-2 (1998).

E. crypticus, reproduced in all soils, showing an intermediate number of juveniles in the OECD soil. The reproduction was significantly lower in IT3 than in the OECD soil, but higher in PRA, PZ and GAN (Figure 6.7). All the soils accomplished the validity requirements of ISO 16387 (2004), as there were above 25 juveniles with a variation

coefficient lower than 50% in all soils (to the exception of BR and IT3, with slightly lower coefficients).

The reproduction in *F. candida* was generally lower in the OECD than in natural soils, although it was only significant for comparisons with IT4, LIT, BR, LUV and PZ (Figure 6.7). This species did not reproduce in PRA, where all the original individuals died, despite this soil being suitable for earthworms' and enchytraeids' reproduction. The remaining soils accomplished the validity requirements of the ISO 11267 (1999), as the number of juveniles was above 100 and the variation coefficient was below 30% (with the exception of RIU, with a slightly higher coefficient).

Figure 6.7. Reproduction of *E. andrei*, *E. crypticus* and *F. candida* in different soils, expressed as number of juveniles. Asterisks indicate significant differences with respect to OECD soil (Bonferroni, $p<0.05$). Bars indicate standard deviation.



In the soil properties PCA, a positive correlation between *E. andrei* reproduction and the third axis was found (Pearson r = 0.774, p = 0.009), which was mainly explained by the organic carbon content. In *E. crypticus*, reproduction was negatively correlated with the first axis (r = -0.581, p = 0.037), mainly explained by the CEC, silt and clay content, and pH. In *F. candida*, reproduction was negatively correlated with the second axis (r = -0.656, p = 0.021), associated with total nitrogen, fine sand and coarse sand content.

6.4. DISCUSSION

6.4.1. Representativeness of the selected soils

The soils studied covered a wide range of soil property values, and hence represented the diversity of natural soils that can be used in ecotoxicological assays. According to the PCA factor scores of their properties, the lack of soil clusters indicates that the values of the different soil properties are well distributed among the different soils, showing their suitability for the purposes of this study.

6.4.2. Influence of soil properties on avoidance behaviour

The avoidance tests are based on the fact that organisms possess chemoreceptors, highly sensitive to chemicals in the environment (Edwards and Bohlen 1992, Stephenson et al. 1998). Since this endpoint reflects the quality of soil as a habitat, it can be used as indicator in risk assessment studies (Lopes et al. 2004, Natal-da-Luz et al. 2004). Avoidance is a key strategy for soil organisms facing pollution, as it has been suggested that this can allow soil organisms to take refuge in relatively clean soil pockets in contaminated sites, thus maintaining soil invertebrate biodiversity (Fountain and Hopkin 2004). This behaviour is also ecologically relevant, as migration of individuals may have an impact on the entire soil ecosystem (Yeardley et al. 1996, Reinecke et al. 2002). However, several limitations have been reported about avoidance methods. First, it has

been shown that some pollutants might inhibit the individuals' locomotion, something that could distort the avoidance patterns, as already observed in earthworms (Capowiez et al. 2003), collembolans (Fabián and Petersen 1994), and suggested for isopods (Loureiro et al. 2005). Second, these methods are only applicable to the pollutants perceived via chemoreceptors (Hund-Rinke et al. 2003). Furthermore, avoidance response to pollutants is also highly dependent on the species (Lukkari and Hari 2005, Natal-da-Luz 2005). Finally, it has been suggested that in practice, and especially in field soils risk assessment, variations in soil properties between the control soil and the tested soil may distort the avoidance due to different pollution burden. For this reason, avoidance tests should only be used if the quality of the control soil is ensured, and if it is guaranteed that the individuals' response is influenced by the pollutant burden rather than by differences in soil properties between the assessed soil and the control (Hund-Rinke and Weichering 2001, Natal-da-Luz 2005). However, not much is known about the soil preferences of the more commonly used test species, indicating the need for generating data on this issue.

Avoidance patterns of our three species appeared in most of the soil combinations tested, showing the high influence of soil properties on this response. Mortality was absent or low in most of the tests. Nevertheless, when enchytraeids or collembolans were used, some soil combinations exceeded the maximum mortality rate for the validity of the test. However, the mortality rates were probably overestimated for these species, since it is likely that some surviving individuals are not recovered in certain soils, considering their fine texture and the small size of the individuals. On the contrary, all earthworms were recovered. Moreover, it was assumed that mortality (or inability to recover) did not affect the distribution of the surviving individuals and hence the avoidance percentages.

In almost half of the soil combinations tested, significant avoidance behaviour could be observed, with *E. andrei* exhibiting the highest percentage of avoidance responses,

followed by *F. candida* and *E. crypticus*. However, differences in avoidance might also reflect their different migration capacities or body size.

Some of the natural soils tested were completely unsuitable for ecotoxicological assays with the species studied. Concerning OECD artificial soil, it was generally not avoided by *E. andrei* and *E. crypticus* (25 and 33% of the combinations), but was frequently avoided by *F. candida* (75%). These results suggest that despite being the soil type most typically used in ecotoxicology, OECD soil has properties which collembolans disfavour. However this soil was suitable for earthworms and enchytraeids.

The soil parameters mainly influencing avoidance were different depending on the species. In *E. andrei* avoidance was high in soils with high silt and clay content and low organic carbon levels. This is consistent with other studies that have indicated the preference of earthworms for high organic level substrates (Bouché 1972, Natal-da-Luz 2005). Avoidance of fine textured soils by some earthworm species has also been reported (Hund-Rinke and Wiechering 2001, Natal-da-Luz 2005), something that some authors have related to the need for sand grains in this group for the grinding of food in the gizzard (Marhan and Scheu 2005). On the contrary, no effects of pH were found in this study, despite some authors having indicated avoidance of acid soils (Curry 1998, Hund-Rinke and Wiechering 2001).

Ecological requirements of *E. crypticus* are unknown, as this species has been described from a composting plant (Römbke 2003) but, like other enchytraeid species, it lives in close association with the pore water (Gejlsbjerg et al. 2001). Results from this study showed that, *E. crypticus* avoids soils with high silt and clay content, as already suggested for *E. albidus* by Amorim et al. (2005d), although it has been claimed that enchytraeids prefer sandy soils (Jänsch et al. 2005b). Some authors have suggested that *E. crypticus* and *E. albidus* avoid acid soils (Amorim et al. 2005d , Jänsch et al. 2005a), and low moisture contents (Kasprzak 1982), but no evidences of this has been found in this study.

The collembolan *F. candida* avoids soils with high water retention capacity and fine sand content, but prefers soils with high silt and clay content. Such behaviour might seem contradictory, as the soils with higher WHC are generally rich in silt and clay. WHC and fine sand quotient values were correlated in this part of the study (Pearson, $r = 0.464$, $p = 0.09$), despite according to Table 6.1 both properties are uncorrelated. Hence, association with water-holding capacity is probably an artefact. Fine sand content might be the main explanation for the avoidance of this species, since there is no logical explanation why collembolans avoided soils with higher WHC, given that in this study the WHC was within the suitable range reported for this species (Pedersen et al. 1997, Van Gestel and van Diepen 1997). Some authors have indicated that *F. candida* has a wider range of tolerance to pH (Van Straalen and Verhoef 1997) and organic matter levels variation (Natal-da-Luz 2005) than other soil organisms. However, it has been reported that extreme soil textures are avoided by this species (Natal-da-Luz 2005). This is not in accordance with the results of this study, in which *F.candida* showed a preference for fine textured soils, in contrast to earthworms and enchytraeids.

6.4.3. Influence of soil properties on reproduction

The influence of soil parameters on the reproduction outcome of soil organisms has been widely reported in the literature (Van Gestel et al. 1992, Sandifer and Hopkin 1996, Gestel and van Diepen 1997, Crouau et al. 1999, Pedersen et al. 1997, Greenslade and Vaughan 2003, Jänsch et al. 2005a, Amorim et al. 2005a, Amorim et al. 2005b, Amorim et al. 2005c, Römbke et al. 2006). The results from our study indicate that most of the natural soils tested are suitable for its use in reproduction assays with enchytraeids and collembolans, as they fulfill the validity criteria established in their respective protocols. On the contrary, earthworms did not accomplish the validity criteria in most soils tested.

Furthermore, some soils were unsuitable for reproduction assays. In earthworms, some soils with low organic carbon content and fine texture were strongly avoided. In collembolans, all the individuals died in one of the soils (PRA) consistent with the higher mortality rates also observed in the avoidance tests when this soil was involved. The high mortality in this soil remains inexplicable, despite its relatively high mineral nitrogen levels suggesting toxic effects related to agrochemical fertilization. It has been reported that ammonia is responsible for short-term reductions of soil fauna density after the application of nitrogenated fertilizer (Seniczak et al. 1994).

As a general trend, reproduction in the different soils is similar in *E. andrei* (20-65 juveniles), but higher variation was observed in *F. candida* (242-881 juveniles), and *E. crypticus* (102-933 juveniles). However, when different soils are compared at least a part of this variability might be also due to individual variability (Crouau and Cazes 2003).

The reproduction of earthworms and enchytraeids in OECD soil showed intermediate values with respect to natural soils. However, in collembolans, reproduction in OECD soil is in the range of other natural soils or lower. This may indicate that OECD artificial soil is within the ecological requirements of earthworms' and enchytraeids' reproduction, but not for collembolans', similar to the pattern found in the avoidance tests.

Soil parameters affecting reproduction, as shown for the avoidance patterns, differed between species. In the earthworm *E. andrei*, reproduction was higher in soils with higher organic carbon levels, which were also one of the main properties that influenced their preferences in the avoidance tests. This indicates that the preference of earthworms for substrates with high organic matter content (Bouché 1972, Natal-da-Luz 2005) is also reflected in higher reproduction levels. No effects of soil texture on reproduction could be found, despite some authors having observed that earthworms present low reproduction rates in soils with extreme textures (Baker et al. 1998, Jänsch et al. 2005a, Marhan and Scheu 2005). The observed avoidance of fine textured soils, together with

the likely drop in reproduction, corresponds with these authors, but in our study no inhibition of reproduction was observed in sandy soils. Furthermore, no significant influence of other soil properties was found, despite some authors having indicated the influence of moisture (Presley et al 1996, Bauer and Römbke 1997, Van Gestel et al. 1992, Jänsch et al. 2005a), and pH (Van Gestel et al. 1992, Jänsch et al. 2005a, Römbke et al. 2006).

In the enchytraeid *E. crypticus*, reproduction was lower in fine textured soils, with higher pH and CEC values, consistent with the observed avoidance of fine textured soils This trend has also been reported for this species (Martikainen 1996) and other related species (Amorim et al. 2005c). Kuperman et al. (in press), testing reproduction of this species in natural soils, found the lower reproduction rate in the finest textured soil. The observed inhibition of enchytraeid reproduction at high pH in this study has not been previously reported, since similar previous work has been based on soils with lower pH. According to several authors, reproduction inhibition in enchytraeids appears at pH below 5 (Achazi et al. 1996, Römbke and Moser 2005, Amorim et al. 2005c, Kuperman et al. in press), but most soils we used showed higher values. Hence, enchytraeid reproduction seems to be both sensitive to acidic and alkaline conditions. The influence of other soil properties on enchytraeid reproduction could not be detected in this study, despite some works having reported differences due to organic matter levels (Jänsch et al. 2005a), or moisture (Puurtinen and Martikainen 1997, Jänsch et al. 2005a).

Reproduction of the collembolan *F. candida* was lower in soils with high nitrogen content and high coarse sand content, but was enhanced in soils with high fine sand contents. This pattern does not fit with the results of avoidance tests, where collembolans avoided soils with high levels of fine sand and preferred silty and clayey soils. Concerning the inhibition of reproduction by total nitrogen content, the most likely explanation is the influence of some nitrogen-derived compounds released during the tests. It is known that, during the organic matter decomposition process, nitrogen losses are mainly as ammonia,

a chemical known to decrease field populations of soil fauna (Seniczak et al. 1994, Neher 1999), and to which survival of *F. candida* is highly sensitive (Domene et al. 2007). The combined favourable conditions for decomposition during the tests (moisture and temperature), together with the limited aeration in test containers, are likely to magnify the release of nitrogenated endproducts and their effects on collembolans. The total mortality observed for this species in PRA soil, with a relative high mineral and total nitrogen content, and with an acid pH which may reduce the loss of ammonium as volatile ammonia may hold up this hypothesis.

In similar studies, soil properties other than texture and nitrogen content explained reproduction in *F. candida*. More precisely, the importance of moisture has been indicated (Van Gestel and van Diepen 1997, Pedersen et al. 1997, Crouau et al. 1999), and also that of pH (Crouau et al. 1999, Sandifer and Hopkin 1996, Greenslade and Vaughan 2003). Despite reproduction inhibition being observed in alkaline and acid soils, we found no effects of this parameter, as already stated by Amorim et al. (2005b) in a similar study. The low influence on reproduction of the more classically studied soil parameters, organic matter and pH, might be interpreted as a high tolerance of this species to soil properties with respect to other collembolans (Amorim et al. 2005b), enchytraeids (Martikainen 1996), and earthworms (Römbke et al. 2006). However, results from this work have shown effects of texture and nitrogen content on reproduction. Other soil parameters are probably influencing avoidance or reproduction, but smaller effects have probably not been detected and only the main contributors could be identified. Furthermore, even though there was a relative variability of soil properties in the soils studied, the limited number of soils used in this work mean that the conclusions of this study should be taken with care. More work is required to identify the main soil properties that might distort the outcomes in ecotoxicological tests.

CONCLUSIONS

OECD artificial soil is generally preferred by *E. andrei* and *E. crypticus* in avoidance tests. On the contrary, this soil is generally rejected by *F. candida*, and reproduction in this artificial substrate is similar or lower than that observed in natural soils. This indicates that its use would be unsuitable if used as control in avoidance tests with natural soils. On the contrary, the use of OECD soil in reproduction tests is suitable with similar outcomes as those obtained with natural soils.

As a general pattern, differences in soil properties strongly influence the distribution of soil organisms in avoidance tests, as in most of the soil combinations tested a significant avoidance response was observed. This finding indicates how important it is to ensure the similarity of the soils used as control in avoidance tests to obtain robust results. The sensitivity to soil property differences is in the order *E. andrei* > *F. candida* > *E. crypticus*.

Regarding the influence of soil properties in reproduction tests, in general, the effects are expected to be low in *E. crypticus* and *F. candida*, as, in nearly all the tested soils, reproduction fulfills the validity requirements for its use in ecotoxicological tests. However, *E. andrei* do not fulfill such requirements in a significant percentage of the soils studied. The main soil properties explaining avoidance differed between species, but generally are consistent with the properties also explaining low reproduction outcomes. *E. andrei* avoids soils with low organic carbon levels, and high silt and clay contents, and shows low reproduction in soils with low organic carbon levels. *E. crypticus* escapes from soils with high levels of silt and clay, which are precisely the soils with lower reproduction rates in this species, together with high pH and CEC values. On the contrary, *F. candida* shows different preferences depending on the endpoint. On the one hand, it avoids soils with high fine sand content, and prefers soils with high silt and clay levels; on the other, it shows lower reproduction in soils with high nitrogen content and coarse sand, and low

fine sand contents. Such results suggest the preference of this species for fine textured soils, as well as the sensitivity of its reproduction to high soil nitrogen contents.

Results from this work also show the unsuitability of soils which are out of the tolerance range of the test species to carry out reproduction tests. This also applies to avoidance tests, as high mortality of individuals may invalidate the results. In the present study this occurred for low organic matter levels for *E. andrei*, and high soil nitrogen content for *F. candida*.

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Chapter 7

The influence of soil properties on the toxicity of organic waste amendments to soil fauna

ABSTRACT

Toxicity results obtained from standardized soil substrates in the laboratory generally neglect most of the interactions that potentially take place in real scenarios, something that might lead to biased results in the extrapolation from the laboratory to field conditions. Among them, soil properties are one of the most important, as in general physico-chemical properties in standard substrates are far from those in natural soils, especially when OECD artificial soil is used. Although some knowledge exists on the representativeness of artificial soil for the field toxicity of pollutants, not much is known about its relevance when used in waste testing.

In this study, the toxicity of sewage sludge to the soil collembolan *Folsomia candida* is assessed in OECD artificial soil together with nine field soils from agricultural, grassland and woodland sites. The OECD soil is shown to be suitable for the extrapolation of effects on the mortality of this species in natural soils, as it presented intermediate LC50. However, when reproduction was used, effects on reproduction were generally overestimated in OECD soil, with the lowest EC50 of all soils. With respect to the main soil properties influencing the sludge toxicity in natural soils, it was shown that toxicity was lower in soils with higher coarse sand and organic carbon contents. Such results suggest that ammonium has a principal role in the toxicity, whith its concentration in soil pore water being strongly influenced by sorption in organic matter and by physico-chemical and bacterial processes influenced by the enhanced aerobic conditions existing in sandy soils.

7. 1. INTRODUCTION

Most soil ecotoxicological knowledge has been developed from tests using standardized soil substrates, with the OECD artificial soil, and the German field standard LUFA 2.2 being the most widely used. The use of standardized substrates allows results to be compared among laboratories and test species (Smit et al. 1998). However, extrapolation to field situations of results obtained in the laboratory from standard soils is limited, as most of the interactions that potentially take place in real scenarios are neglected. Among these, one of the most important is the known influence of soil properties on the bioavailability of pollutants and, as a consequence, their toxicity for soil organisms, which limits the ecological representativeness of standard soils, since their soil properties are usually different from those of field soils.

The bioavailability of pollutants in soils is mainly explained by the sorption equilibrium between the soil solid-phase and the pore water, and more precisely by pollutant concentrations in soil pore-water, which is the main exposure route for chemicals in soil-inhabiting animals (Smit and Van Gestel 1998). As sorption in soil pore water is mediated by the nature and content of clay and humus, the bioavailability and toxicity of pollutants are influenced by soil properties. Such influence has been widely studied for individual pollutants (Lock and Janssen 2001c, Sverdrup et al. 2001, Amorim et al. 2005a, Amorim et al. 2005b), but studies dealing with the influence on toxicity of complex mixtures of pollutants contained in organic matrices have not been published, despite the fact that pollutants bioavailability and leachability in field soils are hardly influenced by soil properties (McBride et al. 2004).

The main aim of this study is to compare the toxicity of an organic waste on the collembolan *Folsomia candida*, when measured in OECD artificial soil and in several natural soils, in order to determine its relevancy for real situations. Furthermore, the main

soil properties are identified which influence toxicity in order to predict which soils are more vulnerable to organic amendments taking into account ecotoxicological criteria.

7.2. METHODS

7.2.1. Soils

Artificial OECD soil was prepared according to OECD 207 (1984). In order to compare the results of toxicity tests in this standard substrate with those using natural soils, nine soils with contrasting properties were collected. All soils were collected from different localities in Catalonia, and came from agricultural land, grassland, and woodland. In agricultural soils, agrochemical treatments were low or absent. Soils were 5-mm sieved, air-dried, and defaunated alternating two freezing-thawing cycles which consisted of placing soils in the freezer (-20°C) for 4 days followed by 4 days at room temperature. The main soil properties of the selected soils are reported in Table 7.1, together with their heavy metals concentrations, determined according to ISO 11885 (1996), in order to detect any concentration over those expected from natural sources.

7.2.2. Sewage sludge

To perform the bioassays, a thermally-dried sewage sludge from Blanes WWTP (Barcelona, Spain) was selected. This organic waste was produced through thermal drying of an aerobically digested and dewatered sludge. Drying was carried out in a heated rotary cylinder with an injected hot air stream at temperatures of around 130-150°C for 45 minutes. Just before the analysis, the sludge was again dried at 60°C for 48 h to guarantee its dryness, and sieved to 2 mm to ensure the homogeneity and accuracy of the test concentrations. The final product was both used for the characterization of the sludge and for the preparation of the soil-mixtures of the bioassays.

Table 7.1. Characterization of the selected soils (topsoil corresponding to A horizons). All values are expressed on dry basis, with the exception of WHC, expressed as water weight on the wet soil. WHC = maximum water holding capacity; CEC = cation exchange capacity.

Soil	**OECD**	**CAM**	**COLL**	**GAN**	**GRA**	**POR**	**PRA2**	**RIU**	**STA**	**VALL**
Site	-	Campdàsens	Collsacreu	Gandesa	La Granadella	Porrera	Prades	Riudellots	Santa Fe	Vallgorguina
Soil use	-	Shrubland	Forest	Vineyard	Olive grove	Vineyard	Grassland	Grainfield	Forest	Forest
WHC, %	44.8	44.9	33.9	37.6	49.8	38.8	28.1	45.0	50.7	29.9
pH, soil:water 1:5	6.2	7.9	4.9	8.3	8.2	6.9	6.5	7.3	5.7	6.4
Coarse sand, %	21.6	13.2	51.1	1.5	2.1	46.2	60.8	23.6	69.3	72.4
Fine sand, %	24.1	18.8	21.6	74.4	25.7	21.4	19.5	34.9	10.9	12.1
Silt, %	31.8	37.9	17.6	12.0	48.5	20.4	16.5	13.8	8.7	8.3
Clay, %	16.3	30.1	9.7	12.0	23.69	11.9	3.2	27.6	11.1	7.2
Organic matter, %	6.9	8.4	3.8	0.6	1.70	4.3	1.9	1.9	12.0	2.2
Organic carbon, %	4.0	4.8	2.2	0.3	0.99	2.5	1.1	1.1	6.9	1.3
Total N, %	0.04	0.3	0.11	0.04	0.11	0.20	0.10	0.13	0.53	0.08
C/N	103.5	16.2	19.9	8.7	9.0	11.3	11.2	8.5	13.1	15.7
CEC, mEq/100g	-	22.3	9.2	6.0	14.2	18.6	7.3	14.9	17.7	9.2
Cd, ppm	-	0.2	<0.1	<0.1	0.2	0.2	0.9	<0.1	<0.1	<0.1
Cu, ppm	-	23.0	8.0	20.0	26.0	92.0	77.0	26.0	11.0	16.0
Cr, ppm	-	39.0	10.0	16.0	19.0	67.0	11.0	22.0	13.0	9.0
Ni, ppm	-	19.0	<10	32.0	28.0	46.0	<10	18.0	<10	<10
Pb, ppm	-	23.0	17.0	11.0	10.0	147.0	48.0	19.0	41.0	14.0
Zn, ppm	-	94.0	62.0	32.0	42.0	420.0	204.0	64.0	145.0	85.0

The physico-chemical properties, heavy metal and organic pollutant contents of the sludge are recorded in Table 7.2. Dry matter, water holding capacity, water pH, electrical conductivity, total nitrogen, and organic matter were measured according to EN 12880 (2000), ISO 11267 (1999), EN 13037 (1999), EN 13038 (1999), EN 13342 (2000) and EN 12879 (2000), respectively. Non-hydrolizable organic matter and non-hydrolyzable nitrogen levels were measured as the percentage of organic matter and nitrogen remaining in the sample residue after acid hydrolysis, as described in Rovira and Vallejo (2002). This treatment removes the more labile fraction of an organic substrate, mainly consisting of polysaccharides and proteins. Hydrolyzable nitrogen was estimated by subtracting the content of nonhydrolyzable nitrogen from the total nitrogen content. $N\text{-}NH_4$ was measured in the distillates obtained from a fresh sample suspension. Elemental analysis of P, K, Cd, Cr, Cu, Hg, Ni, Pb and Zn was carried out by ICP-MS according to ISO 11885 (1996). Polychlorinated dibenzodioxins and dibenzofuranes (PCDD/F) were measured with HRGC-HRMS, polychlorinated biphenyls (PCB) by HRGC-ECD, and i(2-ethylhexyl)phthalate (DEHP) and nonylphenols (NPE) by HRGC-MS. Polycyclic aromatic hydrocarbons (PAH) and linear alkylbenzene sulphonates (LAS) were determined by HPLC with fluorescence and UV detectors, respectively. Concentration values were expressed as recorded in the third draft of the Working Document on Sludge (European Communities 2000). DEHP, LAS, and PCDD/F values corresponded to total values. NPE included nonylphenol and nonylphenol ethoxylates with one or two ethoxy groups; PAH was the sum of acenapthene, phenanthrene, fluorene, fluoranthene, pyrene, benzo(b+j+k)-fluoranthene, benzo(a)-pyrene, benzo(ghi)-perylene, and indeno(1, 2, 3-c, d)-pyrene; PCB was the sum of the polychlorinated biphenyl congeners number 28, 52, 101, 118, 138, 153 and 180.

Pollutant concentration in the sludge was below the limit values of the current legislation on sludge (Directive 86/278/EEC), with the exception of lead, which exceeded the limit value allowing its reuse in soil (50-300 mg Kg^{-1}). NPE values were also slightly above

the 50 mg Kg^{-1} limit value suggested by the third draft of new Directive on sludge (European Communities 2000).

Table 7.2. Physico-chemical properties, heavy metal and organic pollutant contents of the thermally dried sewage sludge; [a] Over the limit value of the current Directive on sludge (86/278/EEC); [b] Over the limit value in the 3rd draft of Working Document on sludge (European Communities, 2000); w.w., wet weight; d.w., dry weight.

Parameter	Units	Value
Dry matter	g kg^{-1} (w.w.)	945
WHC	% (w.w.)	74.7
pH	Water, 1:5 (v/v)	6.9
Electrical conductivity	dS m^{-1}, 25°C	3.57
Organic matter	g kg^{-1} (d.w.)	687
Stable organic matter	%	40.4
N	g kg^{-1}, (d.w.)	60.6
Non-hydrolyzable N	g kg^{-1}, (d.w.)	19.1
Hydrolyzable N	g kg^{-1}, (d.w.)	41.5
NH_4-N	g kg^{-1}, (d.w.)	8.0
P	g kg^{-1}, (d.w.)	20.5
K	g kg^{-1}, (d.w.)	2.2
Cd	mg kg^{-1}, (d.w.)	1.3
Cr	mg kg^{-1}, (d.w.)	30
Cu	mg kg^{-1}, (d.w.)	645
Hg	mg kg^{-1}, (d.w.)	0.95
Ni	mg kg^{-1}, (d.w.)	53
Pb	mg kg^{-1}, (d.w.)	3747[a]
Zn	mg kg^{-1}, (d.w.)	952
DEHP	mg kg^{-1}, (d.w.)	27
LAS	mg kg^{-1}, (d.w.)	331
NPE	mg kg^{-1}, (d.w.)	76[b]
PAH	mg kg^{-1}, (d.w.)	0.3
PCB	mg kg^{-1}, (d.w.)	0.029
PCDD/F	ng TEQ kg^{-1} (d.w.)	13.7

7.2.3. Test organism

The soil collembolan *Folsomia candida* was used as the test organism. The effects of pollutants on this species are considered as representative for collembolans, a group present in all types of soil and a key group in soil ecosystems. Collembolans are exposed to pollutants via the epidermis, ventral tube via water, or gut via food. That

said, the main exposure route is not clear (Fountain and Hopkin 2004a). Individuals were bred in a moistened mixture made of plaster of Paris and charcoal (9:1, w/w) and fed weekly with granulated dry yeast. Cultures were maintained in the dark at 21±1 °C. For the assays, individuals of synchronized age (10 to 12 days) were used, produced as described in ISO 11267 (1999).

7.2.4. Test performance

The water content in each soil was adjusted to the 50% of their maximum water holding capacity (WHC). For soils with higher silt and clay content, this moisture level was difficult to reach without loosing the soil crumbly structure, as soil easily takes a doughy structure. This difficulty was overcome by the addition of small quantities of water during several hours.

Eleven test concentrations were prepared for the assays: 0, 1, 2, 4, 7.9, 15.8, 31.6, 63.1, 125.9, 251.2, and 501.2 g kg^{-1} (w/w) of sludge in a mixture with OECD artificial soil. For each sludge concentration, five replicates were prepared, which consisted of 20 g (dry weigth) of wet soil-waste mixture in sealed 150-mL polyethylene flasks.

As indicated in 11267 (1999), ten individuals 10 to 12 days-old were added to each replicate, together with 5 mg of granulated yeast. Food was again provided after two weeks. Flasks were sealed to avoid water losses but aerated twice a week. The test lasted 28 days and was carried out in the dark and at 21±1 °C.

At the end of the test, the flask content was poured into a bigger container and flooded with water in order to float the adults and juveniles present in the soil. After adding a few drops of a dark dye and gently stirring the soil, a picture of the surface was taken. Adults and juveniles were counted from these pictures with the image treatment software ImageTool 3.0.

7.2.5. Data treatment

The number of surviving individuals and juveniles of each replicate were expressed as a percentage of the mean values of the survival or reproduction rates of their respective controls. For each soil, the LC50 and EC50 for reproduction, together with their 95% confidence intervals, were calculated with Statistica 6.0 using the most suitable regression model in each case (exponential, Gompertz, hormesis, and logistic).

The assessment of the main soil properties influencing toxicity results was carried out with SPSS 13.0. Principal components analysis (PCA) of some of the properties recorded in Table 7.1 was used for this purpose. OECD artificial soil was excluded to carry out the PCA in order to include only relevant soils for field situations.

The selection of soil properties for PCA was based on their biological meaning, and on the lack of high correlation with other properties (Pearson $r<0.8$). Following these criteria, WHC, coarse sand, fine sand, silt, clay, organic carbon and cation exchange capacity (CEC) were selected to carry out the PCA. C/N and organic matter were not taken into account because they were calculated from organic carbon and nitrogen content. Nitrogen was removed since it was highly and positively correlated with organic carbon, and pH was also not taken into account given its negative correlation with coarse sand in this group of soils. Heavy metals were also not used for the analysis, as they were assumed not to influence the toxicity results, since their values were within the usual ranges in non-polluted soils in Catalonia (Tobías et al. 1997, Bech et al. 2005, Sardans and Peñuelas 2005), and below the values expected to affect survival (Crommentuijn et al. 1993, Greenslade and Vaughan 2003, Bongers et al. 2004, Lock and Janssen 2001b) and reproduction (Lock and Janssen 2001a, Lock and Janssen 2002a, Lock and Janssen 2002b, Fountain and Hopkin 2005) in this species.

After this depuration, the selected soil properties were reduced to a few components (axes) through PCA. Factor scores for each soil in each component were then obtained and compared with their respective toxicity values by means of Pearson correlations.

Eventual significant relationships between toxicity and any of the components ($p<0.05$) indicated an influence on the toxicity of the soil properties mainly associated with this component.

7.3. RESULTS

7.3.1. Sludge toxicity in the different soils

Toxicity results for survival (LC50) and reproduction (EC50) are recorded in Table 7.3. The performance of collembolans in test soils fulfilled the validity criteria of ISO 11267 (1999), as mean survival in controls was always over 80%, and more than 100 juveniles were produced per replicate (ranging from 187 juveniles in CAM to 938 in PRA2). The coefficient of variation was also below 30% in all soils, with the exception of VALL (36%) and STA (46%). Despite this, we assumed that the results from these two soils were suitable for the purposes of this study.

Table 7.3. *F. candida* toxicity values for survival (LCx) and reproduction (ECx) in the different soils, expressed as g kg^{-1} together with its 95% confidence intervals enclosed in parentheses.

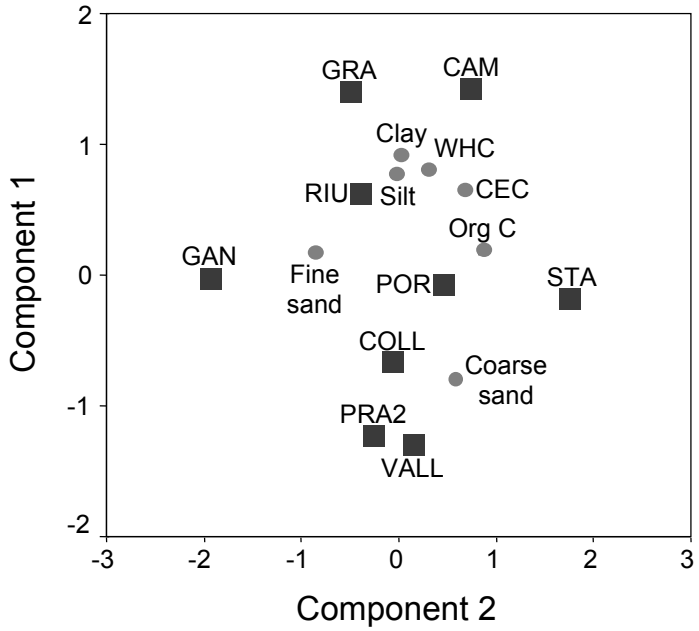
Soil	LC50 (g Kg^{-1})	EC50 (g Kg^{-1})
OECD	32.0 (28.9, 35.2)	2.7 (2.0, 3.4)
CAM	90.7 (82.8, 99.3)	13.3 (11.4, 15.5)
COLL	46.6 (45.3, 48.0)	23.9 (21.7, 26.4)
GAN	20.7 (18.1, 23.4)	5.0 (4.6, 5.5)
GRA	35.7 (29.6, 42.6)	8.7 (7.7, 9.8)
POR	57.3 (51.6, 63.5)	15.6 (13.8, 17.6)
PRA2	29.0 (25.2, 33.2)	10.7 (9.3, 12.2)
RIU	33.2 (29.9, 36.8)	17.1 (15.3, 19.0)
STA	91.7 (87.0, 96.7)	47.2 (44.3, 50.2)
VALL	84.7 (58.5, 120.8)	36.1 (27.5, 46.7)

The sludge toxicity was relatively high, as LC50 were always below 100 g Kg^{-1}, and EC50 were below 50 g Kg^{-1} in all soils. When the different soils were compared for toxicity, it is worth noting that LC50 in OECD artificial soil was slightly below but not far from the median value in the whole set of soils (41.1 g Kg^{-1}). On the contrary, EC50 was the lowest in the OECD soil, far from the median value (14.5 g Kg^{-1}).

7.3.2. Influence of soil properties on sludge toxicity

PCA summarized soil parameters in two main components which explained 47.2, and 33.1% of the variance, respectively. The first component was associated with the clay content (0.897), WHC (0.869), CEC (0.818), and silt content (0.729). The second component was explanatory of the fine sand content (-0.859), organic carbon content (0.788), and coarse sand content (0.784). The scatterplot of the factor scores of each soil and soil property are presented in Figure 7.1.

Figure 7.1. PCA factor scores scatterplot of soils and soil properties.



GRA, CAM and RIU were fine textured soils with high pH, WHC, and CEC. POR and STA were mainly characterized by its high organic carbon content and CEC. COLL, PRA2, and VALL were soils with high coarse sand contents. Finally, GAN showed important fine sand levels.

Pearson correlations of toxicity values with the two components showed a significant positive association of LC50 with the second component ($r=0.806$, $p=0.009$), but also a significant association of EC50 with the same component ($r=0.728$, $p=0.026$).

7.4. DISCUSSION

7.4.1. OECD artificial soil and its representativeness in sludge testing

Ecotoxicological soil studies are usually performed in standard substrates such as the artificial OECD soil or the natural German soil LUFA 2.2. However, when assessing the toxic effects in the environment, the soil properties of natural soils are generally different from those of standard soils, which may lead to different exposure scenarios for the organisms and to a variety of results.

Several authors have pointed out that, when OECD artificial substrate is used, metal and organic pollutant toxicity is underestimated with respect to natural soils (Spurgeon & Hopkin 1995, Lock and Janssen 2001b, Lock and Janssen 2001c, Amorim et al 2005a, Amorim et al. 2005c, Amorim et al. 2005d). The main reason is the high organic content, fine texture, and high CEC of the OECD soil and other similar artificial substrates, which determines a lower pollutant bioavailability especially for cationic and hydrophobic substances (Boyd and Williams 2003, Crouau and Tan Tchiam 2006). For this reason, some authors have suggested to reduce the OECD soil peat content from 10% to 5% in order to increase its field relevance (Amorim et al. 2005c). Furthermore, it has been indicated that OECD soil is not completely representative of real situations, because it includes a clay mineral which is not usually present in most natural soils (kaolin), and

because it lacks aluminium, iron, and manganese oxides, which are important in the bioavailability processes (Römbke et al. 2006). However, other authors have found similar toxicities in the OECD substrate and in natural soils (Martikainen 1996).

Furthermore, it is also worth noting that results in OECD soil may sometimes overestimate the pollutants toxicity, such as when results are compared to those from old-polluted field soils with equivalent pollution. In such soils, long-term chemical processes known as ageing processes, decrease the pollutants bioavailability and toxicity (Lock and Janssen 2003). As an example, Spurgeon & Hopkin (1995) reported that zinc toxicity for *Eisenia fetida* was around ten times greater in OECD artificial substrate when compared to field contaminated soils with equivalent zinc concentrations, given the greater metals bioavailability in the OECD soil. Fountain & Hopkin (2004b) also indicated that toxicity to *F. candida* exposed to equivalent zinc concentrations was higher in OECD soil than in polluted field soils.

Results from this work showed that the toxicity of sewage sludge to the collembolan *F. candida* was generally overestimated in the OECD substrate in comparison with several natural soils. Effects on mortality appeared at similar sludge concentrations to those observed in the agricultural soils, but at much more lower concentrations than in shrubland or forest soils. On the contrary, OECD soil highly overestimated the concentrations affecting reproduction, as this endpoint was inhibited at much lower concentrations than in any of the natural soils.

7.4.2. Influence of soil properties on the sludges toxicity

In the context of deriving general rules for the extrapolation of toxicity data between soils, it is difficult to establish cause-and-effect relationships between soil properties and metal bioavailability, as generally soil properties are inter-correlated. Hence, for instance, CEC is generally related to pH, as cation exchange sites are pH-dependent in soils rich in organic matter, but it is also related to clay and organic carbon content, as

they contain such cation-exchange sites (Dayton et al. 2006). Furthermore, for each pollutant and soil properties combination, the properties with largest influence may differ (Van Gestel 1997, Peijnenburg et al. 1999). However, it is widely accepted that pH, CEC, and clay and organic matter content are the most important soil parameters which affect the toxicity of pollutants (Van Gestel et al. 1995, Lock et al. 2000, Boyd and Williams 2003, Simini et al. 2004).

In this study, sludge showed lower toxicity in soils with low fine sand content, high coarse sand content, and high organic carbon content.

It is known that pollutants generally show lower toxicities to soil fauna in fine textured soils than in sandy soils (Lock and Janssen 2001a, Lock and Janssen 2001b, Simini et al. 2004), given their higher CEC and its influence on bioavailability. However, in this study, the lower sludge toxicity was associated with low fine sand contents and high coarse sand contents. In the studied soils, coarse and fine sand were marginally and negatively correlated ($r = -0.666$, $p = 0.05$), but more importantly, the higher coarse contents were negatively correlated with the clay content ($r = -0.704$, $p = 0.034$). Hence, the toxicity of this sludge was lower in the more coarse sandy soils, but higher in more fine textured soils.

In this study, increasing organic matter contents decreased the sludge toxicity, as has also been shown to occur with individual pollutants in enchytraeids, earthworms, collembolans and plants (Martikainen 1996, Spurgeon and Hopkin 1996, Martikainen and Krogh 1999, Simini et al. 2004, Amorim et al. 2005a, Dayton et al. 2006). Its influence is mediated by the retention of cations on its cation exchange sites, but also through the sorption of hydrophobic compounds. Furthermore, the more fine textured the soil, the higher its organic matter content (Tisdall and Oades 1982, Oades 1988), contributing to the higher CEC in soils with this characteristic and to a lower bioavailability of pollutants. However, in the set of soils used in the present study, both parameters were uncorrelated

($p = 0.736$), something that may explain that organic carbon but not clay content was explanatory of a decrease in the toxicity of sludges.

This study has not been able to detect the influence of other properties known to influence the bioavailability of pollutants (CEC, pH or moisture retention) on the toxicity of the studied sewage sludge. Several authors have suggested that CEC is the best indicator of the bioavailability of metals to soil enchytraeids (Lock et al. 2000, Lock and Janssen 2001a) and plants (Rooney et al. 2006) as it takes into account the type of clay and organic matter, as well as other adsorption phases such as oxyhydroxides. Furthermore, this property has also been signaled as important for the toxicity of metals to collembolans (Römbke et al. 2006). However, in this work, CEC was not explanatory of changes in the toxicity. Some other authors have suggested pH as the main property influencing the bioavailability of soil pollutants for a wide range of soil inhabitants, generally reporting increases of bioavailability under more acidic conditions (Hopkin 1996, Anderson and Christensen 1988, Gestel and Van Dis 1988, Lock et al. 2000, Phillips et al. 2002, Van Gestel and Koolhaas 2004, Amorim et al. 2005b, Bradham et al. 2006, Echevarria et al. 2006, Römbke et al. 2006). In the particular case of sludge amendments, it has been suggested that after soil amendments with sludge, the high dissolved organic matter and the more acidic pH conditions in the treated soil may promote dissolution and allow migration of some pollutants (McBride et al. 2004). Finally, with respect to moisture, it has been indicated that low soil moistures usually increase the toxicity of pollutants, something that has been suggested to be a synergistic interaction between the toxic stress and drought stress (Bauer and Römbke 1997, Van Gestel and Van Diepen 1997, Puurtinen and Martikainen 1997, Højer et al. 2001, Amorim et al. 2005a). No influence of moisture was found in this study, as it was maintained in optimum conditions in all the studied soils.

7.4.3. Comparison of sludge toxicities in different soils

The pollutants toxicity may differ between test organisms, but soil properties also have a great influence on the impact of pollutants on soil organisms through the effects on their bioavailability (Lock and Janssen 2001c, Sverdrup et al. 2001, Amorim et al. 2005a, Amorim et al. 2005b). It is also known that pollutants bioavailability is mainly explained by the sorption equilibrium between soil solid-phase and pore water. This is why toxicity differences among soils for a given pollutant burden are mainly explained by different pollutant concentrations in soil pore-water, the main exposure route to chemicals of soil-inhabiting animals (Smit and Van Gestel 1998). It has been pointed out that bioavailability and leachability of pollutants after amendments in field soils with sewage sludge are highly influenced by soil properties (McBride et al. 2004). However, to the best knowledge of the authors, no research studies on this topic had been carried out using ecotoxicological criteria.

Results from this work seem to support the validity of such assumptions for the studied organic waste, as important differences in toxicity were found between different soils (the OECD artificial soil and agricultural, grassland, and woodland natural soils). Differences in toxicity among soils were four-fold for mortality and seventeen-fold for reproduction. When only agricultural soils were taken in to account, variation was lower but still important, with close to a three-fold impact for mortality and reproduction.

The sewage sludge used in this study presented high concentrations of lead and ethoxylated nonylphenols (NPE), which are expected to have effects on the survival and reproduction of this species according to several studies (Greenslade and Vaughan 2003, Bongers et al. 2004, Scott-Fordsmand and Krogh 2004, Fountain and Hopkin 2005). However, it is also known that the bioavailability of pollutants in sludge is low, as they are strongly associated with organic matter and mineral particles (Jacobsen et al. 2004, Alonso et al. 2006, Dayton et al. 2006). In addition, it has been reported that ammonium is probably one of the main contributors to the short-term toxicity of organic

wastes in this species, over any individual pollutant concentration (Domene et al. 2007). After soil amendments with sewage sludge, ammonium levels in the water soil phase are typically significant during the first weeks, coupled with higher decomposition rates. Later, such releases cease and other nitrogen forms, nitrates and nitrites, become dominant (Qiang et al. 2004, Van Niekerk et al. 2006). Ammonium is water soluble, but in addition it is strongly retained in the soil solid phase by the cation exchange sites of clay, metal oxides, and organic matter (Buss et al. 2004). Furthermore, ammonium levels in the soil pore water are not static, and this compound is oxidized to nitrate by nitrifier bacteria, in a process generally carried out in aerobic conditions (Buss et al. 2004). Additionally, ammonium can be transformed to ammonia under neutral and alkaline conditions, generally associated with decomposition in aerobic environments, and given its high volatility, can be released from soil (Kirchmann and Witter 1989). Hence, although ammonia can accumulate in soil pore water coming from the decomposition of organic wastes, the combined action of sorption, biotransformation, and volatilization can reduce its concentration in the soil and also the observed toxicity.

It has been suggested that, in collembolans, the uptake process is more associated to the soil solid-phase than in the soft-bodied oligochaete species and plants, which are more strongly influenced by pore-water characteristics (Vijver et al. 2001). However, the pore-water pollutant concentrations have been indicated as the main explanatory parameter for the toxicity in collembolans (Martikainen and Krogh 1999, Lock and Janssen 2003), despite the fact that other uptake processes are likely to occur simultaneously, as is the case for this species, which ingest organic wastes during the bioassays (Domene et al. 2007, chapter 3).

The results of this study suggest that, in the studied sludge, the uptake of pollutants from pore water might be significant in collembolans, since important differences in toxicity had been observed in a set of contrasted soils. However, differences in the gut uptake might also influence the observed toxicity in soils with higher organic content, as it has

been demonstrated that this species is able to avoid feeding on polluted organic wastes, especially when clean food sources are available (chapter 3).

No direct measures of the concentrations of pollutants in pore water were carried out, and neither was the feeding behaviour of the individuals in different soils assessed. Whichever the main uptake route, it is likely that toxicity differences were mainly related to the ammonium levels, as demonstrated in a previous work (Domene et al. 2007). The decrease in the sludge toxicity at increasing soil organic carbon contents agrees with the known high sorption of ammonium to organic matter, but also with an eventual avoidance behavior when unpolluted food is available. The decrease in sludge toxicity in soils with higher sand contents (and as consequence lower clay content), however, is contrary to that expected for ammonium, highly retained in clay (Buss et al. 2004). This association suggests a preeminence of soil-pore uptake in this species, as the higher gaseous exchange in sandy soils might explain the lower toxicity of sludge. The maintenance of more aerobic conditions in sandy soils facilitate the bacterial conversion of ammonium to other less toxic nitrogen forms (Buss et al. 2004) but also its volatilization in the form of ammonia (Kirchmann and Witter 1989).

Conclusions drawn in this study should be taken with care, as the soil properties identified as the main predictors for differences in sludge toxicity might be biased by the set of selected soils, which is too small in number to provide robust conclusions.

CONCLUSIONS

The use of the OECD artificial soil provides relevant data for the toxicity assessment of sewage sludge to real agricultural soils when the survival of *F. candida* is used as the endpoint, as effects are displayed at similar ranges as found in natural agricultural soils. However, when reproduction is used, OECD artificial soil can overestimate, sometimes significantly, the toxicity of sludge with respect to agricultural soils. Results with the OECD

artificial soil are not suitable for the estimation of effects in non-agricultural soils, as in this case toxicity was highly overestimated.

The toxicity of sewage sludge to collembolans showed large variation between soils, but especially when reproduction was used as the endpoint. Soils which presented lower toxic effects of sludge were those with higher coarse sand content, lower fine sand content, and higher organic carbon content, thus suggesting a higher resilience to sludge amendments of soils with such properties.

The particular soil properties which mainly influence the relative toxicity of the sludge in different soils suggest that ammonium might play an important role in the sludge toxicity to this species, at least in the short-term after the application.

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Chapter 8

General discussion, conclusions, and further research

8.1. The problem

Correct organic wastes management is one of the main environmental challenges in the European Union. The increasing production of such wastes, and also the growing concerns and legal limitations to incineration and landfilling, indicate waste recycling in soil as the main future management option. Even though benefits of organic wastes amendments to soils are widely documented, it is also know that they tend to accumulate chemicals released from different human activities, posing a risk to soil ecosystems.

Organic wastes' recycling in soils lacks specific legislation, with the exception of sewage sludge, for which any sort of stabilization treatment and low heavy metal content are required. This is why, for most wastes, recycling in soil is exclusively regulated by agronomical criteria and good practices guidelines. For other wastes, use in soil might be indirectly limited by some laws, such as surface waters protection from nitrate pollution in vulnerable regions, or by limit values for heavy metals content when wastes are commercialized as fertilisers.

However, none of these laws include ecotoxicological assays for wastes risk assessment, despite the already known limitations of chemical assays for this purpose in real situations.

The use of bioassays is doubtless a suitable approach to manage the current environmental challenge of defining appropriate tools for the organic waste ecological risk assessment. To date, the most usual approach has been testing waste aqueous eluates with aquatic bioassays. However, this approach is being questioned by

researchers because of its low relevance for soils ecosystems, and because of the increasing availability of bioassays using soil organisms.

8.2. Suitable methodologies for organic waste ecotoxicity assessment

8.2.1. Waste solid-phase versus waste eluate assays

Until recently, the most usual approach to assess a waste's ecotoxicity to soils is to prepare water eluates of the waste and to carry out aquatic ecotoxicity assays with dilutions of this eluate. Since a pollutant's water-soluble fraction is expected to equal the bioavailable fraction, it is assumed that results from such tests on aquatic organisms are also representative of pollutant effects on soil organisms.

Results from this thesis indicate that the wastes' aqueous eluates used in aquatic tests (Microtox, *D. magna* mobility test) are not relevant for predicting sublethal effects of the wastes' solid-phase on most soil organisms (see Annex 1). Results from the *D. magna* tests with aqueous eluates are equivalent to that obtained from wastes' solid-phase tests on *F. candida's* mortality, and with growth inhibition in some plants. On the contrary, no correlations have been found with other lethal or sublethal endpoints of other soil-dwelling organisms. Hence, the use of aqueous eluates is generally not relevant to estimate effects on soil-dwelling organisms, which are more accurately assessed through solid-phase assays on soil organisms.

Waste solid-phase assays show peculiarities that do not occurr in single chemical assays. Increasing waste concentrations in soil-waste mixtures might result in changes in pH, electrical conductivity, water holding capacity and nourishing effect of the mixture. However, these properties are not expected to disturb the observed results at the concentrations presenting noxious effects, and if they do, such peculiarities should be considered a part of the complex effects exerted by wastes in real situations.

8.2.2. Suitable test organisms and endpoints for waste testing

Among aquatic assays with aqueous eluates, both Microtox luminescence inhibition test and *D. magna* mobility inhibition test are sensitive to different wastes but, in general terms, results are not consistent with those obtained from soil assays with the wastes' solid-phase. The only exception is the *D. magna* mobility inhibition test, whose results correlate with those obtained in solid-phase bioassays with *F. candida* (mortality), *B. rapa* (growth) and *L. perenne* (growth) (see Annex 1).

In soil bioassays, earthworms', enchytraeids' and collembolans' mortality follows a consistent pattern among species and correlates with sublethal effects observed both in soil animals and plants (see Annex 1). Thus, mortality may be considered a suitable and sensitive endpoint.

Plant germination and growth, enchytraeid and collembolan reproduction, collembolan feeding inhibition and earthworm body weight also are sensitive endpoints for waste testing. Results of these assays are generally consistent with the exception of enchytraeid and collembolan reproduction. In contrast, collembolan body length and soil microorganisms' subtrate-induced respiration are not useful for waste testing, as they are usually enhanced at increasing waste concentrations (see Annex 1).

Collembolan feeding represents an innovative and promising approach that might be used as an alternative to the endpoints traditionally used for this species (mortality and reproduction), given the lower experimental time required (less than a week) compared to mortality and reproduction (requiring four weeks).

8.3. Main parameters responsible for organic waste toxicity

Chemical assays are not useful to predict organic wastes' ecological risk to soil-dwelling organisms, despite the fact that they are the only criteria included in the current and upcoming European legislation. Neither individual pollutant concentration nor total pollutant concentration correlate with the observed lethal or sublethal toxic effects on

any test organism or endpoint, neither in solid-phase nor in eluate assays (see Annex 2). The exception is feeding inhibition in *F. candida* after 4 days of exposure, which correlates with the total pollutant burden.

However, toxicity on most of the soil inhabitant species and endpoints tested is clearly and significantly correlated with parameters reflecting a low waste stability (high ease of decomposition). Furthermore, the mobility inhibition of *D. magna* by the wastes' water eluates also shows an association with the original wastes' stability (see Annex 3).

Substances released during the wastes' decomposition may be responsible for this relationship. The lower the wastes' stability, the higher is the release of substances (i.e. ammonium). The effect of these substances on toxicity is strong enough to mask small differences in the wastes' pollutant burden, at least in the short-term after the application of the wastes to soils.

8.4. Effect of waste treatments and post-treatments on toxicity

The demonstrated relationship between low organic matter stability and toxicity to soil-dwelling organisms indicates the need for organic wastes' stabilization post-treatments prior to their use in soil. Composting is the most effective post-treatment to reduce the original dewatered sludge toxicity, mainly through enhancing its stability. Therefore this post-treatment, together with low pollutant burden, should be recommended as a requirement for waste application to soil. Furthermore, composting facilitates the reduction of the wastes' water content and transport costs, and removes most of the pathogens present in the original waste.

Results from this thesis also suggest that thermal drying might increase waste toxicity, probably due to the breakdown of the organic waste matrix structures at high temperatures, decreasing waste stability and increasing the release of noxious substances. Hence, despite the advantages of this treatment in terms of hygienization

and transport ease, according to ecotoxicological criteria, thermal drying is the least recommended post-treatment for wastes to be used in soils.

In the last years, the number of specialized pig slurry treatment plants have increased in Europe in order to decrease the nitrogen overfertilization of soils. However, the end product of these plants, thermally-dried pig slurry, is also unsuitable for use in soils, because its low stability is likely to exert harmful effects on soil-dwelling organisms.

8.5. The relevance of laboratory bioassays for the ecological risk assessment of organic waste amendments

Data from a soil test battery and the "species sensitivity distribution" (SSD) method for deriving maximum (ecologically non-harmful) amendment rates (PNEC) are suitable to compare the ecological risk of different organic wastes. The maximum amendment rates derived by the SSD method are in accordance with the non-harmful rates to soil-dwelling organisms reported in different published studies. However, the estimated safe amendment rates have not been validated in field conditions, and therefore further investigations are needed to justify extrapolation.

Most bioassay and endpoint toxicity data obtained from waste solid-phase tests are correlated with the derived maximum amendment rates (PNEC) (see Annex 1), and therefore are also relevant for comparing different wastes for ecotoxicological risk (with the exception of collembolans reproduction). On the other hand, water eluate tests are not suitable for this purpose as they show no correlations with maximum amendment rates.

As already found in the individual bioassays, organic waste pollutant burden is not suitable for estimating maximum amendment rates (Annex 2). However, parameters related to waste stability were clearly correlated with the safe amendment rates. The higher the stability, the higher the amendment rate may be without risk to soil agroecosystems (Annex 3).

Organic waste composting allows safe use in soil. If sludge composts are applied according to the usual amendment criteria, non-harmful effects to soil agroecosystems are expected below 70-110 t ha^{-1} DM, a rate notably higher than the crops N-demand. For the other wastes, harmful effects are likely to appear at around 5 t ha^{-1} DM.

8.6. Influence of soil properties on the bioassays' results

The use of OECD artificial soil is suitable to assess effects on the mortality or reproduction of earthworms, enchytraeids or collembolans. However, collembolans usually evade this substrate in avoidance tests and prefer natural soils, probably due to its high sand content. Thus OECD artificial soil is not recommended as a control for polluted natural soils in avoidance tests with collembolans. Some selected natural soils are also unsuitable in ecotoxicological testing with particular species. More precisely, fine texture and low organic matter soils are not advisable for tests with *E. andrei* and *E. crypticus*. On the other hand, *F. candida* avoids sandy soils with high nitrogen levels.

Collembolan mortality and reproduction was investigated in a risk assessment of an organic waste in OECD artificial soil and natural agricultural soils. Similar mortality was found at similar concentrations of waste in both OECD and agricultural soils. In contrast, effects on reproduction are overestimated in OECD soil. These facts suggest that extrapolations relying exclusively on *F. candida* tests with artificial soil can lead to biased conclusions.

Fine texture and low organic matter content of soils enhance the waste toxic effects on collembolans. Since this sort of soils is widespread in the Mediterranean area, this region may be considered vulnerable to waste amendments. However, these results are only applicable to collembolans, and not to other organisms, for which more work is required.

8.7. General conclusions

A) Selection of suitable methodologies for organic waste ecotoxicity assessment in terms of sample preparation procedures, test organisms and endpoints.

1. The general lack of correlation between the results of eluate assays using aquatic organisms with the results of solid-phase assays using soil-dwelling organisms leads to recommend the latter for the organic waste ecotoxicological assessment to soils.
2. The Microtox luminescence inhibition test is not suitable for predicting organic waste amendment effects on soil-dwelling species. In contrast, the *D. magna* mobility inhibition test provides information equivalent to the waste solid-phase lethality tests on collembolans, and to growth inhibition tests in plants.
3. Lethality is a sensitive endpoint for organic waste testing in earthworms, enchytraeids, and collembolans, and generally correlates with sublethal effects on both soil animals and plants.
4. Plant germination and growth, enchytraeid and collembolan reproduction, collembolan feeding inhibition, and earthworm body weight are sensitive sublethal endpoints suitable for waste testing.
5. Collembolan body length is unsuitable for organic waste testing, as they it is generally stimulated by waste concentration.
6. Collembolan feeding inhibition is a promising tool for organic waste risk assessment as it produces results in less than a week, whereas survival and reproduction inhibition tests require a four week test period for equivalent conclusions.

B) Identification of the main waste parameters explaining the toxicity of waste to soil-dwelling organisms, and also the most suitable waste treatments to decrease toxicity.

7. Chemical assays are unsuitable for predicting a waste's ecotoxicological risk, because the results are usually uncorrelated with results from most current evaluated tests.
8. Lethal and sublethal waste toxicity on most soil-dwelling organisms is mainly explained by the low stability of the waste.
9. Composting post-treatment is better than thermal drying as it, in addition to allow the hygienisation and ease of transport of raw wastes, reduces the waste's ecotoxicity and improves its quality for soil amendment.

C) Assessment of the aptitude of laboratory waste bioassays for the ecological risk assessment of organic waste field amendments.

10. Extrapolation of safe amendment rates from the "species sensitivity distribution" method is suitable for comparative purposes, but its relevance for actual field amendments remains unverified.
11. Composted sludge can be safely used in soil, since the dosages of amendment expected to be harmful are much greater than the usual amendment rates. On the other hand, dewatered and thermally-dried sludge and thermally-dried pig slurry are not hazardous for soils if applied according to crop demands (usually below 3 t ha^{-1} DM). However, slightly higher dosages (around 5 t ha^{-1} DM) are expected to have noxious effects on soil ecosystems.

D) Evaluation of the suitability of OECD artificial soil as a natural soil surrogate for waste bioassays, and the influence of different soil properties on test results.

12. The OECD soil is suitable for waste ecotoxicity risk assessment with collembolan mortality but not with collembolan reproduction, since toxicity is usually overestimated when using this substrate in comparison with natural agricultural soils.
13. Fine textured soils with low organic matter levels are more vulnerable to organic waste amendment toxic effects.

E) Assessment of the effect of different soils (including OECD) and different soil properties on test organisms.

14. Some natural soils are unsuitable for ecotoxicological testing purposes, but this inappropriateness is strongly dependent on the test organism used. Soils with low organic contents and with fine texture are not suitable for ecotoxicity tests with *E. andrei* and *E. crypticus*, while sandy soils with high nitrogen levels are not suitable for *F. candida*.
15. The OECD soil is suitable as a test substrate in reproduction and avoidance tests with earthworms, enchytraeids and collembolans, but is not recommended for avoidance tests with collembolans, since they usually prefer natural soils to the OECD substrate.

8.8. Further research

Results from this thesis suggest the need for more research, which might be outlined as:

- Standardization of sensitive single-species test methods for wastes, taking into account the peculiarities of such materials, in terms of sample and soil-waste mixture preparation and water content selection.
- Validation of the suitability of feeding inhibition and avoidance response as endpoints for waste testing with respect to traditional endpoints (mortality, reproduction, growth).
- Assessment of multispecies systems (terrestrial ecosystem models) as a tool to evaluate more precisely the noxious effects of organic wastes on soil functions and soil communities.
- Validation in field of the ecological relevance of the extrapolations from single-species tests or multispecies systems.
- Quantification of the bias of soil properties on the toxicity of wastes to different soil-dwelling organisms, in order to improve extrapolation from a specific soil to a variety of field soils.

ANNEX 1. Pearson's correlation coefficients among bioassay toxicity results.

	PNEC	*B. rapa* EC20 germination	*B. rapa* EC20 growth	*D. magna* EC50 mobility 48h	*E. andrei* LC20 14d	*E. andrei* LC20 28d	*E. crypticus* EC20 reproduction	*E. crypticus* LC20	*F. candida* EC20 reproduction	*F. candida* EC50 feeding 2d	*F. candida* EC50 feeding 4d	*F. candida* EC50 feeding 7d	*F. candida* LC20	*L. perenne* EC20 germination	*L. perenne* EC20 growth	Microtox	*T. pratense* EC20 germination	*T. pratense* EC20 growth
PNEC	1	0.834(*)	0.857(*)	0.705	0.945(**)	0.921(**)	0.957(**)	0.993(**)	0.462	0.965(**)	0.952(**)	0.971(**)	0.850(*)	0.848(*)	0.875(**)	0.607	0.909(**)	0.943(**)
***B. rapa* EC20 germination**	0.834(*)	1	0.951(**)	0.788	0.942(**)	0.955(**)	0.739	0.824(*)	0.38	0.858(*)	0.899(*)	0.854(*)	0.970(**)	0.978(**)	0.981(**)	0.43	0.940(**)	0.916(**)
***B. rapa EC20* growth**	0.857(*)	0.951(**)	1	0.908(*)	0.920(**)	0.921(**)	0.779(*)	0.836(*)	0.442	0.905(*)	0.935(**)	0.901(*)	0.948(**)	0.919(**)	0.990(**)	0.525	0.871(*)	0.855(*)
***D. magna* EC50 mobility 48h**	0.705	0.788	0.908(*)	1	0.679	0.668	0.539	0.71	0.534	0.832	0.845	0.811	0.909(*)	0.799	0.889(*)	-0.143	0.606	0.605
***E. andrei* LC20 14d**	0.945(**)	0.942(**)	0.920(**)	0.679	1	0.998(**)	0.892(**)	0.932(**)	0.495	0.938(**)	0.957(**)	0.946(**)	0.902(**)	0.911(**)	0.950(**)	0.578	0.948(**)	0.956(**)
***E. andrei* LC20 28d**	0.921(**)	0.955(**)	0.921(**)	0.668	0.998(**)	1	0.863(*)	0.908(**)	0.496	0.920(**)	0.946(**)	0.929(**)	0.905(**)	0.916(**)	0.954(**)	0.549	0.945(**)	0.948(**)
***E. crypticus* EC20 reproduction**	0.957(**)	0.739	0.779(*)	0.539	0.892(**)	0.863(*)	1	0.920(**)	0.311	0.854(*)	0.856(*)	0.895(*)	0.719	0.737	0.780(*)	0.682	0.841(*)	0.902(**)
***E. crypticus* LC20**	0.993(**)	0.824(*)	0.836(*)	0.71	0.932(**)	0.908(**)	0.920(**)	1	0.505	0.971(**)	0.946(**)	0.962(**)	0.856(*)	0.850(*)	0.863(*)	0.578	0.907(**)	0.932(**)
***F. candida* EC20 reproduction**	0.462	0.38	0.442	0.534	0.495	0.496	0.311	0.505	1	0.793	0.767	0.755	0.371	0.292	0.458	0.1	0.275	0.28
***F. candida* EC50 feeding 2d**	0.965(**)	0.858(*)	0.905(*)	0.832	0.938(**)	0.920(**)	0.854(*)	0.971(**)	0.793	1	0.991(**)	0.989(**)	0.902(*)	0.882(*)	0.917(*)	0.59	0.876(*)	0.899(*)
***F. candida* EC50 feeding 4d**	0.952(**)	0.899(*)	0.935(**)	0.845	0.957(**)	0.946(**)	0.856(*)	0.946(**)	0.767	0.991(**)	1	0.994(**)	0.917(*)	0.909(*)	0.944(**)	0.554	0.885(*)	0.914(*)
***F. candida* EC50 feeding 7d**	0.971(**)	0.854(*)	0.901(*)	0.811	0.946(**)	0.929(**)	0.895(*)	0.962(**)	0.755	0.989(**)	0.994(**)	1	0.870(*)	0.864(*)	0.906(*)	0.567	0.865(*)	0.913(*)
***F. candida* LC20**	0.850(*)	0.970(**)	0.948(**)	0.909(*)	0.902(**)	0.905(**)	0.719	0.856(*)	0.371	0.902(*)	0.917(*)	0.870(*)	1	0.988(**)	0.972(**)	0.435	0.935(**)	0.901(**)
***L. perenne* EC20 germination**	0.848(*)	0.978(**)	0.919(**)	0.799	0.911(**)	0.916(**)	0.737	0.850(*)	0.292	0.882(*)	0.909(*)	0.864(*)	0.988(**)	1	0.956(**)	0.421	0.963(**)	0.937(**)
***L. perenne* EC20 growth**	0.875(**)	0.981(**)	0.990(**)	0.889(*)	0.950(**)	0.954(**)	0.780(*)	0.863(*)	0.458	0.917(*)	0.944(**)	0.906(*)	0.972(**)	0.956(**)	1	0.508	0.919(**)	0.898(**)
Microtox	0.607	0.43	0.525	-0.143	0.578	0.549	0.682	0.578	0.1	0.59	0.554	0.567	0.435	0.421	0.508	1	0.58	0.548
***T. pratense* EC20 germination**	0.909(**)	0.940(**)	0.871(*)	0.606	0.948(**)	0.945(**)	0.841(*)	0.907(**)	0.275	0.876(*)	0.886(*)	0.865(*)	0.935(**)	0.963(**)	0.919(**)	0.58	1	0.985(**)
***T. pratense* EC20 growth**	0.943(**)	0.916(**)	0.855(*)	0.605	0.956(**)	0.948(**)	0.902(**)	0.932(**)	0.28	0.899(*)	0.914(*)	0.913(*)	0.901(**)	0.937(**)	0.898(**)	0.548	0.985(**)	1

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

ANNEX 2. Pearson's correlation coefficients among bioassay toxicity results and waste pollutant burdens.

	Heavy metals	Organic pollutants	Persistent organics	Non-pesistent organics	Total burden	Cd	Cr	Cu	Hg	Ni	Pb	Zn	DEHP	LAS	NPE	PAH	PCB	PCDD/F
PNEC	-0.509	-0.024	0.441	-0.03	-0.501	0.256	0.774(*)	-0.55	0.263	0.341	-0.08	-0.458	-0.05	-0.038	0.049	0.155	0.181	0.438
***B. rapa* EC20 germination**	-0.481	-0.081	0.42	-0.092	-0.607	0.43	0.464	-0.281	0.388	0.551	-0.055	-0.386	0.115	-0.111	0.061	0.321	0.318	0.409
***B. rapa* EC20 growth**	-0.699	-0.035	0.28	-0.04	-0.619	0.476	0.46	-0.183	0.322	0.315	-0.298	-0.273	0.023	-0.074	0.139	0.322	0.184	0.265
***D. magna* EC50 mobility 48h**	-0.805	0.527	0.326	0.531	-0.023	0.54	0.725	-0.2	0.444	0.182	-0.297	-0.446	0.347	0.525	0.574	0.34	0.09	0.312
***E. andrei* LC20 14d**	-0.459	-0.212	0.337	-0.22	-0.692	0.21	0.591	-0.505	0.187	0.4	-0.032	-0.326	-0.093	-0.233	-0.096	0.097	0.139	0.334
***E. andrei* LC20 28d**	-0.441	-0.236	0.324	-0.244	-0.712	0.206	0.552	-0.49	0.182	0.421	-0.029	-0.307	-0.095	-0.257	-0.114	0.092	0.138	0.32
***E. crypticus* EC20 reproduction**	-0.386	-0.175	0.373	-0.181	-0.529	0.076	0.722	-0.652	0.098	0.178	0.072	-0.4	-0.16	-0.178	-0.146	-0.018	0.073	0.376
***E. crypticus* LC20**	-0.509	0.025	0.463	0.02	-0.467	0.287	0.789(*)	-0.526	0.305	0.397	-0.07	-0.474	0.044	0.09	0.104	0.197	0.22	0.459
***F. candida* EC20 reproduction**	-0.574	-0.114	-0.307	-0.102	-0.514	-0.068	0.331	-0.23	-0.219	-0.056	-0.502	0.249	-0.303	-0.139	0.049	-0.221	-0.46	-0.314
***F. candida* EC50 feeding 2d**	-0.711	-0.223	0.128	-0.215	-0.771	0.198	0.69	-0.379	-0.019	0.115	-0.485	-0.221	-0.516	-0.266	-0.08	-0.015	-0.312	0.061
***F. candida* EC50 feeding 4d**	-0.694	-0.279	0.061	-0.271	-0.815(*)	0.174	0.652	-0.396	-0.058	0.119	-0.465	-0.191	-0.543	-0.321	-0.062	-0.056	-0.358	0.07
***F. candida* EC50 feeding 7d**	-0.67	-0.277	0.131	-0.269	-0.783	0.104	0.723	-0.468	-0.129	0.046	-0.417	-0.298	-0.586	-0.311	-0.088	-0.126	-0.419	0.082
***F. candida* LC20**	-0.58	0.118	0.51	0.108	-0.464	0.6	0.532	-0.17	0.544	0.589	-0.11	-0.476	0.263	0.082	0.267	0.501	0.442	0.495
***L. perenne* EC20 germination**	-0.458	0.07	0.578	0.058	-0.459	0.542	0.546	-0.257	0.544	0.655	0.031	-0.538	0.275	0.042	0.188	0.463	0.484	0.567
***L. perenne* EC20 growth**	-0.624	-0.054	0.338	-0.061	-0.627	0.456	0.486	-0.237	0.348	0.423	-0.207	-0.319	0.053	-0.09	0.112	0.321	0.238	0.325
Microtox	-0.283	-0.444	-0.028	-0.444	-0.619	0.024	0.12	-0.174	-0.151	-0.116	-0.199	0.103	-0.461	-0.474	-0.33	-0.011	0.05	-0.03
***T. pratense* EC20 germination**	-0.338	-0.083	0.562	-0.096	-0.532	0.37	0.584	-0.419	0.411	0.606	0.137	-0.512	0.125	-0.104	0.07	0.316	0.438	0.557

ANNEX 3. Pearson's correlation coefficients among bioassay toxicity results and waste physico-chemical parameters.

	Organic matter	Organic carbon	Stability	pH	EC	N	Non-hydrolyzable N	Hydrolyzable N	NH4-N
PNEC	-0.387	-0.387	0.838(*)	0.184	-0.399	-0.736	0.359	-0.756(*)	-0.837(*)
***B. rapa* EC20 germination**	-0.707	-0.707	0.885(**)	0.297	-0.436	-0.956(**)	0.145	-0.945(**)	-0.806(*)
***B. rapa* EC20 growth**	-0.758(*)	-0.758(*)	0.932(**)	0.131	-0.231	-0.951(**)	0.071	-0.931(**)	-0.711
***D. magna* EC50 mobility 48h**	-0.613	-0.613	0.949(**)	0.529	-0.387	-0.941(**)	-0.019	-0.925(**)	-0.474
***E. andrei* LC20 14d**	-0.577	-0.577	0.820(*)	0.178	-0.364	-0.847(*)	0.186	-0.853(*)	-0.817(*)
***E. andrei* LC20 28d**	-0.609	-0.609	0.810(*)	0.193	-0.368	-0.861(*)	0.15	-0.862(*)	-0.806(*)
***E. crypticus* EC20 reproduction**	-0.237	-0.237	0.729	-0.019	-0.305	-0.599	0.439	-0.641	-0.830(*)
***E. crypticus* LC20**	-0.387	-0.387	0.830(*)	0.251	-0.43	-0.738	0.346	-0.756(*)	-0.818(*)
***F. candida* EC20 reproduction**	-0.598	-0.598	0.311	0.247	0.079	-0.395	-0.565	-0.289	0.01
***F. candida* EC50 feeding 2d**	-0.654	-0.654	0.897(*)	0.056	-0.109	-0.802	-0.149	-0.78	-0.771
***F. candida* EC50 feeding 4d**	-0.698	-0.698	0.912(*)	0.059	-0.14	-0.829(*)	-0.19	-0.797	-0.802
***F. candida* EC50 feeding 7d**	-0.617	-0.617	0.884(*)	0.032	-0.155	-0.764	-0.112	-0.737	-0.817(*)
***F. candida* LC20**	-0.676	-0.676	0.947(**)	0.352	-0.462	-0.968(**)	0.218	-0.964(**)	-0.801(*)
***L. perenne* EC20 germination**	-0.605	-0.605	0.919(**)	0.374	-0.545	-0.940(**)	0.3	-0.948(**)	-0.867(*)
***L. perenne* EC20 growth**	-0.742	-0.742	0.921(**)	0.21	-0.319	-0.963(**)	0.099	-0.949(**)	-0.756(*)
Microtox	-0.168	-0.168	0.367	-0.587	0.278	-0.423	0.289	-0.547	-0.42
***T. pratense* EC20 germination**	-0.46	-0.46	0.837(*)	0.25	-0.513	-0.856(*)	0.407	-0.90(**)	-0.913(**)
***T. pratense* EC20 growth**	-0.388	-0.388	0.833(*)	0.264	-0.554	-0.796(*)	0.45	-0.833(*)	-0.946(**)





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