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Jean Mathieu Bazenga Renaud

INCREASING THE ECOLOGICAL RELEVANCE OF THE EFFECTS OF METAL MIXTURES IN SOIL

Tese no âmbito do Doutoramento em Biociências, especialização em Ecologia, orientada pelo Doutor Tiago Natal da Luz, Professor Doutor José Paulo Sousa e Professor Doutor Steven Douglas Siciliano e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Novembro de 2020

Faculdade de Ciências e Tecnologia
da Universidade de Coimbra

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Tese apresentada à Universidade de Coimbra para a obtenção do grau de Doutor em Biociências, realizada sob a orientação científica do Doutor Tiago Natal da Luz. Investigador do Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra, Professor Doutor José Paulo Sousa, Professor Associado do Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra e do Professor Doutor Steven Douglas Siciliano, Professor Catedrático do Departamento de Ciências do Solo da Universidade de Saskatchewan

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Nesta secção embora não seja claro nas regras (espero não apanhar um raspanete), sinto-me na obrigação de me expressar de forma bilingue pois acho que alem do Inglês obrigatório, merece umas palavras em Português.

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Resumo

Os estudos sobre toxicidade de metais em solo têm-se focado na realização de ensaios onde espécies são testadas individualmente a cada metal. Esta abordagem contrasta com a maioria dos locais contaminados por metais que contêm misturas complexas que afetam todo o ecossistema, suas funções e onde os organismos interagem entre si. Em ecotoxicologia de solos a maioria dos estudos com misturas de metais considerou apenas misturas binárias ou ternárias, que estão abaixo da complexidade normalmente observada em locais contaminados. Para misturas de metais mais complexas de cinco ou mais elementos, experiências com desenhos fatoriais completos não são viáveis. Assim, nesta tese, foram preparados solos com diferentes doses de rácios fixos de cinco metais. Estes rácios fixos foram definidos com base nas proporções e concentrações de metais estabelecidas na legislação Europeia e Canadiana e em locais contaminados.

A contaminação de solos com metais em ecotoxicologia é geralmente feita com sais metálicos que requerem lixiviação posterior para remover o excesso de sais no solo. Este processo promove a perda de metais em taxas diferentes consoante o elemento, alterando as proporções dos elementos nos rácios. Por esta razão, em experiências com rácios fixos são necessários métodos alternativos de dosagem metais que não necessitem de lixiviação. No capítulo 2 deste trabalho foi testado o uso de óxidos metálicos e cinzas metálicas, que não requerem lixiviação, como alternativas aos sais metálicos. Os métodos de dosagem foram testados quanto à concentração total de metais no solo e à sua toxicidade a três espécies de invertebrados padrão (*Folsomia candida*, *Oppia nitens* e *Enchytraeus crypticus*) utilizando três solos naturais. Comparando com os nitratos metálicos, a dosagem com óxidos e cinzas apresentaram maiores concentrações totais, mais próximas das doses nominais, e mantiveram melhor as proporções de cada rácio. Em termos de toxicidade, os óxidos metálicos foram tóxicos para todas as espécies testadas, embora a inferior à toxicidade dos sais. Por outro lado, as cinzas metálicas não foram tóxicas para nenhuma das espécies. Embora ambos os óxidos e as cinzas tenham mantido melhor a proporção entre elementos, devido ao maior esforço de preparação e à falta de toxicidade das cinzas metálicas, foram utilizados óxidos metálicos nas experiências seguintes.

No capítulo 3, foi estudada a ação conjunta dos cinco elementos metálicos em mistura e os seus desvios ao modelo CA. Nesta experiência, foram estabelecidas 10 misturas com rácios fixos com diferente relevância ambiental. Cada rácio foi testado com 10 doses, em três espécies (*E. crypticus, F. candida e O. nitens*) e com dois solos naturais. Além das misturas, cada metal foi testado individualmente para calcular unidades de toxicidade (TUs) específicas para cada espécie. Os desvios ao modelo CA foram testadas as a diferentes níveis de dose/efeito (EC10 - EC90). A ação conjunta dos metais, foi globalmente aditiva para *F. candida*, enquanto para *E. crypticus* e *O. nitens* apresentaram desvios ao modelo CA, que foram mais pronunciados em doses/efeitos acima e abaixo do EC50. Em particular, foram detetados sinergismos significativos abaixo do EC50, onde são geralmente definidos os limites de proteção ambiental.

No capítulo 4, foram testados três rácios fixos (CSQG, ARL e SUD) de cinco elementos numa experiência de microcosmos com comunidades naturais. Nesta experiência foi estabelecido o conceito de concentração efeito para a comunidade, assumindo que à medida que a contaminação aumenta, a similaridade da comunidade entre os tratamentos e o controlo diminui, produzindo uma curva dose resposta e permitindo o cálculo dos valores de ECx da comunidade. O conceito de ECx da comunidade foi aplicado com sucesso para as três misturas de rácio fixo testadas. Nos rácios baseados na legislação (CSQG e ARL), os EC10 da comunidade foram quatro vezes maiores que os valores limite estabelecidos na lei, demonstrando que a legislação atual pode ser demasiado restritiva. Para a mistura estabelecida com base na proporção de metais de um local contaminado (SUD), as concentrações do local contaminado corresponderam apenas a um efeito de 20% (EC20) mas era esperado que provocassem um nível de efeito maior na comunidade, especialmente considerando a exposição mais homogénea que normalmente ocorre nos microcosmos. Estes dados sugerem que na avaliação de risco retrospetiva de metais devem ser tidas em consideração as propriedades do local de estudo, que podem influenciar o efeito dos contaminantes.

No capítulo 5, as misturas testadas no capítulo 3 foram usadas numa experiência de TMEs. Nesta experiência, os efeitos das misturas foram medidos na comunidade de microartropodes, na atividade enzimática (desidrogenase, nitrificação potencial e fosfatase ácida), na atividade alimentar e na decomposição da matéria orgânica. Foram utilizadas três doses para cada rácio de mistura (baixa, média e alta) estabelecidas com base nos ECx de comunidade do capítulo 4. Nenhum dos tratamentos testados afetou significativamente a abundância da comunidade de invertebrados após 16 semanas de exposição. Nas enzimas do solo, a fosfatase ácida também não foi afetada, mas a

atividade da desidrogenase e a nitrificação potencial foram fortemente afetadas, com uma redução superior a 50% em todos os tratamentos. As misturas também afetaram funcionalidades do ecossistema, mesmo na ausência de efeitos na abundância de microartropodes. Os resultados sugerem que parâmetros estruturais e funcionais nem sempre são corroborados entre si. Na avaliação de risco de metais e das suas misturas devem ser consideradas as propriedades específicas do local, os vários compartimentos do ecossistema e a estrutura e funções do ecossistema para se assegurar uma proteção ambiental adequada e integral.

Palavras chave

Misturas, Metais, Competição, Comunidades, Ecossistemas

Abstract

Current research in soil metal toxicity has mostly focused on the exposure of single species to single metals. This is a stark contrast from real world scenarios where metal contaminated sites contain complex mixtures which affect the whole soil ecosystem where organisms are heavily interconnected. In soil, some ecotoxicological research has been conducted with metal mixtures but the majority has considered simpler binary or ternary mixtures, which are still below the level of complexity observed in metal contaminated sites. For complex metal mixtures with five elements or more, performing full factorial designs is not feasible. As a result, in this thesis dosing of five element metal mixtures was performed using fixed ratios based on environmental regulation and from metal contaminated sites.

Dosing soils with metal salts, the standard for soil ecotoxicology, requires leaching to remove excess salinity, which promotes the loss of metals affecting the intended fixed ratios. Therefore, alternative dosing method which better retain mixture ratios while keeping adequate levels of toxicity to soil invertebrates must be considered. In Chapter 2, metal oxides and annealed metals which do not require leaching were tested as alternatives to dosing with metal nitrate salts. Dosing methods were tested for their total metal concentrations in soil and their toxicity to *Folsomia candida*, *Oppia nitens* and *Enchytraeus crypticus*, using three natural soils. Compared to metal nitrate salts, oxide and annealed metal dosing had higher total metal concentrations, closer to nominal doses and maintained better mixture ratios. Regarding toxicity, metal oxides were toxic to all test species but less toxic than metal nitrate salts while the annealed metal treatments were non-toxic. Considering that annealed metals require a higher dosing effort and had no toxicity, metal oxides were used for dosing metal mixtures in the following experiments.

In Chapter 3, the joint action of metals in mixtures and their deviations from CA was evaluated. In this experiment, ten fixed ratios were established with different environmental and regulatory relevance. Each ratio was tested with 10 mixture doses on three soil invertebrate test species (*E. crypticus*, *F. candida* and *O. nitens*) in an acid sandy forest and a loamy natural soil. In addition to mixtures, each metal was also tested as a single and used to calculate species specific toxic units. Deviations from CA were tested at different dose/effect levels (EC10 – EC90). The joint action of metals was

globally additive for *F. candida*, while both *E. crypticus* and *O. nitens* had deviations from additivity that were more pronounced outside the EC50 dose effect level. In particular significant synergisms were at low dose effect levels, where most environmental thresholds are established.

In Chapter 4, three fixed ratios (CSQG, ARL and SUD) of the five element metal mixtures were tested using a natural community microcosm experiment. In this experiment the community effect concentration was established, which assumes that as contamination increases, the community similarity between test and control treatments decreases producing a dose response curve that allows the calculation of community effect concentrations (ECs). In this experiment it was possible to successfully apply and calculate community ECx values for all three fixed ratio metal mixtures. For regulatory mixture ratios (CSQG and ARL), community EC10s were four times higher than regulatory threshold values and current regulation might be overprotective. For the contaminated site ratio (SUD), the field dose in the contaminated site corresponded to a community EC20 but larger effect level was expected, especially considering the homogenized dosing in microcosms. Results suggest that in retrospective risk assessment site specific properties should be considered to address potential additional stressors.

In Chapter 5, the same mixtures tested in Chapter 4, were used in a terrestrial model ecosystem (TME) experiment. The effects of metal mixtures were measured on the microarthropod community, soil enzymes (dehydrogenase, potential nitrification and acid phosphatase) and on ecosystem functioning (feeding activity and organic matter decomposition). Each ratio was dosed with three mixture doses, (Low, Med and High) established based on the community EC values from the microcosm experiment of Chapter 3. For the invertebrate community, none of the treatments significantly affected invertebrate abundances after 16 weeks of exposure. In soil enzymes, acid phosphatase was not affected but dehydrogenase activity and potential nitrification were severely impacted with a reduction above 50% in all dosed treatments. Metal mixtures also affected ecosystem functioning, even in the absence of effects on microarthropod abundances. The results suggest that structural and functional parameters are not always corroborated. In the risk assessment of metals and their mixtures, site-specific approaches should be pursued across multiple compartments, considering both ecosystem structure and function for a complete and adequate environmental protection.

Keywords

Mixtures, Metals, Competition, Communities, Ecosystem

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Chapter 1 - General introduction

Metals in soil, sources, contamination, and soil guidance values

Soils are formed from the weathering of pedogenic material and, consequently, vary in their metal composition which is highly related to their geological source [1]. Some soils have naturally high metal concentrations, such as serpentine soils extensively studied in plant ecology [2]. The high metal concentrations and low nutrient content typical of serpentine soils forced plants to develop adaptations to these soils, resulting in a high number of endemic plant species in this type of soils [2]. In serpentine soils, soil communities (including fauna and flora communities) had time to adapt and develop functional communities in equilibrium with those soil characteristics. Contrastingly, in metal contaminated sites resulting from anthropogenic activities, the increase of metal concentrations in soil occurs over a short period of time and soil communities have no time to adapt [3,4]. Moreover, metals originated from anthropogenic activities also tend to be more mobile and bioavailable compared to those from lithogenic sources, increasing their potential toxic effects [3]. Within the main anthropogenic activities that lead to metal contamination in soils are the use of organic/sewage wastes and industrial wastes, pesticides and fertilizers, atmospheric deposition, coal combustion, and mining activities [1,3,4]. Mining activities, including extraction and smelting, are one of the most recognizable industries leading to metal contaminated land which can remain degraded for several years, even after mining activities have ended, if remediation is not performed (Figure 1).

Figure 1 – Deserted open pit mine, in southern Portugal (Mina de São Domingos), mine closed in 1966, picture taken in 2018.

In the European Union (EU) mining and quarrying employed in 2017 more than 400,000 people over 17000 enterprises and represented an added value of 40 billion euros [5]. In Canada, in 2016, 200 mines and 7000 quarries, produced metals representing a value of over 43 billion dollars. Either directly or indirectly, in 2016, Canadian mining activities contributed to more than 3% of its gross domestic product (GDP), and employed more than 400.000 people [6]. Mining activities are diverse and Canada is within the top 5 worldwide producers of potash, Uranium, niobium, nickel, cobalt, aluminum, platinum, gold and diamonds [6].

The importance of mining and the environmental risks from metal contamination have led to regulation and environmental protection for metals. In the EU, no generic values are established as threshold metal concentrations for soils. Metals are regulated by the Registration Evaluation Authorisation and Restriction of Chemicals (REACH) regulation which looks to establish predicted no effect concentrations (PNEC) taking into consideration different soil types [7]. Soil PNEC values are established based on soil invertebrates, soil microbial processes and plants whilst wildlife is considered separately. In compliance with the REACH regulation, a soil PNEC calculator has been developed by considering specific soil properties (soil pH, organic carbon content, cation exchange capacity and clay content) affecting bioavailability and the toxicity of metals [8]. Soil PNEC values are determined using ecotoxicity data (NOEC or EC10) corrected for bioavailability using a soil leaching factor. The corrected data is then compiled in species sensitivity distributions (SSD) where the 5^{th} percentile hazardous concentration (HC5) is selected and to which an assessment factor between 1 and 5 is applied depending on the uncertainty associated to the HC calculation [7].

Several EU Member States, such as Germany and the Netherlands, also have their own guidance values for metals in soil, that are usually more restrictive than values of metal limits based on PNEC calculator. In Germany there are three types of legislative values: precautionary values that intend to prevent further contamination of a site, trigger values where further investigation is required and action values where remediation actions must be taken to clean the site [9]. In the Netherlands a similar approach is followed but with only two categories, target values which correspond to remedial goals and intervention values which are concentrations above which remediation actions must be taken [10].

In Canada, there are soil quality guidelines established to protect the soil from the negative impacts of metals known as the Canadian Soil Quality Guidelines (CSQG) [11]. These guidelines are recommended threshold values established by the Canadian Council of Ministers of the Environment and are adapted for different types of land use [11]. Guidelines

are more restrictive for agricultural and residential areas and more permissive for commercial and industrial lands. CSQG are established by determining HC values derived from SSDs using chronic EC25 toxicity data [12]. For residential and agricultural land, the HC25 is used and for industrial and commercial areas the HC50. An uncertainty factor between 5 and 10 may be applied to these HC values depending on the type (chronic vs acute) and quantity of data available. When insufficient chronic EC25 data is available, the lowest observed effect concentration (LOEC) or median effects through EC50 data are used. In risk assessment, soil quality guidelines can be implemented directly by adopting the guideline values as the remediation objective, be modified to a site-specific remediation objective or ultimately disregarded if a full site-specific risk assessment is performed [13]. In addition to national guideline values, many provinces and territories of Canada have their own guidance values and environmental policies [13].

In this thesis, the focus will be on five metals (lead, copper, nickel, zinc and cobalt) which are of concern in some Canadian mining areas, like Sudbury (high Cu and Ni) [14,15], Flin Flon (high Zn, Cu, Pb) [16] and Port Colborne (high Ni, Co and Cu) [17]. Also for cobalt there is little research into its toxic effects on soil invertebrates and for the most common standard test species EC50 data is only known for *Folsomia candida* [18,19]. There are also other metals which occur as high concentrations in these metal contaminated sites, like As, Cd, Hg [15–17], but these elements were not considered within the framework of this thesis as they would increase the complexity of data beyond a reasonable limit. For the selected metals, the CSQG values and the soil PNEC values calculated for reference soils using the soil PNEC calculator are presented in Table 1.

Table 1 – Soil environmental thresholds for the Canadian soil Quality guideline (CSQG) under Agricultural, Residential/Parkland, Commercial or Industrial soil use and derived for different reference soils (Acid sandy forest, acid sandy arable, loamy alluvial, loamy, clay and peaty) in the EU using REACH, approach.

Soil Environmental thresholds						
Environmental Guideline		Lead	Copper	Nickel	Zinc	Cobalt
		mg/kg dry weight				
CSQG	Agricultural	70	63	45	250	40
	Residential/Parkland	140	63	45	250	50
	Commercial	260	91	89	410	300
	Industrial	600	91	89	410	300
EU REACH Soil PNEC	Acid sandy forest soil	127	40	13	35	9
	Acid sandy arable soil	44	26	4	28	2
	Loamy alluvial soil	359	89	50	271	43
	Loamy soil	211	63	23	134	18
	Clay soil	474	144	97	282	87
	Peaty soil	470	176	94	299	84

Toxicity of Single metals

In soil invertebrates, there has been little research on how they regulate internal metal concentrations and most existing research has focused on earthworms. As essential metals, Cu and Zn are generally regulated by an increase in elimination rates through excretion or a decrease in their rate of uptake [20,21]. Additional mechanisms for regulation vary according to species. For instance, in some earthworms species, Cu and Zn can be bound to chloragosomes and cytoplasmic granules and sequestered for storage, detoxification or regulation [20]. However, after prolonged exposure to metal contaminated soil, earthworms can have a decreased ability for Cu and Zn regulation [22]. Cobalt is also an essential metal (at least for earthworms, as Co is included in the required vitamin B12) and its regulation could also be through uptake and excretion but there has been considerably less research into specific internal mechanisms [23,24]. Unlike essential metals, non-essential metals like lead and nickel (nickel essentiality in soil invertebrates is still not clear [25]), are not required for biological processes and organisms must limit their accumulation in cells or store them in non-toxic forms [20]. Specific methods for regulation of Pb and Ni are poorly reported, but in earthworms, Pb can be permanently stored in waste nodules [22].

Above a certain concentration (which tends to be species specific) metals can cause toxicity inhibiting the functioning of soil invertebrates and affecting their reproduction and survival.

The underlying mechanisms for metal toxicity in soil invertebrates are still not fully understood, but in general, metals are known to cause the generation of reactive oxygen species (ROS) leading to oxidative stress. Oxidative damage and lipid peroxidation has been demonstrated, for *Enchytraeus albidus* when exposed to Zn [26] while toxicity of Pb has been linked to both ROS formation and enzyme inactivation in *Eisenia andrei* [27]. In more recent studies using transcriptomic approaches, the toxicity of Zn was linked to the regulation of gene expression, calcium homeostasis and cellular respiration [28]. Taxonomic approaches also confirmed that Cu and Ni toxicity is linked to oxidative stress and organism response to ROS, while Ni specifically also affects *E. albidus* immune response [25]. This study also found that Cu, Ni and Zn (under individual exposure) all have a similar action when compared to Cd [25]. In a more recent study Ni toxicity was linked to increase proteolysis, apoptosis, inflammatory responses and with interference in the nervous system and glutathione synthesis [29].

Extensive research has been done on the ecotoxicity of single metals to standard test species, affecting their survival and reproduction and guidelines depicted above are based on these data. Table 2 compiles all the available EC50 and LC50 data for the five metals of interest in this thesis (Pb, Cu, Ni, Zn, Co). There is significantly more research dealing with these single metals that do not report EC50 or LC50 in soil ecotoxicology and were not presented in Table 2. However, the data presented in Table 2 allows an understanding of where research has been predominantly focused at. In terms of species most research has been performed on *F. candida*, *E. cryptics* and *E. andrei/fetida* compared to the recently standardized reproduction tests with *Oppia nitens* and *Hypoaspis aculeifer*. In terms of metals, most research has been focused on lead, copper and zinc, with considerably less research for nickel and especially cobalt. For cobalt, even for the most studied species, there are still considerable data gaps (*F. candida* LC50, *Enchytraeus albidus/crypticus* EC50, *E. andrei/fetida* LC50 and EC50). The toxicity data gathered is also quite variable not only between metals and species but within the same species for the same metals (Table 2). There are three important factors which can affect metal toxicity: (i) the soil and its properties, (ii) the metal itself and its speciation and (iii) the species and its biological traits. Environmental variables such as temperature and soil moisture can also affect metal toxicity [34,53,92,93] but these are usually controlled under laboratory testing.

Table 2. Median and range of lethal and reproductive 50% effect concentrations (LC50 and EC50) for the standard test species *Folsomia candida*, *Enchytraeus albidus/crypticus*, *Eisenia fetida/andrei*, *Oppia nitens* and *Hypoaspis aculeifer* in the scientific literature for lead, copper, nickel, zinc and cobalt.

		EC50		LC50		
Species	Metal	Median	Range	Median	Range	References
F. candida	Lead	2575	389 - 4256	1700	181 - 2573	[19,30–36]
	Copper	710	45 - 2270	2023	869 - 6840	[30,33,34,37–42]
	Nickel	469	105 - 1148	882	214 - 4025	[41,43–45]
	Zinc	600	50 - 3233	1155	391 - 6282	[19,30,52–54,33,34,46–51]
	Cobalt	409	368 - 1480	-	-	[18,19]
E. albidus/crypticus	Lead	126	81 - 1008	1881	287 - 7040	[55–61]
	Copper	305	73 - 617	271	15 - 778	[38,55–57,62–67]
	Nickel	168	60 - 275	133	-	[25,43]
	Zinc	219	35 - 345	605	73 - 2950	[26,46,48,55,57,64,65,68,69]
	Cobalt	-	-	455	227 - 683	[35]
E. andrei/fetida	Lead	1340	110 - 5080	5211	4480 - 5941	[36,70–73]
	Copper	260	53 - 716	351	72 - 8700	[37,70–72,74–78]
	Nickel	245	159 - 362	913	684 - 1202	[43,71,79–81]
	Zinc	462	136 - 1898	1010	451 - 4147	[46,48,87,70–72,82–86]
	Cobalt	-	-	-	-	-
O. nitens	Lead	1678	-	6761	-	[88]
	Copper	2896	-	3311	-	[88]
	Nickel	-	-	-	-	-
	Zinc	5339	201 - 30882	2291	1805 - 11076	[88,89]
	Cobalt	-	-	-	-	-
H. aculeifer	Lead	-	-	-	-	-
	Copper	2634	2459 - 2814	4482	-	[90,91]
	Nickel	-	-	-	-	-
	Zinc	-	-	-	-	-
	Cobalt	-	-	-	-	-

Soil Properties and Metal Toxicity

Soil properties are known to affect metal toxicity and many of the studies presented in table 2 address the variation in toxicity as a result of different soil properties, [33,36,82,87,92,93,37,38,45,56,59,66,68,79]. Total metal concentrations represent the total metal pool in a soil but soil properties determine their availability to organisms, affecting their toxicity [94,95]. Metal partitioning and consequently availability is a complex interplay of chemical reactions of precipitation/dissolution, adsorption/desorption, and aqueous complexation, dependent on soil composition for adsorbing surfaces (Al/Fe/Mn oxides, clay and organic matter content) and modulated by soil chemical properties (i.e. soil pH, CEC,) [1,95,96]. The complex inter-play between these variables implies that predicting metal availability and toxicity based on soil properties is a complicated task. It is possible to determine a measure of metal bioavailability through chemical extraction with different solvents however these chemical measures tend to be organism and endpoint specific [97].

It is generally thought that it is the metal free-ion which can transverse biological membranes and cause toxicity and consequently metal toxicity would be regulated by free-ion concentrations in soil pore-water [96,98]. In general, pH is considered the master variable in regulating metal availability in pore-water and the most important soil property to predict metal solubility [94,99,100]. However, in literature there are contradictory evidences, with some studies correlating toxicity to solubility and pore-water concentrations [51,77,101] while others, where solubility and pore-water concentrations do not fully explain toxicity [47,48,89,100,102]. Currently it is not possible to predict the toxicity of metals based only on soil chemistry and metals solubility.

Cation exchange capacity, or specifically effective cation exchange capacity (eCEC), has been found to be a good predictor of toxicity to soil organisms and plants and better overall predictor of toxicity than pH [1,37,100]. CEC is the measure of the negatively charged surfaces available for the adsorption of cations [95], thus for cationic metals higher CEC leads to a stronger binding to the soil mineral surfaces and lower availability for uptake by organisms. Some studies have also found that rather than a single regressor, a combination of pH and CEC best explain the ecotoxicity of metals in soil [56]. However, since it is the eCEC rather than CEC that best predicts toxicity, this parameter hides a pH effect in itself, as the eCEC is the CEC measured at the native soil pH [100]. Other properties like Clay content, organic matter, Al/Fe/Mn oxides, as binding surfaces for metals also correlate with bioavailability and uptake but are rarely significant predictors alone and normally only significant parameters in conjunction with pH and CEC, in complex metal specific equations [68,94]. In fact, the soil CEC, as a measure of adsorption surfaces, is closely related and

already incorporates soil clay content, metal oxyhydroxides and organic matter content [37,56,94].

In ecotoxicology, the role of soils and their properties on the toxicity of metals have been extensively studied but mostly considering their role in metal availability (as described above). However, soils are also the habitat for organisms and can affect their performance and the resources/energy they have available to tolerate and resist the toxic effects of contamination. Recently, it was demonstrated that habitat quality affects the tolerance of *O. nitens* to zinc, where *O. nitens* was able to tolerate higher body burdens in high quality soils, compared to low quality soils [89]. Soil habitat quality in this study was positively correlated with soil organic carbon and cation exchange capacity and was determined using the reproduction of invertebrates and plant growth across a range of 47 soils in the absence of contamination.

Metals, metal speciation and toxicity

Metal chemical forms and speciation can affect their toxicity to soil invertebrates [48,53,61,62,87,103] and their availability [104]. Research has found contradicting evidences on the role of chemical forms in the toxicity of metals to soil invertebrates. For instance, zinc chloride salts were found to be more acute [53,87,103] and chronically [53,87] toxic than nano and non-nano zinc oxides which had similar toxicity, whilst in another study oxides and powders were more acutely toxic than chloride salts, but all chemical forms produced similar chronic toxicity [48]. Lead oxides were less toxic than lead nitrate salts [61] while copper nanoparticles were more toxic than chlorides [62]. Interestingly, in these studies on different metal forms only one study performed leaching to correct for salinity when dosing with metal salts [61]. Added salinity is a confounding factor which can in itself produce toxic effects on organisms [105,106] and also affect the availability and toxicity of metals [74,107,108].

Organism traits influencing exposure

Soil organisms not only have different intrinsic sensitivities to metals, but their biological traits can, in addition to affecting the internal regulation of metals, affect their routes of exposure changing the way and the amount of metals to which they are exposed [94]. The routes of exposure for contaminants are generally ingestion, dermal adsorption and respiration but the latter does not seem particularly relevant for most metals [42,99,109,110]. The porosity and permeability of their external membranes can affect exposure through soil pore-water or even direct contact. Using standard test species as an example, they present

very distinct external barriers, *O. nitens* adults have a heavily sclerotic exoskeleton, *F. candida* have an impermeable cuticle while *E. crypticus* are soft-bodied [109–111]. Organism feeding behaviour, and soil ingestion in particular, is expected to play a significant role in their exposure to metals but the degree and importance of soil ingestion is not clearly known [48,111–113]. The body size of organisms can also affect their exposure to metals even with similar exterior barriers. Organisms with smaller body size (i.e. *E. crypticus* vs *E. andrei*) are expected to have a higher exposure due to a higher surface to volume area [114]. Some research has reported the importance of different routes of exposure but many times results are contradictory and research clearly measuring and quantifying the importance exposure routes and their interlink with traits is lacking [42,91,113,115]. Exposure to contaminants and how traits affect them is expected to shift with different contaminants and soil properties affecting contaminant partitioning between soil solid and pore-water phase. While there might not be a universal route of exposure a better understanding of species traits would aid in interpreting differences in exposure for specific scenarios.

Overall, the toxicity of metals is affected by soil properties the metals and their speciation and the organism itself. However, these different "factors" are highly connected in modulating metal toxicity. Soil properties affect the partitioning of metals which in turn is dependent on which metal and its chemical form or speciation. Species traits influence organism exposure and are dependent on the partitioning of metals in soil and soil pore-water. Also, different metals have inherently different toxicities to organisms based on their intrinsic sensitivity and ability to regulate its internal concentrations which, in turn, is affected by habitat quality dictating the available energy to resist against metal presence [89]. Finally, organisms and their traits will dictate which properties define the quality of a habitat.

Metal Mixtures

Modeling metal mixtures

Current environmental regulation and most of the research has focused on the toxicity of single metals. In fact, in the EU REACH approach metal mixtures cannot be considered because effects must be attributed to the individual chemical, or metal under registration [7]. However, in most metal contaminated sites, toxicity results from complex mixtures of several metals rather than single elements [1,14,16,116,117]. Since metals occur mostly as mixtures, guidelines based on single metals will only be useful if the toxicity of a mixture is equal or

lower than the most toxic compound. This is rarely the case and mixtures of chemical contaminants, (including metals) produce toxic effects which are larger than each chemical applied singly [118].

For mixtures there are two general reference models for non-interactive joint-action: the concentration addition (CA) and the independent action (IA) models [118,119]. These models differ in their assumptions regarding the toxic mode of action of the contaminants in a mixture. The IA model assumes that contaminants have a dissimilar mode of action while the CA assumes a similar mode of toxic action from the different contaminants [119,120]. In risk assessment schemes CA model is recommended as the default first tier for metal mixtures [118] and has been demonstrated as a reasonable worst case scenario and more conservative than IA [55].

The concentration addition model assumes that the individual components of a mixture can be added in the form of toxic units (TU) [120]. TUs are estimated by dividing the exposure concentration of the metal element (a) with the concentration which elicits a particular biological response (ECxa), typically the reproductive EC50 or lethal LC50 (equation 1).

$$TU = \frac{[a]}{ECxa} \quad - \; Equation\ 1$$

If a mixture follows concentration addition, the sum of the TUs at a particular ECx, should equal 1 at the same level of biological response (ECx) for the mixture. However, elements within a mixture can interact and produce responses that are higher (synergism) or lower (antagonism) than the added toxicities of the single elements leading to an under or overestimation of risk, respectively, when such interactions are not taken into account. Specifically, if the sum of the TUs of a mixture is significantly larger than 1, then the mixture is less than additive (i.e. antagonistic) and if the sum of TUs if significantly lower than 1, then the mixture is more than additive (i.e. synergistic; equation 2). For risk assessment, synergistic mixtures (those producing more toxicity than the one expected by additive effects) are those that are particularly of concern and that constitute higher environmental threat.

$$\sum TU_{ECx} = \sum_{i=1}^{n} \frac{Ci}{ECxi} \; = \; 1\ additive; > 1\ Antagonistic; < \; 1\ Synergistic - Equation\ 2$$

Similarly, to what occurs with the toxicity of single metals, in mixture toxicity we should consider three aspects that modulate metal interaction affecting their toxicity to organisms. Interactions can occur in the soil, in the process of uptake by the organism and at the site of toxic action [52,121]. In soil, different metal elements can compete for sorption sites and can interact between themselves affecting their precipitation leading to changes in their bioavailability. Interactions can also occur in the moment of uptake by the organism, during their physiological regulation and when binding to receptors in the site of toxic action [52,121].

The TU approach has a serious drawback as it does not consider the full response of organisms to mixtures but rather a single point (generally the EC50) within the dose-response of organisms [120]. In mixtures, deviations from additivity may not be constant or static, even within the same organism and soil and may be both dose-dependant and ratio dependant. These deviations can be studied using surface response models looking at different doses and ratios of mixtures [55,122,123]. These more complex models were particularly studied by Jonker et al. [122]. to address dose and ratio dependant deviations from additivity.

Jonker et al. [122], assumes four different possible outcomes to mixture toxicity: (i) no deviation where mixture toxicity is accurately predicted by concentration addition; (ii) synergism or antagonism (S/A), where all combinations and doses of a mixture produce a consistently synergistic or antagonistic response; (iii) dose-level dependant deviations (DL) where deviations from CA are dependent on the dose of the mixture and (iv) dose ratio-dependant deviation (DR) where the deviations from CA are dependent on the ratio of the different elements in the mixture. These four scenarios are depicted in 3D form in figure 2, for binary mixtures.

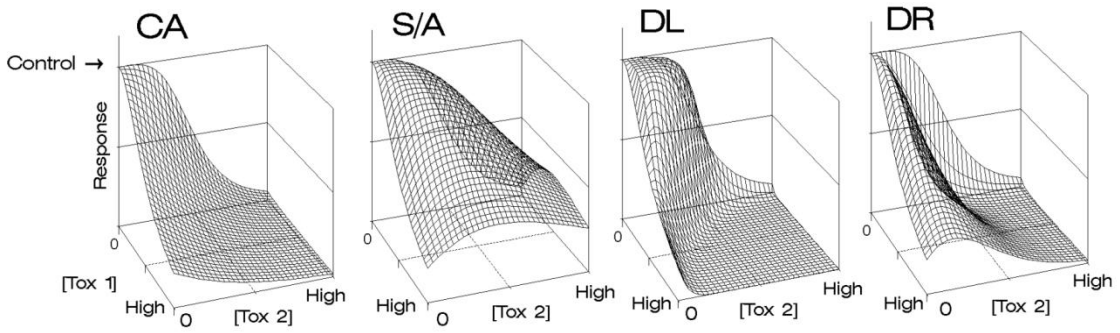

Figure 2 - Binary mixture dose–response relationships illustrating concentration addition and three deviation patterns from this reference in 3D response surfaces: no deviation (CA),

antagonistic deviation (S/A), dose level–dependent deviation (DL), and dose ratio–dependent deviation (DR). The biological response is high in the control group and decreases as doses of toxicants increase (e.g., survival or reproduction) Source: Jonker et al. 2005.

The models proposed by Jonker et al. [113], provide very detailed information on the interaction between metals but are limited to simpler (binary) mixtures because of their high data requirements. For complex mixture modeling, such as surface response models, a full factorial or central composite design should be considered and provide the best information across dose and ratio [55,124]. This is possible with binary mixtures and maybe for ternary mixtures but as mixture complexity increases, the experimental requirements to conduct these test designs increases geometrically making it unfeasible [55,125]. Also, when increasing mixture complexity, the interpretation of deviations using surface response models is complicated as it would require multiple (more than three) dimensional space.

Ecotoxicity of metal mixtures in soil

There are not many studies on the effects of metal mixtures on soil invertebrates and most have focused on simpler binary or ternary mixtures. This section will cover a revision of metal mixture studies in soil, being focused uniquely on studies with collembola, enchytraeids and earthworms. Unfortunately, to date no studies have been reported on the effects of metal mixtures for soil mites. Studies containing metals in mixtures with other contaminants (i.e. pesticides) were not considered.

Collembola

For Collembola, there are four studies on the effects of binary metal mixtures to *F. candida* with some contradicting responses [31,52,124,126]. In the first study, conducted in 1997, mixtures of Cd and Zn were found to be antagonistic when measuring *F. candida* growth but additive when measuring reproduction [52]. This study also found that using total, extractable and organism body concentrations of metals did not change the overall outcomes of mixture toxicity. In a more recent study, the response of *F. candida* reproduction and survival to mixtures of Cd and Zn was dose-level dependant and switched from antagonism at low doses to synergism at high doses [126]. Baas et al. [124], tested binary mixtures of Cd, Cu, Pb and Zn and found no interactions for *F. candida* survival (including Cd and Zn), except for the binary mixture of Cu and Zn which was slightly antagonistic [124]. In another study,

mixtures of Pb and Cd were antagonistic when testing *F. candida* reproduction and growth [31].

Binary mixtures of Cu, Mn and Ni, were also tested on the reproduction of the collembola *Paronychiurus kimi* [127]. The binary mixtures containing Cu, (Cu/Mn and Cu/Ni) were synergistic at the EC50 level whilst the mixture of Mn/Ni was additive. Considering different dose levels, mixtures shifted from antagonism at low dose effect levels to synergism at high dose effect levels.

For Collembola there is only one known study with more than binary mixtures, testing a quaternary mixture of Cr/Cu/Ni and Zn on *F. candida* [128]. In this study soils were dosed either in the laboratory with metal salts or amended with sewage sludges. For both dosed soils (laboratory or sewage sludge), mixture effects were antagonistic for collembola mortality, whilst for reproduction laboratory spiked soils were antagonistic, but sewage sludge amended soils were synergistic.

Enchytraeids

For Enchytraeid worms there are a similar number of studies compared to Collembola, but covering a larger variety of metals (Cd, Cu, Zn, Pb, Ni and Co) [55,64,129–132]. The first metal mixture study on enchytraeids was conducted by Posthuma et al. [64], investigating the effects of Cu and Zn mixtures on *E. crypticus* reproduction and found deviations from additivity changed based on how metal concentrations were determined. When considering total and $CaCl_2$ extractable metal concentrations, toxicity followed concentration addition but for internal body concentrations antagonism was observed. The difference was attributed to interaction between metals for sorption sites within the soil and during the process of uptake. In another study with *E. crypticus*, Weltje [129], also observed that the reproductive effects of Cu and Zn mixtures followed concentration addition whilst Cd and Zn mixtures were significantly antagonistic considering total and extractable metal concentrations. Lock and Janssen [55], tested binary mixtures of Zn, Cd, Cu and Pb on *Enchytraeus albidus* using a more complex central composite design to develop surface response models. All binary mixture combinations in this study produced antagonistic reproductive effects for total metal concentrations in soil, contradicting previous finding on Cu/Zn for *E. crypticus* [55]. Recently He et al. [130,131] studied the effects of Ni and Co mixtures on *E. crypticus* survival. In these studies no deviation from additivity was observed when considering body concentrations of Ni and Co but when considering free ion activity the response was generally antagonistic [130]. In addition to reproduction and mortality the effects of metal

mixtures were also tested on enchytraeid avoidance behaviour where Cd and Zn mixtures were found to be additive across a range of doses [132].

Earthworms

Regarding earthworms, only two studies have observed the effects of binary mixtures of metals [129,133]. In the first study Weltje [129], found that mixtures of Cd/Cu, Cu/Zn and Cd/Zn all had antagonistic interactions when measuring *Eisenia andrei* cocoon production and total metal concentrations in soil. For extractable metal concentrations (only measured for Cd/Zn mixtures) *E. andrei* response was additive. The effects of Cd and Zn mixtures were also tested using *Aporrectodea caliginosa* [133]. Mixture toxicity for *A. caliginosa* was antagonistic but the intensity of antagonism was dose and ratio dependant and increasing concentrations of Cd promoted higher levels of antagonism. There has also been a recent study testing binary and ternary mixtures on *E. andrei* but this study did not model mixture responses and deviations from additivity [134].

Studies on more than binary mixtures were also conducted with earthworms with three studies reporting the effects of ternary mixtures [129,135,136] and two studies on quaternary mixtures [128,129]. The effects of ternary mixtures of Cd/Cu/Zn were investigated using the earthworm *A. caliginosa,* and were additive for growth but antagonistic for cocoon production [135,136]. Weltje [129], also observed the effects of a ternary mixture of Cd/Cu/Zn on *A. caliginosa* and a quaternary mixture of Cd/Cu/Pb/Zn on *E. andrei* and *E. fetida*. Both ternary and quaternary mixtures followed concentration addition, except when testing a contaminated smelter soil (quaternary mixture of Cd/Cu/Zn/Pb) that was significantly antagonistic for *E. fetida*. Finally, the effects of a quaternary mixture of Cr/Cu/Ni/Zn, dosed using metal salts or in organic matrix (sewage sludge amendment), were determined for *E. andrei* [128]. For both spiked and sewage sludge amended soils, effects on *E. andrei* survival were antagonistic. When measuring effects on reproduction, mixtures were antagonistic for sludge amendment but synergistic for metal spiked soils.

Overview of metal mixtures on soil invertebrates

A full summary on the effects of metal mixtures to collembola, enchytraeids and earthworms is presented in table 3. Metal mixture studies have considerable variation in test design, many studies test only at the EC50 level while more recent studies measure deviations across different dose levels. Overall, in the current literature, response to metal mixtures is very variable but seem to either follow concentration addition or antagonism. Synergism, when detected, is most common at high doses. Even for the same mixture combination, responses

are quite variable in the surveyed literature. For instance, with the most tested mixture Cd/Zn responses differed between additive or antagonistic based on the endpoint measured (i.e. reproduction or growth), species tested and dose level of mixtures. As previously mentioned, there are three aspects that modulate the interactions between metals (soil, uptake and site of toxic action), therefore even for the same metal combinations responses are expected to change as the soil and species tested shift. There is still considerable research lacking especially with more relevant and complex mixtures. As the complexity of metal mixture increases, so does the number of potential interactions not only between the metals but also between metals and other factors like soil properties and organism and their traits.

In current metal mixture research in soil, no study so far, tests a range of species within the same experimental design, and it is complicated to extrapolate the differences in species responses to mixtures based on studies conducted with different mixture combinations, soils and test designs. However, it is expected that species respond differently to metal mixtures, due to their different biological traits affecting not only their exposure to metals but also their internal regulation of metals. It is also important to consider that from an ecological perspective species are not isolated and their interactions can lead to unpredictable indirect effects from contamination. In this case it is important to consider the effects of metal mixtures at increasing levels of ecological complexity.

Table 3 – Summary of Metal mixture studies on Collembola, Enchytraeids and Earthworms, R – Reproduction, G – Growth, S – Surival, C – Cocoon Production, J – Juvenile production, TU – Single toxic unit, DR – Dose and ratio deviations, Ant – Antagonistic, CA – Additive, Syn – Synergistic.

	Species	Mixture	Metal type	Soil	Metal concentrations	Endpoint	Estimations	General response	REF
Collembola	*Folsomia candida*	Cd/Zn	Chloride salts	OECD	Total	G R	TU	G - ANT R - CA	[52]
		Binary Cd/Cu/Pb/Zn	Chloride salts Nitrate salts	Lufa 2.2	Body (estimated)	S	DR	Cu/Pb – ANT Rest - CA	[124]
		Pb/Cd	Nitrate salts	Natural soils (3)	Total; Soil Solution; CaCl2	G, R	TU	Cu/Pb - ANT	[31]
		Cd/Zn	Chloride salts	Lufa 2.2	Nominal	S, R	DR	Low doses ANT, High doses SYN	[126]
		Cr/Cu/Ni/Zn	Sludge or spiked nitrate (Cr) chloride (rest)	Natural Soil	Total	S, R	TU	S - ANT(Spiked and sludge) R - ANT (Spiked), SYN (sludge)	[128]
	Paronychiurus kimi	Binary - Cu/Mn/Ni	chloride salts	OECD	Nominal	R	TU	Low doses ANT, High doses SYN	[127]
Enchytraeus	Enchytraeus crypticus	Cu/Zn	chloride salts	OECD	Total, water, CaCl2 Body	R	TU	Total, Water, CaCl2 CA Body – ANT	[64]
		Cd/Zn	chloride salts	OECD	Total and CaCl2	R	TU	ANT	[129]a
		Co/Ni	chloride salts	Quartz sand	Body Free ion activity	S	DR	Body – CA Free ion activity - ANT	[130]
	Enchytraeus albidus	Binary Cd/Cu/Pb/Zn	Nitrate salt (Pb) chloride salts (rest)	OECD	Total	R	DR	ANT	[55]
		Cd/Zn	chloride salts	Lufa 2.2	Nominal	A	DR	CA	[132]
Earthworms	*Eisenia andrei*	Binary Cd/Cu/Zn	chloride salts	OECD	Total CaCl2 (only Cd/Zn)	C J (only Cd/Zn)	TU	C, Total metal - CA C,J - CaCl2 (Cd/Zn) - ANT	[129]a
		Cd/Cu/Pb/Zn	Smelter contamination	Natural soil	Total and CaCl2	C	TU	CA	[129]b
		Cr/Cu/Ni/Zn	Sludge or spiked nitrate (Cr) chloride (rest)	Natural Soil	Total	S,R	TU	S - ANT (Spiked and sludge) R - Synergism (spiked), ANT (sludge)	[128]
	Eisenia fetida	Cd/Cu/Pb/Zn	Smelter contamination nitrate salts	Natural soil OCED	Total and CaCl2 (only smelter soil)	C	TU	Smelter soil - ANT Spiked soil - CA	[129]c
	Aporrectodea Caliginosa	Cd/Zn	acetate salts	Natural Soil	Total, pore-water and CaCl2	S	DR	ANT - Increasing with dose	[133]
		Cd/Cu/Zn	sulphate salts	Natural soil	Total	S, C, G	TU	S, C - ANT G - CA	[135,136]

[a,b,c] Data from Posthuma et al. [137], Weltje et al. [138], Spurgeon and Hopkin [72], respectively, reviewed and collected from Weltje [129].

Tackling the Ecology in Ecotoxicology

In ecological risk assessment, the goal is to protect the ecosystem and its communities. As such, and whilst single species tests are an important first step to evaluate the risk of contaminants, in routine risk analysis, values derived from single species tests require validation from more ecologically relevant and complex semi-field or field studies when they reveal to be unacceptable. Moreover, outside a routine risk assessment procedure, these ecologically more relevant tests can/should also be conducted to calibrate assessment factors used in association to low tier tests. Therefore, threshold values may be improved when using more relevant community research, including more ecological principals in interpreting the effects of contaminants on ecosystem structure [139]. In addition to ecosystem structure, soil systems are responsible for a variety of ecosystem functions, such as nutrient cycling and organic matter decomposition, essential to human life and ecosystem health [140–142]. In this section some of the literature testing the effects of chemicals on species interactions, multi-species, community, and ecosystem responses using laboratory and semi-field experiments will be reviewed.

Two-species tests

To understand the effects of contaminants on species interactions and food webs, some research has been performed on simpler two species predator-prey interactions. A model test system for the predator-prey interactions between *Folsomia fimetaria* and *H. aculeifer* was developed by Axelsen et al. [143] in 1997. This study found that, whilst this setup is much simpler than larger community food webs, it is still complex for experimental testing presenting some caveats. For instance, food placement in the test vessel will promote aggregation of prey, and consequently predators, increasing prey catchability, which changes the natural outcome of this interaction. Also, the starting number of collembolans must be chosen carefully to avoid effects of reduced reproduction for mites due to starvation.

One of the first studies using this system was performed by Hamers and Krogh [144], who observed the predator-prey interactions between *F. fimetaria* and *H. aculeifer* when exposed to sub-lethal concentrations of dimethoate. In this study, *F. fimetaria* juveniles were mostly affected by predation, but dimethoate may affect predator evasion increasing predation levels. The effects of dimethoate were also studied by Baatrup Bayley and Axelsen 2006, which confirmed that *F. fimetaria* had significantly higher predation by *H. aculeifer* when exposed to sub-lethal concentrations of dimethoate [145]. For *F. fimetaria*

adults, they were affected by a combination of dimethoate toxicity and predation, despite preference of *H. aculeifer* on juvenile predation. Regarding the predator, *H. aculeifer* was not affected by dimethoate at any dose tested in the individual exposure, but in the predator-prey experiment, reproduction was reduced in the higher dose because of reduced prey availability. Predator-prey systems with *H. aculeifer* and *F. fimetaria* were also used to assess the effects of the pharmaceutical ivermectin [146] and the polyaromatic hydrocarbon benzo[a]pyrene [147]. For benzo[a]pyrene no effects of the chemical were observed but in the ivermectin experiment, *F. fimetaria* was significantly more affected in the predator/prey combined test than under single exposure. The increased effects in the predator prey system could be due to increased energy investment in predator avoidance, reducing their tolerance to ivermectin or more likely increased predation due to sub-lethal toxic effects of the chemical affecting predator evasion.

In more recent studies, predator prey dynamics were studied still using *H. aculeifer* as a predator but with *F. candida* as a prey species. Predator prey systems with *F. candida* were used to determine the toxicity of Cd [148] and the mobility of microplastics [149]. In the Cd experiment, unlike traditional predatory prey systems no single species exposure was conducted only combined exposure. In the Cd experiment, unlike traditional predatory prey systems no single species exposure was conducted only combined exposure. In this case, the response of *F. candida* in the combined exposure was more sensitive than literature values for single exposure, because of the combined effects of predation and Cd toxicity. Interestingly *H. aculeifer* was also more affected than *F. candida*, however it is unclear if these effects are due to Cd toxicity or reduced prey availability in higher test doses, as no Cd toxicity under single exposure was determined. For microplastics, predator-prey experiments were used to test the mobility of microplastics in soil. In this study, no data was presented on the toxicity of microplastics or the reproductive effect of the predator-prey interaction. Compared to single species exposure, the combined predator-prey exposure increased the dispersion of microplastics by 40% compared to single species exposures. Species interaction may in this case increase the dispersion of microplastics (and potentially other contaminants) in soil increasing the exposure of other soil biota.

Recently, the role of a pre-exposure of prey to contaminant before its use in tests with *H. aculeifer* as food was investigated. Although these tests could not be considered a two-species test in the sense that effects on prey were not measured, these studies demonstrated the importance of considering contaminant accumulation in preys when measuring toxic effects on predator species [91,150].

Unfortunately, research on two-species interactions has mostly focused on predator-prey systems and no research has been performed on other two-species interaction such as competition.

Multi-species Tests

Soil food webs and community responses were also studied in constructed artificial communities with more than two species. These studies increase the level of complexity and species interactions over two species predatory prey systems but are still simpler, less variable and more reproducible than natural community experiments.

Domene et al. [151], constructed a community with *Avena sativa* as the primary producer, several soil invertebrates as consumers (*Porcellinoides sexfasciatus, E. crypticus, F. candida, Ceratophysella denticulata* and *Proisotoma minuta*) and one predatory mite species (*H. aculeifer*), to test the effect of nonylphenol (NP). Microcosms were destructively sampled at three time points (28, 56 and 112 days) after the start of the experiment. Results found that only the highest concentration of NP (270 mg/kg) significantly affected the community at the 28 and 56 day sampling periods. After 112 days, the community recovered from the effects observed at the highest dose, due to the degradation of the contaminant. A community NOEC of 90 mg/kg of NP was derived using an ANOVA to compare the samples scores between tested doses and the control at each time point derived from a principal response curve. However, due to the rapid degradation of this compound and the ability of the community to recover, high concentrations even above the NOEC have a relatively low ecological risk. This community resilience would not be detected in standard single species tests which are generally much shorter in duration and more suitable for the assessment of resistance of test species against a contaminant.

Artificial soil invertebrate communities were also used by Pernin et al. [152], to assess the community effects of a sewage sludge and a copper enriched sludge. In addition to the community effects, this study also included ecosystem functioning by measuring the decomposition of oak leaves. The community consisted of six collembolan species (*F. candida, Isotomurus prasinus, Lepidocyrtus cyaneus, Mesaphorura macrochaeta, Parisotoma notabilis, Protaphorura armata*), two species of oribatid mites (*Archipteria coleoptrata* and *Adoristes sp.*), one predatory mite species (*H. aculeifer*) and one enchytraeid species (*E. crypticus*). Community abundances increased in all sludge treatments due to the higher nutrient and organic matter concentrations, but the rate of increase was lower in the highest doses because of changes in habitat conditions due to

higher sludge application (pH, OM, microbial community) while copper toxicity was considered low due to its complexation with sludge organic matter. For leaf decomposition, no effects were observed for the different treatments, but changes in leaf chemical composition were detected with significant increases in N content in leaves in the highest sludge/copper treatment. and attributed to changes in microbial community structure (not measured) in the highest dose.

Previous research tested the effects of contaminant on a artificial communities with a fixed combination of species but Cortet et al. [153], measured the effects of phenanthrene at a single dose of 43 mg/kg (corresponding to *F. candida* LC50) on five different artificial communities with increasing complexity. The simplest community contained only natural soil microorganisms (that were common in all the five communities), in the second community the collembolan *F. fimetaria* was added, to another community the collembolans species *I. prasinus, Hypogastrura assimilis, M. macrochaeta* and *P. armata were* added, the fourth community contained also the enchytraeid *E. crypticus* and to the final community, with maximum level of complexity, the predatory mite *H. aculeifer* was added. This study found that responses to pollutants are strongly mediated by the interactions within the soil community and predicted effects based on single species were less accurate with increasing community complexity. For instance, in the most complex community, *F. fimetaria* was confronted by three stressors (competition, predation and phenanthrene toxicity), which resulted in a decreased performance. Interestingly, not only did phenanthrene affect the soil community but the fate of phenanthrene was also affected by community composition and degradation rates were higher in the most complex community. The higher degradation of phenanthrene in the most complex community was attributed to a higher functional richness leading to either an increase in the mesofauna activity or changes in the soil microflora composition. However, the effects of functional diversity could not be distinguished from increases in degrader abundances with increasing community complexity.

Recently, the effects of BTEX (mixture of benzene, toluene, ethylbenzene and xylene generated in petrochemical industries) in soil were determined using a community composed of two plant species (*Lactuca sativa* and *Sinapis alba*) and two soil invertebrates (the earthworm *E andrei* and the woodlice *Armadillium vulgare*) [154]. BTEX was tested at a single dose but diluted in either ethanol or water. No effects were observed for BTEX on invertebrate mortality, seedling length or microbial activity. However, when BTEX was delivered through water rather than ethanol, there was a decrease in woodlice weight and plant germination for both plant species. In this case the

authors attributed the effects to the direct toxicity of BTEX to plant germination and woodlice and did not consider indirect effects from species interactions.

In recent years, an attempt has been made to standardize artificial community tests in soil multi-species test systems (SMS) to understand the effects of contaminants on soil communities. This was performed with the goal of introducing community effects in regulatory decisions, with lower variabilities and higher reproducibility than systems using natural communities. These systems generally include 4 to 5 collembolan species, an oligochaete species, either the enchytraeid *E. crypticus* or the earthworm *E. andrei* and the predatory mite *H. aculeifer* [155–159]. SMS systems have been used to assess the effects of copper [155,159–161], ivermectin [156], a-cypermethrin [157] and three biocides esfenvalerate, picoxystrobin and triclosan [158]. Experimental design can vary, but usually SMS microcosms are destructively sampled at certain time periods and the number of organisms from each species is counted.

In the first experiment with copper, Scott-Fordsmand et al. [155] used a SMS to assess the effects of a field copper contaminated soil and a copper sulphate spiked soil in the laboratory with destructive sampling of microcosms after, 28. 56 and 84 days. In both experiments the community was significantly affected by all copper concentrations and no recovery was observed after 84 days. This study also measured individual species response within the community to calculate EC10 and estimate HC5 concentrations via an SSD. The estimated HC5 was estimated between 25 to 35 mg/kg depending on exposure duration and has the advantage of including species interactions over HC5s calculated from single species.

Copper contamination was also studied by Menezes-Oliveira et al. [159] through similar SMS systems as Scott-Fordsmand et al. [155], but in conjunction with changes in exposure temperature. The experiment was conducted at four experimental temperatures (19, 23, 26 and 29ºC) using a control or a copper experimental dose of 100 mg/kg. SMS were sampled after 24, 61 and 84 days. High temperature negatively impacted the soil community structure and function (the latter measured using bait-lamina and litter bags) and obscured the effects of copper contamination. The use of a higher copper concentration could have better demonstrated the combined effect of temperature and copper toxicity but was avoided to reduce the risk of high community mortality. The Negative effects of temperature on the community were more noticeable with increased exposure times, an important indication that recovery was not occurring.

Recently, these SMSs were used to understand the effects of copper nanoparticles in two studies by Mendes et al. [160,161]. In the first study toxicity of copper nanomaterials

(CuO NM) was compared to copper chloride in the SMS with destructive sampling of microcosms at 25, 56 and 84 days. In addition to SMS, individual species exposure was also conducted simultaneously. In the SMS, the magnitude of copper toxic effects increased by exposure time for both CuCl and CuO NM and no recovery was detected. Comparing the toxicity of CuCl and CuO NM, there was no general trend for the EC10 across species in the SMS with some species more sensitive to CuCl and others to CuO NM. Comparing between SMS and single species exposures, for both forms of copper, the rank sensitivity of species differed because of species interactions. The overall sensitivity to copper, decreased when including species interactions (abundance HC5 from SMS for CuCl – 21 mg/kg and CuO NM – 31 mg/kg) compared to the individual species exposure (individual species reproduction HC5 for CuCl – 13 mg/kg and CuO NM – 7 mg/kg). In the second study, the focus was on nanomaterial coating, namely CuO NMs coated with four safer by design coatings. In this study, non-coated CuO NM and citrate (CIT), ascorbate (ASC), polyethylenimine (PEI), polyvinylpyrrolidone (PVP) in a SMS with destructive sampling at 28, 56 and 84 days. In addition to the SMS test, single species exposure was conducted but only on three collembola species (*F. candida*, *P. minuta* and *H. assimilis*). In general, the non-coated CuO NMs had an intermediated toxicity along with PVP coating while CIT and ASC coatings were the most toxic and PEI was the least. As reported for other copper studies, the magnitude of effect increased with increasing exposure times in the SMS. Comparing between SMS and individual species, species interactions significantly contributed to community response and in individual exposure species were less affected than in the SMS exposure (EC50 individual exposure > EC50 SMS). In the previous study by the same authors [160], the opposite was observed where individual exposure was more sensitive than in SMS, however in the current study, only a sub-set of species was tested in the individual exposures and for instance the most affected collembola (*Mesophorura macrochaeta*) in the SMS was not tested separately.

In another study, Jensen and Scott-Fordsmand [156] used the SMS system to assess the effects of the pharmaceutical ivermectin. In addition to effects on invertebrate abundances this study also included a measure of ecosystem function by measuring feeding activity through bait lamina. Similarly to the previous study of Scott-Fordsmand [155], community response was measured as well as individual responses within the community to calculate species EC10 and EC50 values. All treatments significantly affected the community and there was a general decrease in population abundance with increasing concentrations of ivermectin. HC5 concentrations measured from individual species within the SMS increased with exposure time (28 days HC_5 – 0.022 mg/kg and 98

day HC_5 – 0.047 mg/kg) days indicating some level of community recovery possibly due to the degradation of the compound. Regarding feeding activity, significant effects were only observed at the highest tested concentration.

Other researchers investigated the toxicity of cypermethrin in communities but using two slightly different SMS systems [157]. These authors used a community composed by the collembolan species *Heteromurus nitidus, F. fimetaria, P. minuta, Protaphorura fimata* and *Mesaphorura macrochaeta*, the predatory mite *H. aculeifer* and one of two oligochaete species., In one SMS community the enchytraeid *E. crypticus* was used and in the other the earthworm *E. fetida*. Results found that the community with the earthworm had higher abundances of collembolan populations, whilst the predatory mite was more abundant in the community with the enchytraeid. The increase of the predator *H. aculeifer* in the enchytraeid experiment could be due to mite predation on the enchytraeids, and consequently the increase in predator population size could have exerted an increased stress on the collembolan community promoting its decrease. The predation of *H. aculeife*r on enchytraeids could also explain the lower abundances of this species when compared to earthworms.

Finally the SMS system was used by Schnug et al. [158] to measure the effect of the biocides esfenvalerate, picoxystrobin and triclosan with only one sampling period after 8 weeks. In this SMS experiment, the community was composed of four collembola species (*P. minuta, H. nitidus, F. fimetaria, P. fimata*), the predatory mite *H. aculeifer* and the earthworm, *E. fetida*. The authors found that the three biocides affected the community differently due to different species-specific sensitivities to each compound. As a result, communities had different dominance structures when testing the different biocides (i.e. *H. nitidus* was dominant for triclosan SMS but *F. fimetaria* and *P. minuta* were more dominant in the picoxystrobin SMS experiment). Compared to single species exposure data in the literature, only triclosan had a similar toxicity compared to literature (*F. candida* EC50), whilst picoxystrobin EC50 values for species in the SMS was generally higher than the literature (*F. fimetaria* EC50) and esfenvalerate EC50 values for individual species within the SMS were lower than in the literature (*F. candida* EC50). The negative effects of the biocides on the collembola community were also strongly correlated with reductions in feeding activity and especially correlated with negative effects on the earthworm *E. fetida*.

Experiments using natural soil communities

In the previous sections, species interactions in simple two-species tests and more complex artificial communities were reviewed. However there are still some questions regarding the reliability of these systems in assessing the response of natural communities and several researchers defend that natural communities should be used to accurately assess the effect of contaminants in soil systems [162–164]. In this section, research containing at least partial natural communities, including microcosms where the soil is manipulated (i.e. sieved and homogenised) and intact terrestrial model ecosystems (TMEs) where the soil structure and communities are preserved, will be reviewed. Research conducted exclusively on nematode or microbial communities was not considered.

Natural and semi-natural soil community microcosms

In some studies, natural communities were used where in addition to the natural occurring invertebrates, some test species were added, leading to a semi-natural system [165–167]. In these studies, common laboratory species are added (either plants or larger invertebrates like earthworms or isopods) to guarantee a more complete ecosystem response in addition to the natural microorganism and microarthropod community. In theses studies, microcosms were not intact soil cores but rather, soils were field collected, sieved and then loosely packed in cylinder microcosm containers.

The first study following this method was conducted by Bogomolov et al. [165], who assessed the effects of gradient of copper sulphate contamination (0 - 800 mg/kg) with destructive sampling after 5, 10, 20 and 40 days. The microcosm consisted of a natural community of microorganisms and microinvertebrates to which the earthworm *Aporrectodea tuberculata* was added. This study found that copper affected community structure by reducing microbial biomass, nematode and earthworm abundance. In general toxicity thresholds observed in the microcosms were like those previously reported for the different trophic groups in the literature. The effects of copper on the soil community affected ecosystem function by reducing litter decomposition (measured using litter bags) and increasing nitrogen mineralization.

In a similar study, Gunderson et al. [166] observed the effects of explosive contaminated sediment in a microcosm experiment. In this experiment three plant species (Soybean, Radish and Lettuce), their associated symbionts and two soil invertebrates (isopod *Armedillidium vulgare* and earthworm *E. fetida*) were added to the naturally occurring microbial and micro-arthropod (including mites and collembolans) community. In this

experiment soil invertebrates were not significantly affected by the contaminated sediment, whilst germination was affected for all plant species as well as root growth for lettuce.

Finally, Burrows and Edwards [167], observed the effects of the pesticide Derasol (active ingredient carbendazim) in a microcosm experiment. Each microcosm, in addition to its natural mesofauna community, was sown with wheat and had three earthworms of the species *Lumbricus rubellus* added. The measured endpoints were earthworm and nematode populations, plant growth and soil invertebrate feeding activity measured using bait lamina. Results found that all measured endpoints were affected by the pesticide. The LC_{50} for earthworms and nematodes was 6.2 and 13.2 mg active ingredient (a.i.) kg respectively; feeding activity was significantly affected at the two highest concentrations (20.52 and 61.56 mg a.i/kg soil) whilst shoot growth was only affected at the highest concentration of carbendazim. Unfortunately, not community level effects, such as community PNECs or EC_{50} were estimated.

The first study using only the natural community without the addition of other species was performed by Parmelee et al. [162] with two subsequent studies following the same experimental procedures [168,169]. In these microcosms soils were collected from the field, sieved, dosed and then loosely packed into microcosm test vessels. Sampling of soil microarthropod and nematode communities was only performed after 7 days to determine the acute toxicity of these chemicals. In the first study, soil microcosms were used to assess the effects of p-nitrophenol, trinitrotoluene (TNT) and copper separately [162], in the second to test the effects of copper, cadmium, malathion and Aroclor 1254 individually in five separate experiments [169] and in the third effects of a chemical warfare agent [168]. In all studies, test chemicals negatively impacted the soil microarthropod communities with the exception of TNT [162] and Cd [169], however the intensity of responses differed between test chemicals. Nitrophenol was very toxic to soil nematodes significantly affecting all groups [162]. For copper, responses differed between studies: in one study [162] omnivore-predator nematodes and mesostigmata and oribatid mites were the most sensitive groups, but in the following experiment [169] nematodes (fungivore, bacterivore, and omnivore–predator nematodes) were the most affected. Both experiments were conducted using copper sulphate and the same soil and differences are expected to be due to differences in the community structure between experiments. Malathion and Aroclor 1254, both produced similar responses where microarthropods were more sensitive than nematodes, potentially as a result of their low water-solubility [169]. Microarthropods were also more sensitive to the warfare agent compared to nematode communities [168].

Natural mesofauna communities were also used by Andrés and Domene [163] to assess the effects of sewage sludge and its different treatments (dewatered, composted and dry sewage sludge). These microcosms were constructed in a PVC pipe container filled with 400g control or treated soils which were colonized with microarthropods extracted from soil cores from an undisturbed forest. In this experiment the soil used in the microcosms was deficient in organic matter to simulate impoverished soils which would benefit from sewage sludge application. Regarding acute effects, Prostigmata mites were the most sensitive taxon, because most are fungivorous and bacteriophages and expected to be more sensitive to the sludge than predatory or phytophagous mites. At intermediate exposure times, Mesostigmata and Cryptostigmata were the most sensitive groups. At low pollution levels, Mesostigmata abundances were stimulated due to increase in prey availability but at higher concentrations they are very sensitive to the accumulation of contaminants in the prey, while Cryptostigmata mites are known to be sensitive to metal contamination. No results are reported for collembolan and no whole community effects were determined.

In a more recent study, Chelinho et al. [164], proposed a slightly different approach for soil microarthropod community testing. In this experiment soil was dosed with carbofuran (using the commercial formulation Furadan 350 SC) in the laboratory (Portugal) and in the field (Brazil). Then the natural microarthropod communities from the two distinct locations (Portugal and Brazil) were extracted directly into the test vessels with the contaminated soils (plus the uncontaminated control soil). This approach allows for the assessment of the effects on the soil community in a worst case homogeneous contamination (Portuguese experiment) and a realistic field application (Brazilian experiment). Results found that initial communities, as expected, varied greatly between locations (Portugal or Brazil), but despite their differences, both communities were affected by carbofuran exposure. Carbofuran led to a reduction in taxonomic diversity, with decreasing diversity with increasing dose in both experiments. but especially in the Brazilian community species adapted to deeper soil layers were more vulnerable to the toxic effects of this insecticide, and cuticle permeability was found to be an important trait reducing the uptake of the contaminant. Mite populations, on the other hand, tended to increase overall with only a slight decline in the Portuguese community at the highest doses. The increase in mite populations was attributed to a lower sensitivity to carbofuran and was mainly caused by an increase of oribatids, which benefitted from reduced competition for resources due to the toxic effects of carbofuran on collembolans. Predatory mites presented a dose-response decrease due to the lack of their main prey species (collembolans).

Terrestrial model ecosystems

Intact terrestrial mesocosms have been used in research for several years but most of the studies performed in the 1980's, focused on vegetation rather than soil invertebrates [170]. The most important study performed using intact TMEs was an international ring-test which has paved the way for subsequent research and is now an important guideline for TME studies. This international ring-test aimed at standardizing an approach for intact terrestrial model ecosystems, whilst validating with field experiments the use of TMEs as relevant systems to assess the effects of contaminants on natural ecosystems. This study was performed in four different locations selected close to the four different project partners in Germany (ECT Oekotoxikologie GmbH), Netherlands (Vrije Universiteit Amsterdam), United Kingdom (University of Wales in Bangor) and Portugal (Universidade de Coimbra). The selected chemical for testing in the project was the fungicide Derosal (active ingredient Carbendazim). The TMEs consisted of 40 cm long and 17.5 cm wide HDPE tubes with a bottom plate with a drilled whole to collect leachates. These intact cores were collected from the field in the spring using a steel extraction corer and a hydraulic excavator and placed in which simulate below ground conditions using circulated cool air (Figures 3 and 4).

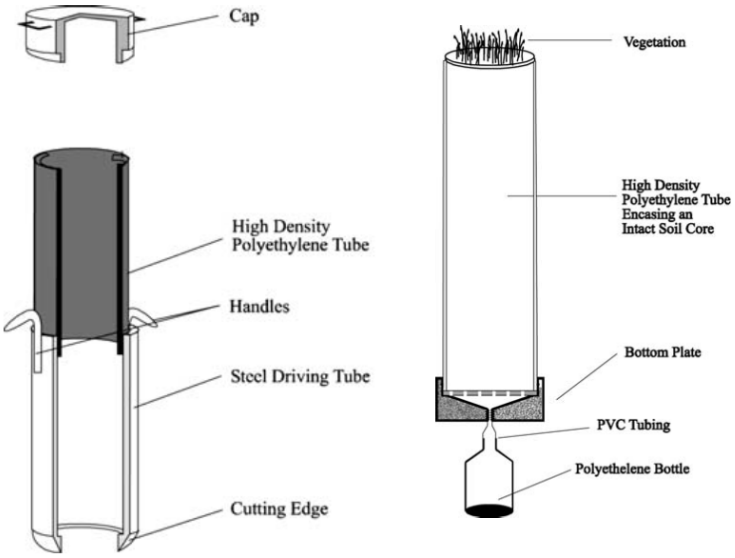

Figure 3 – Soil-extraction apparatus and TME configuration adapted from Knacker et al. [96].

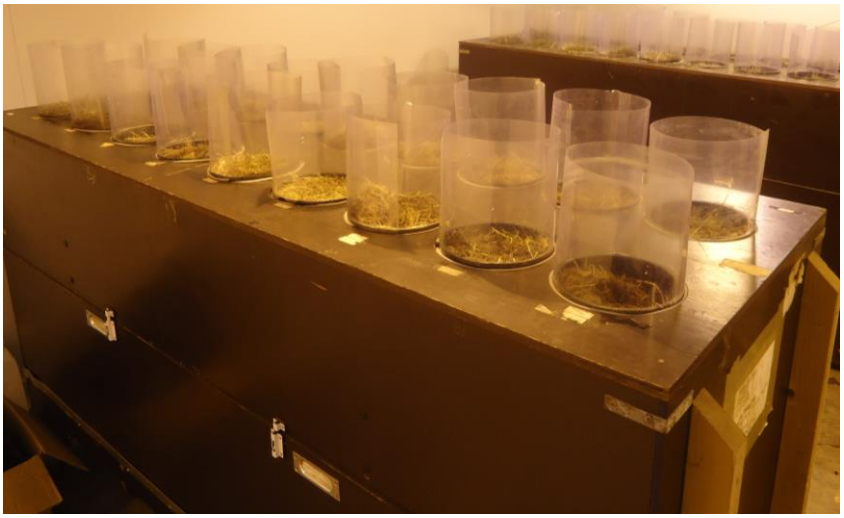

Figure 4 - TME carts from the University of Coimbra depicting the individual soil cores.

In the fate experiment [171] both the TME and field experiments across the different partners showed the same degradation trends (DT50 and DT90) and carbendazim was only detected in the first 15 cm of soil due to its strong binding to soil particles. The one exception found was in the Flörsheim experiment where carbendazim was less persistent in the TMEs than the field experiment, possibly due to changes in environmental conditions in the Flörsheim field validation experiment.

Regarding the effects of Carbendazim exposure, effects were measured on earthworms [172], enchytraeid [173], nematodes [174] and microarthropod communities [175]. For both earthworms and enchytraeids, data was highly variable limiting statistical testing. For earthworm abundance and biomass, it was not possible to determine NOECs but EC50 values ranged between 2.04 and 48.8 kg a.i./ha and 1.02 to 34.6 kg a.i./ha for abundance and biomass respectively. Effects on diversity were hard to distinguish due to low number of individuals per species but the genus *Lumbricus* appeared to be the most sensitive. For enchytraeids EC50-values ranged between 0.5 and 28.4 kg a.i./ha and between 7.2 and 87.4 kg a.i./ha for abundance and species richness, respectively. In more detail, the genus *Achaeta* and *Enchytraeus* which prefer deeper soil layers where carbendazim concentrations are lower were not affected, while species from the *Fridericia* genus demonstrated a dose-response relationship (abundance of Fridericia EC50 - 0.9–24.7 mg/kg). Carbendazim affected the number of nematode families, their trophic structure and their maturity index (MI), but effects were particularly pronounced for the abundance of omnivorous nematodes (EC50 ranged between 0.93 and 7.24 kg a.i./ha) unlike total nematode abundance which was not affected. For microarthropods, data was only presented for three TME experiments, Amsterdam (Preliminary and TME experiment) and Bango (preliminary experiment), experiments conducted in Coimbra and

Flörsheim were not included in the paper. Destructive sampling of TMEs took place after, 1, 4, 8 and 16 weeks for the preliminary experiments and at 1, 8 and 16 weeks for the TME experiment. Microarthropod data was very variable both within and across TME experiments. Using PRC's, significant effects were observed for the Collembola community in only one of the TME experiments (Amsterdam preliminary), while for mites effects were observed in two of the three TME experiments (Amsterdam and Bangor preliminary), with no significant effect of sampling time for either Collembola or Mite communities. NOECs of carbendazim for microarthropods derived from this data ranged from 0.36 to 87.5 kg a.i./ha.

This ring test experiment also measured the effects of carbendazim on ecosystem function by measuring organic matter breakdown (using cellulose paper), soil fauna feeding activity (using bait-lamina), nutrient cycling (nitrate, ammonium, phosphate and sulphate concentrations) and microbial parameters (substrate induced respiration, dehydrogenase activity, phosphatase activity and thymidine incorporation) [176–178]. Organic matter breakdown was affected by carbendazim and followed a clear dose-response relationship (EC50 ranged between 2.1 – 9.5 kg a.i./ha) while feeding activity, was extremely variable and did not follow as clear a dose-response (EC50 ranged between 2.0 - 56 kg a.i./ha). Nutrient cycling was not affected by carbendazim treatments, even at the highest dose tested (97.5 kg a.i./ha). This demonstrates the importance of functional redundancy allowing nutrient cycling to be performed normally even when some aspects of the community structure are affected by a chemical. For microbial parameters (microbial biomass, bacterial growth rates and enzyme activities) carbendazim treatments effects were mild and did not allow the estimation of EC50 values.

The ring-test experiment with TMEs, and the detailed conceptual design paper produced has stimulated subsequent research. Kools et al. [179,180], for instance, used TMEs to assess the structure and function of a metal polluted grassland and the effects of zinc and heat stress to this already polluted system. These studies found that for the metal contaminated grassland, without additional stressors (zinc and heat) nematode community structure, enchytraeid and earthworm diversity were negatively correlated with metal contamination and lower soil pH. In fact, pH was an important factor in all analysis due to both its direct effects on organism performance and its indirect effects on metal availability. This study also found that ecosystem function was more correlated with the structural diversity of the ecosystem than total biomass. When increasing the stress to the ecosystem with the addition of zinc and heat, most severe effects were

observed in the more contaminated soils, indicating that these more degraded systems are less stable and more vulnerable to additional stressors.

The TME approach was also used to assess the fate and effects of ivermectin by Förster et al. [181]. In this study Ivermectin, a veterinary pharmaceutical, was applied to the surface of TMEs in cow dung slurry simulating realistic exposure scenarios. Sampling of the TMEs was performed at three different time intervals, after 7, 28 and 96 days, to assess not only acute and chronic effects but also potential community recovery. No effects were observed on microbial biomass and nematode and mite abundance whilst enchytraeid, collembolans and earthworms were affected but with no clear dose-response. In fact, dose responses allowing the estimation of EC50 values were only possible for feeding activity (using bait-lamina) at different soil layers. This demonstrates that whilst no clear dose-response pattern was observed on soil organisms, there was a dose-response effect on ecosystem function as measured through feeding activity. These results reinforce the importance of accounting not only for ecosystem structure but also ecosystem function in current ecotoxicological practices. Community NOECs estimated using PRCs were 0.33 mg/kg after 7 days of exposure and at 0.78 mg/kg after 96 days, which indicates a slight community recovery for the duration of the experiment.

Terrestrial model ecosystems were also used by Scholz-Starke et al. [182,183], to assess the effects of the pesticide Lindane. One interesting variation in these studies, unlike the previous studies, is that TME systems were kept outdoors under natural environmental conditions. In the first study Lindane was applied to TMEs at a concentration of 10 and 100 mg a.i /kg in the top 5 cm of the TME. Results found a clear dose-response relationship for micro-arthropod communities, whilst earthworms and functional endpoints (bait-lamina feeding activity) were unaffected up to one year after the application of the pesticide. In a second and more extensive, included sampling of fungi, enchytraeids and nematodes not considered in the previous study and a higher range of test concentrations (five concentrations between 0.032 and 3.2 mg a.i./kg). In this case, no significant effects were observed for enchytraeids, nematodes and fungi. For collembolans significant effects were observed in the intermediate and higher doses of Lindane after 3 and 5 months but the community recovered and one year after application no significant effects were observed.

Terrestrial model ecosystems can also be used to investigate the role of environmental variables on the toxicity on chemicals. This was investigated in two recent studies with the fungicide pyrimethanil [184,185]. In a first study Ng et al. [184] observed the effects of pyrimethanil on the soil microbial communities under different and extreme rainfall

regimes. This study found that effects on bacterial biomass were consistent within the fungicide treatments amongst the different rainfall regimes and the same was observed for functional endpoints (i.e. enzyme activity). On the other hand, bacterial community structure was significantly affected by rainfall and rainfall regimes impacted the effects of the fungicide. In the more recent study, Bandow et al. [185], observed the effects of the same fungicide on enchytraeid communities under different soil moisture regimes in two different experiments (conducted in Portugal and Germany). In both experiments, three rainfall regimes were used (low, medium and high) but different approaches were considered for the fungicide application, in the Portuguese experiment three doses of fungicide were used (Control, maximum application rate and 5x the maximum application rate) while in the experiment in Germany a ECx approach was conduced with 11 test doses. In the Portuguese experiment, the enchytraeid communities were affected by soil moisture but not affected by either of the fungicide application doses. In the experiment in Germany the ECx approach allowed the calculation of EC50 values which changed according to rainfall regime and decreased with drier soils. The lower EC50 values in drier soils is an important consideration highlighting the importance of incorporating climate change in risk assessment of plant protection products.

TMEs have also been used to test complex agricultural management strategies [186]. This experiment consisted in five treatment strategies, an intact control with no treatment, a tillage treatment with and without insecticide application (CTI and CT respectively) and no tillage treatments but with herbicide application with and without insecticide application (NTI and NT respectively). Soil tillage did not affect the soil mesofauna communities, however insecticide application affected the soil community. For collembola and enchytraeids the adverse effects of insecticide application were similar in the CTI and NTI treatments, but for mites, tillage (CTI) increased the duration of effects. Results for mites demonstrate that other mechanical stressors (tillage) can increase the effects of insecticide application, but the discrepancy in this response for collembola is not clear.

The effects of non-traditional contaminants or stressors can also be tested in TME experiments such as the salinization of coastal ecosystems [187]. Salinization did not affect Collembola, nematode and earthworm communities but heavily impacted enchytraeids communities. However once salinization effects cease the enchytraeid community fully recovered. Interestingly plant biomass and earthworm communities showed delayed effects only after salinization activities ceased.

Overall TMEs can provide an immense amount of information including fate, ecosystem structural and functional data. They allow the use of realistic exposure scenarios, realistic environmental variables or even the manipulation of these environmental factors to study added stressor effects. The TME ring test, conducted in conjunction with field experiments, also demonstrated that TME results were generally a good predictor of field variability and response. Finally, TMEs allow the testing of persistent contaminants at adequate scales (i.e. metals) whilst protecting the actual environment from unnecessary risk of dosing field sites. Despite the clear advantages of the TME approach, these mesocosms are still closed systems and thus are limited in maximum incubation time, not allowing for very long-term effects (more than one year) due to community collapse. Also, TMEs do not include the process of external recovery and for larger organisms, such as earthworms, these systems might be inadequate and considerably small. Larger TMEs, (30 cm diameter, compared with the 17.5 cm commonly used) are currently being used and developed which can allow more adequate space for larger organisms and for non-destructive sampling over time [188].

Thesis objectives and outline

The major goal of this PhD project is to investigate the environmental risk of metals in soil at real levels of contaminant and ecological complexity.

As demonstrated in the literature review, there is a wealth of knowledge on the effects of single metals to soil invertebrates. Despite their importance these studies do not consider that metals generally occur in the environment as complex mixtures which can have different toxic effects from those assumed by concentration addition. In regard to metal mixtures, whilst some research has been performed for the soil environment, this research is rather scarce and most of the current research has been performed with more simple binary and ternary mixtures which are still not an adequate representation of the mixtures found in contaminated sites. Furthermore, most of the data produced with metal mixtures has been done with laboratory spiked soils using metal salts and have not accounted for the added stress of salinity.

Chapter 2 of this study will address dosing methods for fixed ratio metal mixtures composed of lead, copper, nickel, zinc, cobalt. Current dosing methods using metal salts require leaching to remove excess salinity which may affect metal mixture ratios. As alternative methods, dosing with commercially acquired metal oxides and laboratory fabricated annealed metal compounds will be tested in conjunction with traditional metal

salts. The toxic effects of relevant environmental and regulatory mixture ratios using these three dosing methods will be assessed on *F. candida*, *O. nite*ns and *E. crypticus*.

Once determined the most appropriate dosing method for fixed ratio metal mixture experimentation, the following chapters analysed the effects of metal mixtures at increasing levels of ecological complexity. In Chapter 3, the effects of metal mixtures were tested on three standard soil invertebrate test species with different ecological traits (*E. crypticus*, *F. candida* and *O. nitens*). Individual metals (lead copper, nickel, zinc and cobalt) were tested at 11 doses and ten mixture ratios using 9 mixture doses. Three fixed ratios were based on contaminated sites, five on regulatory thresholds, one on the ecotoxicological response of *F. candida* and an equal ratio between all five metals (equal proportion of each metal 1:1:1:1:1). The joint effect of metal mixtures was determined at different dose/effect levels for each species. Metal mixtures were then tested in more complex community experiments.

In Chapter 4, three mixtures ratios (2 based on regulatory levels and 1 based on a contaminated site) were tested with 9 mixture doses using a natural soil community. Data was evaluated using a novel concept based on community similarity to determine community level effect concentrations.

Finally, in Chapter 5 the same metal mixtures used in microcosm experiments were tested in a TME experiment with three doses based on EC values estimated from the community tests performed in Chapter 4 (Community EC10, EC50 and 2x EC50). In TMEs, effects were determined for microarthropods abundances (similarly to microcosms) in a more realistic exposure scenario but also on different compartments (soil enzymes) and on ecosystem functioning.

The proposed experiments intend to provide guidance on metal dosing methods for future mixture experiments, produce single species data on the joint effect of complex metal mixtures and bridge the gap between the effects of mixtures in single-species tests and at community and ecosystem levels. The results from these experiments are expected to provide clarity into the effects of metals at environmentally relevant scales. At higher ecological levels, this thesis intends to not only understand the effects of metals on the community and ecosystem, but also develop tools to incorporate community data into risk assessment schemes.

Chapter 2 - Metal oxides and annealed metals as alternatives to metal salts for fixed-ratio metal mixture ecotoxicity tests in soil

This chapter is based on the published paper

M. Renaud, M. Cousins, K.F. Awuah, O. Jegede, B. Hale, J.P. Sousa, S.D. Siciliano, Metal oxides and annealed metals as alternatives to metal salts for fixed-ratio metal mixture ecotoxicity tests in soil, PLoS One. 15 (2020) e0229794. doi:10.1371/journal.pone.0229794.

Abstract

In soil metal ecotoxicology research, dosing is usually performed with metal salts, followed by leaching to remove excess salinity. This process also removes some metals, affecting metal mixture ratios as different metals are removed by leaching at different rates. Consequently, alternative dosing methods must be considered for fixed ratio metal mixture research. In this study three different metal mixture dosing methods (nitrate, oxide and annealed metal dosing) were examined for metal concentrations and toxicity. In the nitrate metal dosing method leaching reduced total metal retention and was affected by soil pH and cation exchange capacity (CEC). Acidic soils 3.22 (pH 3.4, CEC 8 meq/100g) and WTRS (pH 4.6, CEC 16 meq/100g) lost more than 75 and 64% of their total metals to leaching respectively while Elora (6.7 pH, CEC 21 meq/100g) and KUBC (pH 5.6, CEC 28 meq/100g) with higher pH and CEC only lost 13.6% and 12.2% total metals, respectively. Metal losses were highest for Ni, Zn and Co (46.0%, 63.7% and 48.4% metal loss respectively) whereas Pb and Cu (5.6% and 20.0% metal loss respectively) were mostly retained, affecting mixture ratios. Comparatively, oxide and annealed metal dosing which do not require leaching had higher total metal concentrations, closer to nominal doses and maintained better mixture ratios (percent of nominal concentrations for the oxide metal dosing were Pb = 109.9%, Cu = 84.6%, Ni = 101.9%, Zn = 82.3% and Co = 97.8% and for the annealed metal dosing were Pb = 81.7%, Cu = 80.3%, Ni = 100.5%, Zn = 89.2% and Co = 101.3%). Relative to their total metal concentrations, nitrate metal dosing (lowest metal concentrations) was the most toxic followed by metal oxides dosing while the annealed dosing method was generally non-toxic. Due to the lack of toxicity of the annealed metals and their higher dosing effort, metal oxides, are the most appropriate of the tested dosing methods, for fixed-ratio metal mixtures studies with soil invertebrates.

Introduction

In soil ecotoxicological research, metal dosing is usually performed using aqueous metal salt solutions in a dilution series [43,55,88,124,135,136]. This approach allows for a high dosing precision, reduced variability and ease in homogenizing the metals within the soil. Despite these advantages, when dosing with metal salt solutions, salinity may be a cause for concern. Salinity not only affects the chemodynamics of metals in soil increasing their mobility, bioavailability and toxicity [32,95,107,108] but can in itself cause toxicity to soil organisms [105,106]. To address these concerns, several authors have proposed and performed the leaching of soils dosed with metal salts to remove the effect of salinity and attempt to increase the realism of laboratory spiked soils compared to contaminated sites [100,101,189]. A consequence of this soil leaching process is that, in addition to salts, some metal is lost in the leachate. Single metal studies can correct for this by expressing biological response to the realized, rather than nominal, dose. In metal mixture studies, looking at specific ratios of different metal elements in the soil, leaching can be disruptive, because different metals are more or less mobile in soil due to differences in soil-metal-water partitioning [190–192].

In research testing metal mixtures, dosing has been performed with metal salts but without the leaching of dosed soils [55,64,124,131,135,136]. In these studies, only Posthuma et al. [64] acknowledged the importance of salinity by adding sodium chloride to control treatments to balance anion concentrations compared with metal dosed treatments. In mixture experiments where leaching is not performed, and salinity is not corrected, toxicity can be much higher than expected not only due to the increased bioavailability of metals, but also because both metal and salt can contribute to toxicity. Consequently, for fixed ratio metal mixture studies, and considering that leaching can affect mixture ratios, alternative methods for dosing soils must be considered.

This research intends to find a suitable alternative to metal salts when dosing complex metal mixtures. To select an adequate dosing method as an alternative to metal salts it is important to satisfy two criteria, namely the selected dosing method must better retain metal mixture ratios and have an adequate toxicity to soil invertebrates. For this goal, we considered three different methods (metal nitrate salts, metal oxides and annealed metal complexes) for dosing fixed ratio metal mixtures. Each method was evaluated for their total and element-specific metal concentrations as well as their toxicity to standard soil invertebrate test species (*Folsomia candida*, *Oppia nitens* and *Enchytraeus crypticus*) with different sensitivities and routes of exposure, in four different test soils.

Metal nitrate salts were selected to represent the standard practice of metal salt dosing, but which require leaching to remove excess salinity. Metal salts have been used extensively in ecotoxicology with a variety of different metal salts (i.e. metal chlorides [18,43,64,130], sulphates [88,135,136]) including nitrates [32–34,60,88]. Unlike some previous mixture studies in soil, which used a combination of chlorides and nitrates salts for different elements [55,124] we selected the same metal salt (nitrate) for all 5 metals tested in this dosing method. Metal oxides are proposed as an alternative dosing method which does not require leaching and which has also previously been used in soil ecotoxicological research mostly in comparison to metal salt toxicity [48,54]. More recently studies have focused on metal oxide nanomaterials (i.e. [103,193]) but which are considered separately from non-nano oxides used in this study. Annealed metal complexes were selected as a dosing method simulating contamination resulting from a smelting operation and have not been previously tested in the scientific literature except for a similar study on the effects of these dosing methods to soil microbial processes [194]. In terms of their realism and environmental relevance, metal contaminated sites can present a variety of different chemical forms [95], but the annealed metal complexes closely resemble the minerals present at a metal contaminated sites like franklinite and willemite [195,196], whereas oxides and metal salts are less representative but still used in routine in laboratory dosing schemes (especially metal salts) for practical reasons.

To address the adequacy of the different dosing methods in terms of their toxicity, three different soil invertebrate species were considered to cover different routes of exposure. The collembolan *F. candida* has been used as a standard test species in soil ecotoxicology for over 50 years and is mostly exposed to contaminants through soil pore-water through the ventral tube and by the ingestion of contaminated pore water and food [110]. Similarly, to *F. candida*, *E. crypticus* is exposed to contaminants through ingestion but also dermally due to their close contact with soil pore water and lack of protective cuticle [197]. *O. nitens* is a relatively new species in soil ecotoxicological research and is exposed to contaminants mostly through ingestion due to their thick sclerotic exoskeleton [198]. However, in juveniles, which lack this exoskeleton, exposure routes can include dermal uptake and affect population performance due to juvenile mortality [88,198]. In terms of sensitivity, *F. candida* has a similar sensitivity to *E. crypticus* whilst *O. nitens* is expected to be less sensitive due to their hard body. Using copper as an example, reproduction EC50 for each species in OECD artificial soil was 477 mg/kg for *E. crypticus* [64], 700 mg/kg for *F. candida* [33], and 2,896 mg/kg for *O. nitens* [88].

Metal solubility and speciation affect the mobility of metals in soils and consequently their bioavailability and toxicity [95]. In this case metal nitrate salts are expected to have

a higher solubility in soil pore water compared to non-soluble oxides or annealed metal complexes [48]. The higher availability in soil pore water (one of the main routes of exposure for invertebrates) implies that nitrate salts should have a higher toxicity than oxides and annealed metal complexes. However, the literature is not always consistent in terms of the relative toxicity of oxides versus salts to soil invertebrates [54,103]. For annealed metal complexes, their toxicity is unknown for soil invertebrates, but this method was designed to incorporate metals in soil directly as a mixture, which is the result of a simulated smelting process, thereby increasing the realism of metal mixture dosing schemes for soil ecotoxicology. When tested using soil enzymes activity (ammonia monooxygenase and acid phosphatases activity), the toxicity of similar dosing methods was both soil and enzyme dependant and did not demonstrate a consistent trend [194].

Soils and their properties can also affect the mobility and availability of metals. The most important soil properties affecting metal bioavailability and toxicity are pH, cation exchange capacity, organic carbon and clay content [37,95,100,199]. Therefore, four different soils covering a range of these different soil properties were selected for method evaluation.

Methods

All experiments were performed with four different Canadian soils. After collection, all soils were air dried and sieved to <2 mm particle size before storage. Soil properties are presented in Table 1. Two of the soils (3.22 and WTRS) were reference soils collected close to mining sites in Flin Flon, Manitoba. Elora soil was collected in Elora, Ontario, while KUBC was a mixed soil from an agricultural research field in Saskatchewan (Kernen) and a soil from Iqaluit, Nunavut (UBC) mixed in a 1:1 ratio. In all cases, private land owners provided permission for soil collection from the sites. Neither endangered or protected ecological species nor humans were sampled for this study.

Table 1 – Soil properties and closest soil type classification in soil PNEC calculator.

Soil	pH-$CaCl_2$	CEC (meq/100g)	Organic C (g/kg)	Clay Content (g/kg)	Water Holding Capacity (ml/g)	Closest soil type in PNEC soil calculator [8]
3.22	3.4	8	17	45	0.3	Acid Sandy Forest
WTRS	4.6	16	25	110	0.35	Acid Sandy Arable
KUBC	5.6	28	12	24	0.48	Loamy
Elora	6.7	21	21	200	0.48	Loamy Alluvial

All four soils (Table 1) were dosed with five different metal mixture ratios (Table 2) at a single mixture dose of 4 toxic units using three different dosing methods: metal nitrate salts, metal oxides and annealed metal complexes. The five mixture ratios were selected based on average metal concentrations for each metal in three contaminated sites in Canada (Flin Flon, Sudbury and Port Colborne), the Canadian soil quality guideline for an agricultural soil use (CSQG) and the estimated PNEC in a Clayey Peaty soil from the Soil PNEC calculator.

Table 2 – Nominal metal mixture ratio compositions in mg/kg dry weight of soil at a dose of 4 toxic units and *Folsomia candida* EC_{50} used in estimating toxic units.

Mixture	Lead (mg/kg)	Copper (mg/kg)	Nickel (mg/kg)	Zinc (mg/kg)	Cobalt (mg/kg)
Ratio 1 - Port Colborne	55.6	380.9	1513	162.6	27.8
Ratio 2 - CSQG	536.2	482.6	344.7	1532	306.4
Ratio 3 - Flin Flon	202.1	618.6	9.2	2223.3	9.2
Ratio 4 - Sudbury	2314.4	160.9	297	1196.4	152.6
Ratio 5 - Clay Peat	612.1	662.5	395.7	1199.2	353.6
F. candida EC50	1600 [33,34]	700 [33,34]	475 [43]	750 [33,34]	1480 [18]

The mixture dose of four toxic units (TU), was calculated based on *F. candida* literature EC50 for each metal element (Table 2). *F. candida* was selected as a standard species to define mixture doses, because literature data is available for all 5 metals. The mixture dose of 4 TU was selected as it was a dose expected to cause toxicity to all invertebrates tested in this study. After each dosing procedure, samples were collected for metal analysis and the remaining soil was used in toxicity assessments. In this study mixture toxicity modeling was not addressed and single metal dosing was not performed, toxicity

testing was performed with a single mixture dose (4 TU) only to determine the suitability of the dosing methods in terms of effects on soil invertebrates.

Nitrate metal dosing

Aqueous nitrate solutions of lead (Sigma-Aldrich, Pb(NO3)2 ACS reagent ≥ 99.0%, #228621), copper (Sigma-Aldrich, Cu(NO3)2 - 2.5H2O ACS reagent, 98%. #223395), nickel (Sigma-Aldrich, Ni(NO3)2 - 6H2O, purum p.a. crystallized, ≥97%, #72253), zinc (Sigma-Aldrich, Zn(NO3)2 - 6H2O, reagent grade, 98%, #228737) and cobalt (Sigma-Aldrich, Co(NO3)2 - 6H2O , reagent grade 98%, #230375) were pipetted individually to each soil from their respective concentrated stock solutions, to reach the intended mixture ratio. Distilled water was then added to each dosed and control soil to adjust soil water content to 50% water holding capacity and all soils were vigorously mixed. Two weeks after dosing soils, the electrical conductivity of soils was measured, and dosed soils were leached using artificial rainwater [200], one pore-volume at a time, until conductivity reached control (non-dosed soil) levels. To account for the loss of fine soil particles from leaching dosed soils, control soils were leached once with one pore-volume of artificial rainwater as well. After leaching, the soils were air dried and lightly macerated to break down aggregates.

Oxide metal dosing

Commercially available metal oxides of lead (Sigma-Aldrich, PbO, ACS reagent ≥ 99.0% #402982), copper (Sigma-Aldrich, CuO, powder <10 µm, 98% #208841), nickel (Sigma-Aldrich, NiO, 325 mesh, 99% #399523), zinc (Sigma-Aldrich, ZnO, ACS Reagent ≥ 99.0%, #96479) and cobalt (Sigma-Aldrich, Co_3O_4powder <10 µm #221643) were used in soil dosing experiments. When necessary, oxides were finely ground to a powder using a mortar and pestle. Once ground, oxides were placed on plastic weigh boats in a sealed glass container with an open beaker of nitric acid. Oxides were left in contact with acid vapours for 48 hours to remove any carbonates and subsequently, air dried in a fume hood for 24 hours. Dried metal oxides were then individually weighed at the appropriate concentration for each metal mixture ratio and added to dry soil. Once all metal oxides were added, soils were thoroughly mixed by stirring and shaking and soil water content was adjusted to 50% water holding capacity.

Annealed metal dosing

Annealed metal complexes were prepared by precipitating and roasting a metal nitrate mixture solution, to simulate the laboratory equivalent of the ash produced from a smelting operation. In this procedure the same individual aqueous metal nitrate stock solutions used for metal salt dosing were combined to create four mixture stock solutions corresponding to each mixture ratio (Table 2). To each mixture solution an iron nitrate solution was added in a 2:1 molar ratio of iron to the sum of the five metals of interest. The addition of iron was performed to increase the precipitation of the 5 metals of interest (Pb, Cu, Ni, Zn and Co). From this mixture solution an initial portion of 25ml was used in the procedure described below to examine metal precipitation rates in the final ash. This preliminary information was used to correct for unprecipitated metals by adjusting their concentration in the mixture stock solution.

Metals were precipitated by increasing solution pH to 7 ± 0.25 with 14.8 M ammonium hydroxide. If the pH rose above 7.25, nitric acid was added to correct for the intended pH value. Once the correct pH was attained the tubes were shaken overnight, after which pH was re-checked and adjusted if needed. The final titrated solutions were centrifuged at 400 g for 30 minutes, after which the supernatant was decanted and resulting precipitates were dried in a fume hood for 12 hours. The resulting pellets were roasted at 600^{o}C for 1 hour in a muffle furnace to decompose the metal nitrate bonds [201–203]. Metal content in ashes was determined to check ratio composition by digesting the samples and analysed through ICP-OES. Sample digestion was performed by stirring 0.05 g of the ash in a heated mixture of HF/HNO_3/$HClO_4$ until dry and the residue was then dissolved in a diluted HNO_3 solution.

Despite initial corrections for unprecipitated metals, in the final annealed material there is always slight deviations from nominal metal ratios. In this case the amount of annealed material applied to each soil was calibrated by using the metal within the ash mixture which best matched the nominal ratio. For example, a target mixture ratio could be 25% lead, 50% copper, and 25% nickel, while the annealed metal complexes were 23% lead, 57% copper, and 20% nickel. In this case the mass balance for dosing each soil would be calculated as per the concentration of lead in the annealed material. After the addition of the metals to soil, these were thoroughly mixed to homogenize and incorporate the metals into the soil and soil water content was adjusted to 50% water holding capacity.

Toxicity tests

The toxicity of the three different dosing methods for each metal mixture and each soil type were assessed using the reproduction of three different soil invertebrate species. These endpoints were determined using standard protocols for *E. crypticus* (ISO 16387 [204]) and *F. candida* (ISO 11267 [205]). *O. nitens* tests were conducted adapting the procedures of Princz et al. [198].

Prior to all invertebrate testing, soil water content was adjusted to 50% of their respective water holding capacity (WHC). A description of experimental conditions for each test species is presented in Table 3. In short, after the addition of soil and test organisms to each test unit, these were incubated in the laboratory for four weeks under a photoperiod of 16h: 8h light:dark. For the duration of the incubation period, soil water content was maintained by adding distilled water to match initial test vessel weight and test units were fed with granular yeast (*F. candida* and *O. nitens*) or rolled oats (*E. crypticus*). After 4 weeks of incubation, for *F. candida* and *O. nitens*, the assays were ended by extracting organisms from each replicate using a heat extractor (previously tested for extraction efficiency (> 90%)) and counted using a binocular microscope. For enchytraeids, organisms from each test vessel were fixed in 70% ethanol and stained with Bengal red (200 to 300 µL of 1% Bengal red in ethanol) for 24h. After staining, samples were wet sieved using a fine mesh (103 µm) and the organisms were counted using a binocular microscope.

Table 3 - Procedures adopted in reproduction tests with *Folsomia candida*, *Enchytraeus crypticus* and *Oppia nitens*.

	F. candida	*E. crypticus*	*O. nitens*
Guideline considered	ISO 11267 [205]	ISO 16387 [204]	Princz et al. [198]
Test period (d)	28	28	28
Test containers (mm)	29 x 80	29 x 80	29 x 80
Number of replicates per treatment	5	5	5 control 4 treatments
Number of organisms per replicate	10	10	15
Food source	Dry yeast	Rolled oats	Dry yeast
Days of food supply	0, 14th	0, 7th, 14th, 21st	0, 7th, 14th, 21st
Days of aeration and moisture reestablishment	7th, 14th, 21st	7th, 14th, 21st	7th, 14th, 21st
Soil per test container (g DW)	30	20	30

Metal analysis

Soil samples were collected from bulk dosed soil for chemical analysis to determine total metal concentrations. Total metal concentrations in soil were determined by reverse *aqua regia* using trace metal grade nitric and hydrochloric acid (3:1; v/v) and following the procedures described by Topper and Kotuby-Amacher [206] and the EPA [207]. For each treatment soil (1 g) was weighed into 60 ml Teflon digestion vessel and 9 ml of nitric acid followed by 3 ml of hydrochloric acid were added to each digestion vessel. The digestion vessels were then swirled every 30 minutes until no acid fumes from organic matter digestion were observed and digested in an oven overnight at 105°C. After digestion was completed the resulting solution was filtered using Whatman 42 paper and analysed using ICP-AES for the metals of interest. In addition to soil samples, analysis was also performed for a standard reference material [208], recoveries for the SRM for all elements were on average 73% and always above 66%. Since SRM values recovered were lower than expected measured metal concentrations in dosed soils were corrected for standard reference material recoveries for each metal element, respectively. Nominal and measured total metal concentrations for each element, dose method, mixture and soil are presented in Annex 1 (Table A1).

Statistical analysis

All data analyses were performed using R version 3.1.3 [209] with the use of organizational packages Rmisc [210] and PMCMR [211] packages.

No statistical analysis is presented in the results (Figures 1 – 3) because different variables were grouped (soils, elements, mixture ratios) to demonstrate the main effects of the dosing methods on metal concentrations and toxicity. These variables have significant interactions between them, and it would be erroneous to present statistical significances for grouped variables. Results are presented as total metal concentrations (grouping all five elements and mixture ratios) in each soil as a percent of the nominal dose to demonstrate the role of soil properties on total metal concentrations according to dosing method (Figure 1). Individual metals concentrations are also presented as a percent of nominal dose for each element across all soils and mixtures to observe the effects of dosing methods on the concentrations of specific elements affecting mixture ratios (Figure 2). Reproduction results from each species are presented as average percent of control response across all mixtures for each soil and dosing method (Figure 3).

Total metal and individual metal concentration were adjusted for background control soil concentrations and then were normalized to their percentage of target dose (nominal) concentration. This was performed to allow comparisons between soils which have different background concentrations. When adjustments for soil background concentrations resulted in negative values as a result of variation or metal loss from leaching, dosed soil concentration was corrected to zero. In toxicity tests, invertebrate reproduction was normalized to each soil's average control reproduction.

Results

Metal concentrations were determined for nitrate metal dosing method before (non-leached nitrate) and after the process of leaching (leached nitrate) to remove salinity, for both oxide and annealed metal dosing methods, no leaching was performed because there was no added salt. Soil had a significant effect on the precision or variability of total metal measurements. All soils suffered the same dosing procedures for each dosing method and the same level of effort in mixing metals within the soil. Differences in the magnitude of variability could be the result of differences in soil properties, such as soil texture, affecting the heterogeneity of metals within the collected soil sample. Elora had the highest variability of the four tested soils followed by WTRS and 3.22 while KUBC was the least variable. Elora and WTRS both had the highest clay content possibly reducing the efficiency of mixing metals within the soil. Excluding non-leached nitrates in the Elora soil, dosing precision was similar between the different dosing methods for each soil. Total metal concentrations for all elements, mixtures and dosing methods are presented in the Annex 1 (Table A1).

When comparing non-leached to leached nitrate dosing, acidic soils (WTRS and 3.22) lost more metals through leaching than soils with higher pH values (Elora and KUBC) (Figure 1). In the lower pH soils, compared to its non-leached counterpart, over 75% (3.22 pH 3.4) and 64% (WTRS pH 4.6) of metals were lost in the leaching process. In contrast, Elora and KUBC with circumneutral pH levels of 6.7 and 5.6 had much lower average percent loss of metals (13.6% and 12.2% respectively) compared to their non-leached treatments. Elora and KUBC also had higher CEC (CEC of 21 and 28 meq/100g respectively) compared to 3.22 and WTRS (CEC of 8 and 16 meq/100g respectively) increasing the binding affinity of metals to soil and reducing metal losses through leaching. For the remaining dosing methods, metal oxide and annealed dosed soils produced similar results to non-leached dosed soils and were not affected by soil except for oxides in Elora where a higher than average recovery was observed. Compared with

leached soils, oxide and annealed metal had higher total metal concentrations and were much closer to the target nominal dose in all soils but especially in the more acidic lower CEC soils 3.22 and WTRS.

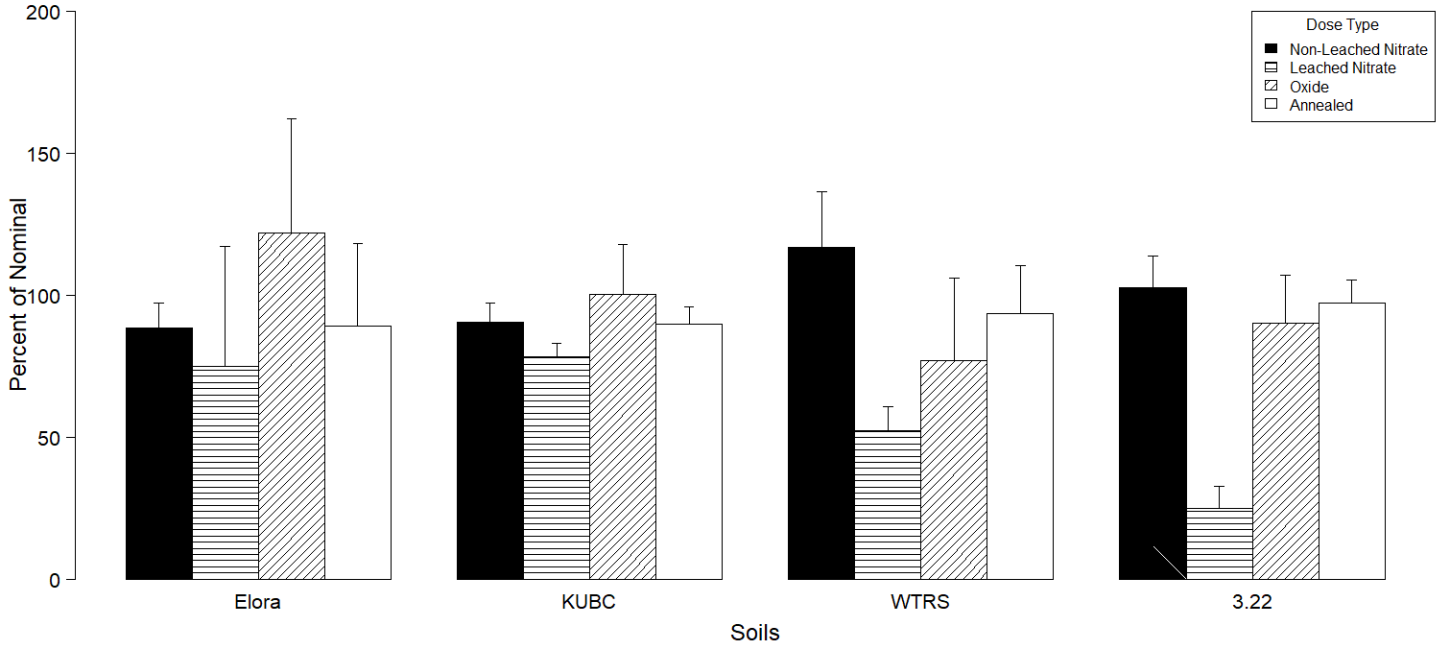

Figure 1 - Effect of dosing method on total metal concentrations by soil. Average percentage of nominal total metal concentration (all elements combined) across all mixture ratios (R1 - Port Colborne, R2 - CSQG, R3 - Flin Flon, R4 - Sudbury and R5 - Clay peat), in each soil according to dosing method. Error bars represent the standard deviation (n = 5).

Leaching selectively removed more than 45% of initially dosed Ni (46.0% lost), Zn (63.7%), and Co (48.4%) (Figure 2). Comparatively, only small losses of lead (5.6%) and copper (20.0%) are observed in the nitrate metal dosing after leaching. In the dosing methods which did not require leaching (oxides and annealed dosing) individual metal concentrations are much more consistent and closer to the target nominal dose, especially for Ni, Zn and Co. For oxide metal dosing, all elements concentrations were higher than leached nitrate dosing, while for annealed lead concentrations were slightly lower. Comparing between oxide and annealed metal dosing, results were similar. Oxides had higher individual concentrations of lead and slightly higher concentrations of copper (Oxide dosing percent of nominal Pb =109.9%, Cu=84.6%, Ni = 101.9%, Zn = 82.3% and Co = 97.8%) but annealed metal dosing was slightly more consistent across different

elements (Annealed dosing percent of nominal Pb = 81.7%, Cu = 80.3%, Ni = 100.5%, Zn = 89.2% and Co = 101.3%). Overall, despite some metals having lower total concentrations towards the target dose, annealed metal dosing had a slightly better consistency for maintaining mixture ratios. Dosing with oxides and annealed complexes produced results similar dosing with metal nitrates prior to leaching the soil (non-leached nitrate metal dosing).

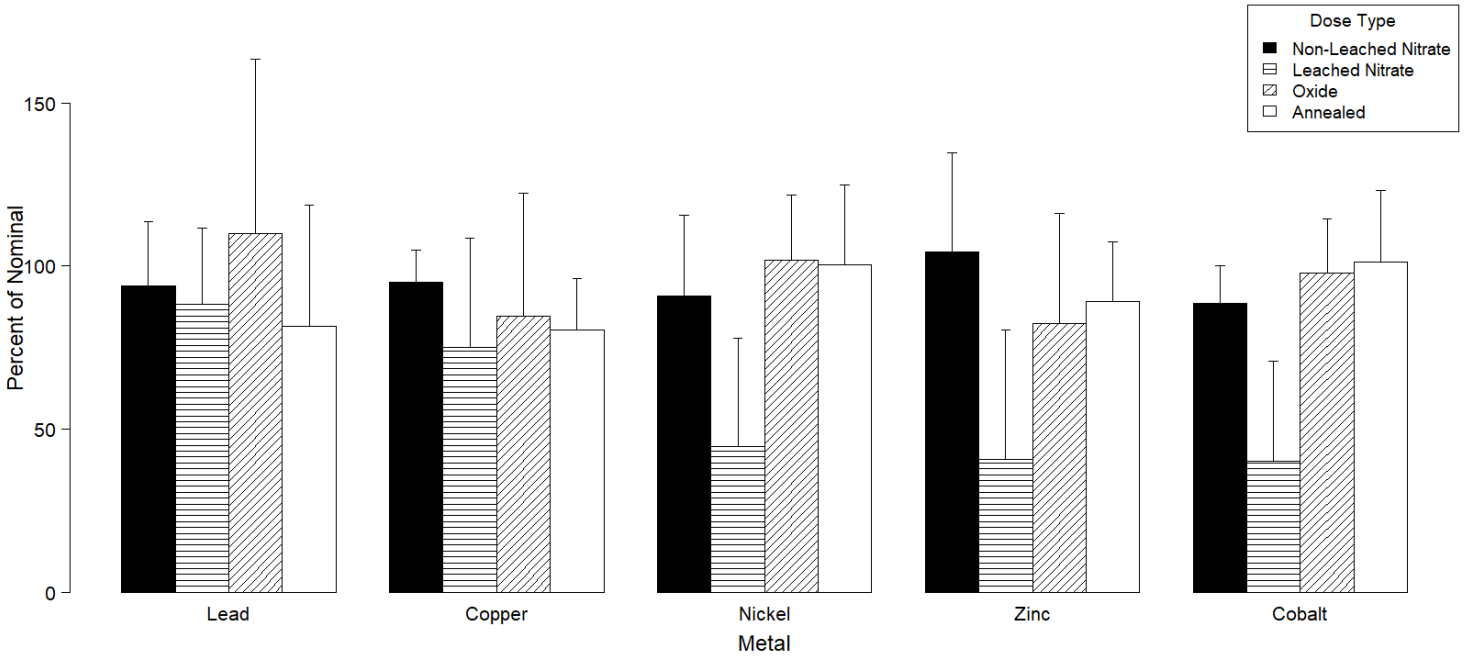

Fig. 2 Effect of dosing method on individual metals concentrations. Average percent of nominal concentration combining all soils (Elora, KUBC, WTRS and 3.22) and mixtures (Port Colborne, CSQG, Flin Flon, Sudbury and Clay Peat) of lead, copper, nickel, zinc and cobalt according to dosing method. Bars represent standard deviation (n = 20).

In terms of invertebrate toxicity tests performance, two soils were excluded for *E. crypticus*, (WTRS and 3.22) due to insufficient reproduction in controls (<10 individuals). In the two remaining soils (KUBC and Elora), validity criteria were met, average mortality was below 10%, reproduction above 340 juveniles and the coefficient of variation (CV) below 34%. For *F. candida*, mortality was always below 20% (average 13%) and juvenile production above 100 (average 558) in controls. In terms of variation, for the annealed dosing method in the Elora soil the CV in controls was above validity requirements (CV = 64%), however, maintaining or excluding this soil did not change the overall outcome of dosing method toxicity as annealed metals were generally non-toxic. In the remaining controls the CV was on average 25% with some soils slightly above the

30% threshold (3.22 annealed – 35%, 3.22 oxides – 31% and KUBC nitrate – 36%). For *O. nitens*, no validity requirements currently exist for this species but the CV in controls was on average 42%, average juvenile production was 83 and adult mortality was always below 29% per treatment (average 10%).

Soil invertebrate reproduction differed among the three dosing methods relative to their metal concentrations (Figure 3). Relative to their metal concentrations, metal nitrates were the most toxic, followed by metal oxides and annealed metal dosing were the least toxic. Annealed metal dosing was non-toxic at the tested mixture dose in all soils and for all three species except for *O. nitens* in 3.22 (average percent control 64%) and *F. candida* in WTRS and 3.22 with very small effects (average percent control 75% and 83%, respectively). Despite their lower total metal concentration, metal nitrates were always more toxic than oxides to *E. crypticus*. *F. candida* was also more sensitive to nitrates than oxides, except in the 3.22 soil where metal loss for nitrate dosing was most notable. *O. nitens* were more sensitive to nitrates over oxides in WTRS and KUBC but in 3.22 and Elora oxides produced a larger toxic effect. Comparing between species, *E. crypticus* was the most sensitive invertebrate species while *O. nitens* and *F. candida* responses were similar with some exceptions. *O. nitens* were more sensitive to oxides and less sensitive to nitrates in the Elora soil compared to *F. candida* and *F. candida* had a higher sensitivity to both oxides and nitrates in the 3.22 soil. Considering both observed toxicity and metal concentrations, the rank toxicity of the dosing methods from highest to lowest toxicity is nitrate metal dosing, oxide metal dosing and annealed metal dosing. In terms of species sensitivity, *E. crypticus* were globally the most sensitive whilst *O. nitens* and *F. candida* had a similar sensitivity.

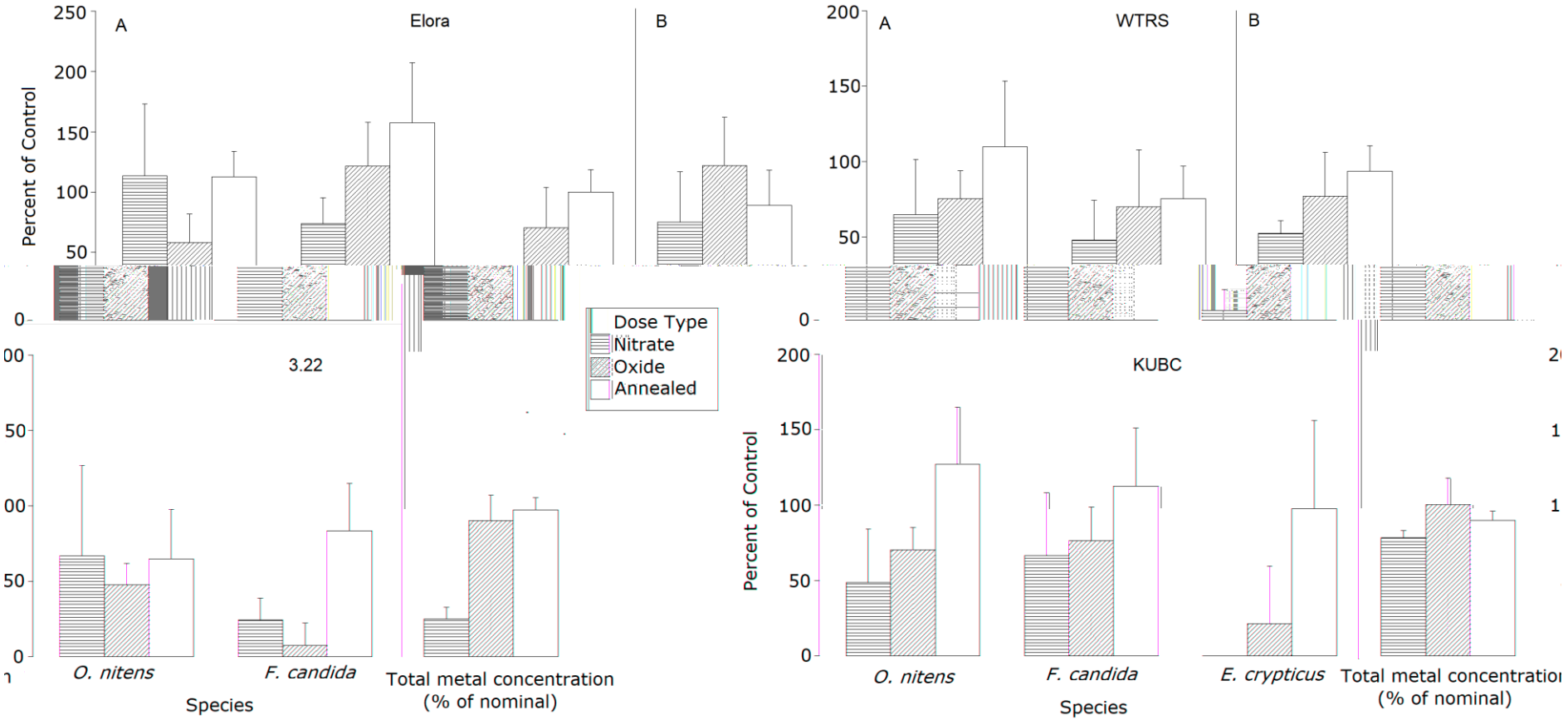

Figure - 3 Effect of dosing method on invertebrate reproduction by soil. Average reproduction (as percent of control) for the three species pooled over all metal mixtures for each dosing method and soil (Panel A); total metal concentration as a percentage of nominal dose for each dosing method over all soils and mixtures (Panel B). Error bars represent the standard deviation of the mean (*Oppia nitens* n = 20, *Enchytraeus crypticus* n = 25, *Folsomia candida* n = 25, Total metals n = 5)

Discussion

Leaching removed metals and the intensity of this loss was affected by soil properties, in particular soil pH and CEC. Acid soils with lower CEC (3.22 and WTRS) lost much more metals than soils with higher pH and CEC values. The role of pH in the mobility of metals has been extensively researched and these results are in accordance with previous literature, where metals, in particular cationic metals have increased mobility in soils with lower pH values [95,189,190]. More recently Dijkstra et al. [212], demonstrated that the pH dependency of metal leaching is in fact a "V-shaped" curve where leaching is lowest at more neutral pH's (like Elora and KUBC) and highest at both extreme soil pH values. Cation exchange capacity could also contribute to the observed differences between soils. Cation exchange capacity, measures the amount of negatively charged sorption sites in a soil, available for cation adsorption [95]. Soils with higher CEC, have a higher capacity to adsorb cationic metals binding them to mineral surfaces leading to lower solubility and bioavailability of metals [37,95,100]. As observed in the mixture results, KUBC and Elora which had a higher CEC, promoted a higher binding of metals to the soil leading to lower metal losses in the leaching process. Soils properties influences the amount of metals lost through leaching but not all metals were equally affected. In this experiment, three elements, Ni, Zn and Co, were lost whereas Pb and Cu were more retained similar to what was previously reported for the mobility of different metal elements in soil [190–192]. Under the current experimental conditions obtaining fixed ratio mixtures is not possible when leaching soils after dosing with nitrate salts.

In this experiment dosed soils were aged for two weeks before leaching, this aging period is short and may have not been long enough to allow metal partitioning within the soil resulting in higher losses during the leaching process. Amorim et al. [38], when testing the role of aging, considered an aging period 60 days following recommendations from a scientific workshop. Using this larger aging period may in fact increase metal retention after leaching, however it would also increase the time required for testing, which can compromise routine testing and be a further disadvantage when compared to oxide and annealed metal dosing methods which do not require extensive aging. Also aging of dosed soils is not commonly considered prior to leaching in soil invertebrate testing even in some research specifically looking at aging and leaching, such as the study of Lock et al. [101], where leaching of $Pb(NO_3)_2$ dosed soils was performed one day after dosing.

Results demonstrated that different elements are lost at different rates as a result of leaching, and the intensity of this loss is dependant on soil properties. Consequently, mixture ratios would change as a function of soil, dose and composition when dosing with the nitrate metal dosing method. While previous research has already demonstrated the differential mobility between metal elements in soils, the goal of this experiment was, not to demonstrate that different elements were lost through leaching at different rates, but rather test if alternative dosing methods would provide an improvement in maintaining fixed metal ratios. In the two alternative methods (oxide and annealed metal dosing) results were more consistent than leached nitrate metal dosing and higher metal concentrations were observed, in particular for Ni, Zn and Co. In both the annealed and the oxide dosing methods there is still some discrepancy towards nominal concentrations (detected values are below 100% of nominal or at times above 100% for individual metals). When dosing soils, there is always an error associated with the dosing procedure and the homogenization of metals within the soil but for both oxide and annealed dosing methods this variation towards nominal concentrations is within the range detected for the standard method of nitrate salt dosing without leaching soils.

Comparing between oxide and annealed dosing methods, both methods performed similarly in terms of total metal concentrations excluding the Elora soil where oxides had higher concentrations than expected. In terms of individual metal elements, responses were also similar with the annealed dosing method being slightly more consistent between elements. However, oxide metal dosing was only slightly less consistent than the annealed metal dosing and was still much less variable than leached nitrate metal dosing. Consistency in the oxide dosing method was mostly affected by the higher than average concentration of lead. When dosing with metal oxides, metals were individually added to the soil while for the annealed metal dosing all metals were added together in a single ash product which could explain the better consistency. Considering both oxide and annealed metal dosing produced similar results, dosing with metal oxides seems a more sensible solution for future research due to the substantially higher effort required to create the annealed metal compounds. Future research dosing with metal oxides, should consider combining individual oxides in a mixture prior to their addition to soil to reduce mixture variability across doses. This is similar to preparing a stock solution, but in this case an "oxide mixture stock", from which all dosing is performed.

In this experiment three different methods were considered: nitrate salts, oxides and annealed materials. Metal nitrate salts were selected to represent a standard dosing procedure with aqueous metal salts. In addition, two other methods were considered which are used in their solid form

and which do not contain salts and therefore do not require leaching. Oxides were selected as an insoluble commercially available laboratory grade metal and annealed metal complexes which were created to simulate smelter ash. The recommendation of using metal oxides in fixed ratio metal mixture experiments is based on this selection, however other metal forms of both soluble metal salts (Chlorides) and insoluble metals (Sulphides, sub-sulphides) should be considered for further testing.

Metal toxicity

Metal nitrate dosing toxicity was similar to oxide and higher than annealed metal dosing despite having the lowest total metal concentrations. In the 3.22 soil, nitrate toxicity was lower than metal oxides, but this is also the soil where the most metals were lost through leaching. The differences in toxicity between dosing methods could be the result of differences in solubility and consequently bioavailability. Compared to metal oxides and annealed metal complexes, nitrate salts were expected to produce higher toxicity. In a similar study, metal salts (after leaching) had similar extractable metal concentrations to metal oxides, in acidic soils, but while oxides only presented extractable Zn concentrations with metal nitrate salts, Zn was the most mobile but a larger proportion of other metals were also mobilized [194]. Annealed metal complexes, the least toxic, also presented mostly Zn and in one case Pb extractable concentrations but only a fraction of the concentrations observed for metal oxides. Also, Awuah et al. [194] found that $CaCl_2$ extractable concentrations for oxide and annealed metal dosing were soil dependant and higher in soils with lower pH, while metal salts had similar extractable concentrations independently of soil. The higher mobilization of metals in lower pH soils does not seem to correspond with toxic response except for the most acidic soil (3.22) where there appears to be a higher toxic response of oxides and annealed complexes compared to the remaining soils, especially for *F. candida*. It is also possible that in addition to metal bioavailability soil properties can affect the toxicodynamics of metals, acting as a confounding factor by improving the resilience of organisms to metals in higher habitat quality soils, independently of metal bioavailability as demonstrated for *O. nitens* [89].

There has not been previous research on the toxicity of annealed complexes to soil invertebrates, but for metal oxides and metal salts, previous research has not been consistent on their relative toxicity. Lock and Janssen [48], using soils dosed with zinc salt, oxide and elemental powders, found a similar chronic toxicity between dosed soils for *Enchytraeus albidus, Eisenia fetida* and *F. candida*. Direct comparisons are difficult as mixtures were tested rather than a single metal,

but at metal mixture dose of 4 TU, metal oxides and salts (after leaching) had a similar toxicity to *F. candida* and *O. nitens* with some variations between soils but nitrates were much more toxic than oxides for *E. crypticus*. Considering metal loss due to leaching, in this experiment, unlike Lock and Janssen [48], metal nitrate salts are more chronically toxic than oxides. The higher toxicity of metal salts to oxides was also reported in another study, where Zn chloride was more toxic than Zn oxides to *F. candida* [53].

These results demonstrate that metal form and speciation affect metal toxicity, independently of their concentration, potentially due to differences in metal solubility and consequently bioavailability. However, it is also important to highlight that bioavailability is modulated by species traits. In this case, Enchytraeids were much more sensitive to metal nitrate salts compared to both *F. candida* and *O. nitens*. The higher sensitivity of Enchytraeids is not surprising considering its exposure routes. In addition to oral exposure through pore water, a common exposure route for *F. candida*, Enchytraeids can also be exposed dermally due to their soft body and lack of a protective cuticle. This can lead to a higher total exposure and consequently to a larger toxic effect. On the other hand, *O. nitens* had a surprisingly similar sensitivity to *F. candida* despite their thick exoskeleton. It is possible that *O. nitens,* juvenile exposure could lead to mortality and lower reproductive outputs [88,198]. Alternatively, ingestion of soil as part of their feeding behaviour could increase their exposure (which has not been reported for *F. candida)* and compensate their more developed external barriers [111] . Further research into the importance of species traits in modulating their exposure to contaminants should be performed specially for more recently standardized test species like *O. nitens*.

In all three species, salts which are more soluble in pore water appear to be more toxic than metal oxides, but the correlation between metal solubility and toxicity is not always clear in the literature. Lock and Janssen [48], found that even measuring pore-water concentrations, Zn salts were still more acutely toxic than oxides. Also, Smolders et al. [100] using a large data set of contaminated soils with Cd, Cu, Co, Ni, Zn and Pb found that, pH was a good general predictor of metal solubility in soils, but a poor predictor of toxicity to organisms, and consequently that metal toxicity cannot be inferred from the solubility of metals. In this case and when mixtures are considered, interactions within the soil, the organism and between the different metals are much more complex and further research is required to better understand the role of different exposure routes and metal solubility on their toxicity [213].

Annealed metals were non-toxic to all test species in the tested mixtures at a dose of 4 TU, with only some exceptions where slight toxicity was observed in the more acidic soils (WTRS and 3.22). The choice to create and use this annealed metal mixture was, to an extent, to simulate a smelting operation. This would allow not only the testing of mixtures created as a single compound but also potentially increase the realism of soil dosing for metal ecotoxicology research. Several authors have highlighted that metal salts currently used in ecotoxicology research are not good representatives of contaminated sites and cause a higher toxicity, leading to the recommendation of both leaching and aging soils [101,189]. In this case creating a metal mixture ash as a potential representative of metal forms found in contaminated sites produced a much lower toxicity than expected, considering the known environmental degradation and toxicity of metal contaminated soils. A possible explanation as to why these annealed metal compounds were non-toxic was the use of an iron solution added to promote metal precipitation. The addition of iron increased precipitation by creating bonds between iron and the different metals but might be reducing the availability of the toxic metals. In fact, in a similar experiment, annealed metal complexes only had a fraction of the extractable metals compared to metal oxides and these were restricted to Zn and in one case to Pb [194]. If this is the case, it raises questions as to why toxicity is observed, for instance in smelter sites where minerals like franklinite (iron and zinc mineral - $ZnFe^{3+}{}_2O_4$) are the dominant form of zinc [196]. One hypothesis is that the difference in toxicity is due to weathering. In a contaminated site and particularly in old legacy mining areas, residues from the smelting procedure are weathered down by rain and changing environmental conditions releasing more bioavailable toxic elements into the environment over large periods of time. It could be that these annealed metal mixtures, unlike salts which have a reduction in toxicity due to aging, could have an increase in toxicity over time due to weathering. Further experiments with the annealed dosed soils, looking at toxicity over time should be performed to validate this hypothesis and demonstrate their potential toxicity.

Overall, the annealed metal complexes which were expected to be the most realistic approach to simulate metal contaminated sites were generally non-toxic at a mixture dose of 4 TU where effects were expected to occur for all test species. There is a potential for increasing toxicity over time with the weathering of the mineral structure of the annealed metal complexes, but these procedures would not be feasible in routine laboratory experiments. Comparatively metal salts and oxides produced a toxic response to all soil invertebrates, but salts appear to be more toxic than oxides relative to total metal concentrations. Dosing with metal oxides and salts while not as representative of metal contaminated sites are much more practical for routine laboratory dosing

regimes. In fact, both metal oxides and salts (in particular) have been previously used in ecotoxicological research and environmental guidelines are currently based on ecotoxicological data with metal salts [8,11].

Conclusion

Considering that dosing with metal nitrate requires leaching and that this leaching disrupts metal mixture ratios and total concentrations, it is not feasible to conduct fixed ratio studies using nitrate salts. The alternative dosing methods (annealed and oxides) which do not require leaching were an improvement in maintaining total and element specific metal concentrations compared to leached metal nitrate dosing and produced similar results to nitrate dosing before leaching is performed. Annealed metal dosing while maintaining more appropriate mixture ratios, did not produce toxic effects within the experimental timeframe of reproduction tests. If our assumptions towards Annealed metals are correct, with the weathering of the annealed complexes an increase in toxicity could be expected over time. In the constraints of standard ecotoxicological tests with fixed ratio metal mixtures, dosing with metal oxides is the most sensible dosing method, retaining fixed mixture ratios and providing adequate levels of toxicity in standard tests. As demonstrated in this study, metal speciation is important in determining the toxicity of metals to soil invertebrates as well as their solubility and mobility in soil. As such, in addition to the dosing methods tested in this study other metal forms, using other aqueous salts (i.e. chlorides) or different insoluble metal forms (Sulphides and sub-sulphides) with lower mobility should be considered in further testing.

Annex 1

Table 1A -. Nominal and measured metal concentrations for each dose method, mixture, and soil tested, corrected for background metal concentrations. Negative concentrations were adjusted to zero. N/A concentrations indicate contaminated/lost sample not used in analysis.

Dose Type	Soil	Mixture	Lead (mg/kg)	Copper (mg/kg)	Nickel (mg/kg)	Zinc (mg/kg)	Cobalt (mg/kg)
Nominal Dose	All	CSQG	536	483	345	1532	306
Nominal Dose	All	Flin_Flon	202	619	9	2223	9
Nominal Dose	All	Sudbury	2314	161	297	1196	153
Nominal Dose	All	Port_Colborne	56	381	1513	163	28
Nominal Dose	All	Clay_Peat	612	662	396	1199	354
Nitrate	KUBC	CSQG	414	393	286	1331	236
Nitrate	3.22	CSQG	554	437	364	1505	333
Nitrate	WTRS	CSQG	418	396	228	3197	263
Nitrate	Elora	CSQG	519	537	339	1477	274
Leached Nitrate	KUBC	CSQG	502	455	261	1124	200
Leached Nitrate	3.22	CSQG	452	167	27	51	13
Leached Nitrate	WTRS	CSQG	553	469	158	319	100
Leached Nitrate	Elora	CSQG	533	497	305	1399	235
Oxide	KUBC	CSQG	452	480	326	1151	318
Oxide	3.22	CSQG	525	224	312	806	296
Oxide	WTRS	CSQG	767	331	331	1213	332
Oxide	Elora	CSQG	930	421	378	1296	346
Annealed	KUBC	CSQG	546	397	357	1354	314
Annealed	S3_22	CSQG	625	428	425	1534	371
Annealed	WTRS	CSQG	642	474	441	1628	384
Annealed	Elora	CSQG	841	602	517	2047	458
Nitrate	KUBC	Flin_Flon	138	549	7	1959	7
Nitrate	3.22	Flin_Flon	178	568	9	1851	7
Nitrate	WTRS	Flin_Flon	209	632	11	3278	8
Nitrate	Elora	Flin_Flon	178	580	0	1986	7
Leached Nitrate	KUBC	Flin_Flon	177	553	5	1482	4
Leached Nitrate	3.22	Flin_Flon	202	318	1	166	0
Leached Nitrate	WTRS	Flin_Flon	214	563	4	579	4
Leached Nitrate	Elora	Flin_Flon	202	624	0	1934	7
Oxide	KUBC	Flin_Flon	242	648	12	2055	8
Oxide	3.22	Flin_Flon	140	430	11	2793	8
Oxide	WTRS	Flin_Flon	N/A	N/A	N/A	N/A	N/A
Oxide	Elora	Flin_Flon	400	559	12	3334	10
Annealed	KUBC	Flin_Flon	166	455	12	1811	12
Annealed	S3_22	Flin_Flon	198	470	9	1901	8
Annealed	WTRS	Flin_Flon	139	384	7	1520	7

Annealed	Elora	Flin_Flon	171	443	4	1768	10
Nitrate	KUBC	Sudbury	2537	152	284	1033	137
Nitrate	3.22	Sudbury	2740	118	315	1180	148
Nitrate	WTRS	Sudbury	2369	163	287	1123	145
Nitrate	Elora	Sudbury	1748	145	237	920	108
Leached Nitrate	KUBC	Sudbury	1897	137	217	805	93
Leached Nitrate	3.22	Sudbury	1523	22	21	0	5
Leached Nitrate	WTRS	Sudbury	2247	126	129	164	45
Leached Nitrate	Elora	Sudbury	1	0	2	4	1
Oxide	KUBC	Sudbury	2131	121	288	1175	156
Oxide	3.22	Sudbury	2693	200	285	921	165
Oxide	WTRS	Sudbury	1177	0	129	0	68
Oxide	Elora	Sudbury	5343	171	361	1456	180
Annealed	KUBC	Sudbury	2251	99	274	863	148
Annealed	S3_22	Sudbury	2612	108	305	1005	167
Annealed	WTRS	Sudbury	2509	124	298	1081	164
Annealed	Elora	Sudbury	2143	116	235	962	135
Nitrate	KUBC	Port_Colborne	38	405	1391	153	24
Nitrate	3.22	Port_Colborne	57	394	1720	221	29
Nitrate	WTRS	Port_Colborne	84	396	1615	204	26
Nitrate	Elora	Port_Colborne	38	334	1251	147	24
Leached Nitrate	KUBC	Port_Colborne	43	343	1163	124	18
Leached Nitrate	3.22	Port_Colborne	58	172	159	0	1
Leached Nitrate	WTRS	Port_Colborne	54	363	755	0	9
Leached Nitrate	Elora	Port_Colborne	53	450	1480	183	26
Oxide	KUBC	Port_Colborne	65	702	1718	176	29
Oxide	3.22	Port_Colborne	11	214	1510	66	24
Oxide	WTRS	Port_Colborne	53	302	1495	112	24
Oxide	Elora	Port_Colborne	19	241	1253	142	24
Annealed	KUBC	Port_Colborne	15	307	1575	153	31
Annealed	S3_22	Port_Colborne	1	283	1630	71	30
Annealed	WTRS	Port_Colborne	27	301	1637	152	34
Annealed	Elora	Port_Colborne	11	213	987	182	20
Nitrate	KUBC	Clay_Peat	564	620	356	1031	292
Nitrate	3.22	Clay_Peat	658	668	440	1292	380
Nitrate	WTRS	Clay_Peat	578	706	405	1193	362
Nitrate	Elora	Clay_Peat	545	655	367	1156	324
Leached Nitrate	KUBC	Clay_Peat	631	670	313	888	236
Leached Nitrate	3.22	Clay_Peat	511	204	29	0	15
Leached Nitrate	WTRS	Clay_Peat	574	560	152	111	98
Leached Nitrate	Elora	Clay_Peat	604	651	324	1035	260
Oxide	KUBC	Clay_Peat	N/A	N/A	N/A	N/A	N/A
Oxide	3.22	Clay_Peat	776	479	399	685	371
Oxide	WTRS	Clay_Peat	682	545	374	895	362
Oxide	Elora	Clay_Peat	625	748	470	1060	389
Annealed	KUBC	Clay_Peat	468	622	403	1127	287
Annealed	S3_22	Clay_Peat	505	678	452	1247	321
Annealed	WTRS	Clay_Peat	454	587	376	1101	269
Annealed	Elora	Clay_Peat	384	511	309	972	227

Chapter 3 - The effects of complex metal oxide mixtures on three soil invertebrates with contrasting biological traits

This chapter is based on the published paper

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Abstract

For regulatory purposes, the concentration addition model is the default first tier for assessing joint-action toxicity of metal mixtures. Although many researchers have evaluated binary and ternary mixtures, fewer have investigated joint-action toxicity in more complex mixtures, where deviations from additivity are more likely due to the greater number of potential interactions. In this study, we tested fixed ratios of five metals (lead, copper, nickel, zinc, cobalt) as metal oxide mixtures on three soil invertebrate species *(Enchytraeus crypticus, Folsomia candida, Oppia nitens*) at different dose effect levels (EC10-EC90) in an acid sandy forest and a loamy soil. Total metal concentrations for mixture ratios in soil did not explain or correlate with species responses. For *F. candida,* toxicity was linked to metal solubility, while for *O. nitens* and *E. crypticus,* toxicity did not correlate with total or extractable metals. In *O. nitens* and *E. crypticus,* however, soil ingestion could be an important route of exposure. Analysing the joint effect of metal mixtures, *F. candida* response was globally additive, while *E. crypticus* and *O. nitens* presented synergistic effects at low-dose effect levels. Estimations at the EC50 level underestimated the deviations from additivity which were larger at higher and especially lower effect levels. Testing across different effect concentrations (EC10-EC90) was an important tool allowing the identification of these larger deviations from additivity outside the EC50 threshold. Considering most protection thresholds are set below the EC50 level, and it was in this low effect range where the highest synergisms were observed, risk assessment schemes should test additivity at the target protection level using representative test organisms.

Introduction

Metals occur naturally in soil from the weathering of parent material. However, they can also accumulate at very high concentrations in soil as a result of anthropogenic activities, such as from organic wastes and fertilizers, coal combustion, and metal mining [3,4]. Metals are severe and persistent chemicals in the environment and are especially concerning because they do not degrade like organic contaminants [3,96]. Although most metal-contaminated sites consist of a complex mixture of metals [16,214], most soil ecotoxicology studies [18,33,34,43] and consequently environmental thresholds used in risk assessment are based on single metals [8,11]. Currently, the concentration addition (CA) model is thought to be a reasonably conservative model and is the default approach for modeling the joint action of chemicals for regulatory purposes [55,118]. However, toxicity responses to metal mixtures often deviate from the CA model [99,118].

In metal mixture ecotoxicological research [118], and in soil ecotoxicology research in particular [52,55,64,123,124,130,131,133,135], most studies focus on binary or ternary mixtures. In soils, binary and ternary mixtures have either additive [52,64,124,130], antagonistic [52,55,64,124,130,133,135] or at high doses (>EC50) synergistic effects [123]. Even within the same study, different patterns of responses might be observed depending on the endpoint measured[52] (e.g., survival, reproduction), measures of metal data (e.g., total, internal body concentrations) [64,130], and the composition of the mixtures [124]. As the complexity of the mixtures increases, it is reasonable to expect more interactions and greater deviations from additivity. Mixture toxicity can be affected by interactions between metals (i) within the soil, (ii) in the process of uptake (i.e., toxicokinetics), and (iii) at the site of toxic action within the organism (i.e., toxicodynamics) [96,121]. The role of the biological compartment in metal interactions (toxicodynamics and toxicokinetics) can lead to differences in the response of organisms to metal mixtures. However, typically only a single species (i.e., *Enchytraeus crypticus* or *Folsomia candida*) is used in mixture studies [52,55,64,123,124,130,131,133]. The use of multiple species, with different traits should be encouraged to better understand and acknowledge the importance of the biological compartment in metal mixture interactions.

In soil, metal availability is determined by total metal concentrations and modified by local soil characteristics [94], while metal partitioning is influenced by chemical reactions within the soil, such as precipitation/dissolution, adsorption/desorption, and aqueous complexation [95]. These

reactions, in turn, depend on metal speciation, soil properties and chemistry [3,213]. In mixtures, the different metals compete and interact, altering metal partitioning causing differences in metal availability. The most common soil properties that modulate the chemical reactions affecting metals are clay content, organic matter, Fe and Mn oxides, cation exchange capacity, calcium carbonate content, redox potential, and most importantly, pH or soil acidity [94,95]. In fact, pH can be considered the master variable for metal availability [99] and has been shown to be a good predictor of availability across a range of soils with differing properties [100].

Metal uptake is organism-specific and correlates with species traits that influence their routes of exposure [94]. In general, routes of exposure for metals in animals include ingestion (soil, pore-water, and contaminated food), dermal adsorption, and respiration [99]. These routes are affected by organism traits such as feeding behaviour and exterior barriers such as the level of sclerotization [115]. Free-metal ions are thought to be the most bioavailable form of metal that can be taken up and that can transverse biological membranes. Consequently, availability is at times considered a consequence of soil pore-water and water chemistry [98]. For soil invertebrates, some studies found that metal toxicity correlates with solubility [51,77] but others found no such correlations [100,102]. As highlighted by Peijnenburg and Jager [98], the relationship between metal chemical properties and bioavailability are not sufficiently understood to predict toxicity, and the correlation between free metal ion activity and uptake may not be as close as initially predicted [98]. The process of uptake may in fact be much more complex and not necessarily mediated by soil pore-water. For instance, *Oppia nitens* exposure to metal oxides was correlated to total rather than extractable metal concentrations and exposure attributed to soil ingestion rather than pore-water [89,111]. In addition to uptake and toxicokinetics, a recent study has demonstrated that soil properties dictate habitat quality and affect the toxicodynamic responses, regulating the energy available for organisms to endure contamination [89].

The objective of this study was to understand the effects of complex five metal oxide mixtures (lead, copper, nickel, zinc, and cobalt) on soil invertebrates with different biological traits. Mixture effects were tested using two natural soils, with contrasting properties known to affect metal availability and toxicity, an acid sandy forest soil (pH 3.4 and CEC 8) and a Loamy soil (pH 5.6 and CEC 28). For the biological compartment three different species were selected, *E. crypticus*, *F. candida* and *O. nitens* with different external barriers and routes of exposure. *E. crypticus* are soft-bodied annelids [109] and exposure to metals, similarly to earthworms, is expected to occur dermally and through soil ingestion [113]. *F. candida* has a protective external

cuticle [110] and exposure is mostly linked to, contaminated soil, porewater and to a lesser extent food [42]. *O. nitens*, have the most developed external barriers with a heavily sclerotized body [111,198] and exposure is expected to occur through the ingestion of contaminated soil or organic matter [89,215] or in juveniles where external barriers are not as developed [198].For the five element metal mixtures, neither a full factorial design [124] nor a central composite design [55] can be used. Therefore, we used a fixed-ratio ray design because it can test for deviations from additivity in ratios of particular interest [125]. Ten fixed mixture ratios were used and deviations from additivity were tested at different dose effect levels ranging from EC10 to EC90. In addition to mixtures, each individual metal was also tested and used to determine toxic units (TU) for each species.

Methods

Soil properties

Two soils (an acid sandy forest soil and a loamy soil) with different soil properties—pH, cation-exchange capacity (CEC), and clay content—were used as test substrate and medium of exposure for the tested metals, both as single elements and complex mixtures. The acid sandy forest soil was a reference soil collected close to a mining area in Flin Flon, Manitoba, and the loamy soil was a 1:1 mixture with soils from an agricultural research field in Saskatchewan and from Iqaluit, Nunavut. Soils were collected from a maximum depth of 30 cm, air dried, and sieved to 2mm. Soil pH was measured in 0.01M $CaCl_2$. CEC was established using the methylene blue method [216], texture was determined by the pipette method [217], organic carbon was determined using a LECO-C632 analyzer [218], and water holding capacity was determined as described in annex C of ISO11268-2 [219]. Soil properties are listed in Table 1.

Table 1. Soil properties and background metal composition measured using X-ray fluorescence

Soil	pH-$CaCl_2$	CEC (meq/100 g)	Organic C (g/kg)	Clay Content (g/kg)	Water Holding Capacity (ml/g)
Acid sandy forest soil	3.4	8	17	45	0.3
Loamy soil	5.6	28	12	24	0.48
Background metal concentration (mg/kg)	Lead	Copper	Nickel	Zinc	Cobalt
Acid sandy forest soil	166	92	0	480	0
Loamy soil	0	0	15	97	0

Metal mixture ratios

A total of 10 fixed metal mixture ratios of lead, copper, nickel, zinc and cobalt were established, each with different environmental and regulatory relevance. Table 2 presents the percent composition in weight and moles of each element for each mixture ratio ray. Five ratios were selected based on regulatory thresholds: One ratio was based on the Canadian Soil Quality Guideline (CSQG) for an agricultural soil, and the remaining four regulatory ratios (Ag Res Loamy, Acid Sand Ara, Clay Peat, and Loam Sand Ind) were the average values for similar ratios, established using a principal component analysis, for different soil PNEC threshold values in the PNEC soil calculator [8] and CSQG under different soil uses. Three environmental ratios were selected to represent the ratios of the five elements as observed in three contaminated sites in Canada (Sudbury, Port Colborne, and Flin Flon). The two final ratios were an ecotoxicological ratio based on *F. candida* literature EC50 values for each metal (lead, copper, nickel, zinc and cobalt) and an equal ratio between all elements.

Table 2. Percent composition of ratios by weight in mg/kg (W) and moles mg/kg (M) of lead, copper, nickel, zinc*, and cobalt in the experimental metal mixture ratio rays.

Ratio Ray		Lead		Copper		Nickel		Zinc		Cobalt	
		(% - M)	(% - W)	(% - M)	(% - W)	(% - M)	(% - W)	(% - M)	(% - W)	(% - M)	(% - W)
CSQG		5.8	16.7	17	15.1	13.1	10.8	52.4	47.8	11.6	9.6
Ag Res Loamy	Regulatory	6.1	17.5	20.9	18.4	14.4	11.8	46.4	42.3	12.2	10
Acid Sand Ara	Regulatory	21.2	46.9	23.2	15.7	10.3	6.4	37.3	26	7.9	5
Clay Peat	Regulatory	6.6	19	23.4	20.6	15.2	12.3	41.3	37.2	13.5	11
Loam Sand Ind	Regulatory	7.8	21.8	18.2	15.5	13.8	10.9	48	42.1	12.2	9.7
Flin Flon	Environmental	2.2	6.6	21.6	20.2	0.3	0.3	75.5	72.6	0.3	0.3
Sudbury	Environmental	28.2	56.2	6.4	3.9	12.8	7.2	46.1	29	6.5	3.7
Port Colborne	Environmental	0.8	2.6	17.1	17.8	73.6	70.7	7.1	7.6	1.3	1.3
EC50		12.4	32.4	18.3	14.7	11.9	8.8	17.8	14.7	39.6	29.4
Equal Ratio		6.9	20	22.5	20	24.4	20	21.9	20	24.3	20

* Regulatory ratios were established in 2016 and do not reflect the changes to the CSQG guideline values for zinc that were revised in 2018.

Dosing and Ecotoxicological testing

Dosing was performed using metal oxides of lead (Sigma-Aldrich, PbO, ACS reagent ≥ 99.0%), copper (Sigma-Aldrich, CuO, powder <10 μm, 98%), nickel (Sigma-Aldrich, NiO, 325 mesh, 99%), zinc (Sigma-Aldrich, ZnO, ACS Reagent ≥99.0%,) and cobalt (Sigma-Aldrich, Co_3O_4powder <10 μm). Before the experiment, oxides were placed for 24h in a desiccator containing concentrated nitric acid in order to remove any carbonates.

Two weeks before dosing, the soil moisture was adjusted to 50% water holding capacity (WHC) to allow the re-establishment of the soil microbiome. Soil was dosed with each metal oxide as a single element 11 times (once each at 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, and 16 toxic units), and each fixed-ratio mixture ray was dosed 9 times (once each at 0.25, 0.5, 1, 1.5, 2, 4, 8, 12, 16 toxic units). To compare single metal and mixture dosing regimes, doses were established in toxic units based on *F. candida* literature toxicity data.

Metal oxides were added to the soil and vigorously mixed to obtain the desired single metal or mixture dose. After allowing the samples to equilibrate for two weeks, reproduction tests were initiated: soil moisture was readjusted to 50% WHC, the soil was transferred to cylindrical glass vials (28 mm diameter by 80 mm height), and test invertebrates were added to their respective test units. The remaining soil from each treatment was collected, air dried, and stored for chemical analysis.

Reproduction test units containing soil and invertebrates were incubated for four weeks (28 days) in a controlled temperature chamber (20 ± 2^{o}C) in a 16:8 h light/dark cycle. The procedures and guidelines followed for each invertebrate reproduction test are listed in Table 3. At the end of the test (i.e., 4-week exposure duration), the total number of surviving adults and juveniles of each species was determined. *O. nitens* and *F. candida* were extracted from the soil using a modified McFayden apparatus for 48 h that had been previously tested for extraction efficiency (>90%) [220] and counted using a binocular microscope. *E. crypticus* organisms were fixed in a 70% ethanol solution and stained by adding a few drops of a 1% bengal red ethanol solution. After staining, samples were wet-sieved (103 μm mesh) to remove fine soil particles, and enchytraeids were counted using a binocular microscope.

Table 3. Procedures and guidelines adopted for soil invertebrate reproduction tests

	Enchytraeus crypticus	*Folsomia candida*	*Oppia nitens*
Guideline	ISO 16387 [204]	ISO11267 [205]	Princz et al. [198]
Number of organisms per test unit	10	10	15
Food source during exposure	Rolled oats	Dry yeast	Dry yeast
Food supply (test days)	Day 1 and 14	Day 1,7,14, and 21	Day 1,7,14, and 21
Aeration and moisture adjustment (test days)	Day 7, 14, and 21	Day 7, 14, and 21	Day 7, 14, and 21
Soil per test unit (g)	20	30	30

Over two weeks, experiments were conducted with randomized treatments containing controls, single-metal treatments (5 elements, 11 doses), mixture ray treatments (10 mixtures rays, 9 doses), and both soils. Once randomized, all three species tests were initiated at the same time for the particular set of treatments for each day (average of 20 treatments per day). Because of the large number of test treatments (292) and because three species were used (total individual test units: 870), replication was only performed on a randomized 10% subset of treatments, with five replicates (total test units with replication: 1, 236).

Chemical analysis

X-Ray Fluorescence (XRF) [221] was used to determine total metal concentrations for all test treatments, including control. Four grams of air-dried soil from one treatment was mixed with 0.8 g of Chemplex SpectroBlend 44 µm adhesive powder. The mixture was then transferred into Chemplex pellet cups, covered with an adhesive polypropylene thin-film, and vacuum-pressed to pellet die sets. Pellet sets were then mounted on a hydraulic press, and a force of 10,000 psi was applied for five minutes to create soil disks. Soil disks were analysed in a Thermofisher ARL Optim-X X-ray analyzer. In data analysis, metal concentrations in dosed soils were estimated after removing the background metal concentrations determined in the non-dosed controls.

Extractable metal concentrations were determined using 0.01 M $CaCl_2$ extraction [222] on a subset of test treatments (31%) to evaluate general metal solubility and availability. For the selected treatments, 2.5 g of soil were placed with 25 ml of 0.01 M $CaCl_2$ in 50ml falcon tubes and shaken for 3 h at 15 rpm. Samples were then centrifuged at 5000 rpm for 10 minutes and filtered through a 0.45 µm syringe filter. The extractable metal concentrations for each metal were measured in an Agilent microwave plasma-atomic emission spectrometer (MP-AES).

Data analysis

No data were collected for *E. crypticus* in the acid sandy forest soil because the organism either could not reproduce or presented very low reproduction, even in the control. In the loamy soil, *F. candida* reproduction was not affected or had very low effects for either single metals or mixtures, which prevented analysis of dose response. For *O. nitens*, effects in the loamy soil were generally low (below the EC50 level) but still allowed analysis of dose response curves; therefore data on *O. nitens* were collected for both test soils.

Single metal toxicity

The effects of each metal (lead, copper, nickel, zinc, and cobalt) on the reproduction of each invertebrate were analysed by creating dose-response curves. Different dose response models (i.e., Weibull, logistic, log-logistic) were selected based on best model fit using Akaike's information criterion and the estimated residual standard error. Models were used to estimate reproductive effect concentrations (ECx; EC10 to EC90), for each metal and invertebrate species. This analysis was conducted using the DRC package in R [223]. See Annex 2 Table 1A for a full list of ECx values.

Mixture analysis

For each fixed mixture ratio-ray, mixture toxic units were established using the ECx for single metals and total metal concentrations. Unlike the traditional approach where toxic units are established based on EC50 data, we calculated toxic units at different effect levels ranging from EC10 to EC90 for each individual species (Equation 1). This approach enabled us to calculate deviations from additivity at different dose/effect levels.

$$\sum TU_{ECx} = \sum_{i=1}^{n} \frac{Ci}{ECxi} = 1\ additive, > 1\ Antagonistic, 1 < Synergistic \quad \textbf{Equation 1}$$

The toxic unit (TU) at an ECx is the sum of the total concentrations of the individual metal (Ci) in the mixture divided by their respective ECx (ECxi). When a particular metal is non-toxic as a single, an arbitrary high value (999999) was selected to calculate mixture toxic units at all effect levels. This was performed to acknowledge the presence of some metals in the mixture which negligibly contribute to toxicity.

The dose-response curves for mixture effects on reproduction were established from the calculated mixture toxic units for each effect level and were analysed using the same procedure described for single metals for each species. Figure 1A in Annex 2 shows the dose response curves for mixture toxic units calculated at different dose/effect levels. Significant deviations from additivity or 1 TU were tested using a single sample *t*-test at $\alpha = 0.05$.

The correlation between total metal mixture ratio and specie response to mixtures (TUs at different ECx) was calculated by converting datasets to distance matrix (using Euclidean distances) and then using a mantel test in R with the package Vegan [251].

Four factors (soil, mixtures, species and dose/effect levels) and their interactions were tested in an analysis of variance (ANOVA). The ANOVA analysis was performed twice, once for all three species in each soil separately and because only *O. nitens* reproduced in both soils, an additional analysis was conducted for *O. nitens* with both soils combined and including soil as a factor. In this analysis, data was log transformed to fulfill assumptions of normality and homoscedasticity, which were determined using analysis of residuals and Q-Q plots.

All statistical analysis was performed in R version 3.5.0 [209], and figures were constructed using the package ggplot2 [224] .

Results

Globally, *E. crypticus* and *O. nitens* responded synergistically to metal mixtures at low dose/effect levels, whereas *F. candida* responded additively ($p > 0.05$) at all dose/effect levels (Figure 1). The largest deviations from 1 toxic unit (TU) for *F. candida* were observed at both low (EC10 = 1.5 TU SE 0.4) and high dose/effect levels (EC90 = 1.5 TU, SE 0.3). However, responses were never high enough or had too large errors to promote significant antagonism. For *E. crypticus*, metal mixtures were globally synergistic at low dose/effect levels (EC10 = 0.47 TU, SE = 0.1; EC20 = 0.7 TU, SE = 0.08; and EC30 = 0.8 TU, SE = 0.07); additive between EC40 (0.9 TU, SE = 0.07), EC50 (1.0 TU, SE = 0.06), and EC60 (1.1 TU, SE = 0.07); and antagonistic at high dose/effect levels (EC70 = 1.2 TU, SE = 0.08; EC80 =1.4 TU, SE = 0.11; EC90 = 1.5 TU, SE = 0.16).

In both soils, *O. nitens* shifted from synergism at low dose/effect to additivity at high dose/effect levels, but never reached significant antagonism in either soil. For *O. nitens*, the differences in deviations from additivity in the two soils were mostly due to higher variability in the acid sandy forest soil (average relative standard error = 1.4) compared to the loamy soil (average relative standard error = 0.5). For example, in the acid sandy forest soil, values at the EC30 and EC40 (EC30 TU = 0.36, SE = 0.36; and EC40 TU = 0.45, SE = 0.31) were non-significantly different from 1, while larger TU responses at the EC50 and EC60 levels (EC50 = 0.50 TU, SE = 0.25; and EC60 = 0.57 TU, SE = 0.21) were significantly synergistic. Furthermore, the responses between soils was dependent on the soil where this variability occurred: for the acid sandy forest soil, where toxicity was higher, the error was higher at lower dose/effect levels (average relative error EC10-EC30 acid sandy forest soil = 2.31, loamy soil = 0.54), while for the loamy soil where toxicity was lower, the error was higher at the highest dose/effect levels (average relative error EC70-EC90 acid sandy forest soil = 0.35, loamy soil = 0.68.

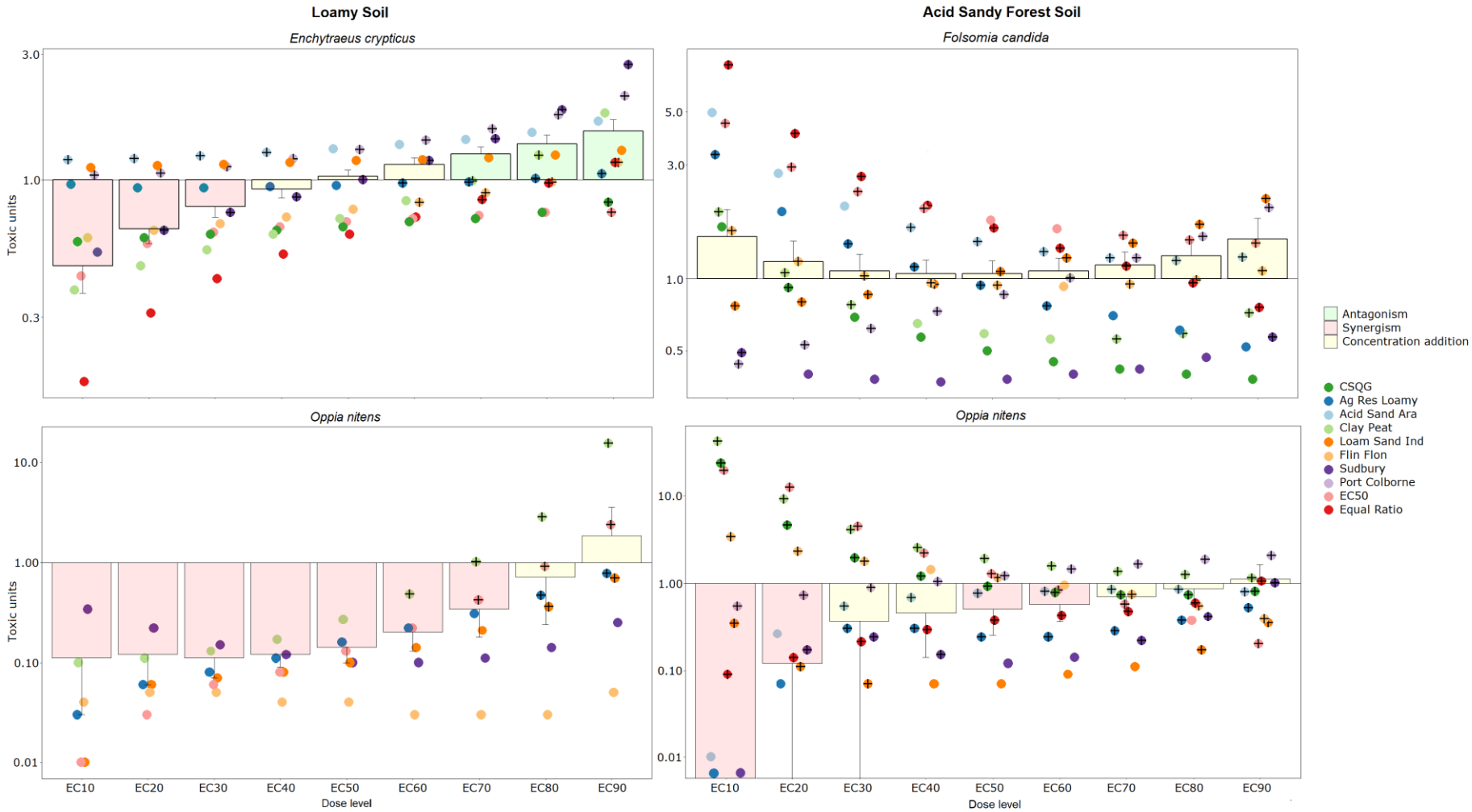

Figure 1 – Global (bars) and individual (points) measured mixture toxic unit (TU) at different dose/effect levels for three test species (Enchytraeus crypticus, Folsomia candida, and Oppia nitens) in the two test soils (loamy and acid sandy forest soils). Bar fills indicate the significance or lack of deviation from additivity (TU = 1). Red bars indicate significant global synergism (TU < 1, $p<0.05$), green bars indicate antagonism (TU > 1, $p<0.05$), and yellow bars indicate concentration addition (TU = 1, $p>0.05$). Full points are significant antagonism or synergism, marked points (black cross) represent concentration addition (value not significantly different from 1, $p<0.05$).

Species responses towards mixtures were significantly different among individual mixtures in both test soils (Table 4, $p<0.001$). Not all individual mixtures presented the same pattern of response between species within the same soil. For example, in the loamy soil, the Flin Flon and Sudbury ratios had different patterns of response between species, and the patterns were the same for Ag/Res/Loamy and equal ratio in the acid sandy forest soil (Figure 1 and Figure 2 - row scaling). In addition to mixtures, species responses differed in magnitude across dose levels, but only in the loamy soil (Table 4, $p<0.01$), where *O. nitens* had higher intensity of synergism than *E. crypticus* at low doses. This synergism also lasted longer in *O. nitens* (EC70) compared to *E. crypticus* (EC40). Although there were differences between dose effect levels ($p=0.02$) in the acid sandy forest soil, the magnitude of response was similar between test species, and the interaction between dose effect levels and species was not significant ($p=1$).

Comparing *O. nitens* response between soils, there was a significant interaction between soil and mixtures (Table 4, $p<0.01$). There was also a difference in magnitude of response across dose levels between the two soils, with a significant interaction between dose/effect level and soil (Table 4, $p<0.01$). As explained above, this could be the result of the higher variability observed at low doses for the acid sandy forest soil (where toxicity was higher) and at high doses in the loamy soil (where toxicity was lower).

It is important that the differences and the contribution of factors presented be interpreted carefully, since the analysis of variance approach, for TU values at each dose/effect level does not include their associated error and consequently may overestimate the differences between responses.

Table 4 – Statistical analysis (ANOVA) of relationships between dose/effect levels, species, and mixtures on the toxic unit responses within the loamy soil and the acid sandy forest soil, and the effect of dose/effect levels, mixtures, and soil on *O. nitens* in both soils.

Factor	Df	Sum Squares	Mean Squares	F Value	Pr(>F)
Loamy Soil					
Dose/Effect level	8	5.89	0.74	8.97	<0.001
Species	1	21.75	21.75	264.68	<0.001
Mixture	9	3.89	0.43	5.26	<0.001
Dose/Effect level:Species	8	2.48	0.31	3.77	0.002
Dose/Effect level:Mixture	72	2.97	0.04	0.5	0.994
Mixture:Species	5	3.17	0.63	7.72	<0.001
Acid Sandy Forest Soil					
Dose/Effect level	8	2.14	0.27	2.42	0.02
Species	1	1.69	1.69	15.27	<0.001
Mixture	9	9.25	1.03	9.31	<0.001
Dose/Effect level:Species	8	0.05	0.01	0.05	1
Dose/Effect level:Mixture	72	8.6	0.12	1.08	0.372
Mixture:Species	9	10.97	1.22	11.04	<0.001
O. nitens in both Soils					
Dose/Effect level	8	2.21	0.28	1.62	0.153
Soil	1	14.79	14.79	86.69	<0.001
Mixture	9	15.24	1.69	9.93	<0.001
Dose/Effect level:Soil	8	6.17	0.77	4.52	0.001
Dose/Effect level:Mixture	72	14.06	0.2	1.15	0.329
Mixture:Soil	5	6.01	1.2	7.04	<0.001

A total metal mixture composition dendrogram (Figure 2) clustering mixtures in terms of their compositional similarity did not group similar species responses. In the case of the loamy soil, clustering by mixture composition did not group similar magnitude of response for *E. crypticus* (Figure 2, No scaling) or pattern of response for *O. nitens* (Figure 2, Row scaling). In the acid sandy forest soil, mixture composition clustering did not group a similar pattern of response (Figure 2, Row scaling) or magnitude of response (Figure 2, No scaling) for either test species. Mixture total metal composition clustering also did not group similar responses when considering significant antagonisms or synergisms (Figure 2, Syn/Ant/CA panels).

The observations in Figure 2, linking mixture clustering and species responses were supported by a Mantel test that determined no correlation between metal mixture composition and species responses (Annex 2, table 5A). No significant correlation was found between species responses

and total metal composition (loamy soil: *E. crypticus* p=0.21, *O. nitens* p=0.44l; acid sandy forest soil: *F. candida* p=0.14, *O. nitens* p=0.87), nominal metal composition (*E. crypticus* p=0.52 , *F. candida* p=0.34 , *O. nitens* acid sandy forest soil p=0.86, loamy soil p=0.77) or available metal mixture composition (loamy soil: *E. crypticus* p=0.21, acid sandy forest soil: *F. candida* p=0.31, *O. nitens* p=0.53). However, the Mantel test showed that total metal mixture composition was correlated between the two soils (p=0.01), confirming mixture dosing regimens were similar and *O. nitens* response was correlated between both soils (p=0.03). No other correlation between species responses was observed either between or within the same soil (p>0.05).

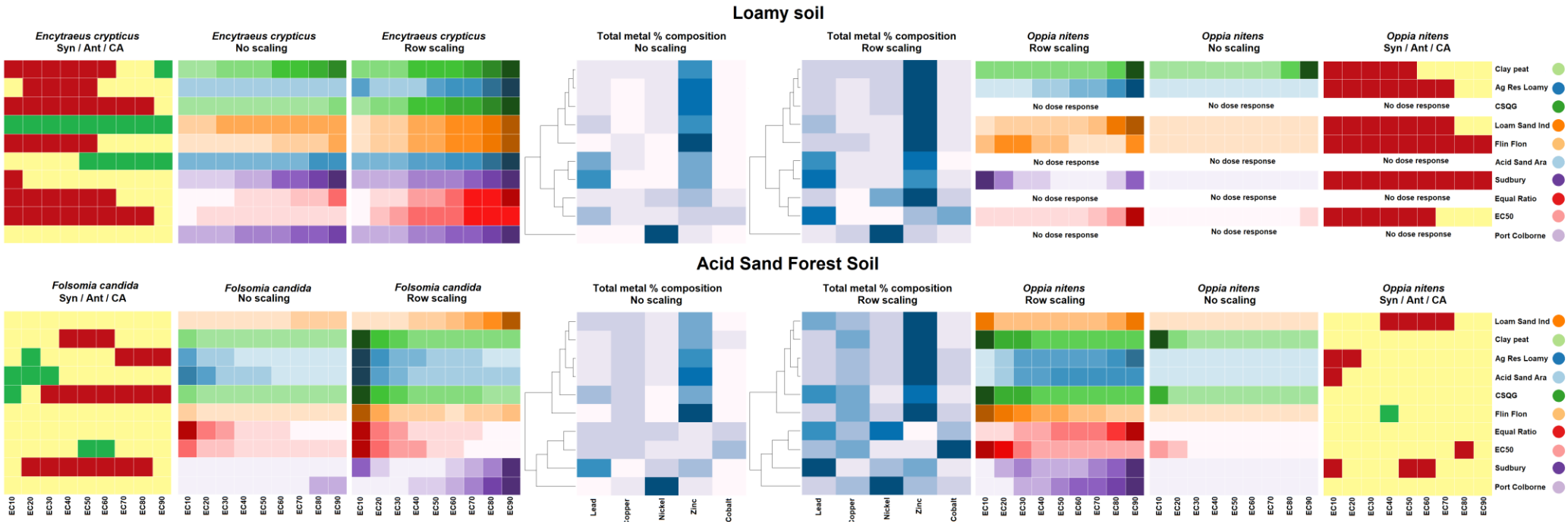

Figure 2. Species response matrixes (with, without scaling by rows and significance of deviation from additivity) ordered by percent total metal mixture composition dendrogram (row scaling represents values scaled for the different doses, while no scaling represents no scaling across mixtures). Color shade correlates with estimate value: darker shade represents larger values, and lighter shade represents smaller values. For the panels reporting significant deviations from additivity (Syn/Ant/CA), yellow represents concentration addition (CA), red represents synergism (Syn), and green represents antagonism (Ant).

The response of *F. candida* to metal mixtures seems to be linked to the more soluble metal fraction. For this species, no toxicity was observed in the loamy soil where extractable metal concentrations were very low (Pb = 0.0003%, Cu = 0.003%, Ni = 0.003%, Co = 0.004%, zinc = 0.57% of total) compared to the acid sandy forest soil (Figure 3). While not correlated with extractable metal concentrations, the response of *F. candida* in the acid sandy forest soil was significantly correlated with percent composition when considering only the ratio of total copper and zinc within the mixtures (Mantel test p=0.038, Table 5A), which are the metals with highest $CaCl_2$ extractable concentrations (zinc = 2.90%, copper = 0.64% of total, Figure 3). For both *O. nitens* and *E. crypticus*, toxicity did not appear to be correlated with metal availability. In the loamy soil, where metal availability is low, mixture toxicity was observed for both species (although lower for *O. nitens*). Furthermore, zinc—the metal with highest extractable concentration (Figure 3)—produced no toxicity as a single metal to *O. nitens* in loamy soil (Annex 2, Tables 1-4).

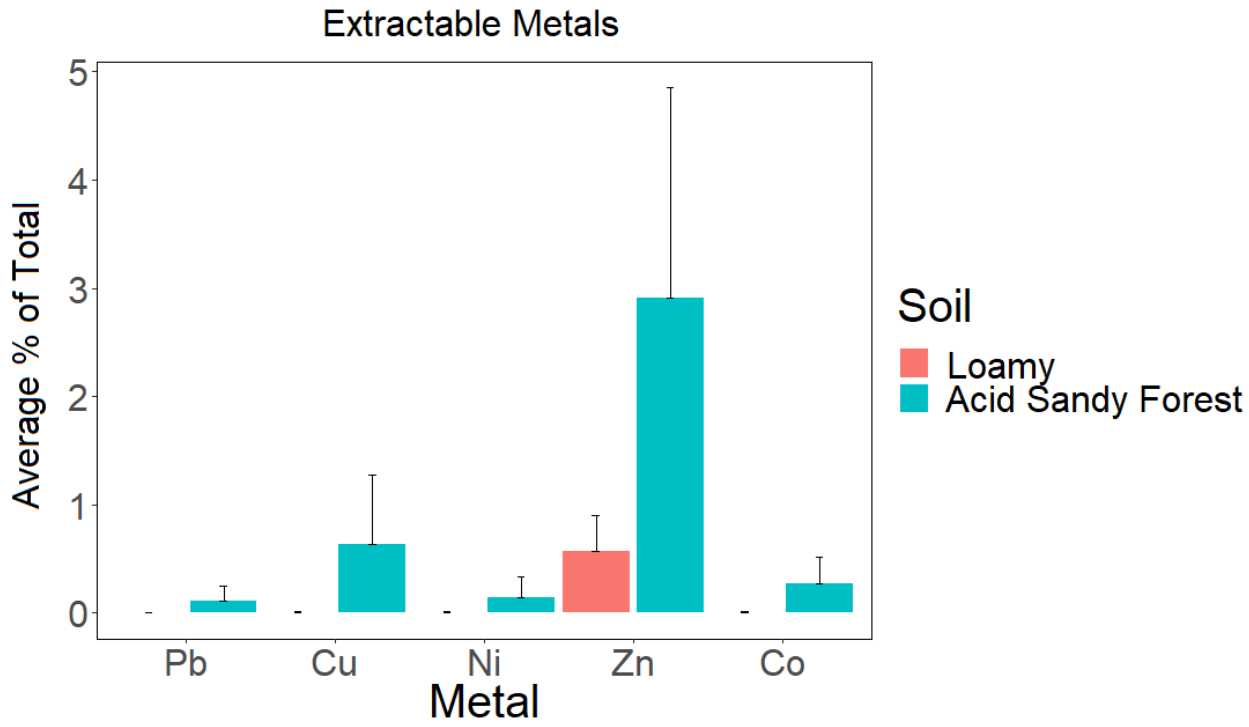

Figure 3. Average percent of total metals extracted by $CaCl_2$ for each metal (lead, copper, nickel, zinc, and cobalt) across all mixture ratios and dose/effect levels for each test soil (loamy and acid sandy forest). Error bars represent the standard deviation from the mean.

Discussion

In general, the results appear to contradict the funnel hypothesis [225] which predicts that as the components of a mixture increases the deviations from additivity decrease. Complex five element metal mixtures deviate from additivity (especially synergism) more than simpler mixtures. In our results, only *F. candida* did not deviate from concentration addition and its response was additive across dose/effect levels. In previous studies, for simpler mixtures, *F. candida* reproduction responses were additive for Cd/Zn [52] or antagonistic for Cd/Pb [31].

In our study *E. crypticus* demonstrated synergism at low dose/effect levels (<EC40) and antagonism at high dose/effect levels (>EC60). For simpler mixtures, enchytraeids EC50 reproduction was additive for Zn/Cd [64] and antagonistic for Zn/Cd and Zn/Cu [129] at the EC50 level, and when considering surface response models for binary mixtures of Zn/Cd/Cu/Pb [55], mixtures were antagonistic. Although researchers have studied Ni and Co mixtures on *E. crypticus*, they have only measured free ion activity where antagonism had been detected or body concentrations where mixtures were additive [130]. For *O. nitens*, there is currently no other research on metal mixture effects. In our study, *O. nitens* had a surprisingly intermediate sensitivity (more sensitive than *F. candida* and less than *E. crypticus*) and an intermediate response to mixtures where synergism occurred in low dose/effect levels (like *E. crypticus*) and additivity occurred at higher dose/effect levels (like *F. candida*).

Estimating mixture additivity at 50 % effect levels underestimates the synergistic potential of metal mixtures. Directly comparing our results with the literature is complicated because previous investigators had considered only simpler mixtures, used metal salts for dosing while we used metal oxides, and many researchers included cadmium in their combinations [31,52,55,129], a metal that we did not include. Furthermore, most of the research on mixture toxicity in soil has considered only the effects at the EC50 level [52,64,129], which does not indicate whether deviations from additivity occur or how their intensity is affected at lower or higher dose/effect levels. Surface response models can and have been considered to evaluate ratio and dose effects for binary mixtures [55]. However, for more than binary mixtures, the difficulties involved with developing surface response models increase exponentially. In these more complex mixture scenarios, our approach to use toxic units (TUs) calculated from different ECx (EC10-90) values could be an important alternative to analysing interactions at different dose/effect levels. In fact, our results demonstrate that the greatest deviations from additivity are

observed at lower or higher dose/effect levels rather than at the EC50 level, where only *O. nitens* deviated from additivity (Figure 2).

Total metal mixture composition in soil does not explain species responses to metal mixtures. In this study, species responses to mixtures differed both globally and to individual mixture ratios but were never correlated with total metal mixture composition (Figure 2, Table 5SD). *F. candida* responses in the acid sandy forest soil were linked to total zinc and copper within mixtures (zinc and copper had the highest extractable concentrations), and no toxicity was observed in the loamy soil where metal availability was very low. While not directly correlated with $CaCl_2$ extractable metal concentrations, the correlation with the highest total Zn and Cu concentrations suggests that metal oxide toxicity is linked to metal solubility and potentially linked to availability in soil pore-water, which was previously reported as the main route of exposure for this species [51,110]. Although this correlation with total Zn and Cu suggests the importance of metal solubility, the lack of correlation with measured $CaCl_2$ concentrations could indicate that this extraction method is not a good direct predictor of availability and toxicity for *F. candida*. In this experiment, considering the range of mixtures and single elements tested there were limitations on the number of analysis to conduct and metal availability was determined only through $CaCl_2$ extractable concentrations and even then were only possible on a sub-set of soil samples. It is also possible that, metal solubility only explains a portion of the mixture effects and that exposure through soil also contributed to toxicity as demonstrated previously for copper [42].

For *E. crypticus* and *O. nitens*, toxic effects do not seem to be driven by metal solubility and soil pore-water. Toxicity was observed in both species (although lower in *O. nitens*) in the loamy soil, which had very low metal availability; as well, zinc, the metal with highest extractable concentrations, was non-toxic to *O. nitens* in the loamy soil. For these species, other routes of exposure that are not mediated by pore-water must be considered. Since clean food was provided in the experimental assays, soil ingestion should be considered an important route of exposure, with metal uptake occurring in the gut and affected by the gut chemistry. The importance of soil ingestion was recently demonstrated in *O. nitens* for cadmium oxides and exposure through pore-water was considered minimal [215]. For *E. crypticus*, there are no studies explicitly demonstrating the contribution of soil ingestion to contamination, however similarly to earthworm, *E. crypticus* actively ingest soil through burrowing. In earthworms, dermal uptake is the most important exposure route, but oral uptake is also a potentially important route, especially for complexed or non-soluble metals (like oxides) that are made more bioavailable

through digestion [113]. If soil ingestion is the main route of exposure, for both *O. nitens* and *E. crypticus* this could explain the similar mixture response at low doses despite their extreme differences in external barriers. The differences in response at high doses (*O. nitens* – CA and *E. crypticus* – antagonism) could be due to the rate of soil ingestion. *E. crypticus's* burrowing is expected to have larger rates of soil ingestion which might lead to a higher competition between metals for uptake in the gut at high doses promoting antagonism, which does not occur due to the lower soil ingestion rates of *O. nitens*.

The link between metal concentrations and exposure to metals and connection to species traits is still a critical data gap in soil ecotoxicity. While several methods have been developed to measure bioavailable fractions of metals there is little consistency and predictive power for toxicity across species and soils [100]. Under the current experimental design looking to test multiple fixed ratios it is not possible to clarify this knowledge gap. More research is needed into chemical methods for deriving bioavailability which link to organism routes of exposure and internal concentrations. For instance, uptake from ingestion of contaminated soil, in the gut mediated by gut chemistry is expected to be considerably different from pore-water concentrations or dermal absorption. Further research is also necessary into test species, how species traits mediate their exposure to metals. Particularly looking at the importance of soil ingestion for invertebrates, how much soil is ingested and the mechanisms of uptake for metals in the gut to understand the variation in invertebrate response to mixtures.

In addition to the biological component, soil significantly affected organisms reproduction not only mediating metal toxicity but also in the absence of metals due to its to its functioning as a habitat. Regarding metal toxicity, soil properties mediate the bioavailability of metals to soil invertebrates. In this experiment, *F. candida* toxicity was related to metal solubility and no toxic effects were observed in the loamy soil that has very low levels of $CaCl_2$ extractable metal concentrations. For *O. nitens*, the only species with results in both test soils, toxicity was also higher in the acid sandy forest soil than in the loamy soil. Comparing both soils, the acid sandy forest soil had lower pH (pH – 3.4), than the close to neutral loamy soil (pH – 5.6), a key variable for metal solubility in soils [100]. Metal solubility in soil is highest at both low and high values of pH and is lowest in intermediate neutral pH values [212]. In addition, the loamy soil had a much higher CEC compared to the acid sandy forest soil, which means that it had a higher availability of sorption surfaces for the binding of metals [37,56]. The eCEC (not measured), while not as strong a predictor of solubility compared to pH, is considered a better overall predictor of toxicity to soil organisms [100]. The lower pH and CEC could have not only

increased the availability of metals in pore-water for *F. candida* but also reduced surface complexation increasing metal release for uptake after ingestion in *O. nitens*. In this experiment while there were large differences in extractable concentrations between soils, the global analysis across mixtures, revealed that Zn and Cu were the most extractable metals. Previous research with metal salts, confirm the high mobility of Zn in soil, contrasting to Cu that is considered immobile and strongly adsorbed to soil particles (compared to Ni and Co) [190,192,220]. In this case either the mobility and adsorption of metal oxides is considerably different than salts for the same elements or interactions between the elements in mixtures might affect competition for adsorption surfaces, reducing the mobility of Ni and Co and increasing the solubility of Cu.

Soil organisms have a range of soil properties which are acceptable for their development, growth and reproduction which in conjunction with environmental variables (i.e. temperature) and ecology (i.e. species competition) define their habitat range. *E. crypticus*, is sensitive to soil pH, has been found to performed poorly in soils with pH below 3.8 [58]. In this experiment, *E. crypticus*, barely reproduces in the selected acid sandy forest soil with low pH, even in the absence of metals and it was not possible to evaluate the effect of mixtures in this soil. For *O. nitens* soil properties could be promoting differences in toxicity not only by regulating metal availability but also through habitat quality affecting the resilience of *O. nitens* to metals. Others have studied *O. nitens* resilience in soils dosed with zinc and found that at similar bioavailable Zn concentrations, soils with higher habitat quality improved the resilience of *O. nitens* with the most important variables defining habitat quality being CEC, OC, and pH [89]. Although the two soils used in this study had similar OC, the acid sandy forest soil had a lower CEC and a higher acidity than the loamy soil, potentially reducing the reproductive resilience of *O. nitens* to metals. Despite the large differences in *O. nitens'* metal sensitivity observed in each soil, the global response towards mixtures was similar (i.e., synergism, decreasing with dose). This suggests that while soil properties greatly affect the magnitude of metal toxicity, they may not as strongly affect the intensity of interactions between metallic elements.

The concentration addition model is the proposed default first tier for assessing joint-action toxicity of metal mixtures [55,118]. However, it may underestimate risks at low dose/effect levels, which are the most important in defining protective thresholds. In both Europe and Canada, protection thresholds are defined below the EC50 level. In Europe, metals are regulated under the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) program. Under REACH soil PNEC values are established using EC10 data compiled in species sensitivity distributions (SSD), and hazardous concentrations are estimated at the 5^{th} percentile

(HC5). In Canada, species EC25 are used, with protective levels for residential and agricultural use set at HC25 and levels for industrial soil use set at HC50 [7,12].

In this study, complex metal oxide mixtures had significant synergism (higher toxicity than predicted) at low dose/effect levels for *O. nitens* and *E. crypticus*. Based on these results, concentration addition would only be protective for *F. candida*, where responses were globally additive at all dose levels. Synergistic effects at low dose/effect levels means that when reducing contaminant concentrations towards a certain protective threshold, an increase in toxicity from what is predicted may be observed. Also, the magnitude of this deviation from additivity increases the lower the dose/effect level considered. At these lower levels, assumptions to correct for deviations from additivity must be made to provide adequate environmental protection for metal mixtures. However, as demonstrated in this study, the degree of deviation from additivity depends on a complex interaction between the species considered, dose/effect level, mixture composition, and soil properties. Ideally, to avoid unpredicted toxic effects from mixture interactions, risk assessors should consider a site-specific approach using fixed ratio rays of the metals present at a contaminated site, across a range of dose/effect levels, while selecting relevant biological endpoints (species) and reference soils from the site of interest. Site-specific risk assessments are not always possible, being many times considered too costly. So instead generic guidelines are adopted, like the Canadian soil quality guidelines (CSQG) and the REACH soil PNEC values [7,12]. Environmental guidelines should be adapted for mixtures using a synergistic assessment factor, based on the strongest estimated synergisms detected in standard test species. Generic guidelines should also include a soil factor (already considered in the EU REACH for single metals in the soil PNEC calculator but only for metal salts [8]). However, soil factors should be quite conservative due to the poor predictive ability of soil properties to the toxic effects on individual test species. In order to be globally protective, soil and mixture synergism factors have to account for a worst-case scenario. This approach while protective might provide very restrictive thresholds and the use of some site-specific properties is recommended to adjust thresholds.

Conclusions

Complex metal mixtures deviate more from additivity than simpler binary and ternary mixtures. Deviations from additivity are greater at high and especially at low dose/effect levels rather than at the EC50 level. However, the majority of simpler mixture studies have only considered the EC50 level, which may be underestimating deviations from additivity.

Total metal mixture composition ratios in soil were found to not correlate with species responses. Although the *F. candida* responses were linked to metal solubility and soil pore-water, *E. crypticus* and *O. nitens* responses were not. For *E. crypticu*s and *O. nitens*, we hypothesize that soil ingestion may be an important route of exposure affecting mixture ratios and uptake of particular elements.

The use of concentration addition may not be appropriate for complex metal oxide mixtures. For two of the tested species (*O. nitens* and *E. crypticus*), significant synergisms were observed at low dose effect levels, producing a higher toxicity than predicted by concentration addition. For complex oxide mixtures, protective thresholds might require refinements, due to differences in toxicity from soil properties using a soil assessment factor and for potential synergistic responses to metal mixtures, using a synergistic assessment factor. The inclusion of these protective assessment factors might render generic guidelines too restrictive for remediation. As a result, site specific approaches might be more appealing, and should test the existing metal mixture ratios at the protective dose effect levels, including both relevant soils and species to adjust protective limits and remedial objectives.

Future mixture ecotoxicological research with complex mixtures should incorporate internal concentrations to investigate how physiology affects metal uptake, explain the differences observed between metal mixture concentrations in soil and species responses, and improve the understanding of soil ingestion and its role as an exposure route for *O. nitens* and *E. crypticus.*

Annex 2

Table 1A - Single metal effect concentrations (10, 20, 30, 40, 50, 60, 70, 80 and 90) of lead, copper, nickel, zinc and cobalt for each species *(E. crypticus, O. nitens* and *F. candida*) and soil (loamy and acid sandy forest soils)

Loamy soil																			
Species	Metal	EC10	STE	EC20	STE	EC30	STE	EC40	STE	EC50	STE	EC60	STE	EC70	STE	EC80	STE	EC90	STE
E. crypticus	Lead	852	162	884	127	912	98	938	70	966	44	997	37	1035	70	1087	134	1176	258
	Copper	652	895	1581	1629	3244	2709	6376	4954	12720	11063	27073	29791	65860	96133	210246	418272	1346814	3893514
	Nickel	4099	5309737	4232	4267025	4315	3584516	4379	3049642	4433	2591033	4483	2174046	4531	1778158	4582	1393587	4646	1057353
	Zinc	465	5	476	4	485	4	493	5	502	7	512	9	524	12	540	16	566	22
	Cobalt	No fitted dose response– assumed value - 9999999																	
O. nitens	Lead	639	748	1827	1607	4280	3260	9536	7593	21625	20814	52947	65288	151880	244290	601300	1286500	5435500	16459000
	Copper	145	1467	1156	5038	4237	6161	11456	31614	26672	132950	57764	411300	123020	1136400	274810	3160500	740790	10595000
	Nickel	2577	344	2918	282	3169	249	3390	241	3607	259	3838	307	4106	389	4459	526	5048	799
	Zinc	No fitted dose response– assumed value - 9999999																	
	Cobalt	10175	3385	11849	2825	13033	2374	14019	1993	14915	1682	15785	1474	16684	1437	17697	1668	19032	2310
Acid Sandy Forest soil																			
F. candida	Lead	No fitted dose response– assumed value - 9999999																	
	Copper	110	87	239	138	388	174	564	202	774	227	1033	259	1372	319	1853	455	2687	806
	Nickel	4752	3492	4940	2855	5061	2439	5156	2113	5238	1837	5314	1588	5390	1358	5471	1143	5573	964
	Zinc	2596	11068	2662	10155	2717	9376	2770	8610	2825	7793	2886	6859	2959	5703	3058	4100	3224	1319
	Cobalt	No fitted dose response – assumed value - 9999999																	
O. nitens	Lead	712	307	828	281	936	324	1050	439	1181	627	1344	909	1564	1348	1907	2125	2618	4006
	Copper	268	195	387	228	523	269	692	343	923	491	1265	791	1832	1427	2971	3022	6442	9245
	Nickel	1	4	6	22	20	58	52	117	119	198	252	307	527	503	1151	1185	3022	4584
	Zinc	126	1000	201	1282	273	1467	351	1597	443	1678	558	1697	719	1607	978	1289	1553	1361
	Cobalt	326	308	482	384	663	457	894	543	1213	667	1695	895	2512	1416	4199	2895	9555	9459

Table 2A – *Enchytraeus crypticus* effect concentrations and standard error in the loamy soil, for each individual mixture ratio and a global model with all mixtures. For effect concentration (ECx) toxic units were calculated using the same effect level (ECX = $TUEC_X$), values closer to 1 represent concentration addition.

Enchytraeus crypticus - Loamy soil																			
Assay	N	EC10	STE	EC20	STE	EC30	STE	EC40	STE	EC50	STE	EC60	STE	EC70	STE	EC80	STE	EC90	STE
CSQG	11	0.58	0.07	0.60	0.06	0.62	0.05	0.64	0.04	0.66	0.04	0.69	0.04	0.71	0.05	0.75	0.08	0.82	0.13
Flin_Flon	11	0.60	0.14	0.64	0.08	0.68	0.05	0.72	0.03	0.77	0.06	0.82	0.11	0.89	0.19	0.98	0.30	1.16	0.54
Sudbury	9	0.53	0.15	0.64	0.16	0.75	0.17	0.86	0.18	1.00	0.22	1.18	0.28	1.43	0.39	1.84	0.63	2.74	1.28
Clay_Peat	12	0.38	0.05	0.47	0.05	0.54	0.04	0.62	0.04	0.71	0.05	0.83	0.06	0.99	0.09	1.24	0.16	1.79	0.35
Port_Colborne	12	1.04	0.60	1.06	0.45	1.12	0.38	1.20	0.34	1.30	0.32	1.41	0.31	1.56	0.34	1.76	0.42	2.08	0.63
EC50	11	0.43	0.25	0.57	0.15	0.63	0.09	0.66	0.06	0.69	0.04	0.71	0.05	0.73	0.07	0.75	0.09	0.75	0.13
Equal_Ratio	9	0.17	0.10	0.31	0.12	0.42	0.12	0.52	0.12	0.62	0.11	0.72	0.12	0.84	0.13	0.97	0.18	1.16	0.27
Ag_Res_Loamy	11	0.96	0.04	0.93	0.03	0.93	0.02	0.94	0.01	0.95	0.02	0.97	0.03	0.98	0.04	1.01	0.06	1.05	0.10
Acid_Sand_Ara	11	1.19	0.16	1.20	0.15	1.23	0.14	1.27	0.13	1.31	0.12	1.36	0.11	1.42	0.11	1.51	0.12	1.67	0.19
Loam_Sand_Ind	9	1.11	0.01	1.13	0.01	1.14	0.01	1.16	0.01	1.18	0.01	1.19	0.01	1.21	0.02	1.24	0.02	1.29	0.03
Global	106	0.47	0.10	0.65	0.08	0.79	0.07	0.92	0.07	1.03	0.06	1.14	0.07	1.25	0.08	1.37	0.11	1.53	0.16

Table 3A – *Oppia nitens* effect concentrations and standard error in the loamy and acid sandy forest soils, for each individual mixture ratio and a global model with all mixtures. For effect concentration (ECx) toxic units were calculated using the same effect level (ECX = $TUEC_X$), values closer to 1 represent concentration addition.

Assay	N	EC10	STE	EC20	STE	EC30	STE	EC40	STE	EC50	STE	EC60	STE	EC70	STE	EC80	STE	EC90	STE
Oppia nitens - Loamy soil																			
CSQG	14	No fitted dose response																	
Flin_Flon	13	0.04	0.06	0.05	0.04	0.05	0.03	0.04	0.02	0.04	0.02	0.03	0.01	0.03	0.02	0.03	0.02	0.05	0.05
Sudbury	14	0.34	0.21	0.22	0.11	0.15	0.07	0.12	0.05	0.10	0.05	0.10	0.05	0.11	0.06	0.14	0.11	0.25	0.28
Clay_Peat	14	0.10	0.17	0.11	0.12	0.13	0.11	0.17	0.14	0.27	0.24	0.48	0.49	1.02	1.30	2.86	4.75	15.67	36.69
Port_Colborne	15	No fitted dose response																	
EC50	12	0.01	0.02	0.03	0.05	0.06	0.06	0.08	0.07	0.13	0.09	0.22	0.18	0.42	0.45	0.91	1.32	2.39	4.70
Equal_Ratio	15	No fitted dose response																	
Ag_Res_Loamy	12	0.03	0.12	0.06	0.13	0.08	0.12	0.11	0.11	0.16	0.11	0.22	0.12	0.31	0.20	0.47	0.39	0.78	0.92
Acid_Sand_Ara	12	No fitted dose response																	
Loam_Sand_Ind	11	0.01	0.07	0.06	0.14	0.07	0.12	0.08	0.10	0.10	0.10	0.14	0.13	0.21	0.22	0.36	0.49	0.70	1.32
Global	76	0.11	0.08	0.12	0.06	0.11	0.04	0.12	0.03	0.14	0.04	0.20	0.07	0.34	0.16	0.71	0.47	1.85	1.73
Oppia nitens - Acid Sandy Forest soil																			
CSQG	11	23.96	35.07	4.60	5.48	1.97	1.97	1.21	1.03	0.91	0.69	0.78	0.59	0.73	0.66	0.73	0.91	0.80	1.56
Flin_Flon	13	3.41	6.15	2.31	2.46	1.77	0.52	1.42	0.18	1.16	0.39	0.94	0.66	0.74	0.71	0.55	2.73	0.37	0.30
Sudbury	15	0.00	0.04	0.17	0.62	0.24	0.64	0.15	0.49	0.12	0.38	0.14	0.40	0.22	0.48	0.41	0.69	1.01	1.83
Clay_Peat	11	42.03	81.71	9.18	12.86	4.12	4.27	2.55	1.96	1.89	1.14	1.57	0.93	1.38	1.05	1.27	1.35	1.16	1.83
Port_Colborne	15	0.54	2.99	0.72	2.88	0.89	2.66	1.05	2.38	1.23	2.06	1.43	1.73	1.64	1.53	1.86	1.81	2.04	2.79
EC50	15	19.66	33.03	12.50	40.40	4.46	8.23	2.19	2.21	1.29	0.91	0.83	0.28	0.57	0.36	0.37	0.07	0.20	25.99
Equal_Ratio	15	0.09	0.51	0.14	0.54	0.21	0.57	0.29	0.55	0.37	0.46	0.42	0.36	0.47	0.40	0.59	0.81	1.07	2.39
Ag_Res_Loamy	15	0.00	0.01	0.07	0.39	0.30	1.14	0.30	1.02	0.24	0.63	0.24	0.51	0.28	0.43	0.37	0.36	0.52	0.74
Acid_Sand_Ara	15	0.01	0.08	0.26	0.77	0.54	1.02	0.68	0.94	0.76	0.79	0.81	0.61	0.84	0.46	0.85	0.44	0.79	0.61
Loam_Sand_Ind	15	0.34	3.12	0.11	0.89	0.07	0.53	0.07	0.43	0.07	0.39	0.09	0.38	0.11	0.38	0.17	0.39	0.35	0.68
Global	140	0.00	0.00	0.12	0.21	0.36	0.36	0.45	0.31	0.50	0.25	0.57	0.21	0.69	0.20	0.85	0.27	1.12	0.51

Table 4A – *Folsomia candida* effect concentrations and standard error in the acid sandy forest soil, for each individual mixture ratio and a global model with all mixtures. For effect concentration (ECx) toxic units were calculated using the same effect level (ECX = $TUEC_X$), values closer to 1 represent concentration addition.

F. candida - Acid Sandy Forest soil																			
Assay	N	EC10	STE	EC20	STE	EC30	STE	EC40	STE	EC50	STE	EC60	STE	EC70	STE	EC80	STE	EC90	STE
CSQG	10	1.65	0.14	0.92	0.06	0.69	0.04	0.57	0.02	0.50	0.02	0.45	0.02	0.42	0.02	0.40	0.03	0.38	0.05
Flin_Flon	10	1.59	0.92	1.18	0.50	1.03	0.34	0.96	0.26	0.94	0.21	0.93	0.19	0.95	0.21	0.99	0.27	1.08	0.41
Sudbury	9	0.49	0.38	0.40	0.22	0.38	0.17	0.37	0.15	0.38	0.14	0.40	0.14	0.42	0.15	0.47	0.19	0.57	0.30
Clay_Peat	9	1.90	0.68	1.06	0.30	0.78	0.19	0.65	0.14	0.59	0.13	0.56	0.15	0.56	0.20	0.59	0.30	0.72	0.55
Port_Colborne	11	0.44	0.49	0.53	0.42	0.62	0.38	0.73	0.34	0.86	0.30	1.01	0.27	1.22	0.26	1.50	0.32	1.98	0.57
EC50	8	4.46	2.44	2.93	1.13	2.31	0.66	1.97	0.43	1.76	0.30	1.62	0.24	1.52	0.23	1.45	0.27	1.41	0.35
Equal_Ratio	12	7.84	36.07	4.05	19.99	2.68	6.28	2.03	4.03	1.63	3.18	1.34	2.16	1.13	1.02	0.96	0.83	0.76	0.53
Ag_Res_Loamy	11	3.30	1.11	1.91	0.37	1.40	0.18	1.12	0.11	0.94	0.08	0.77	0.63	0.70	0.04	0.61	0.03	0.52	0.02
Acid_Sand_Ara	12	4.96	1.4	2.76	0.64	2.01	0.4	1.64	0.3	1.43	0.3	1.3	0.3	1.22	0.3	1.19	0.4	1.23	0.52
Loam_Sand_Ind	11	0.77	1.56	0.80	1.14	0.86	0.93	0.95	0.79	1.07	0.69	1.22	0.59	1.41	0.52	1.69	0.52	2.16	0.80
Global	103	1.5	0.45	1.18	0.26	1.08	0.2	1.05	0.2	1.05	0.1	1.08	0.1	1.14	0.2	1.25	0.2	1.47	0.32

Table 5A– Mantel test for the correlation between different metal mixture and species response

Mantel TEST			
Matrix 1	Matrix 2	Mantel Statistics r	Significance p
Between species			
E. crypticus - Loamy soil	*O. nitens* - Loamy soil	-0.15	0.561
E. crypticus - Loamy soil	*F. candida* - Acid sandy forest soil	0.073	0.287
E. crypticus - Loamy soil	*O. nitens* - Acid sandy forest soil	-0.009	0.442
F. candida - Acid sand forest Soil	*O. nitens* - Loamy soil	-0.346	0.708
F. candida - Acid sand forest Soil	*O. nitens* - Acid sandy forest soil	-0.223	0.94
O. nitens - Loamy Soil	*O. nitens* - Acid sandy forest soil	0.870	0.030
Between Soils			
Loamy Soil - Total metal	Acid sandy forest soil - Total metal	0.945	0.001
Loamy Soil - Avail. metal	Acid sandy forest soil - Avail. metal	0.845	0.006
Species response vs Total metal composition			
E. crypticus	Loamy soil - Total metal	0.131	0.212
O. nitens	Loamy soil - Total metal	-0.053	0.443
F. candida	Acid sandy forest soil - Total metal	0.262	0.141
O. nitens	Acid sandy forest soil - Total metal	-0.267	0.875
Species response vs Available metal composition			
E. crypticus	Loamy soil - Avail. metal	0.117	0.216
O. nitens	Loamy soil - Avail. metal	No compute	
F. candida	Acid sandy forest soil - Avail. metal	0.019	0.310
O. nitens	Acid sandy forest soil - Avail. metal	-0.112	0.529
Species response vs Nominal metal composition			
E. crypticus	Nominal	-0.043	0.522
O. nitens - Loamy Soil	Nominal	-0.238	0.769
F. candida	Nominal	0.008	0.336
O. nitens - Acid sand forest Soil	Nominal	-0.208	0.857
Species responses vs total metal concentrations for most available metals			
F. candida	Acid sandy forest soil – Only Zn and Copper	0.355	0.038
O. nitens	Acid sandy forest soil – Only Zn and Copper	-0.156	0.785

matrices, values in bold represent significant correlations.

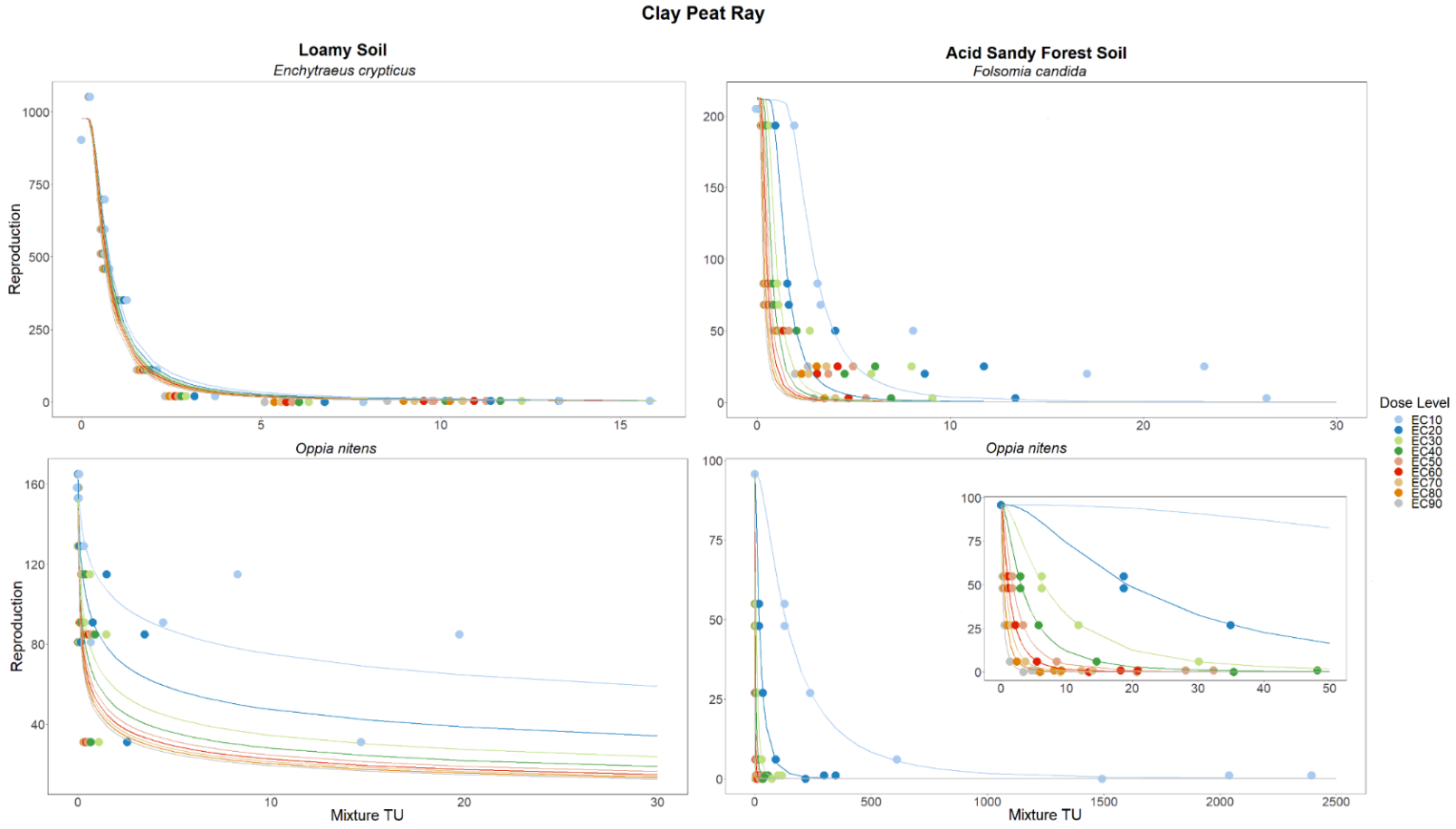

Figure 1A – Clay Peat Mixture ratio ray dose response curves for each species/soil for toxic units calculated at different dose/effect levels, in the last graph (*O. nitens* acid sandy forest soil) an increased resolution at low mixture TU is provided to better visualize differences between TU calculated at different dose/effect levels.

Chapter 4 - Community effect concentrations as new concept to easily incorporate community data in environmental effect assessment of complex metal mixtures.

This chapter is based on the submitted paper

M. Renaud, P.Martins da Silva, T. Natal-da-Luz, S.D. Siciliano, J.P. Sousa. Community effect concentrations as new concept to easily incorporate community data in environmental effect assessment of complex metal mixtures. Submitted to the Journal of Hazardous Materials

Abstract

The goal of this study was to incorporate community data into the effect assessment of environmental and regulatory relevant metal mixtures. In this experiment three fixed mixture ratios (Canadian soil quality guideline ratio - CSQG, Agricultural, Residential and Loamy ratio - ARL and Sudbury ratio - SUD) were tested in a natural community microcosm with 11 doses for each mixture ratio. The effect of metal mixtures on the community was measured using the community effect concentration (EC) concept which assumes that as contamination increases, the community similarity between test and control treatments decreases producing a dose response curve allowing the calculation of community effect concentrations. In regulatory mixture ratios (CSQG and ARL) community EC10s were four times higher than regulatory thresholds and current regulation might be overprotective of the microarthropod communities in some soils. For the contaminated site ratio (SUD), the field dose in the contaminated site corresponded to a community EC20 and if metal concentrations were reduced by 1 TU (from 3.1 TU to 2.1TU) effects would be below a community EC10. Overall, the community EC concept was successfully applied and has the potential for inclusion in risk assessment schemes as a measure of community response.

Introduction

Current environmental guidelines in Canada and Europe use single species data to derive environmental protection thresholds [12,141]. For example, single species data has been used to establish the Canadian soil metal quality guidelines [11] and in the European Union (EU), to comply with the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) through the development of a soil predicted no effect concentration (PNEC) calculator [8]. Standard test species differ in their sensitivity to metals [37,43], and thus environmental guidelines should be conservative when used to extrapolate effects on communities. To improve risk estimates, species sensitivity distributions (SSDs) using data from multiple standard test organisms, can account for inter-species variability. SSDs allow the calculation of hazard concentrations (HC_x) expected to affect a certain proportion of the community. Recently, an environmental threshold calculator for metals in soil was developed using HC_5 values from SSDs [226]. However, unlike the aquatic environment [227], for soil communities, SSD estimates have yet to be validated with higher tier, field or mesocosm data. Finally, while SSDs account for species variability in sensitivity, single species data do not account for indirect effects of contamination resulting from species interactions.

Microcosm experiments test inter-species variability and include species interactions. Rather, than replace more environmentally relevant terrestrial model ecosystems (TMEs) and field experiments, microcosms are suited for intermediate tiers and provide important effect data at the community level, at a lower cost and under less time. In soil ecotoxicology, microcosms using constructed [151,155,158], semi-natural [165,167] and natural communities [164,169,228], evaluated a variety of contaminants including metals. Natural community microcosms measure the direct and indirect effects of a contaminant on a fraction of a real soil community. Currently, natural community microcosms are not considered for legislative purposes. For its inclusion in routine risk assessments more research on method development and data interpretation is required [141].

The interpretation of the effects of contaminants on a soil community can be quite complex. In a soil community, some species can thrive at increasing metal contamination levels (metal tolerant species), while others may disappear (metal sensitive species) [229–233]. Differences in species responses, within a community, means that alpha diversity measures such as total abundances, species richness and evenness indices can be poor predictors of metal toxicity [231,233,234].

Indicator species (metal sensitive and metal tolerant) are an alternative to classify the "health" status of a soil community [234]. However, in contaminated sites the classification of a species in terms of their relative sensitivity is not always consistent. For instance, *Protaphorura armata* has been considered a metal tolerant species [229] but also metal sensitive because it decreases in abundance in more contaminated soil patches [235]. Using indicator species to interpret the effects of contamination is a minimalistic approach, which does not consider the holistic effects of contaminant on a soil community and if focused only on the sensitivity of indicator species disregards, the indirect effects of contamination on community structure and function.

In a natural community microcosm experiment, the goal is to assess if and how increasing doses of a contaminant change the soil community being tested. This could be achieved by using similarity metrics (indices) to measure the level of similarity (or dissimilarity) between communities from different treatments and, when coupled to specific techniques (e.g., analysis of similarities, permutational multivariate analysis of variance), evaluate if the observed differences in community composition are in fact significant. While the use of similarity metrics to assess changes in community composition is widespread in soil ecological research for many years [236–242], their use in soil ecotoxicology is more restrict [116,164,243,244]. However, this approach can be easily used in micro- and mesocosm experiments and field studies to derive community no observed effect concentrations (NOEC) and lowest observed effect concentration (LOEC) values, by directly comparing and assessing significant differences between the community composition of the different contaminant treatments vs the control community (e.g., [164,244,245]). In a more elaborate way, Principal Response Curves are also be used to derive community NOEC values when these studies are conducted over time [151,181,183]. However, it is recognized in ecotoxicology and risk assessment schemes, that effect concentration (ECx) values are more comprehensive toxicity endpoints than NOEC or LOEC values [246]. Therefore, there is increasing importance to develop a method that can easily derive community EC values.

In ecotoxicological community experiments (microcosm, mesocosm or field study), the toxicity caused by a contaminant is expected to change the community composition or structure compared to its control. As such, as contamination increases the community similarity between test and control treatments decreases, producing a similarity dose response curve allowing, similar to traditional metrics (i.e. reproduction, survival), the calculation of community effect concentrations. The community ECx is the concentration of the contaminant causing x percentage of change to the composition of the control community. The community EC approach presented is a new concept that, as far as we know, was never explored in ecotoxicology. The

derivation of community EC values using the simple approach proposed in this study can be a step forward in ecotoxicology and risk assessment research with a strong potential since the derived values can be used to establish environmental protection thresholds, in a transparent manner, supported by community data.

In the same way that most ecotoxicology research is dedicated to single species effects, toxicity of metals, has focused on single compounds rather than mixtures [18,33,34,37,43]. Some metal mixture research has been performed in soil ecotoxicology but mostly with binary mixtures [52,55,64,124,133]. However binary mixtures are still well below the complexity of metal mixtures observed in many metal contaminated sites [229,233,235]. For more complex mixtures, full factorial designs, are complicated as the number of potential combinations increases exponentially with each added element. In this case, testing fixed ratios of individual elements across a dose range, may be preferred. Ratios based on environmental regulations, can test the adequacy of current environmental thresholds, in a mixture setting. When based on contaminated sites, ratios can be used to determine their current risk for reference communities and estimate protective site-specific remedial goals.

In this study, the objective was to determine the effects of complex metal mixtures on soil microarthropod communities using a community effect concentration approach. For this objective, metal mixtures of lead, copper, nickel, zinc and cobalt were tested in a natural microarthropod community in a microcosm experiment using three fixed ratios. Two ratios based on regulatory guidelines and a third ratio based on average metal concentrations in a metal contaminated site. Each ratio was tested using a control and ten mixture doses expressed in toxic units (TU). Metal mixture effects on the soil community were analysed using the proposed community similarity dose response curves. Estimated community EC values were compared to environmental thresholds of Canada and EU, used to derive the regulatory mixture ratios (Canadian soil quality guideline ratio and Agricultural, residential and loamy ratio) as well as average concentrations in the contaminated site in Sudbury, Canada used to define the contaminated site ratio (Sudbury ratio).

Methods

Test soil and natural microarthropod community

Soil was collected from a "Montado" (cork oak forest) area in southern Portugal (38°41'39.7"N 8°18'27.6"W). Collected soil was sieved at 5 mm and stored in the dark until use. The test soil had an organic matter content of 4.0 ± 0.7% (OM; loss on ignition at 500ºC for 6 h), pH 4.02 ± 0.04 (1 M KCl, 1:6, v:v), cation exchange capacity of 9.2 ± 0.4 meq/100g (as described by Chapman [247]), water-holding capacity of 51.1 ± 1.0% (ISO 11267 annex C [205]) and soil texture was 68 ± 1% sand, 24 ± 0.6% silt and 8 ± 1% clay (as described by Ge and Bauder [248]). The natural soil microarthropod community was sampled using 520 individual soil cores collected from an approximate 5 m^2 area at the same site using polyvinylchloride cores (5 cm diameter and depth). This small area was selected to minimize variability in soil fauna composition and abundance of microarthropods within soil cores due to aggregated distribution of theses organisms in space. Collected soil cores were stored in a controlled temperature chamber (20 ± 2ºC) until use, but for no longer than 1 week.

Metal Mixture dosing

Three mixture ratios of lead, copper, nickel, zinc and cobalt were selected for their environmental and legislative relevance (Table 1). The three tested mixtures ratios were selected from a larger set of mixtures previously tested using a single species approach [249]. The Canadian soil quality guideline (CSQG) ratio was based on the threshold value of each metal in the Canadian Soil quality guidelines considering an agricultural soil use. Experiments were conducted before the revision of the Zinc threshold in the Canadian soil quality guidelines altered in 2018. As a result, the CSQG mixture ratio used does not include 2018 revised value for zinc (zinc threshold used – 200 mg/kg; 2018 revised threshold– 250 mg/kg) [11]. The Sudbury (SUD) ratio was based on the average concentration of each element across a range of samples from a contaminated smelter site in Sudbury, Canada. Lastly, the Agricultural, residential and loamy (ARL) ratio was the average values of similar ratios, established using a principal component analysis, for PNEC values for different reference soils (acid sandy forest, acid sandy arable, loamy alluvial, loamy, clay and peaty soil) in the EU REACH PNEC calculator and CSQG values for different soil uses (agricultural, residential, commercial and industrial).

Specifically, ARL is the ratio based on average concentrations between CSQG for agricultural and residential soil use and a loamy reference soil in the EU REACH PNEC calculator [8,11].

Each mixture ratio was tested with 11 mixture doses in toxic units including a control with no metals added. Since no community data was available for each individual metal, *F. candida* EC50's for each metal in the literature [18,33,34,43] were used as a surrogate to calculate mixture TUs (Table 1). The 11 nominal doses in TUs, ranged from 0.065 to 12 TU and were corrected to actual TUs using measured total metal concentrations (Table 2).

The concentration of each metal in the environmental regulation (CSQG and ARL) and contaminated site (SUD), used to define the ratio (Table 1) was also calculated in toxic units and designated as the "field dose". Similarly, to experimental doses, field doses were also calculated using *F. candida* EC50 data. Calculating the field dose for each test ratio (CSQG, ARL and SUD) was performed to allow comparisons between community effects and concentrations in environmental guidelines or contaminated sites when presented as a mixture.

Table 1 – Mixture composition of lead, copper, nickel, zinc and cobalt of the three mixture ratios used in microcosm experiments (CSQG, SUD and ARL), the field dose of each mixture ratio (see definition in the text) and *Folsomia candida* literature EC50 values for each metal.

Mixture	Lead (mg/kg)	Copper (mg/kg)	Nickel (mg/kg)	Zinc (mg/kg)	Cobalt (mg/kg)	Field dose Toxic Units
CSQG	70	63	45	200	40	0.53
SUD	1798	125	231	929	119	3.11
ARL	82	86	55	197	47	0.58
F. candida EC50	1600	700	475	750	1480	-

Soil dosing was performed using lead oxide (Pb_3O_4, purity 99%, Sigma-Aldrich, Darmstadt, Germany), copper oxide (CuO, purity 96%, Panreac Applichem, Barcelona, Spain), nickel oxide (NiO, purity 99.8%, Sigma-Aldrich, Darmstadt, Germany), zinc oxide (ZnO, purity 99%, Panreac Applichem, Barcelona, Spain) and cobalt (Co_3O_4, purity 99.5%, Sigma-Aldrich, St Louis, MO, USA). Metal oxides were selected over metal salts based on previous experiments

where oxides were more appropriate in retaining fixed ratio metal mixtures [220]. Oxides were combined into metal oxide mixtures at the appropriate ratio for each of the three test mixtures. From this oxide mixture the appropriate weight was added to soil for a particular dose and vigorously mixed using a soil hand mixer. After dosing, soil water content was adjusted to 50% water holding capacity and samples were collected for metal analysis.

Table 3 – Nominal and measured total metal concentrations of lead (Pb), copper (Cu), nickel (Ni), zinc (Zn) and cobalt (Co) and toxic units calculated from *Folsomia candida* EC50 literature values using nominal (Nominal dose) and total metal concentrations (Corrected dose) in the three mixture ratios used in microcosm experiments (CSQG, SUD and ARL – for details on mixture ratios and *F. candida* EC50 values, see Table 1)

	Nominal dose	Nominal concentration (mg/kg)					Total metal concentration (mg/kg)					Corrected dose
	Nominal Toxic unit	Pb	Cu	Ni	Zn	Co	Pb	Cu	Ni	Zn	Co	Actual Toxic unit
	0	0	0	0	0	0	0	0	0	0	0	0
	0.065	8.4	7.5	5.4	23.9	4.8	14.5	10.9	2	35.2	2.5	0.1
	0.125	16.8	15.1	10.8	47.9	9.6	31.3	24.2	7.7	85.6	24.5	0.2
	0.25	33.5	30.2	21.5	95.8	19.2	53.4	44	18.8	149.4	16.9	0.3
	0.5	67	60.3	43.1	191.5	38.3	102.4	83.1	41.1	271.8	37.8	0.7
CSQG	1	134.1	120.6	86.2	383	76.6	200	170.4	83.7	502.5	63.3	1.3
	1.5	201.1	181	129.3	574.5	115	287.3	260.9	188.1	792.6	114.2	2.1
	2	268.1	241.3	172.4	766	153	358.9	313.2	188.5	1001	150.6	2.5
	4	536.2	482.6	344.7	1532	306	746.8	626.8	381.1	2072	285.5	5.1
	8	1072.4	965.2	689.4	3064	613	1469.8	1122.7	764.2	4014.3	557.2	9.9
	12	1608.6	1448	1034	4596	919	2066	1511.4	1027.7	5272.6	768.8	13.2
	0	0	0	0	0	0	0	0	0	0	0	0
	0.065	36.2	2.5	4.6	18.7	2.4	63.1	3.3	1.4	35.5	18.9	0.1
	0.125	72.3	5	9.3	37.4	4.8	114.9	5.4	3.9	55	8.4	0.2
	0.25	144.6	10.1	18.6	74.8	9.5	220.4	10.6	18.7	106.5	15.9	0.3
	0.5	289.3	20.1	37.1	149.5	19.1	440.8	22.6	38.8	202.6	20.1	0.7
SUD	1	578.6	40.2	74.3	299.1	38.2	962.1	63.1	86.8	472.7	56.8	1.5
	1.5	867.9	60.3	111.4	448.6	57.2	1283.5	77.1	135.1	588.9	58.2	2.0
	2	1157.2	80.4	148.5	598.2	76.3	2264.8	147.7	250.5	1075	110.6	3.7
	4	2314.4	160.9	297	1196	153	3436.7	222.8	360.6	1689.8	163	5.6
	8	4628.8	321.8	594.1	2393	305	6847.1	407.3	698	3237.9	298.2	10.8
	12	6943.2	482.7	891.1	3589	458	9225.7	505.3	924.3	4324	423.9	14.5
	0	0	0	0	0	0	0	0	0	0	0	0
	0.065	8.7	9.2	5.9	21.1	5	19.1	17.4	3.8	34.1	12.8	0.1
	0.125	17.5	18.4	11.8	42.2	10	33.9	28	11	61.5	3.5	0.2
	0.25	35	36.8	23.5	84.4	19.9	52.8	47.7	21.4	103	25.5	0.3
	0.5	70	73.7	47.1	168.7	39.9	105.9	110.8	44.9	245.4	43.5	0.7
ARL	1	139.9	147.3	94.2	337.5	79.7	208.4	221.4	100.7	482.6	77.5	1.4
	1.5	209.9	221	141.3	506.2	120	367.9	392.3	202.9	827	162.8	2.4
	2	279.8	294.7	188.4	674.9	159	473.7	515.4	250.1	1103.4	196.4	3.2
	4	559.6	589.3	376.7	1350	319	938	977.3	502.4	2143.8	375.1	6.2
	8	1119.2	1179	753.4	2700	638	1625.1	1477.9	900.2	3638.4	657.1	10.3
	12	1678.8	1768	1130	4050	957	2317.7	2070.5	1265.1	4989.5	910.7	14.3

Microcosm experiment

The microcosm experiment was performed following the procedures described in Chelinho et al. [164], with some modifications. Test vessels used were cylindrical plastic pots (11 cm diameter and 12 cm height) each one filled with 300 g of soil (dry-weight equivalent) from a specific mixture ratio dose and control. Control treatments for each mixture ratio were performed with six replicates and dosed treatments with 5 replicates. Afterwards, soil microarthropods from three randomly selected soil cores (collected as described above) were added to each microcosm as follows. They were directly extracted into a portion of soil from a particular microcosm placed in a falcon tube (approximate volume of 10 ml in the falcon tube) using a high gradient MacFayden extractor at 45^{o}C for 72h. During the 72h extraction, falcon tubes containing the test soil and extracted microarthropods were replaced with falcon tubes containing fresh test soil after every 24h cycle. Soil containing extracted microarthropods was placed back into its respective microcosm. Microarthropods from all treatments for a particular mixture ratio (including controls) were extracted at the same time to reduce variability within a mixture ratio microcosm experiment. The renewal of soil in falcon tubes after every 24h cycle was performed to avoid microarthropod excess exposure to heat. Prior to the microcosm experiment, the extraction procedure was tested for its efficiency and at 72h 92.4% of the existing microarthropods were extracted.

After the addition of the extracted soil microarthropod community, test vessels were covered with perforated transparent plastic lids and placed in an incubation chamber at 20 ± 2^{o}C under a photoperiod of 16h light and 8h dark for four months. Incubation conditions are those recommended for rearing standard soil invertebrate test species [205,219]. During incubation, food in the form of granulated dry yeast (approximately 2 mg) was provided and soil water content was adjusted using distilled water on a weekly basis. At the end of the test period, soil microarthropods were extracted under the same conditions as the initial community extraction but for the full 72h period and directly into a 70% ethanol solution.

Soil microarthropods were identified into major groups using a binocular microscope. Mites were further identified to the order, sub-order or cohort level (Prostigmata, Mesostigmata, Oribatida and Astigmata) while collembolans were identified to species level. Average number of individuals per treatment is presented in Annex 3 (Table 1-3A).

Metal analysis

Soil samples collected immediately after soil dosing, from the bulk dosed soil, were used to determine total metal concentrations. Collected samples were air-dried and lightly ground prior to analysis. Initial metal content measurements were performed using X-ray fluorescence (XRF). In this procedure, soils (three samples per soil treatment) were placed in microplate wells until each well was full (approximately 3 g) and analysed by XRF. The collected XRF results were corrected using linear regressions from the analysis of four treatments per mixture ratio (36% of treatments) by ICP-MS. In the ICP-MS analysis, 0.2 g of soil was placed in Teflon vessels and digested with 2ml of distilled nitric acid under pressure in a PDS-6 system (Loftfields analytical solutions, Neu Eichenberg, Germany) at 150^{o}C for 10h. Resulting extracts were diluted with ultra-pure water to an acidity of 3% and analysed by ICP-MS. Metal concentrations in soil samples were determined in kinetic energy discrimination mode (to control cell-formed interferences) after linear calibration for each element.

In addition to soil samples, replicate blanks were performed with each ICP-MS analysis and the accuracy of the analysis was checked by digesting a sample of the standard reference material SRM 2709 (San Joaquin Soil-Standard Reference Material) certified by the National Institute of Standards and Technology (US Department of Commerce). The recovery of the reference standard was 98.2, 99.4, 98.7, 143.3 and 84.1% for lead, copper, nickel, zinc, and cobalt, respectively. Due to the high recovery of zinc in the reference material, zinc values in dosed soils were corrected to reference values. The quantification limits for lead, copper, nickel, zinc and cobalt were 0.27, 0.04, 0.19, 0.55 and 0.03 mg/kg, respectively.

Data analysis

Once the identification of microarthropods was performed, the complete abundance dataset from each mixture ratio was used (i) to assess significant differences between microarthropod community from each tested dose and the respective control treatment and (ii) to derive community EC10, EC20 and EC50 values for that particular mixture ratio. All analyses were done on the Bray-Curtis similarity index applied to raw abundance data without transformation.

The community EC10, EC20 and EC50 were selected to represent a low, medium and high effect of metals to the natural community. This selection was performed considering that these EC values represent community change, including both the increase and decrease of species abundances rather than typical endpoints of mortality or reproduction.

Significant differences in community composition between control and dosed treatments were determined using permutational multivariate analysis of variance (Permanova). The species contribution to significant differences was calculated by similarity of percentages analysis (SIMPER). Analyses were performed using the PRIMER 6 software [250].

Dose response curves were constructed by calculating the similarity within control treatments and between control and each dosed treatment. Metal toxicity will promote changes in the community composition and a reduction in similarity between the control and each increasing metal dosed treatment, producing a dose response curve. Community similarity dose response curves were analysed using dose response models (i.e. Weibull, Logistic, Log-Logistic) selected based on best model fit (Akaike's information criterion, estimated residual standard error and lack-of-fit p value) to estimate community effect concentrations (EC10, EC20 and EC50). Selected models and effect concentrations for community data are provided in Table 3. Significant differences between mixture community effect concentrations, was determined using the generalized ratio test. In addition to community analysis, when possible, the EC_x values of the three most abundant and influent species in the community (i.e. *Hemisotoma thermophila complex*, *Ceratophysella gibbosa* and *Protaphorura armata*) were calculated to evaluate if single species within the community may explain the community response as a whole. These analyses were performed in R version 3.5.0 [209]. Distance matrices were calculated using the packages Vegan [251], dose response curves were analysed using the drc package [223] and all presented figures were constructed with the package ggplot2 [224].

Results

In all three mixture experiments the soil microcosm community was dominated by the Collembola *H. thermophila* complex (Figure 1). Despite some variability, abundances for this species remained high until a large decrease at intermediate mixture doses. In the CSQG and ARL mixtures this decrease was observed at 2.5 and 2.4 TU while in the SUD mixtures, it occurred at a higher mixture dose (5.6 TU). The next most abundant collembolan species in all three mixtures were *C. gibbosa* and *Protaphorura armata*. For all mixtures *C. gibbosa* was largely restricted to low doses and was rare after intermediate dose levels (CSQG – 1.3 TU, SUD – 0.7 TU, ARL - 1.4 TU). The highest abundances of this species occurred sooner in the SUD

(SUD 0 – 0.3 TU) and ARL (ARL 0 - 0.7 TU) mixtures than in the CSQG mixture (CSQG 0.2 - 0.7 TU). The abundances of *P. armata* were not as high as those of *C. gibbosa* but this species was less sensitive to metals and was generally present at all dose levels. For all three mixtures *P. armata* highest abundance occurred at intermediate to high doses (CSQG – 0.7 and 2.5 TU, SUD – 3.7 TU and ARL - 1.4 TU). The four remaining Collembola species, *Pseudosinella octopunctata* (CSQG, ARL and SUD), *Sphaeridia pumilis* (CSQG and SUD), *Ahrropalites caecus* (SUD and ARL) and *Lepidocirtus violaceus* (SUD and ARL) were rare. Only in the SUD mixture, did *P. octopunctata*, register a relatively high abundance at 0.7 and 1.5 TU.

Compared to the three most abundant Collembola species (*H. thermophila*, *C. gibbosa* and *P. armata*) mites had very low abundances. Astigmata was the most abundant group of mites followed by Oribatida, while both Prostigmata and especially Mesostigmata were rare. For the two most abundant groups, Astigmata followed a bell-shaped distribution with its highest abundances observed at intermediate mixture doses while oribatid mites did not present a consistent trend in the three microcosms. The highest abundances for Astigmata were registered at intermediate concentrations (CSQG - 2.1 TU, SUD - 1.5 TU and ARL - 0.7 TU). For Oribatid mites, in the CSQG mixture their highest abundances are registered in intermediate doses (0.1, 0.7 and 1.4 TU), in the SUD mixture in the control and 5.6 TU dose and in the ARL mixture at intermediate (1.4 TU) and high doses (14.3 TU).

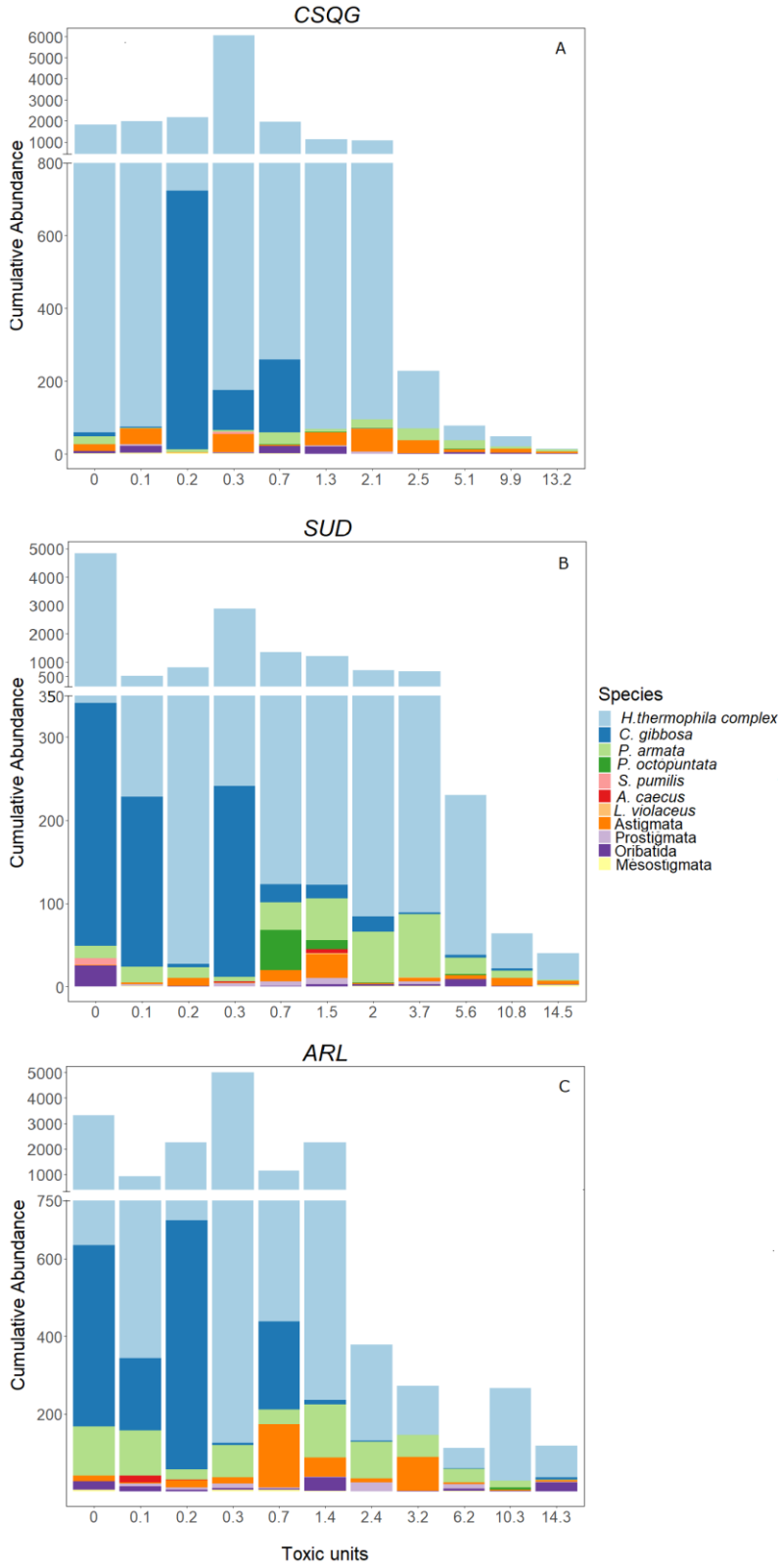

Figure 1 – Collembola species and mite total abundances in all tested doses of the CSQG (Panel A), SUD (Panel B) and ARL (Panel C) mixtures. Y axis scale cut to accommodate high abundances of *H. thermophila complex*. Dose in toxic units corrected for total metal concentrations – Actual Toxic Units. Control (0TU) n = 6 and dosed treatments (>0TU) n = 5.

As a result of increasing metal contamination, community similarity between treatments and control decreased in all three mixtures in response to increasing metal concentrations (Figure 2). In CSQG and SUD mixtures, the community response was comparable with a gradual decrease in similarity. However, in the ARL mixture, a sharp reduction in similarity was observed between 1.4 and 3.2 TU. Despite the resemblance between CSQG and SUD dose response curves, significant differences from control levels were detected at different doses. In the CSQG, only the three highest doses (5.1, 9.9 and 13.2 TU) were significantly different from control levels whilst in the SUD experiment these started at lower doses (from 1.5 TU up to14.5 TU). In the ARL mixture, as expected, significant differences were registered following the large decrease in similarity at 1.4 TU. In CSQG mixture, the NOEC was 2.5 TU and the LOEC was 5.1, for the SUD mixture both estimates were lower (NOEC 0.7 TU and LOEC 1.5 TU) while for the ARL mixture estimates were higher than SUD and lower than CSQG mixtures (ARL NOEC 1.4 TU and LOEC 2.4 TU).

The dominant collembola, *H. thermophila* complex was the most important contributor to significant changes in community composition between control and treatment doses in all three mixture experiments. For secondary contributors there were some mixture specific differences. In the CSQG and SUD mixtures the level of contribution for *H. thermophila complex* was comparable (CSQG – 88.2%, SUD 80.2%) but the next most important contributor was different (CSQG, *P. armata* – 6.6% and SUD, *C. gibbosa* – 16.7%). In the ARL mixture, the contribution of *H. thermophila complex* was lower (71.2%) and both C. *gibbosa* (19.2%) and *P. armata* (1.2%) were important contributors to changes in species composition

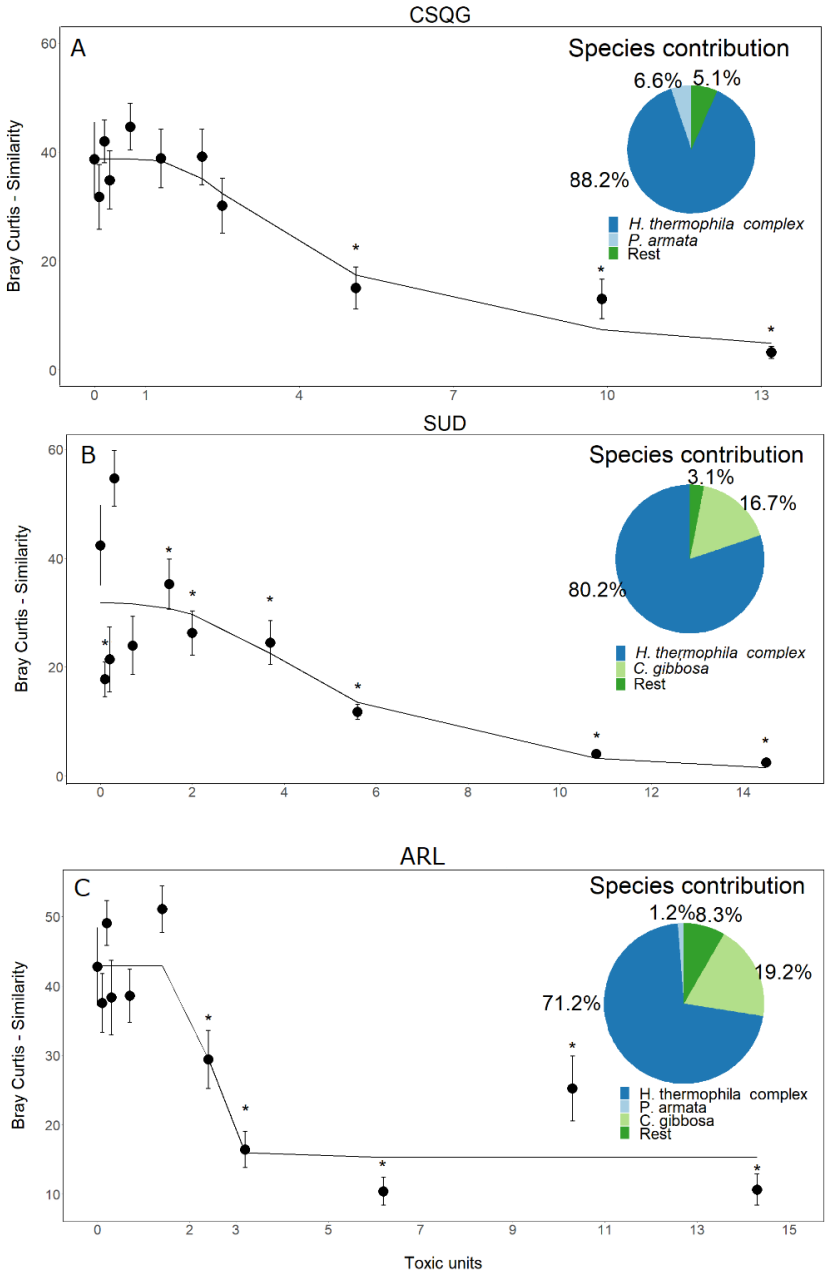

Figure 2. Average community similarity (black dots) within controls (dose 0) (n = 15) and between control and test doses (n=30), predicted values from dose response model (black line) and average species contribution to significant decreases in similarity (pie chart) for the CSQG (Panel A), SUD (Panel B) and ARL (Panel C). Error bars represent the standard error; * significant differences from control (by Permanova, for $p<0.05$); species contribution determined by SIMPER analysis. Dose in toxic units corrected for total metal concentrations – Actual Toxic Units.

Community similarity dose response curves allowed the calculation of effect concentrations which can compare mixture toxicity and risk level of current regulation (CSQG and ARL) and contaminated sites (SUD) (Table 3). CSQG and SUD community EC50 values were not significantly different from each other but were both significantly higher than the ARL EC50. In the two lower effect concentrations (EC10 and EC20) no significant differences were observed between any of the tested mixtures. Compared to their field doses, both CSQG and ARL mixtures based on legislative guidelines are well below the estimated community effect concentrations even for the most conservative EC10. For the SUD mixture, based on a contaminated site, the field dose is within the range of a community EC20.

Table 3 - Estimated community effect concentrations (EC10, EC20 and EC50 and respective 95% confidence intervals), in actual toxic units (corrected for measured total metal concentrations), dose response model, model coefficients and field dose (see Table 1 for details) for each tested mixture.

				Community Effect Concentration		
Mixture	Model	Coef.	Field dose	EC10	EC20	EC50
				(Toxic units)		
CSQG	Three-parameter Weibull $f(x) = 0 + (d - 0)exp^{(-exp^{(b(\log(x)-e)}}$	b = -1.6 d = 38.7 e = 3.7	0.52	2.1 (1.2 - 3.0)	2.7 (1.7 - 3.6)	4.6 (3.1 - 6.1)
SUD	Three-parameter Log logistic $f(x) = 0 + \frac{d - 0}{1 + exp^{(b(\log(x)-\log(e}}$	b = 2.8 d = 31.8 e = 5.0	3.11	2.3 (0.6 – 4.0)	3.1 (1.5 - 4.7)	5.0 (3.6 - 6.5)
ARL	Four-parameter Log logistic $f(x) = c + \frac{(d - c)}{1 + exp^{(b(\log(x)-\log(e}}$	b = 12.9 c =15.3 d = 42.9 e = 2.4	0.58	2.0 (1.2 – 2.8)	2.2 (1.6 – 2.7)	2.4 (2.2 – 2.6)

The individual responses of the three most abundant collembola species (*H. thermophila* complex, *C. gibbosa* and *P. armata*) did not explain community effect concentrations (Table 5). Effect concentrations calculated for individual species confidence was poor and did not allow for the estimation of 95% confidence intervals. For *H. thermophila complex* and *C. gibbosa* effect concentrations calculated (i.e. EC20, *H. thermophila complex* – 0.8, 0.0, 1.8 and *C. gibbosa* – 0.9, 0.1, 0.2 TU for CSQG, SUD and ARL, respectively) were lower than those calculated for the whole community in all three mixture experiments (i.e. EC20, CSQG – 2.7, SUD – 3.1 and ARL – 2.2 TU). In the CSQG and SUD experiments, *P. armata* did not present a dose response allowing the estimation of effect concentrations. Only in the ARL microcosm experiment, did *P. armata* produce EC_{10} values similar to community estimates.

Table 4 - Estimated effect concentrations (EC10, EC20 and EC50) based on the individual dose response of species *Hemisotoma thermophila complex*, *Ceratophysella gibbosa* and *Protaphorura armata* for each mixture experiment (CSQG, SUD and ARL).

Single Species Effect Concentration				
Mixture	Species	EC10	EC20	EC50
			(Toxic units)	
CSQG	*H. thermophila complex*	0.6 (*.)	0.8 (0.02 – 1.5)	1.3 (0.2 – 2.4)
	C. gibbosa		0.8 (*) 0.9 (*) 1.0 (*)	
	P. armata		No observed dose response	
SUD	*H. thermophila complex*	0.0 (*)	0.0 (*)	0.0 (*)
	C. gibbosa	0.0 (*)	0.1 (*)	0.3 (*)
	P. armata		No observed dose response	
ARL	*H. thermophila complex*	1.5 (*)	1.8 (*)	2.1 (*)
	C. gibbosa	0.2 (*)	0.2 (*)	0.3 (*)
	P. armata	2.0 (*)	3.5 (*)	5.8 (*)

*Data did not allow the estimation of confidence intervals

Discussion

Community composition in microcosms

The collembola community in microcosms was heavily dominated by *H. thermophila complex* followed by *C. gibbosa* and *P. armata*, and all other remaining species were rare. While the dominance of few species is not always desirable the community composition it is a reflection of the selected study site, a cork oak forest, where collembola diversity is known to be low where few species dominate (Martins da Silva, personal communication). In example with collembola sampling from litter in the same site, over 15 months, only a total of 37 species were observed of which only 10 species dominated the community (average 82.1% of total abundances across sampling periods) including *H. thermophila* (prev. *Cryptopygus thermophilus*) which was the most dominant and *C. gibbosa* the third most dominant species across sampling periods [252]. The absence of *P. armata* from the most abundant species in the study by Nascimento et al. [252], might be due to litter sampling as this species is more eudaphic living within the soil. Despite the known low richness of collembola species inherent of this study site, microcosms still presented lower total species numbers compared to Nascimento et al. [252]. Lower numbers in microcosms could be due to, differences in litter vs soil communities, to a single sampling period in microcosms compared to the 15 month monitoring over multiple seasons used by Nascimento et al, [252] and the relatively low sampling area used for microcosms (5 m^2). The small sampling area for core collection was selected to reduce variability between treatments but consequently has a lower representation of the total collembola pool of a particular site. Future studies should be conducted on different communities, potential with higher collembola richness and over different seasons to include seasonal variabilities in collembola community structure. Additionally, a plot design could be conducted sampling a larger area of a particular location, or higher tier studies such as TMEs or field experiments to better represent the full microarthropod community of a particular location.

Metal toxicity to microcosm community

Regarding metal sensitivity for the three most abundant collembola species, *P. armata* was the least sensitive, *H. thermophila* had an intermediate sensitivity and *C. gibbosa* was the most sensitive. In metal contaminated sites, *P. armata* (prev. *Onychiurus armatus*) has been described as a metal tolerant species [229,233]. Metal tolerance of this species has been attributed to shifts

in feeding preferences avoiding hyperaccumulating plants, increased moulting for detoxification [229] and metal avoidance [253]. In the microcosm experiments, moulting could be the preferred strategy, as soil contamination was homogeneous and clean food was provided, reducing the importance of avoidance behaviour and feeding strategies. Regarding *H. thermophila* complex (prev. *Cryptopygus thermophilus*), this species can be dominant in metal contaminated soil [231,254]but, according to Fountain and Hopkins [234], only in soils containing between 0 – 2000 ppm of zinc. A concentration of 2000 ppm zinc corresponds to a 2.7 TU dose (using *F. candida* EC50 in Table 1) where a reduction in the abundances of *H. thermophila complex* was observed for all mixtures (CSQG – 2.5 TU, ARL - 2.4 TU) except SUD (5.6 TU). Despite some field results supporting microcosm results for *P. armata* and *H. thermophila* some contradicting data also exist. Decreasing abundances with increasing metal contamination have been observed for *P. armata* [235]. In a different metal contaminated site, *P. armata* was found to avoid the most contaminated edges, where *H. thermophila* was abundant [232]. For the most sensitive Collembola, *C. gibbosa*, literature data on its sensitivity to metals is not known. However, *Ceratophysella denticullata*, a species from the same genus, was described as metal tolerant [231,234,255]. Considering that large differences in sensitivity can occur even within the same genus, extrapolating effects amongst different Collembola species should be avoided or carefully interpreted.

The abundance of mites was lower than the three dominant Collembola. Astigmata were the most abundant group in the community followed by Oribatid mites, while Prostigmata and, in particular, Mesostigmata mites were rare. Astigmata mites seem to follow a bell shaped distribution, a common response of microarthropods to metal stress in contaminated sites [244,256,257]. Oribatid mites did not have a consistent response between mixtures but their high abundance in the highest dose of the ARL could suggest they are not sensitive to metals. Prostigmata and Mesostigmata abundances were too low to determine a pattern of metal response. In the scientific literature, responses of mite groups to metal contamination are not consistent [256,258]. For soil mites, when identified only to the group level the interpretation of effects is complicated due to differences in sensitivity within the same group [244]. For instance, within Oribatid mites, species can have different responses towards metal contamination including both metal tolerant and sensitive species [257].

Community effect concentrations

Using species/group abundances, as described above, to compare between different contamination scenarios and determine environmental risk thresholds is a complicated process. Due to differences in sensitivity and species interactions, community abundances can follow a bell-shaped distribution [2,6,11,14], an increase [1,3,13] and a decrease [16] with increasing metal contamination. The same is observed for species richness which can follow bell-shaped distribution [6], an increase [1,12] or a decrease [8,10,11,17] with increasing contamination. In contrast, using the community similarity approach, all changes to a community contribute to a decrease in similarity towards a control or reference community. In the microcosm experiments, the proposed approach was successfully used to reflect the change in community composition with increasing contamination. For CSQG and SUD mixtures, community response was similar with gradual reductions in similarity compared to the ARL mixture where a steep decrease was observed between 1.4 and 3.2 TU. Results were unexpected considering CSQG and ARL (based on legislative guideline) are much more similar in metal ratio composition than the SUD mixture (based on a contaminated site). The decrease in community similarity with increasing metal mixture dose allowed the calculation of community level effect concentrations. These estimates can be an important tool to establish protective environmental threshold values and allow comparisons between mixtures, literature values, legislation and contaminated sites.

Due to the dominance of few collembola species, it was important to verify if a single species was determining the whole community EC values. In the microcosm experiments, except for *P. armata* EC_{10} in the ARL mixture, individual species ECx values for the three most abundant collembola did not match community level effects. Individual species estimates (except for *P. armata*) were always lower than community ECx values, potentially indicating that community ECx values may not be protective of individual species within the community. Despite some changes in species composition a result of the intrinsic spatial variability occurring in natural communities, demonstrated in the slight changes in species contributions to similarity changes between mixtures. Community estimates between mixtures at similar mixture dose levels were not significantly different (excluding ARL EC50). This means that the natural variability within a community does not highly affect the overall community response at similar dose.

Risk assessment of metal mixtures from Community EC values

Metal mixture toxicity in microcosms was lower than predicted from single species literature data. Using *F. candida* EC50 data and following a TU approach, a community EC50 (CSQG – 4.6 TU, SUD – 5.0 TU, ARL – 2.4 TU) would be expected at a mixture dose of 1 TU. Considering that current environmental regulation is based on single species data, it is not surprising that the mixture dose of regulatory ratios (field dose, CSQG – 0.52 TU and ARL – 0.58 TU) produced much lower toxicity than the calculated soil community EC10 (CSQG – 2.1TU ARL – 2.0TU). In metal contaminated sites, soil communities have also been found to be less affected than predicted from single species ecotoxicity data, with laboratory spiked soils [234,259]. Differences in toxicity could be due to lower metal bioavailability [47,259], species adaptation to metal stress [229] and avoidance behaviours [253]. In microcosms, these factors should be less relevant since, natural communities were from a reference site, not pre-adapted to metal contamination. Dosed soils were sieved and homogenously contaminated reducing the importance of avoidance behaviour and increasing organism exposure. For the microcosm experiments, differences could be due to metal mixture antagonism, detected for some binary mixtures [55,124,133], differences in soil properties known to affect metal toxicity [37], the use of oxides which can be less toxic than salts (Renaud et al. [220]) or, single species reproduction data does not translate to community level effects. Further research is required to clearly determine the cause of this discrepancy between single species and microcosm effects. However, the magnitude of difference between environmental thresholds and community effects is an indicator that current regulation could be overprotective.

For the contaminated site ratio (SUD), the field dose (3.1 TU) is within the range of an EC20 (3.1 TU). Unfortunately, no data is available on soil microarthropod communities from the Sudbury site to correlate with microcosm results. Considering microcosms were conducted using a reference community and homogeneous contamination, the field dose of this contaminated site was expected to produce an effect larger than EC20 on the soil community. Microcosm results indicate that if metal concentrations in Sudbury were reduced from a mixture dose of 3.1 to 2.1 TU, then only minimal community effects (EC10 – 2.3 TU) would be observed. However, in contaminated sites, it is common that habitat quality is impaired not only by metals but multiple other stressors. Using the example of the Sudbury region as reported by Winterhalder [260], in addition to metal deposition, this region has suffered other extensive environmental impacts from lumbering and forest fires. The resulting landscape is denuded of vegetation, has low soil pH and

organic matter, is prone to erosion and loss of nutrients. For these metal contaminated sites, a more realist approach which considers the remaining stressors other than metals may be preferred. For instance, microcosm experiments could test a gradient of field contaminated soils directly with a reference community adjacent to the site, containing its potential colonizers. Microcosm experiments, using field contaminated soils can also test the community effects of different remediation strategies prior to larger scale implementation.

Conclusion

Community similarity dose response analysis may be an important tool to interpret community responses to contamination and community effect concentrations could provide a standardized approach to determine and explore environmental protection thresholds. Using two regulatory ratios, microcosm data suggests that current guidelines for metals, based on single species, could be overprotective for the soil community when presented as mixtures. For contaminated sites, risk estimates were lower than expected and site-specific approaches could be preferred using field contaminated soils to address uncertainties in terms of their environmental risk.

Despite the successful application of community similarity dose response curves and estimation of community level effect concentrations, estimates require a calibration at higher tier. Further testing is also required to validate the overall approach testing different chemicals and soil microarthropod communities.

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Annex 3

Table 1A – Species abundance, diversity indices (Shannon Weiner and Simpson) and species richness, average value (AV) (Control, dose 0 – n = 6, treatments n = 5) per test unit and standard deviation (SD) for the CSQG mixture respective dose levels.

CSQG Mixture																								
Dose	*Hemisotoma thermophila complex*		*Ceratophysella gibbosa*		*Protaphorura armata*		*Sphaeridia pumilis*		*Pseudosinella octopunctata*		Astigmata		Prostigmata		Oribatid		Mesostigmata		Shannon Weiner		Simpson		Species Richness	
Toxic units	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD
0	296.2	285.5	1.8	4.5	3.8	4.8	0.0	0.0	0.0	0.0	3.0	5.9	0	0	1	1.6	0.3	0.5	0.4	0.5	0.2	0.3	3.2	1.5
0.1	381.2	396.0	0.4	0.6	0.4	0.9	0.0	0.0	0.0	0.0	8.8	13.8	0.8	1.79	3.8	8.0	0.6	1.3	0.4	0.5	0.2	0.3	3.0	1.4
0.2	288.4	168.3	142.4	109.4	1.4	1.5	0.0	0.0	0.0	0.0	0.2	0.5	0	0	0.2	0.5	0.6	0.9	0.6	0.3	0.4	0.2	3.2	1.3
0.3	1176.8	1111.3	22.0	44.8	0.8	0.5	1.4	3.1	0.0	0.0	9.8	16.5	0.2	0.45	0.4	0.9	0.4	0.9	0.2	0.5	0.1	0.3	3.6	1.7
0.7	339.2	347.0	39.8	37.6	6.8	11.6	0.0	0.0	0.2	0.5	0.6	0.9	0	0	4	7.8	0.4	0.6	0.5	0.2	0.3	0.1	4.0	1.6
1.3	212.4	254.0	0.0	0.0	1.4	2.1	0.0	0.0	0.2	0.5	7.4	10.1	0.4	0.55	4	7.9	0.2	0.5	0.4	0.3	0.2	0.2	3.4	1.3
2.1	194.2	271.0	0.0	0.0	4.8	7.8	0.4	0.9	0.2	0.5	12.2	16.8	1.2	2.68	0	0.0	0.2	0.5	0.4	0.4	0.3	0.2	2.8	0.8
2.5	31.6	22.7	0.0	0.0	6.6	13.7	0.0	0.0	0.0	0.0	7.0	8.9	0	0	0.2	0.5	0.2	0.5	0.5	0.4	0.3	0.3	2.6	1.5
5.1	8.2	10.1	0.0	0.0	4.6	4.3	0.0	0.0	0.4	0.6	1.4	2.0	0	0	0.8	1.3	0.2	0.5	0.8	0.6	0.4	0.3	3.2	1.8
9.9	7.0	8.4	0.0	0.0	1.3	1.3	0.0	0.0	0.0	0.0	3.0	3.2	0	0	0.5	0.6	0.3	0.5	0.7	0.4	0.6	0.3	3.0	2.2
13.2	0.6	0.9	0.0	0.0	1.2	2.7	0.0	0.0	0.0	0.0	0.8	1.8	0	0	0.2	0.5	0.2	0.5	0.2	0.3	0.3	0.4	1.2	0.8

Table 2A – Species abundance, diversity indices (Shannon Weiner and Simpson) and species richness, average value (AV) (Control, dose 0 – n = 6, treatments n = 5) per test unit and standard deviation (SD) for the Sudbury mixture respective dose levels.

Sudbury Mixture																												
Dose	*Hemisotoma thermophila complex*		*Ceratophysella gibbosa*		*Protaphorura armata*		*Sphaeridia pumilis.*		*Pseudosinella octopunctata.*		*Ahrropalites caecus.*		*Lepidocyrtus violaceus*		Astigmata		Prostigmata		Oribatid		Mesostigmata		Shannon.Weiner		Simpson		Species.Richness	
Toxic units	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD
0	747.8	772.5	48.7	56.9	2.5	3.7	1.3	3.3	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.4	0.0	0.0	4.2	8.8	0.0	0.0	0.3	0.2	0.1	0.1	3.2	0.8
0.1	59.4	57.1	40.8	89.6	3.8	8.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.6	0.4	0.6	0.0	0.0	0.2	0.5	0.2	0.2	0.1	0.1	2.8	0.8
0.2	196.3	348.5	1.0	0.8	3.3	4.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.3	4.5	0.0	0.0	0.3	0.5	0.0	0.0	0.4	0.3	0.2	0.2	3.0	0.8
0.3	527.4	308.9	45.8	63.5	1.2	1.6	0.0	0.0	0.0	0.0	0.2	0.5	0.0	0.0	0.2	0.5	0.8	0.8	0.0	0.0	0.0	0.0	0.3	0.3	0.1	0.2	3.2	1.6
0.7	247.8	404.1	4.4	6.7	6.6	8.1	0.0	0.0	9.6	21.5	0.0	0.0	0.0	0.0	2.8	4.8	1.0	2.2	0.2	0.5	0.0	0.0	0.5	0.3	0.3	0.2	3.4	0.9
1.5	219.6	185.0	3.2	6.6	10.0	18.6	0.0	0.0	2.2	4.9	1.0	2.2	0.2	0.5	5.8	9.8	1.4	3.1	0.6	1.3	0.0	0.0	0.4	0.3	0.2	0.2	3.8	1.3
2.0	127.4	121.6	3.6	4.9	12.2	17.4	0.0	0.0	0.2	0.5	0.0	0.0	0.0	0.0	0.2	0.5	0.0	0.0	0.4	0.6	0.2	0.5	0.4	0.3	0.2	0.2	3.2	1.3
3.7	116.6	102.0	0.4	0.6	15.2	19.9	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.5	0.8	1.3	0.6	1.3	0.4	0.9	0.2	0.5	0.5	0.4	0.3	0.2	3.4	2.1
5.6	38.4	23.1	0.6	0.9	4.0	3.9	0.0	0.0	0.2	0.5	0.0	0.0	0.0	0.0	1.0	1.4	0.0	0.0	1.8	4.0	0.0	0.0	0.6	0.4	0.3	0.2	3.0	0.7
10.8	8.4	7.8	0.6	1.3	1.8	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.8	1.8	0.0	0.0	0.2	0.5	0.0	0.0	0.6	0.4	0.3	0.3	2.6	1.1
14.5	6.4	9.4	0.0	0.0	0.2	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.8	0.0	0.0	0.2	0.5	0.4	0.9	0.4	0.4	0.5	0.4	2.0	1.6

Table 3A – Species abundance, diversity indices (Shannon Weiner and Simpson) and species richness, average value (AV) (Control, dose 0 – n = 6, treatments n = 5) per test unit and standard deviation (SD) for the Ag/Res/Loam mixture respective dose levels.

Ag/Res/Loam mixture																										
Dose	*Hemisotoma thermophila complex*		*Ceratophysella gibbosa*		*Protaphorura armata*		*Pseudosinella octopunctata*		*Ahrropalites caecus*		*Lepidocyrtus violaceus*		Astigmata		Prostigmata		Oribatid		Mesostigmata		Shannon Weiner		Simpson		Species Richness	
Toxic units	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD
0	448.0	462.0	77.3	98.0	21.0	25.6	0.0	0.0	0.0	0.0	0.0	0.0	2.5	3.4	0.0	0.0	3.7	6.7	0.7	1.2	0.6	0.4	0.3	0.3	4.2	1.5
0.1	116.0	113.9	37.4	48.9	23.2	26.3	0.0	0.0	3.6	8.1	0.0	0.0	0.4	0.6	1.4	3.1	2.8	4.1	0.0	0.0	0.7	0.3	0.4	0.2	3.8	1.6
0.2	310.0	246.4	128.2	215.9	5.2	5.7	0.0	0.0	0.2	0.5	0.0	0.0	4.0	5.7	1.2	1.8	0.8	0.8	0.0	0.0	0.4	0.2	0.2	0.1	4.6	0.6
0.3	974.8	723.5	1.2	2.7	16.6	19.2	0.0	0.0	0.0	0.0	0.0	0.0	3.0	5.1	2.4	5.4	0.8	0.8	1.0	2.2	0.3	0.5	0.2	0.3	3.6	1.1
0.7	143.8	106.6	45.4	88.9	7.4	12.8	0.0	0.0	0.0	0.0	0.0	0.0	32.6	61.8	0.8	1.8	0.6	0.9	0.8	1.1	0.7	0.2	0.4	0.1	4.0	2.0
1.4	404.2	288.4	2.4	2.9	27.2	21.6	0.0	0.0	0.0	0.0	0.0	0.0	10.0	19.6	0.4	0.6	7.0	8.9	0.2	0.5	0.4	0.2	0.2	0.1	4.4	1.5
2.4	49.4	40.9	0.4	0.9	19.0	7.5	0.2	0.5	0.0	0.0	0.0	0.0	1.8	3.5	4.8	6.6	0.0	0.0	0.0	0.0	0.8	0.3	0.5	0.2	3.4	0.6
3.2	25.2	17.7	0.0	0.0	11.4	14.1	0.0	0.0	0.0	0.0	0.0	0.0	17.4	36.7	0.2	0.5	0.2	0.5	0.0	0.0	0.7	0.2	0.4	0.2	2.8	0.8
6.2	10.4	14.3	0.4	0.9	6.8	5.4	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0	2.4	4.3	1.2	1.3	0.2	0.5	0.8	0.5	0.4	0.3	3.6	2.0
10.3	47.6	44.6	0.0	0.0	3.6	0.6	1.0	2.2	0.0	0.0	0.0	0.0	0.8	1.1	0.0	0.0	0.2	0.5	0.0	0.0	0.5	0.3	0.3	0.2	2.8	0.8
14.3	16.2	17.0	1.2	1.8	0.2	0.5	0.0	0.0	0.0	0.0	0.2	0.5	0.8	1.3	0.2	0.5	4.8	7.6	0.0	0.0	0.6	0.5	0.3	0.3	3.0	1.6

Chapter 5 - Are structural and functional endpoints of soil communities similarly affected by metal mixtures? – A Terrestrial model ecosystem study

Abstract

Soils are habitat to a variety of flora and fauna and a linked ecosystem which provides essential ecosystem services. Metals can accumulate at high concentrations in soil because of anthropogenic activities, leading to toxic effects threatening the ecosystem and the services it provides. In most contamination scenarios, metals occur as complex mixtures which can interact and produce different toxicity than predicted from individual metal data. Current regulatory guidelines are based on single species responses to individual metals and ignore indirect effects inherent to the inter-linked nature of ecosystems. Also, the evaluation of anthropogenic impacts to soil communities is usually measured through structural endpoints (e.g. total abundance) disregarding functional measurements (e.g. enzymatic activities and organic matter decomposition rates), which are often seen as tightly related and thus similarly affected. In this study we tested three mixture ratios of five metal oxides (lead, copper, nickel, zinc, cobalt) at three dose levels (Low, Med, High) in a terrestrial model ecosystem experiment and measured structural and functional endpoints. Exposure to metal mixtures for 16 weeks did not affect invertebrate community abundances but produced severe effects on soil enzyme activity reducing activity below 50% compared to control levels, in all dosed treatments. Metal contamination also significantly affected feeding activity and organic matter decomposition, but effects were not as pronounced as enzymes. Data suggest that, the in risk assessment of metals and their mixtures effects on ecosystem structure and functions must be considered to provide adequate environmental protection.

Introduction

Soils provide habitat and support for a large assortment of flora and a large diversity of fauna. In-soil organisms encompass two groups, microorganisms and soil fauna including macro (e.g. earthworms) , meso (e.g. Collembola and mites), and micro (e.g. nematodes) fauna [141]. The biodiversity of in-soil organisms is essential not only for its intrinsic value, but also to maintain soil ecosystem functioning. In-soil organisms are key players in many soil functions, such as nutrient cycling and organic matter decomposition, maintenance of soil structure and soil purification, that are intimately linked to the provision of key ecosystem services essential to human life [140,141,176,180,183]. High biodiversity provides functional redundancy, which allows the maintenance of functional systems and provides resistance and/or resilience to natural and/or anthropogenic stressors [141]. To maintain the health of soil systems, both structural and functional aspects should be monitored and protected against environmental stressors [183]. However, these two components are usually considered to be related, similarly reactive against stressors and are usually not measured simultaneously.

The importance of the soil system is recognized but most ecotoxicology research considers contaminant effects to individual components (i.e. single invertebrate species, enzyme activities). For instance, current metal regulatory guidelines and protective soil thresholds are based on single species data on a limited number of organism groups [8,11]. Analysing individual compartments ignores that the components of the ecosystem are interlinked with complex predator/prey and competition relationships (inter and intraspecific relationships). Contamination can affect species directly but also produce indirect effects on non-sensitive populations by affecting the relations between them [175,231,234,258,261,262]. Laboratory testing on single species and structural microbial endpoints also ignore ecosystem functioning which many times does not have a direct relation to structural effects [116,176,263]. In recent years, terrestrial model ecosystems (TMEs) have been proposed for higher tier ecotoxicological testing [141,264]. TMEs have been used to evaluate both structural and functional effects [175–177,180,181,185,243] and also the fate [171] of contaminants in soil ecosystems.

In soil, metals are naturally occurring from the weathering of pedogenic material but can become an environmental concern when resulting from anthropogenic activities (e.g. mining and smelting). These anthropogenic activities increase the accumulation of metals at a much larger rate than in pedogenic processes, leading to high metal concentrations, which produce toxic

effects in the environment [4,265]. In most metal contaminated sites, metals occur in complex mixtures [231,234,258,261,266], but most research [37,38,56,100,267–269] and regulatory threshold values [8,11] focus on single metals using single species data. In mixtures, concentration addition is the proposed default model for the joint action of metals [118], however metals deviate from the additivity assumed by concentration addition [55,118,135]. Testing metals (and metal mixtures) experimentally in the field is a complicated task due to their persistence. Unlike organic contaminants, high experimental doses of metals in field tests will not gradually degrade over time causing an environmental issue which requires remediation or removal actions. In this case, TMEs are particularly well suited to test high doses of complex metal mixtures. In TMEs, the soil is dosed after its collection from the field as intact soil cores that are kept in controlled experimental conditions and can be safely disposed after experimentation has ended. As a more realistic approach, TMEs are currently considered as a calibration tool for lower tiers in risk assessments. Direct comparisons between single species effects and TMEs are scarce but the accuracy of laboratory data (single species) towards TMEs was found to be dependant on depth and less accurate in deeper soil layers where laboratory data had a higher underestimation of effects [141].

Metals do not degrade but their toxicity to organisms is variable and depends on their bioavailability. The bioavailability of a metal depends on chemical reactions involving precipitation/dissolution which are highly dependent on soil properties [95]. Soil pH, cation exchange capacity (CEC), clay content, Fe and Mn oxides and soil organic matter are the properties mostly known to affect the availability of metals in soil [94–96] and their toxicity to soil organisms [37,38,56,100]. Soil pH is particularly important in regulating the chemical reactions of metals in soil [94,96] and is a generally good predictor of metal availability [100]. Also, the chemical form of metals (metal speciation - oxides, chlorides) can itself produce different levels of toxicity to soil invertebrates [48,53,62] and in contaminated sites various chemical forms of metals can be present [196]. In laboratory testing, metal salts are the most commonly used, but for fixed ratio metal mixtures, metal oxides are more adequate as they do not require leaching to remove counter-ions which can affect mixture ratios [220].

In this study we tested the effects of mixtures of five metal oxides (lead, copper, nickel, zinc and cobalt) on the soil ecosystem structure and function using a TME approach. Metal mixtures were tested in three fixed ratios, and each mixture was tested at three doses. The effect of metal mixtures was evaluated by measuring structural and functional parameters. The working hypothesis assumes that the highest doses of the test mixtures will negatively impact the

structural parameters (mite and Collembola abundances). Compared to soil invertebrate abundances, microbial endpoints (enzyme activities) are expected to be more resilient to metal stress due to microorganism's ability to rapidly adapt to metal stress. The expected effects on structural endpoints should also negatively impact ecosystem function (i.e. reducing feeding activity and organic matter decomposition).

Materials and Methods

TME collections

A total of 62 TMEs (17.5 cm diameter and 40 cm height) were collected from an agricultural field under sustainable management and with no pesticide for more than five years. The experimental field is in the city of Coimbra (40°13'01.6"N, 8°26'43.5"W) and the soil properties for the site are in Table 1. Soil pH was measured in 1 M KCL, soil total carbon, cation exchange capacity (CEC), water holding capacity (WHC) and particle size distribution were determined following ISO guidelines [205,270–272]. Soil organic matter was estimated by multiplying total C by 1.724. TMEs were all collected from the same area, spaced at an interval of approximately 20 cm and extracted as described in Knacker et al. [264]. Briefly, vegetation was cut to a homogenous size (approximately 2 cm) and TME cores were carefully extracted using a metal corer and a hydraulic soil excavator.

Table 1 – Physical and chemical properties of the natural soil from the experimental site used for TME core extractions

Soil properties	
OM (%)	3.4
Total C (%)	2.0
pH (1M, KCl)	5.3
CEC (meq/100g)	24.3
WHC (%)	75
Texture	Silty loam
Sand (%)	13.2
Silt (%)	55.0
Clay (%)	24.7

Experimental design

Three fixed ratio metal oxide mixtures of lead, copper, nickel, zinc and cobalt based on environmental (ratio in contaminated site in Sudbury, Canada - SUD) and regulatory guidelines (EU REACH PNEC concentrations for different soil types and Canadian soil quality guideline thresholds - ARL and CSQG) were tested in the TME experiment (for a detailed description on metal mixture ratios see Chapter 3). Commercially available metal oxides of lead (Pb_3O_4, purity 99%, Sigma-Aldrich, Darmstadt, Germany), copper (CuO, purity 96%, Panreac Applichem, Barcelona, Spain), nickel (NiO, purity 99.8%, Sigma-Aldrich, Darmstadt, Germany), zinc (ZnO, purity 99%, Panreac Applichem, Barcelona, Spain) and cobalt (Co_3O_4, purity 99.5%, Sigma-Aldrich, St Louis, MO, USA) were mixed at the appropriate ratio for each of the three test mixtures. Each metal mixture ratio was tested in TMEs at three dose levels: Low (community EC10); Medium (community EC50); and High (2x Community EC50) based on the results obtained on Chapter 3. The appropriate dose of metal oxide mixture to add in each TME, at a depth of 5 cm, was determined based on a calculated soil mass of 1069 g per a 5 cm depth soil layer. This soil mass was calculated based on the internal diameter of the TME (16.5 cm), a depth of 5 cm and assuming a soil bulk density of 1.5 g/cm^3. The nominal concentrations (0-5 cm depth) are presented together with measured total metal concentrations (0-5 and 5-10 cm depth) in the results section (Table 2).

Dosing was performed on the same day as the field collection by carefully transferring the top 5 cm layer of soil from the TME to a plastic vase, breaking down major aggregates by hand and

adding and mixing the metal oxide mixture with a spoon (until a seemingly homogeneous mixture was obtained) for each treatment. Once the metal oxides were mixed into the soil, the dosed soil was gently packed back into its respective TME while avoiding to over compact the soil. The same procedure was performed for control TMEs but without the addition of metal oxides. Each dosed treatment was performed with 6 TME replicates whilst the control had 8 TME replicates. After dosing was completed, TMEs were placed in carts with circulated cool air to simulate conditions belowground (12 ± 2ºC) and were kept in a climate-controlled chamber at a photoperiod of 16:8 ligth:dark cycle, with a light intensity of 8,000 to 12,000 lux, an average temperature of 26 ± 4ºC and an average relative air humidity of 42 ± 8%. The different TME treatments were randomly distributed in each cart (figure 1A, Annex 4). TMEs were incubated in a climate-controlled chamber for 16 weeks starting from the day of dosing. Over the experiment, TMEs were watered three times a week with 100 ml of artificial rainwater modified after Velthrost [273]. Moisture probes were inserted in each TME to monitor soil moisture over the experiment.

One day after dosing, a hard plastic netting (4 mm mesh size) was placed at the soil surface of each TME, and 4 freshly collected soil cores (5 cm diameter and 5 cm depth) were placed inverted on the surface of the plastic mesh (see figure 2A – Annex 4). Soil cores were used to enrich microarthropod abundances after dosing TMEs simulating the recolonization of the impacted area (resulting from soil mobilization and mechanical disturbance) by organisms from the surrounding areas. The soil cores used in this step, were hand collected from the same agricultural site used for TME extractions. During the simulated recolonization, TMEs were watered as described above, but carefully avoiding moisture reaching soil cores. After seven days both the soil cores and plastic net were removed from the TMEs.

In the 16 week incubation period, feeding activity and organic matter decomposition were measured using bait-lamina and litterbags. The procedures adopted in these measurements, as well as those used for the assessment of soil microarthropods, soil enzymes, and soil metal content, are described below. After 16 weeks, the experiment was ended and destructive sampling was performed. In this procedure, soil samples were collected at different depths for each TME core. In the 0-5 cm depth, samples were collected for soil moisture and pH, microarthropod extraction, enzyme activity and total metal concentrations. In the 5 – 10 cm depth, samples were collected for microarthropod extraction and total metal concentrations. Finally, in the 10 – 20 and 20 – 40 cm layers, samples were collected only for total metal analysis. The TME experiment timeline and sampling strategy is summarized in figure 1.

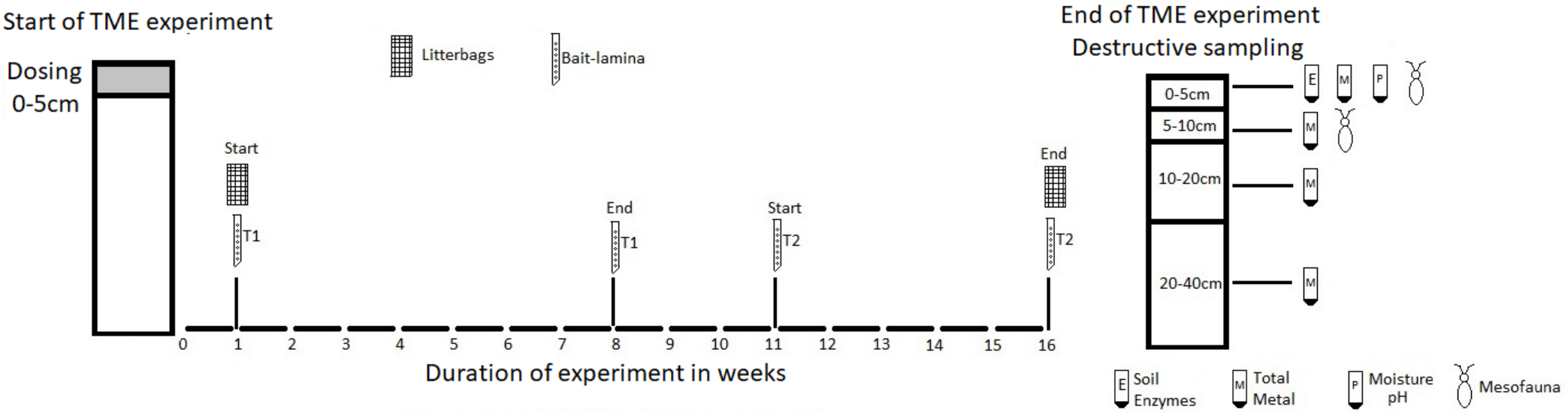


Figure 1 – TME experiment timeline and sampling design

Feeding activity and organic matter decomposition

Bait-lamina were prepared as described in ISO 18311 [274] and 4 bait-lamina sticks were placed in each TME. Two time periods were used for measuring soil fauna feeding activity with bait-lamina. Period 1 (T1) started at week 1 (one week after dosing) and ended at week 8 and period 2 (T2), started at week 11 and ended immediately before TME destructive sampling at week 16 (Figure 1). Once removed from TMEs, bait-lamina were stored at 4ºC (over a period no longer than 8 weeks) until analysis to reduce/stop microbial degradation. The feeding activity in bait-lamina strips was measured by facing each strip against a lamp and classifying the bait in each pore as consumed (more than 50%) or not consumed (less than 50%). For statistical analysis, the average percent consumed baits per TME was used.

Litterbags were constructed following the OECD guideline nº56 [275] with some modifications. Smaller size litterbags (2 cm wide and 5 cm length) with 2 mm mesh size were used and filled with straw litter from the site where TMEs were collected. Litterbags were added to the soil surface of TMEs one week after soil dosing and removed at week 16 immediately before TME destructive sampling. Once removed from TMEs, litterbags were placed in a cold chamber (4ºC) until analysis (over a period no longer than 8 weeks) to interrupt microbial degradation. Litter decomposition was analysed by estimating the percent of initial weight of litter lost through decomposition at the end of the exposure period. This percentage was calculated based on litter ash-free weight values after applying a soil and plant correction factor to the ash-free weight of initial litter as described in the OECD nº 56 [275].

Soil microarthropods

For microarthropods half of the soil of a TME core (approximately 500 g) was collected from each depth layer (0-5 and 5-10 cm) and placed into loosely closed plastic bags. Collected samples were stored in a climate chamber at 20ºC until extraction (over a period no longer than 2 weeks). Microarthropods were extracted from soil samples using a high gradient MacFayden extractor over 7 days at a temperature of 45ºC directly into an 80% ethanol solution. Extracted organisms were sorted into mites and Collembola using a binocular microscope with 40x magnification. Mites were further identified to major groups, Oribatida, Prostigmata, Mesostigmata and Astigmata according to Lindquist et al. [276]. Collembola were identified to the species level when possible (following specific literature [277–281]) using a

stereomicroscope with 400x and 1000x magnification. For Collembola, morphological trait scores (based on ocelli, furca, antenna, pigmentation, scales/hairs) were also determined to calculate the community trait weight mean (mT). The full list of trait scores is presented in Annex 4 (table 1A – Annex 4). For Collembola, species abundance data was also used to calculate species richness and diversity indices (Simpson and Shannon diversity indices) for each mixture and dose treatment.

Enzyme activity

For the measurement of enzyme activities, samples were collected from the 0-5 cm layer (approximately 300 g) in each TME, sieved at 2 mm and stored at 4^{o}C until use (over a period no longer than 4 weeks). Prior to enzyme determinations, soil samples were incubated in the dark for 48h at 20^{o}C to re-establish microbial activity. Three enzymes were analysed in each TME: dehydrogenase activity (DHA), potential nitrification (PNR) and acid phosphatase (AP). DHA is an indicator of overall microbiological activity, PNR is an indicator of the nitrogen cycling function and AP is an enzyme related to the regulation of the phosphorus cycle. Enzyme assays were conducted following standard methods adapted for analysis in a microplate reader as described in Ng et al. [243]. An aliquot of each soil sample was centrifuged at 3000 rpm prior to analysis in a microplate reader (Tecan, Sunrise remote) and 200 µl of supernatant was used per plate well. For DHA and PNR, the supernatant extinctions were measured at 546 nm and for AP at 405 nm.

In the analysis of DHA, three replicates of 1 g were incubated per sample, for 24h at 40^{o}C in 1 ml substrate solution of 1%, 2,3,5-trisphenyltetrazolium chloride (TTC) in pH 7.7, 0.1 M tris-hydroxymethyl aminomethane (THAM) buffer. After incubation, 5 ml of acetone solution was added, and samples were placed on a rotatory shaker for 2h in the dark. A control replicate was also prepared for each sample using only the buffer solution (THAM) during incubation.

For PNR, two replicates of 2 g were incubated per sample for 5 h on a rotatory shaker in 8 ml of 0.1 mM of Ammonium sulphate and 0.1 ml of 1.5 M sodium chlorate. After incubation, 2 ml of potassium chloride were added to each sample and samples were mixed and centrifuged at 3000 rpm. The supernatant (5 ml) was transferred into other test tubes to which 3 ml of ammonium chloride buffer (0.19 M, pH 8.5) and 2 ml of colour reagent composed of 84 mM sulphanilamide, 2 mM N-(1-naph-thyl)-ethylenediamine hydrochloride and 8.5% phosphoric acid were added.

For each sample, a control was also conducted, following the same procedure but frozen during the incubation period.

For AP, three replicates of 1 g were incubated per sample for 2h at 35ºC in 1 ml substrate solution of 720 mM p-nitrophenyl-phosphate solution and 4 ml of 50 mM Acetate buffer. After incubation 4 ml of THAM buffer (0.1 M, pH12) and 1 ml of 0.5 M calcium chloride was added to each tube. For each sample, a control was prepared using only buffer solution (acetate buffer) during incubation.

Metal analysis

For metal analysis, soil samples were collected at four different depths from each TME (0-5, 5-10, 10-20 and 20-40 cm). The soil, from each depth, was homogenized and approximately 150 g of soil was stored into plastic vials. After collection, soil samples were air-dried in the laboratory at approximately 20ºC and ground to a fine powder. Metal digestion was performed by adding 0.1 g of soil sample in a teflon vessel with 2 ml of distilled nitric acid and digested under pressure in a PDS-6 system (Loftfields analytical solutions, Neu Eichenberg, Germany) at 150ºC for 10h. Resulting extracts were diluted with ultra-pure water to an acidity of 3% and analysed in ICP-MS. Replicate blanks were performed with each ICP-MS analysis as well as samples containing the standard reference material SRM 2709 (San Joaquin Soil-Standard Reference Material) certified by the National Institute of Standards and Technology (US Department of Commerce). Resulting metal data was corrected for reference material values and for dosed treatments a background correction was performed to determine the added mixture dose.

Data analysis

The significance of the effects of metal mixtures on enzyme activity, bait-lamina, litter bags, fauna diversity indices, species richness and Collembola traits was determined using generalized or linear (when data distribution was normal) mixed models (GLMM and LMM, respectively). Prior to analysis, data was analysed for the presence of outliers using Cleveland dot-plots and boxplots. Fixed factors in the analyses were the experimental treatment (Control, ARL low, ARL med, ARL high, CSQG low, CSQG med, CSQG high, SUD low, SUD med and SUD high) and the soil moisture measured in the final destructive samplings in each TME. Mixture and dose were not included as separate independent fixed factors in the models because it would require a very high number of replicates per treatment. The carts among which TMEs were distributed

(four carts in total) were used as random factor. Model fit was determined using residuals vs fitted plots and normality of residuals was determined through QQ plots. When QQ plots were insufficient to discern normality, a Shapiro-wilks test was performed. For non-normal distributions, GLMMs with different distributions were considered (Gaussian with log link, Gamma and log-normal) however, when assumptions were not met, or model fit was not adequate, the response variable was transformed directly (Log or square-root transformed) in an LMM. In cases where the influence of soil moisture in samples was non-significant, this variable was removed as a fixed factor. Moreover, the random factor (cart) was removed when its variance was 0 (LMM), or when model fit improved in its absence (GLMM). Significant differences between control and dosed treatments were determined using a post-hoc Dunnett test. In addition to the tests referred above, statistical analysis was also performed on individual collembolan species and mite groups although most did not allow fitting a statistical model. Analysis of individual species was restricted to those with a relatively high abundance, with more than a few punctual occurrences and with low variability (i.e. *Hemisotoma thermophila* and *Paratulbergia macdougalli*).

Significant effects of metal mixtures to microarthropod communities were determined using permutational multivariate analysis of variance (Permanova). This analysis was performed under three different scenarios, using the full dataset, analysing Collembola and mites analysed separately and considering only the most important Collembola species (highest percentage of influence), as determined by similarity of percentages (SIMPER) analysis. The species selected as most important were those present in all treatment vs control comparisons with an average contribution higher than 5%, representing at least a cumulative contribution to community changes above 75%. Permanova and SIMPER analyses were performed using the statistical software PRIMER 6 version 6.1.13 [250] and PERMANOVA+ version 1.0.3 [282].

Except for the multivariate statistics performed in PRIMER 6 and PERMANOVA+, all analyses were performed in R, using different statistical packages. Mixed models were analysed with nlme [283] and multcomp [46], Collembola mean trait scores (mT) were calculated using the package FD [285] and diversity indices were calculated using Vegan [251]. All figures presented were constructed using the ggplot2 package [224].

Results

In the top 0-5 cm layer, soil pH increased with metal dosed treatments, but soil moisture was highly variable independently of metal treatment (Table 2A – Annex 4). Metal mixtures increased soil pH, but this increase was never above 0.65 values of pH compared to control. Soil moisture at the end of the 16-week incubation ranged from 2.2 to 34.3%.

Total metal concentrations measured in the top 0-5 cm soil layer were higher than the target nominal dose (Table 2). One factor that might have contributed to these higher values could be an overestimation of the soil mass (possibly real soil density < 1.5 g/ cm^3) when determining the nominal doses for the TME experiment in the 0-5 cm soil layer. Mixture ratios, between doses were generally maintained with variation in ratio for all mixtures below 5% per element except for zinc (ARL 7.1%, CSQG 6.3% and SUD 7.3%) and in one case for lead (SUD 6.7%) (Table 3A – Annex 4). The average coefficient of variation for each element across treatments and doses was always lower than 35%, at the 0-5 cm layer (Pb = 26.0%, Cu = 22.0%, Ni = 28.8%, Zn = 34.1%, Co = 33.4%). Dosing was only performed in the top 0-5 cm layer but metals were still detected above background values in the 5-10 cm depth (Table 2). The amount of metals present in the 5-10 cm was on average 13% of total metals (after background correction) introduced by dosing in the 0-5 cm layer with some variation between, metals, doses and mixtures but without a particular pattern (Table 3A – Annex 4). In the 10-20 cm layer, only samples from 9 TMEs randomly selected (representing 14.5% of the total) were analysed and concentrations were never higher than the background level. No further samples were analysed at the 20-40 cm layer and it was assumed that metal contamination was restricted to the 0 – 5 and 5 – 10 cm layers.

Table 2 – Nominal and measured total metal concentrations (average ± standard deviation; n = 6 or 8) for each mixture ratio (ARL, CSQG or SUD) and dose (Low, Med or High) at a depth of 0-5 cm and measured metal concentrations at the 5-10 cm depth. For more information regarding mixture ratios and dose see the text.

			Metal concentrations (mg/kg)				
			Pb	Cu	Ni	Zn	Co
Background		Measured	69.9 ± 77.3	21.0 ± 3.8	19.3 ± 4.8	129.4 ± 59.9	8.6 ± 1.6
ARL 0 - 5 cm	Low	Nominal	199.6	210.2	134.4	481.6	113.7
		Measured	241.1 ± 27.5	257.5 ± 26.1	169.7 ± 29.7	818.4 ± 240.6	168.4 ± 39.0
	Med	Nominal	212.5	223.7	143.1	512.6	121.0
		Measured	280.1 ± 71.5	290.5 ± 90.4	191.4 ± 72.9	916.8 ± 371.1	193.4 ± 79.4
	High	Nominal	425.0	447.4	286.1	1025.2	242.1
		Measured	526.6 ± 157.4	515.2 ± 102.0	364.6 ± 92.3	2103.3 ± 610.7	368.5 ± 95.6
CSQG 0 - 5 cm	Low	Nominal	213.0	191.5	136.9	608.3	121.7
		Measured	257.0 ± 54.0	223.5 ± 41.2	156.1 ± 42.2	925.5 ± 272.0	163.0 ± 57.3
	Med	Nominal	361.9	325.4	232.6	1033.5	206.7
		Measured	368.0 ± 123.6	306.50 ± 75.8	211.2 ± 70.6	1405.3 ± 732.4	207.7 ± 86.1
	High	Nominal	723.7	650.8	465.2	2067.0	413.4
		Measured	843.7 ± 171.8	713.9 ± 131.4	517.6 ± 131.1	3009.1 ± 1077.2	505.4 ± 173.8
SUD 0 - 5 cm	Low	Nominal	746.9	51.9	95.9	386.1	49.3
		Measured	963.7 ± 200.5	62.3 ± 12.9	114.9 ± 25.2	677.3 ± 255.9	64.9 ± 22.5
	Med	Nominal	1933.2	134.3	248.3	999.4	127.6
		Measured	2557.1 ± 429.8	169.7 ± 43.8	329.2 ± 115.9	1686.3 ± 417.6	192.8 ± 62.3
	High	Nominal	3866.5	268.6	496.5	1998.7	255.3
		Measured	4369.1 ± 1610.9	325.1 ± 92.6	606.5 ± 216.6	3277.4 ± 918.9	371.2 ± 120.8
ARL 5 - 10 cm	Low	Measured	37.8 ± 50.1	61.3 ± 53.0	35.5 ± 32.5	118.5 ± 127.7	38.0 ± 34.2
	Med	Measured	66.1 ± 93.9	59.2 ± 79.4	40.3 ± 55.1	161.3 ± 206.0	40.2 ± 56.2
	High	Measured	59.6 ± 71.5	70 ± 66.0	44.5 ± 43.4	158.9 ± 153.3	47.4 ± 49.2
CSQG 5 - 10 cm	Low	Measured	9.8 ± 8.7	31.3 ± 17.4	21.8 ± 12.8	81.8 ± 61.2	22.1 ± 12.1
	Med	Measured	82.3 ± 138.3	88.6 ± 109.7	59.5 ± 65.0	258.4 ± 290.1	61.7 ± 66.0
	High	Measured	85.5 ± 53.9	80.7 ± 61.5	59.3 ± 44.3	312.7 ± 229.0	62.3 ± 48.3
SUD 5 - 10 cm	Low	Measured	115.8 ± 167.1	8.3 ± 12.1	14.6 ± 23.1	73 ± 104.8	9.7 ± 15.1
	Med	Measured	538.3 ± 624.4	34.1 ± 37.2	63.1 ± 69.0	257.7 ± 281.6	38.8 ± 41.8
	High	Measured	886.3 ± 951.6	59.1 ± 62.7	102.5 ± 108.2	440.4 ± 481.1	64.6 ± 67.7

The soil microarthropod community in TMEs was dominated by mites which had twice the total abundance of Collembola (mite total abundance 8324, Collembola total abundance 4837; Figures 2 and 3). Abundances were higher in the superficial soil layers (total abundance mites – 6467 and Collembola – 3658 in the 0-5 cm layer) compared to the deeper soil layers (total abundance mites- 1847 and Collembola – 1179 in the 5-10 cm layer). For Collembola a total of 23 species were found in both soil layers but many were rare, contributing less than 1% to the total community abundance (*Ceratophysella sp., Isotomodes productus, Protaphorura juv, Pseudosinella blind, Sinella tenebricosa, Sminthurides juv, Sminthurides parvulus, Sminthurinus juv, Symphypleona juv*). *H. thermophila* was clearly dominant (47.5% of total Collembola) and only three species, excluding *H. thermophila*, contributed more than 5% to the total Collembola abundance (*Protaphorura hortensis* – 17.8%, *Isotomurus c.f. pseudopalustris* – 5.6%, *Mesaphorura sp.* – 5.3%). For mites, the most abundant groups belong to Mesostigmata (33.7% of total) and Astigmata (33% of total), followed by Oribatida (17.5% of total) and Prostigmata (15.9% of total).

Metal oxide mixtures did not affect the soil microarthropod community abundances in any of the tested ratios or doses when compared to controls. Permanova results revealed no significant effects were observed at either measured depths (0-5 and 5-10) when considering the whole community (0-5 cm, *p=0.64*, Pseudo-F=0.94; 5-10 cm, *p=0.22*, Pseudo-F=1.12) or Collembola and mite separately (Collembola 0-5 cm, *p=0.28*, Pseudo-F=1.09; 5-10 cm, *p=0.54*, Pseudo-F=0.97 and mites 0-5 cm, *p=0.58*, Pseudo-F=0.93; 5-10 cm, *p=0.36*, Pseudo-F=1.07). For Collembola, even when considering only the most important species, no significant effect of treatments was observed at both test depths (0-5 cm, *p=0.36*, , pseudo-f=1.05 and 5-10 cm, *p=0.52*, pseudo-f=0.97). Also, no significant effects were observed on Collembola species richness or Shannon and Simpson diversity indices (Figures 3A-5A – Annex 4). Collembola mean traits scores increased because of metal contamination (Figure 6A – Annex 4) but effects were only significant in the high dose of the CSQG mixture (*p=0.002*) for the 0-5 cm soil layer.

Except for *H. thermophila* and *P. macdougalli*, no other individual species of Collembola or group of mites was significantly affected by metal contamination. *H. thermophila* average abundances were always lower than control, except for the SUD – Med treatment in the 5-10 cm layer (Figure 7A – Annex 4). However significant decreases were only observed for three treatments in the 0-5 cm layer (ARL – Med p=0.01, CSQG - Low p=0.04 and CSQG - High p=0.02) and one treatment in the 5-10 cm layer (ARL – Med p=0.01). For *P. macdougalli*,

significant effects were detected for the ARL – Low treatment in the 0-5 cm layer where abundances where much higher than control (Figure 8A – Annex 4).

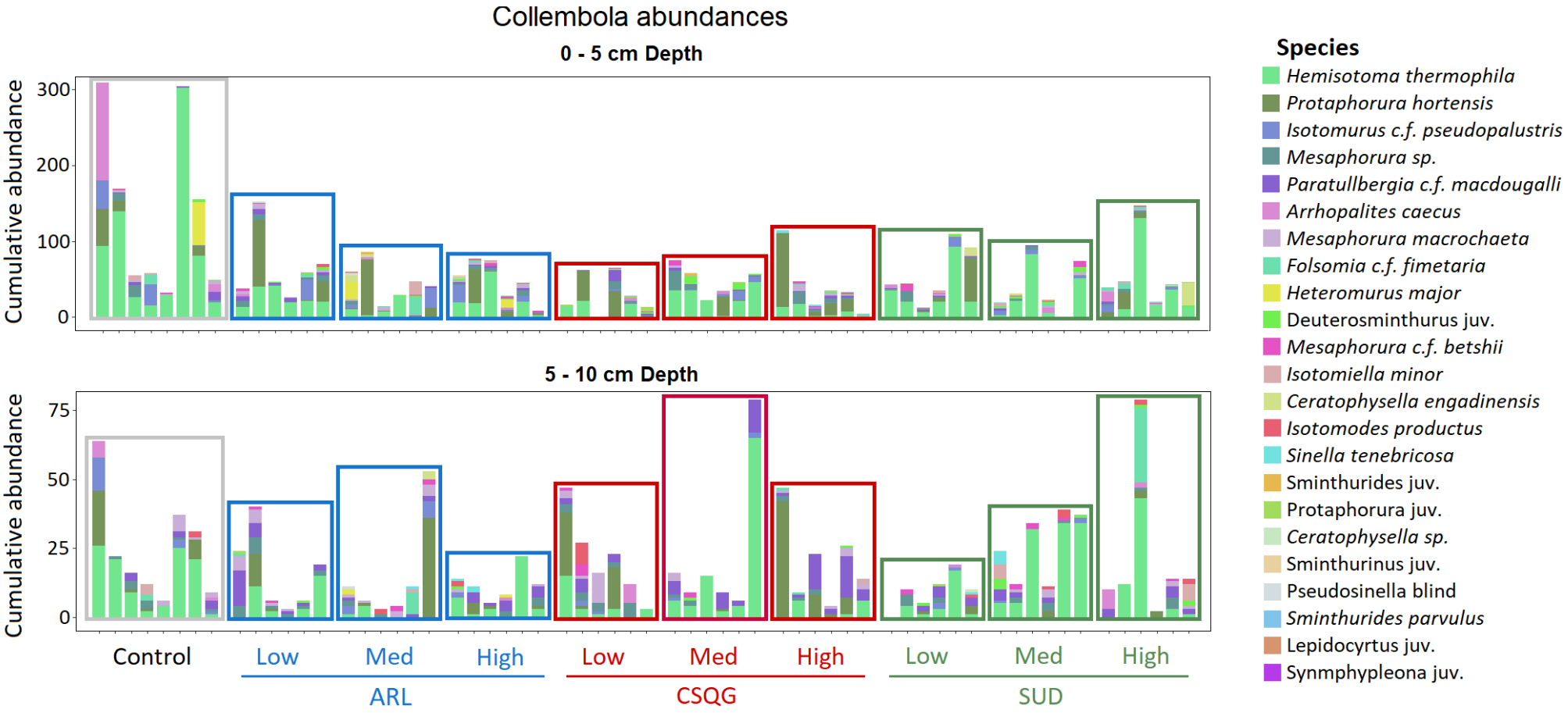


Figure 2 – Cumulative abundance of each Collembola species after sixteen weeks of incubation in each TME of the control and of the three metal mixture ratios (ARL, CSQG and SUD) with three doses per ratio (Low, Med and High).

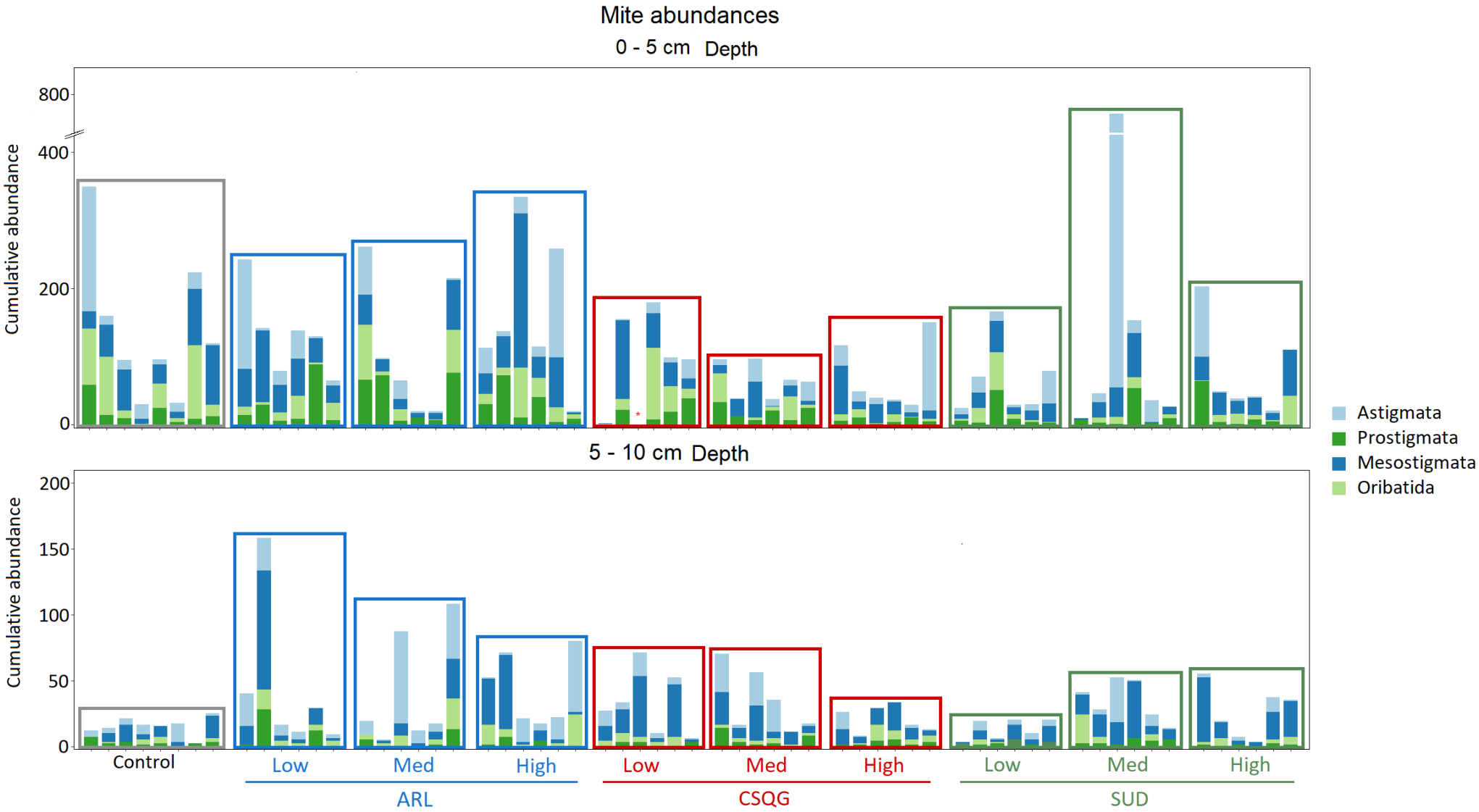


Figure 3 – Cumulative abundance of each mite group after sixteen weeks of incubation in each TME of the control and of the three metal mixture ratios (ARL, CSQG and SUD) with three doses per ratio (Low, Med and High).

Regarding enzyme activities (Figure 5 A-C), DHA and PNR were significantly affected by soil metal contamination at all mixture dose levels ($p<0.001$ for all treatments) but AP was not significantly affected at any test dose ($p=0.07$). For DHA, all treatments reduced activity to below 50% of control levels with activity decreasing with increasing mixture dose (ARL - Low 19.5%, Med 18.3%, High 14.9%, CSQG – Low 20.0, Med 15.5%, High 8.0% and SUD - Low 39.8%, Med 25.4%, High 10.6%). In the ARL mixture, there was no significant differences between doses but in CSQG and SUD mixtures, Low (CSQG Low-High and SUD Low-High $p<0.01$) and Med doses (CSQG Med-High $p=0.03$, SUD Med-High $p<0.01$) were significantly higher than the High dose. For PNR, activity was significantly affected ($p<0.01$ for all treatments) and reduced to below 50% of control in all treatments (highest average value found SUD - Low 44.8%) and a dose response pattern is only evident for the SUD mixture ratio (SUD - Low 44.8%, Med 28.3% and High 19.9%). However, significant differences between doses were never detected for any mixture ratio. Regarding AP, all treatments showed lower values than control, although not treatment was not significantly affected ($p=0.07$), and average activity was never below 75% of control. In the ARL (Low 90.7%, Med 85.9%, High 81.3%) and CSQG (Low 94.0%, Med 81.0%, High 76.1%) mixtures, a slight decrease of activity with increasing doses is observed but for the SUD mixture ratio (Low 80.3%, Med 84.4%, High 87.0%) the opposite dose-response pattern seems to occur.

Soil feeding activity and organic matter decomposition were affected by soil metal contamination even in the absence of effects to microarthropod community abundances (Figure 5A-C). For bait-lamina, in the T1 sampling, all treatments presented lower values than control, but only four treatments were significantly different (ARL – High, $p<0.01$; CSQG – High, $p<0.01$; SUD – Med, $p=0.01$ and SUD – High, $p=0.01$). In the second sampling date (T2), feeding activity recovered and only ARL - Med ($p<0.01$) was significantly lower than control. Compared to bait-lamina data, litterbag results were much more consistent within treatments and all medium (ARL – Med $p<0.01$; CSQG – Med $p=0.01$; SUD – Med $p=0.01$) and high treatments (ARL, CSQG and SUD – High, $p<0.01$) presented a significantly lower organic matter degradation, for all test ratios.

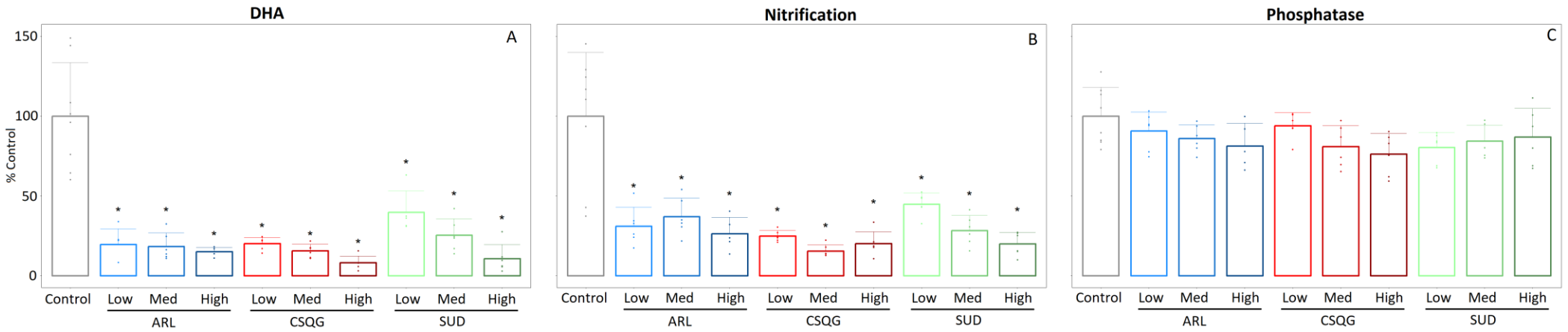


Figure 5 – Dehydrogenase activity (DHA; graph A), Potential nitrification (Nitrification; graph B) and Acid Phosphatase (Phosphatase; graph C) measured in percentage of control after sixteen weeks of exposure to three metal mixture ratios (ARL, CSQG and SUD) with three doses per ratio (Low, Med and High). Bars represent the averages, error bars the standard deviation of the mean (n = 8 Control, n = 6 treatments) and points represent individual observations for each TME. * - Significantly different than control ($p \leq 0.05$)

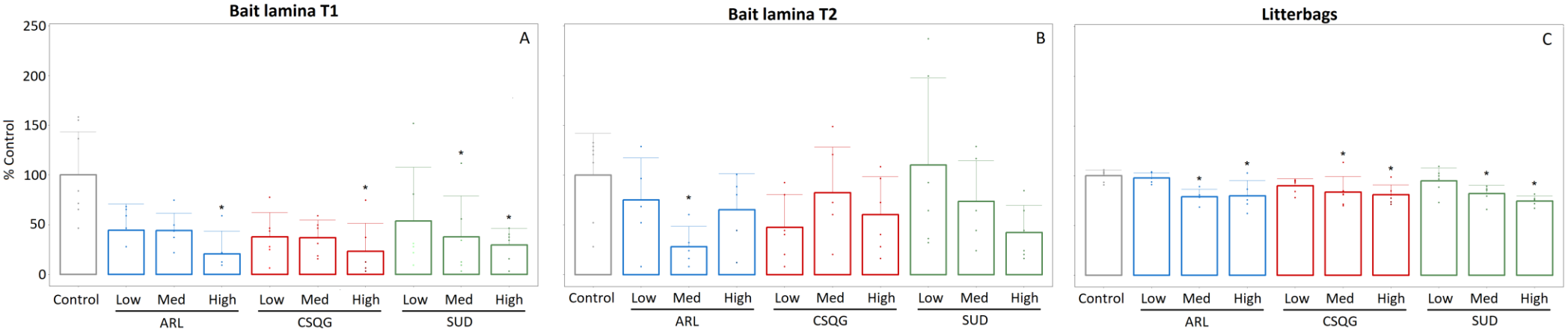


Figure 6 – Percent of control of bait-lamina feeding activity between week one and week eight after dosing (T1 – graph A), between week eleven and week sixteen after dosing (T2 – graph B) and litterbag decomposition sixteen weeks after dosing (graph C) with three metal mixture ratios (ARL, CSQG and SUD) and three doses per ratio (Low, Med and High). Bars represent the averages, error bars the standard deviation of the mean (n = 8 Control, n = 6 treatments) and points represent individual observations for each TME. * - Significantly different than control ($p \leq 0.05$)

Discussion

Considering average total metal concentrations measured per treatment in TMEs, and the ratio doses as predicted invertebrate community effects (community ECx) based on microcosm data, the following effects were expected: ARL low – EC15, Med – EC40, High - >EC90, CSQG low – EC10, Med -EC28, High - EC69 and SUD low – EC6, Med EC48, High – EC84. Despite community effects observed in microcosm tests at these dose levels, results obtained in TMEs showed that microarthropod community abundances were not affect at any tested dose. In the low dose, these results were expectable as the corrected community EC values are ≤EC15, thus possibly not detectable. On the other hand, at the medium and high doses, toxic effects on invertebrate abundances were expected.

There are several factors which could explain the difference between predicted effects based on microcosm tests and effects observed in the TME experiment. The more realistic heterogeneous contamination, in TMEs, compared to the heavily processed and laboratory dosed soil of microcosms, could allow organisms to survive by avoiding higher metal contamination patches within the soil [229,286]. This heterogeneity is difficult to detect because samples for chemical analysis were homogenised to be representative of a particular soil layer. Even though the 0-5 cm soil layers of TMEs were thoroughly homogenised, the 5-10 cm layers maintained their soil structure intact, which could have functioned as a refuge from contamination and/or a population reservoir allowing the recovery of the contaminated 0-5 cm soil layer. Having multiple destructive samples over time could help to understand how or if this recovery process is occurring. Unlike microcosms, TMEs represent a full system with growing plants and plant roots that may serve as refuge for organisms, also TMEs present a larger variety of food options compared to microcosms where food is provided in the form of yeast. As a result, TMEs could provide a better habitat for soil organisms allowing them to cope with larger amounts of contamination. Experimentally, TMEs are much more complex systems, compared to microcosms, which can potentially allow for soil invertebrates to have a higher resistance and resilience to metal contamination, either by allowing them to avoid contamination, providing population niches to allow recovery or directly increasing their tolerance as a result of improved habitat quality.

Excluding differences in test design between microcosms and TMEs, there are two important factors which can also influence the difference in toxicity observed. Each experiment

(microcosm and TMEs) was conducted using soil and microarthropod communities from different field sites. TMEs were collected from an agricultural site while microcosms were performed with soil and communities from a cork oak forest. Both sites present different invertebrate communities and soil properties which can influence the outcome of metal toxicity. In terms of soil communities, TMEs had a higher abundance of mites compared to Collembola while in microcosms the opposite was observed. There is no consistent relative response of mites compared to Collembola towards metal contamination. The differences between groups is expected to depend heavily on their specific compositions and both groups are known to have metal sensitive and tolerant species [229,231,233,258]. Specifically, in the Collembola community, diversity and equitability could have played an important role in the discrepancy between microcosms and TMEs. *H. thermophila* was dominant in both TME and microcosm experiments but TMEs were more diverse and the dominance of *H. thermophila* was not as pronounced. In the TMEs, this species was the only species (of those allowing statistical testing) significantly reduced (0-5 depth, ARL – Med, CSQG – Low, High, 5-10 depth, ARL - Med) by metal contamination. However, because it was not as dominant in the community as in the microcosm experiment, this was not translated into global community effects.

The different soils used in TME and microcosms could have played a significant role in reducing the toxicity predicted from microcosm experiments. It is well known that soil properties can affect the availability and toxicity of metals [37,56,100,269] but also habitat quality for organisms affecting their tolerance of metals [89]. In a recent study, habitat quality for *O. nitens* was found to be positively correlated with soil organic matter and CEC. Soils with higher habitat quality (higher OM and CEC) increased the tolerance of *O. nitens* to zinc contamination, independently of their internal zinc concentrations [89]. Specifically, for metal oxide mixtures, a previous paper found that soil properties drastically affected their toxicity to the Collembola *F. candida*, with significant effects in an acidic soil (pH – 3.4, CEC - 8) but without observed toxicity in a more neutral soil (pH – 4.6, CEC - 28) [249]. In the TMEs, the soil had a higher pH (microcosm pH 4.0 and TME pH 5.3) and, more importantly, had a much higher clay content and CEC (microcosm CEC 9.2, TME CEC - 24). The CEC values for the TME soil are within the range where no toxicity of metal oxide mixtures was observed for *F. candida* [249]. Soils with higher CEC have an increase in the binding of metals reducing metal availability and consequently decreasing the toxic effect of metals to soil invertebrates [37,56,100]. It is possible that the metals in TMEs have a reduced availability in soil pore-water, a major route of exposure for soil invertebrates, which is suggested by their low vertical mobility within TME cores.

It is also important to note, that while discussing the differences between experimental tiers is valuable in understanding the toxicity of mixtures, these different tiers of testing are not expected to be concordant. From the perspective of environmental risk assessments following a tiered approach, lower tiers are expected to be more conservative and provide a more direct exposure than in higher tiers which are more realistic. In this sense, while TME experiments are more realistic exposure than microcosms, the latter are still an essential step in the tiered approach and if no effects were observed in microcosms there would be little reason to proceed with a TME experiment. However, while experiments between tiers do not have to be concordant results which demonstrate low/no toxicity in lower tiers (single species and microcosms) should still be validated at higher tiers (TMEs and field studies) to ensure their environmental estimates are actually protective.

The only parameter measured in collembolans affected by metal contamination was collembolan mean traits scores (mT). As a trend, mT increased in contaminated soil compared to control but this change was only significant in CSQG high treatment for the 0-5 cm soil layer (Figure 6A – Annex 4). The increase in mean trait scores implies an increase in species with traits adapted to deeper soil layers (euedaphic) or inversely a decrease in hemi-edaphic or epiedaphic species [287]. In the TME experiment, the increase in mT appears to be the result of the decrease in abundance of the hemi-edaphic *H. thermophila*, the only individual species negatively affected by metal contamination (Figure 7A – Annex 4) and a known metal sensitive species [231,234]. In the scientific literature there is no consistent relationship between Collembola sensitivity and metal concentrations regarding their habitat traits. Some studies have found hemi-edaphic and epiedaphic species to be more tolerant and less affected by metal contamination [234,261]. On the other hand, there are other studies where metal tolerant euedaphic Collembola species have been identified (i.e. *Mesaphorura macrochaeta*, *Protaphorura armata*) [229,233,288]. Generalizing sensitivity to metal contamination based on habitat traits is difficult and will vary according to which metals are present, their environmental dose and the source of contamination, affecting the distribution of metals between soil layers and also community composition, affecting individual sensitivities and species interaction.

Contrary to soil invertebrate abundances, DHA and PNR were heavily impacted by metal contamination (decreases ≥ 50% compared to control levels). On the other hand, AP was not significantly affected. To the best of our knowledge, there are only three studies including the

measurement of all three enzyme parameters (DHA, PNR and AP) to evaluate metal contamination one study in a mine in southern Portugal (main metals of concern As, Cd, Cr, Cu, Ni and Pb) [289] and two in a metal contaminated site in the tropics (main metals of concern Pb, Cd, Cu, and Zn) [116,290]. Results from the study conducted in Portugal [289], were concordant with those in the TME experiment where, DHA and PNR were the most sensitive enzymes and AP was considered to not be particularly sensitive to metal contamination. For the two studies conducted in the tropics, data were incorporated in a larger risk assessment scheme and the relationship between these activities were not discussed in detail by the authors [116,290]. However, unlike TME results, in these studies [116,290] nitrification increased in metal contaminated soils, which was attributed to imbalances in the N-Cycle while AP (also unlike TME results) and especially DHA were sensitive to metal contamination. These studies [116,290], also found that soil enzymes were poor at detecting gradients of contamination. In the TMEs, PNR was also not sensitive to gradients of contaminations and no significant differences between doses within the same mixture ratio were detected (all doses equally affected). However, DHA was slightly more sensitive to dose, where no dose effect was observed for the ARL mixture but in the CSQG and SUD mixtures, low and Med doses were significantly less affected than High doses. Individually, PNR, DHA and AP have all shown sensitivity to metal toxicity in other research studies [194,268,269,291,292]. Compared to AP, both PNR and DHA have shown higher sensitivity to metal contamination in previous studies [194,268]. When testing different soils, Awuah et al. [194], found that the magnitude of difference in sensitivity between PNR and AP was linked to soil pH and was higher at higher soil pH (pH 4.6 and 6.8) compared to a low pH soil (pH – 3.4). In TMEs the soil pH (pH – 5.3) is within the range where the larger difference in response between both enzymes was previously observed.

Even in the absence of effects to soil invertebrate abundances, organic matter decomposition and feeding activity were both significantly affected by metal contamination. Bait-lamina are known to be a sensitive endpoint to measure the effect of metal contamination and have previously been used in metal contaminated sites [116,293,294]. In a metal contaminated site in the tropics (main metals, Pb, Cd, Cu and Zn), structural parameters (abundance, species richness and diversity indices) were also less affected than functional parameters (litter decomposition and feeding activity through bait-lamina) [116]. In the TME experiment, bait-lamina data was highly variable (compared to litter bags), which is in agreement with previous studies that also reported high variability of bait-lamina data [181]. Despite that, feeding activity was significantly reduced in all high treatments and in the SUD medium treatment in the first sampling period (T1 - week 1 to

week 8), and in the ARL Med in the second sampling period (T2 – week 11 to week 16). Overall, the average difference between all treatments and the control was larger in T1 than in T2 except for ARL med and all high doses that were significantly lower than controls in T1 but not in T2. This suggests that, after a stronger initial impact during the first eight weeks of incubation, the community partially recovered between eight to sixteen weeks, especially in the high doses. Combined with the lack of effects on invertebrate abundances at the end of the experiment, this suggests that either there was an initial impact on invertebrate abundances followed by recovery or that organisms resisted an initial impact (T1) by reducing their activity (i.e. feeding activity measured through bait-lamina) which partially recovered by the end of the experiment (T2). Regarding organic matter decomposition through litterbags, all medium and high treatments were significantly reduced compared to control. In this case the magnitude of effect was not as large as bait-lamina (especially in T1). Metal contamination in field studies (different combinations of Pb, Cu, Ni, Zn, Cd) have previously been demonstrated to negatively impact organic matter decomposition either by direct measurement using litter-bags [116,263] or indirectly due to increased litter accumulation in contaminated soil [229,258,295]. Litter decomposition is an important function provided by soil invertebrates, thus changes in this parameter were expected to correlate with microarthropod community abundances. It is possible, that the reduction in OM decomposition, in litterbags, is a residual effect, after the recovery from an initial impact evidenced from the difference in bait-lamina response between T1 and T2. Overall, it is hypothesised that functional effects are the result of an initial impact to which communities recovered or a reduction in organism activity (possibly for energy conservation) as a tolerance mechanism to metal stress. The reduction in organism activity as tolerance mechanism has not been extensively studied but Creamer et al. [263] also suggested that reduced organic matter decomposition as a result of metal contamination (Zn, Cu and Ni) was a consequence of changes in invertebrate activity rather than structure. The effects of metal mixtures on soil microbial communities, as evidenced by enzyme activities (e.g. DHA), could also have contributed to the observed reduction in feeding activity and organic matter decomposition in metal contaminated test treatments, as microorganisms are also important contributors to the degradation of organic matter [263,294,296].

Implications for the risk assessment of metals in soil

It is very difficult to predict the environmental impact of metals and their mixtures in soil systems. In current regulatory approaches, such as the EU REACH PNEC calculator, soil properties have been considered to reduce the uncertainty of threshold values [8]. However, data considers soils dosed with metal salts and it is unclear if bioavailability models for soil properties are equally valid for different metal forms such as oxides used in TMEs. Moreover, guidelines do not consider other important factors such as community composition, species interaction, ecosystem structure affecting recovery and habitat quality, and metal distribution in different soil/litter layers. Lower tier laboratory testing used to define regulatory guidelines is expected to provide conservative and protective environmental thresholds under a worst-case scenario. However, the discrepancy between regulatory values and TME effects appears to be considerably large, specially under a retrospective risk assessment scenario. In the TME experiment, the low dose used, is already higher than regulatory values (approximately 3 times higher for ARL and 4 times higher for CSQG) and in TMEs even high doses (approximately 7 times higher for ARL and 12 times higher for CSQG than regulatory values) did not affect invertebrate abundances. Even at an intermediate tier with higher complexity, such as microcosms, predicted effects were not representative of TMEs from a different soil and invertebrate community. The number of variables that can influence metal toxicity, including those related to the contaminant (speciation, mixture composition) and inherent to the ecosystem itself (soil properties, habitat quality, community composition) is an indication that adequate environmental protection, which is not too stringent, should be determined using a site-specific approach. Considering the degree of discrepancy observed between lower tier tests used in regulatory guidelines and effects in TMEs, it may be preferable for industry, in retrospective risk assessment, to invest in site-specific risk assessment to provide more accurate levels of environmental protection which are not overly conservative.

To adequately protect soil ecosystems, risk assessments should include not only site- specific properties, but also multiple components of the ecosystem and both structural and functional endpoints in a TRIAD approach (that in the ecotoxicological line of evidence could include both single species and community tests under different tiers). The TME data presented in this study demonstrated that organic matter decomposition, feeding activity and especially enzyme activity can respond very differently than invertebrate communities. In fact, while the low dose was above regulatory thresholds and too conservative for invertebrate community abundances, this

was not the case for enzyme activity where DHA and PNR were reduced below 50% of control values. Considering that different components of the soil ecosystem are interlinked, there is the possibility of stronger effects on fauna and flora over longer time scales due to effects on soil enzymes and the impairment of nutrient cycling. Experiments over longer timescales would be interesting to confirm this hypothesis. Ecosystem function was expected to be equally affected or even more resilient than structure due to functional redundancy, but this does not seem to be the case. In fact, the opposite was observed, where invertebrate abundances were either resistant or resilient (recovering over an initial impact) while feeding activity and organic matter decomposition were not fully recovered at the end of the experiment. TME data demonstrates that structural and functional parameters may not be directly linked or their correlation could be delayed in time and, therefore, when considering the risk assessment of metals and their mixtures, covering both types of parameters should be considered.

The outcome of metal contamination on the different components of the soil ecosystem structure and function cannot be predicted from laboratory generated data. Adequate risk estimates which are protective but not overly conservative can only be determined in a holistic site-specific approach covering structural and functional parameters of the ecosystem. In the literature, there are some site specific studies which include several lines of evidence but these are still rare and most include only tier one or screening data [116,117,266,290,293,297]. Ecotoxicology research will greatly benefit from more site-specific data, improving site-specific risk assessments themselves and our understanding of the minutia involved in metal toxicity in real environmental scenarios. Industry, responsible for remediating contaminated land, may benefit from site-specific data by reducing remediation efforts compared to the potentially over conservative thresholds in current guidelines. While environmental thresholds appear conservative for community abundances, this is not the case for functional parameters (especially enzymes) which must be monitored carefully.

Annex 4

4.1 – Supplementary material

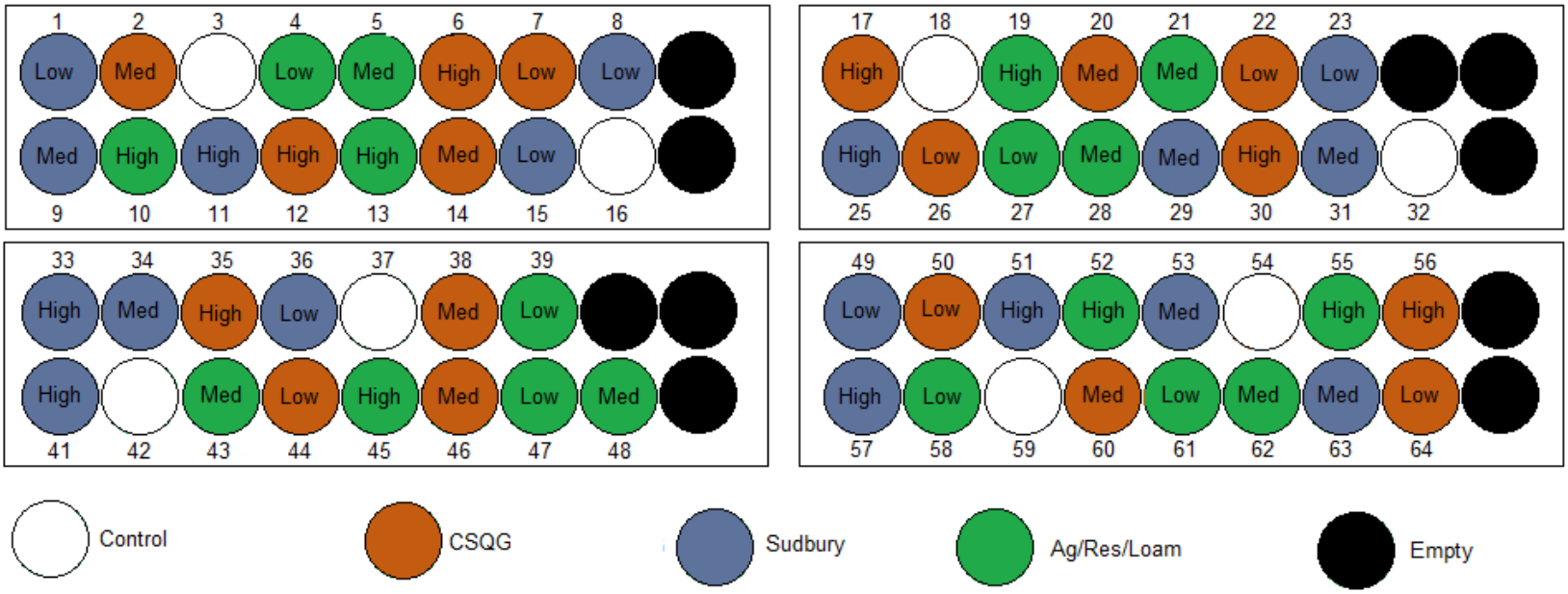
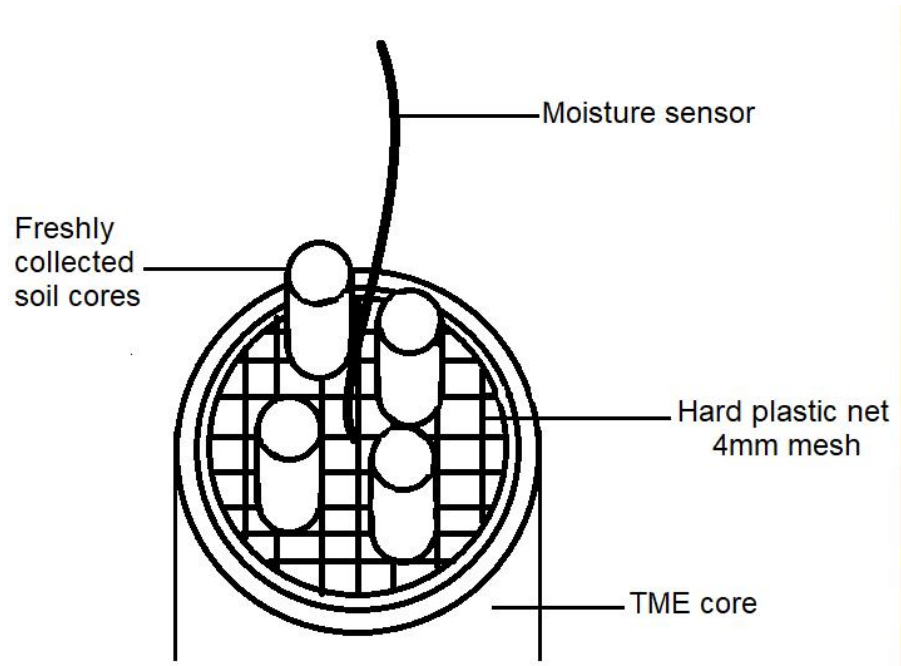


Figure 1A - Layout of TME treatments in each cart.



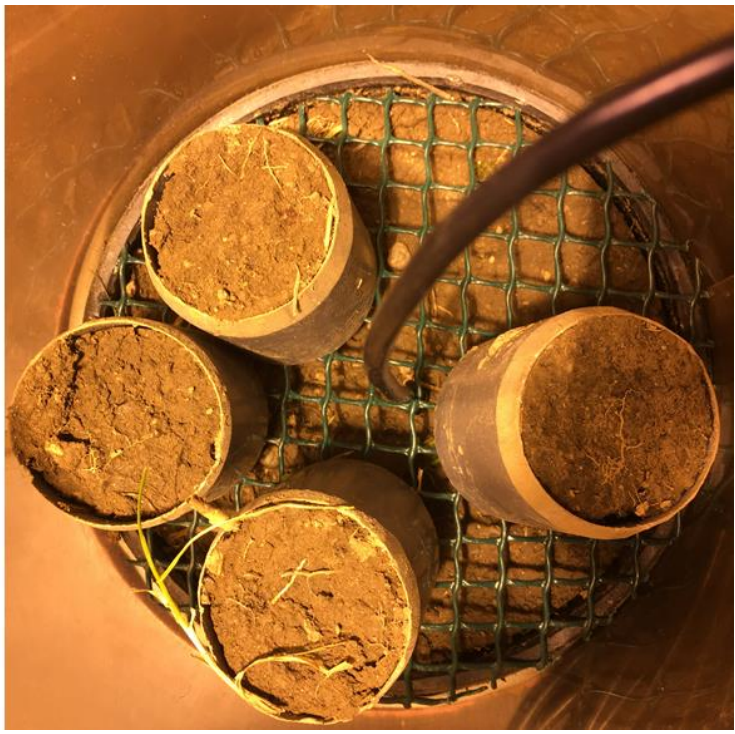


Figure 2A. Placement of soil cores on each TME one day after dosing.

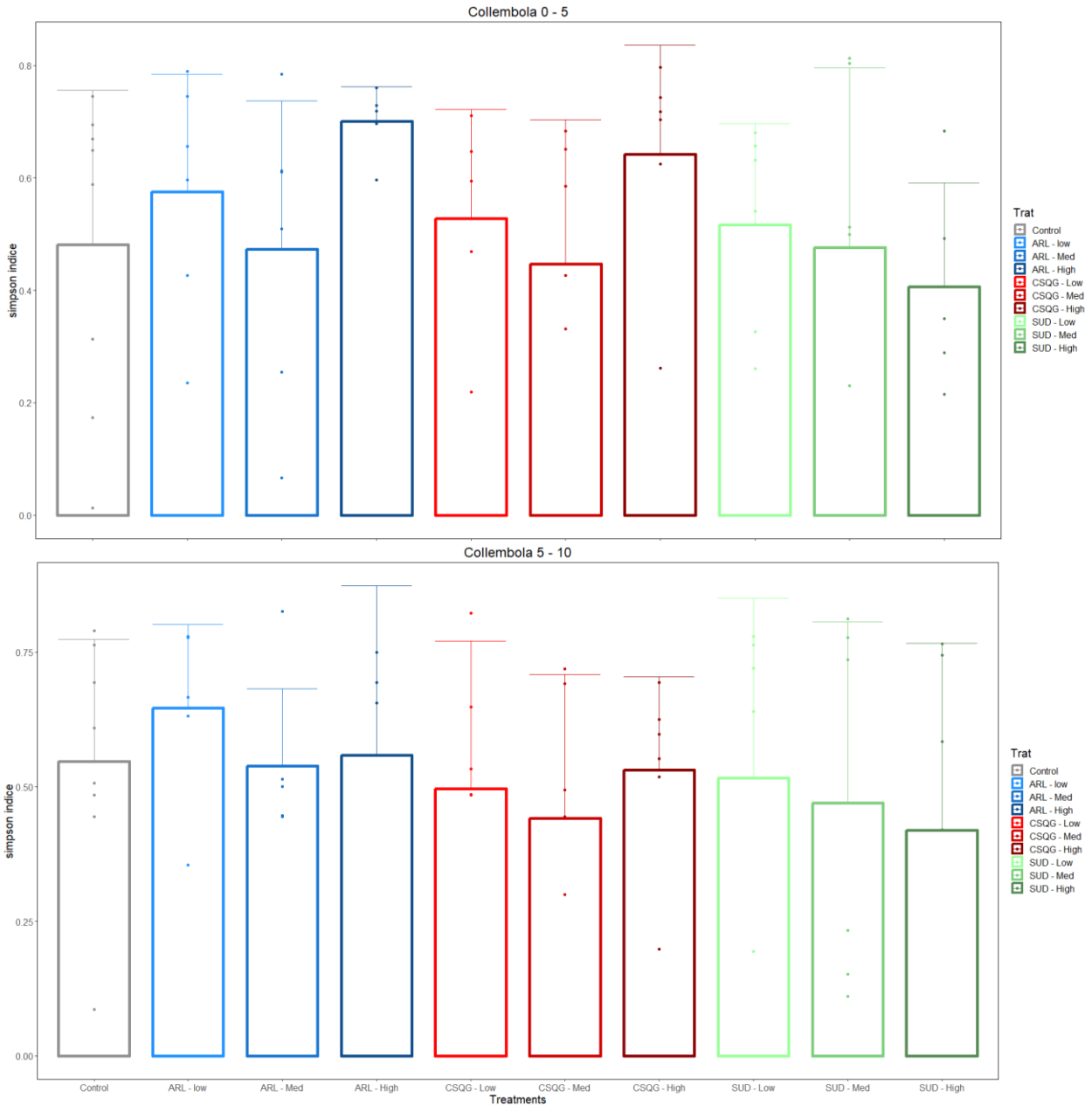


Figure 3A – Simpson diversity index for TMEs treated with metal mixtures (ARL, CSQG and SUD) with three doses per mixture (Low, Med and High) and at a soil depth of 0-5 and 5-10 cm. Bars represent the average value (Treatments n = 6, Control n = 8), error bars the standard deviation of the mean, points represent individual observations for each TME. * Significantly different than control ($p \leq 0.05$)

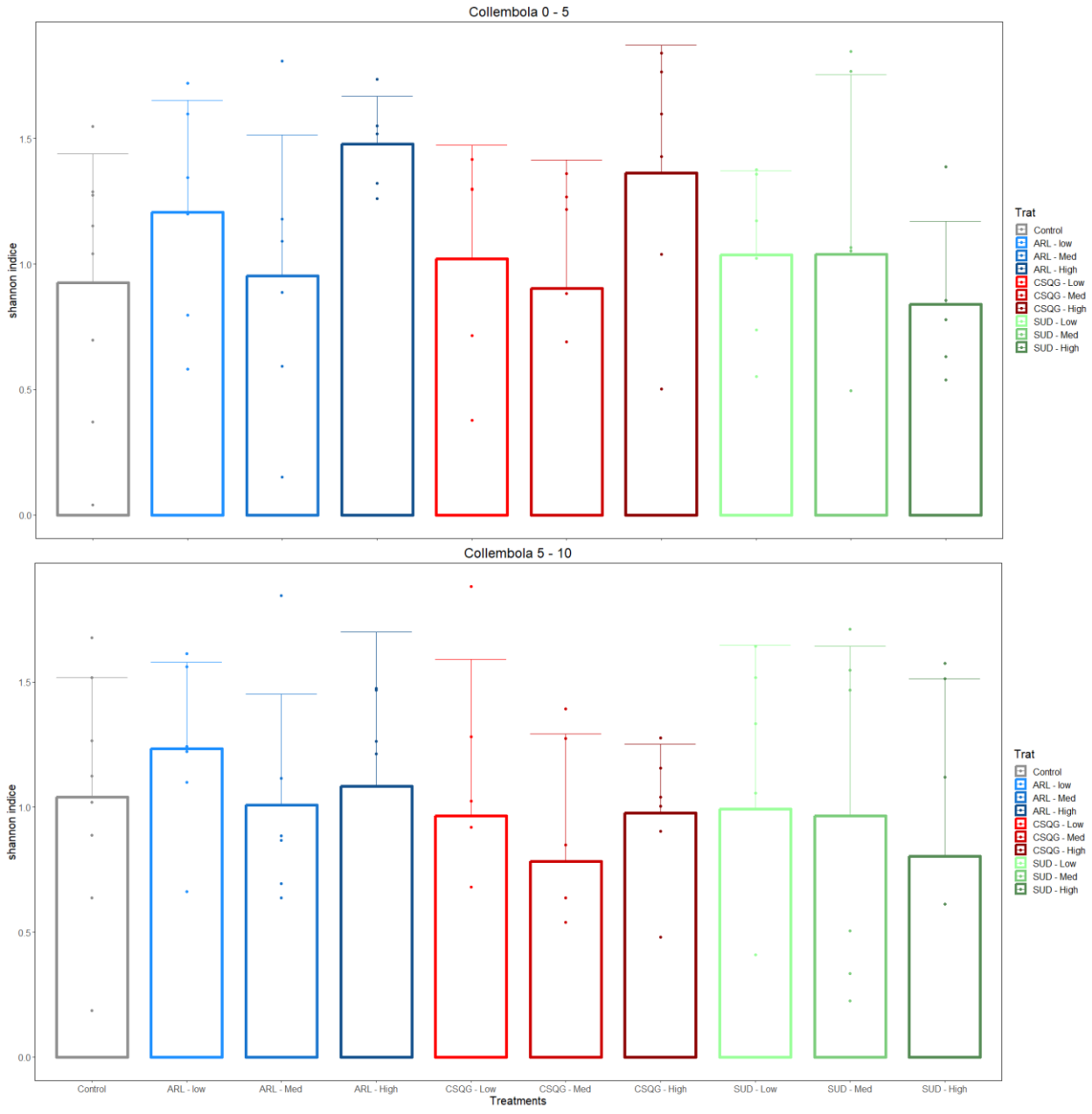


Figure 4A - Shannon diversity index for TMEs treated with metal mixtures (ARL, CSQG and SUD) with three doses per mixture (Low, Med and High) and at a soil depth of 0-5 and 5-10 cm. Bars represent the average value (Treatments n = 6, Control n = 8), error bars the standard deviation of the mean, points represent individual observations for each TME. * Significantly different than control ($p \leq 0.05$)

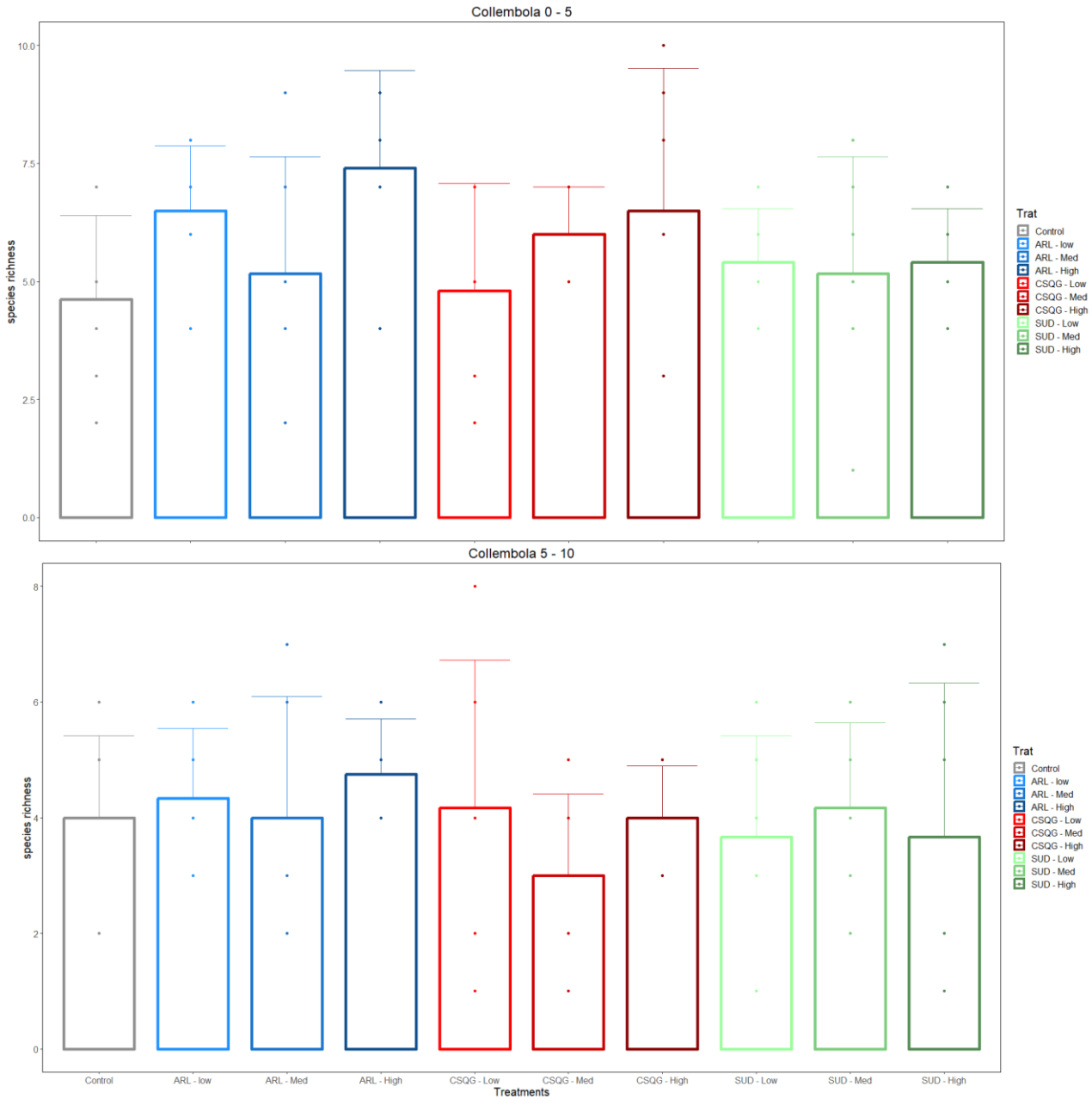


Figure 5A – Collembola species richness for TMEs treated with metal mixtures (ARL, CSQG and SUD) with three doses per mixture (Low, Med and High) and at a soil depth of 0-5 and 5-10 cm. Bars represent the average value (Treatments n = 6, Control n = 8), error bars the standard deviation of the mean, points represent individual observations for each TME. * Significantly different than control ($p \leq 0.05$)

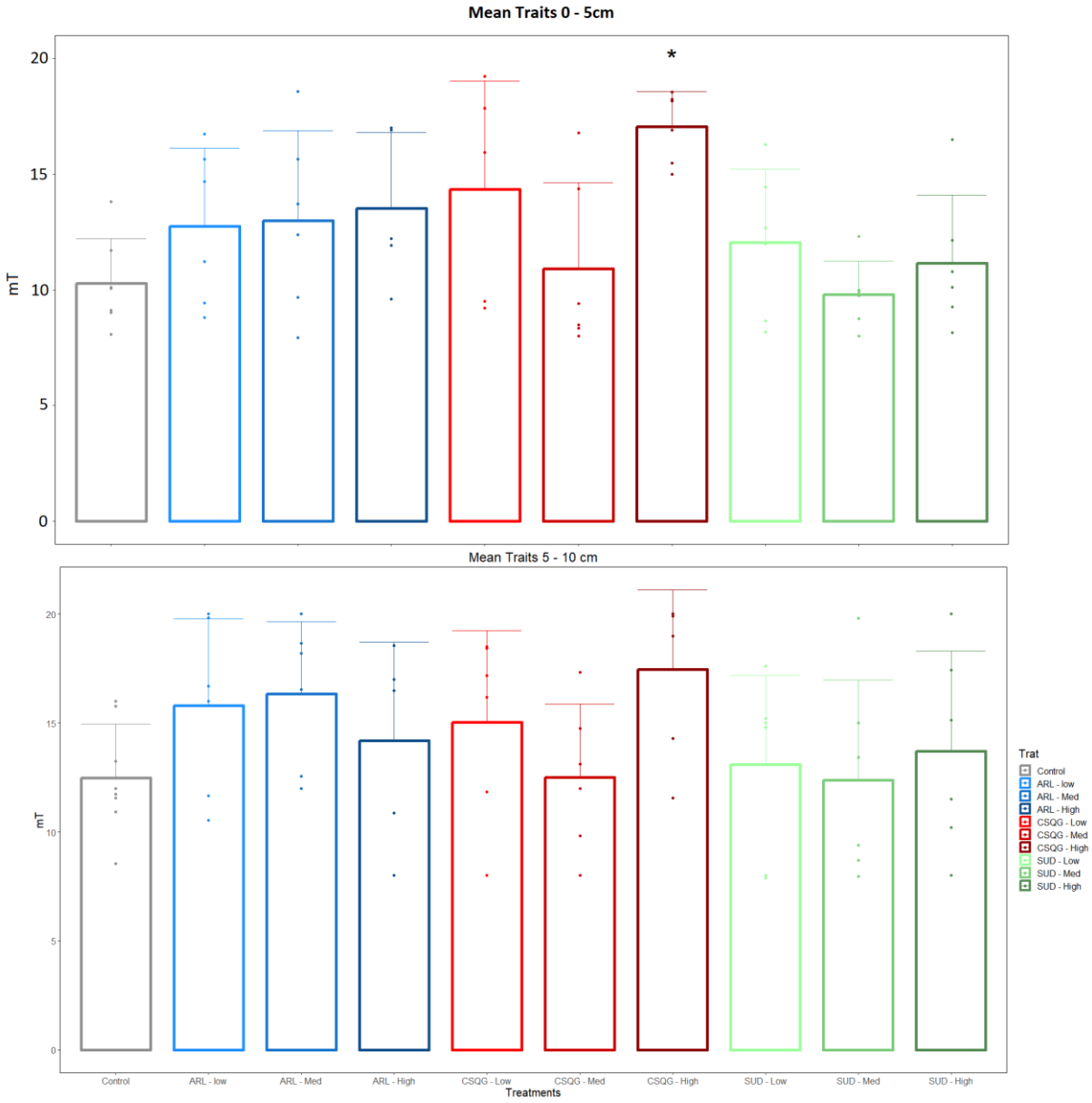


Figure 6A – Mean Collembola community trait (mT) scores of TMEs treated with metal mixtures (ARL, CSQG and SUD) with three doses per mixture (Low, Med and High) and at a soil depth of 0-5 and 5-10 cm. Bars represent the average value (Treatments n = 6, Control n = 8), error bars the standard deviation of the mean, points represent individual observations for each TME. * Significantly different than control ($p \leq 0.05$)

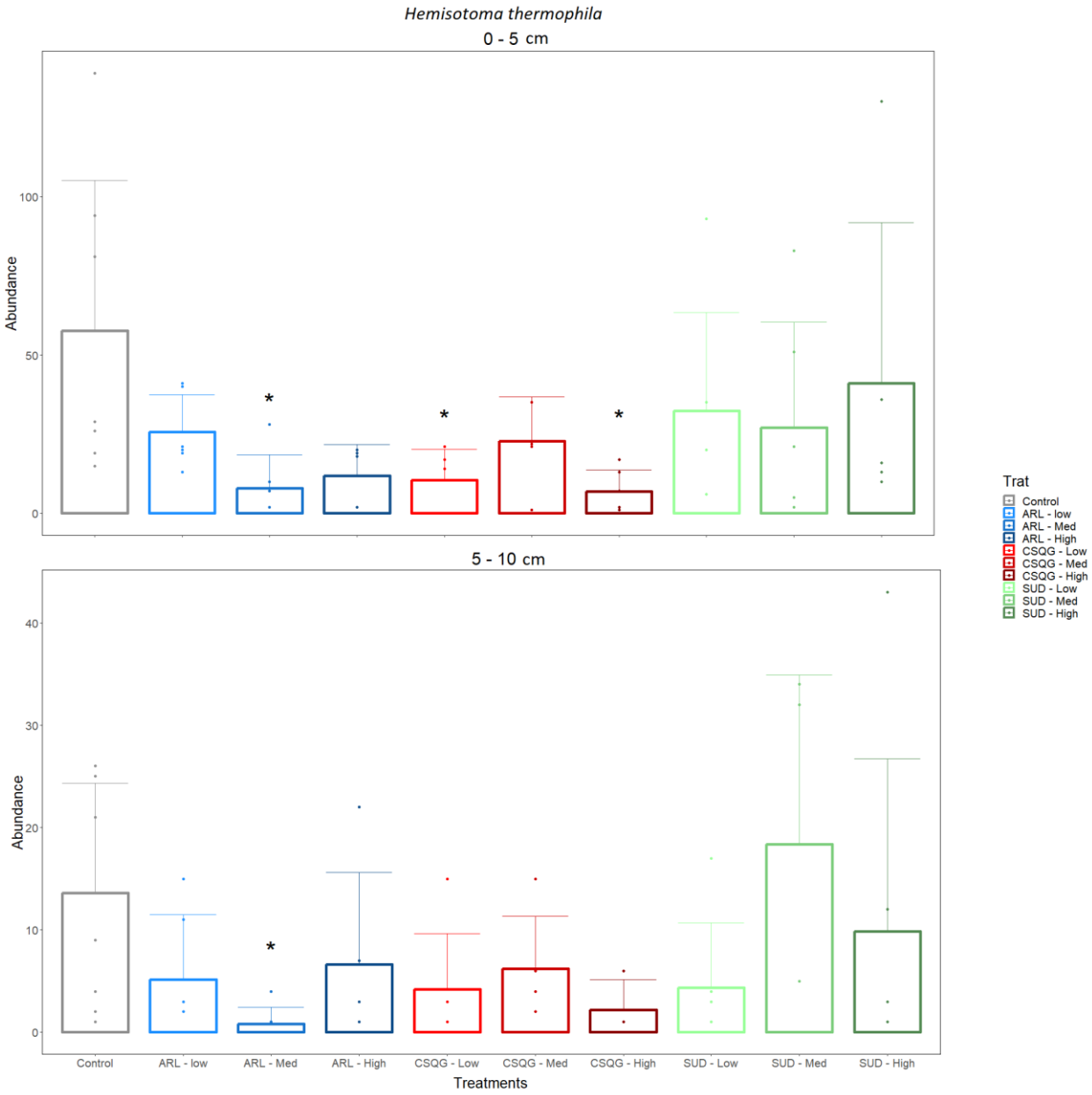


Figure 7A – *Hemisotoma thermophila* abundances in all TME treatments composed by soil dosed with metal mixtures (ARL, CSQG and SUD) with three dilutions per mixture (Low, Med and High) and at 0-5 and 5-10 cm depth. Bars represent the average abundances (Treatments n = 6, Control n = 8), error bars the standard deviation of the mean and points represent individual observations for each TME. * - Significantly different than control ($p \leq 0.05$)

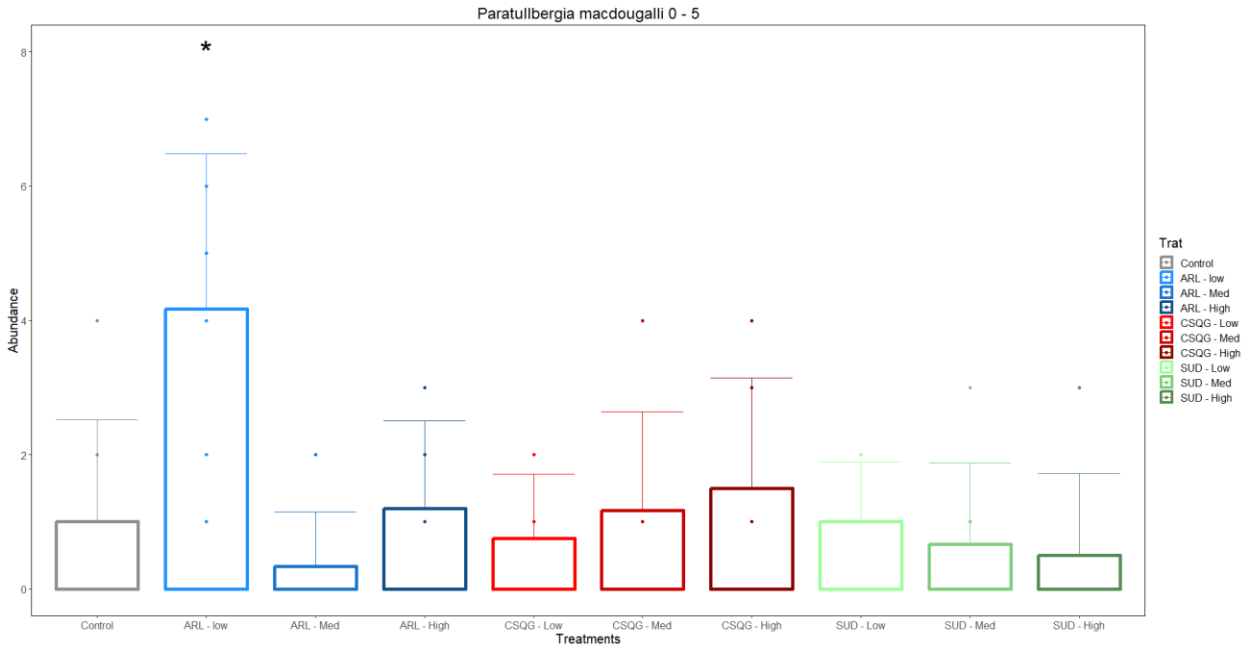


Figure 7A – *Paratulbergia macdougalli* abundances in all TME treatments composed by soil dosed with metal mixtures (ARL, CSQG and SUD) with three dilutions per mixture (Low, Med and High) at 0-5. In the 5-10 cm depth no significant effects were detected. Bars represent the average abundances (Treatments n = 6, Control n = 8), error bars the standard deviation of the mean and points represent individual observations for each TME. * - Significantly different than control ($p \leq 0.05$)

Table 1A – Individual and total trait scores of Collembola species found in the TME experiment. Traits considered: Furca (absent = 4, reduced = 2, developed 0), Antenna (size smaller than half the body length = 4, between half and full body length = 2, larger than body length = 0), Ocelli (absent = 4, present = 0), Pigmentation (absent = 4, present = 2, present with patterns = 0), Hairs and scales (absent = 4, present = 0).

Species	Furca	Antenna	Ocelli	Pigmentation	Hairs/scales	Total
Ahrropalites caecus	0	2	0	0	4	6
Ceratophysella engadinensis	2	4	0	2	4	12
Ceratophysella sp.	2	4	0	2	4	12
Deuterosminthurus juvenile	0	2	0	0	4	6
Folsomia c.f. *fimetaria*	0	4	4	4	4	16
Heteromurus major	0	4	0	2	0	6
Hemisotoma thermophila	0	4	0	0	4	8
Isotomiella minor	0	4	4	4	4	16
Isotomodes productus	2	4	4	4	4	18
Isotomurus c.f. *pseudopalustris*	0	4	0	0	4	8
Lepidocyrtus juvenile	0	4	0	2	0	6
Mesaphorura c.f. *betschii*	4	4	4	4	4	20
Mesaphorura macrochaeta	4	4	4	4	4	20
Mesaphorura sp.	4	4	4	4	4	20
Protaphorura hortensis	4	4	4	4	4	20
Protaphorura juvenile	4	4	4	4	4	20
Paratullbergia c.f. *macdougalli*	4	4	4	4	4	20
Pseudosinella blind	2	4	4	4	0	14
Sinella tenebricosa	2	4	4	4	4	18
Sminthurides juvenile	2	4	0	2	4	12
Sminthurides parvulus	2	4	0	2	4	12
Sminthurinus juvenile	2	4	0	0	4	10
Symphypleona juvenile	2	4	0	0	4	10

Table 2A – Soil moisture and pH for each individual TME at the end of the test experiment (16 weeks) in the top 0-5 cm soil layer.

Treatment	TME number	Soil moisture (%)	pH
Control	3	16.6	5.4
Control	16	12.9	5.3
Control	18	18.7	5.2
Control	32	23.3	5.3
Control	37	13.4	5.4
Control	42	17.6	5.5
Control	54	15.8	5.2
Control	59	21.6	5.2
ARL - low	4	14.5	5.9
ARL - low	27	15.0	5.7
ARL - low	39	12.7	5.8
ARL - low	47	11.0	5.4
ARL - low	58	20.8	5.6
ARL - low	61	23.5	5.5
ARL - Med	5	17.2	5.7
ARL - Med	21	26.1	5.6
ARL - Med	28	2.2	5.8
ARL - Med	43	23.6	5.8
ARL - Med	48	19.2	5.7
ARL - Med	62	26.5	5.7
ARL - High	10	29.7	5.8
ARL - High	13	14.5	5.8
ARL - High	19	8.9	5.9
ARL - High	45	13.3	5.9
ARL - High	52	12.8	5.7
ARL - High	55	27.2	5.7
CSQG - Low	7	11.7	5.5
CSQG - Low	22	7.6	5.6
CSQG - Low	26	19.0	5.7
CSQG - Low	44	21.7	5.8
CSQG - Low	50	17.5	5.7
CSQG - Low	64	34.3	5.7
CSQG - Med	2	26.1	5.9
CSQG - Med	14	15.3	5.8
CSQG - Med	20	15.5	5.8
CSQG - Med	38	15.3	5.8
CSQG - Med	46	11.7	5.8
CSQG - Med	60	14.0	5.9
CSQG - High	6	14.3	5.9
CSQG - High	12	21.7	6.0
CSQG - High	17	20.2	5.9
CSQG - High	30	22.9	5.9
CSQG - High	35	25.2	6.0
CSQG - High	56	29.4	5.9
SUD - Low	1	26.8	5.4
SUD - Low	8	4.5	5.7
SUD - Low	15	8.7	5.8
SUD - Low	23	19.8	5.6
SUD - Low	36	15.0	5.9
SUD - Low	49	17.0	5.7

SUD - Med	9	25.9	5.8
SUD - Med	29	10.7	5.9
SUD - Med	31	12.6	6.0
SUD - Med	34	26.4	5.7
SUD - Med	53	8.1	5.8
SUD - Med	63	22.1	5.8
SUD - High	11	24.4	5.9
SUD - High	25	27.2	6.1
SUD - High	33	28.9	5.9
SUD - High	41	26.6	5.9
SUD - High	51	25.8	6.0
SUD - High	57	27.3	5.6

Table 3A – Average total metal ratio across dosed and its standard deviation of the mean (SD) and the percent of total metals (0-10 cm) present in the lower 5-10 cm depth.

Metal		Pb	Cu	Ni	Zn	Co
		%	%	%	%	%
Total metal mixture ratio						
ARL	Average ratio	14.7	14.9	9.9	50.5	9.9
	SD	3.2	2.1	1.2	7.1	1.4
CSQG	Average ratio	15.5	13.1	9.0	53.7	8.8
	SD	3.2	1.9	1.0	6.3	1.4
SUD	Average ratio	51.0	3.4	6.4	35.4	3.7
	SD	6.7	0.4	0.8	7.3	0.6
Percent of total metals present in 5-10 cm depth						
ARL	Low	13.5	19.2	17.3	12.7	18.4
	Med	19.1	16.9	17.4	15.0	17.2
	High	10.2	12.0	10.9	7.0	11.4
CSQG	Low	3.7	12.3	12.2	8.1	11.9
	Med	18.3	22.4	22.0	15.5	22.9
	High	9.2	10.2	10.3	9.4	11.0
SUD	Low	12.1	1.1	2.7	2.4	1.9
	Med	17.4	16.8	16.1	13.3	16.8
	High	16.9	15.4	14.5	11.8	14.8

Chapter 6 - General discussion

Portions of this discussion are based on the published manuscript:

M. Renaud, J.P. Sousa, S.D. Siciliano, A Dynamic Shift In Soil Metal Risk Assessment, It's Time To Shift From Toxicokinetics To Toxicodynamics. Environmental Toxicology and Chemistry-https://doi.org/10.1002/etc.4735.

The research presented in this thesis has a focus on the toxic effects of metals in soil but deals in its essence with complexity. Complexity in terms of metal contamination, testing mixtures of five metals (lead, copper, nickel, zinc and cobalt) but also ecological complexity. Chapter 2, explored different dosing methods for fixed ratio metal mixture testing. Chapter 3 analyzed the joint action of 5 element metal mixtures using three standard test species and Chapters 4 and 5 tested a select number of mixture ratios at increasing levels of ecological complexity, namely in a natural community microcosm and a terrestrial model ecosystem experiment.

Toxicity of metals and their mixtures

The toxicity of metals to the soil ecosystem is a very complex process which, in its essence, is the result of the complex interplay between three factors: the metals, the soil and the organisms in the ecosystem. The role of these factors on the toxicity of metal mixtures was explored throughout this thesis, either directly or indirectly.

Metals

In terms of metals, Chapter 2 demonstrated, while testing dosing methods, that the chemical form of a metal can drastically affects their toxicity. In this domain, previous research had already explored differences in toxicity between metal salts, oxides and, more recently nanomaterials, but has not always been consistent on their relative toxicity [48,53,54,62,103].

The annealed metal mixtures results were particularly interesting. Annealed metals were specifically designed to simulate contamination from a smelting operation but were non-toxic to soil invertebrates. The lack of toxicity to any of the test species in any of the soils was quite surprising, as similar minerals, such as Franklinite can be a dominant form of metal in contaminated sites [196]. The non-toxicity of annealed metals could in part explain the consistent discrepancy in toxicity between field and laboratory contaminated soils where laboratory spiked soils are known to produce higher toxicity [100,101]. However, for the annealed metals, and considering the environmental impacts of metal contaminated sites, some toxicity (even if lower than salts) was expected. The differences between expected and observed toxicity, for the annealed metals, could be because in metal contaminated sites, weathering constantly breaks down mineral structure releasing more bioavailable forms of metals.

The toxicity of metals also depends on which metals are present and mixtures can drastically affect the toxicity based on what is predicted from single metal toxicity. In Chapter 3, the dose of metals mixtures played an important role in mixture interactions where strongest deviations from concentration addition model were detected at low and high dose/effect levels. Compared to previous research with simpler binary and ternary mixtures, interactions seem larger, contradicting the funnel hypothesis [225]. From an empirical sense, this was expected since as the complexity of mixture increases so do the potential avenues for interactions. Specifically, more synergisms were detected than in previous studies and they occurred at low rather than high dose/effect levels [126,127].

Soils

The role of soils is commonly associated to their function as regulators of the bioavailability of metals. The toxicity of metals is generally considered the result of their free-ions which can transverse biological membranes and cause toxic effects [98]. In this case, the availability of metal free ions in soil pore water, modulated by soil properties is expected to dictate the toxicity of metals.

In Chapter 2, we indirectly measured the solubility of metal salts in soil by measuring metal loss during the leaching process. Confirming previous research, metal solubility was larger in more acidic soils with lower CEC and toxicity was also higher in soils with lower pH and CEC. The role of soil properties could in part explain the large discrepancies in toxicity to microarthropod between the microcosm experiment in Chapter 4 and the TMEs in Chapter 5. Experimental doses used in TMEs were established based on community ECx values from microcosms, but no toxicity on microarthropod abundances was observed in TMEs at any dose (even at 2x the EC50). For logistical reasons, it was not possible to conduct the TME experiment on the same soil as in microcosms and the higher CEC in the TMEs could have produced a higher binding of metals and lower availability to soil invertebrates. However, the link between metal solubility and toxicity has consistently been questioned.

In Chapter 3, soils played an important role in metal mixture toxicity and organism performance but did not affect mixture interactions. Analysis of mixture interactions in both test soils was only possible for *O. nitens* and the pattern of mixture response was correlated in both test soil. For *F. candida*, metal mixture toxicity was correlated with metal solubility and in the soil with higher pH and CEC, mixtures were mostly non-toxic. The response of *F. candida* while

correlated with total metal concentrations of the most soluble metals (zinc and copper) was not correlated with their $CaCl_2$ extractable concentrations. For *O. nitens* and *E. crypticus*, species responses were not correlated with either total or available metal concentrations.

Organisms

Regarding the biological compartment and focusing on soil organisms, their importance has been explored in all four chapters. In Chapter 2, not only was *E. crypticus* more sensitive than *F. candida* and *O. nitens,* but metal salts were quite more toxic than oxides, even after leaching. The soft body of *E. crypticus* might have increased its exposure to the most soluble metal salts due to dermal adsorption. On the other hand, the similar sensitivity for *O. nitens* and *F. candida* was surprising, because compared to *F. candida*, *O. nitens* have much more developed external barriers due to its sclerotic exoskeleton [111]. However, these external barriers are not well developed in juveniles and juvenile mortality could compensate for a lower exposure in adults [198]. In addition, while exposure for *F. candida* is usually associated with soil-porewater, for *O. nitens* exposure to cadmium oxide has been linked to ingestion of contaminated soil and organic matter [215]. Soil ingestion is not typically associated with *F. candida* and could potentially reduce the gap in the exposure to less soluble metal oxides between both species.

The importance of species and their traits was also observed in their response to mixtures, where *F. candida* response was globally additive, *E. crypticus* and *O. nitens* had synergism at low dose effect levels which decreased in intensity at higher dose effect levels. At first glance, the similarity in response of *E. crypticus* and *O. nitens* is quite surprising as they are on opposite ends in terms of their external barriers. *E. crypticus* has very reduced external barriers while *O. nitens* a well-developed exoskeleton. However, for both *E. crypticus* and *O. nitens* responses were not correlated with metal solubility, while *F. candida* responses were linked to the more soluble metals. In dosing soils with metal oxides, dermal adsorption in *E. crypticus* might be reduced and soil ingestion could be the dominant pathway for exposure, increasing the similarity in response to metal mixtures with *O. nitens*.

Finally, in Chapters 4 and 5, there is evidence of the importance of community composition in the response to metal mixtures. The effects of metal mixtures to the microarthropod community in microcosms and TMEs were drastically different. While this discrepancy might by due to differences in soil properties as depicted above, community composition might also have contributed. In both the microcosm and TME experiments, *H. thermophila* was a dominant

Collembola and was sensitive to metal mixture contamination. However, TMEs were more diverse, had higher mite abundances and *H. thermophila* while still a dominant collembolan, this dominance was not as pronounced and did not translate into community level effects.

Toxicodynamics and a more ecological approach to risk assessment

In soil ecotoxicology and risk assessment it is currently not possible to accurately predict the toxicity of metals. The role of metals, soil and organism traits has consistently been viewed, under the scope of toxicokinetics. Even in the research conducted in this thesis, the role of soil properties, metal chemistry and organism traits (affecting exposure), have been related to bioavailability and viewed under metal toxicokinetics. However, when only metal bioavailability and toxicokinetics are considered, it is not possible to accurately predicted toxicity and it may be time, to better protect the soil environment, to consider how these factors (soil/organism/metal) impact toxicodynamics (Figure 1).

Above we argued that soil properties drive the toxicity of metals through toxicokinetics regulating their bioavailability, but soils also function as a habitat and can drive toxicodynamics by modulating the energy organisms have available to endure contamination. This has recently been demonstrated for *O. nitens* which has increased tolerance to metals in high habitat quality soils, compared to those with low habitat quality [89], despite similar Zn bioavailability.

It is also important to consider ecological implications for risk assessment of soil as a habitat. The discrepancy between the toxicity of annealed metals and the environmental impact observed in metal contaminated sites where these mineral forms are dominant, was attributed to increased metal availability over time due to weathering. However, it is important to consider that most metal contaminated sites, as a result of the industrial activities leading to contamination, are multi-stressed environments, impairing habitat quality. In Chapter 4, it was observed that average concentrations in a contaminated site in Sudbury, Canada, (when presented as metal oxides) corresponded to only a 20% change in the community (EC20) and if these metal mixture concentrations were reduced, in this contaminated site from 3.1 TU to 2.1 TU effects would be at the community EC10. However, this assumes that the environmental degradation is caused only by high metal concentrations in soil, this is rarely the case and in many contaminated sites the environment is impacted by a combination of multiple stressors. For instance, Sudbury Canada in addition to high metal concentrations, has a heavily degraded landscape due to low soil pH, a

denuded landscape, low soil organic matter, reducing habitat quality and the resilience of organisms to metals. In this case, if full site-specific studies cannot be conducted, soil microcosm and TME experiment using field contaminated soils and soil cores could be (and have already [179,243]) used to study the impact of these multiple stressors.

In the community experiments (Chapters 4 and 5), the difference in toxicity between microcosm and TMEs, could be due to differences in soil properties and community composition, affecting the availability and sensitivity to metals respectively. However, differences could also be due to experimental design, affecting the habitat, soil structuring and their interaction with organism behaviours. As more complex systems, which provide a more realistic habitat, TME experiments in Chapter 5 could allow soil invertebrates to have higher resistance and resilience to metal contamination when compared to microcosm tests of Chapter 4. In TMEs, under a more realistic contamination, the lower contamination in deeper soil layers, could function as a population reservoir allowing the recovery of the more contaminated soil, closer to the surface. Also, TMEs as a model ecosystem could provide more sources of refuge from contamination, like growing plants and plant roots.

There are also factors which, while not explicitly demonstrated in this thesis, are important to consider as drivers of toxicodynamics. An organism's ecological strategies can affect their responses to metal contamination, for instance in chapter 5, it was hypothesised that organisms could reduce their activities (measured by feeding activity and organic matter decomposition) as a coping mechanism to metal stress allowing them to maintain their abundances. Also, some species could reduce their reproductive output as a strategy to increase energy allocation for metal resistance and survival [298]. While this hypotheses has been suggested [298] the mechanisms behind these strategies are not well known. Finally, climate, which has been a major focus in recent years due to climate change, is known to affect the response of organisms to contamination through temperature and expected to function as an added stressor to the organism's biology. While experimentation has been performed at different temperatures [34,159,179,256], few studies, if any, report the toxicodynamic mechanisms of how temperature affects organism response to metals.

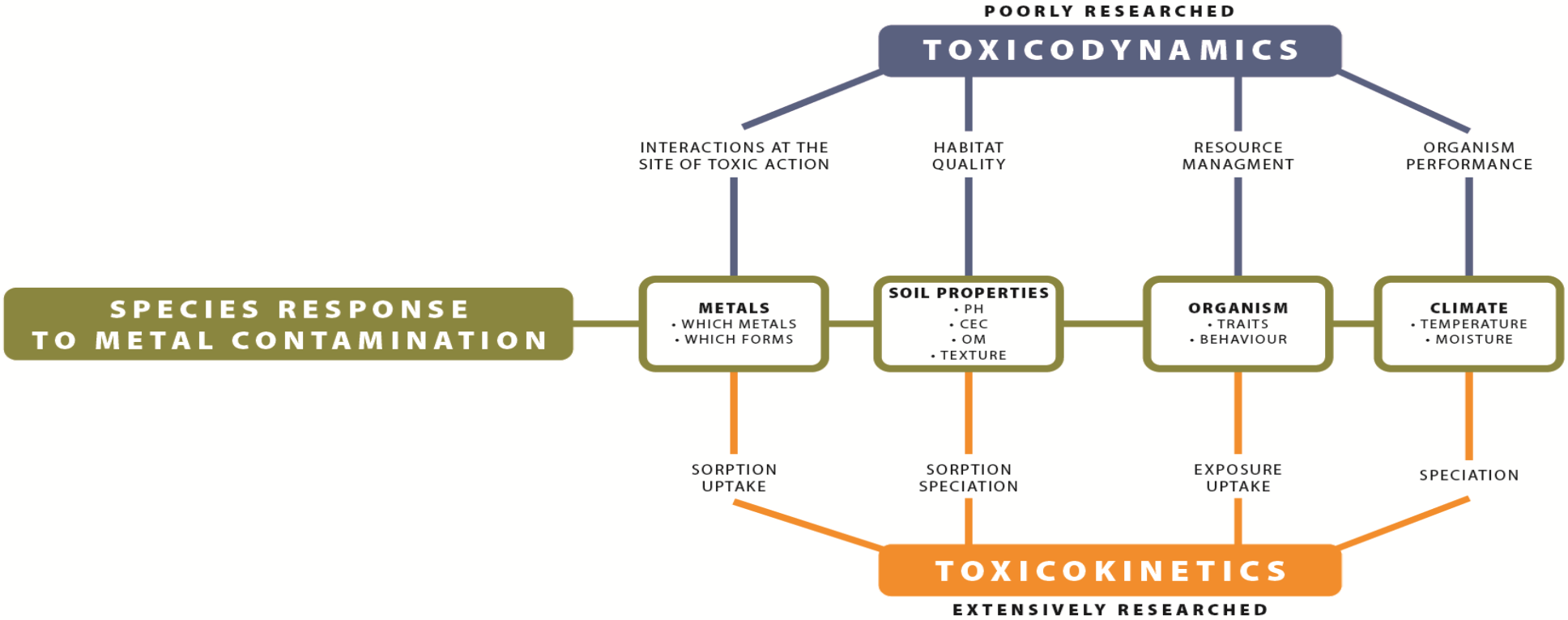


Figure 1. A comparison of toxicodynamic and toxicokinetic influence of soil invertebrate response to metals. The green boxes include examples of research questions, whereas the text breaking the orange/blue pathways indicate fundamental concepts being explored by those questions.

Risk assessment of metals in soil

The research conducted in this thesis demonstrates that, risk assessments need to change in how they deal with metals in soil. If there was a single take home message from this work, it is that risk assessment schemes must be holistic covering multiple compartments and site specific. Site-specific studies covering multiple compartments, while undoubtedly more costly, might be more than compensated in the long term by reducing the cost of remedial actions to current generic guideline values but still maintaining adequate environmental protection. This is especially true considering the magnitude of difference between guideline thresholds and observed effects at higher ecological levels (Chapter 4 and 5).

In more detail, the work developed in this thesis highlights the need of considering site specific information in ERA schemes. Experiments conducted demonstrated that soil properties heavily influence the toxicity of metals and that total metal and $CaCl_2$ metal concentrations are poor predictors of toxicity. Also, risk assessment schemes, should consider soil properties not only in their role affecting toxicokinetics but also toxicodynamics.

For metal mixtures, it is important that appropriate ratios relevant for a particular contaminated site be considered and their joint action evaluated at the target protection threshold. It was also demonstrated in this study that responses to metal mixtures can be species specific and standard test species might not be representative of the global community response to the joint action of metals. One of the factors discussed in this thesis, is the importance of community composition, in affecting the outcome of metal contamination (chapter 4). In this sense, in the restoration of a contaminated site, it is important to define which are the potential colonizing species of a site to better understand their recolonization potential and adapt target protection threshold to these communities. The effects of contamination must also evaluate multiple compartments of the ecosystem and cover ecosystem function as these can respond quite differently between each other. Finally, it is important to consider that the soil functions as a habitat and identify other potential stressors impairing habitat quality in addition to metal contamination.

In this project, important overarching tools were developed with a much wider frame of future use in risk assessment. It is known that mixture interactions can shift at different dose levels, however when using a toxic unit approach, additivity is generally tested only at the EC50 level. Surface response models could be considered to include dose and ratio but are limited in the

complexity of mixtures to which they can be applied. To indirectly account for dose, in Chapter 3, a novel approach was developed by measuring deviations from additivity in a range of dose/effect levels (EC10 – EC90). This approach demonstrated that mixture effects shift at different effect levels and were strongest outside the EC50 range. In this thesis important tools were also established to deal with ecological complexity, especially for the integration of community data in risk assessment schemes by developing community effect concentrations. Developed in Chapter 4 to analyse microcosm data, community effect concentrations measure the percent of community change produced by a contaminant compared to a control community not exposed to contamination. This concept uses community similarity indices and assumes that as contamination increases, the similarity of the community decreases, producing a dose response curve which can be used to derive effect concentrations.

Knowledge gaps and future research

In the risk assessment of metals and ecotoxicology in general there are some knowledge gaps which need further research investment. In addition to a better understanding of the mechanistic toxicity of contaminants (including metals) with recent studies on genomics, proteomics and energy budget, there needs to be an investment in more of a "back to basics" sense in understanding the biology and ecology of, at least, standard test species. In particular, research on soil ingestion, if it occurs, either passively or actively and how much, to explore its role as a route of exposure for soil invertebrates.

There is still the need for a more accurate measure of the availability of metals to organisms. In Chapter 2, the toxicity of mixtures to *F. candida*, which is commonly assumed to be exposed through pore-water, was linked to the most soluble metals, but not correlated with $CaCl_2$ extractable concentrations. Other extractants could have been considered to estimate bioavailability, however there is a lack of guidance over which methods are more appropriate and under which circumstances. For instance, the bioavailable fraction of metal oxides versus salts might require the use of different extractants. Also, different routes of exposure might require different methods for ascertaining availability, where for example soil ingestion, affected by gut chemistry is expected to be different than exposure routes mediated by soil pore-water.

In terms of higher tier testing using natural community and TME experiments the questions are still vast and we just need, more! More data, more experiments, under different conditions, test

designs leading to a better understanding and better tools. Specifically and related to the findings of this thesis, the community ECx concept must be validated with further experiments, at different tiers and with different contaminants, to test its robustness and there needs to be a better understanding of the link between ecosystem structural and functional responses to contaminants.

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