Ecological risk assessment of a tropical metal contaminated area: the case study of Santo Amaro, Bahia, Brazil

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Resumo

O presente estudo, realizado na área abandonada de uma fundição de chumbo no Brasil, teve como principal objetivo contribuir para a aplicação, em ambientes tropicais, de um esquema de análise de risco ecológico em etapas, e avaliar a adequabilidade dos diferentes parâmetros nas etapas dentro do enfoque da Tríade. Buscou-se caracterizar o risco ecológico em vários pontos da área de estudo, uma das mais contaminadas por metais do mundo, em Santo Amaro (BA, Brasil), fornecendo informações que indiquem possíveis medidas de remediação para o local e trazendo uma importante contribuição para decisões futuras. O objetivo deste trabalho é contribuir para o uso da avaliação de risco ecológico em processos de restauração e recuperação de locais contaminados no Brasil, seguindo as atuais tendências mundiais de proteção do solo. O Capítulo 2 apresenta a investigação preliminar, incluindo a fase de formulação do problema e o levantamento de informações científicas disponíveis sobre a área de estudo, resultando na elaboração de um modelo conceitual para o local e um plano de análise para a avaliação de risco. Um esquema em fases é proposto, integrando informações de três linhas de evidência (LoE): química, ecotoxicológica e ecológica. Os objetivos e as ações de cada fase da análise de risco foram estabelecidos para incluir avaliações ecológicas e ecotoxicológicas, não realizadas nos estudos anteriores sobre o local, com foco no compartimento ambiental solo. O plano de análise incluiu duas fases, usando a abordagem da Tríade: a fase 1, sendo uma fase de "varredura", (Capítulo 3); e a fase 2, sendo a avaliação de risco detalhada (Capítulo 7). Na fase 1, a LoE química indicou um alto nível de contaminação por metais relacionados às atividades da antiga fábrica, mostrando uma alta heterogeneidade espacial relacionada aos locais de deposição de escórias e à tentativa de recobrir a escória com solo. Na LoE ecotoxicológica, os ensaios de fuga com organismos de solo indicaram um risco mais alto para o solo, relacionado aos locais dentro da área da fábrica com solos arenosos, do que para a água, relacionado aos ensaios com eluatos (luminescência de *V. fischeri* e ensaio de letalidade com *D. magna*), o que sugere uma alta retenção dos metais no solo da maioria dos pontos analisados. Na LoE ecológica, respiração basal do solo, *bait lamina* e cobertura vegetal apresentaram a mesma tendência nas respostas, apesar da menor sensibilidade deste último parâmetro. Os altos valores de risco encontrados em locais dentro da área da fábrica sugeriram a necessidade de ações de remediação do local, enquanto que algumas incertezas associadas às diferentes respostas nas LoE sugeriram a necessidade de avançar para a fase 2 da avaliação de risco em alguns pontos (cujas informações foram coletadas nos capítulos seguintes à fase 1). O Capítulo 4 apresenta os efeitos dos solos contaminados sobre a

reprodução de *Eisenia andrei*, *Enchytraeus crypticus* e *Folsomia candida,* e uma discussão sobre as diferentes sensibilidades destas species e sobre a performance destes ensaios padronizados em solos tropicais. Os Capítulos 5 e 6 foram dedicados aos parâmetros ecológicos usados na LoE ecológica. O Capítulo 5 apresenta o uso de indicadores microbianos de qualidade de solo na avaliação das condições ecológicas e da atividade biológica na área de estudo. O Capítulo 6 apresenta a aplicação das avaliações ecológicas e ensaios *in situ* para avaliar os efeitos na estrutura e no funcionamento do ecossistema. Os resultados de alguns destes parâmetros indicaram que a cobertura vegetal e a composição de espécies estiveram correlacionadas com algumas funções do solo, tais como a ciclagem de nutrientes e a quebra de material orgânico. Os *endpoints* avaliados foram estrutura da vegetação, invertebrados da superfície do solo, decomposição do material orgânico e alguns parâmetros microbianos. Estes parâmetros indicaram uma clara diferença entre os pontos dentro e fora da área da fábrica, indicando risco ecológico mesmo 17 anos depois do encerramento das atividades da fábrica. No Capítulo 7 são apresentados os valores de risco para cada LoE e os valores de risco integrado, calculados na fase 2 da análise de risco, usando os resultados dos parâmetros apresentados nos Capítulos de 4 a 6, e incluindo os ensaios com plantas e os ensaios crônicos aquáticos com os eluatos. Os altos valores de risco em alguns pontos, confirmando o risco indicado na fase 1, restringem o uso da área mesmo como área industrial, requerendo o encapsulamento do solo. No Capítulo 8 é apresentada uma discussão crítica sobre os resultados e sobre as perspectivas de pesquisas futuras para o local. Em geral, os resultados sugerem que a deposição de escória dentro da área da fábrica e a tentativa falhada de encapsulamento tem impedido o estabelecimento da vegetação, levando a uma mudança e simplificação da estrutura do habitat. Aliado ao efeito tóxico dos metais, a limitação do estabelecimento da vegetação resulta em baixos conteúdos de matéria orgânica no solo para servir de fonte de C e energia para os microrganismos e como proteção para a comunidade microbiana contra os altos níveis de metais no solo. Estas mudanças nas condições microclimáticas no solo e na quantidade de entrada de matéria orgânica causam impactos negativos na atividade microbiana e dos organismos do solo, consequentemente afetando os serviços do ecossistema e outros processos realizados por estes organismos. O encapsulamento adequado dos depósitos de escória, com o paralelo re-estabelecimento da cobertura vegetal, parecem ser essenciais para melhorar a condição ecológica do local.

Keywords: Avaliação de risco ecológico, Ecotoxicologia terrestre, Função de habitat, Função de retenção, Metais

Summary

The present study was carried out in an abandoned lead smelter in Brazil, with the major goal of further contribute to the application of a tiered ecological risk assessment framework to tropical environments, and to evaluate the feasibility and usefulness of different assessment tools to be used in different tiers within a Triad approach. The results aimed at characterizing the ecological risk in one of the most metal contaminated areas in the world, in Santo Amaro (BA, Brazil), ranking sites in the study area, supplying information to indicate remediation measures and bringing an important contribution to support future decisions. This thesis aims at providing important information to help the regular use of the risk assessment process to support site restoration and reclamation decisions in Brazil, following the current trends in soil protection around the world. Chapter 2 presents the preliminary investigation, including the problem formulation phase and the collection of the scientific information available about the study area, that resulted in the conceptual model and the analysis plan for the risk assessment. A tiered approach is proposed integrating information from three lines of evidence (LoE): chemical, ecotoxicological and ecological. Aims and actions of each phase of risk assessment are established, in order to include the ecological and ecotoxicological perspectives missing in previews studies conducted in this area, focusing on the soil compartment. The analysis plan included two tiers using the triad approach: tier 1, the screening phase (Chapter 3), and tier 2, the detailed risk assessment (Chapter 7). At tier 1, chemical LoE indicated a high level of metal contamination in the study area caused by the smelting activities, showing high spatial heterogeneity originated not only by the uneven deposition of residues during smelting activities, but also by the current status of the (pseudo) rehabilitated residue piles. In the ecotoxicological LoE, avoidance tests on soil organisms indicated a higher risk at sites inside the smelter and with sandy soils than tests on eluates (*V. fischeri* luminescence and *D. magna* lethal), suggesting a high retention of metals on soils in most areas. Regarding the ecological LoE, soil respiration, bait lamina and vegetation cover revealed a concordant response, despite the lower sensitivity of this last parameter. Very high risk levels, associated with sandy soils and residue deposits inside the smelter area, suggest the need to proceed with remediation actions, while the uncertainties associated with the contradictory information given by certain LoE at certain sampling points showed the need to confirm potential risks in a tier 2 analysis (which information was collected in the following chapters). Chapter 4 presents the effects of the tested metal contaminated soils on the reproduction of *Eisenia andrei*, *Enchytraeus crypticus* and *Folsomia candida,* and a discussion about the different sensitivities of these

species and about the performance of these standardized tests in tropical soils. Chapters 5 and 6 are dedicated to ecological parameters used within the ecological LoE. Chapter 5 presents the use of microbial soil-quality indicators to evaluate the ecological conditions and biological activity in the study area. Chapter 6 presents the application of ecological evaluations and *in situ* tests following known protocols, evaluating the ecological risk to ecosystem structure and functioning. Results of some microbial parameters showed that vegetation cover and plant species composition were correlated with some soil functions, such as nutrient cycling and organic material breakdown. The endpoints evaluated were vegetation structure, soil ground running invertebrates, decomposition of organic material and some microbial parameters. The ecological parameters indicated a clear distinction between sites inside and outside the smelter area, indicating an ecological risk to soil system even 17 years after the end of smelting activities. In Chapter 7 the integration of the parameters measured on chapters 4 to 6 is presented on calculating the risk values for each line of evidence and the integrated risk values in a tier 2 assessment. The high risk values in some points, confirming the risks pointed at tier 1, restrict the use of the area even to industrial activities, requiring sealed soils. In Chapter 8 a critical discussion of the results obtained and some words about the future perspectives for forthcoming studies on the site are presented. Results suggest that the deposit of highly contaminated tailings within the area and the failed attempt to encapsulate them have impaired the proper establishment of the vegetation, leading to change and simplification of the habitat structure. Allied to direct toxic effect of metals, the limitation of plant reestablishment resulted in low amounts of organic matter inputs into the soil to act as source of C and energy for microbial growth and for acting as protection for microbial community against high levels of heavy-metals in soil. These changes in the microclimatic conditions at the ground level, and in the amount and quality of the potential of organic matter inputs, caused negative impacts on microbial activity and on soil organisms, consequently affecting the ecosystem services and underlying processes carried out by them. The suitable encapsulation of the tailing deposits, with the concomitant re-establishment of a vegetation cover, seems to be essential to improve the ecological conditions at this site.

Keywords: Ecological risk assessment, Soil ecotoxicology, Habitat function, Retention function, Metals

Chapter 1

General introduction

1.1 Soil protection and ecological risk assessment

Soil is a dynamic system essential to the survival of humans and ecosystems. It can be considered a non-renewable resource, due to the high rates of degradation and the slow formation and generation soil processes (EC 2004). The main threats to soil, impairing the provision of different ecosystem services, include erosion, decline in organic matter, soil sealing, compaction, decline in biodiversity, salinisation, floods and landslides, and soil contamination from natural or anthropogenic sources (local and diffuse) (EC 2004; Bone et al. 2010).

Nowadays, soil contamination is no longer perceived as a few severe incidents, but rather recognized as a problem at world scale. Contaminated land has the potential to pose serious environmental and human health risks, including the contamination of other environmental compartments such as surface and groundwater, air and biota (Blum et al. 2004; Balasubramaniam et al. 2007). Just in the US, McKeehan (2011) estimated in over 600,000 the number of brownfields. Brownfields are vacant, abandoned, or underutilized commercial and industrial properties, where real or perceived environmental contamination is an obstacle to redevelopment or utilization. Furthermore, abandoned mines, dump areas, thermoelectric industries, dams and other deactivated structures are considered *brownfields*. In Europe, some countries provided estimates about brownfields: Germany (about 128,000 hectares), The Netherlands (between 9,000 and 11,000 hectares), Belgium/Wallonia (about 9,000 hectares) (Grimski and Ferber 2001). However, these data are not directly comparable because they include different kinds of sites, since there is a lack of consensus about the concept of brownfields in Europe.

The protection of soils, its diversity and ecological functions, has become an objective of environmental agencies around the world (Bone et al. 2010). Legislation aiming to protect soils in Europe includes the EU Soil Thematic Strategy (EC 2006), the European strategy to biodiversity conservation, and the, still under discussion, Soil Framework Directive – SFD (EC 2006). The SFD has the objective of establishing a common strategy for the protection and sustainable use of soil, integrating soil concerns into policy making, preventing threats to soil and mitigating their effects, restoring degraded soils to a level of functionality consistent at

least with the current and future uses. Being approved, the SFD will require identification of risk areas, to be carried out on the basis of common elements (EC 2006).

Some EU member states have legislation focused on soil contamination. The Netherlands, Germany and Belgium are some of the most advanced states for soil protection in EU (Bone et al. 2010). The Netherlands policies include the 1987 Soil Protection Act (amended 2008) (VROM 1986), the Soil Policy Letter (Van Geel 2003) and the Soil Remediation Circular (VROM 2009). The German policies include the Federal Soil Protection Act (Federal Ministry for the Environment Nature Conservation and Nuclear Safety 1998), where reference is made to "harmful soil changes", and the Federal Soil Protection and Contaminated Sites Ordinance (Federal Ministry for the Environment Nature Conservation and Nuclear Safety 1999). Belgium approved the Decree on Soil remediation and Soil Protection (Public Waste Agency of Flanders 2007) and the Order of the Flemish Government establishing the Flemish regulation on soil remediation and soil protection (Flemish Government 2007). In England, the Soil Strategy for England (DEFRA 2009) was built on the First Soil Action Plan for England (2004-2006), aiming to improve the sustainable management of soil and tackle degradation within 20 years. The Soil Strategy for England is focused on four main themes: the sustainable use of agricultural soils; the role of soils in mitigation and adaptation to climate change; protecting soil functions during construction and development; preventing pollution and dealing with historic contamination (DEFRA 2009).

In general, politics and actions on soil protection rest on two basic approaches: (i) Optimization of biodiversity and (ii) Protection of ecosystem services (and their underlying biological/ecological processes) essential for the survival of mankind. Soil is seen as a multifunctional unit, supplying provisioning (food, water, fuel), regulating (soil erosion, flood control), cultural (recreation, spiritual value, sense of place) and essentially supporting (soil formation, nutrient cycling, oxygen from photosynthesis) services simultaneously (Letey et al. 2003; Millennium Ecosystem Assessment 2005; Tallis and Kareiva 2005).

The role of biodiversity on the quality of ecosystem services is very complex and hard to quantify because of the mutually dependent interactions between soil organisms (as in food webs) and their different role in ecosystem processes, such as in nutrient cycling (Griffiths et al. 2001; Swartjes et al. 2011). To Ghilarov (2000), the meaning of biodiversity for ecosystem functioning depends on the definition of "ecosystem function". According to Ghilarov (2000), any decrease in species diversity will be meaningful if we consider ecosystem functioning including the synthesis of all compounds that plants, animals and other organisms contain in

their bodies or release to the external environment; not only considering ecosystem "as a natural factory producing different goods and services", which is an utilitarian human's point of view, but also as a "living stage for a unique evolutionary play". Although the concept of ecosystem services can contribute to develop a framework to the sustainable use of biodiversity and natural resources, final decisions depend on the socio-political and cultural context where they are inserted (Wallace 2007).

Ecosystem services have received explicit attention in the EU Soil Thematic Strategy that pointed the following ecosystem services as protection goals (EC 2006):

a) Production of food and other biomass;

b) Capacity for storing, filtering and transformation of nutrients, substances and water;

c) Reservoir of biodiversity;

d) Physical and cultural environment to human activities;

e) Source of raw material;

f) Reservoir of carbon;

g) Conservation of arqueologic and geologic patrimony.

Discussion on contaminated sites led to the question whether we should protect the multifunctionality of soils, as a resource for future generations, or whether we should make a differentiation between different types of land use, the so called "risk based approach". According to Bardos et al. (2011), there has been a shift from the multifunctional policy approach for land remediation, to "end-use-related" remediation, which is considered a more sustainable approach. Despite the environmental benefits of the rehabilitation of contaminated land required for any future use (multifunctionality), rather than for a specific land use, the former is considered an unsustainable political approach because of its economic and social costs, being an obstacle to the reuse of contaminated land. More sustainable development appraisals, such as the risk-based land management (RBLM), have begun to be applied to remediation projects and debated in sustainable brownfield regeneration, as in UK Sustainable Remediation Forum (SuRF-UK) (Bardos et al. 2011). The concept of RBLM aims to restore the usability and economic value of the land by risk reduction (fitness for use), protection of the environment and reduction of aftercare (Vegter 2001).

The RBLM approach is complex and has generated a demand for decision support tools (Vegter et al. 2003). Therefore risk assessment provides a useful starting point to prioritize actions and to conduct any remediation strategy. Also, land use can be adjusted according to soil quality indicated by a site-specific risk assessment (Vegter et al. 2003; Faber 2006). In this context, the Ecological Risk Assessment (ERA) process has been recognized as a powerful tool for the decision-making process in the management of contaminated sites or sites suspected of contamination (USEPA 1998; Suter et al. 2000; Bardos et al. 2002). Furthermore, purposes for ERA include the estimate of the risk in contaminated sites for public disclosure and damage assessment, when those who contaminated a site must compensate any injury to natural resources (Suter et al. 2000).

ERA is a complex process of collecting, organizing and analyzing environmental data to estimate the probability of adverse effects due to contamination, being expert judgment fundamental in the decision making framework before, during and after the process (Jensen and Mesman 2006). ERA uses data from different environmental compartments collected at different lines of evidence and different sources of information, such as chemical analysis of contaminants, physical properties of the environment, biological surveys, and ecotoxicity tests with environmental samples. Although a general trend of biological responses, according to the type of contamination, can be expected in contaminated areas, the relationships between the sources, exposure, and effects to ecological receptors are complex and often specific to a particular site, a set of environmental condition, and a specific receptor organism, claiming for a site-specific ERA to support decisions about risk management and remediation. Site-specific risk assessments will involve only a single geographical area of concern and, therefore, can incorporate locally relevant aspects of environmental chemistry and species sensitivity, while for regional and national-scale assessments, more general assumptions are taken, frequently producing results that are more conservative in an effort to be protective of sensitive species or locations (USEPA 2007). Rutgers et al. (2000) highlighted the need of defining the intended soil use before the implementation of the site-specific ERA, followed by the selection of what groups of organisms or ecological processes are most likely to be affected given the soil use at that site.

An ERA process should include indicators both of ecosystem structure and functions (Burger et al. 2007). In spite of the recent increase of test systems and standardized protocols developed for the terrestrial compartment (Achazi 2002; Knacker et al. 2004; Roembke et al. 2006a) there is still a lack of information on terrestrial ecosystems including soil biodiversity, biology of species, ecosystems functioning and toxicity database in comparison to the aquatic

compartment (Ahlers 2001; Swartjes et al. 2008). There is still a gap between soil protection and assessment concepts and those currently discussed in ecology, especially those covering both risk assessment and ecosystem functioning (Van Straalen 2002; Filser et al. 2008; Kuperman et al. 2009), since the impact of chemicals on soil functions has mostly been studied in reductionist ecotoxicological approaches, such as spiked soils in laboratory.

The aforementioned challenges are more critical in tropical environments. Although a no distinct tropical ecotoxicology should exists (da Silva and Soares 2010), there is a lack of knowledge about ecotoxicology in tropical environments (Lacher and Goldstein 1997), which may be a challenge to risk assessment in these regions (Chelinho et al. 2012). There are important differences in behavior and distribution of contaminants in tropical areas, once different conditions between environments, such as temperature (Spurgeon et al. 1997; Abdel-Lateif et al. 1998; Garcia 2004) and soil properties (Bradham et al. 2006; Roembke et al. 2006b), can influence the toxicity of contaminants and should be considered during the ERA and management of contaminated environments (Guimarães et al. 2000). For example, high temperatures in humid tropical areas can accelerate microbial and chemical degradation, volatilization, rate of uptake and increased bioconcentration (Castillo et al. 2010). Transport of soil contaminants by surface runoff and groundwater leaching can be favored by rainy conditions prevailing in some tropical areas (Henriques et al. 2007; Castillo et al. 2010; Chelinho et al. 2012). Furthermore, the higher species richness and ecological diversity than in temperate regions can mean a higher number of species at risk, and the possible occurrence of more sensitive species cannot be ignored (Lacher and Goldstein 1997; Castillo et al. 2010).

Any ERA should start with the application of generic and conservative principles for optimum protection (Rutgers and Jensen 2011). In this sense, ecotoxicological data has been used in the derivation of risk limits and environmental quality objectives like the Soil Screening Levels (SSL), also known as benchmarks, guideline values, etc. SSL are normally derived from No Observed Effect Concentration (NOEC) values, e.g. by using Species Sensitivity Distribution (SSD) (Posthuma et al. 2002). In many countries the first stage of the ERA of contaminated soils consists in the comparison of soil concentrations with national SSL. For the majority of sites, this generic risk assessment is sufficient to exclude acceptable risks (when the SSL are not exceeded), being considered very useful screening tools (Rutgers and Jensen 2011). However, when the SSL are exceeded, a more specific assessment is necessary. Since the SSL data are derived from spiked experiments conducted in laboratory, large discrepancies have been observed between the predicted and observed effects (Jensen et al. 1996). In this way, the complement of chemical analysis with direct toxicity testing can tell us whether the

contaminants are bioavailable and at toxic levels, integrating the combined effect of mixtures, including its degradation products and metabolites, and that of contaminants not analyzed or for which soil quality levels do not exist (Weeks et al. 2004; Fernandez et al. 2005; Jensen and Mesman 2006), reducing uncertainties.

The response of biota to environmental conditions is an integrated result of the direct and indirect impacts of contaminants, natural stressors, or a combination of both. Biological systems under stress commonly exhibit a certain sequence of responses; some general patterns include reduction in species diversity, shifts in species composition, simplification of community structure, substitution of sensitive taxa by tolerant ones, changes in nutrient cycling, and changes in primary productivity (e.g. Georgieva et al. 2002, Podgaiski and Rodrigues 2010). In ERA, the effects of stressors should be explored and understood at multiple levels of organization. One same stressor can be beneficial at one level and detrimental at another (Odum 1985). Even at the same level the effects are species dependent due to different routes of exposure or to intrinsically different sensitivity of the species (Breure et al. 2005). For example, in ecotoxicological studies with the usual tested species of soil invertebrates, *Folsomia candida*, *Enchytraeus crypticus* and *Eisenia andrei*, there is not a general ranking of their sensitivity among different contaminants (Kobeticová et al. 2008; Kuperman et al. 2009).

The movement of contaminants between environmental compartments (e.g. soil to water) and between trophic levels should be considered in risk assessment and risk management (Blum et al. 2004). Because of the soil quality implications to other environmental compartments, both habitat function and retention function are commonly evaluated in ERA of contaminated soils. According to the International Standardization Organization (ISO), in the guideline ISO 15799 on the ecotoxicological characterization of soils and soil materials (ISO 2003), the habitat function is the *ability of soils/ soil materials to serve as a habitat for micro-organisms, plants, soil living animals and their interactions (biocenoses)*, while the retention function is the *ability of soils/soil materials to adsorb pollutants in such a way that they cannot be mobilized via the water pathway,* including the *buffer inhibiting movement of water, contaminants or other agents into the ground water.* The habitat function is commonly evaluated through testing the soil matrix using soil invertebrates or plant tests (e.g. Hund-Rinke et al. 2003), while retention function is assessed by testing soil extracts (e.g. eluates) with aquatic organisms, such as algae and cladocerans (e.g. Natal da Luz et al. 2012).

1.2 Regulatory context of the ecological risk assessment

Regulatory agencies around the world are increasingly incorporating risk based approaches into environmental decision making (Pollard et al. 2004). In the US, the study of toxic effects gained prominence with the advent of modern environmental law, especially with the Clean Water Act of 1970 for aquatic systems and the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) of 1981 for terrestrial systems (Kapustka et al. 2004). The application of ecological risk assessment to contaminated sites was stimulated by USEPA (US Environmental Protection Agency) in the Superfund program (USEPA 1989). The practice of ERA took off after 1992 with the publication of the USEPA (1992) framework and other texts.

European cooperation on soil quality assessment started with the CARACAS (Concerted Action on Risk Assessment for Contaminated Sites in the European Union; 1996–1998) and CLARINET (Contaminated Land Rehabilitation Network for Environmental Technologies in Europe; 1998–2000) concerted actions. In 1996 the industrial network NICOLE (Network for Industrially Contaminated Land in Europe) started and, in 2005, after several meetings where ERA was discussed, it was concluded that the industry clearly has a potential to impact ecological processes in soil (Bardos et al. 2005). It was advocated to further develop an intelligent cost-effective tiered approach, possibly along the idea of the triad approach (integrating chemical analyses, ecotoxicity testing and biological surveys) (see section 1.3.2). In 2005 the HERACLES (Human and Ecological Risk Assessment for Contaminated Land in European Member States) network was initiated, with the purpose to promote harmonization of models and frameworks of risk assessment in Europe. Also the EU Soil Thematic Strategy and SFD will further stimulate attention to ERA at contaminated sites, once they predict the exchange of information aiming the harmonization of some elements to develop and improve the methodologies on risk assessment (Rutgers and Jensen 2011). For more details about the history on European cooperation in the field of soil contamination see Swartjes et al. (2008).

In The Netherlands, the Soil Protection Act was introduced in 1986 and extended in 1994, with the addition of a procedure to determine the urgency of remediation based on site-specific risk assessment (Swartjes 2009). Such procedure was significantly improved by the use of the triad approach (Mesman et al. 2007). According to Rutgers and Jensen (2011), the ERA

approach in The Netherlands might be different from many other countries with a soil protection policy, being applied for all sites with a serious soil contamination, and remediation should be seriously considered for all unpaved and uncovered soil, including those at industrial sites. Contaminated sites are first assessed using a set of Soil Quality Standards which take both human and ecological risk into account, applied to all kind of land uses and soil types (Rutgers and Den Besten 2005). This assessment is followed by a calculation of Toxic Pressure of the mixture of contaminants. The Triad is applied in a subsequent step to further improving ERA when it is recommended. In many cases, the goal of a Triad approach is to reduce the surface area to be remediated and hence to reduce costs (Rutgers and Jensen 2011).

In UK, ERA framework is applied only to controlled waters and certain protected habitats, defined in Part IIA of the Environmental Protection Act of 1990 (Weeks 2004).

In many other countries, soil quality is expressed in chemical concentrations as standards to address the potential ecological risks without an established ERA framework. For example, in Brazil, target values and intervention values established for soils in the *Resolução CONAMA 420/09* (BRAZIL, 2009), pre-defined based on fitotoxicity assays, and ecological and human risk, is the guideline to prevention, punition and chemical criteria about soil quality, for management of soil use and remediation. However, until 2011 only the State of São Paulo had reference values of soil quality in Brazil. Environmental agencies of each Brazilian State should list the different soils in their territory and establish reference values (backgrounds) until 2013, providing crucial information to identify contaminated areas and carry out intervention actions. A project of identification and mapping of contaminated sites or under investigation, carried out at São Paulo, registered 2514 sites until December 2008 (CETESB 2007). These data evidenced the importance of detecting and identifying contaminated areas because of the risks associated to future uses, such as residential use.

1.3 Ecological risk assessment schemes – a bird's eye

1.3.1 Main phases of an ERA

Although different schemes of ERA are proposed and applied in different countries, the components of the ERA process are similar among them, generally including the following phases:

1. *Preliminary investigation*: this phase includes the problem formulation of the assessment, including the collection of all the scientific information available for the area; the development of a conceptual model; and an analysis plan for the risk assessment (e.g. Pereira et al. 2004; Weeks et al 2004). Although this phase is basically a desk work, complemented with one or two site visits, it is a critical step for the risk assessment process, since many of the actions to be taken on the following phases are dependent on the information (and gaps of information) identified at this stage. The conceptual model is built involving what is currently known about the site, geographical limits, source and type of contamination, historical use and activities in the site, current pathways of exposure, and observation of perceptible risks and ecological receptors at risk (Weeks 2004, Jensen and Mesman, 2006, Ashton et al. 2008). It should take into account the inherent properties of contaminants involved, such as toxicity, persistence, mobility, potential of bioaccumulation through food chains, etc. In addition, the development of the conceptual model involves site characterization and identification of data gaps, urgency of decisions, current and future land use, and whether the site may be regulated under specific directives (Jensen and Mesman, 2006). After the conceptual model is developed (which can be updated throughout the risk assessment process based on the information being collected), if ecological receptors are able to be exposed to the contaminants given their fate, behavior and proximity, an analysis plan for the risk assessment is proposed.

2. *Exploratory investigation*: this is an optional phase to confirm the existence of unacceptable contamination on the study area by performing a preliminary sampling on the site, followed by chemical analysis and comparison with soil quality guidelines. In some cases where contamination is not so visible, this exploratory investigation is performed with the objective of providing the proof of the absence of contamination.

3. *Main investigation*: this phase is performed in case a contamination is confirmed and a potential ecological risk can occur. This phase is usually done using a tiered approach where in each tier different assessment tools belonging to different lines of evidence are applied, including more detailed chemical analysis, bioassays and ecological field surveys. Ecological receptors potentially at risk and major pathways of exposure identified in the preliminary investigation and described in the conceptual model are the main targets on this main investigation phase. Besides a more detailed evaluation of the spatial extent of the contamination, these assessments usually consider evaluation of effects on soil invertebrates, plants, and wildlife species, and some assessments also examine effects on microbial communities and soil processes (Jensen and Mesman 2006, USEPA 2007, Swartjes et al. 2008). At the end of the assessment, the results are integrated to describe the risk. Moreover, effects

on the retention function of the soil, using assays with aquatic organisms are also applied. The combined information of the different blocks (lines) of information to infer a potential risk for the site is called the triad, and has been used as one promising approach to be applied in this main investigation phase (see 1.3.2.).

The ERA framework designed by USEPA (1998) consists of the following components (Sutter et al. 2000): a) Problem formulation, including the planning phase; b) Analysis, where the technical evaluation of the data concerning the characterization of exposure and effects is carried out; c) Risk characterization, where the results of the analysis phase are integrated to estimate and describe risks.

The Canadian framework for ERA consists of the three tiers: Screening Assessment (basically desktop work), Preliminary Quantitative ERA (field and laboratory quantitative data collection), and Detailed Quantitative ERA (detailed quantitative methods for reduction of uncertainties, if necessary). Each tier includes the following components: receptor characterization, exposure assessment, hazard assessment, and risk characterization though the Detailed Quantitative ERA does not necessarily include all components. More details about the Canadian framework can be obtained from the homepage of the Canadian Council of Ministers of the Environment (CCME, www.ccme.ca) or in CCME (1996, 1997a). Acceptable risks are dependent on land-use type since in Canada, soil quality guidelines are derived for given land-uses, including agricultural, residential/parkland, commercial and industrial (CCME, 1997b, 2006).

The UK framework was based on schemes from US, Australia, Canada and the Netherlands (Weeks 2004), being a tiered framework using the Triad approach, where early tiers are screening phases, and subsequent tiers are intended to make more realistic assessments of the risk to key ecological receptors. This framework includes a first step called “Tier 0” involving the development of a conceptual site model, aiming to determine whether a site falls under the Part IIA conditions (Weeks 2004), where mode detailed studies need to be performed.

An harmonization of ERA tools and in the resulting soil quality standards of these tools within the EU have been discussed since 2005 under the HERACLES, a long term research network to promote the development of common risk assessment tools for soil quality assessment (Swartjes et al. 2008). A promising approach is the Triad (Rutgers and Den Besten 2005) which has been adapted and successfully applied in Europe (Jensen and Mesman 2006; Critto et al. 2007; Semenzin et al. 2007, 2008). In The Netherlands, the EU country with most

experience in the application of site-specific ERA, a practical Triad approach has been developed (Mesman et al. 2007) and it is described in 1.3.2. In other EU countries (e.g., Germany, Spain and Sweden), ERA can be based on additional types of testing to make the triad approach feasible (Rutgers and Jensen 2011). A Decision Support System (DSS-ERAMANIA) for assisting in site-specific ERA was developed based on research at the Acna di Cengio (Italy) contaminated site, using the Triad and the Weight of Evidence (WoE) approaches, and where ecological observations and ecotoxicological tests were compared according to Multi Criteria Decision Analysis (MCDA) (Critto et al. 2007, Semenzin 2007, 2008, 2009).

1.3.2 The Triad approach

The Triad approach was originally developed to evaluate sediment quality (Long and Chapman 1985). Consisting of three lines of evidence (LoE; chemical, ecotoxicological and ecological), the Triad approach is usually applied within a tiered system, i.e., information from each LoE is collected at each tier following a stepwise cost-effective process (Jensen and Mesman 2006). The tiered approach is designed to be efficient in excluding extreme sites, i.e., either sites that pose no risk to ecosystems or sites that pose a high risk and where remediation actions are needed. Moreover it is essential to *gather sufficient evidence of harm, or the possibility of harm, at sites where risk management may be required* (Ashton et al. 2008). The Triad approach relies on the concept of WoE, which is the process of combining information from multiple lines of evidence to reach a conclusion about an environmental system or stressor (Burton et al. 2002). Such approach minimizes the chance of false positive and false negative conclusions.

Tier 1 is essentially a screening phase, aiming to produce a first spatial representation of the risk and to determine whether a site can be excluded from higher tiers and of further testing (either because it is unlikely to pose a risk to the relevant ecological receptors or because a high risk is detected and there could be a need for immediate mitigation actions), or it needs to be further evaluated (Weeks et al. 2004, Critto et al. 2007) at Tier 2. Thus, the tools used in tier 1 to collect information from each LoE should not only be able to indicate effects, but also be rapid, easy to apply and cost-effective (Jensen and Mesman 2006).

Tiers 2 and 3 are performed to reduce uncertainties about the actual risk. In tier 2, the chemical LoE can comprise extraction techniques to assess the bioavailable fraction of

pollutants in soil. This should be complemented with information derived from ecotoxicological tests (ecotoxicological LoE) and ecological surveys (ecological LoE).

The ecotoxicological LoE usually comprises long-term studies focusing on chronic endpoints such as reproduction and growth, and some mineralization processes, since chronic effects can occur at intermediate levels of pollution (between target or baseline values and intervention values, usually representing high contamination levels) (Weeks et al. 2004; Critto et al. 2007). At this LoE, standardized chronic tests with Collembola (ISO 1999) and Oligochaeta (ISO 1998, 2004) to evaluate sub-lethal effects of the soil matrix on the reproduction of soil invertebrates, and plant emergence and growth tests (ISO 2005) to evaluate the habitat function to vegetation, have been recommended. Soil extracts are used to perform widely established tests with aquatic organisms (e.g. cladocerans and microalgae) to evaluate the retention function of soil. The ecological information at tier 2 is collected to get more details about the possible impact on populations and communities of microbial communities, flora and fauna *in situ*. Such surveys are more time consuming and may require specific knowledge, but provide site-specific information about the status of specific taxonomic groups or ecosystem processes (Chapman 1990).

Although standardized ecotoxicity tests were originally designed to assess toxic effects of chemicals added to soils in regulatory risk assessment, they have been used in a WoE approach (Sutter et al. 2000) together with ecological and chemical data in retrospective risk assessments. Some OECD and ISO standard protocols have already been developed that can be used in ERA (Achazi 2002; Roembke et al. 2006a). However, the use of these tests (developed for temperate environments) under different conditions such as with different species, soil types and climatic regimes, can result in differences in the measured toxicity (Weeks et al. 2004). In this way, adequate methodologies should be generated to recognize these differences, adapting the ERA protocols to the specific conditions of different sites. As the vast majority of the world's most threatened biodiversity hotspots are found in the tropics, ecotoxicology in these regions represents an enormous challenge. Therefore, it requires the determination or development of appropriate tools to prevent the loss of valuable ecological services (da Silva and Soares 2010).

On the ecological line of evidence, despite the fact that environmental agencies encourage biological surveys, they are less frequently carried out for contaminated soils than for waters or sediments. Biological surveys include a variety of techniques for characterization of populations, communities and ecosystem processes. For example, microbial biomass, soil

basal respiration, enzyme activities, and nutrient transformations are important attributes related to soil fertility (Edwards 2002) and can be used as bioindicators of soil health as a consequence of contamination (Smejkalova et al., 2003; Castaldi et al. 2004), agricultural use (Araujo et al. 2003), suitable management or success of restoration practices (Balota et al. 2004; Nogueira et al. 2006; Clemente et al. 2007). Soil microorganisms fix carbon and nitrogen by forming new biomass using the energy they obtain from oxidation of carbon sources through respiration or inorganic chemical reactions (Chen et al. 2003), constituting an important reservoir of nutrients (Gregorich et al. 1994), and improving the sustainability of an ecosystem (Kaschuk et al. 2010). Contaminants may affect a variety of microbiological processes in soil thereby affecting the nutrient cycling and the capacity to perform key ecological functions, such as the mineralization of organic compounds and the synthesis of organic matter (Giller et al. 1998, 2009; Moreno et al. 2009). However, because the microbial community may adapt to the novel conditions and surpass the negative effect of contamination (Lejon et al. 2010), microbial data from field surveys should be interpreted with supporting information (e.g. chemical analysis) to avoid misleading interpretations (Sutter et al. 2000).

Below and above ground soil invertebrates surveys are also widely performed in site-specific ERA. Soil invertebrates are recognized regulators of nutrient recycling and organic matter breakdown by fragmenting leaf litter (the litter shredders like earthworms, isopods, diplopods, etc), improving soil structure (manly earthworms) and microbial activity (several groups of soil mesofauna like collembolan and mites) (Dangerfield and Milner 1996). They positively affect soil chemistry for plant nutrition and increase the likelihood of successful plant community restoration (Muys et al. 2003) and consequently play an important role in ecosystem restoration (Snyder and Hendrix 2008).

Vegetation surveys are one of the most used tools to evaluate habitat quality in terrestrial ecosystems (Godinez-Alvarez et al. 2009). Plants are the primary producers, a key structural component of the habitat for all soil inhabitants. Measurements of vegetation cover and composition are important to indicate changes in habitat quality due to stress caused by pollution. Besides, some advantages such as their immobility and easy sampling make them a suitable tool to be used in ERA (Sutter et al. 2000, Weeks et al. 2004, Jensen and Mesman 2006).

One of the most common approaches in biological surveys is the calculation of diversity indexes for biological communities. Although diversity indices are largely used to

evaluate environmental quality because they are easy to interpret, some authors noticed their insensitivity to species differences and abundance (Izsák and Papp 2000) and that they do not necessarily provide direct information on environmental quality or degree of degradation (Thiebaut et al. 2002). A general review about problems with indices is presented by Green and Chapman (2011). The authors recommend to avoid the use of indices because of the loss of information and the likelihood of misleading conclusions; if the use is inevitable, they should be used together with other statistical methods that retain more information in the biological data set (e.g., an appropriate combination of univariate and multivariate analysis) (Green and Chapman 2011).

Risk characterization is the culminating step of the risk assessment process. Risk characterization communicates the key findings and the strengths and weaknesses of the assessment through a conscious and deliberate transparent effort to bring all the important considerations about risk into an integrated analysis by being clear, consistent and reasonable.

According to Rutgers and Jensen (2011), although many tools for a Triad approach in ERA area available, the increasing number of Triad-based Risk Assessment will demand for improved, new, standardized, robust and cost-effective tools. We hope to contribute in this sense with the present work, applying the Triad approach to risk assessment of a tropical metal contaminated area in Brazil.

1.4 Objectives

The major goal of this work is to further contribute to the application of a tiered ERA framework to tropical environments, and to evaluate the feasibility and usefulness of different biological parameters to be used in different tiers. The results aimed at characterizing the ecological risk in one of the most metal contaminated areas in the world, ranking sites in the study area, supplying information to indicate remediation measures and bringing an important contribution to support future decisions. This thesis aims at providing important information to help the regular use of the risk assessment process to support site restoration and reclamation decisions in Brazil, following the current trend in soil protection around the world.

The present study was carried out in an abandoned lead smelter that was operational between 1960 and 1993, located adjacent to the urban area of Santo Amaro, BA, Brazil, about

150 Km away from Salvador. The area presents a high health risk to animals and humans (Costa 2001; Carvalho et al. 2003), due to high levels of metals in soil and water, as well as by tailings and airborne dust from atmospheric deposition through chimneys emission (up to 3 km from the industrial area), while the smelter was operational (Anjos 2003; Machado et al. 2004). This site is a useful demonstration-site to apply the framework for several reasons. Firstly, there is more than 30 years of published research about the smelter, but mainly concerning risks to human health. This site is considered by Brazilian authorities as one of the priority metal contaminated areas to monitor in the country. Although a human risk assessment has already been performed in Santo Amaro by FUNASA (2003), human risk-based criteria are unrelated to the ecological parameters that may be important to sustain soil functions and the provision of ecosystem services (Dawson et al. 2007). Therefore, the implementation of an ecological risk assessment is considered a priority. Secondly, metals are in fact the major source of contamination in the area, facilitating the interpretation of the results obtained.

The specific questions we would like to answer with this study are:

- Does the metal contamination in the smelter area still pose some ecological risk to soil habitat and retention functions 17 years after the closure of the smelter? How is the spatial extension of the risks posed by the smelter area?

- Are the effects detected directly related to the presence of the metals (direct toxicity) or to an indirect effect (habitat disruption)?

- Is a tiered ERA framework suitable to be applied in contaminated sites with this typology of contamination? Does it need (and if so, how) to be modified to generate more sound decisions about soil quality in the tropics?

- Which type of biological (ecotoxicological and ecological) parameters are more sensitive to detect risk? Are they able to discriminate different levels of risk? And at which tier should they be used?

The data gathered on this study, when trying to answer these questions, will provide relevant information that can help risk managers to take better and more sound decisions to mitigate the environmental risks and to rehabilitate the area for specific land-uses.

1.5 The structure of this thesis

This thesis is divided into eight chapters, including this one.

Chapter 2 presents the Preliminary Investigation, including the problem formulation phase and the collection of the scientific information available about the study area, that resulted in the conceptual model and the analysis plan for the risk assessment. A tiered approach is proposed integrating information from three lines of evidence: chemical, ecotoxicological and ecological. Aims and actions of each phase of risk assessment are established, in order to include the ecological and ecotoxicological perspectives missing in previews studies conducted in this area. Although the aquatic compartment is included in the conceptual model, the analysis plan focus only on the soil compartment.

Chapter 3 presents the tier 1 (or screening phase) of the Triad (included in the Main investigation). This phase also intended to calculate risk with the purpose of ranking sites within the study area and to identify those that may need to be further investigated.

Chapter 4 presents the application of laboratorial chronic tests with soil invertebrates to evaluate the ecological risk of soils from the study area to this group of organisms. This chapter presents the effects of the tested metal contaminated soils on the reproduction of *Eisenia andrei*, *Enchytraeus crypticus* and *Folsomia candida,* and a discussion about the different sensitivities of these species and about the performance of these standardized tests in tropical soils.

Chapters 5 and 6 are dedicated to ecological parameters used in the ecological LoE. Chapter 5 presents the use of microbial soil-quality indicators to evaluate the ecological conditions and biological activity in the study area. Chapter 6 presents the application of ecological evaluations and *in situ* tests following known protocols, evaluating the ecological risk to ecosystem structure and functioning. The endpoints evaluated were vegetation structure, soil ground running invertebrates, decomposition of organic material and some microbial parameters. The effect of the historical contamination on the ecosystem and a critical discussion about the use of these tools in risk assessment are presented.

In Chapter 7 the integration of the parameters measured on chapters 4 to 6 is presented on calculating the risk values for each line of evidence and the integrated risk values in a Tier 2 assessment.

Finally, in chapter 8 a critical discussion of the results obtained and some words about the future perspectives for forthcoming studies on the site are presented

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Chapter 2

Conceptual model for the Sto Amaro site and analysis plan

Abstract

The majority of the risk assessment studies carried out are based just on chemical characterization, focused on limit values of contaminants in soils, water or food, usually not including biological and ecotoxicological considerations. However, when these studies are doing aiming at a reclamation of contaminated areas or human risk assessment, approaches related to ecosystem structure and functioning should be considered. The abandoned area of the lead smelter Plumbum in Santo Amaro, Bahia, Brazil, is a historical case of a serious metal contaminated area, being considered as priority in metal monitoring by Brazilian authorities due to human and environment contamination. This study presents the problem formulation phase of the environmental risk assessment, including the scientific information available about the study area, the conceptual model for the contaminated site, and the analysis plan for the risk assessment. A tiered approach is proposed integrating information from three lines of evidence: chemical, ecotoxicological and ecological. Aims and actions of each phase of the risk assessment are established, to include the ecological and ecotoxicological perspectives not included by previews studies conducted in this area, focusing on the soil compartment.

Key words: ecological risk assessment, metals, soil ecotoxicology

2.1 Introduction

Environmental impacts of mining activities have been described for many countries around the world, related to the exploitation of geologic resources, processing of raw material, and generated tailings and wastewater. Such activities release, in first place, metals that were trapped in a mineral form, and that under certain circunstances may become available to biological communities. The effects of metal contamination quite often persist after closure of metal industries because adequate reclamation measures are not taken (Pereira et al. 2004). As microorganisms cannot degrade metals, their impact in the environment can persist for decades, limiting the establishment of vegetation and faunal communities, thus originating an impairment of ecosystem functions and associated ecosystems services. Furthermore, surrounding natural and/or production areas (crops, pastures) and public health may be at risk.

At these areas the assessment of risks to the environment become a priority[1] for local stakeholders as way to better define actions to take, i.e., implement remediation measures to decrease the risks or not. In this context, Ecological Risk Assessment (ERA) becomes a cost-effective management tool, allowing not only to evaluate the risks, but also to discriminate those sites inside the assessment area where (remediation) actions should take place, from those where no action is required (Weeks et al. 2004).

Ecological risk assessment is a complex process of collecting, organizing and analyzing environmental data to estimate the risk of contamination to ecosystems (Jensen and Mesman 2006). The initial phase of an ERA for contaminated sites encompasses a close dialogue with local social actors (e.g., stakeholders, population), aiming to understand their concerns and their plans regarding the future of the site. This dialogue should help defining the protection goals and the level of risk to be accepted for the site according to the intended land-use, overarching aspects of the entire ERA process (Lanno 2003).

In parallel to this dialogue, the collection of all scientific and technical information about the site (complemented by one or more site visits) also takes place at this stage, which usually culminates in the construction of a conceptual model for the site (CSM) and an action

[1] Human health risks are also a priority in these cases. However, since they are not the subject of this study they were no highlighted in the text.

plan that will govern the ERA process (Pereira et al. 2004). The CSM is an essential tool to communicate with stakeholders and should rule the type of actions/decisions taken further down in the ERA process. It is built based on what is currently known about the site. Information about the sources and types of contaminants, extent of contamination, current pathways of exposure, and ecological receptors at risk should be compiled (Weeks 2004, Jensen and Mesman 2006). Furthermore, the CSM should include information about the perceived risks and areas needing urgent action, and should identify data gaps (Jensen and Mesman 2006). At this stage, a decision to implement a full ERA should be taken based on the information gathered and on particular legal requirements.

After this first phase, and if a full ERA process is required, a site specific evaluation starts by collecting information from different lines of evidence – LoEs – usually adopting a Triad approach and taking decisions on the risk following a "weight of evidence" approach. The Triad approach, originally developed to evaluate sediment quality (Long and Chapman 1985), has been recommended and successfully applied in ERA of contaminated soils (Wagelmans et al. 2009). Consisting of three lines of evidence (chemical, ecotoxicological and ecological), is usually applied using a tiered framework (Jensen and Mesman 2006, Rutgers and Jensen 2010). The progression through tiers reflects a refinement in the ecological relevance of information gathered and progressive reduction of uncertainty. This stepwise process allows to eliminate from further investigation such areas within the study site showing no risk (or an acceptable risk) and areas showing a high risk with a high degree of confidence at the initial steps, therefore stopping the ERA and saving resources (Week 2004).

The aim of this chapter is to present the conceptual site model and the analysis plan of a site-specific ERA of a metal-contaminated area in Santo Amaro da Purificação (BA, Brazil), following a tiered framework and adopting the Triad approach, joining information from chemical, ecological and ecotoxicological lines of evidence (LoE). This case of contamination is considered as priority in monitoring of metal contamination in Brazil by Brazilian health authorities (FUNASA 2003), as the area is one of the most metal contaminated sites of the world (Anjos and Sánchez 2001). Although a human risk assessment has already been performed in Santo Amaro (FUNASA 2003), human risk-based criteria are unrelated to the ecological parameters that may be important to sustain soil functions (Dawson et al. 2007). Therefore, a site-specific ERA is necessary to evaluate the risks towards key ecological receptors and processes they mediate. Despite preview works cover soil compartment, there was a lack of systematic sampling and a lack of information about methodology, detection limits, location of sampling, or chemical analysis not covering all main contaminants (not just

Pb and Cd) etc., which were pointed by FUNASA (2003) as a limitation to use these data in ATSDR (Agency for Toxic Substances and Disease Registry) methodology for human risk assessment. FUNASA (2003) carried out complementary analysis to fulfill these gaps focusing on smelter area and urban area under a human risk perspective. Furthermore, none of the preview works analyzed receptors and compartments under an ecological perspective of soil functioning. This ERA will be focused on the soil compartment and its ecological receptors at risk addressing indirectly the risk to ground water and associated fresh water systems.

2.2 Study site: location and history

2.2.1 Location of the study site

The study site belongs to the Plumbum Mining and Metalurgy Ltd (initially called COBRAC, Brazilian Lead Company) and is located Northwest of urban area of the small city of Santo Amaro da Purificação, state of Bahia, Brazil (Lat: 12° 32‘4‘‘ S, Long: 38° 42’ 43‘‘ W). The city is located in the metropolitan region of Salvador, on the right bank of the Subaé River, and comprises an area of 518 km^2 and 58,387 habitants, and elevated indices of poverty (IBGE 2009).

The climate of the region is tropical, characterized by a dry and a rainy season, hot weather with annual mean temperature of 25.4 °C and annual precipitation ranging between 1.000 and 1.600 mm (FUNASA 2003). The rainy season occurs between May and July. Atlantic forest is the dominant biome, but historical occupation since the early days of colonization has modified the landscape considerably by suppression of vegetation.

The smelter area (Fig 2.2) is located 300 m away from the Subaé river, which crosses the city and meets the bay of Todos os Santos ca 10 Km downstream. The river has a recognized historical importance to the economic and commercial development of this region, including food supply. However, it has been strongly impacted by domestic and industrial wastes without treatment, and also by the smelter activities that aggravated this scenario (CRA 2000; FUNASA 2003).

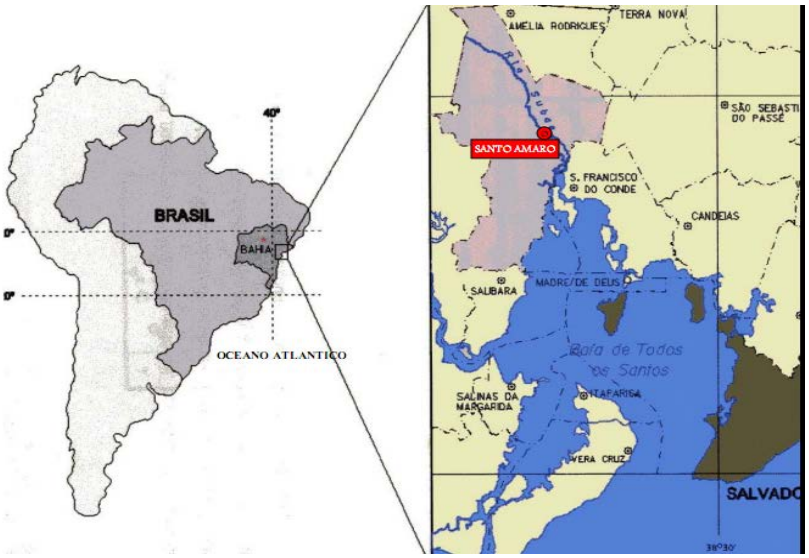

Fig. 2.1 Location of the Santo Amaro city in the State of Bahia, Brazil.

Fig. 2.2 Smelter area of Plumbum metallurgy, Santo Amaro, Bahia, Brazil.

2.2.2 The smelter and the environmental liability

The smelter remained active between 1960 and 1993, producing lead ingots. The raw mineral source used was galena (PbS), containing about 86% lead, and coming mainly from the Boquira mine, 800 km away from the smelter.

In 1960 the COBRAC started its activities and since then there are reports of environmental impacts. In 1961, an investigation carried out by Dr. Hans Dittimar, requested

by cattle breeders, indicated the smelter as responsible by soil, air and water contamination and cattle death (Oliveira 1977). At this date, the first request to close the smelter due to aquatic contamination was made.

In 1980 and 1981, CEPRAM (State Council of Environmental Protection) imposed some measures to reduce environmental pollution and treatment of the residues by establishing the annual limit of refined Pb in 22,000 ton/year (Cunha and Araújo 2001). In order to attend this decision, a reduction of 50% in the production should occur (Tavares 1990). CEPRAM measures also included the monitoring of human health on local population.

In 1988 COBRAC was incorporated to Grupo Trevo and called Plumbum Mining and Metallurgy Ltd, and in 1991 the smelter required the license of operation to the state environmental office (CRA) (Anjos 2003). This agency imposed some conditions to issue the license, among them: (I) a better control of atmospheric emissions; (ii) minimization of the contamination of the Subaé River by effluents or runoff; (iii) characterization of tailings; (iv) monitoring for metals in groundwater and Subaé River; (v) an epidemiological study including measures of prevention, control and treatment of affected population. According to CRA these conditions were never met and the smelter was shut down in 1993 and the area was abandoned (Anjos 1998; FUNASA 2003).

At the end of 1992, it was estimated that about 50% of the particulate matter generated by the smelter was not being captured (Silvany Neto et al. 1996). The mean composition of the tailings generated by this smelter was characterized by Machado et al. (2004) using X- ray fluorescence as: 32.5% SiO_2, 4.19% Al_2O_3, 5.02% MgO, 0.74% SO_3, 18.90% CaO, 1.10% MnO, 7.68% ZnO, 3.78% PbO, 244% Fe_2O_3 and 1.69% others. The slag was classified by Santos (1995) as Class 1 – Hazardous, according to the Brazilian guideline NBR 10.004 (ABNT 1987, posteriorly revised and modified in ABNT 2004). The metal contamination of the nearby town occurred through the deposited tailings and airborne dust from atmospheric deposition through chimney's emission while the smelter was operational (Anjos 2003; Machado et al. 2004). Until 1994 more than 500,000 tons of tailings were generated. It has been reported that approximately 180,000 m^3 of tailings had been deposited around the smelter area from which approximately 55,000 m^3 were buried under roads by municipal authorities or used by the habitants in their house's backyards (Machado et al. 2004).

In 1995, the CRA required the encapsulation of tailings according to standard protocols to mitigate contamination, and following guidelines to disposition of non inert residues or hazardous solid residues (Anjos 2003). However, only part of the total tailings was covered

with a thin layer of soil and an exotic grass (*Brachiaria* sp) was planted to fix the soil. In some areas, tailings were again exposed causing aerial dispersion of tailings dust in the smelter site (Anjos 2003; Machado et al. 2004).

In 2003, the Brazilian Ministry of Health applied the methodology of ATSDR (Agency for Toxic Substance and Disease Registry) to human health risk assessment (FUNASA 2003). This study showed the environmental contamination (soil, groundwater, domiciliary dusts, sediments and food) by metals.

2.2.3 Contamination of the environmental compartments

Despite preview works cover all environmental compartments, there was a lack of systematic sampling and a lack of information about methodology, detection limits, location of sampling, or chemical analysis not covering all main contaminants, pointed by FUNASA (2003) as Pb, Cd, Cu and Zn.

According to Costa (2001) and Carvalho et al. (2003), the site presents a high health risk to humans due to high levels of metals in soil and water, occasioned by the exposed furnace slags and airborne dust, besides the past atmospheric deposition through chimney's emission (up to 3 km from the industrial area) (Anjos 2003; Machado et al. 2004). Several studies showed human contamination by Pb and Cd, especially in children (Sylvany-Neto et al. 1989, 1996; Tavares and Carvalho 1992, Carvalho et al. 1996). A study carried out by the Brazilian health authority (FUNASA 2003) observed the following routes of exposure to humans (pass, present and future): ingestion or dermal contact with superficial soil from the smelter area or streets and backyards, due to tailings deposition, inhalation of domiciliary contaminated dusts, ingestion of food cultivated in contaminated soils or ingestion of mollusks or crustaceans from the estuary of the Subaé River.

High levels of metal contamination have been found in the Subaé River and its estuary system, sediments and the associated biota (Paredes et al. 1995, Cunha and Araújo 2001), and in the sediments and biota of Todos os Santos Bay (Tavares 1990, Hatje et al. 2006, Amado-Filho et al. 2008), related to the smelter contamination, and putting health risk for subsistence fishers and subsistence shellfish consumers (Souza et al. 2011). The Subaé River is one of the main tributaries of the Todos os Santos Bay, the second largest bay in Brazil (Hatje et al. 2006). Hatje et al. 2006, assessing the current trace metal contamination of the sediments and the benthic macrofauna assemblages in Subaé estuarine system, concluded that the inactive Pb

smelter is an important contemporaneous source of trace metals for the Subaé system. Anjos (1998) pointed out that groundwater contamination in the smelter area exceeded the recommended threshold for Pb and Cd. High levels of Pb values in groundwater were also found in monitoring wells distant from the smelter area (Machado et al. 2004), confirming the dispersal contamination of the surrounding of the smelter occasioned probably by atmospherically dispersion of particulate materials at the time of the smelter works.

High mean levels of Pb were found by Costa (2001), in 1995, in blood of cattle grazing in the smelter area (28,4 ± 22,0 μg/dL, n=29) when compared with a control group not belonging to Santo Amaro da Purificação (1,73 ± 0,68 μg/dL, n=17). The usual presence of cattle grazing in the smelter area and drinking water from the basin was recorded also by Carvalho et al. (2003) which stressed the risk for human health, through the consumption of meat and milk from these animals.

Machado et al. (2010) showed a clear persistence of Pb and Cd contamination on the superficial soil around the smelter site, related to the past emissions when the smelter was still active, with some points exceeding by far the agricultural and residential intervention values from CETESB (2005).

Nowadays, the management objectives and strategies to Santo Amaro are related to prevent potential harm effects to human health and to the environment. The main concerns of the current projects are related directly to human health, such as to the possibility of remove the tailings that were used to pave streets, squares and backyards in urban area, the bioaccumulation of metals in plants on these backyards, air dispersion of dusts in urban area, agricultural use of surrounding areas of smelter.

2.2.4 How the site looks today

An initial site visit was made at the start of project to conduct a visual inspection of the site. Nowadays, the entry in the smelter area is forbidden to avoid human exposure to contamination. Our team required a judicial authorization to enter in the smelter area for scientific purpose.

Inside the smelter area, there are the smelter facilities ruins and some piles of furnace slag covered by soil. The site is a grassland area with some herbaceous and shrubs, which were planted in the smelter area after the disposing of soil inside the tailings deposits. In general,

low vegetation cover and evidence of erosion and runoff were noted in these pills. Furthermore, furnace slag can be seen in some points inside the smelter area, evidencing a failed procedure of furnace slag recovery (Fig. 2.3A). It seems that the vegetation is not able to establish in some points where the tailings are visible at the superficial layer, which can favor the dispersion of contaminated dusts mainly in the dry season (Fig. 2.3B).

Fig. 2.3 A) Evidence of runoff and erosion in a pile of furnace slag (gray material) in the smelter area, at rainy season. B) The aspect of the smelter area in the dry season (dusts and lack of vegetation).

Regarding aquatic compartment, two temporary ponds are located inside the smelter area, which were formed by the arrangement from the piling of furnace slags. The Subaé River is located 300 m far away this area. During the rainy season there is drainage from the site to the river through a drainage channel. There is possible risk to these aquatic compartments (the ponds and the river) and consequently the sediment and associated biota, besides occasioned by runoff of the contaminated soil. Ground water could, in principle, to be also at risk due to leaching potential of metals.

A pre-sampling campaign was designed, using six transects on a radial shape to confirm the presence of total metal concentrations of the main contaminants (Pb, Cd, Cu, Zn) in soil and to determine any major gradients of contamination. The transects shared a central point (P0, located next to the smelter facility) and were composed of four or five points each,

located at 20, 50, 150, 400, and 1,000 m from P0. The results for total metals in soil confirmed the high level of contamination by Pb, Cd, Cu and Zn, once some points presented a critical level of contamination, exceeding by far (between one and 200 times) the screening levels of these four metals (corrected Dutch Intervention Values, VROM 2000).

2.3 Conceptual model and analysis plan

2.3.1 Building up the conceptual model

A Conceptual Model is the essential first step in the ERA, beginning with a combination of desk studies and subsequent site visits and explorations to identify potential contaminants, pathways of exposure and ecological receptors, aiming to identify potential significant pollutant-recptor linkages (Weeks et al. 2004). To build up the Conceptual Model in the present work, some steps were followed:

1) To know the management objectives and strategies to the site. In Santo Amaro, they are related to prevent potential harm effects to human health and to the environment, avoiding air dispersion of dust, avoiding the contamination of Subaé system, and ensuring the agricultural use of surrounding areas of smelter.

2) Summarize existing site data, identifying data gaps or inconsistencies.

3) Site visit and exploration, identifying possible contamintant-pathway-receptor linkages *in situ,* visible risks and possible data gaps, and examining the current state of the site.

Based on the available information about the site, including the one obtained in the site visit and the chemical pre-sampling, a conceptual model (Fig. 2.4) and an analysis plan (section 2.3.2) for an ecological risk assessment were developed. This assessment focuses on the soil compartment and aims at:

- Assessing the possible loss of habitat function and retention function of soil inside and surrounding the smelter area, evaluating the adverse effects of historical contamination on soil organisms, and the likelihood of effects to aquatic organisms via leaching;

- Assessing potential ecological risks at the site using the Triad approach, i.e., by integrating information from different lines of evidence (LoE's): ecotoxicological (previous aim), chemical and ecological;

- To combine the information gathered to help prioritizing the management of areas within the site and formulate appropriate action strategies using the information gathered.

The primary contamination source identified was soil contaminated by furnace slag deposition or by aerial deposition (wind-blow of dusts or past chimney emissions). Contaminant-pathway-receptor linkages for the site were identified based on the history of the smelter activities, preview works and on the site visit. The principal source of potential exposure to the ecological receptors is the contaminated soil, through ingestion, cellular absorption, aerial deposition (wind-blow particles) and root uptake. Metal contaminants can be available posing potential risk to primary receptors, such as plants, soil invertebrates and soil microbial communities. In addition, other species can be linked to contaminants through the terrestrial food chain, such invertebrates feeding on plants, and vertebrates, such birds (seed-, plant-eating and invertebrate-feeding species), small mammals, amphibians, reptiles and raptor species.

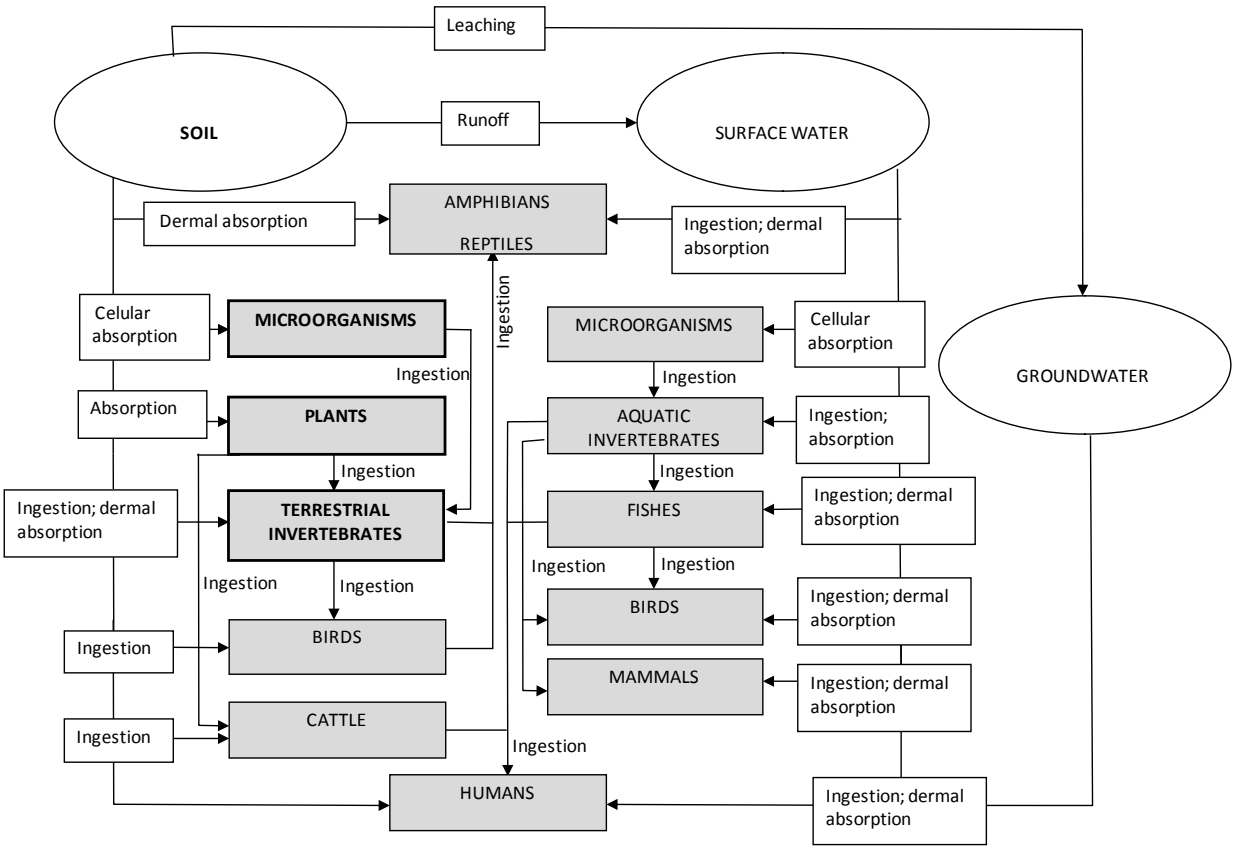

Fig. 2.4 Conceptual model for risk assessment in the contaminated area in Santo Amaro, Bahia, Brazil. Environmental compartments are circles, exposure pathways are arrows and ecological receptors are represented by boxes. The soil is the main source of contaminants (tail deposits and aerial deposition). Receptors in bold were those considered for evaluation in this study.

2.3.2 Analysis plan

The analysis plan for the site-specific risk assessment was focused on the soil compartment, addressing indirectly the risk to groundwater and fresh water systems. Non-soil invertebrates, vertebrates and direct effects on water systems were not covered by this study. A summary of the assessment and measurement endpoints is shown in Table 2.1

The definitive soil sampling strategy was designed based on the soil total metal concentrations of the major metals detected at the site (Pb, Cd, Cu, Zn) and derived from the pre-sampling campaign (see section 2.2.4). Two 1 km transects (T1 and T3) were defined along the two major gradients of contamination detected. The two transects shared a central point

(P0, located next to the smelter facility) and were composed of five points each, located at 20, 50, 150, 400, and 1000 m from P0 (P20T1 till P1000T1 and P20T3 till P1000T3).

Soils samples at each sampling point consisted of a composite sample collected at the top 20 cm. Soil was hand mixed on site to homogenize, transported to the laboratory, sieved (≤5 mm), and processed according to the different analyses (see details at each chapter). Due to the heterogeneity of the soil among the different sampling points, it was necessary to work on a multi-reference basis. Therefore soils were assembled into three groups based on a Factorial analysis. Each group differed mainly in terms of texture, organic matter content and pH. To find matching reference soils, soil from several points in the surroundings of the area were screened, analyzed for metals and soil properties, and three reference soils (the best possible for each identified group of soils) were selected at 9 km (Ref. 1) and 3 km (Refs. 2 and 3) from the site. Details on this process (grouping the soils and finding the reference soils) can be found on Chapter 3.

The analysis plan included two tiers. Tier 1 was essentially a screening phase, aiming to produce a first representation of the risk and to determine whether a site can be excluded from higher tiers of testing (either because it is unlikely to pose a risk to the relevant ecological receptors or because a high risk is detected and there could be a need for immediate mitigation actions), or if it needs to be further evaluated (Weeks et al. 2004; Critto et al. 2007). Thus, the tools used in tier 1 should be also rapid, easy to apply and cost-effective (Jensen and Mesman 2006).

In this phase, the chemical LoE comprised the calculation of the toxic pressure (Rutgers and Jensen 2010) based on the comparison of the total concentrations of metals of the study site with soil screening levels. The ecological information at tier 1 was collected through a quick vegetation survey and by assessing easy measureable functional parameters, such as soil respiration (Jensen and Mesman 2006) and soil faunal feeding activity using bait lamina sticks (Von Törne 1990; Van Gestel et al. 2003; Hamel et al. 2007). These tools have been proposed as relevant for fast ecological assessments (Filzek et al. 2004; André et al. 2009; Van Gestel et al. 2009) and some of them (bait-lamina) have already been successfully tested in tropical soils (Römbke et al. 2006).

Regarding the ecotoxicological LoE at tier 1, short-term cost-effective bioassays evaluating both the habitat and retention functions of the soil were carried out. The later was evaluated using soil extracts (eluates) in tests with cladocerans (*Daphnia magna* acute test) and with the luminescent bacteria *Vibrio fischeri* (Van Gestel et al. 2001; Achazi 2002; Loureiro

et al. 2005). Soil samples were used to evaluate the loss of habitat function through avoidance tests with earthworms (Hund-Rinke et al. 2003; Antunes et al. 2008) and collembolans (Natal-da-Luz et al. 2004).

Tier 2 was performed to reduce uncertainties about the actual risk pointed by tier 1 and contains also information from the three lines of evidence. The chemical LoE at this tier comprised the calculation of the toxic pressure based on total metals in habitat function (as done in tier 1) and the analysis of extractable metals using 0.01 M $CaCl_2$ solution to assess the soil retention function (mainly with the aim of evaluating potential to ground-water contamination).

The ecotoxicological LoE in tier 2 usually comprises long-term studies focusing on chronic endpoints such as reproduction and growth, since sublethal endpoints are usually more sensitive being able to discriminate intermediate levels of potential effects than lethal endpoints (Sutter et al. 2000). At this LoE, standardized chronic tests with Collembola (ISO 1999) and Oligochaeta (ISO 1998, 2004) were performed to evaluate sub-lethal effects of soil matrix on reproduction of soil invertebrates (van Gestel et al. 2001, Loureiro et al 2005, Natal da Luz et al. 2011), furthermore evaluating the toxicity to organisms with different roles in soil processes and exposed to soil contaminants via different exposure routes. Effects towards plants were evaluated by performing plant growth tests using standard species (at least one monocotyledonous and one dicotyledonous species) following ISO 11269-2 (ISO 2005). In addition, soil extracts (eluates) were used to perform widely established tests with cladocerans (OECD 2008) and microalgae (OECD 1984) to evaluate the retention function of soil, thus assessing the indirect risk to aquatic compartment (mainly groundwater) (Jensen and Mesman 2006, Chelinho et al. 2009).

Regarding the ecological LoE at tier 2, information was collected to get more details about the possible impact on selected ecological receptors. Changes in diversity and community composition of plants, soil surface dwelling invertebrates, as well as several functional processes were evaluated.

Microbiological soil-quality indicators considered in this study were microbial biomass, substrate-induced respiration, enzymatic activity and nutrient transformations. These are proxies for important processes related to soil fertility (Edwards 2000) and can be used as bioindicators of soil stress by contamination (Castaldi et al. 2004; Smejkalova et al. 2003, Zimakowska-Gnoinska et al. 2000, Gulser and Erdogan 2008), or to indicate suitable

management and restoration practices (Balota et al. 2004; Nogueira et al. 2006; Clemente et al. 2007).

Pitfall trapping was established in all sampling points and also at the reference sites to determine whether metal pollution exerts effects on community parameters of surface-dwelling invertebrates. The ecological evaluation was complemented with the assessment of effects on organic matter (litter) decomposition, a functional parameter by excellence, which can be used as indicative of negative effects on the soil microbial community, soil fauna or both. The litter bag test is considered the most appropriate method available for assessing organic material breakdown in semi-field or field conditions, and mass loss has been considered the best suited measurement endpoint (Knacker et al. 2003; Römbke et al. 2003; OECD 2006).

Table 2.1 Assessment and measurement endpoints. Adapted from Weeks et al (2004).

Receptor	Relevance for the ecosystem functioning	Assessment endpoint	Measurement endpoints
Plant community	Food and habitat supply for animal species Maintenance of soil structure Supply of nutrients	Habitat function in order to sustain plant germination, growth, biomass and species richness	Plant toxicity test with monocotyledonous and dicotyledonous species Determination of vegetation cover *in situ* Determination of vegetation richness species *in situ*
Soil invertebrate community and activity	Food supply for animal species Predation microfauna Decomposition of organic material Maintenance of soil structure	Habitat function in order to sustain diverse and active invertebrate populations	Avoidance behavior tests with earthworms and springtails Reproduction tests with earthworms, springtails and enchytraeids Composition of soil surface dwelling macroarthropods community collected on pitfall traps (richness, diversity index, differences in community composition) Feeding activity evaluated by bait lamina test *in situ* Decomposition rate of organic material on litter bags *in situ*
Soil microbial community	Nutrients supply to support plant growth Important in maintaining microagregatte soil structure	Habitat function in order to sustain viable and functional microbial populations	Soil basal respiration Soil microbial biomass of carbon and nitrogen Microbial enzymatic activity (dehydrogenase, acid phosphatase and asparaginase) Soil nitrification and ammonification rate Organic material breakdown on litter bags *in situ*

Table 2.1 (Continued).

Receptor	Relevance for the ecosystem functioning	Assessment endpoint	Measurement endpoints
Microorganisms and algae	Primary production Recycling of nutrients	Retention function of soil in order to evaluate risks to aquatic receptors (in this case particularly via groundwater contamination)	*Vibrio fischeri* (bacteria) luminescence test Algae growth test
Aquatic invertebrates	Aquatic food web	Retention function of soil in order to evaluate risks to aquatic receptors (in this case particularly via groundwater contamination)	Cladocerans lethal test Cladocerans reproduction test

2.4 Conclusions

This work showed a conceptual model for the contaminated area of Sto Amaro and an analysis plan for an environmental risk assessment for that area, using the Triad approach (integrated information from chemical, ecotoxicological and ecological lines of evidence). The tiered approach proposed allowed an early screening of risk involving short-term and cost-effective tools in tier 1, and a detailed assessment of risk in tier 2. Integrated risk values will provide information about the actual bioavailability of contaminants, and it will help to prioritize areas to action, besides avoid unnecessary costs in remedial actions.

The framework proposed can be adapted to other scenarios or sources of contamination in order to stimulate the development of site-specific risk assessment to support actions of management and reclamation of contaminated areas.

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Chapter 3

Ecological risk assessment of a metal contaminated area in the tropics.

Tier 1: screening phase

Based on the following manuscript:

Niemeyer JC, Moreira-Santos M, Ribeiro R, Da Silva EM, Sousa JP, 2010. Environmental risk assessment of a metal contaminated area in the tropics. Tier I: screening phase. *J Soils Sediments* 10: 1557-1571.

Abstract

The present study presents data on the screening phase (tier 1) of a site specific ecological risk assessment in a former smelter area heavily contaminated with metals (Santo Amaro, Bahia, Brazil). Joining information from three lines of evidence (LoE), chemical, ecotoxicological and ecological, integrated risk values were calculated to rank sites within the area and identify those that may need further investigation in tier 2. Eleven points were selected up to 1,000 m from the smelter. Three reference points were 3 and 9 Km away from the area. Risk values for the chemical LoE were derived from calculating the toxic pressure based on total metal concentrations. Those for the ecotoxicological LoE were based on avoidance (*Folsomia candida* and *Eisenia andrei*) and eluate tests (*Daphnia magna* acute test and Microtox) whereas for the ecological LoE the bait lamina test, soil basal respiration and vegetation cover were used to derive risk values. The chemical LoE showed high risk in those points inside the area where metal loadings exceeded in much the existing soil screening values. Ecotoxicological tools showed a variable response, with tests on soil organisms inducing a higher risk (again at sites inside the smelter and with sandy soils) than tests on eluates. The three parameters composing the ecological LoE revealed a concordant response, despite the lower sensitivity of the vegetation cover. A high risk on this LoE was also observed on those sampling points where a high chemical risk was calculated. Integrated risk was low outside the smelter area. Inside, a high spatial heterogeneity of risk levels was observed, related to the non homogeneous deposition of smelting residues. Very high risk levels, associated with sandy soils and residue deposits, suggest the need to proceed with remediation actions. However, the uncertainties associated with the contradictory information given by certain LoEs for certain sampling points show the need to confirm potential risks in a tier 2 analysis.

Keywords: Integrated risk values, Lines of evidence, Soil habitat function, Soil retention function, Triad

3.1 Introduction

The triad approach, originally developed to evaluate sediment quality (Long and Chapman 1985), has been recommended and successfully applied in ecological risk assessment (ERA) of contaminated soils (Wagelmans et al. 2009). Consisting of three lines of evidence (LoE; chemical, ecotoxicological and ecological), the triad approach is usually applied within a tiered system, i.e., information from each LoE is collected at each tier following a step-wise cost-effective process (Jensen and Mesman 2006). While tiers 2 and 3 are performed to reduce uncertainties about the actual risk, tier 1 is essentially a screening phase, aiming to produce a first spatial representation of the risk and to determine whether a site can be excluded from higher tiers of testing (either because it is unlikely to pose a risk to the relevant ecological receptors or because a high risk is detected and there could be a need for immediate mitigation actions), or it needs to be further evaluated (Weeks et al. 2004a; Critto et al. 2007). Thus, the tools used in tier 1 to collect information from each LoE should be not only able to indicate effects, but also rapid, easy to apply and inexpensive, i.e., cost-effective (Jensen and Mesman 2006).

In tier 1, the chemical LoE comprises the comparison of the total concentrations of contaminants at the study sites with soil screening levels. This should be complemented with information derived from ecological surveys (ecological LoE) and ecotoxicological tests (ecotoxicological LoE) (Weeks et al. 2004a; Fernandez et al. 2006). The ecological information at tier 1 is often collected through quick soil fauna or vegetation surveys and by measuring quick microbial parameters (e.g. soil respiration) (Jensen and Mesman 2006).In addition, the bait lamina test developed by Von Törne (1990), being a practical tool to assess soil faunal feeding activity *in situ* (Larink and Sommer 2002; van Gestel et al. 2003; Hamel et al. 2007), has been proposed as a relevant tool for ecological assessments (Filsek et al. 2004; André et al. 2009; Van Gestel et al. 2009), and has already been successfully tested in tropical soils (Römbke et al. 2006). Regarding the ecotoxicological LoE, short-term cost-effective bioassays evaluating both the habitat and retention functions of the soil are currently used in tier 1. Such bioassays integrate the combined effect of mixtures and that of contaminants not analyzed or for which soil quality levels do not exist (Weeks et al. 2004a; Fernandez et al. 2005; Spurgeon et al. 2005; Jensen and Mesman 2006). Whereas soil extracts (e.g. eluates) are used to perform

widely established tests with cladocerans, microalgae and the luminescent bacteria *Vibrio fischeri* (van Gestel et al. 2001; Achazi 2002; Loureiro et al. 2005a), soil samples are being increasingly evaluated through avoidance tests with earthworms (Hund-Rinke et al. 2003; Antunes et al. 2008) and collembolans (Natal-da-Luz et al. 2004). Earthworm avoidance tests have been shown to be a useful tool in the screening phase of risk assessment of contaminated soils (Lukkari and Haimi 2005), providing rapid information for future decisions (Schaefer 2003; Loureiro et al. 2005b), while being ecological relevant and of low cost (Yeardley et al. 1996). Although collembola avoidance tests are still under the process of standardization, their use in soil ecotoxicology has been acknowledged (Natal-da-Luz et al. 2004; Aldaya et al. 2006), mainly because the avoidance response of collembola is less influenced by the soil properties than that of earthworms (Natal-da-Luz et al. 2008). However, one of the limitations to use avoidance tests is that some substances are not perceived as repellents by the organisms and consequently are not avoided, leading to an underestimation of the risk (Greenslade and Vaughan, 2003). Moreover, high concentrations of some substances (e,g, pesticides acting as AChE inhibitors) may affect mobility of the organisms to such an extent that they are not able to avoid the contaminated soil, creating also biased results (Natal-da Luz, personal communication).

This study aimed to conduct the first step (tier 1) of a site-specific ERA of a metal contaminated area in Santo Amaro (BA, Brazil), joining information from the three LoE mentioned above. Although a human risk assessment has already been performed in Santo Amaro (FUNASA 2003), human risk-based criteria are unrelated to the ecological parameters that may be important to sustain soil functions (Dawson et al. 2007). Therefore, a site-specific ERA is necessary to evaluate the risks towards key ecological receptors. Besides bringing together chemical and (ecological and biological) effect data, the present study also intended to calculate risk with the purpose of ranking sites within the study area and to identify those that may need to be further investigated. In this way, the present work constitutes an innovative approach in metal contaminated tropical environments, bringing an important contribution to the resolution of a local problem.

3.2 Materials and methods

3.2.1 Study area

This study was carried out in an abandoned lead smelter that was operational between 1960 and 1993, located adjacent to the urban area of Santo Amaro, BA, Brazil (12° 32' 49" S, 38° 42' 43" W). The area presents a high health risk to animals and humans (Costa 2001; Carvalho et al. 2003), due to high levels of metals in soil and water, as well as by tailings and airborne dust from atmospheric deposition through chimneys emission (up to 3 km from the industrial area), while the smelter was operational (Anjos 2003; Machado et al. 2004). It has been reported that approximately 180,000 m^3 of tailings had been deposited around the smelter area from which approximately 55,000 m^3 were buried under roads and house's backyards (Machado et al. 2004). In 1995, the Bahia environmental state agency recommended the encapsulation of tailings with the use of organic matter rich soil to mitigate contamination (Anjos 2003). However, because the process was carried out without following the adequate standard procedure, tailings are still exposed and the aerial dispersion of tailings dust is still occurring within and outside the smelter area (Anjos 2003; Machado et al. 2004).

3.2.2 Soil sampling and selection of reference soils

Based on the soil total metal concentrations (Pb, Cd, Cu, Zn) derived from a pre-sampling campaign using six transects on a radial shape (unpublished data), two 1 Km transects (T1 and T3) were defined along the two major gradients of contamination detected. The two transects shared a central point (P0 – located next to the smelter facility) and were composed of five points each, located at 20, 50, 150, 400, and 1,000 m from P0 (P20T1-P1000T1 and P20T3-P1000T3; see Fig. 3.1).

Soils samples at each sampling point consisted of a composite sample made of four sub-samples collected at the top 20 cm. Soil was hand mixed on site to homogenize, immediately transported to the laboratory, sieved (≤5 mm), and defaunated by one freeze-thawing cycle. After the physico-chemical characterization of each of the 11 soil samples (see next section), a multivariate factor analysis was run using soil properties data (metals

excluded; see next section) aiming to define groups of samples and the main variables defining those groups. Based on this analysis, soil samples were assembled into three groups mainly differing in terms of texture, organic matter content and pH. The obtained results dictated the adoption of a multireference system. Soil from several points in the surrounding of the area were screened, analyzed for metals and soil properties, and three reference soils (the best possible for each identified group of sampling points) were selected at 3 km (Ref. 2 and 3) and 9 Km (Ref. 1) from the area (see Fig. 3.1).

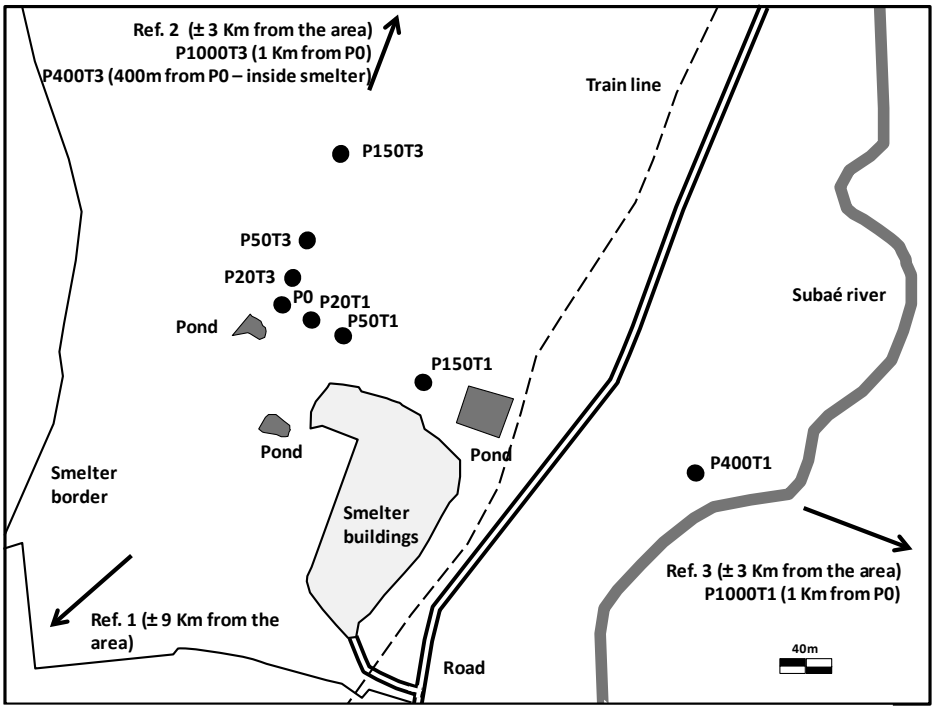

Fig. 3.1 Schematic representation of the study area (an abandoned lead smelter, Santo Amaro, BA, Brazil) showing the location of the 11 sampling points along the two transects and of the three reference points.

3.2.3 Soil physico-chemical characterization

Based on the historical use of the site and on a previous study (FUNASA 2003), soils were analyzed for the four main metals responsible for the contamination of the area (Pb, Cd, Cu, and Zn), and also for Cr, Ni, Fe, Co, and Mn. Metals were quantified in the bulk soil by inductively coupled plasma-atomic spectroscopy. Other soil physico-chemical parameters measured were pH (KCl 1M) (ISO 1994a), water holding capacity (ISO 1998), cation exchange

capacity (ISO 1994b), organic matter (OM) content (loss on ignition at 500 ºC for 6 h) and soil texture (LNEC 1970).

3.2.4 Avoidance tests with *Folsomia candida* and *Eisenia andrei*

Avoidance tests with collembolans and earthworms were conducted using dual combinations of each test soil vs. the corresponding reference soil. To validate the test results, dual control tests using OECD (1984a) artificial soil were performed with both test species. Prior to testing, the water content of each soil (including the OECD soil) was adjusted to 50% of its maximum water holding capacity. Both test species originated from laboratory cultures maintained as described by Natal-da-Luz et al. (2009).

Tests with *F. candida* were carried out based on the ISO draft guideline 17512-2 (ISO 2007a). Cylindrical plastic containers (diameter: 7 cm; height: 6 cm) were divided into two equal sections by a plastic divider introduced vertically. Each container was filled with 30 g fresh weight (FW) of test soil on one side and 30 g FW of the corresponding reference soil on the other side. After removal of the divider, 20 adult collembolans (10 to 12 d old) were placed into the middle line of each test container. After 48 h of incubation, the content of each compartment was emptied into other plastic container to which water and a few drops of blue ink were added. The mixture was gently stirred and the organisms floating on the water surface were counted. Five replicates were prepared for each combination tested, plus one replicate without animals for pH and moisture determination. In the dual control tests with OECD soil the same procedures were adopted but the artificial soil was placed on both sides of the tests container. All tests were performed in a temperature controlled chamber at 25±2ºC and with a photoperiod of 16:8 h (light:dark).

Avoidance tests with *E. andrei* were conducted according to the ISO guideline 17512-1 (ISO 2007b). The test procedures, number of replicates per soil combination and incubation conditions were similar to those used for *F. candida*. However, rectangular plastic boxes (length: 20 cm, width: 12 cm, height: 5 cm) were used, and 250 g FW of (test, or reference) soil were placed in each section of the test container, and ten adult worms were used per replicate The number of organisms in each section was counted and recorded after a 48 h exposure period. For the dual control tests with OECD soil, the same procedures of the avoidance tests were adopted.

3.2.5 *Daphnia magna* lethal test

A 48 h *D. magna* lethal test (OECD 1984b) was conducted on eluates prepared from all soils. Soil eluates were obtained by shaking on a magnetic stirrer a soil:water mixture (ratio 1:4) for 18 h. The obtained soil suspension was left to settle for 24 h, time after which the supernatant was centrifuged for 7 min at 3370*g*, filtered through a Schleicher & Schuell filter paper and stored in plastic bottles at 4ºC and in darkness until use (within 10 days). Although filtration may decrease sample toxicity by removing the fraction of the contaminant adsorbed to suspended particles (Weltens et al. 2000), it was a required procedure to eliminate the potential detrimental effects of the suspended particles per se on the biological responses being measured. The pH was not adjusted. The water used to prepare the eluates was reconstituted hard water (ASTM 2002), since it was the media used for organism culturing in the tests as control and dilution media. Organism used for testing were 24-h old neonates (clone Ircha) from third- to fifth-broods of mothers cultured according to the procedures outlined in Rosa et al. (2010). Four replicates were set up for each treatment with five neonates and 50 mL each. During testing the incubation conditions were similar to those used for the soil organisms, and no food was provided. After 24 and 48 h exposure periods, the immobility/death of the neonates was checked. Measurements of pH, dissolved oxygen and electrical conductivity were measured at the beginning and at the end of all tests. All soil eluates were first tested at 100%. For eluates where immobility was observed, a dilution series of 100, 50, 25, 12.5 and 6.25% of the eluate was tested to determine the median lethal dilution (LC_{50} values).

3.2.6 *Vibrio fischeri* luminescent test

The luminescent test with the marine bacterium *V. fischeri* was carried out on all soil eluates, prepared as described above but using ultra-pure rather than ASTM water, following a previously established protocol (ASTM 2004). All tests were carried out by Cetrel (Camaçari, BA, Brazil). The Microtox toxicity analyzer model 500 (Azur Environmental, Carlsbad, CA, USA) was used to measure the light emission of the bacteria after a 15 min exposure.

3.2.7 Soil fauna feeding activity using bait lamina

Bait material was prepared in a 1/5/14 ratio of finely ground oat, activated charcoal and cellulose powder (Merck). Five groups (samples) of five bait lamina strips were exposed in each sampling point for 14 days. Baits were inserted vertically into the soil, within an area of 15 x 15 cm at each group. In parallel, soil moisture was determined at each point. After the exposure period, each bait lamina was removed from the soil, conditioned together with the baits from the same group and brought to the laboratory. There, after carefully washing it in water, each bait strip was visually assessed by holding it against a light source and counting the number of pierced (= eaten) holes. No distinction was made between partially or fully pierced holes. The feeding activity per sample (group of five strips) at each sampling point was expressed in percentage, dividing the number of eaten holes by the total number of holes.

3.2.8 Basal soil respiration

At each sampling point soil samples for the determination of basal respiration were collected using a different procedure than described in section 2.2. In this case, at each sampling point three parallel transects (5 m apart) were defined. Along each transect, 15 subsamples (10cm depth) were collected and pooled to form a composite sample. After mixing, the samples were sieved (< 5mm), stored at 4 °C and processed within the next 72 h. The methodology to determine the basal soil respiration is described in Alef (1995). Basal soil respiration was measured after 8 days for incubation at 28°C in the dark, with soil moisture adjusted to 60% of water holding capacity. The CO_2 evolved from 100g samples in hermetically sealed containers was trapped in 20 mL of 1 M NaOH. Back-titration with standardized HCl revealed the remaining NaOH and consequently the CO_2-C evolved. Results were expressed on an oven-dried soil basis at 105 °C for 24h.

3.2.9 Vegetation cover

Assessment of percentage of vegetation cover was carried out according to Veiga and Wildner (1993). A plastic grid of 50 cm x 50 cm, subdivided in small 100 squares of 5 cm x 5 cm, was randomly released four times (four samples) at each sampling point. The sum of the

intersections of small squares over vegetation in each grid represents the percentage of vegetation cover at each sample.

3.2.10 Data analysis

3.2.10.1 Ecotoxicological and ecological tests

The avoidance response to each tested soil was calculated according to the ISO guidelines (ISO 2007a, b). Avoidance data for *E. andrei* at each combination tested was corrected for site specific properties using a generalized linear model developed by our team based on data gathered with non-contaminated natural soils (unpublished study). Soils properties considered in the model were texture parameters (sand and silt contents) and soil pH. According to this unpublished study, the avoidance response of *F. candida* showed much less sensitivity to soil properties. Therefore no correction was done for this organism in the current study. The significance of the avoidance responses ($p<0.05$) was evaluated using the Fisher exact test (Zar 1996), as described by Natal-da-Luz et al. (2008). For the avoidance tests, a one-tailed test was chosen, and the null hypothesis assumes that half of the individuals are staying in the soil that is being assessed, meaning that there are no avoiders regarding that soil. For the dual control tests, a two-tailed test was chosen, and the null hypothesis assumes an equal distribution of the organisms on both sides of each test container. The 24 and 48 h LC_{50} values for *D. magna* and respective 95% confidence limits (CL) were computed using the software PriProbit 1.63, with the probit transformation of the proportion of deaths and the log transformation of the dilution values (Sakuma 1998). For the *V. fischeri* test, the EC_{50} values (median effective dilutions) and respective 95% CL were calculated using the Microtox Omni Software 1.18 (Azur Environmental).

For the ecological parameters (bait lamina test, vegetation cover and soil basal respiration) differences between sampling points were evaluated via a one-way analysis of variance followed by the Dunnet test. In these analyses an overall reference value was used, based on the values obtained for each reference sampling point. Soil moisture and organic matter contents were used as covariables in the basal respiration ANOVA. Normality and homoscedasticity were checked via the Kolmogorof-Smirnov and Bartlet tests, respectively. Analyses were done using the Statistica 7.0 software (Stat Soft).

3.2.10.2 Risk calculations

Risk calculations followed the approach proposed by Jensen and Mesman (2006) where risk values are expressed in a scale ranging from zero ("no risk") to one ("high risk"). This method assumes that the risk value of the reference soils is zero and that the risk of the tested soils is calculated in relation to the value of the respective reference soil. This implies that results from the various parameters across the three LoE should be made comparable (expressed under the same scale). For each sampling point, the calculation of the risk values was done through three steps: (1) scaling the results of each test/evaluation within each LoE; (2) integrating scaled information and calculating the contribution of each LoE to the overall risk; (3) integrate the information from the three LOEs and calculate the integrated risk.

In the first step, the results of all determinations within each LoE were scaled between zero and one. For the chemical LoE the total content of each metal was used to calculate the specific Toxic Pressure (PAF - Potential Affected Fraction of species) at each sampling point. This was done based on the mixture model of concentration addition described by De Zwart and Posthuma (2005). The benchmarks ($HC50_{EC50}$ values) and model parameters used for each metal in these calculations can be found in Rutgers et al. (2008). According to these authors the use of HC50 values derived from species sensitivity distributions based on NOEC values ($HC50_{NOEC}$) could originate an overestimation of risk values (many values closer to 1). Since no HC50 values based on EC50 values ($HC50_{EC50}$) are available in literature, they advise to apply the safety factor of 10 to the $HC50_{NOEC}$ and work with those values ($HC50_{EC50}$ = 10 x $HC50_{NOEC}$; Rutgers et al. 2008). Prior to calculations, the $HC50_{EC50}$ values were corrected for sampling site-specific differences (taking into account the organic matter and the clay content of each soil) according to the correction formula described in Boivin et al. (2006). This implies that different $HC50_{EC50}$ values (HC50cor) were used for each metal at each sampling point. Since HC50 values exist only for some metals, risk derived from the chemical LoE was based only on the concentrations of Pb, Cd, Cu, Zn, Cr and Ni.

For the ecotoxicological LoE, the scaling of the avoidance data was done based on the percentage of avoidance, where negative values (no avoidance) were set to zero. Since the avoidance response towards a reference soil (when tested against itself) is zero, percentage values (converted between 0 and 1) were used directly as individual risk values. In the *D. magna* and *V. fischeri* tests, the LC_{50} and EC_{50} values, respectively, expressed as the percentage of dilution of the eluates, were used. For the ecological LoE, the bait lamina (expressed as the percentage of fed holes), the vegetation cover (expressed in percentage

values), and the basal soil respiration (expressed as μg CO_2-C g^{-1} day^{-1}) values were scaled using an overall reference value calculated based on the values from the three reference sampling points.

In the second step, the risk derived from each LoE was calculated by integrating the respective scaled information for each parameter. In the chemical LoE this was achieved by estimating the msPAF (multi-substance PAF) by integrating the individual metal PAF's according to a response addition model described by De Zwart and Posthuma (2005). Finally, in step three, the integrated risk (IR) was calculated for each tested soil (sampling point). To evaluate whether the different lines of evidence contributed differently to the total risk, the standard deviation associated to each IR value was also calculated. More details on the calculation involved in each of the three steps (including formulas for each type of data used) can be seen in Jensen and Mesman (2006).

3.3 Results and discussion

3.3.1 Soil properties and selection of reference soils

Soils from the study area showed low (<2%) to medium (2 to 6%) organic matter content (according to the USEPA 2004), a Cation Exchange Capacity (CEC) mostly between 30 and 40 meq/100 g, and pH values near neutral, with the exception of soils P1000T1 and Ref.2 with a low pH (Table 3.1). These characteristics agrees with those reported by Anjos (2003), who identified basic pH high CEC, high clay percentage and high organic matter content as characteristics of soils from the study area.

Results from the multivariate factor analysis indicated that texture (described by coarse sand, silt and clay contents) was the main soil characteristic determining the separation of the soils (along axis 1, explaining 49.5% of the variation), followed by pH (along axis 2, explaining 24.3%) and organic matter content (along axis 3, explaining 12.8% of total variation). Texture variables separated soils of group 2 (highest sand content and lowest silt and clay contents) from all other soils. The latter were then separated into two groups based mainly on their OM content, with group 1 generally presenting lower values than group 3. The reference soil allocated to each of the three soil groups was selected so that its characteristics matched, to the extent possible, these three soil properties, which are known to influence not

only the bioavailability of contaminants (Kuperman et al. 2009), but also the avoidance response of the two tested soil-dwelling species (Natal-da-Luz et al. 2008). The heterogeneity of the soil inside the smelter area can be related to the failed attempt to encapsulate the tailings by depositing thousands of cubic meters of soil from regions around (Anjos 2003).

3.3.2 Soil metal concentrations

Total metal concentrations for each soil are shown in Table 3.2. For at least one among four metals (Pb, Cd, Cu, and Zn), soils from three sampling points presented levels exceeding the $HC50_{cor}$ values. Among these points, P0 presented a high degree of Zn contamination by exceeding almost three fold the corresponding $HC50_{cor}$ value, whereas points P150T1 and P50T3 presented a critical level of contamination, exceeding by far (between 1.6 and 73.5 times) the screening levels of these four metals.

High levels of metal contamination in the area were also previously reported (Anjos 2003; Machado et al. 2004). Most likely, such contamination levels resulted from the deposition of residues inside the smelting area as well as from the aerial deposition of contaminated particles from the smelter plume while in function, being responsible for the extent of contamination outside the smelter area.

Table 3.1 Physico-chemical characteristics of sampled soils and respective reference soils. USDA – United States Department of Agriculture; CEC – Cation Exchange Capacity

Soil group	Coarse sand (%)	Fine sand (%)	Sand (total) (%)	Silt (%)	Clay (%)	Texture (USDA)	pH (KCl 1:5 v:v)	Organic matter (%)	CEC (meq 100g)	WHC (%)
Group 1										
Ref 1	2.3	8.5	10.9	42.1	47.0	Silty Clay	7.1	1.1	34.16	53.78
P1000T1	2.5	21.8	24.3	19.9	55.8	Clay	3.7	2.0	43.20	59.95
P20T3	11.4	30.0	41.4	22.3	36.3	Clay Loam	6.8	1.9	42.16	67.73
P400T3	6.5	8.6	15.1	52.4	32.5	Silt Clay Loam	7.1	1.9	35.84	56.67
Group 2										
Ref 2	50.9	38.5	89.4	2.8	7.7	Loamy Sand	4.9	1.0	37.60	27.53
P0	43.2	31.3	74.5	11.9	13.6	Sandy Loam	6.7	0.3	38.56	44.12
P20T1	48.0	13.8	61.8	19.0	19.3	Sandy Loam	7.1	0.2	37.28	46.40
P150T1	56.2	21.1	77.4	12.3	10.3	Sandy Loam	6.7	2.1	21.28	28.55
P50T3	69.2	9.1	78.3	10.4	11.3	Sandy Loam	7.2	2.8	16.56	22.05
Group 3										
Ref 3	22.2	15.0	37.2	11.1	51.7	Clay	6.1	3.9	36.48	60.75
P50T1	25.2	13.4	38.6	29.0	32.4	Clay Loam	6.7	1.1	38.16	54.51
P400T1	19.6	23.9	43.5	20.2	36.3	Clay Loam	6.8	5.1	37.44	58.93
P150T3	8.4	15.2	23.5	21.4	55.1	Clay	6.8	2.5	49.20	61.76
P1000T3	10.3	19.5	29.8	29.8	40.4	Clay Loam	7.0	5.7	42.72	57.57

Table 3.2 Total metal concentrations (mg/Kg) in sampled soils and respective references.

Soil group	Pb	Cd	Cu	Zn	Cr	Ni	Fe	Mn
Group 1								
Ref 1	16	<0.2	66	94	77	54	45000	840
P1000T1	23	<0.2	60	80	62	46	48000	360
P20T3	308	<0.2	56	420	78	60	49000	672
P400T3	179	0.3	44	90	59	46	34000	760
Group 2								
Ref 2	13	<0.2	18	24	16	28	2900	34
P0	1264	<0.2	76	3800 (2.8)	72	57	52000	674
P20T1	133	<0.2	56	220	80	56	41000	780
P150T1	37460 (10.4)	771 (9.8)	594 (1.6)	42200 (33.5)	57	70	110000	1720
P50T3	26074 (7.1)	62	3196 (8.2)	95940 (73.5)	80	40	117000	5880
Group 3								
Ref 3	152	<0.2	40	260	59	40	53000	820
P50T1	164	<0.2	60	240	80	58	43000	720
P400T1	961	8.8	60	840	64	48	35000	540
P150T3	2200	12	108	3300	84	58	56000	678
P1000T3	99	<0.2	56	156	84	52	49000	568

Numbers in superscript indicate an exceedance of the corrected Dutch $HC50_{EC50}$ values (after Rutgers et al. 2008) (Ex: the [Pb] at P150T1: 37460 (10.4), indicates that [Pb] was 10.4 times higher than the $HC50_{cor}$Pb).

3.3.3 Avoidance tests with *Eisenia andrei* and *Folsomia candida*

Avoidance tests with *F. candida* fulfilled the validity criteria, since mortality at each combination was less than 20% (ranged between 2% and 18% in avoidance tests, and was 1% in dual control tests), and a homogeneous distribution of individuals in the two compartments was observed in the dual control tests ($p>0.05$, Fischer Exact test). Collembolans avoided significantly most of the test soils (Fig. 3.2). The exception was soil from point P1000T3, where no avoidance was detected. Considering that points P150T1 and P50T3 presented the two most contaminated soils (with the highest concentrations of Cu, Zn, Pb and Cd), a stronger avoidance response was expected relatively to all other soils. *F. candida* is known to avoid copper concentrations well below those that impair survival and reproduction (e.g. a 48 h EC_{50} for avoidance of 61 mg/Kg versus a 28 d EC_{50} for reproduction of 751 mg/Kg; Greenslade and Vaughan 2003), and some evidences point that the same can occur with zinc (Natal-da-Luz et al. 2004). However, according to Greenslade and Vaughan (2003), these organisms do not avoid cadmium. Moreover, *F. candida* seems to be less sensitive to lead than to other metals, as observed by Sandifer and Hopkin (1996) ($EC_{50reproduction}$=2970 µg/g at pH 6.0), and as reported by Fountain and Hopkin (2001), where *F. candida* fed on yeast contaminated with Pb up to 49200 µg/g did not exhibited significant change in mortality at all concentrations. In view of these facts, the weak avoidance response observed in these two soils (22% in P150T1 and 11% in P50T3) was most likely due to other factors than due to the total metal loads. Although there are indications that the avoidance response of *F. candida* is less influenced by soil properties than that of *E. andrei* (Natal-da-Luz et al. 2008), the fact that soils P150T1 and P50T3 had a higher organic matter content and pH compared to Ref 2, may have influenced the availability of metals to the organisms. Moreover, the very low organic matter content in soils from P0 and P20T1 may have caused the higher avoidance response observed.

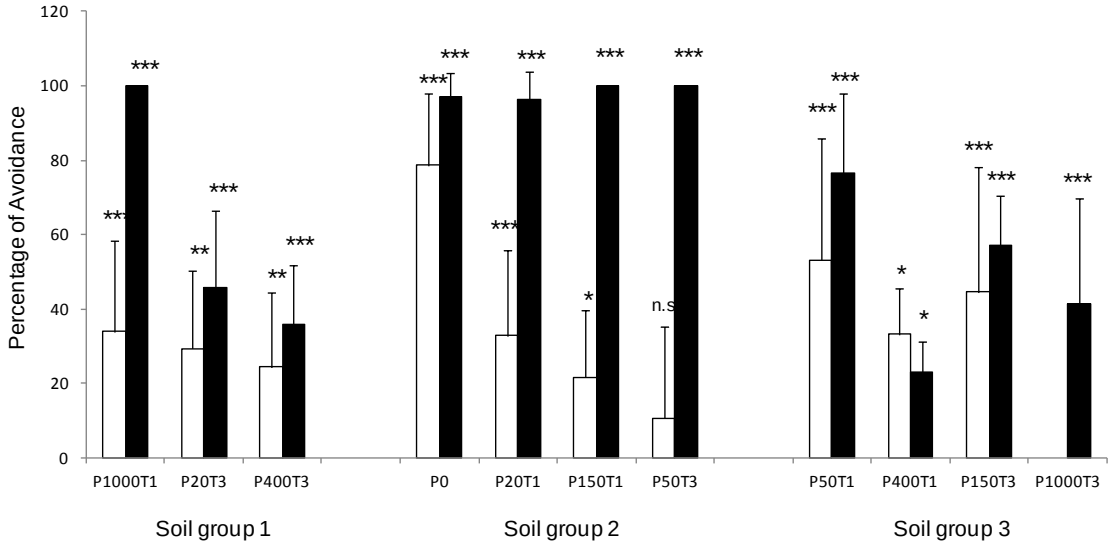

Fig. 3.2 Avoidance percentage (mean + standard deviation) of *Folsomia candida* (white bars) and *Eisenia andrei* (black bars) for each tested soil. Asterisks indicate a significant avoidance using Fisher exact test (* $p \leq 0.05$; ** $p < 0.01$). Negative values were not shown.

Tests with *E. andrei* also fulfilled the validity criteria since no mortality was found in any soil and the organisms were homogeneously distributed between the two compartments in the dual control tests ($p > 0.05$ in Fischer Exact test). All soils were significantly avoided by the earthworms (see Fig. 3.2). An impairment of the habitat function of the soil according to the avoidance criterion proposed by Hund-Rinke and Wiechering (2001), i.e., an avoidance response higher than 60%, was observed for all soils from group 2 and also for soils from points P1000T1 and P50T1. This strong avoidance response may have been induced by the high levels of metals, in some cases exceeding by far the $HC50_{cor}$ values, or by soil related properties known to influence the avoidance behavior of earthworms. Loureiro et al. (2005b) reported avoidance response of *E. andrei* to copper (EC_{50}=181.1 mg/kg Cu) and Lukkari et al. (2005) observed a significant avoidance of *Aporrectodea tuberculata* to 53 mg/Kg of Cu and 92 mg/Kg of Zn. Moreover, Alvarenga et al. (2008) observed avoidance response of *E. fetida* to a mine contaminated soil containing 1250, 362, 264, and 2.6 mg/kg of Pb, Cu, Zn, and Cd, respectively. These findings support our results for soils from group 2, presenting contents of several metals higher than the benchmarks reported by these authors. Besides the high levels of metals, other factors are known to influence the avoidance response of *E. andrei*. According to Natal-da-Luz et al. (2004; 2008), soils with low pH, low organic matter content and fine

texture are avoided by these organisms, which could explain the high avoidance response for soils P1000T1, P20T1 and P50T1 (low pH and low organic matter content, respectively, in comparison with the respective reference soils). In the case of sampling point P1000T1, by being outside the smelter area and in the middle of a pasture, the presence of another type of contamination (e.g., pesticides) eliciting the observed high avoidance response should not be ruled out. The high organic matter content in soil from point P400T1 (5.1%) relatively to the respective reference may explain the lack of a strong avoidance response by *E. andrei*. Organic matter decreases the bioavailability of metals in soil (Lock et al. 2000; Lock and Janssen 2001) and *E. andrei*, being a compost worm, is known to prefer soils with high organic matter content.

Overall, earthworms were generally more sensitive to the metal contamination and presented a less variable response than the collembolans. The present results are thus in accordance with the documented sensitivity of earthworms to metals in avoidance tests (Yeardley et al. 1996; Hund-Rinke and Wiechering 2001; Lukkari et al. 2005) and their more consistent response to metal contaminated soils than collembolans (Natal-da-Luz et al. 2004) confirming their valuable use in ERA (Römbke et al. 2005).

3.3.4 *Daphnia magna* lethal test and *Vibrio fischeri* luminescent test

Lethal effects on *D. magna* were observed in eluates prepared from soils P150T1 and P1000T1; values of 24 h LC_{50} of 88% (95% CL; 82 – 95) and >100% and of 48 h LC_{50} of 68% (95% CL; 63–74) and 91% (95% CL; 85–97), respectively, were registered. Regarding the *V. fischeri* test, eluate from soil P50T3 was toxic to the bacteria, with a 15 min EC_{50} value of 8.6% (95% CL of 1.1–65.3). The present results are in agreement with the fact that metal loads were highest in soils from points P150T1 and P50T3. Although the soil from point P1000T1 was not classified as metal contaminated according to the $HC50_{cor}$ benchmarks used, the response in this soil eluate was most likely related to the low pH (3.7) of the bulk soil, and thus the low pH levels in this soil eluate; pH ranged from 4.84-4.98 to 5.53-6.22 at the start and end of the lethal test, respectively. Actually, lethal effects on cladocerans due to pH alone are likely to occur for values outside the range 6.0-9.0 (USEPA 2002). In agreement, the lack of lethal effects in the eluate prepared from the P0 soil (classified as metal contaminated according to the $HC50_{cor}$ values) was most likely due to the fact that pH level in this eluate was always well above 6.98, i.e., level not only not detrimental for freshwater organisms but at which most metals are not in their dissolved form and thus not bioavailable. The present results suggest that the

retention function of soils at most of the sampling points was enough to prevent the mobilization of metals via the water pathway. Bioavailability of metals in soils may depend on several factors, such as pH, organic matter content, cation exchange capacity and clay content (Van Gestel 1992). Due to soil heterogeneity, the sorption potential might vary considerably, resulting in changes of contaminant availability, sometimes even within a small area. With time, sequestration processes become even more pronounced, a phenomenon generally referred to as "ageing". In general, most of the soils sampled have low organic matter content, however, all soils (except P1000T1 and Ref 2) have an alkaline pH, typical of soils of this region (Anjos 2003), which favors soil adsorption and restricts metal bioavailability. Moreover, soil "ageing" may be occurring in the area between the closure of the smelter in 1993 and the last attempt to rehabilitate the residue piles in 2001 (Anjos 2003).

3.3.5 Bait lamina test

The average feeding activity observed at the sampling points was in general significantly lower than the overall reference value (Table 3.3). Lower feeding activity was registered at sampling points within the smelter area, mainly at those associated to a high degree of contamination (P0, P150T1 and P50T3) or to a low organic matter content (P20T1 and P50T1). Unexpectedly, point P400T3 also presented a low feeding activity, but other soil or habitat parameters must explain these results. With the exception of sampling point P1000T1, bait lamina data was, in general, in good agreement with the *E. andrei* avoidance responses, i.e., high avoidance was usually associated to a low feeding activity. This is in agreement with van Gestel et al. (2003) that found a strong association between soil fauna feeding activity measured by bait lamina and earthworm densities. The observed decline in feeding activity at contaminated sampling points may suggest an impact of metals on the soil fauna, especially earthworms, and eventually on invertebrate abundance and diversity (Weeks et al. 2004b). Similar results were also reported by Filsek et al. (2004) and André et al. (2009) on metal contaminated areas in the UK and Portugal, respectively.

Table 3.3 Ecological parameters (average values ± standard deviation) for the assessed sampling points. The values for the three reference points were averaged to give an overall reference value. Asterisks indicate significant differences (* p<0.05; ** p<0.01; *** p<0.001) for a one-tailed hypothesis of a Dunnet test between each sampling point and the overall reference value (Ref value higher than sampling point value). In the soil respiration ANOVA, soil moisture and soil organic matter contents were used as covariables. N - number of replicates.

Soil groups	Bait lamina (% pierced holes) N=5	Vegetation cover (%) N=4	Respiration (ug CO_2/ g soil/ day) N=3
Overall reference	48.6 ± 13.9	81.3 ± 21.0	139.4 ± 106.4
Group 1			
P1000T1	45.3 ± 16.1	67.5 ± 15.0	164.0 ± 79.1
P20T3	30.4 ± 15.4	32.5 ± 12.6 ***	82.6 ± 15.8
P400T3	10.3 ± 6.7 ***	97.5 ± 5.0	165.2 ± 41.3
Group 2			
P0	18.4 ± 14.3 ***	22.5 ± 22.2 ***	34.9 ± 7.8 ***
P20T1	17.8 ± 10.2 ***	30.0 ± 16.3 ***	35.1 ± 7.1 ***
P150T1	7.3 ± 8.1 ***	30.0 ± 42.4 ***	49.2 ± 6.6 **
P50T3	11.8 ± 5.7 ***	20.0 ± 14.1 ***	52.2 ± 12.6 **
Group 3			
P50T1	19.8 ± 6.8 ***	57.5 ± 12.6	41.4 ± 2.4 **
P400T1	61.5 ± 23.8	100.0 ± 0.0	234.9 ± 83.3
P150T3	5.5 ± 6.9 ***	57.5 ± 9.6	60.5 ± 9.2 *
P1000T3	26.3 ± 17.5 *	100.0 ± 0.0	n.d.

n.d. - not determined

3.3.6 Basal soil respiration

Basal soil respiration was significantly lower in sampling points inside the smelter area, either those with high metal contents (P0, P150T1, P50T3 and P150T3) or those with very low organic matter content (P20T1 and P50T1), despite the correction for this parameter (Table 3.3). These results showed impairment in microbial activity, which indicates a probable negative effects on nutrients cycle in points inside the smelter area. Respiration is a functional parameter widely used to indicate the microbial activity related to nutrient cycling (Araujo and Monteiro, 2007). Results obtained by Zimakowska-Gnoinska et al (2000) confirmed that soil respiration can be used for estimations and comparisons of soil ecological conditions and biological activity of soils. These authors observed a significantly lower oxygen consumption in soil samples from contaminated sites in comparison to uncontaminated sites. Similar results were obtained by Gulser and Erdogan (2008) that presented a negative correlation between soil respiration and metal contents in roadside fields of intensive traffic areas.

3.3.7 Vegetation cover

Vegetation cover ranged between 20-100 % (Table 3.3). Like for the other two ecological parameters, and despite the large variability in the data, a significant reduction of the vegetation cover in comparison to the overall reference situation was observed in most sampling points within the smelter area (P0, P20T1, P150T1, P20T3 and P50T3). These points, together with P50T1 and P150T3 (where a reduction in vegetation cover was also observed), correspond to sites where tailings were deposed and where a non-successful re-vegetating action took place. At these sites vegetation was dominated by one herbaceous species. In some of these points, evidences of erosion were observed, which could have originated the delay of the natural regeneration process.

Our results are in agreement with Salemaa et al (2001). When studying plant diversity and cover along a metal pollution gradient in a smelter area in SW Finland, these authors found that these parameters decreased in soils with metals and sulphur, and increased with distance from the smelter. Similarly to our findings, they also found that a few tolerant species dominated the ground vegetation on the most polluted sites. They also found that the understory vegetation near the smelter was more damaged than trees, which confirms the importance of including understory vegetation in monitoring programmes.

According to Godinez-Alvarez et al (2009) vegetation cover is an important indicator of soil quality. However when used alone (without information on other vegetation parameters like biomass or species richness) data obtained should be interpreted carefully, since a higher cover does not necessarily indicate a good soil quality. In this study vegetation cover showed to be a less sensitive parameter than bait-lamina or microbial basal respiration, being able to detect differences only on those heavy degraded soils. However, due to the extreme simplicity in terms of sampling, can be considered a good parameter for the screening phase when used together with other ecological parameters.

3.3.8 Lines of evidence and integrated risk

Table 3.4 shows the individual contribution and the combined calculated risk values for each LoE. Sampling points presenting very high risk values (above 0.75) derived from the chemical LoE were, as expected, those where the metal concentrations exceed the $HC50_{cor}$ values (P0, P150T1 and P50T3) or were near that threshold (P150T3; see also Table 3.2). Regarding the ecotoxicological LoE, the differences in sensitivity of the screening tests were clearly visible in their contribution for the calculated combined risk, with avoidance tests indicating a higher risk than aquatic tests. The highest risk values (0.60 to 0.82) for this LoE were found in the more sandy soils (sampling points from group 2), with clay and silt soils presenting lower risk values (P1000T1 was the exception to this trend due to the high contribution of the earthworm avoidance test). Despite the differences in sensitivity, the contributions of the three parameters from the ecological LoE were concordant with the other LoE's in indicating greater risk values (above 0.60) for sampling sites from group 2 (sandy soils with high metal content), and for P150T3 (soil with high metal loadings).

The combination of the three lines of evidence into an integrated ecological risk value (IR) for tier 1, showed the spatial heterogeneity of the risk along the study area. However, high levels of risk were found at sampling points within the smelter area, particularly in soils with a coarse texture (soils from group 2; Fig. 3.3). Very high integrated risk values (IR > 0.75) were calculated for sampling points P0, P150T1 and P50T3 which, according to the Dutch limit acceptable values according to land use (Jensen and Mesman 2006) restricts their use to industrial activities and requires sealed soils. Among the latter soils, the weight of evidence was strong, since a high level of risk was indicated by each of the three lines of evidence (as illustrated in Fig.3.3 by the low levels of standard deviation and balanced triangular graphs).

Sampling points P20T1 and P150T3 showed a moderate risk ($0.51 \leq IR \leq 0.75$) (Fig. 3.3). However, the weight of evidence was not as strong as for the three soils described earlier, as seen by the slightly higher standard deviation associated to the IR values. At sampling point P20T1 the low risk indicated by the chemical LoE did not agree with the high risk values for both the ecotoxicological and the ecological LoE's. The observed low metal loadings on this soil, and the consequent low chemical risk values obtained, were unexpected due to the spatial location of the sampling point. However this is another clear sign of the spatial heterogeneity of the contamination within the smelter area and the degree of uncertainty associated to this spatial variation. This type of discrepancy, i.e. high effects on the biological parameters but low risk shown by the chemical parameters, may lead to the discussion about the weighting of the different LoE's. In principle each LoE is equally weighted, but under special circumstances a differential weighting can be attributed. Rutgers and Jensen (2010) mention, as examples, that the lack of proper reference sites or a deficient chemical characterization are situations where a lower weight should be attributed to the ecological and to the chemical LoE's respectively. In this study, the lower and unexpected risk value obtained for the chemical LoE could justify the attribution of a lower weight to this LoE. However, the option of attributing the same weight to each LoE was followed, especially because the existing experiences in attributing different weights to different LoE's are scarce for the terrestrial environment. Moreover this discrepancy should be confirmed on a tier 2 assessment. In sampling point P150T3, the low weight of evidence was originated by the low risk values of the ecotoxicological LoE (0.3 – Table 3.4). The reason behind this low risk value is the very low sensitivity of the aquatic tests performed. Although these findings should be further investigated in a tier 2 evaluation, they trigger the discussion of differential weighting, this time within each LoE.

A low risk ($IR \leq 0.50$) was associated to sampling points P50T1, P400T1, P1000T1, P20T3, P400T3, and P1000T3, all clay based soils. On points P50T1, P20T3, P400T3 and P1000T3 all the lines of evidence pointed to the same direction (a general low risk), despite the high impact on soil fauna feeding activity found in P400T3 (a trend not followed by the other two ecological parameters measured). However for the other two sampling points some uncertainties still persisted (IR value with a high standard deviation). The risk at sampling point P1000T1 was just indicated by the ecotoxicological LoE, as the chemical and ecological LoE indicated no risk. The high toxicity of this soil, mainly indicated by the earthworm avoidance, can either be related to the low pH value of the soil or to the presence of contaminants not analyzed in this study, namely pesticides. Nevertheless the tendency of earthworm avoidance

tests to produce high risk values (near 1) when the percentage avoidance is used directly as individual risk value in the scaling process (see methods section) should not be neglected. One could argue that another type of scaling process could be used instead with this type of data. However this problem can be minimized since avoidance tests with earthworms should be only one of the ecotoxicological tools applied at this tier. For sampling point P400T1 the high standard deviation associated to the IR value was due to the indication of low or no risk by the ecotoxicological and ecological LoE's, respectively, and to the low to moderate risk signaled by the chemical LoE, related to the levels of Pb and Zn. This result may indicate that the metal levels at this point had a low bioavailability, thus, that this degree of uncertainty needs to be further confirmed in a second tier of risk analysis.

These results confirm the added value of deriving risk values in site specific risk assessment not only by adopting the triad approach, but also by obtaining ecotoxicological information using different test organisms, covering different sensitivities and exposure routes. As shown in this study, biological testing is of direct relevance to the principle of *significant harm*, because the organisms will respond to the bioavailable fraction of the contaminant (Spurgeon et al. 2005). Furthermore, ecological data derived also from different parameters give information about the structure and function of soils, linked directly to the aim of diversity protection, complementing and improving the ecological relevance of the risk assessment process. Either a positive response in an ecotoxicological screening test, an exceedance of soil screening levels or evidences of damage to the ecological structure and functioning of soils, is sufficient to warrant progression to the next tier in the process, where a reduction of uncertainties is done through sublethal bioassays, determination of the available fraction of contaminants and inclusion of additional ecological data.

Table 3.4 Individual risk values for each parameter and combined risk for each line of evidence. For each sampling point, values are scaled between 0 and 1 and are given in relation to the respective reference soil (risk for reference soil is set to 0) (see text for details). ChLoE (chemical line of evidence); EcLoE (ecotoxicological line of evidence); ELoE (ecological line of evidence).

	Chemical Line of Evidence (ChLoE)							Ecotoxicological Line of Evidence (EcLoE)					Ecological Line of Evidence (ELoE)			
Soil groups	Pb	Cd	Cu	Zn	Cr	Ni	**Combined ChLoE**	Avoidance *Eisenia*	Avoidance *Folsomia*	*Daphnia* acute	Microtox	**Combined EcLoE**	Bait lamina	Vegetation cover	Respiration	**Combined ELoE**
Group 1																
P1000T1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.33	0.09	0.00	0.72	0.00	0.17	0.00	0.06
P20T3	0.08	0.00	0.00	0.13	0.01	0.02	0.23	0.46	0.30	0.00	0.00	0.21	0.37	0.60	0.41	0.47
P400T3	0.05	0.00	0.00	0.01	0.00	0.01	0.05	0.36	0.25	0.00	0.00	0.17	0.79	0.00	0.00	0.40
Group 2																
P0	0.29	0.00	0.12	0.73	0.08	0.05	0.85	0.97	0.79	0.00	0.00	0.72	0.44	0.72	0.75	0.66
P20T1	0.00	0.00	0.07	0.11	0.08	0.02	0.25	0.96	0.33	0.00	0.00	0.60	0.63	0.63	0.75	0.68
P150T1	0.88	0.84	0.60	0.98	0.07	0.10	1.00	1.00	0.22	0.32	0.00	0.73	0.85	0.63	0.65	0.73
P50T3	0.83	0.45	0.90	1.00	0.10	0.02	1.00	1.00	0.11	0.00	0.89	0.82	0.76	0.75	0.63	0.72
Group 3																
P50T1	0.05	0.00	0.05	0.02	0.04	0.04	0.19	0.76	0.54	0.00	0.00	0.43	0.59	0.29	0.70	0.56
P400T1	0.20	0.13	0.04	0.20	0.02	0.02	0.49	0.23	0.32	0.00	0.00	0.15	0.00	0.00	0.00	0.00
P150T3	0.30	0.15	0.08	0.47	0.02	0.02	0.72	0.57	0.45	0.00	0.00	0.30	0.89	0.29	0.57	0.67
P1000T3	0.00	0.00	0.03	0.00	0.03	0.02	0.06	0.42	0.00	0.00	0.00	0.13	0.46	0.00	n.d.	0.19

n.d. - not determined

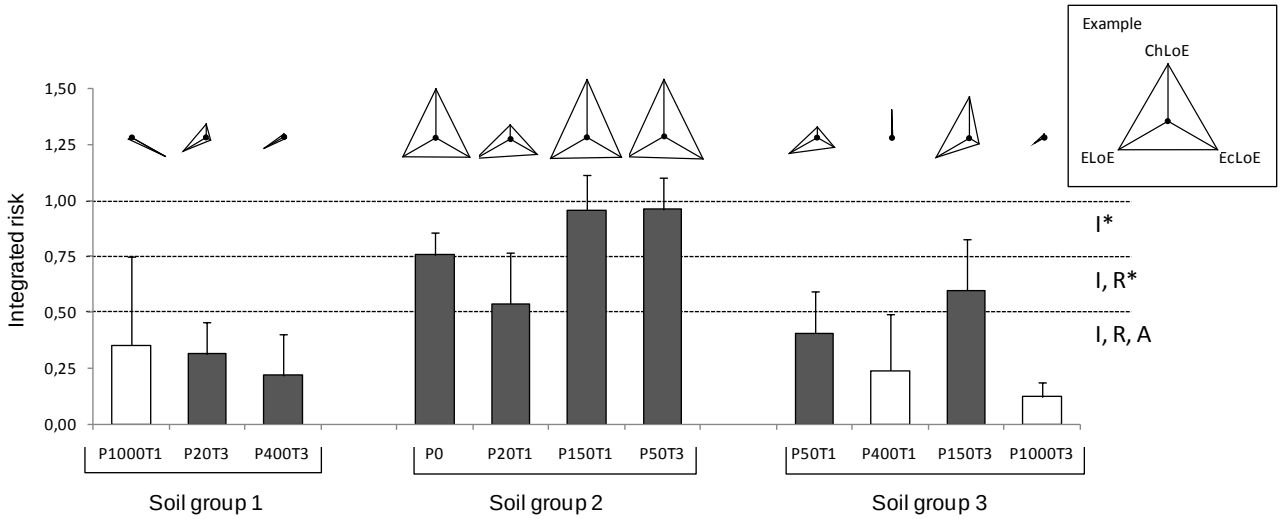

Fig. 3.3 Integrated ecological risk values (+ standard deviation) (Min: 0; Max: 1) for each sampling point, combining information from the chemical, ecotoxicological and ecological lines of evidence. Points with grey bars are located inside of the smelter area. Different bands indicate limits of accepted risk values for different soil uses (A- Agriculture; R- Residential; I-Industrial; asterisks indicate necessity of sealed soils) according to Jensen & Mesman (2006). Triangles on top of each bar represent the contribution of each LoE for the integrated risk value being an indicator of the weight of evidence (on the top right the example the length of each axis of the triangle represent maximum risk (1) from each LoE).

3.4 Conclusions

In general, integrated risk was low outside the smelter area, although some uncertainties were observed that need further investigation on the next tier. Inside the smelter area a high spatial heterogeneity of risk levels was observed, probably related to the non homogeneous deposition of smelting residues. Very high levels of risk were observed mainly in sampling points having sandy soils, and possibly associated to residue deposits. This high risk (above 0.75) may indicate the need to proceed with some remediation action. However, due to several uncertainties associated to the contradictory information given by the lines of evidence in some sampling points, there is a need to confirm the potential risk in a tier 2 analysis. With this aim, further data from the three lines of evidence (including evaluation of metal extractable concentrations, sublethal bioassays and additional ecological surveys) is being collected.

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SECTION 2. DETAILED RISK ASSESSMENT

Chapter 4

Effects of metal contamination of tropical soils on the reproduction of *Folsomia candida, Eisenia andrei* and *Enchytraeus crypticus*

Abstract

The present study evaluated the ecotoxicity of metal contaminated soils nearby to an abandoned lead smelter, on the reproduction of the oligochaete species *Eisenia andrei* and *Enchytraeus crypticus* and the collembolan *Folsomia candida*. Eleven points were selected up to 1,000 m from the smelter, and the reference points were 3 and 9 km far away from the area. Reproduction tests were conducted according to ISO guidelines, under a temperature of 25°C. Reproduction of oligochaetes and collembolans was affected in several soils; however, these organisms showed different sensitivities. Oligochaete species were the most sensitive, and impairment on reproduction was highly correlated to soil contamination and slag content. Reproduction of Collembola was not correlated to metal concentrations. The results reinforced the importance of ecotoxicity tests to assess soil toxicity as a complement of physical and chemical analyses. These tests integrated the combined effect of mixtures of contaminants and their actual bioavailability. Data obtained still highlighted the importance of using different test species in environmental risk assessment, since species may be affected in a different way due to the fact that they represent different routes of exposure to the contaminants.

Keywords: Soil invertebrates, Reproduction, Metals, *Folsomia candida*, *Enchytraeus crypticus*, *Eisenia andrei*

4.1 Introduction

Mining activities have contributed to metal contamination of soil systems (Natal-da-Luz et al. 2004) often resulting in acid mine drainage formation (e.g. Lopes et al. 1999; Huang et al. 2010). Toxicity of soil due to metal contamination is usually evaluated based on its total metal concentrations. However, it is widely recognized that chemical quantification of metals in soil *per si* is not sufficient to evaluate potential risks. Joint effects of metal mixtures to soil organisms may be different than expected from the effect of single chemical exposures, depending on factors such as the nature of the chemicals and multi-contaminant interactions (De Zwartz and Posthuma 2005). On the other hand, ecotoxicological assays integrate the impact of contaminants as a whole (including those not considered or detected by chemical analyses) (Weeks et al. 2004; Natal-da-Luz et al. 2009). Due to that reason, ecotoxicological assays have been recommended to evaluate the ecological risk of contaminated soils as a complement of chemical analyses (Fernández et al. 2005; Antunes et al. 2008; Lors et al. 2009). In fact, ecotoxicological tests with soil invertebrates using sublethal endpoints are suitable for assessing the toxicity of polluted soils (van Gestel et al. 2001) and reproduction is an endpoint with high ecological meaning due to its relevance at the population level (van Gestel 2012).

Moreover, soil invertebrates play an important role in the provision of ecological processes in soil (Jansch et al. 2005; Römbke et al. 2005; Lavelle et al. 2006). For instance the importance of earthworm populations on soil aggregates formation and organic matter decomposition (Marinissen and Hillernaar 1997) and their influence on resource availability to other species ("ecosystem engineers" [Jones et al. 1994]) has been reported. Mesofauna species, like collembolan, play an important role on soil structure as well, namely influencing surface roughness at fine scales (Schrader et al. 1997).

Due to the key-role of these soil fauna groups, over the last decade, earthworm (Ávila et al. 2009; Natal-da-Luz et al. 2011), enchytraeid (Römbke 2003; Amorim et al. 2005) and collembola species (Domene et al. 2007; Crouau and Pinelli 2008) have been used in ecotoxicological laboratory tests to evaluate the toxicity of metal-contaminated soils. These species have shown to be sensitive towards chemical stressors (Römbke 2003; Fountain and Hopkin 2005; Römbke et al. 2005), and represent different routes of exposure to soil contaminants. Several standardized guidelines advice the use of key-species of earthworms,

collembolans, and enchytraeids as suitable organisms to be used in laboratory ecotoxicological tests to evaluate the toxicity of soil pollutants (ISO 1998a; ISO 1999; ISO 2004). Therefore makes reproduction laboratory tests suitable to be integrated in high tier levels of ecological risk assessment (ERA) schemes as proposed by Jensen and Mesman (2006). Integrated on the tier 2 of an ongoing site specific ERA of a metal contaminated area in Brazil (Santo Amaro, Bahia), laboratory reproduction tests with soil species were performed. These ecotoxicological tests were achieved in metal contaminated tropical soils collected along an abandoned mining area using the earthworm *Eisenia andrei*, the potworm *Enchytraeus crypticus* and the collembolan *Folsomia candida* as test organisms. The working hypothesize was that the reproductive output of the test species was lower with increases in the metal contamination of the test soils.

4.2 Materials and methods

4.2.1 Study area

The present study was carried out in an abandoned lead smelter (operational between 1960 and 1993), located in an area adjacent to the urban area of Santo Amaro, BA, Brazil (12° 32 ′49 ″S, 38° 42 ′43 ″W). This area presents a high risk to animals and humans health (Costa 2001; Carvalho et al. 2003) and to the environment, due to the high metal contamination of soil and water, and tailings and airborne dust from atmospheric deposition (up to 3 km from the industrial area), resulting from the smelter activity (Anjos 2003; Machado et al. 2004). In 1995, the Bahia environmental state agency recommended the encapsulation of tailings to mitigate contamination. However, the process failed because it was conducted inappropriately (Anjos 2003; Machado et al. 2004). Due to that reason, there are signs of soil erosion and tailings are exposed and the aerial and runoff derived dispersion of contaminated material is still occurring in some areas within the smelter area (Anjos 2003; Machado et al. 2004). A full characterization of the study site can be found in Chapter 3 (Niemeyer et al. 2010).

4.2.2 Soil sampling

Based on soil total metal concentrations (Pb, Cd, Cu, Zn) derived from a pre-sampling campaign using six transepts on a radial shape, two 1 km transepts (T1 and T3) shared a central point (P0 – located next to the smelter facility) and were composed of 5 points each (at 20, 50, 150, 400, and 1000 m from P0). Tested soils were divided into three groups according to main soil properties. Three reference soils were selected at 9 km (Ref. 1) and 3 km (Ref. 2 and 3) from the study area (Fig.4.1). The reference soils were selected so that their characteristics matched each of the three soil groups, to the extent possible, in what regards texture, pH and organic matter, which are known to influence not only the bioavailability of contaminants (Kuperman et al. 2009), but also the response of the tested species (Chelinho et al. 2011). Detailed information regarding soil sampling and choice of reference soils can be found in Chapter 3 (Niemeyer et al. 2010).

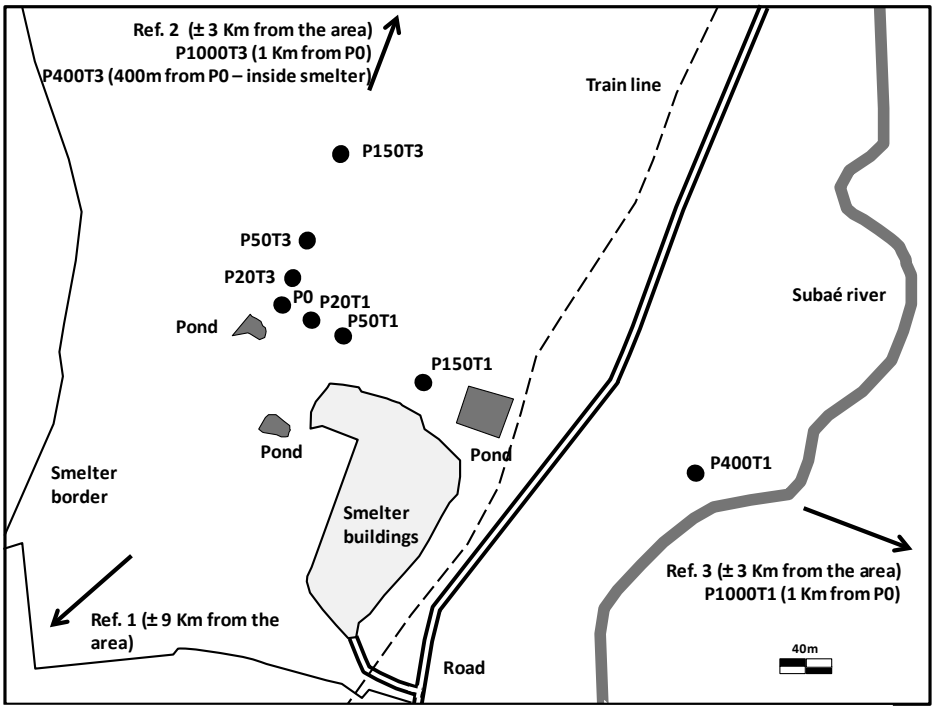

Fig. 4.1 Schematic representation of the study area (an abandoned lead smelter, Santo Amaro, BA, Brazil) showing the location of the 11 sampling points along the two transects and of the three reference points.

4.2.3 Chemical analysis

Based on the historical use of the site and on a previous study (FUNASA 2003), soils were analyzed for the main metals responsible for the contamination of the area (Pb, Cd, Cu, and Zn) and also for Cr, Ni, Fe, Co, and Mn. Metals were quantified in the bulk soil by inductively coupled plasma-atomic spectroscopy. The other soil physico-chemical parameters measured were pH (KCl 1M) (ISO 1994a), water holding capacity (WHC; ISO 1998b), cation exchange capacity (CEC; ISO 1994b), organic matter (OM) content (loss on ignition at 500°C for 6 h) and soil texture (LNEC 1970).

4.2.4 Test organisms

The collembolan *F. candida*, the earthworm *E. andrei* and the potworm *E. crypticus* were used as test organisms. *F. candida* laboratory cultures were maintained in plastic culture containers with a moist substrate of 10:1 (w/w) plaster of Paris:activated charcoal, using granulated yeast as food supply once a week (ISO 1999). Laboratory cultures of *E. crypticus* were kept in plastic culture containers using the standard natural soil Lufa 2.2 as substrate and feeding them daily with finely grounded oat meal (ISO 2004). In the *E. andrei* laboratory cultures a mixture of horse manure and *Sphagnum* peat (1:1, w/w) was used as substrate in plastic culture containers (36 cm length, 22 cm width, and 11 cm height), and cooked oat meal was given once a week as food (ISO 1998a). All species were maintained in laboratory for many generations at 20 ± 2°C and under a photoperiod of 16:8 h light:dark.

4.2.5 Reproduction tests

The procedures adopted in reproduction laboratory tests followed the respective ISO guidelines. The tests were performed at a photoperiod of 16:8 h light:dark, but the temperature was adjusted to 25 ± 1°C, a more realistic condition considering tropical areas. Both soil pH and moisture were measured at the beginning and the end of the tests. The moisture of the test soils was always adjusted to 50% of the WHC before being used in the tests. OECD artificial soil was used as control in all tests to confirm the quality of the test organisms used. This soil consisted of 10% *Sphagnum* peat (previously air dried and sieved at 5 mm), 20% kaolinite clay and 70% quartz sand (OECD 1984). All tested soils were evaluated only at the 100% dilutions.

4.2.5.1 Reproduction tests with *E. andrei*

The procedure adopted in reproduction tests with *E. andrei* followed the ISO guideline 11268-2 (ISO 1998a). One week before starting the test, adult worms (with a well-developed clitellum) were selected and acclimatized in OECD artificial soil (with the addition of fine horse manure as food supply). Four replicates were prepared per each test soil, each one consisting of a cylindrical plastic box with 500 g of soil (wet mass). At the beginning of the test, ten acclimatized worms, weighting between 250 and 500 mg were washed, weighted and then introduced in each replicate. Horse manure was added as food supply once a week. After 28 d, surviving earthworms were counted to assess mortality. After 56 d, at the end of the assay, the test boxes were placed into a water-bath at 60 °C to force juveniles to reach the surface and to be counted.

4.2.5.2 Reproduction tests with *E. crypticus*

The procedure adopted in reproduction tests with *E. crypticus* followed the ISO guidelines 16387 (ISO 2004). Four replicates were used per test soil. The test vessels consisted of glass vessels (100 ml capacity) filled with 30 g of soil (wet mass). Ten organisms with a well-developed clitellum were introduced in each vessel and finely ground oat was given as food after 0 and 14 d of beginning of the test. The test vessels were opened twice a week to aerate the soil and to adjust moisture by weighting the vessels and compensating the weight loss by the addition of distilled water. After 28 d, each test container was filled with alcohol 80% to kill the organisms. Some drops of Bengal red (1% solution in ethanol) were added and the mixture was shaken to homogenize. After 24 h, the content of each vessel was sieved (250 µm) and then the organisms red colored were observed under the binocular (40x) for counting the number of juveniles born during the test period. An additional replicate without organisms was prepared per each test soil to measure the moisture and the soil pH at the end of the test.

4.2.5.3 Reproduction tests with *F. candida*

The procedure adopted in reproduction tests with *F. candida* followed the ISO guideline 11267 (ISO 1999). Springtails 10 to 12 d old obtained from synchronized cultures were used in the experiment. Five test glass vessels (100 ml capacity) with 30 g of soil (wet mass) were prepared per each test soil. Ten springtails 10 to 12 d old, obtained from

synchronized cultures, were introduced in each replicate. Granulated dry yeast (approximately 2 mg) was added as food at the beginning and after 14 d of experiment. Twice a week, the test vessels were opened to allow soil aeration and once a week the water loss by evaporation was compensated (water loss determined by the weight loss of the test vessels). After 28 d, the content of each test vessel was transferred to a larger vessel, filled up with water and gently stirred, leading organisms (adults and juveniles) to float into the surface. Afterwards, some drops of a dark ink were added to the water surface to increase contrast and facilitate counting of living organisms. The number of surviving adults was recorded. The water surface was photographed and the number of juveniles was counted using UTHSCSA Image Tool for Windows, version 3.0. As for enchytraeids, an additional replicate without springtails was prepared per each test soil for measuring of soil moisture and pH at the end of the test period.

4.2.6 Data analysis

For each test, differences in the number of juveniles produced between contaminated soils and the respective reference soil was evaluated by one-way analysis of variance (ANOVA) followed by Dunnet's post-hoc test, using organic matter as covariable in the analysis. Data normality and homoscedasticity were previously evaluated by Kolmogorov-Smirnov and Bartlett's tests, respectively.

To investigate the relationship between total metal concentrations and reproduction of the test species, partial correlation analyses (using also organic matter as co-variable) were conducted using the Pearson's correlation coefficient. Moreover, partial correlations were also performed between the reproduction of the tests species and the index of metal pollution (W) proposed by Widianarko et al. (2000), which indicates how much the background concentration is exceeded by the metal concentrations of the test soil. For a tested soil, this index is the logarithm of the multiplication of a factor for each metal, which is calculated as the ratio between the metal concentration in the tested soil by the mean concentration of the metal at the reference sites. The W index was calculated for each sampling point, aiming to obtain a unique value representing all metals analyzed. Initially developed for sediment samples, the W index can be used to synthesize the metal loading of any environmental sample in comparison to metal basal levels, including soils (Timmermans et al. 2007; Janssens et al. 2008). This index is negative when metal loadings are below the basal levels, whereas the positive values indicate metal loadings higher than the basal levels. All analyses were performed using Statistica 6.0 (StatSoft 2001).

4.3 Results

4.3.1 Soils characterization

A full characterization of the collected soils is given in Chapter 3 (Niemeyer et al. 2010). Soils from the study area showed an organic carbon content ranging from 0.12 to 3.31%, a CEC between 30 and 40 meq/100 g, and pH values between 6.1 and 7.2, except the soils P1000T1 and Ref.2 that presented pH values of 3.7 and 4.9, respectively (Table 4.1).

4.3.2 Chemical analysis

Table 4.2 shows the total metal concentrations measured in the tested soils and the corresponding index W of metal pollution.

Sandy soils showed metal concentrations exceeding the Dutch $HC50_{cor}$ screening levels, at list for one metal, which indicate high ecological risk (Rutgers et al. 2008). P0 presented a high Zn contamination exceeding by almost three times the corresponding $HC50_{cor}$ value, whereas P150T1 and P50T3 presented a critical level of contamination, exceeding by far (between 1.6 and 73.5 times) the screening levels; please see Chapter 3 for detail explanation on these screening levels. The W index values estimated were slightly positive (or even negative) in the reference soils or in soils collected outside the smelter area (e.g. P1000T1), contrasting to highly positive W values in most of the soil collected the smelter area. According to the W index, P150T1 and P50T3 soils were the most contaminated ones.

Table 4.1 Physico-chemical characteristics of sampled soils and respective reference soils. USDA – United States Department of Agriculture; CEC – Cation Exchange Capacity; WHC – Water Holding Capacity.

Soil group	Coarse sand (%)	Fine sand (%)	Sand (total) (%)	Silt (%)	Clay (%)	Texture (USDA)	pH (KCl 1:5 v:v)	Organic matter (%)	CEC (meq 100g)	WHC (%)
Group 1										
Ref 1	2.3	8.5	10.9	42.1	47.0	Silty Clay	7.1	1.1	34.16	53.78
P1000T1	2.5	21.8	24.3	19.9	55.8	Clay	3.7	2.0	43.20	59.95
P20T3	11.4	30.0	41.4	22.3	36.3	Clay Loam	6.8	1.9	42.16	67.73
P400T3	6.5	8.6	15.1	52.4	32.5	Silt Clay Loam	7.1	1.9	35.84	56.67
Group 2										
Ref 2	50.9	38.5	89.4	2.8	7.7	Loamy Sand	4.9	1.0	37.60	27.53
P0	43.2	31.3	74.5	11.9	13.6	Sandy Loam	6.7	0.3	38.56	44.12
P20T1	48.0	13.8	61.8	19.0	19.3	Sandy Loam	7.1	0.2	37.28	46.40
P150T1	56.2	21.1	77.4	12.3	10.3	Sandy Loam	6.7	2.1	21.28	28.55
P50T3	69.2	9.1	78.3	10.4	11.3	Sandy Loam	7.2	2.8	16.56	22.05
Group 3										
Ref 3	22.2	15.0	37.2	11.1	51.7	Clay	6.1	3.9	36.48	60.75
P50T1	25.2	13.4	38.6	29.0	32.4	Clay Loam	6.7	1.1	38.16	54.51
P400T1	19.6	23.9	43.5	20.2	36.3	Clay Loam	6.8	5.1	37.44	58.93
P150T3	8.4	15.2	23.5	21.4	55.1	Clay	6.8	2.5	49.20	61.76
P1000T3	10.3	19.5	29.8	29.8	40.4	Clay Loam	7.0	5.7	42.72	57.57

Table 4.2 Total metal concentrations (mg/Kg) and the metal pollution index in tested and respective reference soils. This table was adapted from Niemeyer at al. 2010.

Sites	Total (mg/kg)								Metal pollution index
	Pb	Cd	Cu	Zn	Cr	Ni	Fe	Mn	W
Group 1									
Ref. 1	16	<0.2	66	94	77	54	45000	840	1.26
P1000T1	23	<0.2	60	80	62	46	48000	360	0.80
P20T3	308	<0.2	56	420	78	60	49000	672	3.12
P400T3	179	0.3	44	90	59	46	34000	760	2.76
Group 2									
Ref. 2	13	<0.2	18	24	16	28	2900	34	-0.89
P0	1264	<0.2	76	3800 (2.8)	72	57	52000	674	5.61
P20T1	133	<0.2	56	220	80	56	41000	780	2.44
P150T1	37460 (10.4)	771 (9.8)	594 (1.6)	42200 (33.5)	57	70	110000	1720	13.33
P50T3	26074 (7.1)	62	3196 (8.2)	95940 (73.5)	80	40	117000	5880	13.63
Group 3									
Ref. 3	152	<0.2	40	260	59	40	53000	820	-0.37
P50T1	164	<0.2	60	240	80	58	43000	720	2.60
P400T1	961	8.8	60	840	64	48	35000	540	5.98
P150T3	2200	12	108	3300	84	58	56000	678	7.83
P1000T3	99	<0.2	56	156	84	52	49000	568	2.09

Numbers in superscript indicate an exceedance of the corrected Dutch $HC50_{EC50}$ values (after Rutgers et al. 2008) (Ex: the [Pb] at P150T1: 37460 (10.4), indicates that [Pb] was 10.4 times higher than the $HC50_{cor}$Pb). See Chapter 3 (Niemeyer et al. 2010) for details on this correction.

4.3.3 Reproduction tests with *E. andrei* and *E. crypticus*

Reproduction tests with *E. andrei* fulfilled the validity criteria of ≥ 80% of survival, > 30 juveniles per test vessel and coefficient of variation < 30% between replicates in OECD artificial soil and reference soils (ISO 1998a). Considering all tested soils, survival ranged between 90 and 100%, except in P1000T1 soil where the survival was 85 ± 17.3%. The reproduction found in the reference soils was always higher or equal to that found in the OECD artificial soil and the Ref 2 soil showed the highest reproduction mean ± standard deviation [SD] of 132.3 ± 24.7 (n = 4). The reproduction of earthworms significantly decreased (compared to the respective reference soils) in treatments composed by soils with high (P50T3, P150T1 and P150T3) and low (P1000T1, P1000T3 and P20T1) indices of metal contamination. The P1000T1 soil, that was the test soil with the lowest index of metal contamination, was the soil that showed the lowest number of juveniles (mean ± SD of 5 ± 7; n = 4) (Fig. 4.2).

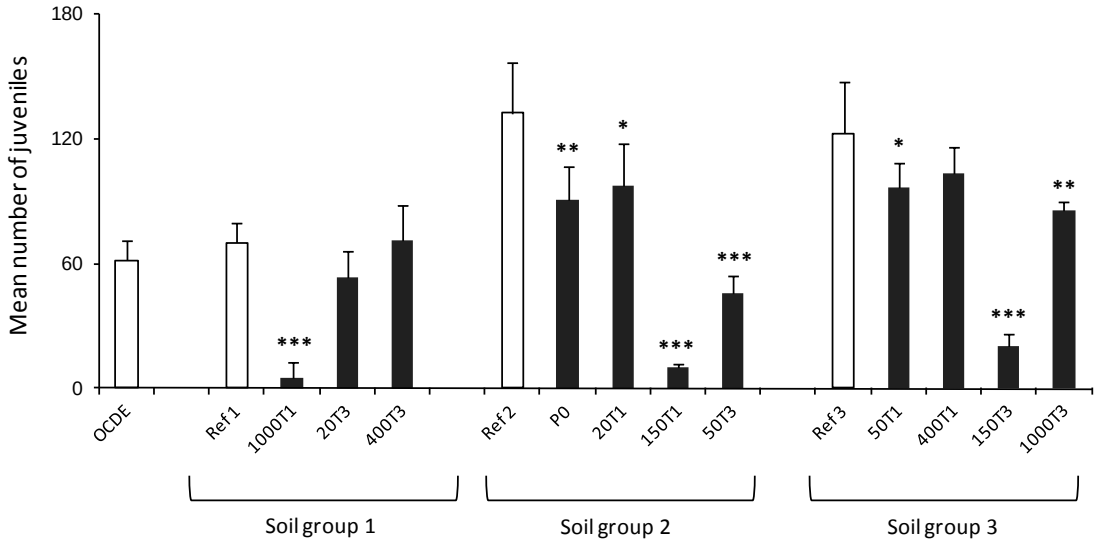

Fig. 4.2 Number of *Eisenia andrei* juveniles (average + standard deviation, *n* = 4) in OECD artificial soil, reference soils (white bars) and test soils (black bars) within each soil group. Asterisks indicate significant differences (*p≤0.05; **p≤0.01; ***p≤0.001) compared to the respective reference soil.

Reproduction test with *E. crypticus* fulfilled the validity criteria of > 25 juveniles per test box and coefficient of variation < 50% between replicates in OECD artificial soil and in natural reference soils (ISO 2004). The number of juveniles found in OECD soil was generally in the same order of magnitude to that found in the reference soils and, similarly to the

reproduction of *E. andrei*, Ref 2 was the reference soil that showed the highest reproduction on average (1089 ± 86; *n* = 4). As for the earthworms, the reproduction of *E. crypticus* was significantly lower (compared to the respective reference soil) for soils with high (P50T3, P150T1 and P0) and low (P1000T1 and P20T1) index of metal contamination. However, for potworms, the soil that showed the lowest reproduction was the test soils with the second highest index of metal contamination (P150T1), where 7 ± 0.5 organisms were observed on average (*n* = 4; Fig 4.3).

Highly significant negative correlations were found between the reproduction of both oligochaete species and metal contamination, represented by the Widianarko index (*E. andrei,* r= - 0.72, p<0.001; *E. crypticus*, r= - 0.61, p<0.001) (Table 4.3).

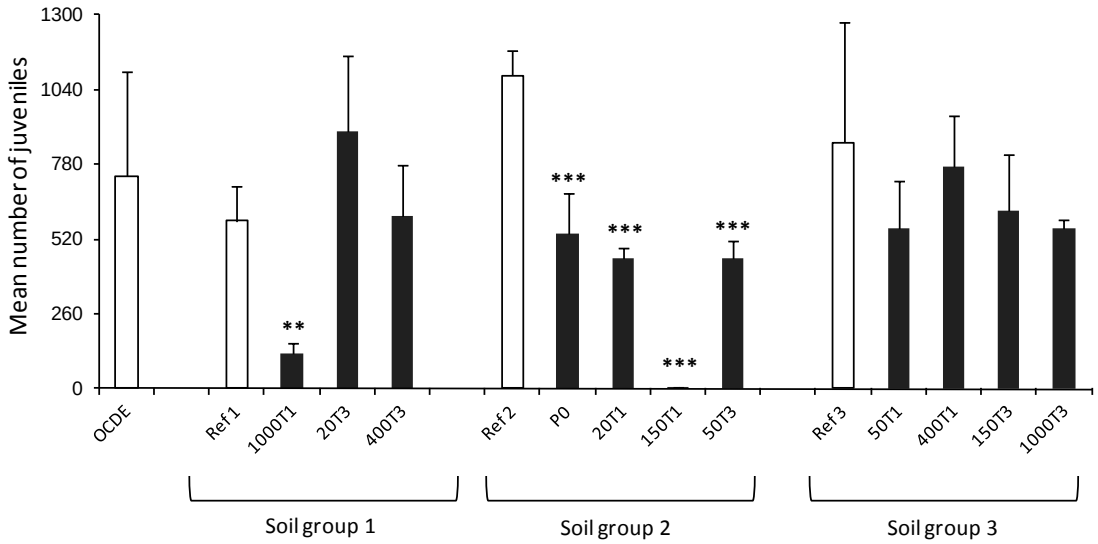

Fig. 4.3 Number of *Enchytraeus crypticus* juveniles (average + standard deviation, *n* = 4) in OECD artificial soil and reference soils (white bars) and test soils (black bars) for each soil group. Asterisks indicate significant differences (*p≤0.05; **p≤0.01; ***p≤0.001) compared to the respective reference soil.

Table 4.3 Pearson's partial correlations between invertebrates reproduction and total metal concentrations, and overall metal loading (Widianarko's index - W).

Invertebrate reproduction	Total metal concentrations								Metal pollution index
	Pb	Cd	Cu	Zn	Cr	Ni	Fe	Mn	W
Eisenia andrei	-0.60***	-0.54***	-0.34*	-0.45**	-0.41**	-0.60***	-0.68***	-0.39**	-0.72***
Enchytraeus crypticus	-0.62***	-0.62***	n.s.	-0.41**	-0.37**	-0.58***	-0.66***	-0.34*	-0.61***
Folsomia candida	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

n.s. - not significant ($p>0.05$)

*$p<0.05$; **$p<0.01$; ***$p<0.001$

4.3.4 Reproduction of *F. candida*

Reproduction test with collembolan fulfilled the validity criteria of ≥ 80% of survival, >100 juveniles per test box and coefficient of variation <30% between replicates (ISO 1999) in OECD and in reference soils. The collembolan reproduction found in OECD soil was in the same order of magnitude of that found in Ref 1, but lower than that found in Ref 3. The Ref 2 soil showed lower reproduction when compared to that in OECD and test soils from the same group (Fig. 4.4). Significant decreases on reproduction were observed (compared to respective reference soil) in soils with W indices of metal contamination between 2.09 and 7.83 (Fig. 4.4). The contaminated soils that showed the highest toxicity were P50T1 and P150T3, where 351 ± 141 and 344 ± 43 (*n* = 5) were found, respectively. No significant correlations were found between reproduction of *F. candida* and metal contamination (Table 4.3).

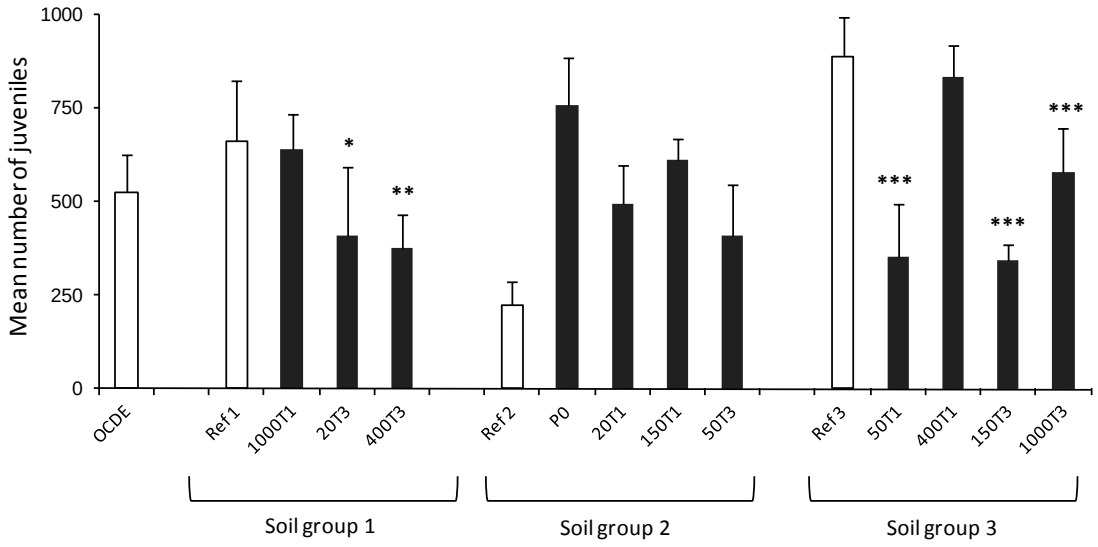

Fig.4.4 Number of *F. candida* juveniles (average + standard deviation) in OECD artificial soil and reference soils (white bars) and test soils (black bars) for each soil group. Asterisks indicate significant differences (*p≤0.05; **p≤0.01; ***p≤0.001) compared to the respective reference soil.

4.4 Discussion

The high metal contamination found in the tested soils agrees with the data previously reported by Anjos (2003) and Machado et al. (2004). According to these authors, such contamination results from the deposition of residues inside the smelting area as well as from the aerial deposition of contaminated particles from the smelter plume while in function. Soil heterogeneity found in the test soils is most probably related to the spatial distribution of different actions recently undertaken in the study area for local rehabilitation, namely the failed attempt to encapsulate the tailings by covering with nearby soil (Anjos 2003).

Soils P0, P50T3 and P150T1 from Group 2, the most metal contaminated soils, affected the reproduction of both oligochaete species. In these soils, at least one metal (Pb, Cd, Cu or Zn) exceeded the reported EC50 values that cause negative effects on the reproduction of oligochaetes (Table 4.4). However, no significant effects on the reproduction of colembolans were found in these soils, despite the fact that they exceeded in much the reported EC50 values that cause negative effects on the reproduction of *F. candida* (Table 4.4).

Effects of Pb contamination on collembolan reproduction could be expected at P50T3 and P150T1, since the Pb concentrations (26074 and 37460 mg Pb/kg soil, respectively) were one order of magnitude higher than the EC50 of 2970 mg Pb/kg found by Sandifer and Hopkin (1996) in OECD soil with pH 6.0, and were similar to an EC50 of 2560 mg Pb/kg found by Greenslade and Vaughan (2003). Moreover, toxic effects of Cu could be expected at P50T3 (with 3196 mg Cu/kg soil) that exceeded in one order of magnitude the EC50 values found by Sandifer and Hopkin (1996) and Greenslade and Vaughan (2003), 700 and 751 mg Cu/kg soil, respectively. According to data from Sandifer and Hopkin (1996) and Greenslade and Vaughan (2003), EC50 values of cadmium for the reproduction of *F. candida*, derived from standard tests in OECD soil, were 590 and 351 mg Cd/kg soil, respectively. Based on these data, in P150T1 soil some effects of cadmium could be expected. Menta et al. (2006) found an EC50 of 47.4 mg Cd/kg soil; however, the test duration was 2 weeks longer, not being comparable with our data.

Lock and Janssen (2003), using *F. candida* to evaluate the toxicity of zinc chloride, zinc oxide and zinc powder in OECD artificial soil, found EC50 values of 391, 461, and 393 mg Zn/kg soil, respectively. Considering these data, effects of Zn on collembolan reproduction could be

expected in P0, P50T3 and P150T1, but these soils did not affect the reproduction of *F. candida*. On the other hand, zinc toxicity could be an explanation to the toxic effects observed on P20T3 and P150T3 soils.

Ageing processes and soil-related factors, such as organic matter or clay content play an important role in the toxicity of metals to soil invertebrates. According to Smit and van Gestel (1998) zinc toxicity to *F. candida* was related to organic matter and clay content of soil in freshly contaminated soils. However, the authors highlighted that the use of spiked soils overestimated the effects of zinc by a factor of 5 to 8 compared to a test soil that was subjected to ageing under field conditions for 1.5 years. Thus, care should be taken in extrapolating the results of laboratory toxicity tests on metals in OECD soil to field soils, in which the biological availability of contaminants is likely to be lower (Fountain and Hopkin 2004). These reasons could explain the absence of effects on reproduction when it should be expected due to metal contents.

Despite the fact that total Zn concentration in P400T1 exceeded the EC50 values reported in the literature about effects on the reproduction of *E. andrei* and E. crypticus (see Table 4.4), no effects were observed on the reproduction of oligochaete species when compared to the respective reference soil. Concerning soil properties, it has been shown that the effective concentrations for soil oligochaetes exposed to zinc, cadmium, copper and lead varied over more than two orders of magnitude depending on the soil characteristics (Lock et al. 2000; Lock and Janssen 2001). According to Lock and Janssen (2001), a 14 d LC50 for *Enchytraeus albidus* exposed to contaminated soils with zinc and cadmium may vary from 83.0 to 1140 mg Zn/kg and from 55.2 to 704 mg Cd/kg, respectively, depending on the type of clay and on the organic matter content used in the artificial soil. These authors also showed that pH and cation exchange capacity (CEC) were the most important parameters affecting zinc and cadmium toxicity; high pH and CEC capacity decreased metals bioavailability.

Table 4.4 Some literature data on toxic effect on the reproduction of *Eisenia fetida* (cocoon production), *Enchytraeus* sp. and *Folsomia candida* (number of juveniles) for metals Pb, Cd, Cu, Zn, Cr, Ni and Mn.

Species	Metal	Exposure time (d)	Soil	EC50 - mg/kg (95% CI)	Reference
Eisenia fetida	Pb	56	OECD 10% O.M.	1940 (-)	Spurgeon et al (1994)
	Cd	56	OECD 10% O.M.	46.3 (25.4-91.4)	Spurgeon et al (1994)
	Cu	56	OECD 10% O.M.	53.3 (32.5-186)	Spurgeon et al (1994)
	Zn	56	OECD 5% O.M.	136	Spurgeon and Hopkin (1996)
		56	OECD 10% O.M.	276 (202-375)	Spurgeon et al (1994)
		21	OECD 10% O.M.	705 (551–1050)	Lock and Janssen (2003)
	Cr	21	OECD 10% O.M.	892 (679 -1110)	Lock and Janssen (2002a)
	Ni	21	OECD 10% O.M.	362 (241-508)	Lock and Janssen (2002b)
	Mn	56	Sandy loam soil	927	Kuperman et al (2004)
Enchytraeus albidus	Pb	42	OECD 10% O.M.	320 (272-371)	Lock and Janssen (2002c)
Enchytraeus crypticus	Cd	21	Lufa 2.2	35 (31 -38)	Castro-Ferreira et al (2012)
	Cu	28	OECD 10% O.M.	477 (345-658)	Posthuma et al (1997)
	Zn	28	OECD 10% O.M.	336 (266-425)	Posthuma et al (1997)
Enchytraeus albidus	Cr	42	OECD 10% O.M.	637 (355-791)	Lock and Janssen (2002a)
Enchytraeus albidus	Ni	42	OECD 10% O.M.	275 (217-346)	Lock and Janssen (2002b)
Enchytraeus crypticus	Mn	28	Sandy loam soil	192 (147-238)	Kuperman et al (2004)
Folsomia candida	Pb	28	OECD 10% O.M.	2970 (-)	Sandifer and Hopkin (1996)
		28	OECD 10% O.M.	2560 (-)	Greenslade and Vaughan (2003)
	Cd	28	OECD 10% O.M.	590 (-)	Sandifer and Hopkin (1996)
		28	OECD 10% O.M.	351 (290-410)	Greenslade and Vaughan (2003)
		45	OECD 10% O.M.	47.4 (-)	Menta et al (2006)
	Cu	28	OECD 10% O.M.	700 (-)	Sandifer and Hopkin (1996)
		28	OECD 10% O.M.	751 (624-905)	Greenslade and Vaughan (2003)
	Zn	28	OECD 10% O.M.	900 (-)	Sandifer and Hopkin (1996)
		28	OECD 10% O.M.	865 (811-924)	Greenslade and Vaughan (2003)
		28	OECD 10% O.M.	391 (266–660)	Lock and Janssen (2003)
	Cr	28	OECD 10% O.M.	604 (254-3380)	Lock and Janssen (2002a)
	Ni	28	OECD 10% O.M.	476 (347-671)	Lock and Janssen (2002b)
	Mn	28	Sandy loam soil	1663 (1491-1834)	Kuperman et al (2004)

The effects to *E. andrei* in P150T3 can be related both to Zn and Pb concentrations (3300 mg/kg and 2200 mg/kg, respectively), and to high clay content (55.1%). Spurgeon and Hopkin (1996) found an EC50 value of 136 mg Zn/kg soil to cocoon production of *E. fetida* in artificial soil with 5% OM content and pH of 6.0. The same authors in other work (Spurgeon and Hopkin 1997), studying the effect of different temperatures (15, 20 and 25°C) on the acute and chronic toxicity of Zn to *E. fetida*, recorded the lowest EC50 on cocoon production of 234 mg Zn/kg at 25°C, suggesting that there is an increase of the toxicity of zinc at higher temperatures. Concerning Pb, the concentration in P150T3 is higher than the EC50 of 1940 mg Pb/kg soil on cocoon production of *E. fetida*, reported by Spurgeon et al (1994). Furthermore, Bradham et al. (2006), exposing the earthworm *E. andrei* for 28 d to different soil types spiked with 2000 mg Pb/kg soil, observed even mortality in some soils.

Besides the influence that soil properties may have on the bioavailability of metals, they can also influence the response of soil organisms (Amorim et al. 2005), which can explain the significantly reduced reproduction of oligochaete species in soils where the metal concentrations were low. The reproduction of both oligochaete species was significantly reduced in soils P20T1 and P1000T1, while the reproduction of *E. andrei* and *F. candida* was reduced in P50T1. This decrease could be related to the low organic matter content in P20T1 (0.2%), low organic content in P50T1 (1.1%) in combination with high clay content (32.4%); and to low organic matter (2.0 %), high clay content (55.8%) and low pH (3.7) in P1000T1 soil. Such soil characteristics probably constituted unsuitable conditions to the reproduction of oligochaetes, according to Chelinho et al. (2011) that reported limitations on the use of *E. andrei* in soils with a pH lower than 4.2 or low organic matter content (e.g., <2%). The same limitations were reported by the authors for *E. crypticus*, although these species presented a wider range of tolerance concerning soil pH (between 4.2 and 7.7) and organic matter content (between 0.6 and 4.8%). Low reproduction of *E. andrei* in soils with a combination of high clay content (33%) and low organic carbon content (0.6%) was also observed by van Gestel et al. (2011). The low organic matter content on P50T1 (1.1 %) could be an explanation for the lower reproduction of *F. candida* on this soil than in its respective reference soil (Ref 3; 3.9 %), since some authors (e.g., Crommentuijn et al. 1997) pointed that low organic matter content can act as stressor to *F. candida.*

On soil P1000T3, localized outside the smelter area, the impairment on reproduction of *E. andrei* and *F. candida* cannot be explained by metal concentrations neither by soil properties. Probably another source of contamination (agrochemicals, natural chemicals), not analyzed in the present study, is the responsible by the effect. Previous results showed that this soil was significantly avoided by *E. andrei* (Chapter 3), which can reinforce the indicative of an unknown source of contamination.

No plausible explanation was found for the effects on the reproduction of *F. candida* in P400T3 soil. Crouau and Pinelli (2008), comparing ecotoxicity of three polluted industrial soils for the *F. candida,* reported how complex and difficult is to interpret results of ecotoxicological tests with soils from polluted sites. Vasseur et al. (2008) investigating the toxicity of metal and PAHs contaminated soils on the reproduction of soil invertebrates, found that toxicity cannot simply be extrapolated from pollutant concentrations in a complex matrix in which bioavailability of pollutants may be reduced by ageing. On the other hand, interactions between the effects of a natural stressor and a toxicant can sometimes result in greater effects than expected from either of the stress types alone, as can be seen in the review of Holmstrup et al. (2010). Stimulatory effect of low levels of contaminants can also be present, as pointed by Smit and van Gestel (1998) to the reproduction of *F. candida* at 160 and 256 mg Zn/kg soil in OECD soil, and at 160, 256 and 410 mg Zn/kg soil in a natural soil with an organic matter content of 2.4% and clay content of 1.9%.

Maybe the lack of statistically significant effects on the reproduction on some soils from group 2 was related to the low reproduction of *F. candida* on the respective reference soil (Ref 2 - 224 ± 62 juveniles), used for comparisons. The high percentage of fine sand and the low WHC in Ref 2 when compared to the other soils might be the cause of the lower performance of *F. candida* in this soil. Our results are in accordance to Domene et al. (2011) that reported a significantly lower reproduction of this species in the more fine textured soils (with higher silt and fine sand content and with higher CEC values), and positive and significant effects of moisture on reproduction.

Despite significant negative effects ($p<0.05$) on the reproduction were observed in some soils, no significant correlations were found between reproduction of *F. candida* and total metal concentrations. In general, *F. candida* appeared to be more tolerant to metal contamination than *E. andrei* and *E. crypticus*. Similar results were also found by Schultz et al. (2004), where *Enchytraeus* sp. was more sensitive than *F. candida* in metal-contaminated soils. van Gestel et al. (2001) observed that collembolan appeared to be less sensitive than

earthworms and plants to assess soils with oil and metal pollution. van Gestel et al. (2011), considering reproduction of soil invertebrates in soils contaminated with molybdenum, observed that earthworms were most sensitive to Mo, followed by the enchytraeids and the Collembola, this last showing absence of toxicity in most soils.

Differences in sensitivity of collembolans and oligochaetes on metal contaminated soils could be explained in part by differences in exposure (Achazi 2002), since solid soil phases are more important for uptake process of springtails, while soft-bodies oligochaete species are more influenced by porewater characteristics (Vijver et al. 2001). Furthermore, it is suggested that Collembola can avoid contaminated food, and are able to excrete assimilated metals during moulting (Fountain and Hopkin 2001), which can be related to their more resistant to metal contaminantion.

Our results suggest that toxicity data obtained in spiked OECD soil should not be used directly in risk assessment for metals in natural contaminated soils. Bioavailability of metals to soil invertebrates may depends on some factors, such as soil pH, OM content, CEC and clay content (van Gestel 1992). According to Peijnenburg et al. (1997), clay minerals are important natural ion-exchange materials, while pH is the most important factor controlling metal partitioning for most metals in soil. Due to the heterogeneity of soil materials, the sorption potential varied considerably and this may lead to changes in contaminant availability, sometimes within a small area. Along a temporal gradient, the process of ageing occurs, and metal sequestration by soil increases, as pointed out by VROM (2000), thus reducing bioavailability. So, the challenge should be to have EC50 values used for comparison derived based on natural soils or corrected for natural soil properties.

Currently, the study area in Santo Amaro is probably a case of strong metal adsorption process, probably due to the type of clay content and more alkaline pH values of *massapê* soil, and also due to ageing processes, since the factory stopped its activities in 1993. The soils at the sampling sites were classified as Vertisols and Inceptisols (Soil Taxonomy, USDA) originated from carbonaceous shale, rich in expansive clay (montmorillonite), with generally low porosity and consequently low permeability (Machado et al. 2002). Furthermore, the form in which the contaminant is present in the soil has environmental relevance and should be considered in interpreting results from ecotoxicity tests (Davies et al. 2003). Andrade Lima and Bernardez (2011), studying the leaching of the slag in the Plumbum smelter area in Santo Amaro, found that the Pb, Zn, Cd and other potentially toxic elements were relatively stable in a weak acidic environment for short contact times, which can be

explained by the low leachability of the metallic Pb and the Zn-bearing species. Furthermore, results on metal extracts from this study area are presented in Chapter 7 (Niemeyer et al. 2012) showing the low availability of metals in the 0.01 M $CaCl_2$ extracts, which could be an indicative of low bioavailability of metals to plants and invertebrates. However, the bioavailability of metals to soil invertebrates are not predicted just by water soluble concentrations (Crommentuijn et al. 1997; Peijnenburg et al. 1999; Vijver et al. 2001), since the uptake of metals could occur through a different exposure route (Fountain and Hopkin 2001) and because soil properties can act as stressors themselves (Crommentuijn et al. 1997).

4.5. Conclusions

The outcome of the reproduction tests indicated some loss of habitat function for the tested species in the majority of the sites analyzed, which can indicate risk to soil functioning once these invertebrates play an important role in maintaining the structure and fertility of soil and are an important part of soil food web. Reproduction of oligochaete species was impaired mainly in sites corresponding to the deposition of tailings inside the area, and significant negative correlations were found between reproduction and total metal concentrations, suggesting that the toxic effects were caused directly by contamination.

No significant correlations were found between reproduction of *F. candida* and metal contamination. The lack of significant effects on the most contaminated soils can be related not only to ageing process, but also to the low reproduction in a reference soil used in comparisons, probably because a high content of fine sand and low WHC.

In general, Oligochaeta was the most sensitive group in reproduction tests. Our results reinforce the importance of using a battery of tests in environmental risk assessment, since tested species can be affected in a different way. Furthermore, data of chemical determinations and soil properties should be used together in the interpretation of reproduction results in natural contaminated soils.

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Chapter 5

Microbiological soil quality indicators as tools in ecological risk assessment of a metal contaminated area in the tropics

Based on the following manuscript:

Niemeyer JC, Lolata GB, Carvalho GM, Da Silva EM, Sousa JP, Nogueira MA, 2012. Microbial indicators of soil health as tools for ecological risk assessment of a metal contaminated site in Brazil. *Appl Soil Ecol* 59: 96 – 105.

Abstract

Microbial and biochemical indicators of soil health were used to assess the ecological conditions and biological activity of soils contaminated with metals at a lead smelter plant and surrounding area in northeast Brazil. Soil respiration, microbial biomass of C and N, acid phosphatase, asparaginase, and density of ammonifying and ammonium-oxidizing microorganisms were positively correlated with soil organic carbon and/or water content, but showed negative correlations with metal contents in soil. Nitrification rate and metabolic quotient (qCO_2) were positively correlated with metal contamination, suggesting favorable conditions for N loss and microbial stress, respectively. No significant correlations were found between metal concentrations in soil and dehydrogenase activity or ammonification rate, considering water content and soil organic carbon as covariables. Soil respiration, microbial biomasses of C and N, dehydrogenase, acid phosphatase, asparaginase activities, and ammonifying microorganisms were positively correlated with percentage vegetation cover, while nitrification and ammonification rates were negatively correlated with this parameter. In general, soil respiration, microbial biomass of C and N, acid phophatase, asparaginase, density of ammonifying and ammonium oxidizing microorganisms, nitrification rate and qCO_2 indicated high ecological risk for soil functions mediated by microorganisms (concerning to C and nutrient cycling) due to deposition of tailing contaminated with metals, even 17 years after the smelter activities had stopped. Besides direct effect of metal toxicity on microbial biomass and activity, there are indirect effects related to changes in vegetation cover, soil organic carbon, pH, and nutrient availability, and consequently changes in the soil microclimate and physical-chemical properties that may lead to losses of habitat function for soil microorganisms and the key processes they play. However, a multivariate decomposition of variance indicated that vegetation cover explained only 3.1%, whereas metals explained 26.9% of the variation associated to the microbial/biochemical indicators, showing a stronger effect of metals.

Keywords: Microbial activity, Microbial biomass, Metals, Soil enzymes, Soil quality

5.1 Introduction

Contaminants may affect a variety of microbial processes in soil, thereby affecting the nutrient cycling and the capacity to perform key ecological functions, such as mineralization of organic compounds and synthesis of organic matter (Giller et al. 1998, 2009; Moreno et al. 2009). Microbial biomass, soil basal respiration, enzyme activities, and nutrient transformations are important attributes related to soil fertility (Edwards 2002) and can be used as indicators of soil health to monitor soil contamination (Castaldi et al. 2004; Smejkalova et al. 2003), agricultural use (Araujo et al. 2003; Tu et al. 2006), suitable management or success of restoration practices (Balota et al. 2004; Nogueira et al. 2006; Clemente et al. 2007). These biological indicators have the advantage of being easy and relatively fast to measure, thus being cost-effective tools for monitoring (Alkorta et al. 2003). Moreover, they provide an integrative biological assessment of soil health (Alkorta et al. 2003; Epelde et al. 2006).

Microbiologically-mediated processes, catalyzed by enzymes, are essential to soil functioning, providing the basis of carbon, nitrogen, phosphorus, and sulfur cycling in soil (Alkorta et al. 2003). Microbial biomass is an important constituent of the soil biological fertility, involved in the biogeochemical cycle of nutrients and carbon. In addition, it is an important reservoir of nutrients in ecosystems. Soil microorganisms immobilize carbon and nitrogen by forming new biomass using the energy they obtain from oxidation of carbon sources through respiration, or inorganic chemical reactions (Chen et al. 2003). Therefore, more microbial biomass can stock and cycle more nutrients (Gregorich et al. 1994), improving the sustainability of an ecosystem (Kaschuk et al. 2010). Soil enzymes can be used as indicative of biological activity on a given biochemical processes in soil, being sensitive to alteration of soil health as a consequence of use and management (Balota et al. 2004; Bastida et al. 2006; Nayak et al. 2007). They also have been used as responsive indicators of contamination with metals on soil biochemical properties involved in carbon and nutrient cycling (Kuperman and Carreiro 1997; Dias-Júnior 1998; Gülsen and Erdogan 2008).

Effects derived from a long-term exposure of microbial communities to metals cannot be predicted by recent addition of metal salts into the soil because microbial communities respond differently to chronic or acute exposures (Giller et al. 1998; Renella et al. 2002). In addition, metal bioavailability may change according to ageing after contamination as a

consequence of physical-chemical interactions with the soil matrix (McGrath 2002; Vig et al. 2003). Moreover, the microbial community may adapt to the novel condition (Sobolev and Begonia 2008) overcoming the negative effect of contamination (Lejon et al. 2010). Thus, long-term metal contaminated sites represent good conditions for studying chronic exposition of microbial communities. In general, there have been observed negative effects of metal contamination on soil microbial community size and diversity (Kelly et al. 2003), enzymatic activities (Begonia et al. 2004; Zeng et al. 2007), microbial biomass (Yuangen et al. 2004), N mineralization, and microbial respiration (Rost et al. 2001).

Microorganisms in soil under stress may be metabolically less effective because they need to invest more energy for cell maintenance, resulting in increased C-CO_2 release per unit of microbial biomass (Epelde et al. 2006), a ratio also known as microbial metabolic quotient, qCO_2 (Anderson and Domsch 1993). This coefficient has been proposed as indicator of microbial stress caused by metal contamination in soil (Zhang et al. 2008), where the higher values, the higher stress.

This study is integrated in a broader project dealing with assessing the ecological risk of a metal multicontaminated area at Santo Amaro, Bahia, Brazil. Time- and cost-effective microbial and biochemical indicators of soil health were assessed aiming at investigating the extent to which a long-term contamination changed the ecological status of a former lead smelting area. We hypothesized that each microbial or biochemical indicators respond to the metal contamination gradient, being impaired in highly contaminated sites as compared to sites at greater distances from the source of contamination.

5.2 Materials and methods

5.2.1 Study area

This study was carried out within and around an abandoned lead smelter that operated between 1960 and 1993, near to the urban area of Santo Amaro, BA, Brazil (12° 32 ′49 ″S, 38° 42 ′43 ″W). The site presents a high health risk for humans (Costa 2001; Carvalho et al. 2003) due to high levels of metals in soil and water. A total of 500.000 t of tailings were deposited inside and in the surroundings of the smelter, and buried under roads and house's backyards (aprox. 55.000 m^3) (Machado et al. 2004). In addition, airborne

contaminated dust from atmospheric deposition through chimney emissions reached up to 3 km away from the industrial area during the period the smelter operated (Anjos 2003; Machado et al. 2004). In 1995, the Bahia State environmental agency recommended the encapsulation of tailings with soil rich in organic matter to mitigate contamination. However, the process failed and currently, in some areas, tailings are still exposed and the aerial dispersion by dust is still occurring within and outside the smelter area (Anjos 2003; Machado et al. 2004), leading to risks of soil and water contamination.

The soils at the sampling sites were classified as Vertisols and Inceptisols (Soil Taxonomy, USDA) originated from carbonaceous shale, rich in expansive clay (montmorillonite), with generally low porosity and consequently low permeability (Machado et al. 2002).

5.2.2 Soil sampling and estimation of vegetation cover

Two 1-km transects (T1 and T3) were defined along the two major detected gradients of contamination (Fig. 5.1). The two transects shared a central point (P0 – located close to the smelter plant) and comprised 5 sampling points each (at 20, 50, 150, 400, and 1000 m from P0).

Soil sampling was done in April 2009 at 0-10 cm topsoil layer. At each point, three parallel transects of 10 m long were defined 2 m apart. Along each parallel transect 10 subsamples were collected and pooled to form a composite sample. After mixing, the samples were sieved (< 5 mm), stored at 4 °C, and processed within 72 h.

Sites in the surrounding area were screened, analyzed for metals, soil properties and vegetation. Three reference sites were selected at 3 km (Ref. 2 and 3) and 9 km (Ref. 1) away from the central sampling site (P0) (Fig.5.1) aiming not only to represent the diversity of habitat composition of the area surrounding the smelter, but also to match the properties of soils and vegetation from sites inside the smelter area.

Assessment of vegetation cover was carried out according to Veiga and Wildner (1993). Briefly, a plastic grid with 50 cm x 50 cm size, subdivided in 100 small squares of 5 x 5 cm, was randomly released four times on each sampling site. The sum of the intersections of small squares over vegetation in each grid represents the percentage of vegetation cover.

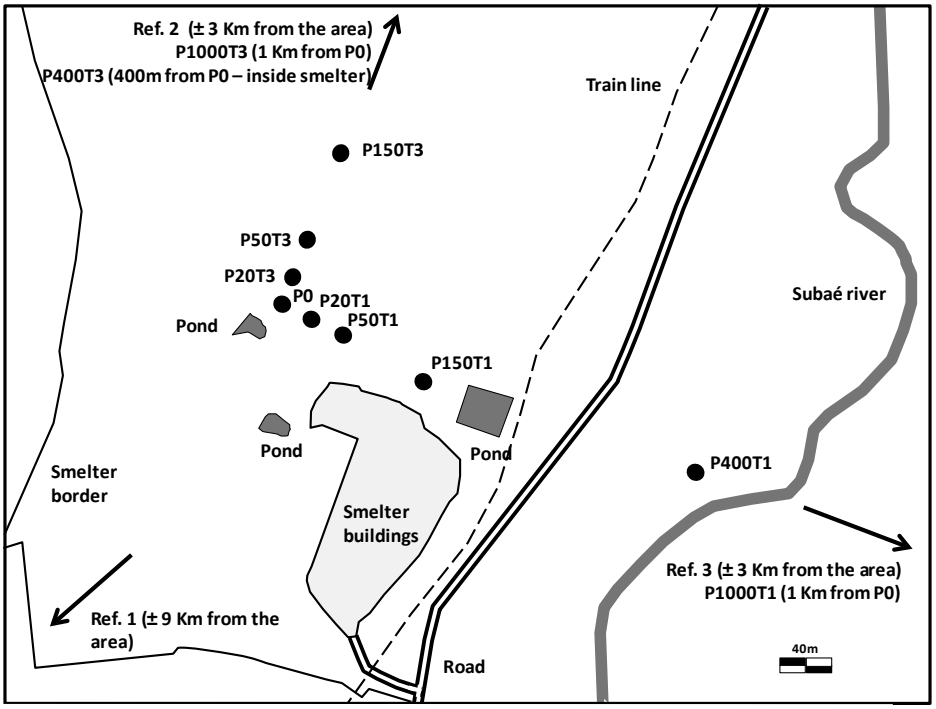

Fig. 5.1 Schematic representation of the study area (an abandoned lead smelter, Santo Amaro, BA, Brazil) showing the location of the 11 sampling points along the two transects and of the three reference points.

5.2.3 Soil metals concentration and physico-chemical analyses

Soil samples were analyzed for the main four metals causing contamination in the smelter area and proximities (Pb, Cd, Cu, and Zn) and also for Cr, Ni, Fe, Co, and Mn. Metals were quantified in the bulk soil and in 0.01 M $CaCl_2$ extracts by inductively coupled plasma-atomic spectroscopy. Extractions using 0.01 M $CaCl_2$ have been proposed as a suitable technique for determination of available fraction of metals in soil (Houba et al. 1996). The extracts were obtained by shaking 15 g of soil (dry weight) for 2.5 h at 200 rpm with 150 mL of a 0.01 M $CaCl_2$ solution. The slurry was then centrifuged for 5 min at 3000 rpm and extracts (supernatants) were filtered through a Schleicher & Schuell filter paper (Dassel, Germany, Reference nº 595).

Other soil physical-chemical parameters measured were pH (1M KCl) (ISO 1994a), soil moisture after oven drying at 105 °C overnight, water holding capacity (ISO 1998), cation exchange capacity (ISO 1994b), organic matter content (mass loss on ignition at 500 °C for 6 h),

and soil texture (LNEC 1970). Mineral N (NO_3^--N and NH_4^+-N) was quantified in aqueous extracts by titration with 0.01 N sulfuric acid (Chapman and Pratt 1978).

5.2.4. Soil microbial and biochemical analyses

For nitrogen transformation rates (nitrification and ammonification) each sample was divided into three aliquots. One was used to measure the initial NO_3^--N and NH_4^+-N concentrations as described above. The second aliquot received 125 µg g^{-1} of NH_4^+-N as ammonium sulfate, while the third one was left with no N addition before being both incubated at 28°C for 21 days in the dark. NO_3^--N and NH_4^+-N concentrations were again determined and values obtained before and after incubation were used to calculate the nitrification and ammonification rates (Schuster and Schroder 1990).

The ammonifying and ammonium oxidizing microorganisms were estimated by most probable number (MPN) (Woomer 1994) after serial dilution of soil samples in sterile 0.85% saline, and inoculation in multiple 5-replication vials containing the respective liquid culture medium. For ammonifying microorganisms, hydrolyzed casein was used as source of organic N (Sarathchandra 1978), while for ammonium oxidizers, NH_4^+-N was used as energy source in the mineral medium (Schmidt and Belser 1994). After appropriated incubation time at 28 ^oC in the dark, the positive vials were counted, confronted to a most probable number table, and results expressed as log MPN g^{-1} dry soil.

Dehydrogenase activity was assessed in field-moist soil samples incubated with 1.5% triphenyl tetrazolium chlorine (TTC) for 24 h at 37 ^oC in the dark and expressed as µg of triphenyl tetrazolium formazan (TTF) g^{-1} d^{-1} at 37 ºC (Casida et al. 1964). Asparaginase activity was estimated by incubation at 37 ^oC for 2 h in sodium acetate buffer pH 10 and L-asparagine as substrate. The NH_4^+-N produced was quantified by steam distillation in KCl-$AgSO_4$ extracts (Frankenberger and Tabatabai 1991) and expressed as µg N-NH_4^+ g^{-1} h^{-1} at 37ºC. Acid phosphatase activity was determined using 0.05 M sodium p-nitrophenyl phosphate as substrate in samples incubated in modified universal buffer pH 6.5 at 37ºC for 1 h; the color intensity was measured colorimetrically and the activity expressed as µg of p-nitrophenol (PNP) g^{-1} h^{-1} at 37ºC (Tabatabai and Bremner 1969).

Microbial biomass of carbon (MBC) and nitrogen (MBN) were estimated by fumigation-extraction method. Two 25 g aliquots of field-moist soil samples were weighed and one of them was fumigated for 24 h at 28 ^oC with ethanol-free chloroform in the dark. Afterwards,

fumigated and non-fumigated samples were extracted with 0.5 M K_2SO_4 and the organic C was quantified (Anderson and Ingram 1993). MBC was estimated considering the difference between C concentrations in the fumigated and non-fumigated extracts, by using a k_C = 0.33 (Vance et al. 1987). MBN was estimated in the same extracts after sulfuric digestion of an aliquot of the extract and determination of N content by semi-micro Kjeldahl method considering a k_N = 0.68 (Brookes et al. 1985). The metabolic quotient (qCO_2) was obtained by the ratio between the C-CO_2 evolved from soil samples (data from Niemeyer et al. 2010) and the respective MBC (Anderson and Domsch 1993), expressed as mg C-CO_2 g^{-1} MBC h^{-1} The qCO_2 values are inversely related to the efficiency to which the microbial biomass uses the substrates, i.e., higher values indicate higher stress and less efficiency.

5.2.5 Data analysis

Statistical differences on microbial parameters between sites were evaluated using a one-way ANOVA followed by a Dunnet's test. Soil moisture, total organic C and mineral N were used as covariables in all analyses. A partial Principal Component Analysis (pPCA) was used to visualize the major response pattern of microbial indicators, using the same soil parameters as covariables.

Partial correlations between microbial parameters and total and extractable metal concentrations, and vegetation cover were done using the Pearson's correlation coefficient. Moreover, partial correlations were also performed between the assessed microbial variables and the index of metal pollution (W) proposed by Widianarko et al. (2000). This index is the ratio between the metal concentration in each site by the corresponding background concentration (reference value or basal level). The result indicates how much the background concentration is exceeded at a given site. The factors for each metal were then multiplied by one another and the logarithm of the product was taken. In this study, the basal level for each metal was calculated as the geometric mean from the three reference sites. This index was calculated for each sampling point, aiming at synthesizing the information on metal contamination joining information from all metals analyzed. Initially developed for sediment samples, the W index can be used to synthesize the metal loading of any environmental sample in comparison to metal basal levels, including soils (Timmermans et al. 2007; Janssens et al. 2008). This index can take negative values in sites where metal loadings are below the basal levels, whereas positive values indicate metal loadings higher than the basal levels.

In order to separate the contribution of metals and vegetation cover in explaining the differences for microbial parameters, a multivariate decomposition of variance was performed. This was done via several redundancy analyses using the microbial parameters as response variables, and metals and/or vegetation cover (depending on the analysis) as explanatory variables, in addition to soil moisture, soil organic carbon, and mineral nitrogen contents as covariables. The significance of the percentage of variation explained by metals alone, vegetation cover alone, and the interaction between both factors was assessed by the Monte Carlo's permutation tests. All analyses of variance and correlations were performed on the Statistica 7.0 package. All multivariate analyses were done using CANOCO 4.0 software.

5.3 Results

5.3.1 Characterization of the sampling sites

Soils from the study sites showed low to medium organic carbon content (USEPA 2004), ranging from 0.12 to 3.31%, a Cation Exchange Capacity (CEC) mostly between 30 and 40 meq/100 g, and pH values near to neutral, except in the sites P1000T1 and Ref.2 that presented low values (Table 5.1).

Vegetation cover ranged between 20-100 % (Table 5.1). In general, a significant reduction of the vegetation cover in comparison to the reference sites was observed in most of the sampling points within the smelter area (P0, P20T1, P150T1, P20T3, and P50T3). These points, together with P50T1 and P150T3, correspond to sites in which tailings were deposited and where the unsuccessful revegetation can be observed. At these sites, vegetation was dominated by one herbaceous species (*Brachiaria* sp.). In some of these sites, there were evidences of erosion, which could have delayed the natural regeneration process. Further discussion on this issue is given in Chapter 3 (Niemeyer et al. 2010).

5.3.2 Soil metal concentrations

Total and 0.01 M $CaCl_2$-extractable metal concentrations are shown in Table 5.2. For at least one out of four metals (Pb, Cd, Cu, and Zn), soils from three sampling points inside the smelter area (P0, P50T3, and P150T1) presented critical levels of contamination with total

metal levels exceeding the Dutch benchmark values for ecological assessment, as defined by Rutgers et al. (2008). Results of Co (not included in Table 5.2) were below the detection limit in all sites: <24 mg/kg (total) and <10.8 mg/kg (extracts).

5.3.3 Soil microbial and biochemical parameters – differences among sites

Soil microbial properties varied among sites, being conditioned mainly by the metal loadings and degree of habitat disturbance, as measured by vegetation cover. The basal respiration and microbial biomass of carbon and nitrogen presented significantly lower values especially at sites nearby the central point (P0) when compared to the overall reference (Table 5.3). Contrary to expected, the metabolic quotient qCO_2 only presented high values at P20T3 and P150T1, but with high variability, and no significant differences from the overall control. The activities of dehydrogenase, phosphatase, and asparaginase had significant decreases in the soils inside the smelter.

Some other soil microbial properties related to N cycling, namely ammonification and nitrification rates, presented higher values in sites inside the smelter area. However, significant differences against the overall reference were found only for nitrification rate (Table 5.3). Regarding the number of ammonifiers, significantly lower numbers were found in sites inside the smelter area, whereas no differences were found among sites for the nitrifiers (ammonium oxidizers).

The partial principal component analysis permitted to see how the attributes were correlated to the sampling sites (Fig. 5.2). Axis 1 separated the sites with lower metal loadings (the 3 references, P1000T1 and P400T3), and located them in the positive side of the axis. These were mostly located outside the smelter area, and were associated to higher levels of microbial respiration, microbial biomass C and N, ammonifiers, and higher phosphatase and asparaginase activities. Points in the negative side of the axis 1, presenting higher W index (Table 5.2) values and located mostly inside the smelter area, were mainly characterized by higher ammonification and nitrification rates.

Table 5.1 Soil physico-chemical characteristics and vegetation cover (%) in the sites within and around the lead smelter area, and in the three reference (Ref) sites.

Sites	Coarse sand (%)	Fine sand (%)	Sand (total) (%)	Silt (%)	Clay (%)	Texture (USDA)	CEC (meq/ 100 g)	pH (KCl 1:5 v:v)	P (mg/kg)	Organic carbon (%)	Mineral N (mg/kg)	Water content (%)	WHC (g/100 g)	Vegetation cover (%)
Ref 1	2.3	8.5	10.9	42.1	47.0	Silty Clay	34.16	7.1	72	0.64	42	19.54	53.78	
Ref 2	50.9	38.5	89.4	2.8	7.7	Loamy Sand	37.60	4.9	1	0.58	42	13.21	27.53	81.3 ± 21.0
Ref 3	22.2	15.0	37.2	11.1	51.7	Clay	36.48	6.1	52	2.26	56	47.20	60.75	
P0	43.2	31.3	74.5	11.9	13.6	Sandy Loam	38.56	6.7	47	0.17	70	31.04	44.12	22.5 ±22.2
P20T1	48.0	13.8	61.8	19.0	19.3	Sandy Loam	37.28	7.1	58	0.12	42	32.67	46.40	30.0 ± 16.3
P20T3	11.4	30,0	41.4	22.3	36.3	Clay Loam	42.16	6.8	106	1.10	42	35.04	67.73	32.5 ± 12.6
P50T1	25.2	13.4	38.6	29.0	32.4	Clay Loam	38.16	6.7	63	0.64	56	28.59	54.51	57.5 ± 12.6
P50T3	69.2	9.1	78.3	10.4	11.3	Sandy Loam	16.56	7.2	>200	1.62	56	39.48	22.05	20.0 ± 14.1
P150T1	56.2	21.1	77.4	12.3	10.3	Sandy Loam	21.28	6.7	>200	1.22	42	29.41	28.55	30.0 ± 42.4
P150T3	8.4	15.2	23.5	21.4	55.1	Clay	49.20	6.8	16	1.45	42	40.71	61.76	57.5 ± 9.6
P400T1	19.6	23.9	43.5	20.2	36.3	Clay Loam	37.44	6.8	>200	2.96	56	24.43	58.93	100.0 ± 0.0
P400T3	6.5	8.6	15.1	52.4	32.5	Silt Clay Loam	35.84	7.1	1	1.10	70	45.48	56.67	97.5 ± 5.0
P1000T1	2.5	21.8	24.3	19.9	55.8	Clay	43.20	3.7	35	1.16	56	28.74	59.95	67.5 ± 15.0
P1000T3	10.3	19.5	29.8	29.8	40.4	Clay Loam	42.72	7.0	>200	3.31	42	n.d.	57.57	n.d.

Table 5.2 Total and 0.01 M $CaCl_2$ extractable metal concentrations, and pollution index (W) in the soil within and around the lead smelter area, and in the three reference (Ref.) sites.

Sites	Total (mg/kg)								Pollution index	Extractable (mg/kg) – (0.01 M CaCl2; 1:10 v:v)							
	Pb	Cd	Cu	Zn	Cr	Ni	Fe	Mn	W	Pb	Cd	Cu	Zn	Cr	Ni	Fe	Mn
Ref. 1	16	<0.2	66	94	77	54	45000	840	1.26	<0.9	<0.09	<7.2	<1.8	<7.2	<12.6	<9.9	<4.5
Ref. 2	13	<0.2	18	24	16	28	2900	34	-0.89	<0.9	<0.09	<7.2	1.8	<7.2	<12.6	<9.9	7.2
Ref. 3	152	<0.2	40	260	59	40	53000	820	-0.37	<0.9	<2.52	<7.2	<1.8	<7.2	<12.6	<9.9	11.7
P0	1264	<0.2	76	3800 (2.8)	72	57	52000	674	5.61	<0.9	<0.09	<7.2	<1.8	<7.2	<12.6	<9.9	<4.5
P20T1	133	<0.2	56	220	80	56	41000	780	2.44	<0.9	<0.09	<7.2	<1.8	<7.2	<12.6	<9.9	<4.5
P20T3	308	<0.2	56	420	78	60	49000	672	3.12	<0.9	<0.09	<7.2	<1.8	<7.2	<12.6	<9.9	<4.5
P50T1	164	<0.2	60	240	80	58	43000	720	2.60	<0.9	<0.09	<7.2	<1.8	<7.2	<12.6	<9.9	<4.5
P50T3	26074 (7.1)	62	3196 (8.2)	95940 (73.5)	80	40	117000	5880	13.63	<0.9	<0.09	<7.2	9	<7.2	<12.6	<9.9	<4.5
P150T1	37460 (10.4)	771 (9.8)	594 (1.6)	42200 (33.5)	57	70	110000	1720	13.33	19.8	65.7	<7.2	11.7	<7.2	<12.6	<9.9	<4.5
P150T3	2200	12	108	3300	84	58	56000	678	7.83	<0.9	<0.09	<7.2	1.8	<7.2	<12.6	<9.9	<4.5
P400T1	961	8.8	60	840	64	48	35000	540	5.98	<0.9	<0.09	<7.2	<1.8	<7.2	<12.6	<9.9	<4.5
P400T3	179	0.3	44	90	59	46	34000	760	2.76	<0.9	<0.09	<7.2	<1.8	<7.2	<12.6	<9.9	<4.5
P1000T1	23	<0.2	60	80	62	46	48000	360	0.80	0.9	3.6	<7.2	17.1	<7.2	14.4	<9.9	63.9
P1000T3	99	<0.2	56	156	84	52	49000	568	2.09	<0.9	<0.09	<7.2	<1.8	<7.2	<12.6	<9.9	8.1

Numbers in brackets indicate an excess of the corrected Dutch $HC50_{EC50}$ values (after Rutgers et al. 2008) (Ex: the [Pb] at P150T1: 37460 (10.4), indicates that [Pb] was 10.4 times higher than the $HC50_{cor}$Pb).

Table 5.3 (Continued)

Sites	Respiration (µg CO_2/ g soil/ day)	MBC (µg/g)	MBN (µg/g)	C/N	qCO_2 (mg CO_2-C/ g biomass C/ h)	Dehydrogenase (µg PNP/ g/ d)	Acid phosphatase (ug PNF/g/h)	Asparaginase (µg N-NH_4^+/g/h)	Ammonification (µg N /g /day)	Ammonifiers (log of MPN/ g)	Nitrification rate (%)	Nitrifiers NH_4^+ oxidizers (log MPN/g)
P50T1	41.4 ± 2.4*	412.9 ± 31.4	11.0 ± 4.2***	41.10 ± 15.23	1.1 ± 0.1	1.2 ± 2.0**	235.7 ± 50.3**	11.2 ± 19.5***	1.8 ± 0.3	5.5 ± 0.5***	17.1 ± 4.9***	2.6 ± 0.1
P50T3	52.2 ± 12.6*	461.7 ± 20.1	22.0 ± 4.5*	21.62 ± 4.76	1.3 ± 0.3	2.1 ± 0.5*	450.3 ± 45.4	32.5 ± 35.1**	1.8 ± 0.3	5.6 ± 0.5***	13.5 ± 4.5*	2.2 ± 0.5
P150T1	49.2 ± 6.6*	115.5 ± 87.0***	9.3 ± 1.3***	12.03 ± 9.24	12.6 ± 15.9	3.3 ± 0.5	355.3 ± 166.0*	22.8 ± 11.8***	0.4 ± 0.2	5.6 ± 0.1***	8.7 ± 2.8	2.4 ± 0.3
P150T3	60.5 ± 9.2*	543.6 ± 160.8	26.6 ± 3.1**	20.85 ± 7.62	1.3 ± 0.4	2.1 ± 1.1*	651.2 ± 150.7	37.0 ± 12.4*	1.5 ± 0.3	6.6 ± 0.6	10.2 ± 0.5	2.3 ± 0.0
P400T1	234.9 ± 83.3	797.3 ± 193.3	83.0 ± 21.2	9.62 ± 0.43	3.3 ± 0.4	16.8 ± 3.7	573.1 ± 133.3	91.7 ± 32.9	0.8 ± 0.1	7.0 ± 0.3	-0.2 ± 5.9	2.8 ± 0.0
P400T3	165.2 ± 41.3	805.3 ± 216.2	59.7 ± 26.5	14.18 ± 2.82	2.3 ± 0.3	1.5 ± 1.1**	792.0 ± 34.5	97.8 ± 16.6	0.4 ± 1.0	6.4 ± 0.1	-3.3 ± 3.17	2.9 ± 0.1
P1000T1	164.0 ± 79.1	1098.1 ± 184.1	51.1 ± 22.0	23.79 ± 9.79	1.71 ± 0.7	4.8 ± 6.2	515.6 ± 353.5	71.57 ± 18.8	0.6 ± 1.8	6.4 ± 0.4	1.9 ± 7.4	2.8 ± 0.0
P1000T3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Ref – Reference soil
MBC – Microbial biomass Carbon
MBN – Microbial biomass Nitrogen
C/N – C to N ratio of the microbial biomass
n.d. - not determined

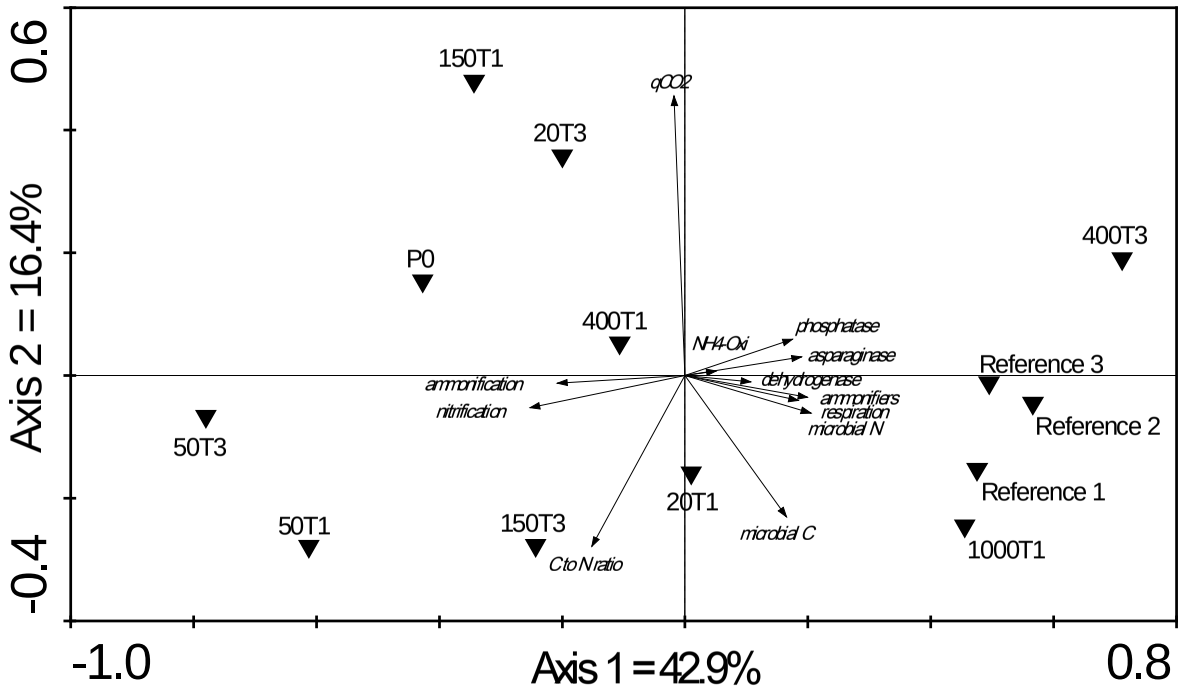

Fig. 5.2 Partial Principal Component Analysis (pPCA) ordination diagram, using the parameters of Table 3. Soil moisture, soil organic carbon, and mineral nitrogen contents were used as covariables in the analysis.

5.3.4 Soil microbial and biochemical parameters – relationship with metal contamination and vegetation cover

Most microbial parameters presented significant partial correlations (using soil moisture, soil organic carbon, and mineral nitrogen as covariables) with metal loadings in soil given by the Widianarko's pollution index (W). Negative relations were found for basal respiration, microbial biomass (C and N), phosphatase and asparaginase activities, and number of ammonifiers, whereas a significant positive relation was observed for nitrification rate (Table 5.4). No significant correlations were observed for microbial C/N ratio, qCO_2, dehydrogenase activity, and ammonification rate.

Similar trends can also be seen when looking at individual metals, especially when considering the total metal concentrations (Table 5.4), given that the correlations with extractable metal were weaker. Significant negative correlations were observed for microbial biomass C with Pb, and number of ammonifiers with total Fe, Pb and Ni. Significant positive correlation with extractable concentrations of Cd and Pb were found for qCO_2.

Microbial and biochemical parameters also presented significant correlations with vegetation cover (Table 5.4). Positive correlations were mostly found with basal respiration, microbial biomass (C and N), enzyme activities (dehydrogenase, phosphatase, and asparaginase), and number of ammonifiers, whereas negative correlations were observed with nitrogen transformation rates (ammonification and nitrification).

As an attempt to decipher the different contribution of metals and vegetation cover in explaining the variation of microbial and biochemical parameters, the multivariate decomposition of variance (partial RDAs using soil moisture, soil organic carbon, and mineral nitrogen contents as covariables) showed that total metal concentrations explained a considerable portion of the variation (49.1%), whereas vegetation cover explained only 25.3% (Table 5.5). However much of the variation in these values correspond to shared variance between both variables (22.2%), indicating that vegetation alone explains only 3.1% against 26.9% of the variation explained by metals alone (Table 5.5).

Table 5.4 Pearson's partial correlations between microbial parameters and metal concentration (total and extractable), overall metal loading (Widianarko's index - W) and vegetation cover – VEG.

Microbial parameters	Total metal concentrations								Extractable metal concentrations				W index	VEG
	Cu	Fe	Mn	Zn	Cd	Cr	Pb	Ni	Mn	Cd	Pb	Ni		
Basal respiration	-0.54***	-0.53**	-0.53**	-0.57**	n.s.	n.s.	-0.50**	n.s.	n.s.	n.s.	n.s.	n.s.	-0.70***	0.61***
MBC	n.s.	-0.44**	-0.35*	-0.40*	-0.38*	n.s.	-0.47**	-0.45**	0.60***	n.s.	-0.36*	0.60***	-0.64***	0.58***
MBN	-0.47**	-0.64***	-0.48**	-0.54**	-0.39*	-0.39*	-0.55***	-0.45**	n.s.	n.s.	-0.35*	n.s.	-0.72***	0.73***
C/N	n.s.	n.s.	n.s.	n.s.	n.s.	0.39*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
qCO_2	n.s.	n.s.	n.s.	n.s.	0.44**	n.s.	n.s.	0.36*	n.s.	0.45**	0.45**	n.s.	n.s.	n.s.
Dehydrogenase	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.42*
Acid Phosphatase	n.s.	-0.51**	-0.34*	n.s.	n.s.	-0.65***	n.s.	-0.56***	n.s.	n.s.	n.s.	n.s.	-0.47**	0.56***
Asparaginase	-0.47**	-0.48**	-0.46**	-0.51**	n.s.	n.s.	-0.48**	n.s.	n.s.	n.s.	n.s.	n.s.	-0.65***	0.55**
Ammonification rate	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.42*
Nitrification rate	0.35*	0.43**	0.38**	0.35*	n.s.	0.54**	n.s.	0.35*	n.s.	n.s.	n.s.	n.s.	0.43**	-0.62***
Ammonifiers	-0.37*	-0.61***	-0.41*	-0.45**	-0.40*	-0.40*	-0.51**	-0.47**	n.s.	-0.36*	-0.37*	n.s.	-0.61***	0.73***
NH_4^+ Oxidizers	n.s.	n.s.	-0.33*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

MBC – Microbial biomass Carbon
MBN – Microbial biomass Nitrogen
C/N – C to N ratio of the microbial biomass
NH_4^+ Oxidizers – Nitrifiers ammonium oxidizers
n.s. - not significant (p>0.05)
*p<0.05; **p<0.01; ***p<0.001

Table 5.5 Variance partitioning of microbial and biochemical data according to total metal content and vegetation cover. Values expressed in percentage of total variation excluding covariables (soil moisture, soil organic carbon, and mineral nitrogen contents).

Variables	Variation explained (%)	P
Variation of microbial and biochemical parameters (excluding covariables)	66.8*	
Covariables	33.2	
Metals and Vegetation (Total)	52.2	0.002
Metals	49.1	0.002
Vegetation	25.3	0.002
Metals (pure)	26.9	0.002
Vegetation (pure)	3.1	0.002
Shared	22.2	

* expressed as % of total variation

5.4 Discussion

The high levels of metals in the soil of smelting area have been previously reported (Anjos 2003; Machado et al. 2004), and resulted both from deposition of residues inside the smelting area and aerial deposition of contaminated particles from the smelter plume during the smelting activity. The plume was also responsible for the extent of contamination outside the smelter area. The heterogeneity of the soil inside the smelter area can be attributed to heterogeneous deposition of tailings and the partially unsuccessful attempt to encapsulate some of the piles by depositing thousands of cubic meters of nearby soil (Anjos 2003).

This study has revealed significant differences in soil microbial and biochemical attributes among the sampling sites differently affected by the lead smelter activity. This can be attributed to disturbances caused by deposition of tailings in the area and to their unsuccessful encapsulation using soil brought from nearby areas, which affected the soil (Niemeyer et al. 2010), and its microbial and biochemical properties. Negative correlations between the W index with some microbial and biochemical attributes illustrate the negative effects of metal contamination on the soil microbial community and some essential role they play in the biogeochemical cycles. Under such condition, the sustainability of the vegetation in the metal-contaminated sites has not been reached, and may lead to more environmental risks in the future. As key microbial processes on C, N and P cycling have been impaired under such condition, in addition to higher contents of metals, the maintenance of vegetation in these heavily-contaminated sites can be progressively difficult, leading to intensification of erosive processes and dispersion of pollutants (Broos et al. 2005).

There have been some controversial findings regarding the effect of metal contamination on soil respiration, as some works have observed increased respiration rates whereas others a decrease with increasing metal concentrations in soil (Smejkalova et al. 2003; Rajapaksha et al. 2004; Khan and Joergensen 2006). In the present work, the soil basal respiration rate was lower in the metal-contaminated soils inside the smelter area and correlated negatively with total soil metal concentrations. These results agree with those obtained by Zimakowska-Gnoinska et al. (2000) who observed less oxygen consumption in soils from metal-contaminated sites in comparison to uncontaminated samples, in addition to strong negative correlations between soil respiration and soil pollution levels. Gulser and

Erdogan (2008) also observed that soil respiration correlated negatively with contents of several metals in roadside fields near to intensive traffic; soil respiration significantly increased with decreasing the levels of metal contents according to the distance from the roadside. In the present work, the negative correlations between soil respiration and metal concentrations, and the positive correlations between soil respiration and other microbial indicators confirmed that soil respiration can be used for estimations and comparisons between soil ecological conditions and biological activity (Zimakowska-Gnoinska et al. 2000).

Microbial biomass is involved in the control of soil organic matter decomposition and synthesis, besides acting as easy-release storage of nutrient in ecosystems. Therefore, sites with high microbial biomass can stock and recycle more nutrients (Gregorich et al. 1994; Kaschuk et al. 2010) to be used for plant nutrition and thus improving the sustainability of a particular ecosystem. Consequently, sites with low microbial biomass can have these functions impaired, as observed in some sites inside the smelter area around the tail deposits. These sites also showed a low vegetation cover, indicating that the soil functions have still not been reestablished and that further actions are needed for reclamation of the degraded sites.

MBC, MBN, and basal respiration were positively correlated with soil organic C, while MBC was also positively correlated to soil moisture and mineral N (supplementary material), showing that microbial indicators can be impacted due to changes in carbon and nitrogen in soil as consequence of soil pollution or management (Monokrousos et al. 2006; Nogueira et al. 2006; Jiang et al. 2010). Given that most of the soil microbial community is composed by chemorganotrophic microorganisms, improvement of soil organic carbon usually stimulates microbial activity and biomass (Kaschuk et al. 2010). In addition, soil organic matter brings indirect beneficial effects to the soil microbial community by improving the soil capacity for water retention and metal complexation (Giller et al. 2009; Moreno et al. 2009).

Enzyme activities were positively correlated with MBC, MBN, and basal respiration (data not shown), indicating that they are associated with active microorganisms, which are the major source of enzymes in soil. Thus, the probable impact on enzyme activities was caused by direct suppression of microbial growth due to negative conditions in the metal-contaminated sites (Kuperman and Carreiro 1997). As indicated by the variance partitioning, metal concentrations were the most responsible for changes in these attributes than vegetation cover and the large portion explained by vegetation is shared with metals, showing their strong indirect effect on microbial parameters. The significant correlations between MBC, MBN, and basal respiration with organic C (supplementary material) indicate that higher

organic C levels in soil are supporting greater microbial biomass and enzyme activities, not only by acting as C and energy sources for soil microbial community, but also due to a chelating effect protecting microorganisms and soil enzymes from excessive levels of metals in soil (Balota et al. 2004; Moreno et al. 2009; Lejon et al. 2010).

Nitrification and ammonification rates have key roles in the nitrogen cycling in soil. While nitrification is considered one of the most sensitive soil microbial processes regarding to metal stress (Broos et al. 2005), some studies have shown adaptation of nitrifying populations to metal-contaminated sites (Mertens et al. 2006). In the present work, nitrification rate was positively correlated with metal concentrations, but in this case, part of that behavior can be explained by the high pH in the highly contaminated sites. It is known that nitrification is favored under high pH, which, at the same time, makes metals less available and thus less toxic to the microbial community. Soil moisture, pH and ammonium contents in soil are generally the main factors affecting nitrification (Krave et al. 2002). In fact, soil pH showed the greatest positive correlation with nitrification in the present work ($r=0.43$, $p<0.01$), emphasizing the importance of soil pH on this attribute (Sarathchandra 1978; Sauvé et al. 1999). In addition, in long-term contaminated sites, adaptation or selection of specific microbial groups or (sub) populations resistant or tolerant to metal contamination is likely to occur (Giller et al. 2009). Sobolev and Begonia (2008) suggested that denitrifying microorganisms were adapted to elevated levels of Pb by selecting for metal-resistant enzymes. Adaptation not only of nitrifying populations in contaminated sites but also other microbial communities is also known to occur (Lejon et al. 2010), and this may have occurred in the present work. A long-term exposure to a heavily Zn-contaminated soil induced structural changes and tolerance of the nitrifying microorganisms to Zn, as compared to the nitrifying community in an uncontaminated control soil (Mertens et al. 2006).

Significant correlations were found between qCO_2 and total Cd and Ni, and extractable Pb and Cd. Once this parameter indicates the energetic demand of heterotrophic microorganisms, integrating MBC and basal respiration, these results can indicate metal stress to soil microorganisms, evidencing the need for more C to supply their energetic demand per unit of microbial biomass (Bardgett and Saggar 1994; Fliessbach et al. 1994; Valsecchi et al. 1995). Soil microbial biomass and activity (*sensu lato*) are closely related to vegetation cover.

In the present work, positive correlations were observed between vegetation cover and MBC, MBN, soil basal respiration, enzyme activities, and ammonifier microorganisms. Vegetation cover can contribute to reduce metal toxicity to microbial community because they

offer favorable conditions not only in the rhizosphere region but also in the bulk soil due to inputs of plant residues that will run humification (Tordoff et al. 2000). On the other hand, vegetation cover showed significantly negative correlations with individual metal contents and the pollution index (W) (data not shown). These relationships can explain the low percentage of the variation of microbial data explained by vegetation cover alone, and the high percentage explained by the interaction with total metals. This does not mean that vegetation is not important for microbial communities, but that, in this case, the vegetation cover was highly conditioned by the metal loadings. The failed establishment of the vegetation in metal-contaminated sites, conditioning microbial parameters, resulted mainly from direct metal contamination (Tordoff et al. 2000).

Vegetated soils have been reported to have both higher microbial biomass and microbial activity when compared to bare soils (Epelde et al. 2006). Hernández-Allica et al. (2006) and Epelde et al. (2010), in studies on phytoextration with *Thlaspi caerulescens* in metal polluted soils, the revegetation activated the soil microbial activity and their functionality. This positive response can be attributed to the improvement of soil conditions, such as organic compounds released by the plant roots and the presence of additional surfaces for microbial colonization (Delorme et al. 2001).

Soil metal contamination in Santo Amaro has impaired the vegetation cover in the smelter area and modified the plant species composition and invertebrates, changing and simplifying the ecosystem structure (Niemeyer et al. submitted*/ecological parameters*). Contamination had detrimental effects on soil properties, modifying the microclimatic conditions at the ground level, and the amount and quality of the potential organic inputs into the soil. Our results showed that these changes caused negative impacts to the soil microorganisms and processes inside the smelter area, where worse values were generally observed. The main negative effects seem to be due to limitation of plant reestablishment that results in low amounts of organic matter inputs into the soil to be used as source of C and energy for microbial growth and also on the protection of microbial community against high levels of metals in soil. Nogueira et al. (2006) recommended providing plant covering in degraded areas to prevent the advance of soil degradation by erosion and to increase microbial activity and diversity, which also contribute to decrease the nutrient losses by leaching. This could also be recommended as a management strategy to be applied in the smelter area in Santo Amaro, improving the soil health and preventing the dust dispersion by wind, which is a current problem for human health, once this area is located at the neighborhood of the city.

5.5 Conclusions

Microbial indicators were positively correlated with soil organic carbon and vegetation cover, while negatively correlated to soil metal levels. In general, microbial indicators showed high ecological risks to soil functions related to tailing deposition even 17 years after the lead smelter have stopped its activities. The main negative effects seem to be due to limitation of plant reestablishment that results in low amounts of organic matter inputs into the soil to be used as source of C and energy for microbial growth and also on the protection of microbial community against high levels of metals in soil.

We can conclude that, besides direct effect of metal toxicity on microbial biomass and activity, there are indirect effects related to changes in the vegetation cover, soil organic carbon, pH, and nutrient availability. These attributes have changed the soil microclimate and physical-chemical properties that may have lead to losses of habitat function for soil microorganisms and the key processes they play, as in carbon and nutrient cycling.

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Chapter 6

Functional and structural parameters to assess the ecological status of a metal contaminated area in the tropics

Based on the following manuscript:

Niemeyer JC, Nogueira MA, Carvalho GM, Cohin-De-Pinho SJ, Outeiro US, Rodrigues GG, Da Silva EM, Sousa JP. (Accepted with minor revisor). Functional and structural parameters to assess the ecological status of a metal contaminated area in the tropics. *Ecotoxicol Environ Saf.*

Abstract

Ecological parameters (soil invertebrates, microbial activity, and plant community) were assessed in a metal contaminated site in an abandoned lead smelter and non-contaminated reference sites, as part of an ecological risk assessment (ERA). Vegetation cover inside the smelter area was lower and presented a homogenous species composition than outside. Failure in vegetation establishment caused an impoverishment of habitat conditions, which allied to metal toxicity, originated a significant impairment of soil microbial and faunal communities in the contaminated sites. A significant reduction in the number of species (and species assemblages) of surface dwelling macroarthropods and feeding activity was observed. Moreover, basal respiration, microbial biomass C and phosphatase activity also decreased. As a result, a significant impairment of organic material decomposition in the most contaminated sites was observed. Metal contamination affected the ecological status of the site, leading to a risk for ecosystem functioning and provisioning of ecosystem services like organic matter decomposition and nutrient cycling, even 17 years after the end of smelting activities. Regarding the sensitivity of the ecological parameters assessed, most were able to distinguish sites within the smelter are from those outside. However only bait lamina, basal respiration and microbial biomass carbon presented high capacity to distinguish the level of soil contamination being promising candidates to integrate the Ecological Line of Evidence of an ERA.

Keywords: Bait lamina, Litter decomposition, Soil invertebrate communities, Vegetation, Microbial activity, Risk assessment

6.1 Introduction

Ecological parameters have been recommended to be used in ecological risk assessment (ERA) of contaminated sites (Sprenger and Charters 1997; Jensen and Mesman 2006; Rutgers and Jensen 2011). Either used independently or integrated in a TRIAD approach, underpinning the ecological line of evidence, ecological parameters are integrative indicators of adverse impacts resulting from contaminant exposure.

Ecological effects of contaminants in soil functions can be assessed both from a structural and a functional perspective (Rutgers 2008; Van Straalen 2002; Semenzin et al. 2009). However, data on how biological processes are impaired due to soil pollution is still scarce since most of assessed ecological parameters are structural (e.g. vegetation surveys, soil faunal density and taxonomic composition). Therefore, links between pollutant effects on soil organisms and on soil functions should be deeply investigated (Cortet et al. 1999) by simultaneously assessing ecological structural and functional parameters in a site evaluation.

In ERA schemes for contaminated soils, ecological parameters can be measured at different tiers and include different groups of organisms (from microorganisms to soil macrofauna) and different biological levels (populations, communities, processes). Soil fauna is a key component of soil environments, involved in many aspects of organic matter decomposition and nutrient cycling (acting mainly as regulators of microbial activity), and on the contribution for maintenance of soil structure (Lavelle 1996). Different studies on soil fauna showed the suitability of these organisms in indicating soil ecological status when comparing areas with different levels of contamination (Gongalsky 2003; Creamer et al. 2008). In addition to the evaluation of soil faunal communities (usually done on a later phase of the assessment) the use of bait-lamina sticks has been proposed as a relevant tool for ecological assessments and was successfully tested in temperate (e.g. Hamel et al. 2007) and tropical soils (e.g. Römbke et al. 2003).

Vegetation surveys are one of the most used tools to evaluate habitat quality in terrestrial ecosystems (Godínez-Alvarez et al. 2009) because plants are the primary producers, key structural component of the habitat for all soil inhabitants. Measurements of vegetation cover and composition are important to indicate changes in habitat quality due to stress

caused by pollution. Besides, some advantages such as their immobility and easy sampling make them a suitable tool to be used in ERA (Suter et al. 2000).

Regarding functional parameters, microbial endpoints are the most used. Because the crucial role of soil microorganisms in carbon and nutrient cycling (Nannipieri et al. 2002) and on decontamination processes, indices related to microbial diversity, biomass and activity can provide important information about the functional impairment or improvement in the soil (Giller et al. 1998; Nogueira et al. 2006). These features make microbial parameters suitable for using in risk assessment of impacted areas (Moreno et al. 2009; Jiang et al. 2010). However, being probably the most integrative indicator of several processes occurring in soil, the rate of organic material decomposition can be used as indicative of negative effects on the soil microbial community, soil fauna or both. Although some studies reported a low sensitivity of this parameter when assessing risks of contaminants, showing none or transient effects (Dinter et al. 2008; Van Gestel et al. 2009), other studies on metal contaminated sites showed effects (Creamer et al. 2008).

This study aimed to investigate the ecological status of a former smelting area with a long-term history of metal contamination located at Santo Amaro (Bahia, Brazil). Moreover, the different sensitivity of each parameter to metal contamination will also be evaluated aiming to propose the most suitable methods in these contamination scenarios. We hypothesized that each ecological endpoint responds to the metal contamination gradient, indicating a decrease in ecological status in the highly contaminated sites as compared to the reference sites.

6.2 Materials and methods

6.2.1 Study area

The study was carried out in an abandoned lead smelter that operated between 1960 and 1993, in the neighborhood of Santo Amaro city, BA, Brazil (12° 32’ 49” S, 38° 42’ 43” W) (Fig. 6.1). Tailings and airborne dust containing metals were spread in the region, leading to soil contamination (Costa 2001; Carvalho et al. 2003). And despite the attempt, in 1995, to encapsulate the residues with soil rich in organic matter, signs of habitat degradation due to

contamination are still visible. A more detailed description of the study area is given in Chapter 3 (Niemeyer et al. 2010).

Based on the total metal concentrations in soil (Pb, Cd, Cu, Zn) derived from a pre-sampling strategy using six radial transects (unpublished data), two 1-km transects (T1 and T3) were chosen along the two major gradients of contamination. Starting in a shared central point (P0 – located next to the smelter facility) five extra sampling points were established on each transect at 20, 50, 150, 400, and 1000 m (P20T1-P1000T1 and P20T3-P1000T3; see Fig.6.1). Three reference sites were selected at 3 km (Ref. 2 and 3) and 9 km (Ref. 1) away from P0 (Fig.6.1) with soil properties similar to the soils along the transects, but without metal contamination. Further details about sampling, soil physical-chemical characterization (including metal analysis), and selection of the reference sites are given in Chapter 3 (Niemeyer et al. 2010).

All surveys were carried out in July 2008, with exception of litter bag test that was conducted between October 2009 and February 2010.

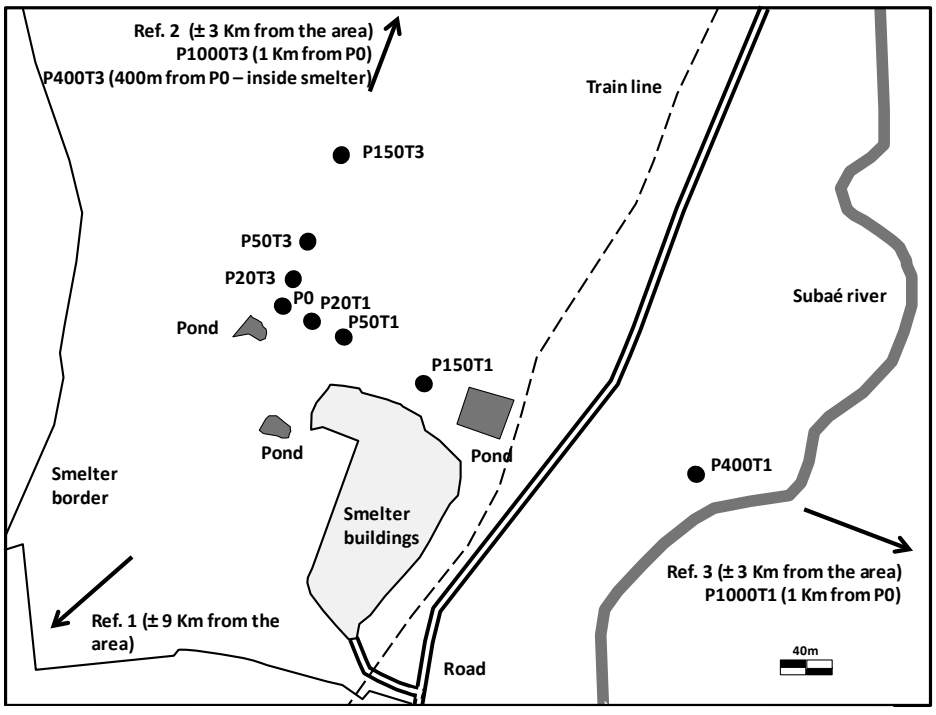

Fig. 6.1 Schematic representation of the study area (an abandoned lead smelter, Santo Amaro, BA, Brazil) showing the location of 11 sampling sites along two transects and three reference sites. Sites outside the area are: P400T1, P1000T1, P1000T3 and the three references sites.

6.2.2 Vegetation cover and succession stage

Vegetation assessment was carried out according to Veiga and Wildner (1993). Further details are given in Chapter 5 (Niemeyer et al. 2012). Simultaneously, the plant species within 10 m radius were inventoried. Classification of successional plant community stage followed the Brazilian criteria established by the Environmental National Council (Brazil 1994).

6.2.3 Surface dwelling invertebrates

Surface dwelling invertebrates were sampled using pitfall traps (plastic cups, Ø 8 cm, 11 cm depth) containing 50% ethanol and some drops of neutral detergent. Three traps were set up in each sampling point, separated 5 m apart in a triangular disposition, over one week in July/2008. After collection, specimens were preserved in 70% ethanol until identification at morphospecies level.

The total number of individuals of each morphospecies at each site was obtained by pooling the results from the three traps. Number of species, abundance, species richness (Margalef index), species diversity (Shannon index), evenness (Pielou index) and dominance (Berger-Parker index) were calculated.

6.2.4 Soil fauna feeding activity (bait lamina method-BLT)

The BLT was prepared using a 1:5:14 ratio of finely ground oat, activated charcoal and cellulose powder (Merck), respectively. Five groups (samples) of five bait-lamina strips were exposed in each sampling point for 14 days, in July/2008. Bait strips were inserted vertically into the soil, each group occupying an area of 15 cm x 15 cm. In parallel, soil moisture was determined in each point. After the exposure period, bait-lamina were removed and taken to the laboratory. After careful washing in tap water, each bait strip was assessed visually against light and counting the number of pierced (= eaten) holes. No distinction was made between partially or fully pierced holes. The feeding activity per sample (group of five strips) at each sampling point was expressed in percentage.

6.2.5 Microbial parameters

Soil samples for assessment of microbial parameters were taken in three parallel transects (2 m apart, and 10 m long), defined at each sampling point. Along each transect, 15 subsamples (0-10 cm depth) were taken and pooled to form a composite sample that was sieved (<5 mm), stored at 4 °C and processed within 72 h.

Basal respiration was determined according to Alef (1995) for 8 days using 1 M NaOH as CO_2 trap. Microbial biomass carbon (MBC) was estimated by fumigation-extraction method (Vance et al. 1987) and C determination in the extracts according to Anderson and Ingram (1993), using a k_C = 0.33.Dehydrogenase activity (DHA), was assessed in field-moist samples incubated with 1.5% triphenyl tetrazolium chlorine (TTC) for 24 h at 37^{o}C (Casida Jr et al. 1964). Acid phosphatase activity was determined using 0.05 M sodium p-nitrophenyl phosphate as substrate (Tabatabai and Bremner 1969). For nitrification rate, samples received 125 µg g^{-1} of NH_4^+-N as ammonium sulfate or left with no N addition and incubated at 28°C for 21 days in the dark. After determinations of NO_3^--N and NH_4^+-N concentrations before and after incubation (Keeney and Nelson, 1982), the nitrification rate was calculated (Schuster and Schroder 1990).

6.2.6 Litter breakdown

Litter bags were used to measure leaf breakdown. Nylon bags with size of 30 cm × 20 cm and a large mesh size (1.0 cm × 0.2 cm) were used to allow activity both by macro- and microorganisms (Cortez 1998). Dried leaves of *Schinus terebinthifolius* Raddi (Anacardiaceae), a native tree species, were collected in a non-contaminated area and used as substrate in the litter bags (4 g in each bag). This species is quite frequent at the study site and is palatable to the soil macrofauna (Podgaiski and Rodrigues 2010). Litter bags were placed on the soil surface. At each sampling point 4 small areas (4 m apart on a quadrangular shape) were defined and 4 bags were placed in each area (total of 16 bags per sampling site). One litter bag of each small area was collected randomly at each period (15, 43, 83 and 131 days) and processed immediately. Any visible plant material from other species, organisms, or soil were removed manually. The material was dried at 60 °C and weight was recorded. Afterwards, the ash-free dry weight (AFDW) was calculated by subtracting the mass of the ignited residue at 600 °C for 1 hour. Litter mass loss was calculated by subtracting the AFDW of the remaining

litter from the AFDW of the initial input, and using soil and litter correction factor according to EPFES protocol (Römbke et al., 2003). Results were expressed as % of mass lost. The monthly decay rate constant was calculated by using the single negative exponential decay model $m_t/m_0 = e^{-kt}$, where m_t/m_0 is the proportion of mass remaining at time *t*, and *t* is the time elapsed in days (months), and *k* is the derived daily (monthly) decay constant. Further details are given in OECD (2006) and Römbke et al. (2003).

6.2.7 Data analysis

The Widianarko's pollution index (WPI) was calculated for each sampling point (Widianarko et al., 2000) to pool the information from all assessed metals. Details are shown in Chapter 4. The geometric mean of each metal concentration in the 3 reference sites was used as base level for each metal. This index can take negative values where metal loadings are below the base levels, while positive values increase with the metal loading above the base levels.

For BLT, arthropod abundance and richness, vegetation cover, soil microbial respiration, MBC, DHA, phosphatase activity and nitrification rate, differences between sampling points were tested with one-way ANOVA (analysis of variance) followed by Dunnet´s test against the overall reference (the average of the values obtained for all reference points was used as overall reference). Soil moisture and organic matter contents were used as covariables in the ANOVA for basal respiration and phosphatase activity. For MBC, DHA and nitrification rate, mineral N was also included as covariable. Differences in abundance and morphospecies richness of surface dwelling arthropods from sites inside vs. sites outside the smelter area was tested by t-test for independent samples. In all analyses, normality and homogeneity of variances were checked with Kolmogorov-Smirnov and Bartlett's tests (Fisher test in case of t-test), respectively, Transformations were applied whenever necessary and according to the type of data. Differences between decomposition rates were tested with ANCOVA (analysis of covariance). Additionally, ecological parameters were correlated (Pearson's correlation coefficient) with WPI to find which parameters best respond to the contamination gradient. Partial correlations were run for microbial parameters using soil moisture, organic matter and mineral nitrogen contents as covariables. Analyses were carried out using the Statistica 7.0 software (Statsoft, Tulsa, OK, USA).

Principal component analysis (PCA) was used to display similarities in vegetation composition, surface dwelling arthropod community, and microbial parameters among sites. Soil moisture, organic matter and mineral nitrogen contents were used as covariables in the microbial PCA, using Canoco for Windows® v.4. To check whether the differences for microbial parameters, surface dwelling arthropods and vegetation were different between sites inside and outside the smelter area, an Analysis of Similarity (ANOSIM) followed by a Similarity Percentage Analysis (SIMPER) were run for each matrix, based on a Bray-Curtis similarity matrix using the Primer v.5 software.

6.3 Results

6.3.1 Sites characterization and metal concentrations in soil

A full characterization of sites is given in Chapter 3 (Niemeyer et al. 2010). Soils from the study sites showed low (<2%) to medium (2 to 6%) organic matter content (USEPA 2004), a cation exchange capacity (CEC) mostly between 30 and 40 meq/100 g, and pH near to 7, with the exception of sites P1000T1 and Ref.2, with pH at the acidic range (Table 6.1). Soil heterogeneity within the smelter area is attributed to spatial distribution of different actions undertaken to rehabilitate the area, namely the failed attempt to encapsulate the tailings by covering with nearby soil (Anjos 2003).

For at least one metal (Pb, Cd, Cu, or Zn), sandy soils presented levels exceeding the Dutch $HC50_{cor}$ benchmarks (Table 6.2), indicating high ecological risk (Rutgers et al., 2008). P0 presented a high Zn contamination exceeding by almost three times the corresponding $HC50_{cor}$ value, whereas P150T1 and P50T3 presented a critical level of contamination, exceeding by far (between 1.6 and 73.5 times) the screening levels. WPI corroborates these data, with slightly positive (or even negative) values in the reference sites or sites outside the smelter areas, in contrast to highly positive values in most of the contaminated sites.

Table 6.1. Physical-chemical characteristics of the three groups of soils sampled at the Santo Amaro (BA, Brazil) study area and respective reference soils. USDA – United States Department of Agriculture; CEC – Cation Exchange Capacity; WHC – Water Holding Capacity.

Soil group	Coarse sand (%)	Fine sand (%)	Sand (total) (%)	Silt (%)	Clay (%)	Texture (USDA)	CEC (meq 100g)	pH (KCl 1:5 v:v)	P (mg/kg)	Organic matter (%)	Mineral N (mg/kg)	Water content (%) (1)	WHC (g/100g)
Group 1													
Ref 1	2.3	8.5	10.9	42.1	47.0	Silty Clay	34.16	7.1	72	1.1	42	19.54	53.78
P1000T1	2.5	21.8	24.3	19.9	55.8	Clay	43.20	3.7	35	2.0	56	28.74	59.95
P20T3	11.4	30,0	41.4	22.3	36.3	Clay Loam	42.16	6.8	106	1.9	42	35.04	67.73
P400T3	6.5	8.6	15.1	52.4	32.5	Silt Clay Loam	35.84	7.1	1	1.9	70	45.48	56.67
Group 2													
Ref 2	50.9	38.5	89.4	2.8	7.7	Loamy Sand	37.60	4.9	1	1.0	42	13.21	27.53
P0	43.2	31.3	74.5	11.9	13.6	Sandy Loam	38.56	6.7	47	0.3	70	31.04	44.12
P20T1	48.0	13.8	61.8	19.0	19.3	Sandy Loam	37.28	7.1	58	0.2	42	32.67	46.40
P150T1	56.2	21.1	77.4	12.3	10.3	Sandy Loam	21.28	6.7	>200	2.1	42	29.41	28.55
P50T3	69.2	9.1	78.3	10.4	11.3	Sandy Loam	16.56	7.2	>200	2.8	56	39.48	22.05
Group 3													
Ref 3	22.2	15.0	37.2	11.1	51.7	Clay	36.48	6.1	52	3.9	56	47.20	60.75
P50T1	25.2	13.4	38.6	29.0	32.4	Clay Loam	38.16	6.7	63	1.1	56	28.59	54.51
P400T1	19.6	23.9	43.5	20.2	36.3	Clay Loam	37.44	6.8	>200	5.1	56	24.43	58.93
P150T3	8.4	15.2	23.5	21.4	55.1	Clay	49.20	6.8	16	2.5	42	40.71	61.76
P1000T3	10.3	19.5	29.8	29.8	40.4	Clay Loam	42.72	7.0	>200	5.7	42	n.d.	57.57

(1) Soil moisture in the samples used for microbial assessments.

Table 6.2 Total metal concentrations (mg/Kg) and the pollution index in sampled and respective reference soils.

Sites	Total (mg/kg)								Metal pollution index
	Pb	Cd	Cu	Zn	Cr	Ni	Fe	Mn	W
Ref. 1	16	<0.2	66	94	77	54	45000	840	1.26
Ref. 2	13	<0.2	18	24	16	28	2900	34	-0.89
Ref. 3	152	<0.2	40	260	59	40	53000	820	-0.37
P0	1264	<0.2	76	3800 (2.8)	72	57	52000	674	5.61
P20T1	133	<0.2	56	220	80	56	41000	780	2.44
P20T3	308	<0.2	56	420	78	60	49000	672	3.12
P50T1	164	<0.2	60	240	80	58	43000	720	2.60
P50T3	26074 (7.1)	62	3196 (8.2)	95940 (73.5)	80	40	117000	5880	13.63
P150T1	37460 (10.4)	771 (9.8)	594 (1.6)	42200 (33.5)	57	70	110000	1720	13.33
P150T3	2200	12	108	3300	84	58	56000	678	7.83
P400T1	961	8.8	60	840	64	48	35000	540	5.98
P400T3	179	0.3	44	90	59	46	34000	760	2.76
P1000T1	23	<0.2	60	80	62	46	48000	360	0.80
P1000T3	99	<0.2	56	156	84	52	49000	568	2.09

Numbers in superscript indicate an exceedance of the corrected Dutch $HC50_{EC50}$ values (after Rutgers et al. 2008) (Ex: the [Pb] at P150T1: 37460 (10.4), indicates that [Pb] was 10.4 times higher than the $HC50_{cor}Pb$).

6.3.2 Vegetation cover and successional stage

The highest vegetation cover were found outside the smelter area (Ref 1, Ref 2, P400T3, P1000T3, P1000T1 and P400T1; 67.5-100%, average = 86.0%, SD = 16.1%), whereas the lowest values were found close the smelter plant (P0, P20T1, P50T1, P150T1, P20T3, and P50T3, 20-100 %, average = 43.4%, SD = 26.9%) (Table 6.3). Vegetation cover correlated negatively with Widinarko's index ($r=-0.59$, $p<0.05$).

A total of 53 plant species were identified; the full list for each site is shown in supplementary material (Table A.1 in the Annex A). Sites P1000T3 and Ref2 were in a more advanced successional stage, with arboreal cover predominating over herbaceous, with occurrence of climbing plants, litter and mean diameter at breast high (DBH) of 8-18 cm. The other sites showed secondary vegetation in an initial stage of succession.

The axis 1 in the PCA represents a clear gradient of contamination, showing a clear separation between sites outside the area (including reference sites – right side of the axis) and contaminated sites inside (left side of the axis), which grouped showing a high similarity in plant composition (Fig. 6.2). The dissimilarity between plant community in sites inside and outside was 63.98% (SIMPER analysis) with both communities being significantly different (ANOSIM - global $R=0.36$, $p<0.01$). The homogeneity of vegetation composition inside the smelter was much higher (77.9% similarity) than outside (20.1% similarity).

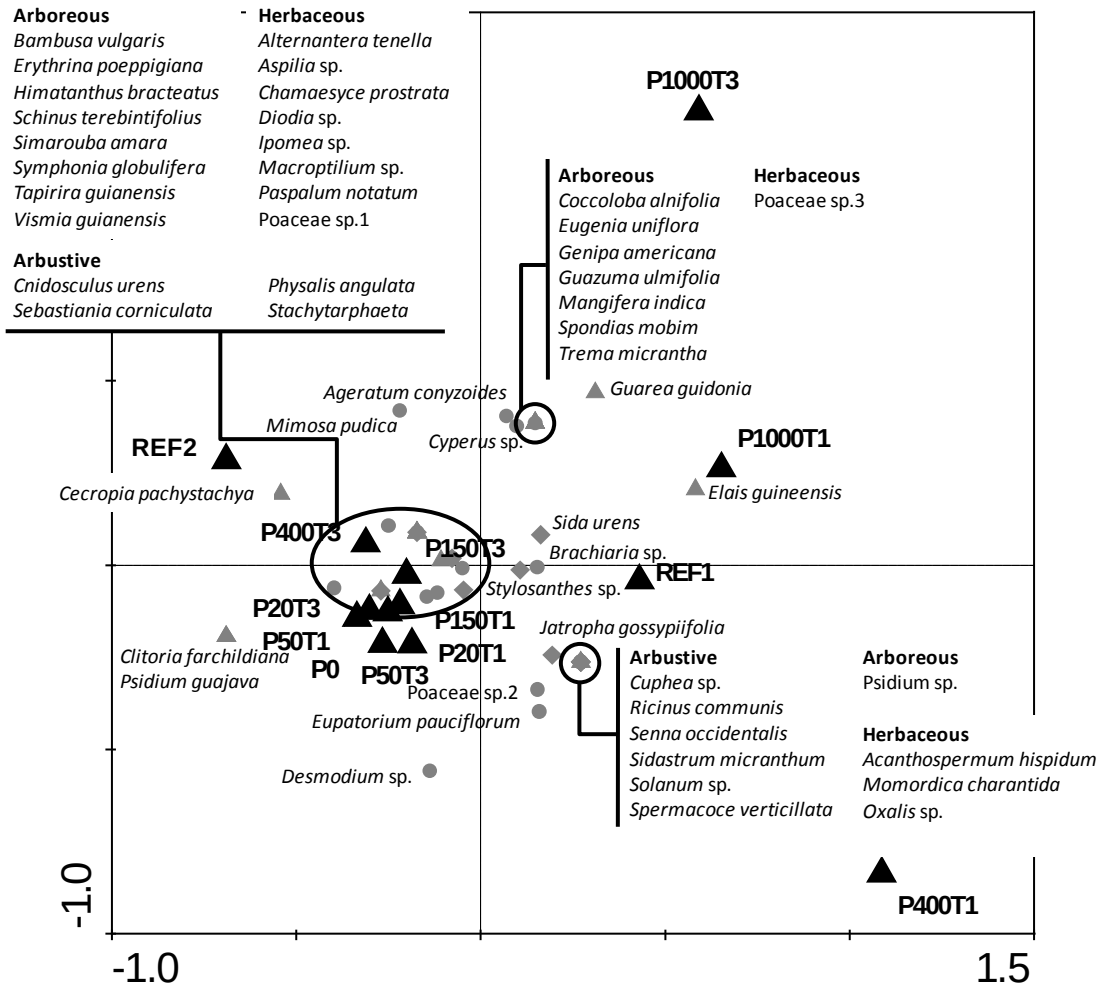

Fig. 6.2 Principal component analysis (PCA) diagram on plant community composition. Legend: grey upper triangles - arboreous species; grey diamonds – shrub species; grey circles – herbaceous species; black upper triangles – sampling points (no survey was possible in Ref3 due to logistic constrains). Variance explained: Axis 1- 24.4%; Axis 2 – 19.4%.

Table 6.3 Ecological parameters (average values ± standard deviation) for the assessed sampling points. The values for the three reference points were geometrically averaged to give an overall reference value. Asterisks indicate significant differences (* p<0.05; ** p<0.01; *** p<0.001) for a one-tailed hypothesis of a Dunnet's test between each sampling point and the overall reference value (assuming that Ref value higher than sampling point value and lower for Potential Nitrification). In the ANOVAs for soil microbial parameters, soil moisture, soil organic matter and soil nitrogen contents were used as co-variables (see text for more details). n - number of replicates.

Soil groups	Bait lamina (% pierced holes) n=5	Vegetation cover (%) n=4	Respiration (µg CO_2/ g soil/ day) n=3	MBC (µg/g) n=3	Dehydrogenase (µg PNP/ g/ d) n=3	Acid phosphatase (ug PNF/g/h) n=3	Nitrification (%) n=3	Decomposition rate [a] *k* (monthly) n=4
Overall reference	48.6 ± 13.9	81.3 ± 21.0	139.4 ± 106.4	642.4 ± 416.1	7.2 ± 2.3	617.1 ± 233.2	3.9 ± 2.2	0.2656 ± 0.1438
P0	18.4 ± 14.3 ***	22.5 ± 22.2 ***	34.9 ± 7.8***	178.1 ± 55.1***	0.7 ± 0.4 **	269.0 ± 22.1**	15.2 ± 9.6**	0.04667***
P20T1	17.8 ± 10.2 ***	30.0 ± 16.3 ***	35.1 ± 7.1***	252.4 ± 142.3**	1.3 ± 1.9**	196.4 ± 33.9***	12.4 ± 2.1*	n.d.
P20T3	30.4 ± 15.4	32.5 ± 12.6 ***	82.6 ± 15.8	170.3 ± 174.1 ***	1.4 ± 1.0**	443.4 ± 9.3	13.3 ± 3.5*	n.d.
P50T1	19.8 ± 6.8 ***	57.5 ± 12.6	41.4 ± 2.4**	412.9 ± 31.4	1.2 ± 2.0**	235.7 ± 50.3**	17.1 ± 4.9***	0.0632***
P50T3	11.8 ± 5.7 ***	20.0 ± 14.1 ***	52.2 ± 12.6**	461.7 ± 20.1	2.1 ± 0.5*	450.3 ± 45.4	13.5 ± 4.5*	0.0412***
P150T1	7.3 ± 8.1 ***	30.0 ± 42.4 ***	49.2 ± 6.6**	115.5 ± 87.0***	3.3 ± 0.5	355.3 ± 166.0*	8.7 ± 2.8	0.0435***
P150T3	5.5 ± 6.9 ***	57.5 ± 9.6	60.5 ± 9.2*	543.6 ± 160.8	2.1 ± 1.1*	651.2 ± 150.7	10.2 ± 0.5	0.0248***
P400T1	61.5 ± 23.8	100.0 ± 0.0	234.9 ± 83.3	797.3 ± 193.3	16.8 ± 3.7	573.1 ± 133.3	-0.2 ± 5.9	0.166*
P400T3	10.3 ± 6.7 ***	97.5 ± 5.0	165.2 ± 41.3	805.3 ± 216.2	1.5 ± 1.1**	792.0 ± 34.5	-3.3 ± 3.17	0.0423***
P1000T1	45.3 ± 16.1	67.5 ± 15.0	164.0 ± 79.1	1098.1 ± 184.1	4.8 ± 6.2	515.6 ± 353.5	1.9 ± 7.4	0.4515
P1000T3	26.3 ± 17.5 *	100.0 ± 0.0	n.d.	n.d.	n.d.	n.d.	n.d.	0.3826

MBC – Microbial biomass Carbon
n.d. - not determined
a after log of percentages

6.3.3 Surface dwelling invertebrates

A total of 1,277 individuals, grouped into 72 morphospecies, were collected in the pitfall traps. Hymenoptera, Coleoptera and Orthoptera occured in all sites, Araneae and Dermaptera in 92.3%, and Opiliones in 69% of the sites (Table 6.4). Isopoda (30.8% of sites), Diplopoda and Hemiptera (15.4%), and Mantodea (1 site) were less frequent. Coleoptera and Araneae presented the highest morphospecies richness (20 and 19, respectively), followed by Hymenoptera with 16 morphospecies. Hymenoptera was the most abundant group in terms of individuals (n = 459), followed by Coleoptera and Araneae (n = 265 each).

Differences in abundance, morphospecies richness or biodiversity descriptors were only found when comparing sites inside and outside the smelter area. Sites outside the area (namely Ref2, P400T3 and P1000T3) presented more morphospecies than sites inside ($t=2.48$, $p<0.05$). However, no significant differences were detected for abundance or any other descriptor of biodiversity (Table 6.4). Analyzing each major faunal group individually, the aforementioned trend was found for abundance and richness of spiders ($t=2.51$, $p<0.05$ and $t=2.63$, $p<0.05$, respectively), and abundance of opilionids ($t=2.61$, $p<0.05$). In contrast, hymenopterans followed the inverse trend for number of individuals ($t=2.72$, $p<0.05$). No correlation was found between none of the invertebrate parameters and WPI.

The PCA showed marked dissimilarity between sites inside and outside the smelter area (Fig. 6.3), with sites outside (400 m or more beyond P0) located in the positive side of axis 1. The ANOSIM revealed significant differences between these two groups (Global $R=0.239$, $p<0.01$). This separation pattern was mainly attributed to 40 morphospecies (SIMPER analysis, average dissimilarity 74,6%), with the highest contributions (up to 50%) attributed to morphospecies of Hymenopeta (2), Coleoptera (3), Orthoptera (2), Dermaptera (1) and Opilionidae (1).

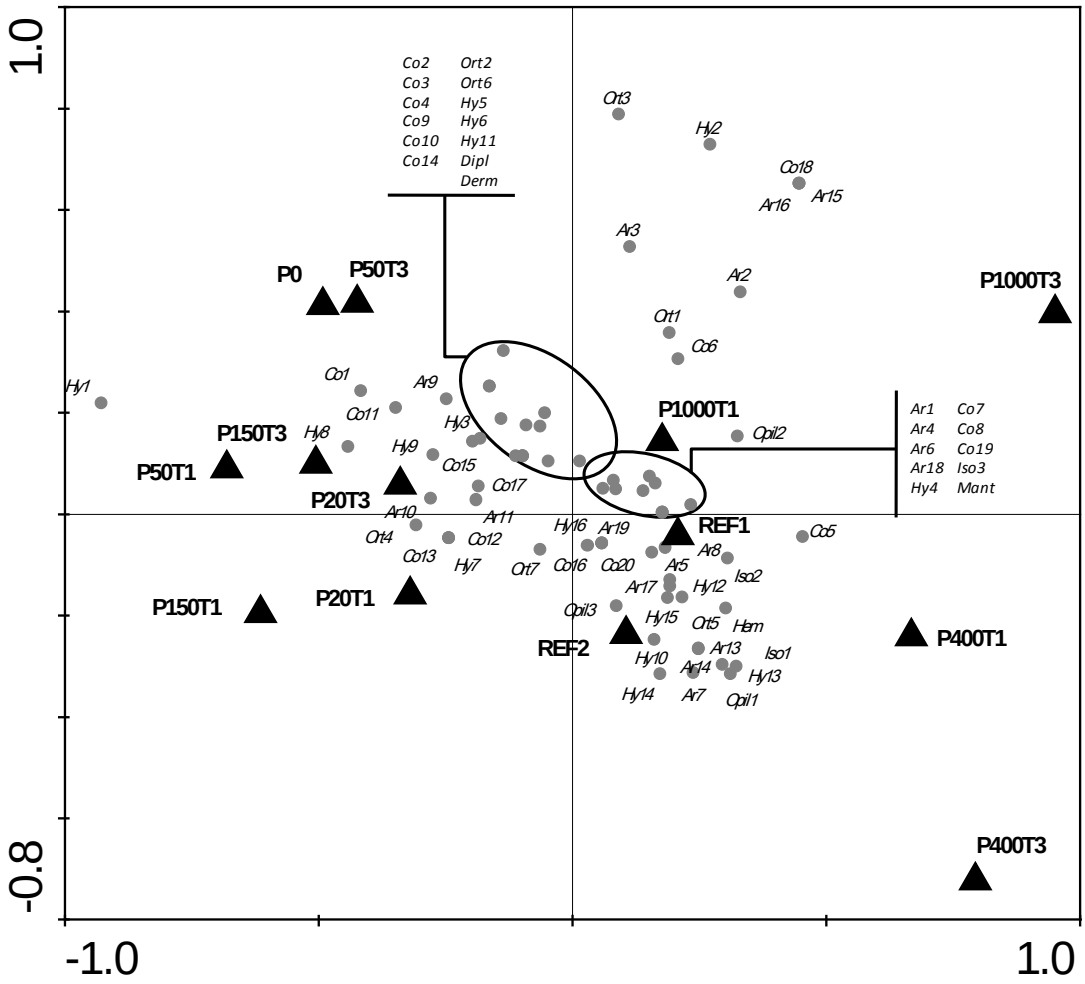

Fig. 6.3 Principal component analysis (PCA) diagram on surface dwelling arthropods. Legend: grey circles – morphospecies; black upper triangles – centroids of the sampling points (no survey was possible in Ref3 due to logistic constrains). Variance explained: Axis 1- 17.7%; Axis 2 – 13.6%.

Table 6.4 Number of individuals and morphospecies (shown in brackets) of each Order of surface dwelling invertebrates caught in pitfall traps (n=3) at each site, and the ecological descriptors for each site, considering all groups together.

	Sites														
	Ref 1	Ref 2	P0	P20T1	P20T3	P50T1	P50T3	P150T1	P150T3	P400T1	P400T3	P1000T1	P1000T3	Sites inside smelter	Sites outside smelter
Orders															
Araneae (Ar)	73 (5)	9 (4)	4 (3)	2 (2)	7 (4)	2 (1)	3 (1)	5 (4)		14 (4)	5 (5)	2 (2)	139 (7)	228 (15)	37 (9)
Hymenoptera (Hy)	15 (5)	15 (5)	31 (3)	26 (3)	48 (4)	76 (3)	57 (5)	33 (2)	52 (4)	15 (3)	16 (6)	45 (5)	30 (3)	121 (13)	338 (10)
Coleoptera (Co)	11 (4)	13 (6)	8 (4)	2 (2)	28 (7)	16 (5)	32 (7)	2 (1)	14 (5)	88 (4)	13 (3)	7 (4)	31 (4)	75 (11)	190 (17)
Orthoptera (Ort)	14 (3)	10 (3)	16 (4)	8 (3)	21 (3)	13 (2)	21 (6)	4 (2)	9 (2)	10 (2)	16 (2)	24 (2)	23 (4)	87 (5)	102 (7)
Isopoda (Iso)			1 (1)							5 (2)	11 (1)	1 (1)		12 (2)	6 (3)
Opiliones (Opil)		4 (2)		1 (1)	2 (2)	2 (1)			1 (1)	6 (1)	7 (3)	10 (1)	2 (2)	23 (3)	12 (3)
Dermaptera (Derm)	3 (1)	2 (1)		1 (1)	9 (1)	4 (1)	4 (1)	1 (1)	6 (1)	2 (1)	5 (1)	2 (1)	1 (1)	13 (1)	27 (1)
Hemiptera (Hem)		1 (1)								1 (1)				1 (1)	1 (1)
Diplopoda (Dipl)				1 (1)								2 (1)		2 (1)	1 (1)
Mantodea (Mant)		1 (1)												1 (1)	0
Biodiversity descriptors															
ABUNDANCE (total)	116	55	60	41	115	113	117	45	82	141	73	93	226		
TAXA (total)	18	23	15	13	21	13	20	10	13	18	21	17	21		
SHANNON	2.59	4.06	2.83	2.85	3.67	2.48	3.38	1.86	2.32	3.05	3.71	3.33	2.24		
PIELOU	0.62	0.90	0.72	0.77	0.84	0.67	0.78	0.56	0.63	0.73	0.85	0.81	0.51		
MARGALEF	3.58	5.49	3.42	3.23	4.22	2.54	3.99	2.36	2.72	3.44	4.66	3.53	3.69		
BERGER-PARKER	0.58	0.18	0.45	0.37	0.22	0.52	0.33	0.69	0.56	0.40	0.15	0.19	0.58		

6.3.4 Soil fauna feeding activity (BLT)

Lower feeding activity was registered in sites within the smelter area, usually associated with contamination (P0, P150T1, P50T3 and P150T3) or low organic matter content (P20T1 and P50T1) (Table 6.3). Point P400T3 also presented low feeding activity, however other soil or habitat parameters (not assessed) must explain these results. A significant negative correlation ($r = -0.72$, $p<0.01$) was found between feeding activity and metal loading, given by WPI.

6.3.5 Microbial parameters

Microbial parameters generally decreased inside the smelter area in relation to the overall reference, except for nitrification, which showed inverse trend (Table 6.3).

Most of parameters correlated negatively with the metal loadings (WPI), like microbial respiration ($r=-0.70$, $p<0.001$), microbial biomass carbon ($r=-0.64$, $p<0.001$), phosphatase activity ($r=-0.47$, $p<0.05$) and nitrification ($r=0.43$, $p<0.05$), while no correlation was found for DHA.

The *p*PCA showed a clear separation of the sites outside the smelter area, except P400T1 (Fig. 6.4). Such separation was confirmed by an ANOSIM (Global $R=0.091$, $p<0.05$) and by a SIMPER analysis, where microbial respiration, microbial biomass carbon and phosphatase activity contributed to over 90% of the dissimilarity between both groups.

6.3.6 Organic material breakdown

The validity criterion of 60 % of mass loss in the reference site at the end of the study (Römbke et al. 2003) was fulfilled. The monthly decay rate in contaminated sites within the smelter area was lower than in the overall reference sites (Table 6.3). Only sites located 1000 m away from P0 presented higher decay rates than the reference sites. Considering the threshold value proposed by Römbke et al. (2003) of >25% difference in mass loss between contaminated and the reference sites at the end of the study, all sites within the smelter area showed an unacceptable risk, where differences compared to the overall reference ranged between 30.5% and 64.1% after 131 days of exposure. A significant and negative correlation was found between the monthly decay rate and WPI ($r=-0.61$, $p<0.05$).

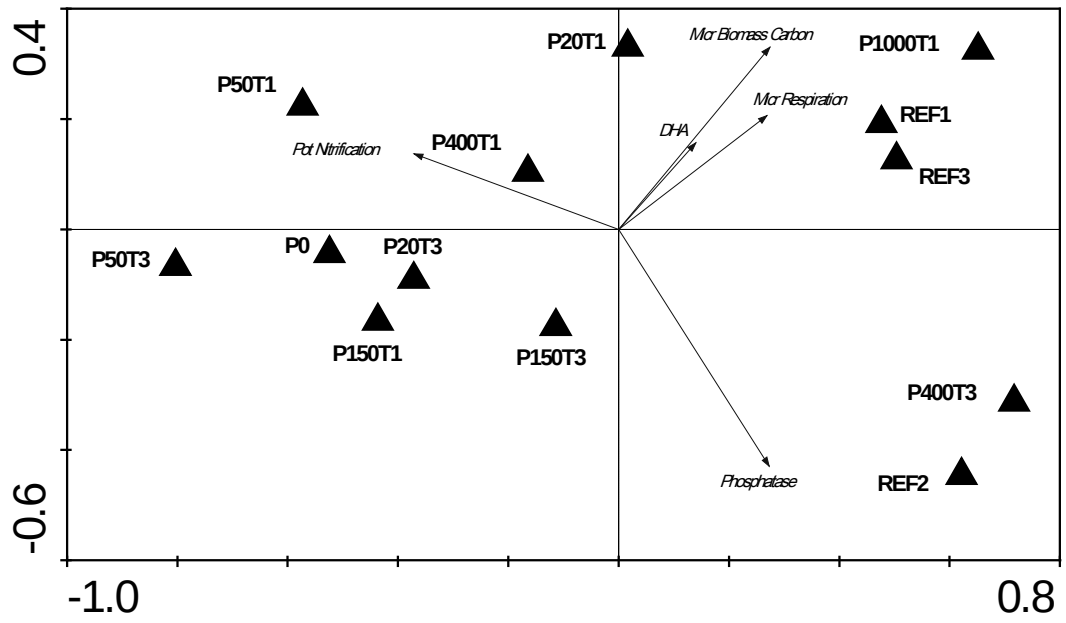

Fig. 6.4 Partial Principal component analysis (pPCA) ordination on the microbial parameters. Black upper triangles represent centroids of the sampling points (no survey was possible in P1000T3 due to logistic constrains). Variance explained: Axis 1- 60.1%; Axis 2 – 16.2%.

6.4 Discussion

6.4.1 Impairment of ecological parameters

The smelting activity caused a high level of metal contamination in the study area, besides moderate to high levels of some metals (namely Pb and Zn) in the vicinity due to deposition of tailings and contaminated dust. No clear contamination gradient could be found along the two transects. This spatial heterogeneity is caused not only by unequal deposition of residues, but also by erosion on the (pseudo) rehabilitated tailings, leaving the pile partially exposed.

Metal contamination allied to loss of habitat affected the different ecological parameters. The general trend was an impaired ecological response inside the smelter area in comparison with the sites outside.

The vegetation cover inside the smelter area decreased and was more homogeneous, with a high frequency of cropped arboreal and invasive herbaceous species in an initial successional stage. This is attributed to metal toxicity, even 17 years after the smelting activity stopped, in addition to the failure in rehabilitating the area. Van Assche and Clijsters (1990) also found potential phytotoxicity in the soil surrounding a zinc smelter more than 20 years after the smelter closure. In addition, nutrient imbalances and a reduced water-holding capacity restrict the plant recolonization on degraded sites (Tordoff et al. 2000; Salemaa et al. 2001), conditions observed in the sites where soil encapsulation was not effective.

A diverse and structured plant community helps to maintain essential functions in soil like cycling processes, providing food and habitat to a highly active and diverse community of decomposing organisms (Balvanera et al. 2006; Jensen and Mesman 2006). However, contamination mostly impaired plant community and habitat conditions inside the smelter area, affecting other ecological parameters, namely the soil fauna feeding activity and surface dwelling invertebrates. In fact, data from the BLT showed a strong impairment of feeding activity in sites within the smelter area associated to a high contamination or to a low organic matter content, in addition to a low vegetation cover. Similar results have also been shown in grasslands with gradient of metal contamination (Filsek et al. 2004) and abandoned uranium mine in Portugal (André et al. 2009), where the abundance and diversity of key detritivore groups decreased. In fact, the feeding activity measured via BLT seems to be related with the abundance of different faunal groups, namely earthworms (van Gestel et al. 2003) and collembolans (Helling et al. 1998; Birkhofer et al. 2011). Although not assessed in this study, an impact on such organisms would also be expected due to the high metal contamination and the loss of habitat function (Fountain and Hopkin 2004).

Besides a significant decrease in the total morphospecies richness inside the smelting area, changes in community composition of surface dwelling invertebrates also occurred. Community outside was more abundant and rich in morphospecies of spiders and opilionids, whereas the community inside the area presented more abundance of hymenopterans. Ferreira (2010) reported a decrease in the abundance of spiders with the increase of metals in soil in a copper mine, showing that different guilds responded differently to contamination, in which the ground hunters were most affected. Read et al. (1998), studying epigeic macroarthropods along a metal contamination gradient, reported that few species were able to adapt to contamination and a larger number was found at non-contaminated sites. Besides the direct effects of contamination, the decrease in abundance (241 vs. 23 individuals) and species richness (16 vs. 8) inside the smelter area can also be attributed to indirect effects. Depletion of preys for specialist species, and the impoverishment of the habitat structure, may impair the trophic requirements for many species.

The inverse trend observed for abundance of ants is in agreement with Grzes (2009), who found an increase in species richness along a metal contamination gradient. Metals may be negative to several

ecological parameters, mainly colony size and relative abundance (Grzes 2010), but ants have the ability to regulate and resist in metal contaminated sites. In this case indirect effects on and population may have occurred. Ants may have benefited from spider and/or coleopteran decreases in abundance or richness as these groups can act as their predators or competitors. In addition, metal pollution affected the habitat structure, creating patches of low vegetation cover, resulting in increase in soil temperature and decrease in moisture, which may have favored the thermophilic species (Grzes 2009).

Metal contamination highly impaired the microbial community and processes, as all parameters inside the smelter area significantly differed from the reference sites. Moreover, significant negative correlations with metal loadings were found. Kapustka (1999) has not advised the inclusion of microbial parameters for ecological assessments due to their strong functional redundancy, rapid change across a small spatial scale, and high influence of confounding factors (e.g. moisture, nutrients). However, several other authors have reported a decrease in enzyme activity, microbial biomass carbon and basal respiration (Zimakowska-Gnoinska et al. 2000; Gülser and Erdogan 2008; Jiang et al. 2010) in impacted soils, pointing out its usefulness in assessing metal effects on microbial functions in contaminated areas. Soil microbial communities and the key processes they mediate are closely related to vegetation and soil use (Zak 2003; Nogueira et al. 2006; Fagotti et al. 2012). Vegetation contribute to reduce metal toxicity by offering favorable conditions in the rhizosphere (Dias-Junior et al. 1998) and in the bulk soil due to inputs of organic residues that act as carbon and energy supply, in addition to a protective effect against metals by chelation. The failure in establishment of vegetation inside the smelter area, especially in tailing deposits, also contributed to decrease the microbial activity and biomass.

Nitrification was the only microbial parameter that increased significantly among the most contaminated sites inside the smelter area. Although considered sensitive to metal stress (Broos et al. 2005), adaptation of nitrifying populations to metal-contaminated sites have been shown (Mertens et al. 2006). High nitrification rates may indicate unbalance in the N-cycling, leading to N losses by leaching or denitrification. The nitrification rate tends to decrease along the successional stages in a forest (Singh et al. 2001), and thus recently disturbed ecological systems show higher nitrification rates, but decreases along the successional status (Montagnini et al. 1989).

Litter breakdown was also significantly impaired within the smelter area, with decay rate being negatively correlated with metal loadings. Although showing only transient or no effects under some stressors (e.g., Dinter et al. 2008; van Gestel et al. 2009; Podgaiski and Rodrigues 2010), some studies have revealed significant effect of metal (e.g. Creamer et al. 2008) or pesticide contamination (Förster et al. 2006) in this parameter.

The litter bag study showed low decay rates in the most contaminated sites. The reduced microbial activity, faunal feeding activity and density of detritivores, allied to a low moisture and high temperature in the more exposed sites due to the low vegetation cover, may have contributed to reduce the litter decomposition in these sites. Thus, the effects on litter decomposition is attributed not only to a direct effect of metals on microbial and faunal communities, but also to indirect effects leading to non-suitable habitat conditions for soil fauna and microbial communities as mentioned earlier.

6.4.2 Sensitivity of ecological parameters for risk assessment

The sensitivity analysis took into account not only the ability of each parameter to detect differences between contaminated and non-contaminated sites, but also the ability to detect a gradient of contamination and time necessary to obtain the parameter (Table 6.5).

All microbial parameters were able to differentiate the contaminated from the reference sites. Except for DHA, they tended to give similar information because showed significant correlations among them. However, only two of them (basal respiration and microbial biomass carbon) presented a high capacity to distinguish the level of soil contamination. Since these two parameters were highly correlated ($r=0.82$, $p<0.001$), assessing only one is enough to have information relative to microbial activity. .

Conversely to microbial parameters, soil fauna structural parameters were not able to detect contamination gradients when considering each site individually. . However, abundance and taxonomic richness were able to differentiate the sites inside and outside the smelter. These results were somehow expected since we sampled highly mobile surface dwelling organisms, which are not associated to properties of a particular site, but with features of a large area around the site. Semezin et al. (2008) found that soil dwelling invertebrate abundance, taxonomic richness and the QBS index (a measure of soil quality based on microarthropods morphotypes) were sensitive parameters for assessing effects of soil contamination with metals and PAHs. Similarly to our findings, diversity indices were not sensitive to contamination. More elaborated conclusions could be taken, namely in terms of effects to particular functional groups and to find better cause-effect relationships, if identification would to be done at species level. However, the separation into morphospecies seems to be sensitive enough for a first evaluation of contamination or habitat disruption. Therefore, this metric should be incorporated in the ecological line of evidence when soil fauna is an ecological receptor under potential risk.

Contrary to structural parameters of fauna, the feeding activity was sensitive to soil metal contamination. This high sensitivity, allied to the fact that several studies showed a relation between BLT and abundance of microarthropods and lumbricids (Birkhofer et al. 2011), and the possibility to have data

from a large number of sampling points over a short period of time, makes the BLT a definitive parameter to be included in the Ecological LoE in site specific assessments (particularly in tier 1).

Despite significantly different in sites inside and outside the smelter area, vegetation cover and composition were not able to detect gradients of contamination. Critto et al. (2007) presented a low rank for vegetation related parameters in Tier 1, mainly due to their cost. However in higher tiers (2 or 3) such parameters presented higher ranking mainly related to their site-specific relevance. In this case, we consider relevant the parameters on vegetation since they showed/explained important derived effects of contamination, especially those related to habitat disruption. So, at different levels, both assessed parameters (vegetation cover and species composition) should be incorporated in the Ecological LoE. Plant litter decomposition showed high sensitivity to contamination and habitat disruption, but presented low capacity to differentiate the level of contamination. Despite being sensitive, it gave similar information as the BLT, thus not being a priority parameter to integrate a tiered scheme, also due to the long time needed for obtaining data.

6.5 Conclusion

In general, the ecological parameters indicated a clear distinction between sites inside and outside the smelter area, indicating an ecological risk to soil system even 17 years after the smelting activities stopped. Metal-rich tailings within the area and the failed attempt to encapsulate them have impaired the proper establishment of vegetation, leading to a simplification of the habitat structure, which conducted to low organic matter input into the soil to act as source of carbon and energy for microbial growth and for acting as protection for microbial community against high levels of metals in soil. Moreover, these changes caused negative impacts on microbial activity and on soil organisms (feeding activity and species composition of surface dwelling organisms), consequently affecting the ecosystem processes that they mediate.

The suitable encapsulation of the smelter residues, with the concomitant re-establishment of a vegetation cover, seems to be essential to improve the ecological conditions at this site, preventing further soil loss and contributing to improve the soil function, minimizing the risk of air and water pollution.

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Annex A

Table A.1 Plant species identified in the study area at each sampling point.

Species	Family	Habit	Ref 1	Ref 2	P0	P20T1	P20T3	P50T1	P50T3	P150T1	P150T3	P400T1	P400T3	P1000T1	P1000T3
Acanthospermum hispidum DC.	Asteraceae	Herbaceous										X			
Ageratum conyzoides L.	Asteraceae	Herbaceous											X		X
Alternantera tenella Colla	Amaranthaceae	Herbaceous		X											
Aspilia sp.	Asteraceae	Herbaceous			X			X							
Bambusa vulgaris Schrad	Poaceae	arborescent		X	X	X	X	X	X	X	X	X	X	X	X
Brachiaria sp.	Poaceae	Herbaceous	X					X		X				X	
Cecropia pachystachya Trécul	Urticaceae	Arboreous		X	X	X	X	X	X	X	X		X		X
Chamaesyce prostrata (Aiton) Small	Euphorbiaceae	Herbaceous						X		X					
Clitoria farchildiana Howard	Leguminosae	Arboreous		X	X	X	X	X	X	X	X		X		
Cnidosculus urens (L.) Arthur	Euphorbiaceae	Arbustive		X	X	X	X	X	X	X	X	X	X		X
Coccoloba alnifolia Casar	Polygonaceae	Arboreous													X
Cuphea sp.	Lythraceae	Arbustive										X			
Cyperus sp.	Cyperaceae	Herbaceous									X				X
Desmodium sp.	Leguminosae	Herbaceous	X		X	X	X	X	X	X	X	X	X		
Diodia sp.	Rubiaceae	Herbaceous		X									X		
Elais guineensis Jacq.	Arecaceae	arborescent										X		X	X
Erythrina poeppigiana (Walpers) O.F.Cook	Leguminosae	Arboreous		X											
Eugenia uniflora L.	Myrtaceae	Arboreous													X
Eupatorium pauciflorum Kunth	Asteraceae	Herbaceous				X			X			X			
Genipa americana L.	Rubiaceae	Arboreous													X
Guarea guidonia (L.) Sleumer	Meliaceae	Arboreous												X	X
Guazuma ulmifolia Lam.	Malvaceae	Arboreous													X
Himatanthus bracteatus (A. DC.) Wood.	Apocynaceae	Arboreous		X											
Ipomea sp.	Convolvulaceae	Herbaceous											X		
Jatropha gossypiifolia L.	Euphorbiaceae	Arbustive										X	X		
Macroptilium sp.	Leguminosae	Herbaceous											X		
Mangifera indica L.	Anacardiaceae	Arboreous													X
Mimosa pudica L.	Leguminosae	Herbaceous		X				X		X			X		X
Momordica charantida L.	Cucurbitaceae	Herbaceous										X			

Table A.1 (Continued)

Species	Family	Habit	Ref 1	Ref 2	P0	P20T1	P20T3	P50T1	P50T3	P150T1	P150T3	P400T1	P400T3	P1000T1	P1000T3
Oxalis sp.	Oxalidaceae	Herbaceous										X			
Paspalum notatum Flügge	Poaceae	Herbaceous		X	X		X		X						
Physalis angulata L.	Solanaceae	Arbustive		X											
Poaceae sp.1	Poaceae	Herbaceous									X				
Poaceae sp.2	Poaceae	Herbaceous						X		X		X			
Poaceae sp.3	Poaceae	Herbaceous													X
Psidium guajava L.	Myrtaceae	Arboreous		X	X	X	X	X	X	X	X		X		
Psidium sp.	Myrtaceae	Arboreous										X			
Ricinus communis L.	Euphorbiaceae	Arbustive										X			
Schinus terebintifolius Raddi	Anacardiaceae	Arboreous		X	X	X	X	X	X	X	X	X	X		X
Sebastiania corniculata (Vah.) Mull. Arg.	Euphorbiaceae	Arbustive				X									
Senna occidentalis (L.) Link	Leguminosae	Arbustive										X			
Sida urens L.	Malvaceae	Arbustive												X	
Sidastrum micranthum (St.-Hil.) Fryxell	Malvaceae	Arbustive										X			
Simarouba amara Aubl.	Simaroubaceae	Arboreous		X											
Solanum sp.	Solanaceae	Arbustive										X			
Spermacoce verticillata L.	Rubiaceae	Arbustive										X			
Spondias mobim L.	Anacardiaceae	Arboreous													X
Stachytarphaeta sp.	Verbenaceae	Arbustive											X		
Stylosanthes sp.	Leguminosae	Arbustive	X												
Symphonia globulifera L.	Clusiaceae	Arboreous		X											
Tapirira guianensis Aubl.	Anacardiaceae	Arboreous		X											
Trema micrantha (L.) Blume	Cannabaceae	Arboreous													X
Vismia guianensis (Aubl.) Choisy	Clusiaceae	Arboreous		X											

Chapter 7

Ecological risk assessment of a metal-contaminated area in the tropics. Tier II: detailed assessment.

Abstract

The present study presents data on the detailed evaluation (tier 2) of a site-specific ecological risk assessment in a former smelter area heavily contaminated with metals (Santo Amaro, Bahia, Brazil). Joining information from three lines of evidence (LoE), chemical, ecotoxicological and ecological, integrated risk values were calculated to rank sites within the area and confirm the potential risk pointed in tier 1. Risk values were calculated separately for the habitat and for the retention functions in each point. Habitat function included the chemical LoE calculated based on total metal concentrations. The ecotoxicological LoE based on reproduction tests with terrestrial invertebrates (reproduction of *Folsomia candida*, *Enchytraeus crypticus*, *Eisenia andrei*), growth and plant biomass (*Avena sativa* and *Brassica rapa*). For the ecological LoE, ecological parameters, embracing microbial, soil invertebrates and litter breakdown were used to derive risk values. Retention function included the chemical LoE, calculated based on extractable metal concentrations, and the ecotoxicological LoE based on eluate tests with aquatic organisms (reproduction of *Daphnia magna* and growth of *Pseudokirchneriella subcapitata*). Ecological and ecotoxicological results in habitat function indicated that the metal residues are sufficient to cause risk to biota. The most affected endpoints in ecotoxicity tests were reproduction of *E. crypticus* and *E. andrei*, and plant biomas. The ecological LoE, based on microbial parameters, litter breakdown and arthropods diversity, indicated damage to soil structure and function in several sites. In spite of the high total metal concentrations in soil, the low metal levels in extracts and the lack of toxicity in aquatic tests using eluates indicated a high soil retention function in most of the selected sites. Integrated risk was low outside the smelter area. Inside, a high spatial heterogeneity of risk levels was observed, related to the non homogeneous deposition of smelting residues. Integrated risk of tier 2 showed the same trend of tier 1, with no risk to retention function in most of the selected sites, but a loss of habitat function in some of them. High risk levels, associated with sandy soils and residue deposits, suggest the need to proceed with remediation actions. These high risk levels observed inside the smelter due to metals were not only of a direct nature (e.g., strong toxicity to some tested organisms), but also of indirect nature, with the failure of the establishment of vegetation and the consequent loss of habitat quality of microorganisms and soil fauna. This study point some light in the selection of tools for the tier 2 of the ERA in a tropical metal contaminated site, focusing on ecological receptors in risk and using available chemical methods, ecological surveys and ecotoxicity tests.

Keywords: Integrated risk values, Lines of evidence, Soil habitat function, Soil retention function, Triad

7.1 Introduction

Ecological risk assessment (ERA) is a process of collecting, organizing and analyzing environmental exposure and effect data to estimate the risk of contamination to ecosystems, being a useful tool, for instance, in managing the risk of contaminated lands (Jensen and Mesman 2006). Only a site specific risk assessment integrating contaminant exposure and biological effects, either through bioassays or in situ surveys, may reveal potential adverse effects of specific (point or diffuse) pollution problems (Posthuma et al. 2008), since toxicity cannot simply be extrapolated from mixtures of contaminants measured in soil due to interactions between them and potential alterations in their bioavailability caused mainly by soil properties and ageing (Vasseur et al. 2008). Thus, chemical analysis need to be complemented with bioassays, which have the key advantage of assessing the toxicity of the whole soil matrix, i.e., of the bioavailable mixture of contaminants, including degradation products and metabolites. Moreover, indirect effects of chemicals, like changes in food availability, habitat and soil structure, may be more important in ERA than direct toxicity (Heimbach 1997), and such impacts can best be evaluated through *in situ* ecological surveys.

For the process of risk characterization, the Triad approach, which consists of integrating three lines of evidence (LoE), chemical, ecotoxicological and ecological (Long and Chapman 1985), has widely been recommended and successfully applied in site specific ERA of contaminated soils (Jensen and Mesman 2006; Wagelmans et al. 2009). The Triad approach is usually applied within a tiered system, i.e., information from each LoE is collected at each tier following a step-wise cost-effective process (Jensen and Mesman 2006). While tier 1 is essentially a screening phase, tier 2 is performed to reduce uncertainties about the actual risk. Thus, the tools used in tier 2 to collect information of each LoE should be directed to indicate long-term effects, derived from habitat disruption, while being more ecologically relevant and of a high capacity to differentiate levels of contamination (Jensen and Mesman 2006; Critto et al. 2007).

In tier 2, the chemical LoE should comprise extraction techniques to quantify the available fraction of the contaminants in soil, complementing the data obtained with the total contaminant concentrations. This LoE should be complemented with information derived from ecotoxicological tests (ecotoxicological LoE) and ecological surveys (ecological LoE). At this

phase, the ecotoxicological LoE usually comprises long-term tests to assess both the habitat and retention functions of the soil (ISO 2003) focusing on sublethal endpoints (Weeks et al. 2004; Critto et al. 2007).

For the soil matrix, standardized reproduction tests with Oligochaeta (ISO 1998a, 2004) and Collembola (ISO 1999) have been recommended to evaluate sub-lethal effects on soil fauna (e.g., Gonzalez et al. 2011; Natal-da-Luz et al. 2011). Standard tests with plants (ISO 2005) are also recommended as part of tests batteries for the ecotoxicological characterization of soils within ERA processes (van Gestel et al. 2001; Achazi 2002; Pandard et al. 2006) and, in particular, they have been widely used in toxicity assessments in metal contaminated areas (Öncel et al. 2000; Everhart et al. 2006; Loureiro et al. 2006; Alvarenga et al. 2008). To evaluate the soil retention function, soil extracts are prepared to perform widely established standardized tests with cladocerans and microalgae to evaluate the retention function of soil (e.g., OECD 1984a, 1998; Chelinho et al. 2010; Natal-da-Luz et al. 2012), as recommended by ISO guidelines on the ecotoxicological characterization of soils (ISO 2003). Finally, the ecological information collected at tier 2 will provide information on the actual impacts on populations and communities of flora and fauna at the study sites (Jensen and Mesman 2006). Surveys of species diversity and population structure of soil invertebrates and fauna and soil microbial parameters and decomposition rates are often applied at this LoE. Compared with the other LoE, the latter has the disadvantage that is generally very time consuming and may require more specialized knowledge (Suter et al. 2000).

This study aimed to conduct the tier 2 of a site-specific ERA of a metal contaminated area in Santo Amaro (BA, Brazil), following the Triad approach, i.e., joining information from the three LoE mentioned above, and complementing the analysis (trying to reduce some uncertainties) done during tier 1 (Chapter 3; Niemeyer et al. 2010). The results obtained in the screening phase (tier 1) indicated very high risk levels at some sampling points, associated with residue deposits, which suggested the need to proceed with remediation actions. However, uncertainties generated by contradictory information among LoE at certain sampling points indicated the need to further elucidate potential risks through a more detailed assessment, to rank sites within the study and fully identify those that need remediation actions (tier 2). To more comprehensively perform tier 2, the present study proposes, within each LoE, to separately calculate the ecological risk for the habitat function and retention function of the soil at each sampling point.

7.2 Materials and methods

7.2.1 Study area

The present study was carried out in an abandoned industrial area in Santo Amaro, BA, Brazil, presenting a severe metal contamination originated from a lead smelter that was operational for 33 years (1960-1993). Human and animal contamination (Costa 2001; Carvalho et al. 2003), and very high levels of metals in soil and water (Anjos 2003; Machado et al. 2004) have been reported, caused by the tons of contaminated debris deposited around the smelter area (aprox. 180,000 m^3) and under roads and house´s backyards (aprox. 55,000 m^3), as well as by the aerial dispersion and deposition of dusts (covering a larger area up to 3 Km from the area) while the smelter was operational. More details are in Niemeyer et al. (2010). The soils in the sampling sites are Vertisols and Inceptisols (Soil Taxonomy, USDA) originated from carbonaceous shale, rich in expansive clay (montmorilonite), with generally low porosity and consequently low permeability (Machado et al. 2002).

7.2.2 Soil sampling and selection of reference soils

Two 1-km transects (T1 and T3) were defined along the two major detected gradients of contamination (Fig. 7.1). The two transects shared a central point (P0 – located close to the smelter plant) and comprised 5 sampling points, each at 20, 50, 150, 400, and 1000 m from P0.

Based on a multivariate factor analysis using soil properties data (metals excluded), soil samples were assembled into three groups mainly differing in terms of texture, organic matter content and pH. Soils from several points in the surrounding of the area were screened, analyzed for metals and soil properties, and three reference soils (the best possible for each identified group of sampling points) were selected at 3 km (Ref. 2 and 3) and 9 km (Ref. 1) from P0 Details about soil sampling and groups are shown in Chapter 3 (Niemeyer et al. 2010).

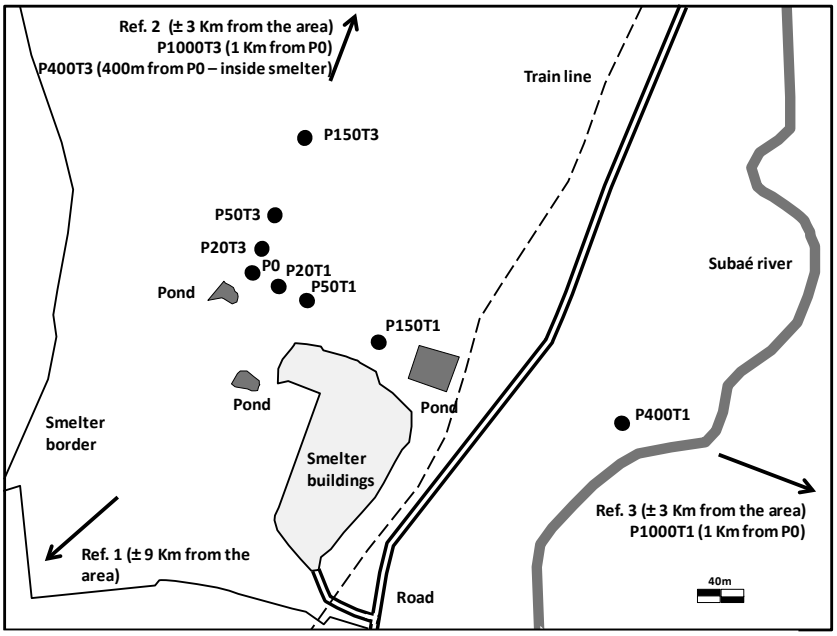

Fig. 7.1 Schematic representation of the study area (an abandoned lead smelter, Santo Amaro, BA, Brazil) showing the location of the 11 sampling points along the two transects and of the three reference points.

7.2.3 Chemical analysis (Chemical LoE)

Based on the historical use of the site and on a previous study (FUNASA 2003), soils were analyzed for the four main metals responsible for the contamination of the area (Pb, Cd, Cu, and Zn), and also for Cr, Ni, Fe, Co, and Mn. Metals were quantified in the bulk soil and in extracts, obtained by shaking 15 g of soil (dry weight) for 2 h:30 min at 200 rpm with 150 ml of a 0.01 M $CaCl_2$ solution. The slurry was then centrifuged for 5 min at 3,000 rpm and the extracts (supernatants) were filtered through a Schleicher & Schuell filter paper (Dassel, Germany, Reference nº 595). Metals were quantified in the bulk soil and in extracts by inductively coupled plasma-atomic spectroscopy.

Other soil physico-chemical parameters measured were pH (KCl 1M) (ISO 1994a), water holding capacity (WHC; ISO 1998b), cation exchange capacity (ISO 1994b), organic matter (OM) content (loss on ignition at 500 ºC for 6 h) and soil texture (LNEC 1970).

Total metal concentrations were compared with the benchmarks median hazard concentration ($HC50_{EC50}$ values; see explanation in Chapter 3) and Ecological Soil Screening Levels (Eco-SSLs) for plants (USEPA 2004). The latter consist in the geometric mean of the

maximum acceptable toxicant concentration for several species under different test conditions of pH and percentage of organic matter, developed to be used in the screening phase of the Superfund ERA process.

7.2.4 Soil invertebrates reproduction tests (Ecotoxicological LoE)

Reproduction tests with *Enchytraeus crypticus* (28 d), *Eisenia andrei* (56 d) and *Folsomia candida* (28 d) were conducted following the ISO standard guidelines (ISO 2004, 1998a, 1999), except for the test temperature which was adjusted for 25ºC, rather than for 20 ºC. All soils were tested at 100%. At the end of the exposure period, reproduction was estimated as the mean number of juveniles per replicate. All detailed procedures are described in Chapter 4 of the present thesis.

7.2.5 Soil plant growth tests (Ecotoxicological LoE)

Plant tests followed the ISO guidelines (ISO 2005), with minor modifications, to evaluate the effects on seeds germination, shoot length and biomass of two plant species. The monocotyledonous *Avena sativa* (oat) and the dicotyledonous *Brassica rapa* (rape) were selected, according to a list of species recommended by the ISO guideline. OECD artificial soil (OECD 1984b) and the reference soils were used as controls. All tests were carried out on 100% soil samples on plastic boxes (12 cm x 9 cm x 6 cm) filled with approximately 450 g moistened soil (about 50% of the soil WHC), with four replicates per soil. A number of 10 seeds were planted on each replicate with the help of a pair of tweezers. Each plastic box was placed inside a similar box filled with distilled water, and the maintenance of soil moisture was guaranteed by capillarity through a fiberglass rope. Twice a week, the position of the test boxes was rearranged according to a randomization scheme, within a plant growth chamber at 23°C with a 16:8-h light:dark cycle (8000-14000 lx) and relative humidity of 60%. No fertilizer was added. Seed germination was determined by visual seed emergence and was recorded at day four. After 50% of the seeds in the control soil germinated, the number of seedling per replicate was reduced to 5 evenly distributed plants. After an exposure period of 14 d for *A. sativa* and 21 d for *B. rapa,* growth was estimated as shoot length (in fresh material) and dry biomass after oven drying the living matter at 70°C until constant weight.

7.2.6 Cladoceran reproduction tests (Ecotoxicological LoE)

The 21-d *Daphnia magna* reproduction tests were conducted on soil eluates prepared from each tested soils as described in Chapter 3 (Niemeyer et al. 2010); using reconstituted hard water (ASTM 2002), the same used as control and dilution medium in tests. The tests were carried out according to the OECD guideline (OECD 1998), with 24-h old neonates (clone A *sensu* Baird et al. (1989)) from third- to fifth-brood. Ten replicates were set up for each treatment, each with 1 organism and 50 ml of medium and incubated at 19 to 21°C under a 14:10-h light:dark cycle (4000 lx). Daily the organisms were fed with *Pseudokirchneriella subcapitata* (3 x 10^5 cells/ml) and newborn neonates were recorded and removed from the vessels. Parent organisms were transferred to new medium every two days, times at which pH, dissolved oxygen and electrical conductivity were measured in new and old medium. All soil eluates were first tested at 100%. At the end of the 21-d exposure reproduction was estimated as the mean number of offspring per live parent animal. Cases where strong lethal effects were observed (P150T1 and P1000T1), a dilution series of 100, 80, 64, 51, and 40% and 100, 83, 69, 58 and 48%, respectively, of the eluate was tested in an attempt to determine the respective median effective dilutions (EC50 values).

7.2.7 Microalgae growth tests (Ecotoxicological LoE)

The 72-h *P. subcapitata* (Korshikov) Hindak growth tests were conducted on eluates prepared from all soils as described in Chapter 3 (Niemeyer et al. 2010); using distilled water. The tests were carried out following standard guidelines (OECD 1984a; Environmental Canada 1992), on 24-well sterile microplates, at 21°C to 23°C and under continuous cool-white fluorescent illumination (8000 lx). Woods Hole MBL growth medium (Stein 1973) diluted 2.5 times, to keep the required N/P levels, was used as control medium. To exclude the potential confounding effect of differences in nutrients levels across eluates on algae growth, all tests were performed on eluates supplemented with the same amounts of nutrients as in the control medium. Three and six 900-µl replicate cultures were set up per each soil eluate (only tested at 100%) and control, respectively, and each was inoculated with 100 µl of algal inoculum, so that cell concentration at the start of the tests was 10^4 cells/ml. For further details on testing procedures see Rosa et al. (2010). At the end of the 72-h exposure, algal growth was estimated as the mean specific growth rate per day. Conductivity and pH were measured at the start of the test.

7.2.8 Surface dwelling invertebrates (Ecological LoE)

Surface dwelling invertebrates were sampled using pitfall traps, which consisted of plastic cups (8 cm in diameter and 11 cm deep) filled with alcohol (at 50%) and a few drops of neutral detergent. Three replicate traps were set up at each sampling point, distant from each other by 5 m in a triangular arrangement, during one week. After collection, specimens were brought to the laboratory and preserved in alcohol (at 70%). Collected invertebrates were identified at morphospecies level. For each Order, invertebrate abundance was estimated as the total number of individuals and invertebrate richness as the total number of taxa (morphospecies), after summing the results of the three replicates. Details are shown in Chapter 6 (Niemeyer et al. *submitted*).

7.2.9 Soil microbial parameters (Ecological LoE)

Details about soil sampling and determination of soil microbial parameters are outlined in Niemeyer et al. (2012) (Chapter 5). The following parameters, involving microbial biomass, enzymes activities, and nitrogen transformation rates were determined and used in the risk calculation: microbial biomass of carbon (µg/g), microbial biomass of nitrogen (µg/g), asparaginase activity (µg N-NH_4^+/g/h), dehydrogenase activity (µg PNP/g/d), acid phosphatase activity (ug PNF/g/h), ammonification rate (ug N/g/d), and nitrification rate (%).

7.2.10 Organic material decomposition rate (litter mass loss) (Ecological LoE)

Litter bags were used to measure litter mass loss. Nylon bags with a size of 30 cm × 20 cm and a mesh size of 1.0 cm × 0.2 cm were used to allow the decomposition activity both by macro- and microorganisms (Cortez 1998). Dried leaves of *Schinus terebinthifolius* Raddi (Anacardiaceae), a native tree species, were collected in a non-contaminated area and used as substrate in the litter bags (4 g in each bag). This species is quite frequent at the study site and is palatable to the soil macrofauna (Podgaiski and Rodrigues 2010). Litter bags were placed on the soil surface. At each sampling point 4 areas (4 m apart on a quadrangular arrangement) were defined and 4 bags were placed in each area (a total of 16 bags per sampling site). Four litter bags per sampling point (one per area) were collected randomly after exposure periods of 15, 43, 83, and 131 d, and processed immediately. The material was dried at 60°C and

weight was recorded. Afterwards, the ash-free dry weight (AFDW) was calculated by subtracting the mass of the ignited residue at 600°C for 1 h. Litter mass loss was calculated by subtracting the AFDW of the remaining litter from the AFDW of the initial input, and using soil and litter correction factor according to EPFES protocol (Römbke et al. 2003). The monthly decay rate constant was calculated by using the single negative exponential decay model $m_t/m_0 = e^{-kt}$, where m_t/m_0 is the proportion of mass remaining at time *t*, and *t* is the time elapsed in days (months), and *k* is the derived daily (monthly) decay constant. The methodology followed the recommendations of OECD (2006) and Römbke et al (2003). Details about methodology and calculations are described in Chapter 6 (Niemeyer et al. submitted). This parameter was not evaluated in points P20T1 and P20T3 due to the the short proximity with P0 and P50T1 and P50T3.

7. 2.11 Data analysis

7. 2.11.1 Ecotoxicological and ecological evaluations

To avoid repetition with data analysis sections in previous chapters, in this section, the detailed analyses comparing the performance of indicators at each site with the respective reference (basically using ANOVA, T-tests, or ANOSIM approaches according to the parameters) was only performed for results that were not presented previously. This is the case for sub-lethal tests with aquatic organisms and the plant assays. However, even not describing in this section the detailed analyses of the other ecotoxicological and ecological parameters used in this chapter, they are described in the corresponding previous chapters (Chapter 4 for soil invertebrate ecotoxicological tests; Chapter 5 for microbial parameters, Chapter 6 for ecological parameters). Of course all these parameters were used in this chapter for the calculation of risk values for each LoE and integrated risk values (see section 7.2.11.2).

For the ecotoxicological tests (plant growth, cladoceran reproduction and microalgae growth), differences among contaminated soils and the respective reference soil were evaluated by one-way analysis of variance (ANOVA), followed by one-tailed Dunnet's test when necessary; organic matter content was used as covariable when analysin plant biomass and shoot length . For the cladoceran reproduction tests on a range of eluate dilutions the median and 20% effective dilutions (EC50 and EC20, respectively) and respective 95% confidence limits (CL) were obtained by fitting organism responses to a logistic model using the least squares method (OECD 1998).

Prior to all analysis, normality and homoscedasticity were checked via the Kolmogorof-Smirnov and Bartlet tests, respectively. When homoscedasticity was not fulfilled, an equivalent non-parametric tests was used, namely the Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons test. All statistical analyses were carried out using the Statistica 7.0 software (Staf Soft).

7.2.11.2 Risk calculations

Risk calculations followed the approach proposed by Janssen and Mesman (2006), where risk values are expressed in a scale ranging from zero ("no risk") to one ("highest risk"). This method assumes that the risk value of reference soils is zero, thus the risk of test soils is always given in relation to the value of the respective reference soil. It implies that all results from the different tests should be made comparable (expressed the same scale) across the various lines of evidence (LoEs).

For each sampling point, risk values were calculated following three steps: (1) scale the results (between 0 and 1) of each test/evaluation within each LoE; (2) integrate all scaled information of all parameters within each LoE to calculate the risk derived from each LoE; (3) integrate the information from the three LoE and calculate the integrated risk. In the present study, the integrated risks to the soil habitat and retention function were calculated separately.

In the first step, the results of all tests/evaluation within each LoE were scaled between 0 and 1. For the chemical LoE of the habitat function, the total content of each metal was used to calculate the specific Toxic Pressure (PAF - Potential Affected Fraction of species) at each sampling point, in the same way as done in tier 1 (see Chapter 3). Benchmarks ($HC50_{EC50}$ values) and model parameters used for each metal in these calculations can be found in Rutgers et al. (2008). For the chemical LoE of retention function, results from each extractable metal were compared to water quality objectives extracted from VROM (2000) and then scaled against metal values from eluates from the respective reference soils according to Jensen and Mesman (2006).

For the ecotoxicological LoE (EcLoE) of both the habitat and retention function, results of the ecotoxicological tests were used and scaled between 0 and 1. Negative values were set to zero. For habitat function, data on reproduction of *E. andrei*, *E. crypticus* and *F. candida*, and on growth, both as shoot length and biomass, of *A. sativa* and *B. rapa* were scaled; negative

values (increase relatively to reference) were also set to zero. For the retention function, the EC50 values of *D*. *magna* reproduction and *P. subcapitata* growth, expressed as the percentage of dilution of the eluates, were used.

For the ecological line of evidence (ELoE), only the risk to the habitat function was calculated, by scaling the data on surface dwelling invertebrates, soil microbial parameters and organic matter decomposition relatively to the overall reference value. Data on abundance and morphospecies richness of the most frequently soil surface dwelling groups (Araneae, Hymenoptera, Coleoptera, Orthoptera) were used separately, while data on other groups, including Isopoda, Dermaptera, Hemiptera, Diplopoda and Mantodea, were pooled. In this case, since both abundance and number of morphospecies are the result of a the same survey, the BKX_Triad method (Jensen and Mesman, 2006) was used. This method allows integrating information from different ecological observations into a single risk value, even if the original data has different units.

In the second step, the risk from each LoE was calculated by integrating the respective scaled information for each parameter. In the chemical LoE for the habitat function this was achieved by integrating the individual metal risk according to a response addition model described by De Zwart and Posthuma (2005). For the retention function this was done according the calculations suggested by Jensen and Mesman (2006).

Finally, in step three, the integrated risk (IR) for habitat function and retention function was calculated for each tested soil (sampling point) independently and using the risk values from each LoE (chemical, ecotoxicological and ecological LoEs in the case of habitat function, but only chemical and ecotoxicological LoEs in the case of retention function). To evaluate whether the different lines of evidence contributed differently to the total risk, the standard deviation associated to each IR value was also calculated. More details on the calculation involved in each of the three steps (including formulas for each type of data used) can be seen in Jensen and Mesman (2006).

7.3 Results and discussion

7.3.1 Sites characterization and metal concentrations

A full physico-chemical characterization of all sampling points and details on the establishment of three main soil groups according to the three reference points are shown in Niemeyer et al. (2010) (Chapter 3). Overall, soils from the study sites showed high clay percentages (above 30%, except for group 2 soils with values close to 10%) low (<2%) to medium (2 to 6%) organic matter content (according to USEPA 2004), a cation exchange capacity (CEC) mostly between 30 and 40 meq/100 g, and pH values near to neutral, with the exception of sites P1000T1 and Ref. 2 with a low pH of 3.7 and 4.9, respectively (Table 1). These characteristics agree with those reported by Anjos (2003), who identified basic pH, high CEC, high clay percentage, and high organic matter content as characteristics of soils from the study area.

Total and extractable metal concentrations are shown in Table 2. For at least one among four metals (Pb, Cd, Cu, and Zn), soils from three sampling points, within group 2 soils, presented levels exceeding by far the benchmark $HC50_{cor}$ values. However, low metal concentrations, the vast majority below detection levels, were found in 0.01 M $CaCl_2$ extracts (Table 2), indicating a probable high metal adsorption to soil particles, which is in accordance to the type of expansive clay (montmorillonite) of high plasticity found in this region (Machado et al. 2002), which probably has increased by ageing (since the smelter ended its activities in 1993).

7.3.2 Soil invertebrates reproduction tests (Ecotoxicological LoE)

All three soil invertebtates showed to be suitable test organisms to be used in tests with soils from that region and at 25°C, since in all three reference soils selected within the study area fulfilled the validity criteria established in the test guidelines for control soils. Results on the reproduction of *E. crypticus, E. andrei* and *F. candida* are shown in Table 3.

Table 7.1 Physico-chemical characteristics of the three groups of soils sampled at the Santo Amaro (BA, Brazil) study area and respective reference soils. USDA – United States Department of Agriculture; CEC – Cation Exchange Capacity; WHC – Water Holding Capacity.

Soil group	Coarse sand (%)	Fine sand (%)	Sand (total) (%)	Silt (%)	Clay (%)	Texture (USDA)	CEC (meq 100g)	pH (KCl 1:5 v:v)	P (mg/kg)	Organic matter (%)	Mineral N (mg/kg)	Water content (%) (1)	WHC (g/100g)
Group 1													
Ref 1	2.3	8.5	10.9	42.1	47.0	Silty Clay	34.16	7.1	72	1.1	42	19.54	53.78
P1000T1	2.5	21.8	24.3	19.9	55.8	Clay	43.20	3.7	35	2.0	56	28.74	59.95
P20T3	11.4	30,0	41.4	22.3	36.3	Clay Loam	42.16	6.8	106	1.9	42	35.04	67.73
P400T3	6.5	8.6	15.1	52.4	32.5	Silt Clay Loam	35.84	7.1	1	1.9	70	45.48	56.67
Group 2													
Ref 2	50.9	38.5	89.4	2.8	7.7	Loamy Sand	37.60	4.9	1	1.0	42	13.21	27.53
P0	43.2	31.3	74.5	11.9	13.6	Sandy Loam	38.56	6.7	47	0.3	70	31.04	44.12
P20T1	48.0	13.8	61.8	19.0	19.3	Sandy Loam	37.28	7.1	58	0.2	42	32.67	46.40
P150T1	56.2	21.1	77.4	12.3	10.3	Sandy Loam	21.28	6.7	>200	2.1	42	29.41	28.55
P50T3	69.2	9.1	78.3	10.4	11.3	Sandy Loam	16.56	7.2	>200	2.8	56	39.48	22.05
Group 3													
Ref 3	22.2	15.0	37.2	11.1	51.7	Clay	36.48	6.1	52	3.9	56	47.20	60.75
P50T1	25.2	13.4	38.6	29.0	32.4	Clay Loam	38.16	6.7	63	1.1	56	28.59	54.51
P400T1	19.6	23.9	43.5	20.2	36.3	Clay Loam	37.44	6.8	>200	5.1	56	24.43	58.93
P150T3	8.4	15.2	23.5	21.4	55.1	Clay	49.20	6.8	16	2.5	42	40.71	61.76
P1000T3	10.3	19.5	29.8	29.8	40.4	Clay Loam	42.72	7.0	>200	5.7	42	n.d.	57.57

(1) Soil moisture in the samples used for microbial assessments.

Table 7.2 Total and extractable metal concentrations in the three groups of soils sampled at the Santo Amaro (BA, Brazil) study area and respective reference soils.

Sites	Total (mg/kg)								Extractable (mg/l) in 0.01 M $CaCl_2$ (1:10; v:v)							
	Pb[a]	Cd[a]	Cu[a]	Zn[a]	Cr	Ni	Fe	Mn	Pb	Cd	Cu	Zn	Cr	Ni	Fe	Mn
Group 1																
Ref 1	16	<0.2	66	94	77	54	45000	840	<0.1	<0.01	<0.8	<0.2	<0.8	<1.4	<1.1	<0.5
P1000T1	23	<0.2	60	80	62	46	48000	360	0.1	0.4	<0.8	1.9	<0.8	1.6	<1.1	71
P20T3	308	<0.2	56	420	78	60	49000	672	<0.1	<0.01	<0.8	<0.2	<0.8	<1.4	<1.1	<0.5
P400T3	179	0.3	44	90	59	46	34000	760	<0.1	<0.01	<0.8	<0.2	<0.8	<1.4	<1.1	<0.5
Group 2																
Ref 2	13	<0.2	18	24	16	28	2900	34	<0.1	<0.01	<0.8	0.2	<0.8	<1.4	<1.1	0.8
P0	1264	<0.2	76	3800 (2.8)	72	57	52000	674	<0.1	<0.01	<0.8	<0.2	<0.8	<1.4	<1.1	<0.5
P20T1	133	<0.2	56	220	80	56	41000	780	<0.1	<0.01	<0.8	<0.2	<0.8	<1.4	<1.1	<0.5
P150T1	37460 (10.4)	771 (9.8)	594 (1.6)	42200 (33.5)	57	70	110000	1720	2.2	7.3	<0.8	1.3	<0.8	<1.4	<1.1	<0.5
P50T3	26074 (7.1)	62	3196 (8.2)	95940 (73.5)	80	40	117000	5880	<0.1	<0.01	<0.8	1.0	<0.8	<1.4	<1.1	<0.5
Group 3																
Ref 3	152	<0.2	40	260	59	40	53000	820	<0.1	0.28	<0.8	<0.2	<0.8	<1.4	<1.1	1.3
P50T1	164	<0.2	60	240	80	58	43000	720	<0.1	<0.01	<0.8	<0.2	<0.8	<1.4	<1.1	<0.5
P400T1	961	8.8	60	840	64	48	35000	540	<0.1	<0.01	<0.8	<0.2	<0.8	<1.4	<1.1	<0.5
P150T3	2200	12	108	3300	84	58	56000	678	<0.1	<0.01	<0.8	0.2	<0.8	<1.4	<1.1	<0.5
P1000T3	99	<0.2	56	156	84	52	49000	568	<0.1	<0.01	<0.8	<0.2	<0.8	<1.4	<1.1	0.9

[a] Numbers in superscript indicate the exceedance (multiplication factor) relatively to the corrected Dutch $HC50_{EC50}$ values (after Rutgers et al. 2008) (Ex: the [Pb] at P150T1: 37460 (10.4), indicates that [Pb] was 10.4 times higher than the HC50cor for Pb). Total metal concentrations in several sampling points exceeded the Eco-SSL for plants proposed by USEPA (2003): Pb: 120 mg/kg soil; Mn: 220 mg/kg soil; Cd: 32 mg/kg soil; Cu: 70 mg/kg soil; Ni: 38 mg/kg soil; Zn: 160 mg/kg soil.

Table 3. Mean number of juveniles of soil invertebrates, and growth of plants (average values ± standard deviation) in ecotoxicity tests for the assessed sampling points. Asterisks indicate significant differences (* p<0.05; ** p<0.01; *** p<0.001) for a one-tailed hypothesis of a Dunnet's test between each sampling point and the respective reference soil. In the ANOVAs for *E. andrei* and for plants, soil organic matter was used as co-variable. n - number of replicates.

Soil groups	Reproduction tests (mean number of juveniles)			Shoot length of plants (cm)		Biomass of plants (g; dry weight)	
	E. crypticus n=4	*E. andrei* n=5	*F. candida* n=5	*A. sativa* n=4	*B. rapa* n=4	*A. sativa* n=4	*B. rapa* n=4
Ref 1	583.0 ± 121.1	70.3 ± 9.5	662 ± 161.3	31.7 ± 1.8	3.3 ± 0.2	0.17 ± 0.01	0.17 ± 0.04
P1000T1	121.8 ± 34.9**	5.3 ± 7.1***	642 ± 91.9	39.5 ± 5.3	2.7 ± 0.2**	0.31 ± 0.04	0.07 ± 0.02***
P20T3	896.0 ± 263.5	53.8 ± 12.5	408 ± 185.9*	18.4 ± 1.0***	2.7 ± 0.2**	0.11 ± 0.01**	0.07 ± 0.01***
P400T3	599.3 ± 180.0	71.0 ± 17.5	377 ± 89.3**	23.0 ± 1.9**	2.3 ± 0.3***	0.16 ± 0.01	0.06 ± 0.01***
Ref 2	1089.3 ± 86.4	132.3 ± 24.7	224 ± 61.8	25.2 ± 3.9	2.7 ±0.2	0.17 ± 0.02	0.09 ± 0.02
P0	536.5 ± 144.6***	91.0 ± 15.4**	760 ± 124.1	26.3 ± 2.1	3.7 ±0.4	0.23 ± 0.02	0.24 ± 0.02
P20T1	452.0± 36.0***	97.3 ± 20.5*	494 ± 105.1	25.2 ± 2.3	2.9 ± 0.0	0.20 ± 0.03	0.14 ± 0.01
P150T1	7.3 ±0.5***	10.3 ± 1.7***	613 ± 55.4	19.8 ± 1.9*	2.7 ± 0.2	0.14 ± 0.04	0.04 ± 0.01**
P50T3	450.8 ± 64.6***	45.5 ± 8.3***	411 ± 135.8	19.5 ± 3.3*	2.9 ± 0.2	0.17 ± 0.01	0.06 ± 0.01
Ref 3	854.7 ± 421.3	122.5 ± 25.0	890 ± 103.2	34.8 ± 3.7	4.7 ±1.2	0.26 ± 0.05	0.45 ± 0.17
P50T1	560.0 ± 164.3	97.0 ± 11.2*	351 ± 141.5***	25.3 ± 2.9**	4.3 ±0.2	0.17 ± 0.03*	0.32 ± 0.05
P400T1	773.5 ± 175.4	103.0 ± 12.8	831 ± 87.9	42.7 ± 3.0	7.3 ± 0.4	0.29 ± 0.05	0.91 ± 0.08
P150T3	615.8 ± 196.1	20.3 ± 5.7***	344 ± 42.9***	28.9 ± 1.0*	3.1 ± 0.1**	0.16 ± 0.03*	0.16 ± 0.02***
P1000T3	555.0 ± 34.5	85.3 ± 4.6**	577 ± 121.9***	30.9 ± 5.3	4.8 ± 0.2	0.24 ± 0.05	0.30 ± 0.04*

Concerning the reproduction of *E. crypticus*, significant effects were observed in soils P1000T1, P0, P20T1, P150T1, and P50T3 (all belonging to the second soil group , except P1000T1) when compared to the respective natural reference soil. The higher mean (± SD) number of juveniles was found in Ref 2, 1089 (±86), while the most toxic soil was P150T1, with just 7 (±0.5) juveniles/adult. Significant effects on reproduction of *E. andrei* were observed in the soils P1000T1, P20T1, P0, P50T3, P150T1, P50T1, P150T3 and P1000T3. The higher mean (± SD) number of juveniles was found in Ref 2, 132 (±25); while the most toxic soil was P1000T1, where just 5 organisms were observed.

The inhibition of the reproduction of both oligochaete species in soils P0, P150T1 and P50T3 was highly expected, as these are the most metal contaminated soils, exceeding not only the benchmark $HC50_{cor}$ values, but also the reported EC50 values that inhibit the reproduction of oligochaetes (see Chapter 4). However, the impairment of reproduction in soil P1000T1, not contaminated by metals, can most likely be related to properties of the soil acting as limiting factors for these species, namely low pH (3.7) combined with low OM (2.0%) and high clay (55.8%) contents. The limitations on the use of *E. andrei* in strongly acid soils or soils with low organic matter content has been previously reposted (Jansch et al. 2005; Römbke et al. 2006; Chelinho et al. 2011). Although *E. crypticus* presents a broader tolerance than *E. andrei* to different soil properties (e.g. range of 4.2–7.7 for pH, 0.6–4.8% for OM, and 3–49% for clay) (Chelinho et al. 2011), the characteristics of P1000T1 soil were out of its range of tolerance. Also, the effects on the reproduction of both oligochaete species observed in P20T1 soil could be related to the low OM content in this soil (0.2%).

A different trend was observed for the reproduction of *F. candida*. Significant effects on its reproduction were observed in soils P20T3, P400T3 (both from Ref 1 group), P50T1, P150T3, and P1000T3 (all from Ref 3 group), when compared to the respective natural reference soil. The highest and lowest mean (±SD) number of juveniles was found in Ref 3, 890 (±103) and Ref 2, 224 (±62). The latter low mean was probably one of the reasons for the absence of significant effects on the reproduction of *F. candida* in the most metal contaminated soils in the study area (Ref 2 group: P0, P150T1 and P50T3). In effect, in these soils at least one metal (Pb, Cd, Cu, or Zn) exceeded in its concentration the reported EC50 values that cause negative effects on reproduction of *F. candida* (see Chapter 4).

In general, *F. candida* appeared to be less sensitive to metal contamination than *E. andrei* and *E. crypticus*. Similar results were also found by Schultz et al. (2004), reporting *Enchytraeus* sp. to be more sensitive than *F. candida* in metal-contaminated soils. Van Gestel et al. (2001) observed that

collembolan appeared to be less sensitive than earthworms and plants to assess soils toxicity with oil and metal contamination. Differences in the sensitivity of collembolans and oligochaetes on metal contaminated soils could be explained in part by differences in exposure (Achazi 2002), since soil solid phases are more important for uptake process of collembolans, while soft-body oligochaete species are more influenced by porewater characteristics (Vijver et al. 2001). It is also suggested that Collembola can avoid contaminated food, and are able to excrete assimilated metals at moulting (Fountain and Hopkin 2001), which can be related to their low sensitivity to metal contaminantion.

A more detailed discussion on the effects of metal loadings on the reproduction of soil invertebrates is shown in Chapter 4. In brief, a decline on the reproduction of these organisms in metal contaminated sites suggests impacts on habitat function for these groups, which can affect soil functions related to fertility, as cycling of soil organic matter and aeration.

7.3.3 Soil plants growth tests (Ecotoxicological LoE)

The growth results, both as shoot length and biomass, of *A. sativa* and *B. rapa* in all tested soils and respective references are shown in Table 3. Species and endpoints were affected differently, though generally soils from the first group were found to be more toxic. Significant negative effects on plant growth were observed in soils P1000T1, P20T3 and P400T3 (from the first group of soils), soils P150T1 and P50T3 (from the second group) and soils P50T1, P150T3 and P1000T3 (from the third group). These were expected results since total concentrations for some of the metals in all these soils exceeded several metal Eco-SSL for plants (Table 2).

However, although a few metal Eco-SSLs were also exceeded for soils from P0, P20T1 and P400T1 no toxic effects on plant growth were observed. These results highlight the fact that exceedence of Eco-SSL does not necessarily mean risk, most likely due to modifications in the bioavailability of metals by the soil properties and to the complex effect of mixtures of contaminants (Weeks et al. 2004). In effect, as shown also by the present results, these benchmark values aim to be protective, indicating that below these concentrations risk is not expected.

The results of the present study also show that the combined effects of metals were specie-specific. This finding is in agreement with the study of An (2006) investigating the toxicity of Pb and Cu to four plant species, *Sorghum bicolor*, *Cucumis sativus*, *Triticum aestivum*, and *Zea* mays. The latter author found that Pb and Cu showed either antagonistic or synergistic toxic effects depending

on the plant species. Also Ben Ghnaya et al. (2009) stated detrimental effects of the metals Zn and Cd on the growth, *chlorophyll* and carotenoid content and metal accumulation on four varieties of *Brassica napus* depending on the metal and plant variety.

Nevertheless, it should be pointed out that toxic effect on plants can be related not only to metal contamination, but also to a lack of soil nutrients and/or modified soil physical properties, which are common problems in mined areas and tail deposits. For instance, Gong et al. (2001), evaluating four plant species in 15 soils including five mineral oil-contaminated soils, concluded that soil nutrient status rather than soil texture significantly affected both seedling emergence and shoot biomass. Also, results obtained by Alvarenga et al. (2008) showed that negative effects on the growth of *Lepidium sativum* in mine soils were probably due not only to metals and soil acidity, but also to the lack of porosity and proper soil structure. Thus, in the present study, the low organic matter content and low WHC at most sampling points combined with metal levels, could well be responsible for the observed detrimental effects on plant growth.

7.3.4 Cladoceran reproduction and microalgae growth tests (Ecotoxicological LoE)

All tests conducted with the eluates from all the soils fulfilled the validity criteria established in the guidelines for cladoceran and microalgae control performance. For the cladoceran tests, significant effects on reproduction were only found with eluates from soils P1000T1 and P150T1, for which mortality at the 100% dilution was 100 and 30%, respectively; in all other tested dilutions of the latter eluates and 100% dilution of all the remaining soil eluates mortality was below the validity criterion of 20%. For P1000T1 eluate EC20 and EC50 values were much higher than the 100% dilution, whereas for the eluate from P150T1 soil the EC50 value was also higher than 100% and the EC20 value was 88% (95%CL: 60 – 115). Given that tier 1 results showed 48 h LC50 values of 91 and 68% for P1000T1 and P150T1 eluates, respectively (see Chapter 3; Niemeyer et al. 2010), in general the acute toxicity of both eluates appeared to be lower in the reproduction than in the lethal test. This fact could be explained by the adsorption of metals to the surface of microalgae cells in the reproduction test, where food (microalgae) is added daily, while in the lethal test no food is provided during exposure. In a study on the influence of algal biomass on metal adsorption, Roy et al. (1993) demonstrated that the green alga *Chlorella minutissima* adsorbed at a fast rate more than 90% of the initial Pb concentration, with the Pb concentration in solution reached the equilibrium within minutes. Kaulbach et al. (2005) described the adsorption of Cd onto the cell wall of *P. subcapitata,*

showing the importance of microalgae in the control of transport and fate of metals in the environment.

Concerning effects on microalgae growth, *P. subcapitata* showed increases in cell density by at least a 40-fold factor with coefficients of variation of the mean specific growth rate lower than 4%, in all tested eluates, suggesting the absence of toxicity. However, when growth rates in eluates of tested soils were compared to those of the respective reference, significant inhibitions in growth (higher than a 10% acceptable threshold) were observed in 1000T1 and P150T1 eluates (16 and 20% inhibition, respectively). These results corroborate those of the lethal and reproduction tests with *D. magna* in tier 1 (Niemeyer et al. 2010) and tier 2 (present chapter), respectively, pointing to the toxicity of both eluates. For P150T1 eluate these were expected results since extracted metal loads were the highest in P150T1. However, as already pointed for tier 1 (Chapter 3; Niemeyer et al. 2010), since the P1000T1 was not classified as contaminated, the toxic responses observed in its eluate were most likely related either to the low soil pH (3.7), or even to other not quantified contaminants, as this sampling point was located in a pasture area.

In general, there was a lack of toxic responses in aquatic tests, suggesting that the metals were not bioavailable and, thus, that the retention function of soils at most sampling points was enough to prevent the mobilization of metals via the water pathway, especially to groundwater. Such finding is in agreement with the results of the chemical LoE reporting low amounts of extractable metals.

7.3.5 Surface dwelling invertebrates (Ecological LoE)

A total of 1,277 individuals, separated into 72 morphospecies of soil invertebrates were collected in the pitfall traps. From these individuals, Hymenoptera, Coleoptera and Orthoptera were found at 100% of the total of 13 sampling points were pitfalls were used, Araneae at 92%, while the group including the pooling of Isopoda, Dermaptera, Hemiptera, Diplopoda, and Mantodea was also found at all sampling points (Table 4; for more detailed results please see Chapter 6).

Araneae presented the highest abundance (139 individuals) and number of taxa (7 morphospecies) in P1000T3 soil, but its abundance and richness was generally low at all other sampling points and no organisms from this Order were found in P150T3 soil. For both Coleoptera and Orthoptera as well as for the Others grouping, the lowest abundance and richness were found in

P150T1 soil (2 and 1, 4 and 2, and 1 and 1, respectively). On the contrary, Hymenoptera presented the highest abundance (76 individuals) inside the smelter area, at point P50T1, though the number of taxa (6) was registered at point P400T3. The lowest abundance and richness within this Order was found at point P400T1. For the latter three groups the highest invertebrate abundance and richness were found either in transect T1 but far from the smelter area or in transect T3. Overall, more abundance of Araneae, Coleoptera and Orthoptera was observed outside rather than inside the smelter area, while the loweest values were found at contaminated sites inside the smelter area, especially in transect T1. Additional analysis and more details are shown in Chapter 6 (Niemeyer et al. *submitted*).

7.3.6 Soil microbial parameters (Ecological LoE)

Microbial community was highly impaired by metal contamination, since all microbial parameters inside the smelter area were significantly affected relatively to the overall reference value, whereas outside the smelter area such effects were rarely observed; except potential nitrification which increased within the smelter area (Table 5). In effect, the observed significant negative correlations between all except one of the microbial parameters and the metal loadings (details shown in Chapter 5; Niemeyer et al. 2012) illustrated the detrimental effects of metal contamination on the soil microbial community, and, thus, the essential role they play on biogeochemical cycles. Therefore, in general the present results of tier 2 corroborate those found in tier 1, where the soil basal respiration rate was lowest in the metal contaminated soils inside the smelter area (Chapter 3; Niemeyer et al. 2010) and correlated negatively with total soil metal concentrations (Chapter 5; Niemeyer et al. 2012).

The only microbial parameter that increased significantly among the most contaminated sites inside the area was potential nitrification. Although being considered one of the most sensitive soil microbial processes regarding metal stress (Broos et al. 2005), some studies have shown adaptation of nitrifying populations at metal-contaminated sites (Mertens et al. 2006), which may be the case in the present study. Nevertheless, high nitrification rates may indicate an unbalance in the N-cycling, which may result in losses of N from the system by leaching or denitrification. Recently disturbed ecological systems tend to show greater nitrification rates, which decreases along the successional status (Montagnini et al. 1989).

Although some authors (e.g., Kapustka 1999) do not recommend the inclusion of microbial parameters for ecological assessments, because microbial communities present strong functional redundancy, rapid changes across small spatial scales, and are highly influenced by confounding factors (e.g., moisture, nutrients), the present study is in agreement with reports on decreases in microbial enzyme activity (Gülser and Erdogan 2008), carbon biomass and basal respiration (Gülser and Erdogan 2008; Zimakowska-Gnoinska et al. 2000; Jian et al. 2010) in impacted soils, strongly point these parameters as useful tools for assessing metal effects on microbial functions in contaminated areas.

On the other hand, soil microbial communities and the key biological processes they mediate are closely related to vegetation and soil use (Nogueira et al. 2006; Zak et al. 2003). The failure in the vegetation establishment inside the smelter area, especially in the tailing deposits, due not only to metal toxicity but also to inappropriate soil physical and chemical properties, may also contribute to the observed decreases in microbial activity and biomass. Given that key microbial processes on C, N and P cycling have most likely been impaired due to such conditions as well as to the high metal contents, the maintenance of vegetation in these heavily-contaminated sites will be progressively more difficult, leading to the intensification of erosive processes and dispersion of pollutants (Broos et al. 2005).

7.3.7 Organic material decomposition (Ecological LoE)

When evaluating litter breakdown, the validity criterion of 60% mass loss in the reference treatment at the end of the study (Römbke et al. 2003) was fulfilled. As for the decomposition of the organic material, the monthly decay rate in the contaminated sites within the smelter area was significantly lower than in the overall reference (Table 5). Only sites located 1000 m away from P0 presented significantly higher monthly decay rates than the overall reference. Moreover, a significant negative correlation was found between the monthly decay rate and metal contamination (details shown in Chapter 6). Considering the threshold value proposed by Römbke et al. (2003) of more than 25% difference in mass loss between reference and contaminated sites to signal the presence of risk, all sites within the smelter area showed an unacceptable risk; differences in mass loss relatively to the overall reference ranged between 31% and 64% after 131 d of exposure.

The present results corroborate those reported in previous studies revealing significant effects of metal (e.g., Creamer et al. 2008) or pesticide contamination (Förster et al. 2006) on the

monthly decay rate of organic material in soil, even though other studies showed transient or no effects under some stressors, (e.g., Dinter et al. 2008; van Gestel et al. 2009; Podgaiski and Rodrigues 2010).

Also, the reduced microbial activity, faunal feeding activity and density of detritivores, allied to the low moisture and high temperatures at the more exposed sites than at those distant from the smelter area, due to the low vegetation cover, may have contributed to reduce litter decomposition within the smelter area (Zak et al. 2003). Thus, the effects on litter decomposition observed in the present study may be attributed not only to a direct toxic effect caused by metal contamination on microbial and faunal communities, but also to indirect effects leading to non-suitable habitat conditions for soil fauna and microbial communities.

Table 7.4 Number of individuals and morphospecies (shown in brackets) of surface dwelling invertebrates caught in pitfall traps (N=3). Main Orders are presented individually while other less abundant orders (Isopoda, Dermaptera, Hemiptera, Diplopoda and Mantodea) were pooled.

	Ref 1	Ref 2	Overall reference	P0	P20T1	P20T3	P50T1	P50T3	P150T1	P150T3	P400T1	P400T3	P1000T1	P1000T3
Orders														
Araneae (Ar)	73 (5)	9 (4)	41 (5)	4 (3)	2 (2)	7 (4)	2 (1)	3 (1)	5 (4)	0 (0)	14 (4)	5 (5)	2 (2)	139 (7)
Hymenoptera (Hy)	15 (5)	15 (5)	15 (5)	31 (3)	26 (3)	48 (4)	76 (3)	57 (5)	33 (2)	52 (4)	15 (3)	16 (6)	45 (5)	30 (3)
Coleoptera (Co)	11 (4)	13 (6)	12 (5)	8 (4)	2 (2)	28 (7)	16 (5)	32 (7)	2 (1)	14 (5)	88 (4)	13 (3)	7 (4)	31 (4)
Orthoptera (Ort)	14 (3)	10 (3)	12 (3)	16 (4)	8 (3)	21 (3)	13 (2)	21 (6)	4 (2)	9 (2)	10 (2)	16 (2)	24 (2)	23 (4)
Others	3 (1)	8 (5)	6 (3)	1 (1)	3 (3)	11 (3)	6 (2)	4 (1)	1 (1)	7 (2)	14 (5)	23 (5)	15 (4)	3 (3)

Table 5. Soil microbial parameters and organic material decomposition (average values ± standard deviation) for the assessed sampling points. The values for the three reference points were geometrically averaged to give an overall reference value. Asterisks indicate significant differences (* p<0.05; ** p<0.01; *** p<0.001) for a one-tailed hypothesis of a Dunnet's test between each sampling point and the overall reference value (assuming that Ref value higher than sampling point value and lower for Potential Nitrification). In the ANOVA for soil microbial parameters, soil moisture, soil organic carbon and soil nitrogen contents were used as covariables (for details, see Chapter 5; Niemeyer et al 2012). n - number of replicates.

Soil groups	MBC (µg/g) n=3	MBN (µg/g) n=3	Asparaginase (µg N-NH_4^+/g/h) n=3	Dehydrogenase (µg PNP/ g/ d) n=3	Acid phosphatase (ug PNF/g/h) n=3	Ammonification (ug N g^{-1} day^{-1}) n=3	Nitrification (%) n=3	Decomposition rate [a] *k* (monthly) n=4
Overall reference	642.4 ± 416.1	50.1 ± 16.2	84.9 ± 53.2	7.2 ± 2.3	617.1 ± 233.2	0.7 ± 0.3	3.9 ± 2.2	0.2656 ± 0.1438
P0	178.1 ± 55.1***	5.4 ± 2.8***	53.8 ± 29.5	0.7 ± 0.4 **	269.0 ± 22.1**	1.7 ± 0.2	15.2 ± 9.6**	0.04667***
P20T1	252.4 ± 142.3**	12.8 ± 4.6***	15.9 ± 18.4***	1.3 ± 1.9**	196.4 ± 33.9***	1.1 ± 0.5	12.4 ± 2.1*	n.d.
P20T3	170.3 ± 174.1 ***	18.6 ± 3.5***	71.0 ± 12.7	1.4 ± 1.0**	443.4 ± 9.3	1.5 ± 0.3	13.3 ± 3.5*	n.d.
P50T1	412.9 ± 31.4	11.0 ± 4.2***	11.2 ± 19.5***	1.2 ± 2.0**	235.7 ± 50.3**	1.8 ± 0.3	17.1 ± 4.9***	0.0632***
P50T3	461.7 ± 20.1	22.0 ± 4.5*	32.5 ± 35.1**	2.1 ± 0.5*	450.3 ± 45.4	1.8 ± 0.3	13.5 ± 4.5*	0.0412***
P150T1	115.5 ± 87.0***	9.3 ± 1.3***	22.8 ± 11.8***	3.3 ± 0.5	355.3 ± 166.0*	0.4 ± 0.2	8.7 ± 2.8	0.0435***
P150T3	543.6 ± 160.8	26.6 ± 3.1**	37.0 ± 12.4*	2.1 ± 1.1*	651.2 ± 150.7	1.5 ± 0.3	10.2 ± 0.5	0.0248***
P400T1	797.3 ± 193.3	83.0 ± 21.2	91.7 ± 32.9	16.8 ± 3.7	573.1 ± 133.3	0.8 ± 0.1	-0.2 ± 5.9	0.166*
P400T3	805.3 ± 216.2	59.7 ± 26.5	97.8 ± 16.6	1.5 ± 1.1**	792.0 ± 34.5	0.4 ± 1.0	-3.3 ± 3.17	0.0423***
P1000T1	1098.1 ± 184.1	51.1 ± 22.0	71.57 ± 18.8	4.8 ± 6.2	515.6 ± 353.5	0.6 ± 1.8	1.9 ± 7.4	0.4515
P1000T3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.3826

MBC – Microbial biomass Carbon, MBN – Microbial biomass nitrogen
n.d. - not determined
a after log of percentages

7.3.8 Lines of evidence and integrated risk

7.3.8.1 Risk to retention function

Table 6 shows the individual contribution and the combined calculated risk values from each LoE (chemical and ecotoxicological) for the soil retention function. Low risk values were found from the Chemical LoE, except at sampling points P1000T1 and P150T1. However, no risk was indicated by the Ecotoxicological LoE, most likely due to the low metal contents in the soil extracts from all except the latter two soils. As a result, a risk for the retention function of the soil was only found at sampling points P150T1 and P1000T1. P150T1 was the most contaminated point, and the metal bioavailabity at this point associated to the low soil retention function was previously demonstrated in tier 1 through the *D.* magna lethal test. In the case of sampling point P1000T1, although levels of extractable metals were low, by being in the middle of a pasture area outside the smelter area, the presence of another type of contamination (e.g., fertilizers, pesticides) causing effect on aquatic tests should not be ruled out.

Low risk for the retention function indicates low mobility of metals from soil to water. This may be favored by the type of soil in the region, which is rich in expansive clay (montmorillonite) (Machado et al. 2002) with a probable high adsorption potential, accentuated by neutral pH values and ageing. Also, Anjos (1998) pointed out that soil characteristics in this smelter area were efficient in the capture of the metals, especially in a wetland zone, not allowing their mobilization into groundwater.

Furthermore, the low metal extractability could be related to the metal form, as pointed out by Andrade Lima and Bernardez (2011). These authors, studying the leaching of the slag in the Plumbum smelter area in Santo Amaro, found that the Pb, Zn, Cd, and other potentially toxic elements were relatively stable in a weak acidic environment for short contact times, which can be explained by the low leachability of the metallic Pb and the Zn-bearing species.

However, in some sites inside the area, or in neighboring areas where groundwater could be used for human consumption, a groundwater monitoring would be advisable, especially on those sites where metal concentrations are very high and where soil characteristics could be more permeable.

7.3.8.2 Risk to habitat function

Tables 7 and 8 show the individual contribution and the combined calculated risk values for each LoE in habitat function. Sampling points presenting very high habitat function risk values (above 0.75) or moderate risk values (between 0.50 and 0.75) were those where the metal concentrations exceeded the $HC50_{cor}$ values (P0, P150T1 and P50T3) or were near that threshold (P150T3) as pointed in tier 1.

The high risk values in the chemical LoE were related to the high total metal concentrations in soil. Regarding the ecotoxicological LoE, the differences in sensitivity of the test species and endpoints were clearly visible. Reproduction tests with Oligochaeta species *E. andrei* and *E. crypticus* were the most sensitive tests. Both oligochaete species indicated high risk values (>0.75) in points P150T1 and P1000T1, and moderated risks in P50T3. High risks could be expected in P150T1 and P50T3, once they are the most metal contaminated soils and exceeded the reported EC50 values that cause negative effects on reproduction of oligochaetes (see Chapter 4). However, the ecotoxicological LoE integrating these results with reproduction of *F. candida* and plants endpoints presented low risk values (≤0.50), except in P150T1 and P1000T1, which showed moderate ecotoxicological risk. The highest risk value was found in P150T1, the most contaminated soil and a sandy soils (sampling point from group 2). In the case of sampling point P1000T1, these results also indicated (as in retention function and in avoidance tests on tier 1) a possible presence of another type of contamination.

Among the parameters from the ecological LoE, some microbial parameters, namely BMC, BMN, acid phosphatase, , asparaginase, and nitrification rate were the most sensitive endpoints in discriminating contaminated sites (this statement was also based on previous analyses made on chapters 5 and 6). Bacterial growth/biomass was highly rated by Critto et al. (2007) as parameters to be assessed in all Triad tiers, mainly due to their rapidity and low cost.

Regarding soil surface dwelling invertebrates, high risk values (>75) were indicated only by Araneae in points P0, 20T1, 50T1, 50T3 and 1000T1, and Others (pooled data of other groups) in points P0 and P150T1. In general, these soil fauna parameters did not present the same level of sensitivity of the others ecological parameters. Abundance and morphospecies richness of main groups of surface running invertebrates were not sensitive parameters to discriminate metal contaminated sites. Similarly to our findings, abundance and number of taxa, as far as diversity

indices (see also Chapter 6; Niemeyer et al. *submitted*), were also not sensitive to contamination in Semenzin et al. (2008). This can be explained by the high mobility of surface dwelling organisms in comparison to soil dwelling invertebrates, not presenting a relation with properties of a particular site but rather with characteristics of a larger area around the point. More elaborated conclusions could be taken, namely in terms of effects to particular functional groups and to find better cause-effect relationships, if identification would to be done at family level or at lower taxonomic level. Besides being more detailed this could also lead to look for specific traits that could help understand better possible effects on these groups of organisms.

The low risk (IR≤0.50) pointed by tier 1 in sampling points P50T1, P400T1, P1000T1, P20T3, P400T3, and P1000T3, all clay based soils, was confirmed in tier 2. On points P400T1, P20T3, P400T3, and P1000T3 all the lines of evidence pointed into the same direction, a general low risk, as also indicated in tier 1. However, in P50T1, both chemical and ecotoxicological LoEs pointed no risk, which did not agree with the moderate risk pointed by ecological LoE.

In P150T3, the same level of moderate risk (0.51≤IR≤0.75) indicated in tier 1 was observed in tier 2, but with a lower standard deviation than in tier 1, which means a reduction of uncertainties. The difference with results from tier 1 is that the ecotoxicological tests used in tier 2 revealed to be more sensitive (EcLoE). The opposite response was obtained in the ecological LoE (ELoE). As in P150T3, sampling point P20T1 showed a moderate risk (0.51≤IR≤0.75) in tier 1, also with a slightly higher level of uncertainty, while in tier 2 it was considered as low risk (IR 0.4), but the same level of uncertainty remained. This can be explained by the low risk indicated both by the chemical LoE and the ecotoxicological LoE in tier 2, which did not agree with the moderate risk pointed by ecological LoE. The higher risk pointed in tier 1 could be related to the type of tests used, especially the risk value obtained with the avoidance test with Eisenia andrei. Despite using corrected values for soil properties (check Chapter 3), this could result in an overestimation of ecotoxicological risk, which did not occur in results obtained with tests used in tier 2.

As in tier 1, the risk at sampling point P1000T1 was just indicated by the ecotoxicological LoE, as the chemical and ecological LoE indicated no risk. High toxicity was indicated by reproduction test with oligochaete species (0.9), and moderate risk by *B. rapa* dry weight (0.6), which can either be related to the low pH value of the soil or to the presence of contaminants not analyzed in this study. As this point is located in the middle of a pasture area outside the smelter area, the presence of another type of contamination (e.g., fertilizers, pesticides) should not be ruled out. This hypothesis was raised in tier 1 because the avoidance response of *E. andrei* observed in this test soil.

The habitat function integrated risk on tier 2 confirmed the spatial heterogeneity of the risk along the study area, pointed by results of tier 1. In the same way, high levels of risk were found at sampling points within the smelter area, particularly in soils with a coarse texture (soils from group 2; Fig. 2). Very high integrated risk values (IR>0.75) were calculated for sampling points P150T1 and P50T3, corresponding to tail deposits. According to the Dutch limit acceptable values to land use (Jensen and Mesman 2006), these high risk values restrict the use of the area even to industrial activities, requiring sealed soils. The relatively large deviation found in the final risk number for the habitat function in some points is related to the high risk pointed by chemical analysis, to the low toxicity indicated in plant endpoints and reproduction of *F. candida*, and the inability of some ecological parameters, namely those related to surface dwelling invertebrates, in discriminating ecological risk levels. These results confirm the added value of not only integrating information from different lines of evidence, but also in using different indicators inside each LoE. This will provide more detailed information about the risk and the inability of the chemical analysis alone in predicting the true risk of a contaminated site. Moreover, these results reinforce that information from the Triad can be used as a basis to take decisions about remediation actions and management concerning the future of the site.

Table 7.6 Individual and combined risk values from the chemical and ecotoxicological lines of evidence for the soil retention function.

	Chem LoE (Extractable metals)	Growth *P. subcapitata*	Reprod. *D. magna*	**Combined EcLoE**	**IR Retention Function**
Group 1					
1000T1	0.99	0.00	0.00	0.00	0.88
20T3	0.00	0.00	0.00	0.00	0.00
400T3	0.00	0.00	0.00	0.00	0.00
Group 2					
P. Zero	0.00	0.00	0.00	0.00	0.00
20T1	0.00	0.00	0.00	0.00	0.00
150T1	1.00	0.00	0.00	0.00	0.99
50T3	0.44	0.00	0.00	0.00	0.25
Group 3					
50T1	0.00	0.00	0.00	0.00	0.00
400T1	0.00	0.00	0.00	0.00	0.00
150T3	0.00	0.00	0.00	0.00	0.00
1000T3	0.00	0.00	0.00	0.00	0.00

Table7. Individual and combined risk values from the ecological line of evidence for the soil habitat function.

Soil groups	Microbial parameters							Surface dewlling arthropods					Decomp	**Combined ELoE**
	MBC	MBN	Asparaginase	DHA	Ac Fosf	Amon	Nitrif	Araneae	Hymenoptera	Coleoptera	Orthoptera	Other Orders		
Group 1														
1000T1	0.41	0.02	0.16	0.34	0.16	0.15	0.00	0.86	0.42	0.32	0.42	0.45	0.41	0.37
20T3	0.73	0.63	0.16	0.81	0.28	0.54	0.00	0.63	0.50	0.45	0.24	0.26	n.d.	0.46
400T3	0.20	0.16	0.13	0.80	0.22	0.39	0.00	0.65	0.12	0.26	0.29	0.60	0.84	0.44
Group 2														
P. Zero	0.72	0.89	0.37	0.90	0.56	0.60	0.74	0.76	0.41	0.27	0.25	0.76	0.82	0.69
20T1	0.61	0.74	0.81	0.82	0.68	0.34	0.69	0.86	0.57	0.74	0.18	0.29	n.d.	0.63
150T1	0.82	0.81	0.73	0.54	0.42	0.48	0.55	0.69	0.57	0.82	0.53	0.76	0.84	0.69
50T3	0.28	0.56	0.62	0.70	0.27	0.62	0.71	0.88	0.49	0.48	0.47	0.53	0.84	0.62
Group 3														
50T1	0.36	0.78	0.87	0.83	0.62	0.61	0.77	0.90	0.66	0.13	0.22	0.18	0.76	0.67
400T1	0.19	0.40	0.57	0.57	0.07	0.12	0.00	0.48	0.23	0.67	0.25	0.49	0.38	0.37
150T3	0.15	0.47	0.56	0.70	0.05	0.53	0.62	0.99	0.52	0.07	0.29	0.18	0.91	0.47
1000T3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.54	0.45	0.44	0.13	0.29	0.31	0.38

MBC – Microbial biomass Carbon. MBN – Microbial biomass nitrogen. DHA – Dehydrogenase. Ac Fosf - Acid phosphatase. Amon – Ammonification. Nitrif – Nitrification rate. Decomp – Decomposition of organic material
n.d. - not determined

Table 8. Individual and combined risk values from the chemical and ecotoxicological lines of evidence for the soil habitat function.

	Chem LoE (total metals)	Reprod. *F. candida*	Reprod. *E. crypticus*	Reprod. *E. andrei*	Shoot lenght of *A. sativa*	Shoot lenght of *B. rapa*	Dry W *A. sativa*	Dry W *B. rapa*	**Combined EcLoE**
Group 1									
1000T1	0.01	0.03	0.96	0.93	0.00	0.17	0.00	0.58	0.63
20T3	0.56	0.38	0.00	0.23	0.42	0.15	0.32	0.59	0.32
400T3	0.27	0.43	0.00	0.00	0.28	0.28	0.08	0.63	0.28
Group 2									
P0	0.98	0.00	0.39	0.31	0.00	0.00	0.00	0.00	0.12
20T1	0.58	0.00	0.59	0.26	0.00	0.00	0.00	0.00	0.16
150T1	1.00	0.00	0.99	0.92	0.17	0.00	0.14	0.53	0.71
50T3	1.00	0.00	0.59	0.66	0.14	0.00	0.00	0.27	0.29
Group 3									
50T1	0.35	0.04	0.00	0.21	0.27	0.09	0.36	0.17	0.17
400T1	0.86	0.00	0.00	0.16	0.00	0.00	0.00	0.00	0.02
150T3	0.96	0.06	0.00	0.83	0.17	0.33	0.29	0.58	0.41
1000T3	0.18	0.61	0.00	0.30	0.11	0.00	0.00	0.21	0.21

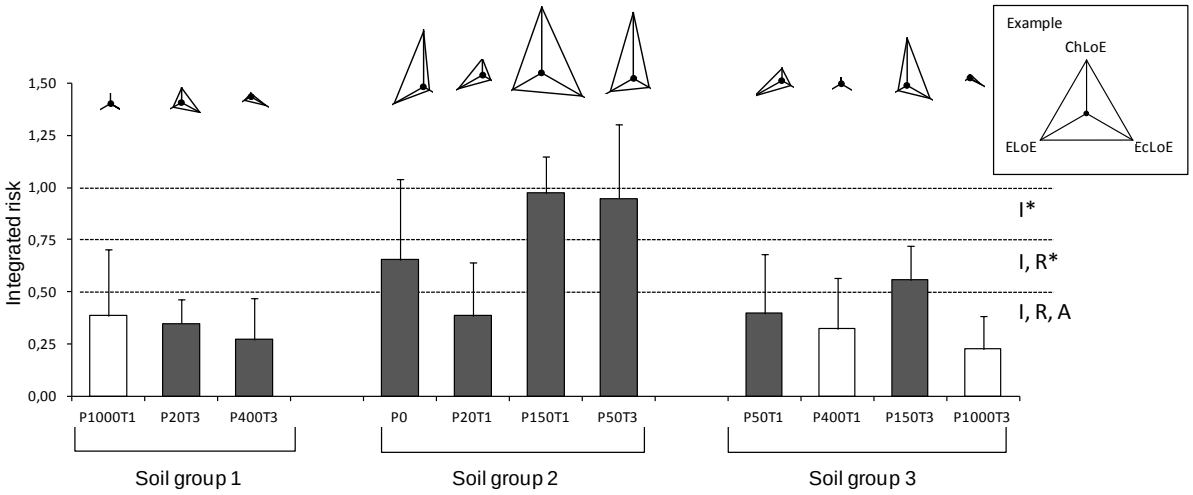

Fig. 7.2 Integrated ecological risk values for habitat function (+ standard deviation) (Min, 0; Max, 1) for each sampling point, combining information from the chemical, ecotoxicological, and ecological lines of evidence. Points with grey bars are located inside of the smelter area. Different bands indicate limits of accepted risk values for different soil uses (A agriculture, R residential, I industrial; asterisks indicate necessity of sealed soils) according to Jensen and Mesman (2006). Triangles on top of each bar represent the contribution of each LoE for the integrated risk value being an indicator of the weight of evidence (on the top right the example with the length of each axis of the triangle representing maximum risk (1) from each LoE).

7.4 Conclusions

In general, results on tier 2 confirmed the risk pointed by tier 1, with points outside the smelter area presenting low or environmentally acceptable risk values, and points inside the smelter area presenting high, or very high risk values, especially in sites associated with tail deposits. In the same way as in tier 1, the low toxicity in eluate tests indicated high adsorption of metals in soil, probably favored by neutral pH, content and type of clay, and ageing, and consequently no risk on retention function in most of points. Results of chemical analysis of extracts confirmed the low mobility of metals from soil to water.

In general, the present results indicated that the failed recovery of the area by covering tails deposits with soil from another site, and the consequently failure in revegetating the area, created inappropriate conditions for the establishment of plant, microbial and animal communities in some of the sites inside the area. So, besides the direct effects of metal contamination seen on ecotoxicological effects, also indirect effects are visible from the presence of these contaminants, compromising the functioning of the ecosystem inside the smelter area.

High risk values in habitat function above 0.75 inside the smelter area indicate the need to proceed with some remediation action, such as encapsulation of tailing and recovery of vegetation of the smelter area. These actions not only could improve soil conditions and ecosystem functioning, but they could mainly avoide the transport of contaminants to other environmental compartments, namely via dust dispersal to outside the area, or via surface runoff to the existing temporary ponds and the Subaé river.

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Chapter 8

General discussion

8.1 Initial risk assessment phases

Chapter 2 aimed to present the conceptual site model and the analysis plan of the site-specific ecological risk assessment (ERA), following a tiered framework and adopting the Triad approach integrating three lines of evidence (LoE): chemical, ecotoxicological and ecological. The conceptual site model described what is already known about the site and the likely source-pathway-receptor linkages, based on published and unpublished works and by a visit to the site. The primary contamination source identified was soil contaminated by furnace slag deposition and by aerial deposition (wind-blow of dusts mainly from past chimney emissions), possibly linked to primary ecological receptors through the pathways of ingestion, cellular absorption, aerial deposition (wind-blow particles) and root uptake.

The analysis plan for the site-specific risk assessment was focused on the soil compartment and its potential risk to primary ecological receptors, such as plants, soil invertebrates and soil microorganisms. Potential risks to groundwater and indirect risks to freshwater systems (namely the temporary lagoons inside the area and the Subaé river) were also addressed by evaluating the soil retention function, i.e., the ability of contaminants to be mobilized via the soil water pathway. Although no direct assessment was done on water bodies, information about the retention function gave some hints on possible contamination of groundwater (via leaching) and surface water (via runoff events).

The analysis plan included two tiers using the triad approach: tier 1, the screening phase (Chapter 3), and tier 2, the detailed risk assessment (Chapter 7). Chemical data at tier 1 showed a high level of metal contamination in the study area caused by the smelting activities. Although moderate to high levels of some metals (namely Pb and Zn) could also be found in the vicinity of the smelting area, due to the deposition of smelting residues and aerial deposition of contaminated dusts, the very high levels of contamination are located within the smelting area. No clear contamination gradient could be found along the two transepts due to the spatial heterogeneity originated, not only by the uneven deposition of residues during smelting activities, but also by the current status of the (pseudo) rehabilitated residue piles. In some cases the layer of soil used to encapsulate the residues was removed by erosion and made the pile exposed, showing a depleted habitat.

In the tier 1 ecotoxicological LoE, avoidance behavior tests with earthworms were generally more sensitive to metal contamination and presented a less variable response than those with collembolans. Avoidance tests on soil organisms induced a higher risk (again at sites inside the smelter and with sandy soils) than tests on eluates (*V. fischeri* luminescence and *D. magna* lethal tests), suggesting a high retention of metals on the soil in most areas. Regarding the tier 1 ecological LoE, soil respiration, bait lamina and vegetation cover revealed a concordant response, despite the lower sensitivity of this last parameter.

Usually in ERA schemes, tier 1 comprises basically a comparison between concentrations of potential contaminants present in soil against thresholds for individual chemicals, concentrations below which no adverse effects are expected on receptors. However, one challenge to assess exposure and effects of contaminants in multi-contaminated areas is the interactions between them (Renella et al. 2002). Some contaminants act additively, others act independently of each other, or have antagonistic or synergistic actions. In this study we have followed Rutgers et al. (2008) and assumed that each metal had an independent toxic mode of action. So, the content of each metal was used to calculate the specific Toxic Pressure (Potential affect fraction of species – PAF) for each metal individually and then the msPAF (multi-substance PAF) of the mixture was calculated using the response addition model described by De Zwart and Posthuma (2005). In this way we could grasp potential risks posed by the metal mixtures

Even so, such chemical analysis *per se* do not incorporate contaminants not analyzed or for which threshold values do not exist, neither the join effects of mixtures. Thus, results obtained here at tier 1 showed the added value of using different LoE and different indicators inside each LoE covering different sensitivities and exposure routes to have a better perception of the potential risks. The parameters selected seemed promising to be used in such screening phase.

In general, in tier 1, very high risk levels, associated with sandy soils and residue deposits inside the smelter area, suggested the need to proceed with remediation actions, while the uncertainties associated with the contradictory information given by certain LoEs for certain sampling points showed the need to confirm potential risks in a tier 2 analysis. In this case, being an academic study, tier 2 analysis was done at all sampling points.

8.2 Detailed risk assessment

The results of the chemical analysis of tier 2 (Chapter 7) demonstrated that, despite the high total metal concentrations in several areas, the extractable fractions were very small, which is reported by other authors in soils contaminated with mine tailings (Alvarenga et al. 2008). And is in accordance with previews works carried out in the study area, e.g., Andrade Lima and Bernardez (2011) that characterized the slag disposed on the smelter site in Santo Amaro in a campaign performed in 2002. In the latter work, the results of the leaching study, using TCLP, SPLP and SWEP, showed that the slag was stable at a pH greater than 2.8, and only in an extremely acid environment was the solubilization of the Pb enhanced significantly. These results can be explained by the limited leachability of the metallic Pb and Zn-bearing compounds. However, the authors pointed that the long-term stability of slag cannot be ensured.

One open issue in ERA is how bioavailability can be properly included in the Triad approach (Swartjes et al. 2008). The availability of metals tends to decrease with the duration of its contact with soil (Naidu et al. 2003), and it is controlled by some factors mainly pH, cation exchange capacity, organic matter content, etc. Weak extractions have been suggested as a way to take bioavailability into account in chemical analysis, considering that the uptake from the water phase is most important for soil organisms (van Gestel and Koolhaas 2004). In the present study, the 0.01M $CaCl_2$ extractions showed low extractability of metals, in accordance to preview works carried out at the site (e.g. Andrade Lima and Bernardes 2011). However, these extractions did not foresee the ecological risks neither reduced the uncertainties. This may be related to the additional uptake from ingestion of soil particles (e.g., by earthworms), ingestion of food (e.g., by arthropods), and changes in bioavailability in earthworm gut or in plant rizosphere, not predicted by these type of traditional testing approaches.

In fact, the ability of soil invertebrate reproduction tests to assess the metal contamination is demonstrated in Chapter 4. The tests with Collembola and Oligochaeta species were carried out at 25 °C and showed to fulfill the validity criteria recommended by the respective ISO guidelines, which indicate that, while novel studies are under development with autochthones species, the standard species can be used in these tropical conditions in

schemes of risk assessment. Some points presented extreme conditions to tested species (e.g., 1000T1, 20T1; low pH and low organic matter content), as discussed in Chapter 4, which should be taken into account when planning and/or interpreting results from a site-specific ERA. Results on reproduction tests reinforced the importance of using ecotoxicological information obtained from different test organisms, covering different sensitivities and exposure routes, as the tested species were affected in a different way. *E. andrei* and *E. crypticus* reported significant reproduction effects for eight and five soil samples, respectively, both including all Soil Group 2 (highest risk values pointed by tier 1), while *F. candida* showed significant reproduction effect for five soils, not included in the Soil Group 2. The lack of significant effects to *F. candida* in Group 2 can be related to the low reproduction in Ref 2, used to perform the statistical comparisons of this group. Although fulfilling the validity criteria, reproduction on Ref 2 was lower than in the contaminated soils from Group 2. In general, results indicated impairment of soil invertebrates reproduction across the area sampled, mainly in sites corresponding to the deposition of tailings inside the smelter area, indicating the possible bioavailability of the metals and consequently posing some risk to ecosystem functioning. Declines in the reproduction of these organisms in metal contaminated sites suggests impact of metals on abundance and diversity of these groups (Spurgeon and Hopkin 1996), which can affect soil functions related to fertility, as cycling of soil organic matter and aeration.

Results from ecotoxicological tests with plants (Chapter 7) confirmed the negative effects of all tested soils (except P0, 20T1 and 400T1) on plant growth and/or biomass, affecting the tested species *A. sativa* and *B. rapa* in a different way. These effects can be related both to metal contamination and lack of organic matter and nutrients, as discussed in Chapter 7. We considered that germination of *A. sativa* and *B. rapa* was not a good endpoint because it takes a longer time in some reference soils than in contaminated soils, which can be related to soil properties (e.g., high clay content) as pointed by Saterbak et al. (1999).

Results of some microbial parameters (Chapter 5) showed that vegetation cover and plant species composition, indicators commonly used in the monitoring of terrestrial ecosystems (Godínez-Alvarez et al. 2009), were correlated with some soil functions, such as nutrient cycling and organic material breakdown. All points where microbial biomass or microbial activities were affected are located inside the smelter area. Several microbiological parameters were positively correlated with vegetation cover (soil respiration, biomass C, biomass N, dehydrogenase activity, acid phosphatase activity, asparaginase and ammonification rate), and negatively correlated with metal loadings (all cited except

dehydrogenase and ammonification rate). However, nitrification rate and metabolic quotient were positively correlated with metal contamination, suggesting favorable conditions for N loss and microbial stress, respectively.

Vegetation enhances favorable conditions to soil biota, such as maintenance of microclimate, availability of habitat structure, and supply of resources to biota (through litter input). The decomposition process, mainly carried out by microorganisms and soil fauna, is the gateway for the soil system, releasing nutrients for plants and supporting a plethora of soil organisms and their food-webs. Therefore, Impacts that alter plant community structure and abundance can induce changes in soil food webs and decomposition rates. In former mine or smelting areas, besides elevated levels of total metals, mine tailings often contain low levels of nutrients and organic matter, and are subject to wind and water erosion, being a difficult medium for plant establishment (Clark and Hutchinson 2005), and consequently the natural succession processes is generally very slow, requiring many years for changes to become apparent (Shu et al. 2005).

Results on litter bags tests (Chapter 6) showed the impairment of leaf-litter decomposition, which is probably occasioned by the absence of favorable conditions to soil biota, reducing microbial activity (showed by soil respiration), faunal feeding activity (showed by bait lamina) and the density of detritivores, allied to the low moisture content and higher temperature in the more exposed sites (caused by a low vegetation cover). Environments with metal contamination show decreases in litter decomposition rates (Giller et al. 1998; McEnroe and Helmisaari 2001) mainly due to inhibited abundance, the diversity and feeding performance of soil detritivores and the microbial activity (Filzek et al. 2004; Loureiro et al. 2006). As pointed by Hooper et al (2005), alterations of biota can modify ecosystem goods and services, which are very difficult to revert. In our work, results on pitfall traps showed that, directly or indirectly, contamination inside the smelter area seemed to had selected distinct communities of soil macroinvertebrates. This is agreement with Podgaiski and Rodrigues (2010) that found the existence of differences in invertebrate community composition in coal ash disposal sites in south of Brazil, due mainly to singular environmental conditions causing the loss of habitat quality in a long term polluted environment. In the present study, besides a significant decrease in the total number of morphospecies richness in the points inside the smelting area, changes in community composition were also significant. Community outside the area was characterized by a higher abundance and morphospecies richness of spiders and opilionids, whereas the community from inside the area presented higher abundance of Hymenoptera.

Regarding ecotoxicity tests on eluates, in general there was a lack of toxic response in aquatic tests, suggesting that the metals were not bioavailable in extracts, in spite of the high metal concentrations in soil. Thus, results indicated that the retention function of soils at most of the sampling points was enough to prevent the mobilization of metals via the water pathway, which is in agreement with the ICP-AES analysis that reported a low amount of extractable metals. However, a recent study carried out in a channel linking the smelter area to Subaé River, draining the excess of water of smelter area in raining season, presented metal contaminated sediment, indicating a present route of transport of contaminated material to the river (Niemeyer et al. unpublished). In addition, tier 1 aquatic tests showed lethal toxicity towards D. magna in two sites of Group 2 corresponding to tailing deposits, and in tier 2 eluates from both sites inhibited the growth of microalgae when compared to the respective reference, even though the observed lack of a marked toxic effect on the *D. magna* reproduction test can be related to metal adsorption by algae (see discussion in Chapter 7). These findings should be considered in future decisions about the site.

8.3 Comparing tier 1 and tier 2 results

The integrated risk (IR) on tier 2 confirmed the spatial heterogeneity of the risk along the study area, pointed by results of tier 1, as well as high levels of risk at sampling points within the smelter area, particularly in soils with a coarse texture (soils from Group 2), with P150T1 and P50T3 (corresponding to tailing deposits) showing an IR>0.75. According to the Dutch limit acceptable values for land use (Jensen and Mesman 2006), these high risk values restrict the use of the area even to industrial activities, requiring sealed soils. Results on integrated risk suggest the need to proceed with remediation actions in the smelter area. The relatively large deviation found in the final risk values in some points is related to the high risk pointed by chemical analysis, to the lack of toxicity in tests with eluates (indicating low mobility of metals), and the inability of some ecological parameters, namely the indexes from surface dwelling invertebrates, to identify the ecological risk.

The high risk values in the chemical LoE were related to the high total metal concentrations in soil, once low metal concentrations were detected in the soil extracts. The exceptions were points P150T1 and P1000T1. P150T1 is the most contaminated point, and such bioavailabity of metals and compromise of retention function was demonstrated in

immobilization *Daphnia* test in Tier 1. In the case of sampling point P1000T1, by being in the middle of a pasture area outside the smelter area, the presence of another type of contamination (e.g., pesticides) causing effect on aquatic tests should not be ruled out. This hypothesis was raised in Tier 1 because the avoidance response of *E. andrei* and *F. candida* observed in this test soil.

Ultimately, in this study, results from tier 2 revealed a similar picture than the one shown from tier 1. With a few exceptions, especially the reduction of some uncertainties, the ranking of risk values among the sampling points was similar. This confirms the added value of integrating, even at early stages of an ERA investigation, information from different lines of evidence and in getting more detailed information about the true potential risks in contaminated sites, and not to put all the investment only in chemical analysis. Information from the Triad can be used as a basis to take decisions about remediation actions and management concerning the future of the site. Of course each site specific ERA is case dependent and results from a tier 2 can give better insights about the real risks in the area. Although not by any means we are advocating to stop an ERA process after tier 1, as indicated above, in this study area, however, both tiers gave similar trends.

8.4 Sensitivity of ecotoxicological and ecological parameters for risk assessment

Aiming at evaluating how sensitive and cost-effective the different ecotoxicological and ecological parameters in the risk assessment could be, a sensitivity analysis was conducted taking into account not only the ability of each parameter to detect differences between contaminated and non-contaminated points (outside the area), but also their ability to detect a gradient of contamination (Tables 8.1 and 8.2). The time necessary to obtain the parameter was also estimated.

The ability of the avoidance behavior (Chapter 3) to detect toxicity within a short test period and at low costs makes this type of tests suitable for use in decision processes, as pointed by several works in the literature. The results obtained were quite promising since, in general, the risk values pointed by them in tier 1 were confirmed in tier 2 with the sub-lethal (reproduction) ecotoxicological tests. However, some care should be taken in the choice of reference soils (similar in properties except contamination) and it is recommended the use of at least one oligoquete and one collembolan species. If finding matching reference soils

becomes a difficult task, models are available to correct for the influence of soil properties (Chelinho et al. 2011).

Chapter 4 demonstrated the high sensitivity of oligochaeta reproduction tests to evaluate the contaminated sites; however, results obtained with *F.candida* were not sufficient for an adequate assessment of metal contaminated soils. As soil invertebrates species were affected in a different way, it is recommended the use of several species from different ecological groups, representing distinct routes of exposure to contaminants is crucial for a suitable evaluation of the risk.

Microbial community was highly impaired by metal contamination. Most microbial parameters presented significant partial correlations (using soil moisture, soil organic carbon, and mineral nitrogen as covariables) with metal loading given by the Widianarko's metal pollution index (W). Negative relations were found for basal respiration, microbial biomass (C and N), phosphatase and asparaginase activities, and number of ammonifiers, whereas a significant positive relation was observed for nitrification rate, which can be related to adaptation of nitrifying populations in metal-contaminated sites (see discussion in Chapters 5 and 6). However, only basal respiration, microbial biomass (C),acid phosphatase activity and nitrification presented a high to medium capacity to distinguish the level of soil contamination. Since the two first parameters were highly correlated ($r=0.82$, $p<0.001$), assessing only one is enough to give information relative to microbial activity. Bacterial parameters related to community structure (not assessed in this study) and bacterial growth/biomass were highly rated by Critto et al. (2007) as parameters to assess in all Triad tiers, mainly due to their rapidity and low cost. Based on these findings only one parameter seems not to be sufficient to give information about general microbial activity, and it should be complemented with other parameters related to microbial genetic diversity (e.g., DGGE) or metabolic diversity (e.g., Biolog) and with other specific activity parameters if processes involving particular nutrients are of interest.

The high sensitivity of feeding activity of soil fauna, allied to the fact that several studies showed the relation between bait-lamina data and abundance of several microarthropod groups and lumbricids (Birkhofer et al. 2011), make the bait-lamina test a definitive parameter to include in the ecological LoE in site specific assessments. Due to its ease and practicability, allowing to process the information from a large number of sampling points over a short time, it is a parameter to use in tier 1 of a Triad approach. Regarding abundance and normal biodiversity descriptors of surface dwelling arthropods, results did not show very promising results in distinguishing different levels of contamination. However, as

shown in Chapter 6, community composition could be a more promising parameter. Although not performed in this study, identification to family and/or trophic group, including the use of vulnerability traits, could be more useful to better decipher the true risks to this group of organisms. Another aspect, also not contemplated in this study is to sample not only surface dwelling organisms but true soil dwelling organisms, that could have a completely different response (as also mentioned in Chapter 6).

Vegetation cover and changes in plant composition were able to detect differences between points inside and outside the smelter area. However, their ability to detect gradients of contamination was not met in this case. Critto et al. (2007) presented a low rank for vegetation surveys related parameters in tier 1, mainly due to their cost. However, in higher tiers (tiers 2 and 3) these parameters presented higher ranking mainly related to their site specific relevance. In this case, we consider relevant the measured parameters on vegetation since they were able to show/explain important derived effects from contamination, namely those related to habitat disruption. So, at different levels, both assessed parameters should be incorporated in the ecological LoE.

Plant litter decomposition showed a high sensitivity to contamination and derived habitat disruption, but presented a low capacity to differentiate the level of contamination. In this case, it gave a similar information as the bait lamina test ($r=0.83$, $p<0.01$), thus not being a priority parameter to integrate in a tiered scheme (also due to the long time needed to obtain results).

Table 8.1 Valuation of the sensitivity of each ecotoxicological parameter assessed at the smelter area. Dark grey - parameters selected based on sensitivity criteria (see text for details).

Organism group	Category	Parameter	Significant response in contaminated sites (1)	Ability to differentiate the level of contamination (2)	Days needed to obtain the parameter (time of exposure)	Use in ERA (Tier)
Soil invertebrates	Habitat function for soil invertebrates	Reproduction of *Eisenia andrei*	High	High	56	2
		Reproduction of *Enchytraeus crypticus*	High	High	28	2
		Reproduction of *Folsomia candida*	No differences	No	28	2
Plants	Habitat function for plants	Growth of *Avena sativa*	Low	Medium	14-21	2
		Dry weight of *Avena sativa*	No	No	14-21	2
		Growth of *Brassica rapa*	No	No	14-21	2
		Dry weight of *Brassica rapa*	No to medium	No	14-21	2

(1) Iinformation based on observed significant differences against reference points (ANOVA): high ($p<0.001$); medium ($p<0.01$); low ($p<0.05$)
(2) Information based on significant correlations with metal loadings (Widianarko index): high ($p<0.001$); medium ($p<0.01$); low ($p<0.05$)

Table 8.2 (Extracted from Chapter 6) Valuation of the sensitivity of each ecological parameter assessed at the smelter area. Light grey - parameters derived from multivariate analysis based on individual parameters from the corresponding organism group; Dark grey - parameters selected based on sensitivity criteria (see text for details).

Organism group	Category	Parameter	Significant response in contaminated sites (1)	Ability to differentiate the level of contamination (2)	Days needed to obtain the parameter (estimated number of working days)	Use in ERA (Tier)
Microrganisms	Community activity	Microbial respiration	Low to High	High	8 (4)	1-2
	Community structure	Microbial biomass C	Low to High	High	2 (2)	1-2
	Community activity	Dehydrogenase activity	Low to Medium	No	1 (1)	1-2
	Community activity	Acid phosphatase activity	Low to High	Medium	1 (1)	1-2
	Biological process	Nitrification rate	Low to High	Medium	21 (3)	1-2
		Multivariate analysis with all microbial parameters	Medium			1-2
Invertebrates	Community activity	Feeding activity (bait lamina)	High	Medium	14 (4)	1
	Community structure	Abundance	Group dependent; low	No	37 (30)	2
	Community structure	(Morpho)Species richness (3)	Group dependent; low	No		2
	Community structure	Shannon diversity index (3)	No differences	No		2
	Community structure	Pielou evenness index (3)	No differences	No		2
	Community structure	Margalef richness index (3)	No differences	No		2
	Community structure	Berger-Parker index (3)	No differences	No		2
	Community structure	Changes in species composition (3)	High			2
Plants	Community structure	% of vegetation cover	High	Low	1 (1)	1
	Community structure	Species richness (3)	No differences	No	2 (2)	1-2
	Community structure	Changes in species composition (3)	High			2
Microrganisms and invertebrates	Ecosystem function	Litter breakdown (decay rate)	High	Low	140 (25)	2

(1) For individual parameters, information based on observed significant differences against reference points (ANOVA); for soil fauna abundance and taxonomic richness and for integrated multivariate analysis (ANOSIM), based on significant differences between sampling points outside the area (non or low contamination) from points inside the area (high contamination); high ($p<0.001$); medium ($p<0.01$); low ($p<0.05$)

(2) Information based on significant correlations with metal loadings (Widianarko index): high ($p<0.001$); medium ($p<0.01$); low ($p<0.05$)

(3) Parameters that require specific taxonomic knowledge

8.5 Final conclusion about the ecological risk at Santo Amaro

The ecological parameters indicated a clear distinction between sites inside and outside the smelter area, indicating an ecological risk to soil system even 17 years after the end of smelting activities. The deposit of highly contaminated tailings within the area and the failed attempt to encapsulate them have impaired the proper establishment of the vegetation, leading to changes and simplifications of the habitat structure. Allied to direct toxic effect of metals, the limitation of plant reestablishment resulted in low amounts of organic matter inputs into the soil to act as source of C and energy for microbial growth and for acting as protection for microbial community against high levels of heavy-metals in soil. Moreover, these changes in the microclimatic conditions at the ground level, and in the amount and quality of the potential of organic matter inputs, caused negative impacts on microbial activity and on soil organisms (feeding activity and species composition of surface dwelling organisms), consequently affecting the ecosystem services and underlying processes carried out by them.

8.6 Recommendations for future actions on site

The suitable encapsulation of the tailing deposits, with the concomitant re-establishment of a vegetation cover, seems to be essential to improve the ecological conditions at this site. The improvement of the vegetation cover can be effective in providing the necessary surface stability to prevent wind-blow of contaminated soil particles, preventing erosion, and reducing water pollution by interception of a substantial proportion of incident precipitation (Tordoff et al. 2000, Wong 2003). Furthermore, the choice of appropriate vegetation is crucial to remediate the adverse physical and chemical properties of the site and to reestablish the ecosystem functioning (Wong 2003), besides the aesthetical improvement of the site. Organic matter, soil nutrients and species diversity generally increase with community development during succession (Wang et al. 2011). It is important to consider the use of locally adapted species which are tolerant not only of physical and chemical conditions of tailings, but also to the climatic conditions of the site (Clark and Hutchinson 2005). Furthermore, plant species with different traits can increment the heterogeneity of soil habitats (Podgaiski and

Rodrigues 2010), thus can support soil communities that demand different requirements of food and shelters (Wardle et al. 2004, 2006). A study developed by Sydner and Hendrix (2008) shown that soil fauna development at a deraded site, especially of detritivorous species, has brought clear benefits to the ecological restoration processes because these organisms greatly affect soil structure and chemistry, and facilitate the ecosystem processes.

Habitat preservation and restoration should be the first priority for conservation of ecosystem, considering the whole landscape (Akçakaya 2001, Fahrig 2001). Considering the ecosystem complexity, besides the complexity involving the landscape use, the ERA should include both structure and functions ecosystem indicators (Burger e Gochfeld 2007). The aims of the ERA should be the protection of populations rather than individuals (European Comission 2002). For this purpose, as pointed by Filser et al. (2008), ecotoxicologists should make better use of basic ecology when establishing new tests or risk assessment schemes and convince regulatory authorities of the necessity of such approach. According to Rutgers and Jensen (2010), these approaches led the Dutch regulators to become less hesitant with respect to interpreting Triad approach-based results.

In Brazil, the resolution 420/2009 of CONAMA (BRAZIL 2009) states target and intervention values of chemical substances in soils, including guidelines for management of contaminated areas by anthropic activities. In contaminated areas under intervention or areas under monitoring for restoration, the risk management can be based on results of an ERA, depending on environmental authorities' criterion. In this sense, environmental authorities should establish procedures and actions for preliminary investigations of soil quality in suspected areas, and actions to eliminate or reduce the human and ecological risk in sites posing some risk. Experience can be acquired with the system by testing the basic approach in practical situations at a number of characteristic sites, as proposed by Rutgers et al. (2000) and as done in the present work, aiming to provide important information to help the regular utilization of the risk assessment process to support site restoration and reclamation decisions in Brazil.

To conclude, regarding the foreseeable future actions for the area, although the area of Santo Amaro is not large, a clear strategy of which areas should be a priority for rehabilitation is necessary. For that it is crucial to construct a risk map that can help identify those priority areas. The information gathered with this study, by relating the level of effects on selected ecological receptors with the level of contamination and some soil properties (mainly texture, pH and organic matter content), can provide crucial information for the

development of spatial models with the goal to produce such risk map. In fact, ongoing activities in the area (Niemeyer, personal information) already collected spatially based information on metal contamination and on soil properties on over 60 points on the entire smelter area. The mechanistic relation between these variables and effect/risk values will be done in a near future.

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