Ecological risk screening of metal (Pb and Zn) contaminated acidic soil using a triad approach

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Abstract

Lead (Pb) and zinc (Zn) are common metal contaminants in terrestrial environments. Decisions on remediation of metal contaminated soil are often based on risk estimates derived from generic guideline values. Guideline values are used at the screening stage of Ecological Risk Assessments (ERA) and have been developed to represent "safe" levels of contaminants applicable over large geographical areas (usually countries). If levels of contaminants exceed these guideline values, the risk is deemed as unacceptable and remediation is often initiated. However, it is now widely known that guideline values often are not effective in estimating true risk to humans or the environment. Using generic guideline values can lead to overly conservative remedial decisions, resulting in costly clean-ups that may not be necessary. Excavation of soil can also increase the risk of exposure to contamination and destroy native ecosystems. A "weight of evidence" or "triad" approach including information on soil chemistry, soil ecotoxicity and information on the ecological state of the site, taking bioavailability of the contaminants into account, could improve site specific risk screening estimates. These separate lines of evidence complement each other with chemical tests identifying contaminants of concern, bioassays confirming toxicity of the field samples, and ecological tests confirming actual effects in the field. However, current standardized tests usually require extensive handling of the field collected soil, including drying, homogenization and sieving. Handling of soil in this way may change the speciation of metals in the soil and thus the bioavailability. Risk estimates based on these tests may thus be inaccurate. To overcome this problem, undisturbed soil cores are proposed. However, if natural conditions of the soil are not within acceptable conditions for the organisms in toxicity tests, they will not survive in controls. This is particularly the case in very acidic soils. The sensitivity of many standardized test organisms to low pH is an important factor to consider, as naturally acidic soils have been estimated to occupy 30% of the world's ice free land area.

The overall objective of this thesis, which is based on papers I-IV, was to recommend tests that can be included in a triad approach at the screening level of ERA at metal (Pb and Zn) contaminated sites with acidic soils. A variety of bioassays and test organisms from three taxonomic groups (papers I, III, IV) as well as chemical speciation methods (papers I-II) and ecological methods (paper III) have been evaluated for use in undisturbed acidic metal contaminated soil cores. A risk characterization method combining the lines of evidence into a risk estimate has also been suggested.

Diffusive gradients in thin films (DGT)-labile metal concentrations and metal concentration in soil leachates from undisturbed soil cores were better predictors of accumulation of Pb and Zn in wheat (*Triticum aestivum*) than total metal concentrations in soil (paper II) and are therefore proposed as possible tools for the chemical assessment line. The wheat bioassay test in soil cores as outlined in papers (I, II) was relatively tolerant of low pH soils but insensitive to the metals of concern (Pb, Zn, Cd and Cu). The *Daphnia magna* test using leachate from the soil cores (paper I) appeared more sensitive to naturally occurring metals in the soil such as Al and Fe as well as low pH. The bioassays with lettuce (*Lactuca sativa)* in paper (I) and (III) appeared sensitive to the metals of concern but also displayed sensitivity to leachate pH below 6. In addition, Microtox, *Hyalella azteca,* and red fescue (*Festuca rubra*) showed similar or higher sensitivity to low pH than to Zn concentrations (III) and are therefore not recommended bioassays for risk screening of acidic soils. The MetSTICK test and growth tests with red clover (*Trifolium pratense*) were confirmed to be suited for risk screening of Zn contaminated acidic soils (paper III). Also, the plant species *Brassica rapa*, , *Allium cepa, Quercus rubra* and *Acer rubrum* were confirmed to be tolerant of low pH soils as well as showed potential to be sensitive to metals. (IV). *Dendrobaena octaedra, Folsomia candida*, *Caenorhabditis elegans*, *Oppia nitens*, were identified as possible invertebrate candidate species (IV) for the ecotoxicity line of evidence. *Colpoda inflata* from the microorganism group may be useful for assessing leachates from the soil cores (IV). For the ecological line of evidence, the screening test Bait Lamina may be suitable for soils with pH above 3.7 (paper III).

In conclusion, bioassay test species, chemical tests and ecological tests have been identified that could be suitable for risk screening of acidic undisturbed soil cores in a triad approach. This approach should result in improved risk estimates based on bioavailable concentrations of metals in soil in comparison with only relying on generic guideline values.

Keywords: Ecological risk assessment (ERA), risk screening, metal contaminated soil, undisturbed soil cores, weight of evidence, triad, bioassays, acid soils, Zn, Pb, diffusive gradients in thin films (DGT)

Sammanfattning (Summary in Swedish)

Bly (Pb) och zink (Zn) är vanligt förekommande metallföroreningar i mark. Beslut om sanering av metallförorenad jord baseras ofta på riskuppskattningar som härrör från generella riktvärden. Dessa riktvärden används i ett tidigt skede (screening) av den ekologiska riskbedömningen och har tagits fram med syftet att representera nivåer av föroreningar som kan anses acceptabla på de flesta platser. Om nivåer av föroreningar finns som överskriver dessa riktvärden påbörjas ofta sanering. Tyvärr har det visat sig att riktvärden ofta inte är effektiva för att uppskatta verklig risk för människor eller miljön. Använding av generella riktvärden som riskbedömingsverktyg leder ofta till överdrivet konservativa saneringsbeslut vilket resulterar i onödigt stora saneringsprojekt. Bortgrävning av jorden är inte bara dyrt utan kan också leda till ökad exponering av föroreningarna samt skada ekosystemet på platsen. Ett ”weight of evidence” eller "triad" angreppssätt som innefattar information om markkemi, marktoxicitet och uppgifter om det ekologiska tillståndet på den förorenade platsen, vilket också tar hänsyn till biotillgänglighet av föroreningarna, skulle ge en bättre platsspecifik riskbedömning. Informationen från de olika bevisleden kompletterar varandra med kemiska tester som identifierar föroreningarna, toxitetstester som bekräftar toxiteten av det platsspecifika provet samt ekologiska tester som bekräftar att effekter finns på platsen. Nuvarande standardiserade ekotoxitetstester och kemiska specierings metoder kräver dock ofta omfattande hantering av jordproverna, inklusive torkning, och siktning. Hantering av jorden kan ändra biotillgängligheten av föroreningar i jorden. Testresultaten kan således bli missvisande. För att övervinna detta problem har det föreslagits att intakta jordkärnor från den förorenade platsen kan användas i testen. Om testorganismerna då inte tål jordens naturliga egenskaper finns det risk att de inte överlever, även i de rena kontrollproverna. Detta fenomen är speciellt vanligt i sur jord. Testorganismers känslighet mot naturligt sur jord är en viktig faktor att beakta eftersom sur jord har uppskattats täcka 30% av jordens isfria yta.

Det övergripande syftet med denna avhandling, som baseras på fyra artiklar (I-V), var att fastställa vilka kemiska, ekotoxikologiska och ekologiska tester som kan användas i en triad på screening nivå för ekologisk riskbedömning av metallförorenad (Pb och Zn) sur mark. Olika biologiska testsystem och organismer från tre taxonomiska grupper (artikel I, III, IV) samt kemiska test metoder (artikel I-II) och ekologiska metoder (artikel III) har bedömts. En metod för att kombinera bevisleden för riskuppskattning har också föreslagits.

Diffusive gradients in thin-films (DGT)-labila metallkoncentrationer och metallkoncentrationer i lakvatten från intakta jordkärnor gav bättre uppskattning av Pb och Zn ackumulering i vete (*Triticum aestivum*) än totala metallkoncentrationer i jord (artikel II) och anses därför vara lämpliga kemiska verktyg för bedömning av miljörisk. Testet med vete som beskrivs i artikel I och II, visade sig vara tåligt vad gäller sur jord men inte känsligt mot de metaller som fanns i jordproven. Testet med *Daphnia magna* i lakvatten från jordkärnorna, som beskrivs i artikel I, var mer känsligt mot naturligt förekommande metaller i jorden, så som Al och Fe, samt mot det låga pH-värdet i lakvattnet. Saladstestet (*Lactuca sativa*) (artikel I och III), var känsligt mot metallföroreningarna i jorden och lakvattnet, men även mot det låga pH värdet (under 6.0). Microtox, *Hyalella azteca,* och *Festuca rubra* som användes som testorganismer i artikel III var också mer känsliga mot det låga pH-värdet än mot metalföroreningarna i jorden, och de rekommenderas därför inte som marktoxitetstester i sur mark. Rekommenderade marktoxitetstester är istället MetSTICK testet i jord och växttestet med *Trifolium pratense* i jord eller lakvatten. Båda dessa test visade sig vara känsliga mot metallföroreningar i jorden och toleranta mot lågt pH (artikel III). Även växtarterna *Brassica rapa*, *Allium cepa, Quercus rubra* and *Acer rubrum* är lovande kandidater i detta avseenede (artikel IV). *Dendrobaena octaedra, Folsomia candida*, *Caenorhabditis elegans* and *Oppia nitens*, har bekräftats som lovande evertebrater för toxitetstest av metallförorenad sur mark (IV). Test med *Colpoda inflata* kan vara lämpligt i lakvatten från jordkärnor (IV). Vad gäller ekolgiska testmetoder kan Bait Lamina testet vara lämpligt i jord med pH-värde över 3.7 (III) .

Sammanfattningsvis kan sägas att metoder för biologisk, kemisk och ekologisk bedöming av sur metal förorenad mark som tillsammans kan användas i en risk screenings triad har identifierats i detta arbete. Denna riskscreeningmetod kan ge en bättre uppskattning av biotillgängligheten av metallerna och därför även en bättre riskuppskattning än en metod baserad enbart på generella riktvärden.

Sökord: Ekologisk risk bedömning, risksceening, metallförorenad mark, intakta jordkärnor, triad, toxitetstester, sur jord, Zn, Pb, diffusive gradients in thin-fims (DGT)

List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals. These papers appear after the summarizing chapter of this compilation thesis.

I. Chapman, E., Dave, G., Murimboh, J., 2010. Ecotoxicological risk assessment of undisturbed metal contaminated soil at two remote lighthouse sites. Ecotoxicology and Environmental Safety 73 (5):961-969.

II. Chapman, E., Dave, G., Murimboh, J., 2012. Bioavailability as a factor in risk assessment of metal contaminated soil. Water Air and Soil Pollution 223 (6), 2907-2922.

III. Chapman, E, Hedrei Helmer, S., Dave, G, Murimboh, J., 2012 Utility of bioassays (lettuce, red clover, red fescue, Microtox, MetSTICK, *Hyalella,* Bait Lamina) in ecological risk screening of acid metal (Zn) contaminated soil. Ecotoxicology and Environmental Safety 80, 161-171.

IV. Chapman, E., Dave, G., Murimboh, J. 2013. A review of metal (Pb and Zn) sensitive and pH tolerant bioassay organisms for risk screening of metal-contaminated acidic soils (accepted for publication on April 17, 2013 in Environmental Pollution).

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1. Background

Following a century of industrialization, contaminated sites lie abandoned or underutilized all over the world. These sites degrade the environment and undermine the economic life of towns and cities. Many countries have developed strategies to tackle these problems proactively through legislative measures, assessment procedures, remediation, and funding (Prokop et al., 2000). However, research and regulations that support and protect terrestrial environments to date have lagged behind water quality research and policy (Van Straalen and Løkke, 1997; O'Halloran, 2006). Damaged soil recovers more slowly than water and the consequence of its contamination may have longer lasting effect (O'Halloran, 2006). Soil quality is critically important to sustain the soil functions that are crucial to terrestrial life (O'Halloran, 2006). These functions include the fixation of nitrogen, decomposition and recycling of nutrients from dead plant and animal tissues, maintenance of soil structure, and detoxification of pollutants (Pankhurst et al., 1997).

It is common for human health risks to drive most management decisions in relation to contaminants in soil. Ecological risks of contaminants in soil are inherently much more difficult to assess compared with human health risks. Ecotoxicology concerns the protection and well-being of several million-species scattered over a variety of habitats, all of which may be subject to an infinite variety of environmental variables and interactions. Since contaminants can degrade ecosystems to the extent that the environment may no longer sustain human needs, then ecotoxicology also indirectly addresses the well-being of humans (Persoone and Gillett, 1990).

To effectively assess and monitor the ecological risks of contaminants in soil, exposure and effects of contaminants to biota in the soil needs to be considered (Vasseur et al., 2008). This is often done in a structured approach referred to as an Ecological Risk Assessment (ERA). If unacceptable risks are identified during this procedure, remedial and other risk management options are considered. Until fairly recently, remediation efforts for contaminated areas have mainly focussed on removal of the contaminated soil. This is largely because of the historically poor understanding of contaminant fate processes in soils. In addition to being an expensive technique, the excavation of soil can increase the risk of exposure to contamination and destroy native ecosystems (Committee on Technologies for Cleanup of Subsurface Contaminants in the DOE Weapons Complex and National Research Council, 1999). Recently, *in-situ* remediation techniques have become more common, especially for large metal contaminated areas or remote areas, where excavation of the material would be too expensive or not feasible. New *in-situ* remediation techniques for metals alter the chemistry of the soil contaminants, making metals less soluble, less mobile, and less bioavailable. These types of immobilization techniques do not affect the total contaminant concentration, but reduce the risk of harm to a target organism (humans, plants, animals, etc.) by decreasing the biological activity of the contaminants (ITRC and Metals in soils Work Team, 1997). Following the immobilization of contaminants, any attempt to assess the risk of contaminants looking only at the total concentrations would fail as the total concentration has not changed. Presently, there is a lack of guidance on approaches for long-term monitoring that specifically target the stability of the contaminant “form” instead of total contaminant concentration (National Research Council, 2003).

1.1 Metal fate and bioavailability in soil

Metals are common contaminants in terrestrial environments, and the most common metal contaminant in soils is Pb (United States Department of Agriculture, 2000; Wuana and Okieimen, 2011). Mining, smelting, and associated activities are one of the most important sources by which soils, plants, and surface waters are contaminated with metals (Jung, 2008). Unfortunately, information on the total concentration of metals in contaminated soil is not sufficient for estimating the risk of metals in soil (Sanz-Medel, 1998; Kong and Bitton, 2003; D'Amore et al., 2005). Whereas reliable techniques for routine measurement of total metal concentrations are readily available, few techniques are available to separate and measure metal speciation in soils. Furthermore, no single tool has been developed that can universally describe or measure metal “bioavailability”. Consequently, the definition of “bioavailability” and the concepts on which it is based are currently operationally defined (Tack and Verloo, 1995).

Many organic compounds are biodegradable, reducing the potential for human and ecological exposure over time. Although metals can be transformed from one form to another (e.g. complexation, redox state, etc.), they are persistent and cannot be destroyed. Pb has been estimated to have a soil retention time from 150 to 5000 years (Kumar et al., 1995). Speciation of the metals effects bioavailability which is determined by both soil chemical and physical characteristics, as well as organism characteristics (Chapman et al., 2003). In the soil pore water, metals can exist in free hydrated forms, inorganic or organometallic complexes, or bound to small dissolved particles (i.e. clay mineral and organic matter). Metals in the soil matrix can be adsorbed on the surface or exist inside of particles and colloids (Slaveykova and Wilkinson, 2005). In soils, most of the metal is insoluble, but some is reversibly bound to the soil particles (Sauvé et al., 1997). The most important soil properties affecting the speciation of metals in soils are acidity, redox conditions, salinity, competing ions, nature of sorbent phases and their surface areas, surface site densities and colloid formation (Peijnenburg and Vijver, 2007). The metal in solution, and particularly the free metal ion, is the most bioavailable and mobile form of metal in soil (Tack and Verloo, 1995; Kong and Bitton, 2003; Berggren Kleja et al., 2006; Zhang and Young, 2006). However, certain portions of the solid phase pool of metals can replenish the soil solution and thus become remobilized and bioavailable (Tack and Verloo, 1995; Zhang and Young, 2006, McBride and Martínez, 2000). This fraction of metals can be referred to as the labile fraction (Lam et al., 1996; Young et al., 2005). Becquer et al. (2005) found that the water extractable fractions of Zn and Pb were poorly correlated with metal accumulation in the earthworms *Aporrectodea caliginosa* and *Lumbricus rubellus*. However, the moderately labile forms (acid soluble, bound to iron oxides and organic matter) of Zn and Pb were well correlated with metal accumulation in the earthworms. Vijver et al., (2001) also noted that concentrations of metals and toxic effects in invertebrates such as isopods do not always correspond well with the soil solution concentrations of metals. Invertebrates are not only exposed to the soil pore water concentration through the dermal pathway, but are also exposed to the bound metals in soil particles when ingesting these particles (Becquer et al., 2005).

Once the metal has entered an organism through a cellular membrane, it will not necessarily result in a toxic effect. Within the organism the metal can be transformed, transported, deposited into inert tissues (such as fat, hair or bone) or excreted (National Research Council, 2003). If, during any of these processes, the metal reaches the site of toxic action, effects will occur. In terms of lead, the Pb^{2+} ion can react with cellular membranes and have a direct toxic effect. In plants typical symptoms of lead toxicity include reduced leaf size, chlorotic and reddish leaves with necrosis, short black roots, and stunted growth (CCME, 1999a). With increasing lead concentrations, exposed plants generally also exhibit decreasing photosynthetic and transpiration rates. Similar symptoms have been reported for plants affected by high levels of zinc in the soil. Chlorosis, mainly in new leaves, and depressed plant growth are the most common symptoms of zinc toxicity in plants (Kabata Pendias and Pendias, 1992).

In the field, contaminants exist in the soil in different mixtures. These contaminants can have independent, additive, synergistic or antagonistic effects on the organisms living in the soil (Amorim et al., 2012). Fulladosa et al. (2005) reported effects of different combinations of Co, Cu, Zn and Pb in terms of toxicity to *Vibrio fischeri* bacteria. Antagonistic effects were observed between Co-Cd, Cd-Zn, Cd-Pb and Cu-Pb. Synergistic effects were found between Co-Cu and Zn-Pb and additive effects in all other cases. Effects on organisms from mixtures can differ between different organisms (Amorim et al., 2012). Preston et al. (2000) reported synergistic effects of Zn and Cd in terms of toxicity to *Esherichia coli* while additive effects were reported by Weltje (1998) for the combination of Cd, Cu, Pb and Zn toxicity to earth worms. Wah Chu and Chow (2002) reported synergistic effects of 10 heavy metals in terms of toxicity to nematodes. Zn has been reported to likely be responsible for the majority of ecological effects observed (to invertebrates) in a mixed (Cu, Pb, Cd and Zn) contaminated soil (Spurgeon and Hopkin, 1995; Laskowski and Hopkin, 1996; Fountain and Hopkin, 2004; Jensen and Pedersen, 2006). The Canadian Network of Toxicology Centre's Metals in the Environment Research Network (MITE-RN) program, conducted a literature review on the toxicity of metal mixtures in June 2004 (Canadian Network of Toxicology Centres, 2004). Their findings concluded that assumptions of additivity were overprotective in 43% of cases (less than additive), appropriately protective in 27% of cases (additive), and under protective in 29% of cases (more than additive). To assess risks of metal mixtures, this research group suggested using bioassays of the mixture as present in the environment. O'Halloran (2006) also emphasized the usefulness of direct toxicity assessment of the contaminated soil in determining the effects of mixtures.

1.2 Ecological risk assessment (ERA) of metal contaminated sites

Ecological Risk Assessment (ERA) has been defined as the "prediction and evaluation of the effects of chemicals, and often other stressors, on ecosystems, usually in specific environmental management situations" (Connell, 1999). The concept of ERA was developed in the 1980s to provide basis for environmental decision making and was derived from practises in human health risk assessment (Suter II, 2007).

Different frameworks have been developed for ERAs in various countries, but in general the steps involved in the structured approach that makes up ERA include: (1) problem formulation, (2) analysis (exposure and effects assessment), (3) risk characterization, and (4) risk management (Figure 1). During the first of these steps, problem formulation, the objectives and overall scope of the assessment are defined. The analysis step consists of data collection to characterize exposure and effects on ecological systems. Information on exposure and effects are then fused as estimates of risk in the risk characterization step.

The majority of ERA frameworks are also based on a tiered approach with different levels representing increased details of assessment (Oregon Department of Environmental Quality, 1998; EPA, 2001; Jensen et al., 2006; Jones, 2006). Each level or tier includes all the steps presented in the previous paragraph (problem formulation, analysis, risk characterization and risk management). Although the number of tiers and names of tiers differs between different frameworks, the common factor is that each subsequent tier has increasing detail in terms of chemical and ecotoxicological data. This approach ensures that resources are used wisely and detailed assessments are only initiated when screening assessments do not provide enough foundation for decision making. For example, the Canadian framework (CCME, 1996) consists of three tiers: (1) screening assessment, (2) preliminary quantitative ERA, and (3) detailed quantitative ERA. During Tier 1 screening assessments, it is common to present exposure as the highest measured concentration of the contaminant(s) of concern. This concentration is then compared with screening levels or guideline values for different contaminants. These guideline values are usually based on standardized toxicity testing results from the literature and safety factors. In later tiers of the ERA, more detailed descriptions of location, extent, transport and accumulation of contaminants are required as well as a complete assessment of effects including the use of bioassays.

In the Recommended Guidance and Checklist for Tier 1 Ecological Risk Assessment of Contaminated sites in British Columbia, Canada (Landis et al., 1998), it was estimated that over 90% of all contaminated sites in British Columbia are assessed and addressed using Tier 1 screening ERA. Only 1% progress to the third tier, detailed quantitative ERA. Jones (2006) also noted that decisions on remediation are most commonly based on information gathered during the screening stage of ERA.

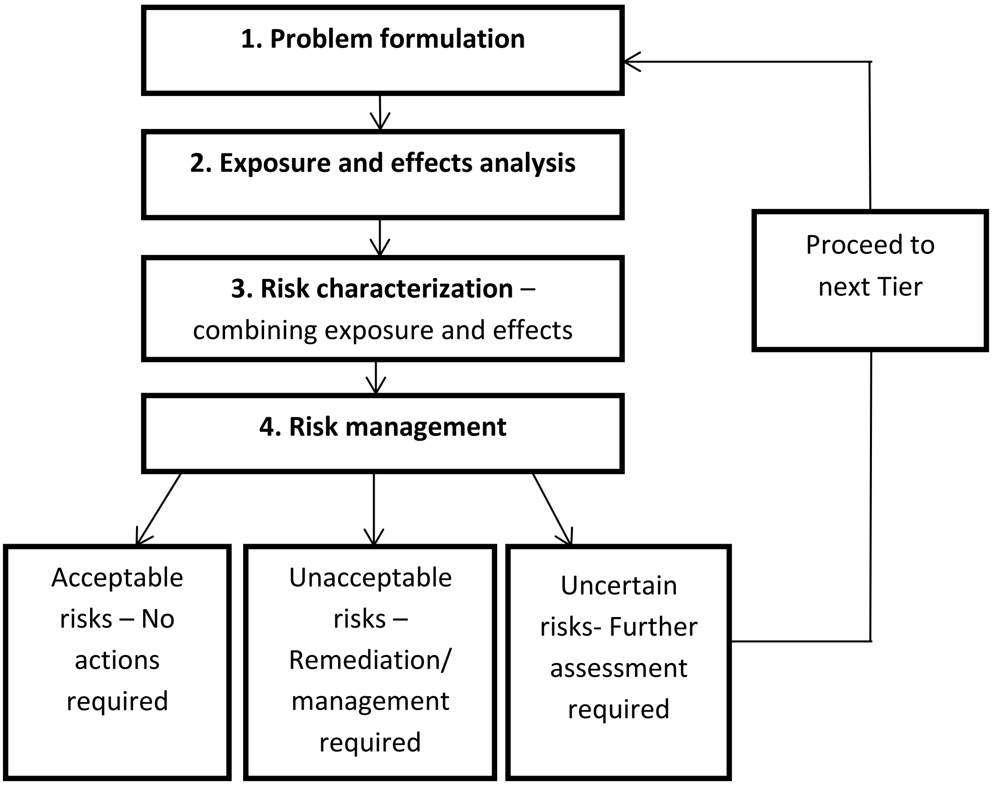

Figure 1. Flow-chart: General ERA framework (created based on (CCME, 1996; US Environmental Protection Agency (EPA), 1998; Jensen et al., 2006; Suter II, 2007))

1.3 Guideline values as risk screening tool

The use of guideline values in the ecological risk screening process of contaminated land is currently the most common practice around the world. Several steps are involved in developing these guideline values. A literature review is generally the first step in this process. In most cases, environmental soil guideline values are based on dose-response data from single species tests (traditionally plant or earthworm tests), or tests on ecological processes in the laboratory. Chronic No Observed Effect Concentrations (NOEC) or Lowest Observed Effect Concentrations (LOEC) data are used when the purpose of the guideline value is to indicate levels where no harmful effects are expected (Jones, 2006). Provided there is enough information available, statistical extrapolation procedures are then used on the data set in order to derive the soil guideline values (Jones, 2006; O'Halloran, 2006). Extrapolation methods can be divided into three different groups: (1) methods that are based on statistical distributions such as species sensitivity distributions (Swartjes et al., 2012) when data are abundant, (2) "safety factor methods" that are used if data are scarce and (3) the use of equilibrium constants from aquatic toxicity data if terrestrial toxicity data are absent (Jones, 2006; O'Halloran, 2006). This third method extrapolates from aquatic toxicity data to terrestrial toxicity. This technique has been questioned for the lack of proven correlation between aquatic and terrestrial data. Consequently, some countries such as Canada and the USA do not use this method to derive soil criteria. Instead they identify data gaps that require further research (US Environmental Protection Agency (EPA), 2005b; CCME, 2006; O'Halloran, 2006).

Soil guideline values to protect the environment vary between different countries and are not easily transferable. In some countries the values represent remediation goals while in other countries they may represent investigation levels (O'Halloran, 2006). Some countries have different ecological guideline values for different organisms, while other countries have different values for different land uses. Table 1 summarizes ecological screening levels or guideline values for Pb and Zn developed by five different organizations: Swedish Environmental Protection Agency, the Canadian Council of Ministers of the Environment, US Environmental Protection Agency, Oak Ridge National Laboratory, and the National Institute for Public Health and the Environment (Netherlands). The guideline values differ significantly between different organizations. This is partly due to different guideline development methods.

Table 1. Examples of guidelines (mg/kg dry wt) for Pb and Zn contamination in soil

Organization, guideline name, objective		**Guideline value (mg/kg dry wt)**	
		Pb	**Zn**
Swedish EPA general guideline values (Naturvårdsverket, 2008)	KM[a]	50	250
	MKM[b]	400	500
CCME[c], SQG[e]s[d] (CCME, 1999a; b) Canada	Agricultural	70	200
	Residential/ Parkland	300	200
	Commercial	600	360
	Industrial	600	360
US EPA, ECO SSL[e] (US Environmental Protection Agency (EPA), 2005a; 2007)	Plants	120	160
	Soil Invertebrates	1700	120
	Wildlife Avian	11	46
	Mammalian	56	79
ORNL[f], Bench mark, (Efroymson et al., 1997a; Efroymson et al., 1997b) USA.	Plant	50	50
	Earthworm	500	200
	Microbial	900	100
RIVM[g,] (Verbruggen et al., 2001) Netherlands	Target value	85	140
	Intervention value	530	720

Explanation of Table Abbreviations: a/ KM: Sensitive land use, b/ MKM: Less sensitive land use, c/ CCME: Canadian Council of Ministers of the Environment, d/ SQG[e]: Soil Quality Guidelines for environmental health, e/ ECO SSL: Ecological Soil Screening Levels, f/ORNL: Oak Ridge National Laboratory (United States of America), g/ RIVM: National Institute for Public Health and the Environment (Netherlands).

In addition to the statistical extrapolations, there are several other limitations with using guideline values as a screening tool in assessing ecological risk to terrestrial organisms. Many of these limitations have been discussed in detail in paper IV and will be summarized briefly here.

- Guideline values are based on total concentrations of contaminants in soil. However, the total concentration in soil has little or no meaning for the protection of soil ecosystems. The contaminant bioavailability must be taken into account when assessing the risk of metal contaminants in soil (Allard et al., 2002; Chapman et al., 2003; Gorsuch et al., 2006; Jensen et al., 2006; Jensen and Pedersen, 2006). In an effort to account for varying metal bioavailability in different soils, some countries allow development of site specific guideline values when generic guideline assumptions on soil properties are not applicable. The Dutch generic guideline values can be corrected to account for site specific soil properties such as percent clay and organic material in the soil (Swartjes et al 2012). However, no standardized procedure for the determination of the bioavailable fraction has been incorporated in Dutch soil policy, and no consideration has been given to bioavailability issues related to different species and processes (Swartjes et al. 2012).

- Guideline values are typically derived from laboratory toxicity studies with very soluble metal salts. The metals in these salts are likely to be more available and hence toxic at lower concentrations than the mixture of aged metals encountered in field soils (Efroymson et al., 1997b; ESTCP-ER, 2005),
- Multiple stresses in the field such as climatic stress, predators, competition, food shortage and site specific metal background concentrations (Chapman et al., 2003; Nowack et al., 2004) are ignored with the guideline value screening approach,
- Contaminated sites are rarely contaminated with just a single contaminant but often contain a complex mixture of contaminants. Analyzing the total concentrations of individual contaminants and comparing them to single contaminant guideline values may oversimplify the risk and exclude synergistic and antagonistic effects resulting from interactions between chemicals, and
- Chemicals not covered by the analysis can be overlooked during the exposure assessment (Efroymson et al., 1997b; O'Halloran, 2006).

1.4 Triad (weight of evidence) approach as risk screening tool

Chapman et al. (2003) argued that the generic ecological risk assessment (ERA) procedure does not apply to metal contaminants and the procedures associated with the generic ERA process should be modified for these contaminants. However, there is no agreement on how this should be done and there is a need for further research into the factors controlling the release of metals from soil. Ideally, ERAs for metals should include information on the speciation of metals, information on physical, chemical and biological factors influencing the speciation, presence of potential receptors, potential pathways for uptake, and information on possible effects on organisms, populations and communities due to the metal fractions present.

In order to deal with conceptual uncertainties, it has been proposed by several researchers to use weight of evidence (WoE) approaches for ecological risk assessments of metal contaminated soil (Burton et al., 2002; Chapman et al., 2002; Jensen et al., 2006, Sovari et al., 2013; Ribé et al., 2012; Gruiz et al., 2007). The research project Liberation (Jensen et al., 2006) supported by the European Commission under the Energy, Environment and Sustainable Development Program , developed a tiered decision support system for ecological risk assessment of contaminated sites including a weight of evidence approach; the triad method. The triad approach is based on the Sediment Quality Triad (Long and Chapman, 1985), developed in the late 1980s for sediment quality assessment. It integrates site-specific toxicological, chemical, and ecological information in the risk assessment (Figure 2). The assumption is that several lines of evidence in three independent disciplines will lead to a more precise answer than an approach, which is solely based on, for example, the total concentrations of pollutants at the site. A multidisciplinary approach also gives acknowledgement to the fact that ecosystems are too complex to analyze in one-factorial approaches.

The Netherlands use a WoE approach at their third tier (detailed site specific risk assessment) of the ERA (Swartjes et al., 2012). Other countries that have incorporated WoE approaches in their official ERA guidelines for contaminated soil management include USA, Canada (British Columbia) and the UK (US EPA, 1998; Science Advisory Board for Contaminated Sites in British Columbia 2008; Environment Agency 2003).

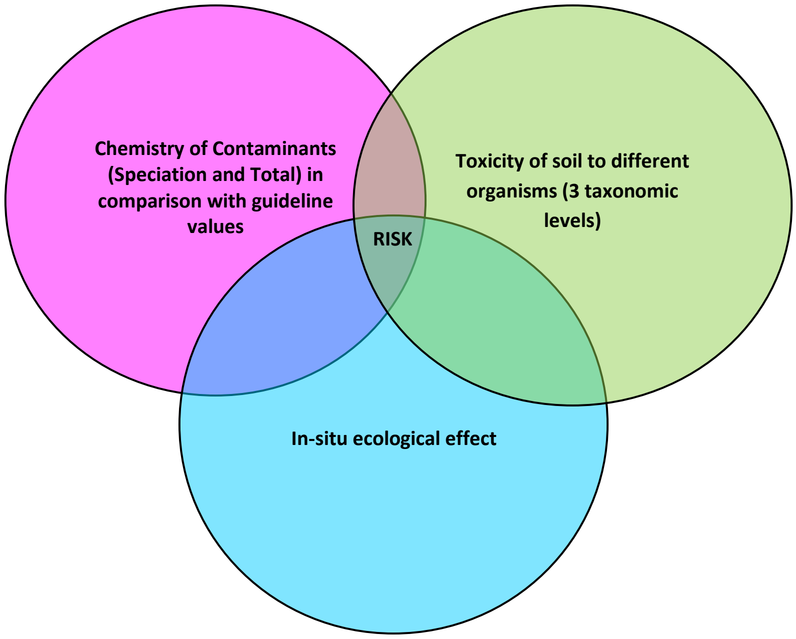

Figure 2. Illustration of the different types of information that could be incorporated into a triad framework for the assessment of ecological risks of contaminants in soil (modified from Jensen et al., 2006).

The chemical line of evidence provides information on the presence and levels of different contaminants in the medium to be tested and chemicals of potential ecological concern (COPEC) are identified. However, proof of actual toxic activity cannot be confirmed. This is where the toxicity line of evidence will help. Species for the toxicity tests should ideally represent the broad diversity of ecological niches associated with the soil environment (Allard et al., 2002) and when possible, standardized tests should be used (Achazi, 2002). This line of evidence will provide information on the toxicity of the test medium to the test organism. It cannot, however, identify the cause of the toxicity. When information from the chemical and toxicity line of evidence is combined with information from the in-situ ecological effect line of evidence, it is possible to confirm if the receiving ecosystem has been affected. Jensen et al. (2006) as well as Weeks and Comber (2005) promote the use of a tiered weight of evidence system in the risk assessment of contaminated soil. The first tier of the assessment process is a simple screening, followed by refined screening, detailed assessment and final assessment. Several methods are suggested to be used in conjunction with one another during each of these tiers. Methods increase in length and complexity for each tier. The screening methods are simple and fast but should still cover chemistry, toxicology and ecology to appropriately assess the risk. Jensen et al. (2006), provided a comprehensive list of different tools that can be used in a weight of evidence risk assessment approach for organic contaminants at different tiers of the ERA. The Microtox test was suggested as a possible bioassay screening tool to be used in addition to guideline values in a WoE system by Weeks and Comber (2005). Other bioassay and ecological screening tools that have been used in ERA of metal contaminated soils include seed germination, *Daphnia magna* immobilization, plant growth tests, *Eisenia fetida* mortality, avoidance behaviour of *Folsomia candida* and *Eisenia andrei*, bait lamina test, soil basal respiration, and vegetation cover (Niemeyer et al., 2010; Alvarenga et al., 2012). However, Niemeyer et al. (2010) noted that the common test organism *E. andrei* was affected by low soil pH. This is supported by Jänsch et al. (2005) who concluded that most of the common standardized bioassay organisms are sensitive to naturally acidic soils and alternative species are required for this specific soil type.

1.5 Specific issues with metal contaminated acidic soils

Over 30% of the planet's ice free land area is estimated to contain soils that are naturally acidic (Von Uexküll and Mutert, 1995). During risk screening of naturally acidic, metal contaminated soil samples using the triad method, there are several specific issues to contend with. It is imperative that soil pH is not changed during the assessment process of the samples as changing pH may inadvertently change the bioavailability and toxicity of contaminants. Therefore, any chemical, ecotoxicological or ecological assessment method aimed at estimating bioavailable concentrations must minimize soil handling. The majority of chemical speciation methods and standardized toxicity methods require extensive handling of soil samples prior to the tests. Some of these tests, such as the Microtox test and the *Daphnia magna* immobilization test, use soil elutriates and require alterations of soil pH if the pH is not within acceptable ranges for the test organism. In certain cases, test organisms are more sensitive to the low pH of the soil than to the contaminants of concern (Jänsch et al., 2005). To overcome the issue with metal bioavailability changes in soil samples, it has been suggested that intact undisturbed soil cores from the field be used for the tests (Van Straalen, 2002; Kuperman et al., 2009; Scroggins et al., 2010). Tests with intact contaminated soil cores have been referred to in the literature as terrestrial microcosm tests (Burrows and Edwards, 2002). For this approach to be effective, test organisms that are tolerant of naturally acidic soil samples as well as being sensitive to metal contaminants must be identified. In addition, chemical speciation techniques that minimize soil handling should be prioritized and assessed for use in undisturbed soil cores.

1.6 Objective

The overall objective of this thesis was to determine how the triad approach best can be applied to the screening level of ecological risk assessments at metal contaminated sites with acidic soils, while focusing on the bioavailability of the metals. Testing directly in undisturbed contaminated soil cores, according to the methodology proposed here, minimizes changes in metal bioavailability that could affect test results and risk estimates. Tests suitable for use directly in undisturbed soil cores therefore need to be identified. The decision was made to focus on Pb and Zn as soil contaminants. This limitation was necessary in order to reach conclusions on this vast topic within the limited time-frame available. However, Pb has been reported to be the most common soil metal contaminant (United States Department of Agriculture, 2000; Wuana and Okieimen, 2011) and Zn has been reported to be the metal most likely responsible for ecological effects in invertebrates in a mixed (Pb, Zn, Cu, Cd) metal contaminated soil (Spurgeon and Hopkin, 1995; Laskowski and Hopkin, 1996; Fountain and Hopkin, 2004; Jensen and Pedersen, 2006).

In order to fulfill the above stated objective, the following specific questions were addressed in different sub-projects:

Chemical assessment:

- What existing chemical methods for assessing and evaluating the bioavailability of metals in soil could be used in undisturbed soil cores, together with information about background concentrations, total concentrations and guideline values at the risk screening stage? (I,II)
- How do the selected chemical methods relate to actual biological uptake and toxicity in undisturbed metal contaminated acidic soils? (II)

Ecotoxicological assessment:

- Can the standardized bioassay tests be adjusted to undisturbed metal contaminated acidic soil cores to effectively screen for ecological risk? (IV)
- What bioassay test organisms from three different taxonomic groups (invertebrates, plants and microorganisms) can withstand naturally acidic soils while being sensitive to the metals of concern? (I, III, IV)

Ecological assessment:

- What ecological screening tools can be used in undisturbed metal contaminated acidic soil cores to effectively screen for ecological risk? (III, IV)

2. Methodological considerations

The methods used are described in detail in the different papers, but are summarized and discussed here.

2.1 Literature review

Initially a literature review was completed. The purpose of this review was to compile information on existing standardized and non-standardized tests that could be used in a triad approach at the screening tier of ERA. Ecotoxicity tests (for the toxicity line of the triad), ecology assessment methods (for the ecology line of the triad) and chemical methods for estimation of the bioavailable fraction of contaminants in soil (for the chemical assessment line of the triad) were evaluated. When standard test methods were available, European methods (ISO-methods, OECD) were compared with Canadian (Environment Canada) and American methods (US EPA and ASTM). Tests with durations longer than 10 days and tests that are exclusively for assessing single spiked chemicals in artificial soil were excluded to ascertain applicability at the screening stage of ERA at historically contaminated sites. A summary of compiled methods are provided in the Results section, Table 3 (chemical methods), Table 5 (biological methods) and Table 8 (ecological methods). Following the review it was determined that no standardized methods exist for assessment of undisturbed contaminated soil cores. All of the standardized toxicity tests require handling of the soil including sieving, homogenization and drying. In certain instances, changes in pH of the test medium are also recommended if the pH is outside of the test organism's tolerance level. It was therefore deemed necessary to investigate different standardized and non-standardized species' sensitivity to pH and metals to establish what species are able to withstand the conditions in an undisturbed acidic soil while being sensitive to the metal contaminants of concern.

A few bioassay test organisms as well as chemical tools to estimate metal bioavailability and an ecology assessment method were selected for the experimental tests with intact contaminated soil cores. Only methods that ensured minimal disturbance of the soil core were considered. This choice was also limited by available resources and time constraints.

2.2 Soil sampling, soil handling and test design

Soils included in the tests were undisturbed soil cores from two different contaminated sites (paper I and II) and Zn spiked acidic soil cores (paper III). The purpose of testing the spiked samples was to establish how sensitive certain tests (and species) were in relation to Zn and pH, keeping all other variables as constant as possible.

The soil samples collected for paper I and II, were obtained in a metal concentration gradient (four from each site) from the contaminant source (lighthouse structures in Nova Scotia and New Brunswick), with highly contaminated samples in close proximity to the structures, to background concentrations at a distance of 35 m and 50 m away from the structures. Five replicate soil samples were collected from each sampling location using a hand corer to minimize soil disturbance (Figure 3). The soil cores were directly transferred into 250-mL soil sample glass jars for transportation to the laboratory. Each soil core was then carefully transferred into a 265-mL plastic pot with minimal disturbance of the soil core. A filter paper was inserted at the bottom to reduce soil particle migration. Transparent plastic beakers were placed underneath each pot to collect the leachate (Figure 3). The pots were irrigated three times a week with Milli-Q water using a spray bottle to mimic precipitation. Each pot was sprayed continuously until approximately 70 mL of water had drained into the container below. In addition to the reference samples collected outside the area of contamination, two external control soils were used in these tests; Garden Soil, Original Grower Mix from ASB Green World and Seed Starting Soil from Miracle-gro. The ASB soil contained sphagnum peat moss, dolomitic limestone and natural wetting agent. The Miracle-gro soil contained sphagnum, peat and perlite.

The soil used for spiking purposes (paper III) was obtained from a pristine site with acidic soils using a hand corer. The clean soil samples were collected and handled using identical procedures as for the aged contaminated samples in paper I and II. For the experiment with spiked soil samples (paper III), the clean acid soil cores were watered to saturation with solutions of $ZnCl_2$ aiming for concentrations in the soil of 100, 200, 400, 800, 1600, 3200, 6400 and 12 800 mg Zn/kg dry weight. This concentration range was deemed appropriate based on background concentrations and a real contamination gradient at a nearby site (paper I and II). The spiking solutions were added to the soil using a spray bottle. Dilution water for the spiking solutions was Milli-Q water. Three replicates of each concentration were prepared. A similar concentration series was prepared with $CaCl_2$ as a non-toxic reference, using 3 replicates aiming for concentrations of 400, 800, 1600, 3200, 6400 and 12 800 mg Ca/kg dry weight in soil.

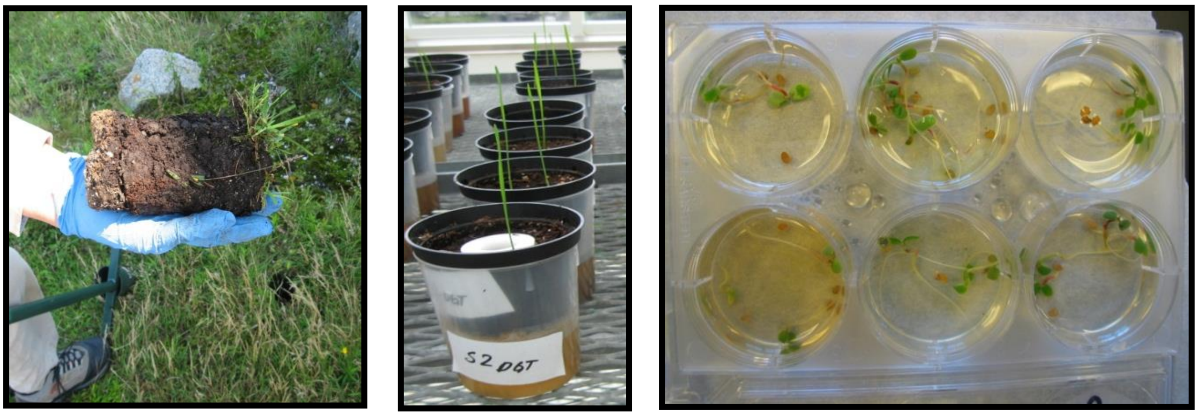

Figure 3. Soil core collected in the field (left), soil cores in pots with wheat seedlings, DGT and plastic beakers collecting leachate (middle) and 6-well plates with soil core leachate and lettuce seeds/seedlings (right). Photos by Emily Chapman.

2.3 Chemical tests and analysis

For the chemical assessment line of the triad, three potential screening tools were evaluated; 1) total concentrations of metals in the soil cores compared with guideline values (traditional approach), 2) diffusive gradients in thin films (DGT) labile concentrations in soil cores, and 3) total concentrations of metals in leachates from soil cores. DGT labile concentrations and concentrations of metals in the soil core leachates were evaluated based on how well they corresponded with actual bioavailability of metal contaminants to wheat grown in the same soil cores.

The DGT samplers were prepared according to the directions provided by DGT Research Ltd (paper II). DGT samplers were then positioned in the same pots as were used for the bioassay growth test with wheat. One DGT sampler was positioned in one of the soil core replicates for each sample location. The first DGT device remained in the soil for the full length of the first week (paper II) and was then removed to minimize the risk of resin overloading. The second DGT device was positioned in the soil for the full length of the last (sixth) week. After retrieval of the DGT samplers, the resins were eluted with 1 mL of 1 *M* Optima nitric acid (Fisher Scientific). The samples were then analyzed with an ELAN DRC-*e* inductively coupled plasma – mass spectrometry (ICP-MS) (PerkinElmer SCIEX). QA/QC protocol incorporated a procedure blank which was carried through all stages of preparation to analysis. In addition, a laboratory control sample and reagent blank were analyzed once every 10 samples. Recoveries were within ±20% of the accepted value of a fortified water sample that was prepared in house and periodically checked against a certified reference material (NIST 1634e).

To evaluate soil core leachate metal concentrations in relation to toxicity and bioavailability of metals (paper I, II and III), each week 12 mL of accumulated leachate from the plastic beakers (under the pots) was filtered through a 0.45 µm polyvinvylidene fluoride syringe filter (Whatman) and acidified to 1% (v/v) with Optima nitric acid (Fisher Scientific). The metal concentration in the leachate was then analyzed using an ELAN DRC-*e* ICP-MS (PerkinElmer SCIEX). Total metal concentrations in the leachates were also compared with the Canadian Council of Ministers of the Environment (CCME) guidelines for protection of aquatic life.

Upon the completion of all the bioassay tests including paper I, II and III, the soil samples were sent to ACME Analytical Laboratories Ltd. in Vancouver, British Columbia, Canada, for 36 element analysis of the soil (0.5 g samples) by digestion with hot (95°C) aqua regia and analysis with an ELAN 9000 ICP-MS (PerkinElmer SCIEX). Total metal concentrations in the soils were compared with the Canadian Council of Ministers of the Environment (CCME) guidelines for protection of environmental health at residential/parkland properties.

Following the completion of the wheat growth test (paper II), the wheat shoots were harvested and forwarded to ACME laboratories for total metal analysis. The wheat samples were composite samples of four replicates. The shoots were analyzed using a 0.5 g split digested in HNO_3, then in *aqua regia* (freshly made mixture (1:3 by volume) of nitric acid and hydrochloric acid) and analysed with an ELAN 9000 ICP-MS (PerkinElmer SCIEX).

2.4 Bioassays and ecological assessment testing

Table 2 summarizes the different bioassay and ecological tests selected for evaluation using intact contaminated soil cores and leachates from soil cores. It should be noted again that some of these tests were completed in aged contaminated soil cores (or leachate from these soil cores) (paper I and II), and others in Zn spiked contaminated soil cores (or leachate from these soil cores) (paper III), which has been indicated in Table 2. Several different taxonomic levels of organisms (plants, microorganism and invertebrates) were evaluated as species used in a battery of tests (for the ecotoxicity line of the triad) should represent the broad diversity of ecological niches associated with the soil environment (Allard et al., 2002; ISO, 2003). The following sections briefly outline the methodology of the tests.

Table 2. Summary of bioassay and ecological screening tests completed in soil cores or soil core leachates

Test/organism	**Paper #**	**Aged contamination or Zn spiked soil?**	**Duration of test**
Soil core			
Growth (shoot) and emergence of wheat (*Triticum aestivum*) (Figure 3)	I,II	Aged contamination	6 weeks
Growth (root and shoot length) and emergence of red clover (*Trifolium pratense*), red fescue (*Festuca rubra*) and lettuce (*Lactuca sativa*)	III	Zn spiked soil	4 weeks
MetSTICK, enzyme (beta-galactosidase) activity in *E. coli*.	III	Zn spiked soil	4 hours
Bait lamina (soil microbial and soil invertebrate activity).	III	Zn spiked soil	2 weeks
Leachate from soil cores			
Root growth of lettuce (Figure 3)	I,III	Aged contamination and Zn spiked soil	1 week (6 tests over 6 weeks, paper I and 4 tests over 4 weeks, paper III)
Root growth of red clover	III	Zn spiked soil	1 week (4 tests over 4 weeks)
Mobility of *Daphnia magna*	I	Aged contamination	24, 48 hours
Microtox, acute luminescent bacteria test (*Vibrio Fischeri*)	III	Zn spiked soil	5 minutes, 15 minutes (pH adjusted and not-pH adjusted)
Mobility of *Hyallella azteca*	III	Zn spiked soil	96 hours (pH adjusted)

2.4.1 Tests in soil cores

Bioassays were completed directly in the soil cores using different plant species and microorganism tests. For the plant tests with aged contaminated soil cores, four seeds of wheat, (paper I and II) were sown in each pot. For the plant tests with Zn spiked soil, ten seeds of lettuce, red fescue and red clover (paper III)) were sown in each pot. A light coating of Miracle-Gro seed starter soil was sprinkled over the seeds. For the test with wheat (aged contaminated samples), the pots were positioned in a phytotron with controlled temperature at 20°C. The length and number of shoots were recorded after 7, 14, 21, 28, 35 and 42 days. All shoots except for the first one were culled from each pot (paper I and II). For the tests with lettuce, red fescue and red clover (Zn spiked soil), the pots were stored randomly in growth chambers set to a standard 16-h day at 24°C with full-spectrum fluorescent > 7000 lux, and an 8-h night at 15°C and ≥ 50% relative humidity. The length and number of shoots were recorded after one week. All shoots except for the longest of each species were then removed from the pot. The length of the shoot was then measured again after 14, 21, and 28 days. After 28 days the plants were removed from the soil and the root length was measured (paper III).

From the microorganism taxonomic group the MetSTICK test was selected as well as the ecological functional test with Bait lamina, as both of these tests can be completed with minimal disturbance of the soil core. The MetSTICK test measures enzyme (beta-galactosidase) activity in *E. coli* and was completed according to directions outlined in (Bitton, 2010) (paper III). The Bait Lamina test measures the feeding activity of microorganisms and invertebrates (Kratz, 1998) and was completed in the soil cores during the first two weeks in conjunction with the plant growth tests (paper III). This test represents a possible ecological assessment method of metal contaminated acidic soil cores. One Bait Lamina stick per core replicate (3 replicates) was inserted into the soil samples (Terra Protecta, 1999).

Many of the standardized acute toxicity tests with soil dwelling invertebrates were found to have long durations (greater than or equal to 14 days) and therefore were not suitable for screening purposes (ISO, 1993; ASTM, 2004; Environment Canada, 2004a; ISO, 2004a). There are a few exceptions to this, including the avoidance tests with earthworms and springtails (Environment Canada, 2004b; ISO, 2008, 2011a) and the toxicity test with *Caenorhabditis elegans* (ASTM, 2001). These tests last for 24-72 hours depending on the species. However, (Jänsch et al., 2005), pointed out that the common terrestrial invertebrate test species *Eisenia fetida* and *Eisenia Andrei* are sensitive to strongly acidic soils and not suitable test organisms in that type of soil. The *Caenorhabditis elegans* toxicity test seemed promising as this organism is tolerant of acidic soil. However, the test requires a very small amount of soil (2.33 g) and could not be used directly in the collected soil cores, without disturbing the soil. Due to these limitations with the terrestrial invertebrates, aquatic test species for use in leachates from soil cores were considered to cover this taxonomic level. Aquatic tests with soil elutriates have been used previously to assess the toxicity of contaminated soil (Loureiro et al., 2005; Sheenan et al., 2003; Hund-Rinke et al., 2002) and can account for effects of metals leaching to surface water or groundwater.

2.4.2 Tests in soil core leachates

The leachate draining from the aged contaminated soil cores was used in toxicity tests with lettuce (paper I) as well as *Daphnia magna* (paper I). Leachate draining from the Zn spiked soil cores was used in toxicity tests with lettuce (paper III), red clover (paper III), *Hyallela azteca* (paper III) and Microtox (paper III). In preparation for these tests, both leachate types were removed from the beakers under the pots once every week (for four (Zn spiked soil) and six (aged contaminated soil) weeks) and inserted into 6-well test plates (10 mL/well) (Figure 3).

For the plant tests, each week five seeds from each plant species were placed in separate wells for each concentration/sample. Milli-Q water was used as a negative control. For the plant test in leachate from aged contaminated samples (paper I) the test wells were incubated for seven days in a phytotron at 20°C. For the tests in leachate from Zn spiked soil cores (paper III), the wells were placed in a growth chamber for seven days. Humidity, lighting and temperature were set at the same conditions as the growth test in Zn spiked soil.

For the tests with invertebrates in soil leachate from aged contaminated soil cores, as outlined in paper (I), five *Daphnia magna* neonates were added to each well. The mobility of the *Daphnia magna* was recorded after 24 h and 48 h. Culture water (US Environmental Protection Agency (EPA), 1985) was used as a negative control. For the tests with invertebrates in leachate from Zn spiked soil cores, five *Hyalella azteca* were added to each well (paper III). The number of live amphipods was counted after 96 h. Due to the sensitivity of *H. azteca* to low pH (leachate pH ranged 2.4-4.4), the pH was adjusted to pH 6-8 with 0.1 M NaOH (paper III). Standard culture water (ISO, 1996) was used as a negative control.

For the microorganism taxonomic group, the Microtox test using the luminescent bacteria (*Vibrio fischeri*) was carried out according to Environment Canada (1992) with incubation times of 5 and 15 min (paper III) in leachate from Zn spiked soil cores. The test was run with both non-pH adjusted samples (leachate from week 4) and with pH adjusted samples (leachate from weeks 2, 3 and 4). The pH was adjusted to 6.0-8.0.

2.4.3 pH and metal sensitivity of other potential bioassay species

To supplement information gained from the experimental bioassay tests outlined above, other potential metal sensitive and pH tolerant invertebrate, plant and microorganism species for use in metal contaminated acidic soil cores and leachates from soil cores were identified in a review paper (IV). Information on sensitivity to metals with associated endpoints, as well as tolerance of low pH was collected from database searches (SCOPUS and ECOTOX). Search terms used included combinations of species name, common name, "toxicity", "Zn", "Pb" and pH tolerance/range. For searches in the ECOTOX database, the query parameters included both plants and animal taxonomic groups. Pb and Zn were chosen as chemicals of concern and only concentration based endpoints were selected. All effect measurements and all soil types were included in the search. Only the most sensitive endpoints and endpoints reporting concentrations of metal in soil were included. Also, as the purpose of this review was to determine bioassay test species sensitivity to Pb and Zn specifically, no studies with mixed metal contamination were included.

3. Results and discussion

In this chapter, the results from papers I-IV and the initial literature review are summarized and discussed. Papers I-III are experimental research papers and paper IV is a review paper outlining tests and species sensitivities to metals and pH. This chapter has been divided into four sections with the first three sections representing the different lines of evidence of the triad; chemical, biological and ecological methods, respectively. The fourth and final section of this chapter discusses a method for combining the lines of evidence into a risk estimate.

3.1 Chemical methods

The literature review provided the foundation for choosing appropriate chemical speciation methods to test for metal bioavailability in undisturbed soil cores. Below is a summary of literature review findings and experimental results.

3.1.1 Literature review

There are different chemical speciation methods available to determine the "bioavailable fraction" of contaminants in soil. Speciation of toxic metals is carried out at present almost exclusively in research laboratories (Sanz-Medel, 1998). With these methods, the bioavailability of metals is operationally defined. Each chemical speciation method defines the species differently. Some of the methods will simply divide the species into three groups; free, labile or bound. Others, like the sequential extraction methods will divide the species into fractions based on the extractants utilized. Some of the methods can only be used to measure free ion activity or labile forms within the solution, while others have been developed specifically to measure the labile form of metal bound to particles in the soil. Different techniques to assess bioavailability of metals in soil using chemical tools are presented in Table 3.

Table 3.A selection of chemical methods to assess bioavailability of contaminants in soil

Method	Metal species	Intact soil cores?	Link to metal uptake/ toxicity	Advantages	Limitations
One step extractions	Opera-tionally/ extractant defined	No	Plant uptake, invertebrate toxicity (Conder et al., 2001; Menzies et al., 2007)	Inexpensive and easy (D'Amore et al., 2005)	Sample handing can effect metal speciation (D'Amore et al., 2005)
Sequential extractions	Opera-tionally/ extractant defined	No	Plant uptake, microorganism toxicity, accumulation earthworms (Kong and Bitton, 2003; Mbila and Thompson, 2004; Becquer et al., 2005);.	Standardized method - Community Bureau of Reference (BCR).(Ure et al., 1993)	Sample handling can affect results (Nirel and Morel, 1990; Scheckel et al., 2003)
Donnan Dialysis	Free ion concen-tration	No	Plant uptake (Nolan et al., 2005)	Several elements measured simultaneously. (Weng et al., 2001)	Large volume of soil solution as donor. Low instrumental sensitivity. Sample handling can affect results (Zhang and Young, 2006)
Ion Selective Electrode (ISE)	Free ion activity – Ag Cd, Cu, Pb and Hg	No	Toxicity in maize (McBride, 2001)	Low cost, user friendly (New Mexico State University, 2006)	Ionic strength of the solution can affect results. Organic molecules can block membranes. For soil solutions, only probes for free Cu, Cd, Pb, Ag and Hg ions. One element is measured at a time.(Sauvé et al., 1997; New Mexico State University, 2006)
Differential pulse anodic stripping voltam-metry	Free ion activity, dissolved labile metal activity	No	Metal uptake in maize (McBride and Martínez, 2000)	Sensitive, dynamic technique with potential (TRACE DETECT; Christidis et al., 2007)	Soil handling may affect metal speciation. Fluvic and humic complexes may contribute to the total measurement (Hooda, 2010)
DGT: Diffuse Gradient in Thin films	Labile metal concen-trations in soil	Yes	Plant uptake (Nowack et al., 2004; Nolan et al., 2005; Almås et al., 2006; Cornu and Denaix, 2006)	User-friendly, low contamination risk, in-situ use and high sensitivity (Harper et al., 1999; International Network for Acid Prevention, 2002; Koster et al., 2005)	Results dependent on gel and soil properties, experimental settings and user (Hooda et al., 1999; Cornu and Denaix, 2006; Conesa et al., 2010)
Leachate/ porewater concen-trations	Porewater concen-trations	Leach-ate from soil cores	Uptake in earthworms (Veltman et al., 2007)	Leachate directly from the undisturbed soil cores can be used.	Concentrations of metals in the sample will depend on leaching procedures (Beesley et al., 2010).

For screening purposes there are several chemical speciation methods that seem promising. Using Diffusive Gradients in Thin-films (DGTs) to assess the bioavailability offers several advantages. DGTs can be deployed directly into the soil without disturbing the soil core, DGT accumulated metal concentrations have been shown to correlate well with plant uptake and measurements can be made relatively quickly. However, Conesa et al. (2010) pointed out that DGTs should be used cautiously in acidic soils as resins can become overloaded. Thicker resin devices would provide better reliability and help this issue by increasing capacity. Simple extraction methods using weak salts also appear promising in terms of predicting the bioavailability of contaminants to soil organisms. Standard procedures using weak extractions with 0.01 M $CaCl_2$ representing actual exposure concentrations in the field, and acid extraction with 0.43 M HNO_3 representing potential exposure concentrations have been proposed for use in higher tier risk assessments (Brand et al., 2009). Extractions are easy and fast, but the soil is disturbed during this procedure which may lead to changes in metal speciation. Measuring metal concentrations in pore water or leachate from soil cores offers the advantage of minimizing soil sample handling and total metal concentrations in pore water have been associated with the fraction available for invertebrate uptake (Veltman et al., 2007).

3.1.2. Total metal concentrations in soil as prediction of bioavailability

In ecological risk assessment, the current most common screening approach is to determine total concentrations of metals of concern and compare these concentrations with guideline values. This technique is endorsed by many regulatory agencies across the globe. It was therefore necessary as a point of reference to include this technique in addition to the other potential chemical speciation techniques to assess risk of metal contaminated soil (paper II). In paper II, significant positive correlations were found between total concentrations of Cd, Cu, Zn, Ca and Mo in soil and accumulated metal concentrations in wheat (table 4). Other metals did not show a significant correlation between metal concentrations in soil and concentrations accumulated in wheat shoots. Although metal concentrations in soil exceeded CCME guideline values, no significant correlation was observed between wheat shoot growth and total metal concentrations in soil. It is possible that the bioavailability of metals in the most contaminated samples was low, preventing toxic effect in the test. Estimating ecological risk of each contaminated sample, looking at total concentrations only, the samples containing highest concentrations for the majority of metals, theoretically representing the highest risk, were the samples collected closest to the former lighthouse structures in New Brunswick and Nova Scotia. However, the highest concentrations actually accumulated in wheat shoots was found in the sample collected 1 m from the light house structure in Nova Scotia, even though this soil sample contained lower total concentrations of most metals in comparison with the sample collected closer to the structure. In general, the soil samples collected immediately adjacent the lighthouse structures had higher soil pH than the soil samples collected further away, possibly related to the construction fill material closer to the structures. This could have significantly affected the bioavailability of the contaminants in the samples and measurements of total concentrations of the metals of concern in these samples would not account for this affect. This is specifically the case for the sample collected closest to the former lighthouse structure in New Brunswick. This soil sample had a pH of 7.3 and contained total metal concentrations (Ba, Cu, Pb and Zn) above guideline values. However, actual wheat accumulated concentrations from this sample were similar and sometimes even below concentrations accumulated in samples collected 10 or 20 m away from the structure with a pH of approximately 4.7. As already confirmed by many others, total concentrations in soil is thus not an adequate predictor of actual ecological risk.

3.1.3. DGT-labile metal concentrations as prediction of bioavailability

DGT-labile concentrations were selected for assessment of metal contaminated intact soil cores as this technique minimizes disturbance of the soil core. Labile concentrations of Cd, Cu, Pb and Zn correlated significantly with concentrations of these metals accumulated in wheat (paper II) (table 4). In terms of assessing potential for uptake by wheat, DGT was successful in predicting relative concentrations of metals of concern. DGT-labile metal concentrations from week 1 were better predictors of accumulation of the metals of concern in wheat than total metal concentrations in soil, especially for Pb. The results are in agreement with those of Degryse et al. (2009) who demonstrated a link between DGT-labile concentrations and plant uptake under non-toxic conditions (i.e. high affinity uptake, low concentrations). This is also consistent with our results which indicated no toxicity in any of the soils as measured by wheat growth. No significant correlation could be found between DGT-labile metal concentrations of concern in soil and wheat shoot growth. Estimating ecological risk looking at the DGT labile concentrations from week 1 only, the sample containing highest concentrations of labile metals was the sample collected 1 m from the structure in Nova Scotia (pH 4.8). This corresponds well with results from the wheat accumulation study. DGT labile concentrations in undisturbed soil cores from week 1, identified this sample as the sample with highest metal bioavailability to wheat and thus highest ecological risk.

3.1.4 Soil core leachate metal concentrations as prediction of bioavailability

Soil core leachate concentrations of metals were chosen for evaluation as this technique uses leachate from the soil without disturbance of the soil core. Total concentrations of metals of concern in soil leachate from most weeks were positively correlated with uptake in wheat (paper II) (table 4).

Metal concentrations in soil leachate were better predictors of metal accumulation in wheat than total metal concentrations in soil. Week 3 showed the best correlation for Pb. However, Cd concentrations in leachate were not correlated with Cd accumulated in wheat during most weeks (with the exception of week 2). Weak significant positive correlations were found during week 6 between Cd and Mo concentrations in soil leachate and wheat growth. No other significant correlations were found between concentrations of metals in soil leachate and wheat growth. Estimating ecological risk of each specific sample looking at the leachate concentrations, the sample containing highest concentrations was the sample collected 1 m from the structure in Nova Scotia (pH 4.8). This corresponds well with results from the wheat accumulation study and the DGT labile measurements.

Table 4. Significant correlations (Spearman two-tailed R; $p < 0.05$; N = 12) between metals accumulated in wheat shoots (after 6 weeks) and total concentrations in soil, DGT labile measurements (week 1 and 6) and total concentrations in soil leachate (week 1-6). (ns = not significant ($p>0.05$)). Only metals of concern (soil concentrations above guideline values) have been included. (Modified from paper II)

Metal	Soil	DGT week no .		Total conc. in soil leachate after week no.					
		1	6	1	2	3	4	5	6
Cd	0.78	0.97	0.62	ns	0.68	ns	ns	ns	ns
Cu	0.82	0.96	ns	ns	0.65	ns	0.82	0.81	0.92
Pb	ns	0.89	0.65	0.86	0.85	0.91	0.86	0.75	0.82
Zn	0.86	0.97	0.86	0.68	0.92	0.96	0.96	0.97	0.97

3.2 Biological methods

The literature review provided the foundation for choosing bioassay organisms for the experimental test with intact soil cores. Below is a summary of literature review findings and experimental results.

3.2.1 Literature review

Ideally bioassays to assess contaminated soil should use the most sensitive and ecologically relevant species as well as the most sensitive endpoint (Markwiese et al., 2001). The test organisms must also tolerate the natural properties of the soil under assessment and be able to survive and thrive in control soils (Lanno, 2003). A collection of standardized toxicity tests for invertebrates, plants and microorganisms for risk screening of metal contaminated soil samples are presented in Table 5. Comments have been made in regards to the tests' soil handling procedures. Tests with durations longer than 10 days and tests that are exclusively for testing single spiked chemicals in artificial soil have been excluded to ascertain applicability at the screening stage of ERA.

Table 5. Standardized toxicity methods for risk screening assessment of soil. Modified from paper IV.

Method/Standard	Duration (days)	Handling of field collected soil and sample volume	Endpoint
INVERTEBRATES			
ISO, 2008b. Avoidance test for testing the quality of soils and effects of chemicals on behavior -- earth worms (*Eisenia fetida* and *Eisenia Andrei*). ISO 17512-1.	2	Homogenized field soils– volume not specified.	Avoidance
Environment Canada, 2004. Biological Test Method: Tests for Toxicity of Contaminated Soil to Earthworms - Acute Avoidance Test. EPS 1/RM/43. Environment Canada.	2-3	Homogenized field soils, 350 mL	Avoidance
ASTM, 2001. Standard Guide for Conducting Laboratory Soil Toxicity Tests with the Nematode *Caenorhabditis elegans* .ASTM E2172-01. American Society for Testing and Materials.	1-2	Mixed field soil, 2.33 g	Mortality
ISO, 2010. Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of Caenorhabditis elegans (Nematoda), ISO 10872.	4	Contaminated whole fresh soil and waste, as well as pore water, elutriates and aqueous extracts obtained from contaminated sediment, soil and waste.	Growth and reproduction
ISO, 2011a. Avoidance test for determining the quality of soils and effects of chemicals on behaviour -- Part 2: Test with collembolans (*Folsomia candida*). ISO 17512-2.	2	Homogenized field soils or spiked soils, 30g	Avoidance
ISO, 2005d. -Effects of pollutants on insect larvae (*Oxythyrea funesta*) -- Determination of acute toxicity. ISO 20963.	10	Field soil diluted with uncontaminated soil	Mortality
PLANTS			
ISO, 2012. Determination of the effects of pollutants on soil flora -- Part 1: Method for the measurement of inhibition of root growth. ISO 11269-1.	5	Homogenized field soil, 88 cm^3	Root length
ISO, 2005c. Determination of the effects of pollutants on soil flora -- Screening test for emergence of lettuce seedlings (*Lactuca sativa L.)* , ISO17126.	Max 7	Field soil mixed with "growth medium". 100 g soil	Emergence
US Environmental Protection Agency (EPA), 1988. Protocol for short term toxicity screening of hazardous waste sites, EPA 600/3-88 029	5	Field soil mixed with "artificial soil". 100 g soil	Emergence and root elongation
ASTM, 2002. Standard Guide for Conducting Terrestrial Plant Toxicity Tests. ASTM E1963-02. American Society for Testing and Materials.	Min 4	Field soils, 100-300 g (soil handling not specified)	Emergence, root length
MICROORGANISMS			
ASTM, 1995. Standard Test Method for Assessing the Microbial Detoxification of Chemically Contaminated Water and Soil Using a Toxicity Test with a Luminescent Marine Bacterium. ASTM D5660. American Society for Testing and Materials.	5-30 min	Aqueous suspensions of soil	Light intensity
Environment Canada, 1992. Biological test method: Toxicity test using luminescent bacteria. EPS 1/RM/24	5, 15, 30 min	Aqueous suspensions of soil	Light intensity
DIN 2002. Toxicity test with *Arthrobacter globiformis* for contaminated solids (L 48), DIN 2002 38412-48:2002-09	2 hours	Soil sample (0.6 g) mixed with distilled water	Dehydrogenase activity

Unfortunately none of the tests presented in Table 5 provide methods for toxicity testing in undisturbed soil cores. Most of the methods require at least homogenization of the field collected soil, as well as dilution of field collected soil. In some cases, pH adjusted soil elutriates are used in the toxicity tests. Handling of the soil and elutriates in this way may change the metal speciation in the samples and thus provide erroneous risk estimates. pH adjustments are, however, necessary as many of the standardized test organisms cannot survive in acidic samples. The following sections present the results in terms of metal sensitivity and pH tolerance with selected invertebrates, plants and microorganisms in undisturbed acidic metal contaminated soil cores or leachate from soil cores.

3.2.2 Invertebrate tests

Two aquatic invertebrate species were tested in soil core leachate; *Daphnia magna* (paper I) and *Hyallela azteca* (paper III). Both of these species are sensitive to metal contaminants. In paper I, *Daphnia magna* was also found to be more sensitive to naturally occurring metals in the soil such as Al and Fe than to the metal contaminants of concern. However, it was evident during the tests that these species are also significantly affected by soil leachate pH. Neither of these species was thus found to be suitable for assessment of metal contaminated acidic soil core leachates.

Paper IV aimed at identifying suitable invertebrate species for use in metal contaminated acidic soil and/or soil core leachate, using existing information in the literature. The most common earthworm species in soil toxicity testing are the compost worms *Eisenia fetida* and *Eisenia andrei.* However, these species are not particularly acid tolerant. A more pH tolerant earthworm species is *Dendrobaena octaedra*, which can tolerate a pH ranging down to 3.0 (Reynolds, 1977). This species also show a higher sensitivity to Zn than most of the more common test species, even in comparison with chronic endpoints (Bindesbøl et al., 2009). *Dendrobaena octaedra* is both acid tolerant, ecologically relevant and sensitive to metal contamination and a good candidate for toxicity testing of acidic metal contaminated soil. Another good candidate for this purpose is the test organism *Folsomia candida* which is the most frequently used Collembola species in toxicity testing of contaminated soil. This species can withstand pH 3.2-7.6, but prefers a pH of 6.0 (Jänsch et al., 2005). In addition, it has been demonstrated to be sensitive to metal contaminants with a NOEC at 18 mg Zn/kg (reproduction) in a low pH soil (pH 4.5). From the invertebrate group of potworms (Enchytraeidae), no metal sensitive and pH tolerant species could be identified. However, a possibility in this organism class is the acid tolerant species *E. norvegicus*. This species has wide tolerances for pH (Jänsch et al., 2005), but is yet to be studied for its sensitivity to metal contaminants. The most common nematode used in toxicity studies is *Caenorhabditis elegans*. It is acid tolerant (down to pH 3.1 (Khanna et al., 1997)) and relatively sensitive to Pb and Zn in soils, with LC50 values after 1 days of exposure of 399 and 142 mg/kg, respectively. This species also appears to be a good choice in metal contaminated acidic soils. From the invertebrate organism group of mites, *Oppia nitens* was identified as the most suitable. This mite is acid tolerant and has been used in avoidance screening tests. EC50 values (avoidance with 24 hour test duration) for Zn was 1585mg/kg with this species. Other potential acid tolerant invertebrate species were identified in all groups; however, limited metal toxicity data were available to assess the species suitability in risk assessment of metal contaminated soils.

3.2.3 Plant tests

The plant species selected for the bioassay tests with intact soil cores and/or leachate from soil cores included; wheat (*Triticum aestivum*), lettuce (*Lactuca sativa)*, red clover (*Trifolium pratense*) and red fescue (*Festuca rubra*).

Wheat was chosen as it has been shown to accumulate heavy metals in the roots, shoots and grains (Liu et al., 2009). This was of importance as one of the objectives of this thesis was to determine how well chemical methods predict actual biological metal uptake. However, no significant correlation was found between wheat shoot growth and total metal concentrations in soil ($p < 0.05$; $n = 12$) (paper I and II). This is not surprising as wheat has been identified as metal tolerant by others

(Fjällborg, 2005). Wheat shoot growth was not significantly affected even in the samples containing the highest total metal concentrations. The highest concentration of Pb measured in any of the lighthouse soil samples was 9714 mg/kg. This sample was collected immediately adjacent to the former lighthouse structure at the New Brunswick site with a pH of 7.3. The highest concentration of Zn (>10000 mg/kg) was encountered in the sample collected immediately adjacent to the light house structure at the Nova Scotia site with a pH of 5.9. Also, no significant correlation was found between soil pH and wheat shoot growth or between organic carbon content and wheat shoot growth. To summarize, the test with wheat in undisturbed acidic soil cores was not sensitive to the contaminants of concern at the levels present; however, wheat appears to be tolerant of low pH soils. Based on the low sensitivity to the metal contaminants in this soil, wheat is not a recommended species for assessing risks of metal contaminants to plants.

Lettuce was chosen as a test species due to its documented sensitivity to metal contamination (Wang and Keturi, 1990; Di Salvatore et al., 2008). Also, a standard method for risk screening exists using this species and it is a common choice in bioassays of contaminated soil. Two tests with lettuce were assessed using different test media. In paper I and III, lettuce seeds were exposed to soil core leachates from aged contaminated soil cores and from Zn spiked soil cores, respectively. Paper III also assessed lettuce for emergence and growth directly in undisturbed soil cores. In paper I, it was determined that lettuce root length was a more sensitive and appropriate measure of response to contaminants in soil leachate than lettuce shoot length. However, lettuce root lengths in soil core leachates were affected by low pH, with no lettuce root lengths above 35 mm observed in any of the samples below pH 6. This was confirmed in paper III where the lettuce root length bioassay in leachate from the soil cores exhibited equal sensitivity to low pH and Zn. In terms of the test with intact soil cores, there was a clear indication of Zn sensitivity in comparison with controls and the Ca spiked samples when observing the EC50 values of 72 mg Zn/kg and 6400 mg Ca/kg. Spearman correlation also showed a slightly higher sensitivity to Zn than low pH. However, after 4 weeks there was no significant difference in effects between Zn spiked samples and controls due to high variance between replicates. Due to the difficulty in distinguishing pH effects from metal contaminant effects, lettuce should be used with caution in acidic soils and other more pH tolerant species should be considered.

Red clover was chosen as it is tolerant to acidic soils (Environment Canada, 2005) and can be grown directly in undisturbed soil cores. In addition, it is ecologically relevant at contaminated sites and has been used extensively in accumulation and toxicity studies (Sinnett et al., 2011). Two tests were assessed using red clover in different test media (Zn spiked soil cores and leachate from Zn spiked soil cores). In paper III, consistent shoot growth was observed in the soil cores every week up to 400 mg Zn/kg (d.w.). The EC50 for shoot length after 4 weeks was nominal 400 mg Zn/kg (d.w.). The EC50 for Ca was estimated to be above a nominal concentration 6400 mg Ca/kg (d.w.). Altogether, this indicates a higher sensitivity to Zn than to low pH. The root length results (in the soil cores) after 4 weeks displayed a similar pattern as the shoot length in the soil cores with the same NOEC (400 mg Zn /kg (d.w)) and LOEC (800 mg Zn /kg (d.w)) values for the spiked Zn concentrations, but root length appeared to be more sensitive to low pH than shoot length. To summarize, red clover is tolerant to low pH and is relatively sensitive to Zn and would thus be suitable to use in a bioassay screening test for Zn contaminated acidic soils. It should be noted here that the growth test with red clover in the undisturbed soil cores had a duration of 4 weeks (paper III). This may not be practical for screening assessments and a test with shorter duration would be preferable. To assess if a shorter duration test would be possible, NOEC and LOEC values after 1, 2 and 3 weeks were calculated for the endpoint shoot height. NOEC and LOEC values for shoot height after 2 and 3 weeks were determined to be similar to results achieved after 4 weeks, indicating similar sensitivity for shorter test duration. However, NOEC and LOEC values after 1 week were determined to be higher than after 4 weeks, indicating lower sensitivity of the test. Based on these results, the shoot height test with red clover could be shortened to at least 14 days with similar results as the 28 day-test. Alternatively, the soil core leachate test measuring red clover root lengths after 1 week could be used. Similar findings in terms of NOEC, LOEC and EC50 were observed for the red clover root growth leachate tests from the first week, compared with the shoot height 4 week test in the soil cores.

Red fescue was also grown in undisturbed soil cores (paper III). Red fescue has been identified by Environment Canada as being pH tolerant (Environment Canada, 2005). However, this species seemed more sensitive to the low pH than to Zn (paper III). With a shoot length EC50 above 4000 mg Zn /kg (Table 4) and higher sensitivity to low pH than to Zn in the soil, the red fescue bioassay does not appear to be suitable for hazard screening of Zn contamination in undisturbed acid soil.

Paper IV aimed at identifying additional suitable plant species for use in metal contaminated acidic soil using existing information in the literature. It was determined that not one specific plant species is sensitive to all metals and different plant species have very different tolerance to pH. Of the crop species commonly used in toxicity testing, oat (*Avena sativa*) has been shown to be relatively sensitive to Pb concentrations in soils with a EC50 at 55,78 mg/kg (root length endpoint after 5 days in silica sand) (Chang et al., 1997) and has also been reported to be acid tolerant (Small and Jackson, 1949). Some varieties can tolerate a pH down to 3.8 (Bilski and Foy, 1987). However, it should be noted that oat has also shown low sensitivity to mixed metal contamination in soil (Loureiro et al., 2006) and has been reported to poorly reflect phytotoxicity of contaminated soils in comparison with other plants such as turnips (*Brassica rapa*) (Wilke et al., 1998). Despite being pH tolerant, oat may therefore not be the ideal choice of plant species for a mixed metal contaminated soil. Other commonly used plant species in toxicity studies that were identified as acid tolerant and metal sensitive were *Brassica rapa* with an LOEC for Zn at 50 mg/kg soil (stem height at soil pH 7.2) (Sheppard et al., 1993) and *Allium cepa* with an EC10 for Pb at 50 mg/kg soil (biomass in an alkaline clay loam soil, pH 8.3, CEC:12.6 cmol/kg) (Dang et al., 1990) (paper III). More ecologically relevant and acid tolerant test species choices such as *Quercus rubra* (oak) and *Acer rubrum* (maple) have confirmed sensitivity to Zn, with concentrations above 100 mg Zn/kg in a sandy medium being lethal to seedlings of these species (Buchauer, 1971).

3.2.4 Microorganism toxicity tests

Two microorganism toxicity tests, Microtox and MetSTICK were assessed in laboratory experimental studies (paper III) for use in undisturbed contaminated soil cores/leachate from soil cores. The Microtox test was used to assess the toxicity of Zn spiked soil core leachates and the MetSTICK test was used directly in the Zn spiked soil cores to test for toxicity.

The Microtox test uses the response of luminescent bacteria (*Vibrio fischeri*). Correlations with most metals were stronger with the more sensitive 15 minute bioassay compared to the 5 minute bioassay. The EC50 values for the pH unadjusted test were lower for the Zn samples than for the Ca samples, indicating higher sensitivity to Zn than to pH. This also indicates that pH adjustments may not be necessary to distinguish an effect from Zn contamination. However, it should be noted that correlations between response and Zn concentrations were the same as between response and pH of the Zn spiked samples. Also, the response was significantly correlated to Cu concentrations in the Zn spiked samples, which could have affected the results. The major disadvantage with this test, regardless of the need to adjust the pH, is the need to adjust salinity of the leachate samples, which could alter metal speciation and thus bioavailability.

MetSTICK utilizes immobilized bacteria (*E. coli*) to measure the metal toxicity of the soil by looking at enzyme (beta-galactosidase) activity in these bacteria. The responses were not significantly correlated to spiked Ca soil concentrations, but they were significantly correlated to spiked Zn concentrations ($R = -0.40$, $N = 27$), indicating a lack of sensitivity to low pH. The NOEC value for the Zn spiked samples was low, close to the CCME guideline value of 200 mg Zn/kg. The results from paper III indicate that MetSTICK is a promising tool for assessing Zn contaminated acidic soils.

Paper IV aimed at identifying additional suitable microorganism toxicity tests for use in metal contaminated acidic soil cores/leachate from soil cores, using existing information in the literature. Interesting microorganisms that may be suitable for use in acidic soil toxicity testing are the ciliates *Colpoda inflata* and *Colpoda steini*. However, no data on the sensitivity of these organisms to Pb and Zn in whole soil could be found. The ciliate test is not standardized and it requires soil elutriates and thus cannot be completed in undisturbed soil cores. However, it may be useful for assessing leachates from the soil cores.

3.2.5 Summary of bioassay results

The tests with different organisms directly in undisturbed contaminated (Pb, Zn, Cd, Cu, As, Ba) or spiked (Zn) soil cores indicated different sensitivity to pH and metals as presented in the sections above. The following table (Table 6) summarizes these findings and indicates which of the tests were deemed suitable for use in acidic metal contaminated soil cores or leachate from soil cores.

Table 6. Summary of bioassay results in undisturbed soil cores/leachate from soil cores. (Tests with Zn spiked soil have been indicated with (Zn))

Test/organism and test medium	pH tolerant?	Sensitive to metal contamination?	Suitable for use in undisturbed metal contaminated acidic soils/leachate
Soil cores			
Growth (shoot) of wheat (*Triticum aestivum)*	Yes	No	No
Growth (root and shoot length) of red clover (*Trifolium pratense*).	Yes	Yes (Zn)	Yes
Growth (root and shoot length) of red fescue (*Festuca rubra*)	No	No (Zn)	No
Growth (root and shoot length) of lettuce (*Lactuca sativa*)	No	Yes	No
MetSTICK, enzyme (beta-galactosidase) activity in *E. coli*.	Yes	Yes (Zn)	Yes
Leachate from soil cores			
Mobility of *Daphnia magna*	No	Yes	No
Mobility of *Hyallella azteca*	No	Yes (Zn)	No
Root growth of lettuce (*Lactuca sativa L.*)	No	Yes	No
Root growth of red clover (*Trifolium pratense L.*)	Yes	Yes (Zn)	Yes
Microtox, acute luminescent bacteria test (*Vibrio Fischeri*)	No	Yes (Zn)	No

Paper IV suggests additional potential organisms from three taxonomic groups for assessment of metal contaminated acidic soils, as outlined in above sections and summarized in Table 7. It should be noted that these organisms have not yet been tested in undisturbed contaminated soil cores but were predicted to be suitable for this purpose based on available information. The methodology with intact soil cores in pots as described in this thesis could be used as a starting point to assess these organisms and endpoints (Table 7) for suitability as screening tools in acidic metal contaminated soils. Growth tests with the plant species suggested below should assess appropriate and realistic duration of the tests. It should be noted that for the endpoint avoidance, a different methodology would be necessary as avoidance tests require organisms to move freely between contaminated soils and reference soils.

Table 7. Summary of findings from paper IV. Acid tolerant species suitable for bioassays with undisturbed acidic metal contaminated soil cores (S) or leachate from soil cores (L).

Organisms	**Endpoint**	**Test substrate**	**Confirmed metal sensitivity**	**Reference(s)**
Invertebrates				
Dendrobaena octaedra	Avoidance	S	Pb and Zn	Lukkari and Haimi, 2005; Bindesbøl et al., 2009
Folsomia candida	Avoidance (sensitivity not confirmed)	S	Zn (sensitivity confirmed for reproduction)	ISO, 2011a, Lock and Janssen, 2001b
Caenorhabditis elegans	Mortality	S or L	Pb and Zn	Peredney and Williams, 2000
Oppia nitens	Avoidance	S	Zn	Owojori et al., 2011
Plants				
Brassica rapa	Growth	S or L	Zn	Sheppard et al., 1993
Allium cepa	Growth	S or L	Pb, Zn	Dang et al., 1990
Trifolium pratense	Growth	S or L	Zn	Paper III
Acer rubrum	Seedling growth	S	Zn	Buchauer, 1971
Quercus rubra	Seedling growth	S	Zn	Buchauer, 1971
Microorganims				
Colpoda inflata	Cell and cyst growth	L	Zn	Pratt et al, 1997

3.3 Ecological methods

The literature review provided the foundation for choosing appropriate ecological methods for assessment of metal contaminated acidic undisturbed soils. Below is a summary of literature review findings and experimental results.

3.3.1 Literature review

By comparing ecological observations in the field or the biological state of the soil of a contaminated site with the biological state of a reference soil or ecological condition of a carefully selected control site, a better understanding of the actual ecological effect of the contaminants *in situ* can be evaluated. When assessing biological parameters it is important to keep in mind that these parameters can be considerably affected by the vegetation, land use, as well as the soil physical and chemical properties, irrespective of contamination levels. Thus, it is of great importance to select a reference soil as similar to the test soil as possible (Yang et al., 2006). Jensen et al., (2006) pointed out the importance of identifying and describing any confounding parameters before a cause-effect relationship can be established in ecological field surveys. These parameters should be characterized both at the test site and at reference sites that are to be utilized for ecological field surveys. The factors include soil type, pH, salinity, hydrology, nutrient and organic matter content and the presence of other contaminants. Ecological field surveys are often time consuming, costly and dependent on ecologically, taxonomically and statistical expertise. Examples of biological indicators in terrestrial ecosystems include assessment of earthworms, mites, enchytraeids, nematodes and protozoa. Surveys of soil biota in order to evaluate the effect of various sources of contamination on soil communities are rarely completed on a larger scale by consultants or authority assessors (Jensen et al., 2006). However, research is ongoing in this field and several methods have been developed to extract organisms from the soil for identification and counting. Some of these methods have been included in Table 8. It should be noted that plant community parameters like plant cover, aboveground plant biomass, plant shoot/root ratio, species diversity and the binary occurrence (presence/absence) of specific indicator species like metal-tolerant species also can be utilized to assess effects of contaminants in soil (Strandberg et al., 2006).

It has been suggested by van Beelen and Doelman (1997) that microorganisms, due to their function and presence, can act as an environmentally relevant indicator of pollution. Several standardized methods exist for determining different types of microbial parameters and activity in soil (refer to Table 8).

Table 8. Selection of methods for assessment of ecological effects from contaminants in soil.

Method	Standard
Abundance and activity of soil microflora using respiration curves	ISO, 2012b
Bait Lamina	Not standardized
Berlese funnel	Not standardized
Carbon Transformation	OECD, 2000b
CLPP (community-level physiological profiling)	Not standardized
Dehydrogenase activity in soils	ISO, 2005a; b
Earthworms survey.	ISO, 2006a
Echytraeids survey.	ISO, 2007a
Microarthropods (Collembola and Acarina) survey.	ISO, 2006b
Microbial biomass	ISO, 1997a; b
Microbial soil respiration	ISO, 2002
Nematode maturity index	Not standardized
Nematodes survey	ISO, 2007b
Nitrogen mineralization and nitrification in soils	ISO, 2012a
Nitrogen Transformation	OECD, 2000a
Phospholipid fatty acid compostion (PLFA)	ISO, 2011b
Pollution-induced community tolerance (PICT)	Not standardized
Potential nitrification and inhibition of nitrification	ISO, 2004b

In terms of evaluating ecological effects in the field during the screening stage of the risk assessment, many of the methods presented in Table 8 would be too time consuming. This is, however, an important line of evidence and should not be ignored during the screening stage. Several of the microorganism activity tests are rapid and easy to use and can provide important information about the ecological state of a site at this stage of the risk assessment. Paper IV included a review of many of the microorganism activity tests listed in Table 8. The microbial biomass test as well as the microbial soil respiration test, have both been standardized. The microbial soil respiration test was found to be sensitive to both Pb and Zn. An EC20 for Zn was reported at 10 mg/kg soil in a loamy sand soil with pH 4.9 (Cornfield, 1977). An EC10 for Pb was reported at 26.8 mg/kg soil in a silt loam with pH 6.9 (Chang and Broadbent, 1982). However, Van Beelen and Doelman (1997) suggested that the respiration rate per unit of biomass is a more sensitive indicator of toxicity than the respiration rate or the amount of biomass alone. Dehydrogenase activity (ISO, 2005b) as well as nitrification (ISO, 2004b) are other metal sensitive activity tests with NOECs of 30 mg Zn/kg soil (Rogers and Li, 1985) and 10 mg Zn/kg soil (Wilson, 1977), respectively. However, both of these parameters have been shown to be affected by soil pH (Sauve et al., 1999; Quilchano and Maranon, 2002). Community level physiological profiling (CLPP), pollution induced community tolerance (PICT) and phospholipid fatty acid composition (PFLA) are also sensitive techniques that have been demonstrated to be useful for assessing heavy metal gradients in field collected soil. It should be noted that the CLPP and PICT methods have not been standardized. A standard method is available for the PLFA test (ISO, 2011b). Svedrup et al. (2006) reported a LOEC for PLFA composition at 135 mg Zn/kg soil. However, Rousk et al. (2010) concluded that soil pH also has a profound influence on the PLFA composition.

Of the microorganism activity tests, the Bait lamina test seems promising for use in undisturbed soil cores or *in situ* at the contaminated site without the need for disturbing the soil. The bait-lamina test, proposed by von Törne (1990) reflects the feeding activity of soil animals. The test consists of vertically inserting 16-hole-bearing plastic strips filled with a mixture of cellulose, bran flakes and active carbon into the soil (Terra Protecta, 1999). After a period of time, the bait-lamina strips are removed and examined for evidence of feeding. Filzek et al. (2004) used the Bait lamina test at six sites located along a metal contamination gradient at a smelter at Avonmouth (Southwest England). Results indicated highest bait perforation rate at sites furthest from the smelter and extremely low activities at the two sites closest to the factory.

3.3.2 Bait lamina test

Paper III examined the Bait lamina test as a potential method for the ecological assessment line of the triad in a Zn spiked acidic soil. Although the EC 50 value indicated that this was a sensitive test, it appeared to be due to a pH lower than 3.7. Activity was observed in the soil in all the controls (Ca and Zn series). However, at the 100 and 200 mg/kg Zn spike level, very little activity was observed. By the 400 mg/kg Zn and Ca there was no activity in the soil. This indicates a sensitivity of organisms in this soil to low pH (pH<3.7) rather than to the Zn levels. The original pH of this soil (prior to spiking) was 3.9. It is possible that the organisms in this soil could not withstand the increased acidity caused by the spiking procedure. However, not enough data points were collected to disregard the utility of this test a hazard screening tool in acidic soils.

3.4 Combining lines of evidence into a risk estimate

Figure 4 illustrates a suggested Ecological Risk screening framework for metal contaminated acidic soil using a triad approach based on the results outlined above.

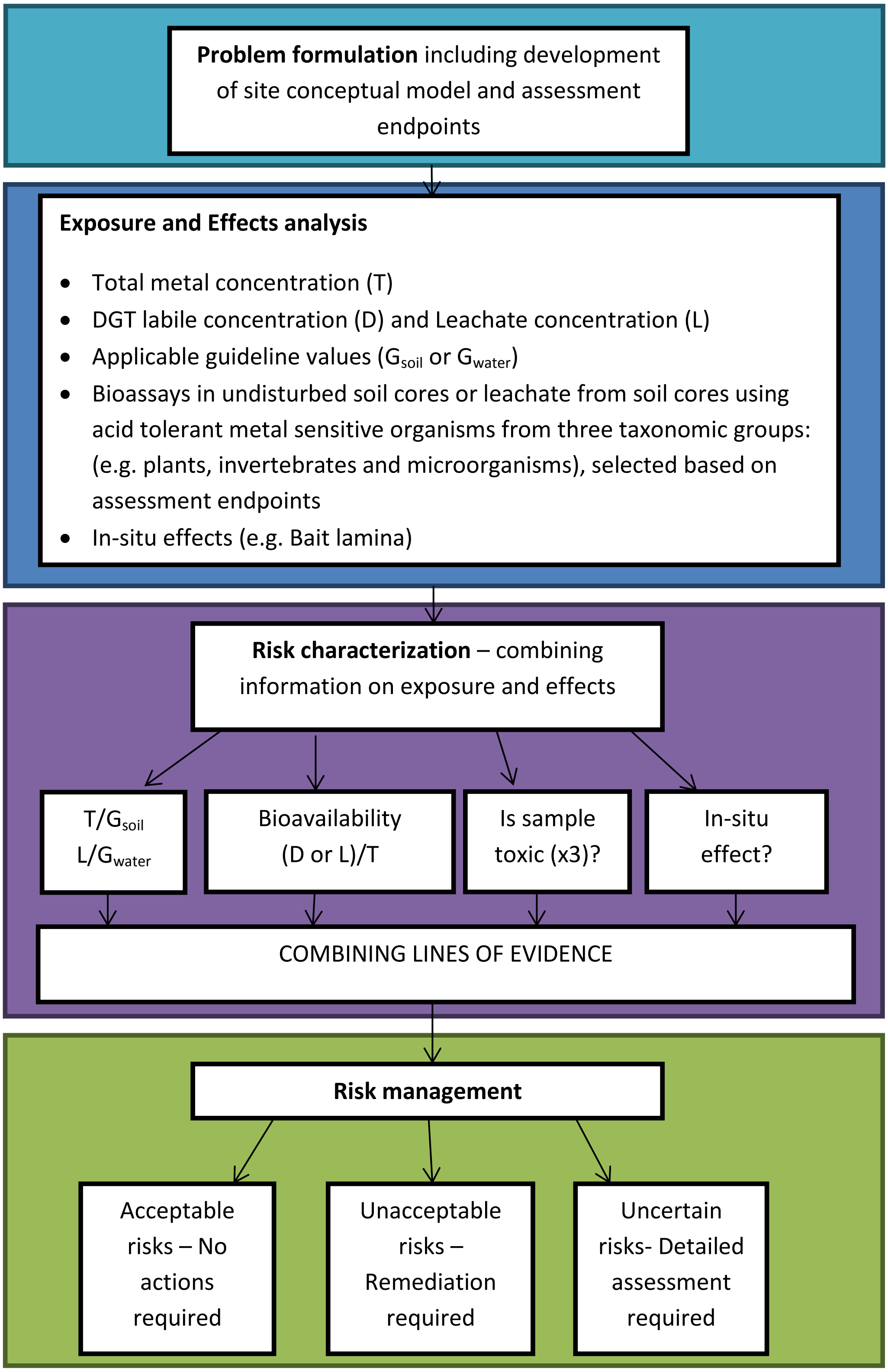

Figure 4. Suggested ecological risk screening framework of acidic metal contaminated soils using a triad approach

A variety of methods for assessing the various lines of evidence in relation and in combination to one another have been proposed by a number of authors. Both quantitative and qualitative risk characterizations have been presented. Chapman (1996) suggested using a simple (+) and (–) approach to illustrate potential exposure or effects and then creating a qualitative risk characterization based on the (+) and (–). Jensen et al. (2006) argued that too much information is lost when assigning a simple (+) to an effect. Instead it is suggested that each of the lines of evidence is scaled from 0 (no effect/exposure) to 1 (100% exposure or effect). By scaling each line of evidence, these can be combined into a final risk estimate between 0-1 for each sample. Using this technique, important differences in effect and exposure are not lost. However, it is noted that the meaning of the final risk estimate number must be determined on a case by case basis. Jensen et al. (2006) suggested that certain land uses be accepted based on certain risk estimate numbers. When screening for contaminants, the assessor generally only wants a rapid snapshot of the potential risk of each sample location. Following screening, there are basically three decisions that can be made: (1) no further assessment/remediation is required (risk is estimated to be low), (2) further assessment is required (risk is uncertain or estimated to be low-medium), or (3) further assessment and remediation is required (risk is estimated to be medium-high). If further assessment is needed, it is important to weigh this need against the cost of initiating remediation without further assessment. If assessment costs exceed remediation costs, immediate remediation should be considered. However, in this context the intrusiveness of the remediation should also be considered. In certain cases remediation may be more damaging to the local ecology than leaving the contaminants in place (Lock and Janssen, 2003). If intrusive remediation is required, further assessment may be needed to establish a more detailed risk characterization of the site than what a screening assessment can provide.

For risk screening assessments, the relative metal bioavailability between samples can be evaluated looking at (D or L)/T (Figure 4). Diffusive gradients in thin-film labile concentrations (D) and soil core leachate metal concentrations (L) were significantly correlated with each other for Pb and Zn. D and L also correlated with Pb and Zn uptake in wheat (paper II). DGT measurements during week 1 only showed slightly better correlations with wheat metal uptake than soil leachate concentrations and therefore is probably the slightly better choice. However, it should be noted that the difference in R values were not large and leachate concentrations may provide a convenient and effective alternative. Ideally soil properties affecting metal bioavailability should be similar in all the samples collected, including the reference sample. However, in reality, as contaminated sites are often large, and samples for the risk assessment need to be collected where contamination is suspected and not based on soil properties, this is difficult to achieve. The (D or L)/T data can help identifying such differences, and will form an integral part of the risk assessment. It should be noted that a high bioavailability score in the reference sample does not necessarily mean that this sample is high risk. Metals at hazardous concentrations need to be present first which is assessed with other lines of evidence. The reference sample selected for the assessment must, however, be free from contamination and representative of background metal concentrations at the site.

Below is a discussion of how the different lines of evidence presented in Figure 4 can be combined into a risk characterization number based on the method presented by Jensen et al. (2006). The different lines of evidence in the triad should have the same weight in the risk assessment, unless special considerations call for different weight or importance (Jensen et al. 2006).

Using T/G as an example (also applicable to L/G and (D or L)/T data), the scaled number (from 0-1) can be calculated by using the equation:

1. $X_{scaled} = 1 - (1 / (1 + T/G))$.

To correct the scaled number (X_{scaled}) for background concentrations, the scaled number from the reference sample (X_{REF_scaled}) can be used in the following equation:

2. $X_{\text{scaled and corrected}} = (X_{\text{scaled}} - X_{\text{REF\_scaled}})/(1 - X_{\text{REF\_scaled}})$

Once the scaled (and background corrected) number has been calculated for each contaminant, the numbers can be combined for all contaminants using the following equation assuming dissimilar toxic modes of action of the contaminants:

3. $X_{\text{combined}} = 1 - ((1 - X_{\text{scaled Pb}}) \times (1 - X_{\text{scaled Zn}}))$

For the bioassay data, different scaling methods are needed. The first technique for scaling presented below works for bioassays with a positive response in the control, such as survival, luminescence (MetSTICK) or feeding activity (Bait lamina test):

4. $X_{\text{scaled}} = (100 - X_{\text{response}}) / 100$

For bioassays with negative response in the control, such as avoidance and mortality, the following equation can be used to scale the results:

5. $X_{\text{scaled}} = X_{\text{response}} / 100$

To scale the difference between the response in sample X and response in the reference sample (Ref), the following equation can be used:

6. $X_{\text{scaled}} = (X_{\text{response}} - \text{Ref}) / (1 - \text{Ref})$

For bioassays with plant growth as response, another method of scaling is necessary:

7. $X_{\text{scaled}} = \log(X_{\text{response}}/\text{Ref})$

Once both the exposure and effects data have been scaled the data can be combined into one risk estimate number with standard deviation using the following equations (as suggested by Jensen et al. 2006): First, the log (1-scaled response) is calculated:

8. $R1 = \log(1 - X_{\text{scaled}})$

Once R1 has been calculated for all lines of evidence, the average R1 value for each sample (R2) is calculated, then R2 values are transformed into a risk estimate for each sample:

9. $R3 = 1 - (10^{R2})$

Finally the standard deviations or coefficients of variation for the three lines of evidence representing each sample can be calculated to indicate how similar or different the lines of evidence are. Jensen et al. (2006) suggested that further assessments should be recommended if the standard deviation exceeds 0.4. The variation between lines of evidence is just as important as the combined risk estimate numbers calculated above. For example, if bioassays and in-situ tests indicate acceptable risks while total concentrations of metals exceed guideline values, logical weighing of the evidence needs to be done, and the basis for the exceeded guideline values needs to be investigated. Questions to be explored include; what toxicity tests are the guidelines based on, what organisms where used, what exposure routes are the most critical and what type of soil (pH, cation exchange capacity, organic carbon content, etc) were used in the experiments? Is it possible that certain exposure pathways important for the guideline value are not applicable on-site? Are site specific pH values higher than in the guideline toxicity test? Are the site specific bioassay organisms relevant to the site of concern? Conversely, if bioassays and/or in-situ tests indicates unacceptable risks while guideline values do not, the same questions should be asked. In addition, it is possible that there is an effect from a contaminant not suspected or measured or there is an effect from a mixture of contaminants. It should be noted that well conducted field surveys that takes into account all limitations of this kind of data indicate the actual state of the receiving environment, so other lines of evidence contradicting field surveys are incorrect.

Despite providing a method for calculating a combined risk estimate number above, it should be noted that this step is not necessary. Each line of evidence provides information that may be lost in a combined risk estimate number. It is suggested here that each line of evidence together with the combined risk estimate number be evaluated before any decisions on remediation are made.

4. Conclusions

This thesis investigates how the triad approach best can be applied to the screening level of ecological risk assessments at metal contaminated sites with acidic soils, using undisturbed contaminated soil cores for the tests.

Chemical assessment:

- DGT-labile metal concentrations in soil cores and metal concentrations in soil leachates from soil cores were identified as suitable tools for risk screening assessment of metal (Pb and Zn) contaminated acidic soil (paper I and II).

- DGT-labile metal concentrations and metal concentration in soil leachates were better predictors of accumulation of Pb and Zn in wheat than total metal concentrations in soil (paper II).

Ecotoxicological assessment:

- No standardized screening method for bioassays in undisturbed contaminated soil cores could be found (paper III, IV). Metal sensitive species that are tolerant of site specific soil properties such as low pH needed to be identified (paper I-IV).

- The wheat (*Triticum aestivum*) bioassay test in soil cores as outlined in paper (I, II) was relatively insensitive to the metals of concern. The *Daphnia magna* test using leachate from the soil cores, paper (I), appeared more sensitive to naturally occurring metals in the soil such as Al and Fe as well as low pH. Therefore these commonly used organisms for toxicity testing do not appear appropriate in terms of testing in undisturbed acidic soil cores/leachate from soil cores.

- The bioassay with lettuce (*Lactuca sativa*) in paper (I, III) appeared sensitive to the metals of concern but also displayed sensitivity to leachate pH below 6 paper (I) and this species is therefore not suitable for risk assessment of acidic soils

- Microtox, *Hyalella azteca* and red fescue (*Festuca rubra*) showed similar or higher sensitivity to low pH than to Zn concentrations and are therefore not recommended bioassays for acidic soils. (paper III)

- MetSTICK in soil cores, and plant growth tests with red clover (*Trifolium pratense*) in soil cores and soil leachate are well suited for use in a risk screening process of Zn contaminated acidic soils (paper (III)) and are thus suitable tools for the ecotoxicity line of evidence.

- Of the more common plant species currently in use during toxicity testing, *Brassica rapa* and *Allium cepa* could be suitable for use in acid undisturbed soil cores for assessment of metal contamination. Other promising species, which are currently less common in toxicity studies, include *Quercus rubra* (oak) and *Acer rubrum* (maple). These species have all shown to be sensitive to metals as well as tolerant of low pH soils (paper IV).

- *Dendrobaena octaedra, Folsomia candida*, *Caenorhabditis elegans* and *Oppia nitens* are suitable candidate invertebrate species for use in bioassays of acidic metal contaminated soils. Some of these species have already been used successfully in avoidance tests. Other promising acid tolerant species exist in all invertebrate groups, but additional testing is required to assess these species sensitivity to metals (paper IV).

- *Colpoda inflata* may be useful for assessing leachates from the soil cores (paper IV).

Ecological assessment:

- The screening test Bait Lamina may be a suitable tool for soils with pH above 3.7 (paper III).

5. Future directions

Risk screening assessments of metal contaminated sites would benefit from becoming more focussed on actual site specific effects of contaminant mixtures and other stressors occurring in the field rather than on generic numerical targets for separate metal concentrations. Including bioassays at the screening stage could be a start to better account for effects of contaminant mixtures. Inevitably metal bioavailability will have to become a standard factor to consider in contaminated land assessment regulations, especially at remote or large sites where excavation of the contaminated soil may not be financially or physically possible. It is important to note that with increasing pressure on our ecosystems in the form of increased human populations and demand for land, moving metal contaminated soil from a location and depositing at a landfill is only transferring the problem from one location to another. Risk screening techniques, such as the one presented in this thesis, could improve the ecological and chemical relevance of the tests and as a consequence could provide better risk estimates. These types of tools can also be used to assess and monitor the success of in-situ remediation techniques using chemical stabilization techniques. The triad approach for screening assessments presented here needs additional refinement and would benefit from the inclusion of a soil invertebrate test. In addition, standardized test procedures will need to be developed for screening testing of undisturbed metal contaminated soil cores from the field.

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