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

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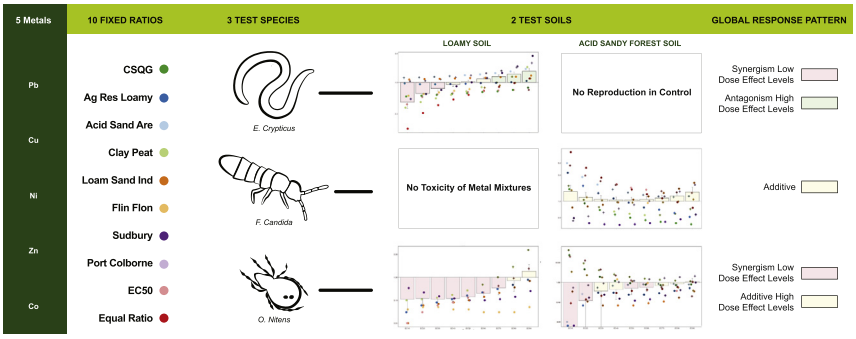
HIGHLIGHTS

- Total metal concentrations did not correlate with species responses to mixtures.
- Joint action only at the EC50 level would underestimate deviations from additivity.
- Highest synergisms were detected below the EC50 level.
- Risk assessment schemes should test additivity at the target protection level.

GRAPHICAL ABSTRACT



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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* both 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.

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1. 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 (Boumc et al., 1988; Wuana and Okieimen, 2014). Metals are severe and persistent chemicals in the environment and are especially concerning because they do not degrade like organic contaminants (Wuana and Okieimen, 2014; Peijnenburg and Vijver, 2007). Although most metal-contaminated sites consist of a complex mixture of metals (McMartin et al., 1999; Gratton et al., 2000), most soil ecotoxicology studies (Sandifer and Hopkin, 1996; Sandifer and Hopkin, 1997; Lock et al., 2004; Lock and Janssen, 2002a) and consequently environmental thresholds used in risk assessment are based on single metals (CCME, Canadian Council of Ministers of the Environment, 2019; Arche-Consulting, n.d.). 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 (Kortenkamp et al., 2009; Lock and Janssen, 2002b). However toxicity responses to metal mixtures often deviate from the CA model (Kortenkamp et al., 2009; Chapman, 2008).

In metal mixture ecotoxicological research (Kortenkamp et al., 2009), and in soil ecotoxicology research in particular (Lock and Janssen, 2002b; Jonker et al., 2004; He and Van Gestel, 2015; Baas et al., 2007; Posthuma et al., 1997; Qiu et al., 2011; Van Gestel and Hensbergen, 1997; He et al., 2015; Khalil et al., 1996), most studies focus on binary or ternary mixtures. In soils, binary and ternary mixtures have either additive (Baas et al., 2007; Posthuma et al., 1997; Van Gestel and Hensbergen, 1997; He et al., 2015), antagonistic (Lock and Janssen, 2002b; Baas et al., 2007; Posthuma et al., 1997; Qiu et al., 2011; Van Gestel and Hensbergen, 1997; He et al., 2015; Khalil et al., 1996) or at high doses (>EC50) synergistic effects (Jonker et al., 2004). Even within the same study, different patterns of responses might be observed depending on the endpoint measured (Van Gestel and Hensbergen, 1997) (e.g., survival, reproduction), measures of metal data (e.g., total, internal body concentrations) (Posthuma et al., 1997; He et al., 2015), and the composition of the mixtures (Baas et al., 2007). 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) (Peijnenburg and Vijver, 2007; Calamari and Alabaster, 1980). 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., *E. crypticus* or *F. candida*) is used in mixture studies (Lock and Janssen, 2002b; Jonker et al., 2004; He and Van Gestel, 2015; Baas et al., 2007; Posthuma et al., 1997; Qiu et al., 2011; Van Gestel and Hensbergen, 1997; He et al., 2015). 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 (Peijnenburg et al., 1999), while metal partitioning is influenced by chemical reactions within the soil, such as precipitation/dissolution, adsorption/desorption, and aqueous complexation (McLean and Bledsoe, 1992). These reactions, in turn, depend on metal speciation, soil properties and chemistry (Wuana and Okieimen, 2014; Cipullo et al., 2018). 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 (Peijnenburg et al., 1999; McLean and Bledsoe, 1992). In fact, pH can be considered the master variable for metal availability (Chapman, 2008) and has been shown to be a good predictor of availability across a range of soils with differing properties (Smolders et al., 2009).

Metal uptake is organism-specific and correlates with species traits that influence their routes of exposure (Peijnenburg et al., 1999). In general, routes of exposure for metals in animals include ingestion (soil, pore-water, and contaminated food), dermal adsorption, and respiration (Chapman, 2008). These routes are affected by organism traits such as feeding behaviour and exterior barriers such as the level of sclerotization (Hedde et al., 2012). 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 (Peijnenburg and Jager, 2003). For soil invertebrates, some studies found that metal toxicity correlates with solubility (Arnold et al., 2003; Smit and Van Gestel, 2009), but others found no such correlation (Smolders et al., 2009; Crommentuijn et al., 1997). As highlighted by Peijnenburg and Jager (2003), 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 (Peijnenburg and Jager, 2003). The process of uptake may in fact be much more complex and not necessarily mediated by soil pore-water. For instance *O. nitens* exposure to metal oxides was correlated to total rather than extractable metal concentrations and exposure attributed to soil ingestion rather than pore-water (Jegede et al., 2019; Fajana et al., 2020). 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 (Jegede et al., 2019).

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, *Enchytraeus crypticus*, *Folsomia candida* and *Oppia nitens* with different external barriers and routes of exposure. *E. crypticus* are soft-bodied annelids (Castro-Ferreira et al., 2012) and exposure to metals, similarly to earthworms, is expected to occur dermally and through soil ingestion (Vijver et al., 2003). *F. candida* has a protective external cuticle (Fountain and Hopkin, 2005) and exposure is mostly linked to, contaminated soil, porewater and to a lesser extent food (Pedersen et al., 2000). *O. nitens*, have the most developed external barriers with a heavily sclerotized body (Princz et al., 2010; Fajana et al., 2019) and exposure is expected to occur through the ingestion of contaminated soil or organic matter (Jegede et al., 2019; Fajana et al., 2020) or in juveniles where external barriers are not as developed (Princz et al., 2010).For the five element metal mixtures, neither a full factorial design (Baas et al., 2007) nor a central composite design (Lock and Janssen, 2002b) can be used. Therefore, we used a fixed-ratio ray design because it can test for deviations from additivity in ratios of particular interest (Coffey et al., 2005). 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.

2. Methods

2.1. 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 2 mm. Soil pH was measured in 0.01 M $CaCl_2$. CEC was established using the methylene blue method (Yukselen and Kaya, 2008), texture was determined by the pipette method (Bouyoucos, 1962), organic carbon was determined using a LECO-C632 analyzer (Wang and Anderson, 1998), and water holding capacity was determined as described in annex C of ISO11268-2 (ISO 11268-2, 1998). Soil properties are listed in Table 1.

2.2. 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 (Arche-Consulting, n.d.) 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 EC_{50} values for each metal (lead, copper, nickel, zinc and cobalt) and an equal ratio between all elements.

2.3. 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_4 powder < 10 μm). Before the experiment, oxides were placed for 24 h 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 regimens, 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 °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%) (Renaud et al., 2020) 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.

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).

2.4. Chemical analysis

X-Ray Fluorescence (XRF) (Marguí et al., 2009) 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 discs. Soil discs 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 (Quevauviller, 1998) 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 50 ml falcon tubes and shaken for 3 h at 15 rpm. Samples were then centrifuged at 5000 rpm for 10 min 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).

2.5. 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

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

Soil	pH-CaCl	CEC	Organic C	Clay content	Water holding capacity
		(meq 100 g^{-1})	(g kg^{-1})	(g kg^{-1})	(ml g^{-1})
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

Table 2
Percent composition of ratios by weight in mg/kg (W) and moles/kg (M) of lead, copper, nickel, zinc[a], 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

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

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.

2.5.1. 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 (Ritz and Strebig, 2016). See Supplementary material Table 1SD for a full list of ECx values.

2.5.2. 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 (Eq. (1)). This approach enabled us to calculate deviations from additivity at different dose/effect levels.

$$\sum TU_{ECx} = \sum_{i=1}^{n} \frac{Ci}{ECxi} = 1\ additive, >1\ Antagonistic, 1<Synergistic \qquad (1)$$

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. Fig. 1SD in the Supplementary material shows the dose response curves for mixture toxic units calculated at different dose/effect levels. Significant deviations from additivity or 1TU 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 (Ritz and Strebig, 2016).

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 (R Development Core Team, 2008), and figures were constructed using the package ggplot2 (Wickham et al., 2018).

3. 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 (Fig. 1). The largest deviations from 1 toxic unit (TU) for *F. candida* were observed at both low

Table 3
Procedures and guidelines adopted for each soil invertebrate reproduction test.

	Enchytraeus crypticus	*Folsomia candida*	*Oppia nitens*
Guideline	ISO 16387, 2016	ISO 11267, 1999	Princz et al., 2010
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

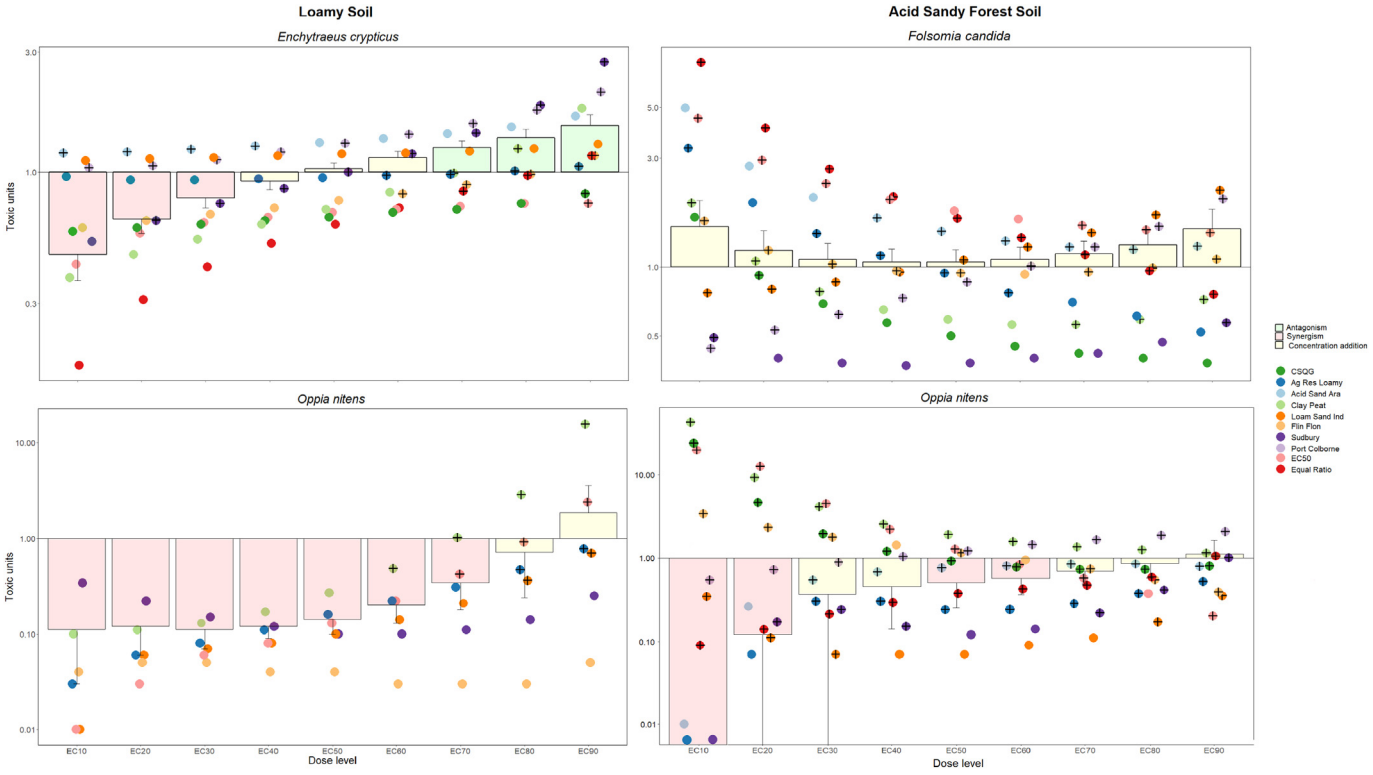

Fig. 1. Global (bars) and individual (points) measured mixture toxic unit (TU) at different dose/effect levels for three test species (*E. crypticus*, *F. candida*, *and O. nitens*) in the two test soils (loamy and acid sandy forest soils). Bar fills indicate the significance or lack of deviation from 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, $p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(EC10 = 1.5 TU ±0.4) and high dose/effect levels (EC90 = 1.5 TU ± 0.3). However, responses were never high enough or had too large an error 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 differed in 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).

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 (Fig. 1 and Fig. 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

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

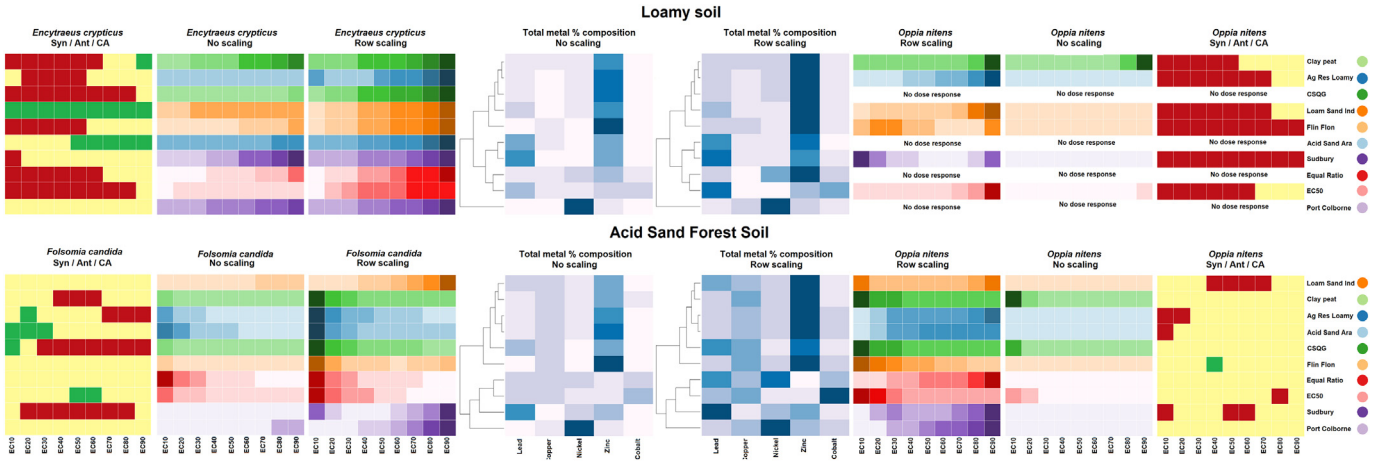

Fig. 2. Species response matrixes (with, without scaling by rows and significance of deviation from additivity) ordered by percent total metal mixture composition dendrogram (row scaling represents values scaled for the different doses, while no scaling represents no scaling across mixtures). Color shade 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). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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.

A total metal mixture composition dendrogram (Fig. 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* (Fig. 2, No scaling) or pattern of response for *O. nitens* (Fig. 2, Row scaling). In the acid sandy forest soil, mixture composition clustering did not group a similar pattern of response (Fig. 2, Row scaling) or magnitude of response (Fig. 2, No scaling) for either test species. Mixture total metal composition clustering also did not group similar responses when considering significant antagonisms or synergisms (Fig. 2, Syn/Ant/CA panels).

The observations in Fig. 2, linking mixture clustering and species responses were supported by a Mantel test that determined no correlation between metal mixture composition and species responses (Supplementary data Table 5SD). No significant correlation was found between species responses and total metal composition (loamy soil: *E. crypticus* $p = 0.21$, *O. nitens* $p = 0.44$ l; 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 ($p > 0.05$).

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 (Fig. 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 5SD), which are the metals with highest CaCl2 extractable concentrations (zinc = 2.90, copper = 0.64% of total, Fig. 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 (Fig. 3)—produced no toxicity as a single metal to *O. nitens* in loamy soil (Supplementary material Tables 1–4SD).

4. Discussion

In general, the results appear to contradict the funnel hypothesis (Warne and Hawker, 1995) 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

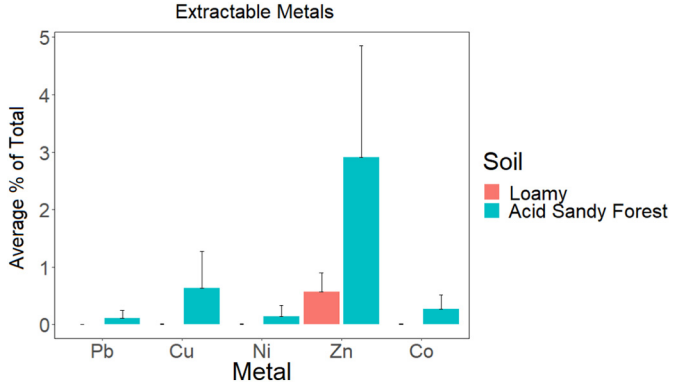

Fig. 3. Average percent of total metals extracted by CaCl2 for each metal (lead, copper, nickel, zinc, and cobalt) across all mixture ratios and dose/effect levels for each test soil (loamy and acid sandy forest). Error bars represent the standard deviation from the mean.

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 (Van Gestel and Hensbergen, 1997) or antagonistic for Cd/Pb (Bur et al., 2012).

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 (Posthuma et al., 1997) and antagonistic for Zn/Cd and Zn/Cu (Weltje, 1998) at the EC50 level, and when considering surface response models for binary mixtures of Zn/Cd/Cu/Pb (Lock and Janssen, 2002b), 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 (He et al., 2015). 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 (Lock and Janssen, 2002b; Van Gestel and Hensbergen, 1997; Bur et al., 2012; Weltje, 1998), 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 (Posthuma et al., 1997; Van Gestel and Hensbergen, 1997; Weltje, 1998), 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 (Lock and Janssen, 2002b). 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 (Fig. 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 (Fig. 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 (Smit and Van Gestel, 2009; Fountain and Hopkin, 2005). 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 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 (Pedersen et al., 2000).

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 (Fajana et al., 2020). 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 (Vijver et al., 2003). 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 (Smolders et al., 2009). 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 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 (Smolders et al., 2009). Metal solubility in soil is highest at both low and high values of pH and is lowest in intermediate neutral pH values (Dijkstra et al., 2004). 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 (Lock and Janssen, 2001; Criel et al., 2008). 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 (Smolders et al., 2009). 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) (Renaud et al., 2020; Korte et al., 1976; Gomes et al., 2001). 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 perform poorly in soils with pH below 3.8 (Luo et al., 2014). 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 (Jegede et al., 2019). 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 (Kortenkamp et al., 2009; Lock and Janssen, 2002b). 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 5th 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 (Checkai et al., 2014; CCME, 2006).

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 (Checkai et al., 2014; CCME, 2006). 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 (Arche-Consulting, n.d.)). 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.

5. 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. crypticus* 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*.

CRediT authorship contribution statement

Mathieu Renaud: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Mark Cousins:** Conceptualization, Investigation, Methodology. **Kobby Fred Awuah:** Conceptualization, Investigation, Methodology, Writing - review & editing. **Olukayode Jegede:** Conceptualization, Investigation, Methodology, Writing - review & editing. **José Paulo Sousa:** Supervision, Writing - review & editing. **Steven Douglas Siciliano:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.139921.

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