IMPROVING THE DOSING METHOD AND ANALYSIS OF METAL MIXTURE DOSE RESPONSE IN SOIL

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ABSTRACT

Examination of a stressor's toxic effects on terrestrial organisms is a relatively new field compared to human health and aquatic toxicity, and the toxicity of multiple stressors on terrestrial organisms is newer still. Dose-response functions and statistical analyses have crossed over from aquatic toxicity research, however the effect of soil properties on soil toxicity is still being researched extensively.

Being home to about 3.5 million contaminated sites (Swartjes, 2011), the European Union has a large incentive to research the effect of stressors in soil. Smolders et al. (2009) used this research to develop the basis for the EU REACH PNEC calculator. The calculator uses pH, eCEC, clay content, and background metal concentrations to estimate the Predicted No Effect Concentration (PNEC) of a stressor in soil: the upper concentration where there should be no significant effect on a soil ecosystem. The calculator is limited to estimating the PNEC of single stressors, and combinations of are assumed to follow Concentration Addition.

Rather than delving into the chemical and biological complexities of how stressors in soil interact with one another and test organisms, Jonker et al. (2005) developed a statistics based tool to evaluate mixture toxicity. Their method uses the concepts of Concentration Addition and the sigmoid dose-response function paired with a deviation parameter. The deviation parameter changes depending on whether a user wants to test perfect Concentration Addition, synergistic or antagonistic effects, dose-ratio dependent deviation, or dose-level deviation. The methods in Jonker et al. (2005) were adapted to analyse binary mixture behaviour using a Microsoft Excel spreadsheet. While this worksheet may work well for small mixtures, it has not been used for larger combinations of stressors.

To improve the results found using the Jonker et al. (2005) methods, I created scripts using the optim() function in R (R Development Core Team, 2008). The scripts will evaluate toxicity data for mixtures of up to five stressors for any of the four types of deviation from Concentration Addition. Rather than using an iterative method as developed in the Excel spreadsheet, the R scripts use the uniroot.all() function in the rootSolve R package (Soetaert,

2009). The uniroot.all() function simultaneously evaluates all data points to make the Jonker et al. (2005) equations valid. The optim() function then changes the individual stressors' dose response curves to minimize the differences between the predicted response and the observed response. Using synthetic data with varying data point spread and starting parameters, the R script produced lower sum of square values than the Excel sheet when modified to evaluate mixtures of five stressors. In addition to finding a lower sum of squares, the annotated R scripts offer a solution to researchers examining the effects of mixtures larger than binary without needing to develop the tools themselves and reduces the "black box" of custom Visual Basic for Applications (VBA) functions if they are not familiar with that language.

Prior to analyzing mixture toxicity data, one must ensure that one's data is useful. In some cases, a stressor may be applied directly to a test medium and a researcher can observe its effect on an organism. In the case of metal effects on soil organisms, however, a problem may occur. The standard application of metal salts to a soil and subsequent leaching can remove a different proportion of each stressor from the test medium. Dosing strategies that require specific combinations of metals, such as fixed-ratio rays, can pose a challenge to a researcher.

To find an alternative to metal salts, five soils were dosed with five different metal mixtures in three different ways. Metals were applied as aqueous nitrate salts and leached, as dry powdered commercially available metal oxides, and as spinel-like minerals. The spinel-like minerals were made by mixing aqueous nitrate salts, adding iron nitrate in a 2:1 molar ratio of iron to all other metals, precipitated, and annealed in a muffle furnace to remove nitrates. The minerals were intended to resemble franklinite, which is commonly found in contaminated smelter sites. *Folsomia candida*, an extensively studied and easy to culture hard-bodied soil invertebrate, was used as a response organism.

The spinel-like minerals and oxides were both more effective at retaining the relative proportions of each metal to one another. In some cases, over 60% of total metal nitrates added to soils was removed during leaching in addition to losing different amounts of each metal. Even though the metal concentrations were lower in nitrate dosed soils, the average reproduction was similar to the oxide dosed soils, where the spinel dosed soils showed no effect on reproduction. The research here shows that, with some more research, dosing soils using metal oxides is a promising alternative to aqueous nitrate dose method.

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LIST OF ABBREVIATIONS

BFGS	Broyden, Fletcher, Garb and Shanno
CA	Concentration Addition
CG	Conjugate Gradients
EC50	Effective Concentration of a stressor to create 50% of a response
eCEC	Effective Cation Exchange Capacity
DLD	Dose-level Dependence
DRD	Dose-Ratio Dependence
HC	Hazardous Concentration for Sensitive Species
IA	Independent Action
L-BFGS-B	Limited memory Broyden, Fletcher, Garb, and Shanno Boxed method
NM	Nelder and Mead (1965)
PNEC	Predicted No-Effect Concentration
S/A	Synergistic or Antagonistic
SANN	Simulated-Annealing
SSD	Species Sensitivity Distribution
SSE	Sum of Squared Errors
TU	Toxic Units
VBA	Visual Basic for Applications

1.0 INTRODUCTION

There have been many new developments in risk assessment tools for metals and other environmental stressors in recent years. Arche Consulting's Soil PNEC Calculator (ARCHE, 2017) is one of those tools. By inputting certain soil parameters and a metal of interest it will calculate a Predicted No-Effect Concentration (PNEC) based on European Union Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) values. At concentrations below the PNEC, effects to the soil ecosystem are not likely to be significant. If there are several metals in a mixture, users are to assume that the contaminants follow Concentration Addition (CA). A new mixture PNEC would be calculated based on the mixture ratio and individual PNECs for each metal.

Mixture toxicity is not a settled matter however. While some mixtures may follow CA, some may be closer to Independent Action (IA) (Cedergreen et al., 2008), or more complex relationships as described in Jonker et. al, 2005. There is also a lack of information on the behavior of metal mixtures in larger than binary combinations (Kortenkamp et al., 2009). Mixture toxicity can be tested as a fixed-ratio ray dose design. This design keeps the relative ratio of each stressor in a mixture constant while increasing the total dosage (Casey et al., 2004). While in the process of conducting a large scale five-metal mixture experiment to examine the mixtures' behavior compared to CA, we encountered problems with the typical dosing method of applying aqueous metal salts to soils.

When the soils had been spiked and subsequently leached to return to control soil conductivity levels, we found that each metal had been leached from the soil in different

quantities. While this is a fairly easily remedied problem with single metal assays, it would prevent us from maintaining a fixed-ratio mixture over increasing dose levels. If a solution to this issue is found, analysis of metal mixture toxicity assays could be much easier.

The behavior of metal mixture assays can be done using methods in Jonker et al. (2005). Rather than depending on toxicological principles to explain interactions, Jonker et al. (2005) used statistical tools to account for synergism or antagonism, dose-ratio dependence, or dose-level dependence. A Microsoft Excel spreadsheet designed for binary mixtures accompanied the research. When the sheet was adapted for five stressor mixtures and a large data set, calculations became slow and needed to be ran several times because the search algorithms would often become trapped in local minima. Developing a more powerful, and easier to use tool would encourage researchers to make use of the methods in Jonker et al. (2005).

1.1 Hypotheses and Objectives

Hypothesis 1: Creating R scripts to determine how organisms respond to a mixture of stressors, as laid out by Jonker et al. (2005), will increase analysis speed and accuracy compared to using Microsoft Excel.

Objective 1: Create R scripts for Concentration Addition, synergism and antagonism, dose-ratio dependence, and dose-level dependence.

Objective 2: Modify these scripts to allow users to test mixtures of 2 to 5 stressors.

Hypothesis 2: Dosing soils with powdered metal oxides will improve the consistency of metal to metal concentrationsover using aqueous metal nitrates and leaching.

Objective 3: Compare soil invertebrate response and metal retention across soils dosed using aqueous metal nitrates, powdered metal oxides, and spinel-like metal compounds.

2.0 LITERATURE REVIEW

2.1 Concentration Addition Theory

Concentration Addition is summarized by Berenbaum (1985) where he describes the effects of multiple stimuli on an individual based on the original theory in Bliss (1939). The basis for CA is the use of toxic units (TU). A toxic unit is the ratio of the concentration of a stressor to a reference toxicity threshold. Such ratios can be calculated using Equation 1 (Ginebreda et al., 2014).

$$\sum_{i=1}^{n} \frac{x_i}{X_i} = TU \qquad \text{(Eq. 1)}$$

Where x_i is the concentration of contaminant i, X_i is the reference dose for contaminant i, and TU is the number of toxic units. When working with some toxicity thresholds, X_i is analogous to an Effective Concentration where x percent of an effect is observed (ECx) (Bhattacharya et al., 2011).

Prior to determining the number of toxic units, however, the X_i must be determined. There are many curve-fitting methods with which to do this including the two-parameter Weibull, probit, logit, and logarithmic distributions (Christensen, 1984; Christensen and Nyholm, 1984; Krogh, 2008). In this case I will be using the two-parameter logarithmic distribution, also known as the sigmoid model. The sigmoid model is shown in Equation 2.

$$R = \frac{k}{1 + \left(\frac{c}{EC50}\right)^{\beta}} \qquad \text{(Eq. 2)}$$

Where R is the biological response of an organism, k is the maximum biological response, c is the concentration of the stressor being examined, $EC50$ is the concentration where a 50% response occurs, and β controls the shape of the curve.

In cases where the concentration of a single metal is equal to the toxicity threshold, the result is a TU of 1. If there are combinations of contaminants, the ratios of x_i to X_i are summed to obtain the amount of TU at the site. At a TU greater than or equal to 1, the environment must be remediated to reduce contaminant concentration to below the specified endpoint (von der Ohe and de Swart, 2013). TU were originally used for acute endpoints (e.g. mortality), but their basic principle has been adapted to other endpoints (von der Ohe and de Swart, 2013).

Adapting TU to find a toxicity threshold for CA, assuming it is valid, is a simple process. Viewing it as the addition of parallel resistors (Anderson and Duffin, 1969), each toxicity threshold can be thought of as an individual resistor. Instead of each threshold (resistor) having equal input, the thresholds are weighted by the relative amount of each stressor in the mixture (Kortenkamp et al., 2009). This relationship is defined in Equation 3.

$$\left(\sum_{i=1}^{n} \frac{p_i}{ECx_i}\right)^{-1} = ECx_{mix} \qquad \text{(Eq. 3)}$$

Where p_i is the fraction of a mixture constituted by stressor i and ECx_i is the concentration of stressor i in isolation that will produce an x% response. The ECx_{mix} is the concentration where an x% response occurs. The concentration ECx_{mix} represents the sum of i stressors in their respective proportions p.

Mixture toxicity can act according to CA or differ in three ways: synergism/antagonism, dose level-dependent deviation, or dose ratio-dependent deviation (Jonker et al., 2005). In order

to properly test how metal mixtures may affect soil differently than CA, all ratios and dose levels must be equally covered (Jonker et al., 2005). This can be accomplished through factorial, central composite, fixed ratio, and D-optimal designs (Jonker et al., 2005).

2.2 Modeling Deviations from Concentration Addition

The method proposed by Jonker et al., 2005 uses a modified form of Equation 1, where the sum of TU does not necessarily equal 1 at the chosen reference dose, i.e. not necessarily following the assumptions of CA. The right side of Equation 1 is replaced by the value e^G (Equation 4).

$$\sum_{i=1}^{n} \frac{c_i}{f^{-1}(Y)_i} = e^G \qquad \text{(Eq. 4)}$$

Where c_i is the concentration of metal i, $f^{-1}(Y)_i$ is the inverse dose response as a function of Y for metal i, G is a function that determines how the model is assumed to deviate from CA, and Y is an artificial biological response fitted to each data point (Jonker et al., 2005).The inverse dose response curve value is the concentration determined from rearranging the dose response function expanded from Equation 2 to obtain a concentration, as shown in Equation 5:

$$f^{-1}(Y) = EC50 * \left(\frac{k - Y}{Y}\right)^{\frac{1}{\beta}} \qquad \text{(Eq. 5)}$$

In this case, Y is analogous to the biological response R, except that it will be used to satisfy the constraints of CA and the 3 types of deviation explained later. k is the maximum biological response as per Equation 2. $f^{-1}(Y)$ replaces c in Equation 2 to define itself more as a function than simply curve interpolation, but is essentially the concentration of a stressor where the response Y will occur.

Equation 5 can be used to determine the $f^{-1}(Y)$ at any point along a single stressor dose response curve. This is, essentially, determining an EC5, EC10, or any other reference dose required from the single metal dose-response curves. Using the concentrations of each metal at each dose, an ECx will be found for each metal that satisfies the constraints of the deviation function being modeled.

2.2.1 Ideal Concentration Addition

Initially, we use the data from the single metal toxicity assays to generate the EC50 and other fitting parameters of the dose response curve. Using the fitting parameters from the single metal exposures, we then assume perfect concentration addition and generate new synthetic values for the responses which cause the right side of Equation 3 (e^G) to become 1 (i.e. Equation 2). The value of G will vary to accommodate the type of deviation for subsequent models. The simplest way to use the inverse dose response function is with the ideal CA adaptation. The following process explains how the predicted biological response at each mixture dose is calculated:

1. Toxic units are calculated as the ratio of contaminant concentration to the reference dose.
2. Any concentration where a response Y is found can be used as a reference dose EC_Y.
3. Creating dose response curves for single stressors allows for interpolation of the biological response Y at any concentration.
4. The inverse dose response function then calculates the concentration EC_Y where a biological response Y occurs.
5. The sum of TU calculated with a reference dose EC_Y at a biological response Y will equal 1.
6. Initially we do not know what the biological response Y should be in the mixture, and therefore do not know the EC_Y where it will occur.
7. Equation 1 therefore has the unknown values Y and n EC_Y values, where n is the number of stressors in the mixture.

8. The predicted *Y* is changed in iterations and the concentrations of the *n* EC_Y for each stressor where the response *Y* would occur is interpolated.
9. The value of *Y* where the left side of Equation 1 is equal to 1 is the predicted biological response at each dose if CA is valid.

The parameters of each dose-response curve are then modified to produce the least sum of squared errors (SSE) between the predicted response *Y* and the observed responses are minimized. Single metal response data are included when minimizing error, however they are only calculated using single metal response curves.

CA is not validated until all other tests have been completed. If adjusting to CA provides the lowest SSE after testing for synergism/antagonism, dose-ratio dependence, and dose-level dependence, it may be considered the dominant mechanism controlling mixture toxicity for this method and combination of metals.

2.2.2 Synergism or Antagonism

The toxicity of each stressor in the mixture can be quantified by its TU, which can then be used to estimate the relative toxicity *z* of each metal in a mixture. This is accomplished by dividing each individual TU by the sum of TU in the mixture. This is summarized by Equation 6:

$$z_i = \frac{TU_i}{\sum_{i=1}^{n} TU} \qquad \text{(Eq.6)}$$

Where z_i is the relative toxicity of stressor *i* in the mixture and TU_i is the toxic units of stressor i.

Jonker et al. (2005) indicates that overall synergism and antagonism of a mixture can be accounted for by multiplying the parameter *a* by the product of relative toxicities z_i calculated by Equation 5 to create Equation 7:

$$G(z_1, z_2, \ldots z_n) = a\prod_{i=1}^{n} z_i \qquad \text{(Eq. 7)}$$

Where *a* is the level of synergism or antagonism.

If *a* is positive there is an overall antagonistic effect and if *a* is negative there is overall synergism. To fit the data, responses of *Y* are calculated assuming $a = 0$ ($G = 0$). The values of *a*, *k*, and individual EC50 and β values are then modified concurrently to reduce the SSE to a minimum.

The fit of observed biological response to fitted response *Y* is compared between CA and the synergism/antagonism (S/A) model using the Chi-Square distribution. The degrees of freedom for the Chi-Square analysis is determined by the number of additional variables in the current deviation type compared to the deviation type being tested against. In this case the degrees of freedom would be 1, as the *a* parameter is the only variable in the S/A equation that is completely independent from the CA equation. The point along the Chi-Square distribution to be evaluated is determined by Equation 8:

$$\chi^2 = n * ln\left(\frac{\sum SS_O}{\sum SS_T}\right) \qquad \text{(Eq.8)}$$

Where χ^2 represents the chi-square statistic, *n* indicated the number of data points, SS_o is the sum of squared errors from the original data fit, CA in this case, and SS_T is the sum of squared errors from the current deviation method being tested.

Using the degrees of freedom and the value of χ^2, a *p* value can be calculated to determine if two of the models are significantly different from one another. If the S/A model produces a lower SSE and is significantly different from CA, S/A can be adopted as the preferred mixture toxicity model.

2.2.3 Dose Ratio Dependent Deviation

Testing for dose-ratio dependence (DRD) is another step past synergism and antagonism analysis where the biological response is examined for effects by individual stressors as well as overall effects. Jonker et al. (2005) describes this new *G* in Equation 8:

$$G(z_1, \dots, z_n) = (a_{DR} + b_{DR1}z_1 + \cdots + b_{DRn-1}z_{n-1}) \prod_{i=1}^{n} z_i \qquad \text{(Eq. 8)}$$

Where a_{DR} is a factor accounting for non-significant effects of metals not included in the model and b_{DRi} is the level of effect for *i* significant metals.

The value of a_{DR} is similar in mechanism to *a* in the S/A dependent *G* (Equation 7). It accounts for overall effects of the metals in a mixture which are not significant enough to be characterized by a significant *b*. In complex mixtures (i.e. greater than binary) each *b* is tested individually with each stressor for an improvement in fit. If the introduction of the b_{DR} value(s) produces a better fit of *Y* to observed responses and is significantly different than CA or S/A according to the Chi-Square distribution, it is likely that the toxicity of the mixture is dependent on the ratio of stressor *i* used in the model. DRD would then be assumed to be the mechanism for this mixture.

For example, in a mixture of copper, nickel, and lead the G could be evaluated first using Equation 9:

$$G(z_{Cu}, z_{Ni}, z_{Pb}) = (a_{DR} + b_{Cu}z_{Cu}) * z_{Cu} * z_{Ni} * z_{Pb} \qquad \text{(Eq. 9)}$$

Again, artificial responses are calculated, and k, β, a_{DR}, and b_{Cu} are changed to determine the least sum of squares between the actual responses and the mixture.

The fit from this equation would be compared to the next, where $b_{Cu}z_{Cu}$ would be replaced by $b_{Ni}z_{Ni}$ and then finally $b_{Pb}z_{Pb}$. The b_i that produces the best fit and is significantly better than S/A analysis would then be adopted into the model.

Next, the model is tested for the next significant b_{DR} value. If, for example, $b_{Ni}z_{Ni}$ produced a significantly better fit than synergism or antagonism alone and had the lowest SSE from all tests, the model would then be analysed for improvement by the addition of more *b* values, such as:

$$G(z_{Cu}, z_{Ni}, z_{Pb}) = (a_{DR} + b_{Ni}z_{Ni} + b_{Cu}z_{Cu}) * z_{Cu} * z_{Ni} * z_{Pb} \quad \text{(Eq. 10)}$$

This process will continue until there is no longer a significant improvement to the model.

2.2.4 Dose-Level Dependent Deviation

Transforming concentrations to TU, and then TU to *z* values is used again for dose-level dependence (DRD). Rather than using the modifier b_{DR} for every z_i, a new b_{DL} is used to examine if synergism/antagonism occurs unevenly along the dose-response curve. Equation 11 shows the general equation for *G* in this method.

$$G(z_1, z_2, \ldots z_n) = a_{DL}\left(1 - b_{DL}\sum_{i=1}^{n} TU_i\right)\prod_{i=1}^{n} z_i \quad \text{(Eq. 11)}$$

In this method a positive a_{DL} indicates antagonism at low doses and synergism at high doses, and a negative *a* indicates the opposite. The b_{DL} parameter indicates what point the mixture changes from synergism/antagonism to the opposite. A b_{DL} between 0 and 1 exclusive indicates a change at higher than the EC50, 1 indicates a change at the EC50, and greater than 1 lower than the EC50. If b_{DL} equals 0 then Equation 11 simplifies to Equation 7, S/A. The point at which the change between synergism and antagonism occurs can be estimated by Equation 12:

$$EC_{\Delta synant} = \frac{EC50}{b_{DL}} \quad \text{(Eq. 12)}$$

Where $EC_{\Delta synant}$ is the concentration where the change between synergism and antagonism occurs. The fitting method for DLD is identical to the previous models. Fitted responses of *Y* are found, and the values of a_{DL}, b_{DL}, *k*, and individual EC50 and β values are optimized to produce the lowest SSE between *Y* and the observed responses. If this curve creates a lower sum of squares than and significantly different than CA or S/A, the mixture toxicity is most likely dependent on the dose level of the mixture. When fitting dose-response data to DLD, b_{DL} should generally have the opposite sign of a_{DL} and be constrained to be less than 1 ($0 > a_{DL}$), or greater than -1 ($a_{DL} > 0$). If the value of b_{DL} is outside of these constraints, an inflection point will occur on the dose-response curve, returning the predicted response to the control value at high stressor concentrations. This consequence would likely prevent the determination of a b_{DL} outside of its normal range, but its potential effects should be noted. A summary of all parameter definitions and effects for CA are shown in Table 2.1.

Table 2.1: Summary of Concentration Addition Deviation Parameters (modified from Jonker et al. (2005))

Fitting Method	Parameter	Value	Meaning
Synergism/ Antagonism	a	> 0	Antagonism
		< 0	Synergism
Dose Ratio Dependence	a	> 0	Antagonism [Except for significantly (-) b_is]
		< 0	Synergism [Except for significantly (+) b_is]
	b_{DRi}	> 0	Antagonism significantly affected by stressor i
		< 0	Synergism significantly affected by stressor i
Dose Level Dependence	a_{DL}	> 0	Antagonism at low dose, synergism at high dose
		< 0	Synergism at low dose, antagonism at high dose
	b_{DL}	> 0	S/A change at less than EC50
		1	S/A change at EC50
		$0 < b_{DL} < 1$	S/A change at greater than EC50
		< 0	No change in S/A, but the amount of S/A is dose dependent

2.3 Effects of Soil and Environmental Properties on Metal Properties and Toxicity

During Chapter 2, the toxic effects of five metals of interest – lead, cobalt, nickel, zinc and cobalt – on *folsomia candida*, *oppia nitens*, and *enchytraeus crypticus* will be tested. The dose levels assigned later are based on literature values for *folsomia candida*,

2.3.1 Limitations of Jonker Excel model

While the Microsoft® Excel file provided by Jonker et al. (2005) appears to work well on binary mixture data, there are several limitations that were encountered during our toxicity analysis. The Solver Add-In was developed in part by Frontline Solvers®, Optimal Methods, Inc., and several university groups since 1989 (Frontline Solvers, 2015a). Solver is limited to 200 decision variables, is susceptible to finding local minimums, and takes longer to run than other modern solving tools, especially when dealing with complex equations (Frontline Solvers, 2015b; c).

2.3.2 Cobalt

Cobalt toxicity has not been measured to the extent that some other metals have been. It is usually extracted as a by-product of copper and zinc smelting (Lide, 2005). Naturally occurring cobalt minerals include cobaltite, skutterudite, and erythrite (Royal Society of Chemistry, 2017a). The EC50 of cobalt for *F. candida* is approximately 1480 mg kg^{-1}, and the Canadian Soil Quality Guidelines limit is 300 mg kg^{-1} for industrial sites (CCME, 1991; Lock et al., 2004). In general, the CCME Soil Quality Guidelines are going to have a relatively low limit as they consider humans a target receptor in industrial sites (CCME, 1996). Cobalt appears to be extracted fairly easily from soil by $CaCl_2$ at 22.5% of total metal content (Hsiao et al., 2009; Lago-Vila et al., 2015). Cobalt (II) nitrate can be dissolved in water at a rate of 103 g 100 mL^{-1} at 25ºC.

2.3.3 Copper

The majority of the world's copper deposits are in the form of chalcopyrite and bornite (Royal Society of Chemistry, 2017b). Refined copper is commonly used for building electric motors and some plumbing applications (Royal Society of Chemistry, 2017b). Copper's EC50 for *F. candida* is 700 mg kg^{-1} while the CCME guideline for industrial sites is only 91 mg kg^{-1} (CCME, 1991; Sandifer and Hopkin, 1996a, 1997). Only about 0.3% of total copper can be extracted by $CaCl_2$, so very little is expected to enter porewater after initial sorption on soil particles. (Pueyo et al., 2004). Copper(II) nitrate is soluble at 145 g 100 mL^{-1}, higher than most of the metals listed here (Lide, 2005).

2.3.4 Lead

Lead, a commonly encountered and studied metal, binds to soil quickly but remains available to soil organisms. Being used in radiation protection, batteries, and, at one time, water pipes, lead has been ubiquitous to us for much of human history (Royal Society of Chemistry, 2017c). Lead's EC50 is closer to cobalt's with an approximate EC50 of 1600 mg kg^{-1}, however the industrial CCME guideline is much lower at 600 mg kg^{-1} (CCME, 1991; Sandifer and Hopkin, 1996a, 1997). Lead(II) nitrate is significantly less soluble than the previous metals at only 60 g 100 mL^{-1} (Lide, 2005). Similar to copper, lead is about 0.2% extractable by $CaCl_2$ (Pueyo et al., 2004; Schreck et al., 2011; Houben et al., 2013).

2.3.5 Nickel

Canada is one of the largest producers of nickel in the world, creating a need to examine its properties in soil. The majority of mined nickel is found in nickel sulfides such as pentlandite, pyrrhotite and chalcopyrite (Cornwall, 1966; Royal Society of Chemistry, 2017d). The EC50 of nickel is near 475 mg kg^{-1} and is limited to 89 mg kg^{-1} by the CCME (CCME, 1991; Lock and Janssen, 2002a). Approximately 8.6% of total nickel can be removed from soil

by $CaCl_2$ for an extractable EC50 of 41 mg kg^{-1} (Lago-Vila et al., 2015). Nickel(II) nitrate can be dissolved in water at approximately 99 g 100 mL^{-1} (Lide, 2005).

2.3.6 Zinc

While not as large of an industry as copper or nickel currently, zinc mines and smelters as well as their impact are prolific across Canada. With applications ranging from batteries, soaps, and to galvanization, zinc has been an essential resource for human development (Royal Society of Chemistry, 2017e). The EC50 for zinc is near 750 mg kg^{-1} with an industrial CCME guideline of 360 mg kg^{-1} (CCME, 1991; Sandifer and Hopkin, 1996a, 1997). Zinc and nickel have close extractable EC50 values with zinc at 40 mg kg^{-1} due to an approximate 5% fraction extractable by $CaCl_2$ (Lock and Janssen, 2003a; Pueyo et al., 2004; Houben et al., 2013). Zinc(II) nitrate is tied with copper for the most soluble compound of the metals of interest at 145 g 100 mL^{-1} (Lide, 2005). A summary of the previously described metals' properties can be found in Table 2.2.

Table 2.2: Summary of threshold values for *F. candida*

Metal	EC50 (mg kg^{-1})	CCME Industrial Guideline Concentration (mg kg^{-1})	Percent Extractable by $CaCl_2$ (%)
Cobalt	1480[†]	300[‡]	22.5[§,¶]
Copper	700[#,††]	91[‡]	0.3[‡‡]
Lead	1600[#,††]	600[‡]	0.2[‡‡,§§,¶¶]
Nickel	475[##]	89[‡]	8.6[¶]
Zinc	750[#,††]	360[‡]	5.0[‡‡,¶¶]

[†]Lock et al., 2004
[‡]CCME, 1991
[§]Hsiao et al., 2009
[¶]Lago-Vila et al., 2015
[#]Sandifer and Hopkin, 1996a
[††]Sandifer and Hopkin, 1997
[‡‡]Pueyo et al., 2004
[§§]Schreck et al., 2011
[¶¶]Houben et al., 2013
[##]Lock and Janssen, 2002a

2.3.7 Nitrates

Soils are regularly dosed with metal nitrate solutions for single metal toxicity assays but can cause problems with metal mixture doses due to leaching. Leaching soil after dosing is a recently developed step to remove excess salt from metal nitrate dosed soils (Stevens et al.,

2003). Salts, whether chlorides, nitrates, or sulfates, are added to soil as a by-product of adding metals of interest. These anions compete for sorption sites in soil, increasing the availability of the dosed metals (Stevens et al., 2003). Single metal toxicity assays usually correct for leaching by testing the metal content of the soil after the leaching process (Smolders et al., 2009; Li et al., 2010). Nitrate structures, when dissolved, for lead, copper, cobalt, nickel and zinc are similar to Zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot (H_2O)_6$, with varying hydrates (Fig. 2.1). The amount of hydration does not cause any effects when dissolved other than changing the amount of reagent required to dose a soil to a designated dose level. All Fischer diagrams were built using ChemSketch (Advanced Chemistry Development Inc., 2019)

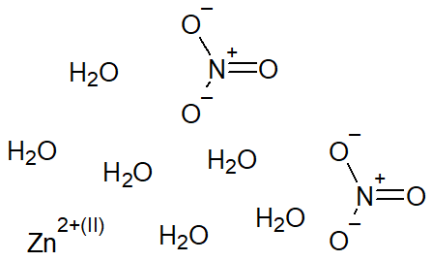

Figure 2.1: Fischer diagram of zinc nitrate hexahydrate

2.3.8 Oxides

Oxides are formed when oxygen and one other element chemically bond. In this case, we are using Co_3O_4, CuO, PbO, NiO, and ZnO. There toxicities of these compounds vary by route of entry, such as how ZnO is Generally Recognized As Safe by the U.S. Food and Drug Administration, but inhalation of ZnO fumes can be deadly (Heng et al., 2010; United States Food and Drug Administration, 2017). Adding these commercially available metal oxides to soil does not appear to significantly change the salinity of soils, indicating that it may show promise as an alternative to metal nitrate dosing. Most of the metal oxides used are double bonded

oxides, like lead(ii) oxide (Fig. 2.2), with the exception of cobalt which was dosed as Co_3O_4 (Fig. 2.3).

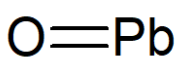

Figure 2.2: Fischer diagram of lead(II) oxide

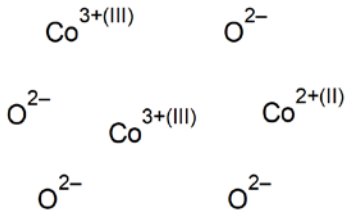

Figure 2.3: Fischer diagram of cobalt(II,III) oxide

2.3.9 Spinel-like metal minerals

The spinel-like minerals that we create are based on the structure of franklinite, a common zinc mineral in the Spinel group. Franklinite is commonly found near zinc smelters due to zinc-iron bonding during the smelting process (Roberts et al., 2002; Hamilton et al., 2016; Ren et al., 2017). Coprecipitation and annealing of cobalt, copper, lead, and nickel with iron is well researched, albeit using slightly different methods than in this project (Manova et al., 2004; Banerjee et al., 2011; Mao et al., 2014). Smelter sites appear to commonly produce spinel group minerals, indicating that this could be a more representative dose method (Piatak et al., 2004; Rozendaal and Horn, 2013). Spinels of each metal vary greatly but are all fairly well documented with the exception of cobalt. Suspected formations of lead (plumboferrite, Fig 2.4)(Holtstam et al., 1995), zinc (franklinite, Fig 2.5)(Pavese et al., 2000), copper (cuproferrite, Fig 2.6)(Prince and Treuting, 1956), and nickel (trevorite, Fig 2.7)(Blesa et al., 1993) are shown below. Cobalt structures are not as well documented but the basic structure is similar to other elements. A basic Fischer diagram is shown in Fig 2.8. All three dimensional diagrams were

created using Jmol modeling software (Cass et al., 2005; “Jmol: an open-source Java viewer for chemical structures in 3D with features for chemicals, crystals, materials and biomolecules,” 2019) using information from the American Mineralogist Crystal Structure Database (Downs and Hall-Wallace, 2003).

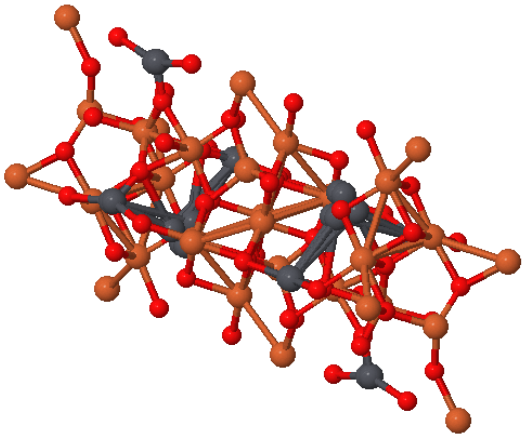

Figure 2.4: Three dimensional model of plumboferrite modeled using Jmol. Lead is dark grey, iron is brown, and oxygen is red

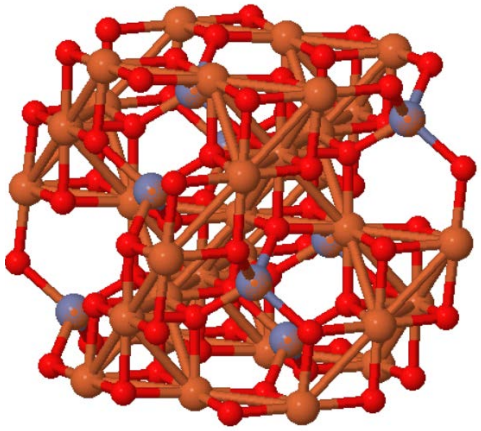

Figure 2.5: Three dimensional model of franklinite modeled using Jmol. Zinc is grey, iron is grey, and oxygen is red

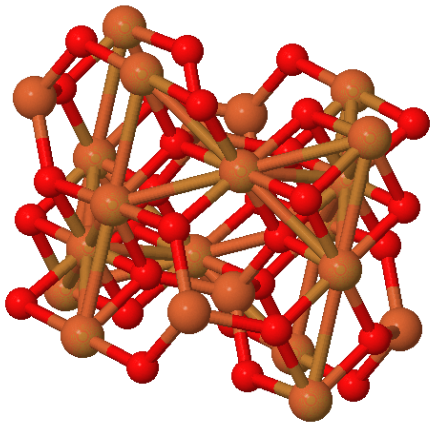

Figure 2.6: Crystal structure of cuprospinel modeled using Jmol. Copper is brown, overlaid on a darker brown iron, while oxygen is red

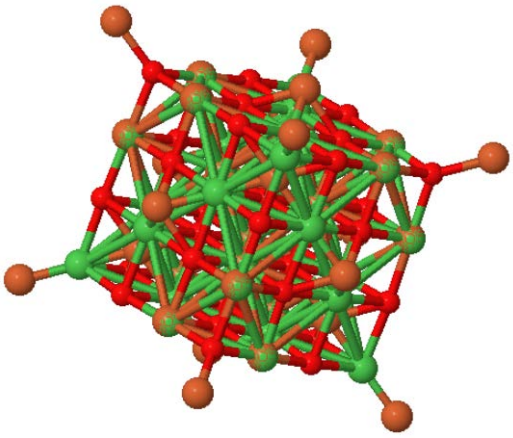

Figure 2.7: Crystal Structure of trevorite modeled using Jmol. Nickel is green, iron is brown, and oxygen is red

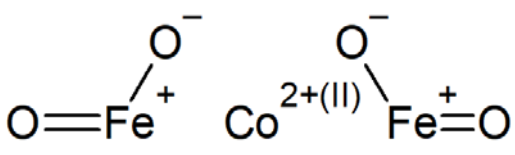

Figure 2.8: Fischer diagram of $CoFe_2O_4$ created using ChemSketch

2.3.10 *Folsomia candida* as an indicator species

F. candida has been extensively used in soil toxicity assays allowing us to compare our results to the mass of data already available (Fountain and Hopkin, 2005). These organisms are quite easy to culture and synchronize as they are largely asexual and reproduce quickly via parthenogenesis, making them ideal for labs to use them for testing the effects of environmental stressors. (Krogh, 2008). The International Organization for Standardization as well as the Government of Canada provide protocols for synchronizing and using *F. candida* (ISO 11267, 1999; Environment Canada, 2014).

F. candida synchronization involves separation of 200-300 individuals into a separate culture. Seven to eight days after the new culture begins laying eggs, the eggs are transferred to a new, empty culture. Two days after *F. candida* start hatching, the eggs are removed from the culture. After *F. candida* grow for ten days, they can be used for testing (Environment Canada, 2014). Assays are started by adding ten age-synchronized *F. candida* to 30 g wet weight of soil. Yeast is added and moistened at the start of the test and on day 14. The tests are terminated on day 28 by heating the soil, causing the invertebrates to drop into an extraction vessel (Environment Canada, 2014).

2.3.11 Soil Properties Affecting Invertebrate Toxicity

Soil toxicity assays are performed to examine the effects of a stressor on an organism in a particular soil. Each soil needs to be examined individually as pH, eCEC, organic carbon and texture have an influence on soil toxicity (Smolders et al., 2009; Checkai et al., 2014).
Soil pH is an established driver of metal toxicity in soils. Lower soil pH levels generally increase metal bioavailability to soil organisms (Sandifer and Hopkin, 1996a; Lock and Janssen, 2003a; Son et al., 2007; Heggelund et al., 2014). Contributing factors for increasing toxicity

may be pH regulated free ion activity and increases in metal mobility at lower pH levels (Smolders et al., 2009; Houben et al., 2013; Checkai et al., 2014). This can influence laboratory toxicity assay results as dosing with metal salts decreases the soil pH over increasing dose levels.

Soil organic carbon has been known to decrease metal toxicity to soil invertebrates for many years (van Gestel and van Dis, 1988). Carbon forms bonds with trace metals in soil, reducing the amount of metal available for uptake by soil organisms (Romero-Freire et al., 2016). This has a compounding effect with pH on metal availability where 99% of copper near neutral pH levels is bound to dissolved organic carbon (Temminghoff et al., 1997). Not only that, but organic carbon attached to *F. candida* can fix copper prior to uptake (Ardestani et al., 2014a). Accounting for organic carbon when evaluating toxicity by total metal concentration should provide more accurate endpoints similar to the results found when using a biotic-ligand model (Thakali et al., 2006).

The eCEC is similar to the standard cation exchange capacity in that it is a sum of the positive charge of base cations (Ca^{2+}, Na^{+}, etc.) in soil. eCEC takes into account the change in cation dominance to H^{+}, Mn^{2+}, and Al^{3+} in acidic soils (Soil Quality Pty Ltd., 2018). The eCEC behaves in a similar manner to organic matter, where eCEC changes pH effects on metal uptake (Ardestani and Van Gestel, 2013). This is mainly due to the additional cations in porewater alongside the metals of interest, increasing competition to interact with binding sites on or in soil organisms (Di Toro et al., 2001). In recent years, the eCEC of a soil has been shown to possibly be even more influential than pH on metal toxicity in soils (Smolders et al., 2009; Ardestani et al., 2014b).

Soil texture can also influence toxicity of metals in soil (Peijnenburg et al., 1999; Smolders et al., 2004). Higher clay fractions increase a soil's eCEC due to their typically

negative charge and high surface area to volume ratio (Doelman and Haanstra, 1984). Generally, this results in a decrease in metal toxicity, however it may be associated with lead bioaccumulation (Ardestani et al., 2014b).

2.3.12 ARCHE Consulting PNEC Calculator

Total metal concentration is not a particularly accurate indicator of toxicity as it is heavily influenced by soil properties as previously described. Laboratory results tend to indicate much lower endpoints than those observed in the environment as well (Smolders et al., 2009). To help resolve these differences, researchers developed a bioavailability model resulting in the ARCHE Consulting PNEC Calculator (Smolders et al., 2009; ARCHE Consulting, 2017). Soils from across Europe were selected across pH 4.0 to 7.5 in different soil types. Some soils from North America were included when examining cobalt toxicity (Smolders et al., 2009). After evaluating the effects of pH, organic carbon, eCEC, clay content and background metal concentrations the researchers were able to develop adjustment factors for cobalt, copper, lead, nickel and zinc (Smolders et al., 2009). Their process was adapted to create the PNEC calculator for many contaminants in the REACH database (ARCHE Consulting, 2017).

The PNEC Calculator determines a PNEC using Species Sensitivity Distributions (SSD) (Smolders et al., 2009). Species Sensitivity Distributions are created by performing toxicity assays on several test species in a single soil (van Straalen and Denneman, 1989). The response of each species is plotted and the endpoint of interest, in this case the EC5, is inferred from each curve (Posthuma et al., 2002). The EC5 of each species is log-transformed and plotted on the X axis, while the Y axis is the cumulative probability that a species will be significantly affected by a concentration of a stressor (Posthuma et al., 2002). The acceptable percent of species to be affected is selected and the concentration corresponding to that percent is chosen, known as the

hazardous concentration for sensitive species (HC) (van Straalen and van Leeuwen, 2002). The PNEC is the concentration where five percent of species are affected, or HC5. The HC5, where approximately 95% of plant and soil invertebrate species are not likely to be affected, is regularly used to develop environmental standards in North America and Europe (Suter II et al., 2002).

In order to create a robust SSD for the PNEC Calculator, previous toxicity assays needed to be normalized by background concentration, leaching/ageing, and bioavailibility before adding their data to an SSD (Smolders et al., 2009). Initially, EC5 values were selected from literature dose response curves. The background concentration of the each tested soil was subtracted from the EC5, resulting in an "added metal threshold concentrations" (Smolders et al., 2009). Many of these adjusted EC5 values were obtained from studies that did not leach or age test soils. The EC5 values from these tests were subsequently adjusted using Equation 13.

$$\boldsymbol{PNEC = C_B * PNEC_{add} * L/A} \qquad \text{(Eq. 13)}$$

(Adapted from Smolders et al., 2009)

Where $PNEC_{add}$ represents the added metal threshold PNEC, C_B is the background concentration, *L/A* is the element specific leaching and ageing factor, and *PNEC* is the non-bioavailibility adjusted PNEC value (Smolders et al., 2009).

The HC5 produced by an SSD typically cannot be adjusted for different sites (Suter II et al., 2002). The PNEC Calculator processes site specific soil data and returns a PNEC for that site (Smolders et al., 2009). The required site specific data varies between stressors. For example, calculating a PNEC for zinc requires eCEC, background zinc concentration, and pH, while cobalt requires eCEC only and lead does not require any soil parameters (Smolders et al., 2009). Adjustment of a PNEC to a specific site can be partly accomplished using Equation 14.

$$\boldsymbol{PNEC_{ref} = PNEC_{test}\left(\frac{abiotic\ factor_{ref}}{abiotic\ factor_{test}}\right)^{slope}} \quad \text{(Eq. 14)}$$

(Adapted from Smolders et al., 2009)

Where $PNEC_{ref}$ and $abiotic\ factor_{ref}$ represent the PNEC and adjustment parameter at a specific site, and $PNEC_{test}$ and $abiotic\ factor_{test}$ represent the known existing SSD PNEC and adjustment parameter, respectively. The *slope* variable is the slope of the relationship between the abiotic factors and changes in toxicity (Smolders et al., 2009).

After the PNEC has been adjusted by each abiotic factor, the PNEC is put through a final Assessement Factor (Smolders et al., 2009). The Assessment Factor is not necessarily based on science and varies between substances, although it is somewhat determined by the level of certainty in the PNEC (Smolders et al., 2009; Checkai et al., 2014). The leaching/aging and bioavailability adjusted PNEC is divided by the Assessment Factor for a final PNEC for use at a specific site.

3.0 DEVELOPMENT OF A MORE VERSATILE R ADAPTATION FOR MEASURING DEVIATIONS FROM CONCENTRATION ADDITION

3.1 Preface

The following chapter is intended to improve on the work of Jonker et al. (2005). By adding explanations as annotations to R scripts rather than an Excel spreadsheet with Visual Basic functions allows researchers to better understand and use the process more easily.

3.2 Abstract

The work of Jonker et al. (2005) stands apart from other methods for analyzing the effects of mixtures of stressors by using statistical tools rather than toxicological principles. A spreadsheet was provided along with their research for other scientists to easily fit their data to the four deviation types developed by Jonker et al. (2005). The custom functions created for the spreadsheet were developed using Visual Basic (VBA), and so the use of their method for higher level mixtures or large data sets could be limited to those with experience in VBA or researchers willing to transfer the functions to another program. This chapter describes a set of scripts created in R using the methods in Jonker et al. (2005) to test mixtures of up to five stressors, along with instructions for using the code to lower the barrier of entry to this form of analysis. When using five stressors the scripts were able to obtain deviation parameters that better fit highly variable data compared to the Excel spreadsheet and also return to the intended parameters when the starting parameters were double or half the value of the intended parameters. The goal of this chapter is to increase the use of Jonker et al. (2005) by making its analysis more accurate than the provided Jonker et al. (2005) Excel spreadsheet when modelling data sets with high variation.

3.3 Introduction

The original Jonker Excel spreadsheet provided in the supplemental information of Jonker et al., 2005 is designed for binary stressor mixtures and makes use of the Microsoft Excel Solver Add-In. While examining deviations from Concentration Addition (CA) for a separate five-component mixture we found that the original sheet (with the functions modified to fit a five-stressor mixture) does not easily handle higher order mixtures with hundreds of data points. The Solver Add-In needed to be run several times to obtain a minimum sum of squares and,

depending on the computer used, each operation could last 5 to 15 minutes. I hypothesized that R (R Development Core Team, 2008) could provide a better, faster solution to these problems.

Other researchers have previously adapted the methods in Jonker et al., 2005 to R for binary mixtures successfully (Asselman et al., 2013). These analyses' scripts were tailored for the researchers' own data and lacked annotations explaining their process. In order to help other investigators make use of the methods outlined by Jonker et al. (2005), R scripts would need to be developed to provide a more general solution for any data set.

3.4 Materials and Methods

As mentioned prior, R (R Development Core Team, 2008) was used to develop a relatively easy to use platform to perform analysis as per Jonker et al., 2005. Similar to the method used by Asselman et al. (2013) and Hochmuth et al. (2014), the script makes use of the rootSolve (Soetaert, 2009) and plyr (Wickham, 2011) packages. The rootSolve package includes the uniroot.all() function, which changes values in an equation to make it equal zero. In this application, Equation 5 is substituted into Equation 4, and e^G moved to the left side of the equation, leading to Equation 14:

$$\sum_{i=1}^{n} \frac{c_i}{EC50 * \left(\frac{k-Y}{Y}\right)^{\frac{1}{\beta}}} - e^G = 0 \qquad \text{(Eq. 14)}$$

Applying uniroot.all() to the inverse dose response function's Y solves the equation so that e^G is equal to the sum of the toxic units at each data point, indicated by the concentration c_i. The value of e^G changes according to the deviation, or lack thereof, from concentration addition. The plyr package (Wickham, 2007) is required to use the function adply(). The adply() function uses matrix manipulation to apply another function to all data points at once, the matrix being the user-supplied data. Matrix manipulation is both faster and less prone to errors than a for-next

loop, which is how the spreadsheet supplied by Jonker et al., 2005 solves Equation 14. As will be shown later in the script, adply() will be used in conjunction with Equation 14 and uniroot.all() to fit both the user supplied curve parameters and to find the least sum of squares. The optim() function, standard in R, will be used to find the least sum of squares. Optim() can minimize a function using six different methods designated as NM, BFGS, CG, L-BFGS-B, SANN, and Brent (R Development Core Team, 2008). The NM method is a Nelder-Mead (1965), or double simplex method, which is generally slower but fairly robust (R Development Core Team, 2008). The Nelder-Mead method is the default solve method used in the optim() function. Broyden (1970a; b), Fletcher (1970), Goldfarb (1970), and Shanno (1970) each produced the variable metric algorithm, represented by BFGS, and CG represents the conjugate gradients method by Fletcher (1964). L-BFGS-B is a limited memory Broyden, Fletcher, Goldfarb, and Shanno boxed method developed by Byrd et al. (1995), which adds upper and lower limits to the variables being changed inside optim() (R Development Core Team, 2008). SANN, short for simulated annealing, was developed by Bélisle (1992). It is a global optimization method which works slowly but can be effective at determining a minimum value over a highly variable response surface (R Development Core Team, 2008). If the user wishes to use functions other than NM, it must be designated within the optim() function after entering the function to solve and the initial guesses using the context ‘,method = “solvemethod”’, where solvemethod is replaced by BFGS, CG, etc.

The last optimization method, Brent, is only used for one-dimensional problems (R Development Core Team, 2008), so it will be excluded from this section.

3.5 Results

Throughout this section I will be referring to Appendix A. Appendix A contains the five-stressor, multiple iteration dose-ratio dependent script according to Jonker et al., 2005 with numbered lines. Other forms of the script are of equal or lesser complexity and have been included in the supplementary information. From this point onwards, blocks of code will be referred to by their line numbers to allow the reader to follow explanations of script functions.

3.5.1 Setting up the script for use

Lines 1 – 36 are introductory annotations describing how instructions are annotated as well as how to format data inputs in the code.

The rm(list= ls()) command in lines 37-43 is intended to clear any stored variables in R. This is mainly to prevent any previous variables created while using R from interfering with new ones created during analysis. It is highly recommended to run line 42 every time the script is executed.

As mentioned previously, the plyr and rootSolve packages are required for the script to run. These packages are known to function properly up to plyr version 1.8.4 and rootsolve version 1.7. If your R library does not have these packages, the # must be removed from lines 55 and 56 to allow R to read them as functions. Run each line for R to automatically fetch these packages from online databases. After installing, add the # to the start of lines 55 and 56 again to avoid updating the packages every time the script is ran.

Once the packages are installed they must be loaded into the current environment. To do this, run lines 62 and 69 to add plyr and rootSolve to the environment. This allows R to access the functions inside each package while the script is running. At this point the tools required for analysis have been loaded and data preparation can start.

To access the toxicity data R needs to know where the information is stored as well as where the output of the script will be placed. That location will be the working directory of the script, placed in the parenthesis and quotes of setwd(“”). It is recommended that a new folder is created for the working directory. Once the folder is created, simply copy and paste the directory into setwd(“”) on line 100, however the backslashes (\) in Windows directories need to be replaced by a forward slash (/). After the working directory is set data can be loaded into the R environment.

3.5.2 Data Organization

Lines 104 – 192 must be followed exactly for the script to work in its current form. The format of the data in lines 110 – 126 is slightly distorted in Appendix A, and is simplified to two stressors in Table 3.1 for clarity. The *x*, *y*, and *z* variables indicate the number of samples in assays 1, 2 and 3 respectively. The Mixture column contains a T (true) or F (false) value depending on whether the assay in that row is a mixture (T) or a single stressor (F). Under the Stressor_1 column, C1 represents the concentration of Stressor 1 at one dose level, C2 represents the concentration of Stressor 2 at a particular dose level, etc. Under the Response column, the value of R 1 represents the biological response produced at a dose of C1 1, C2 1, C3 1, C4 1, and C5 1. R 2 the response at C1 2, C2 2, etc. A sample data table is shown in Table 3.2.

Table 3.1: Clarification of Appendix A, lines 110 to 126

Assay	Mixture	Stressor_1	Stressor_2	Stressor_3	Stressor_4	Stressor_5	Response
Assay 1	T/F	C1 1	C2 1	C3 1	C4 1	C5 1	R 1
Assay 1	T/F	C1 2	C2 2	C3 2	C4 2	C5 2	R 2
Assay 1	T/F	C1 x	C2 x	C3 x	C4 x	C5 x	R x
Assay 2	T/F	C1 x+1	C2 x+1	C3 x+1	C4 x+1	C5 x+1	R x+1
Assay 2	T/F	C1 x+2	C2 x+2	C3 x+2	C4 x+2	C5 x+2	R x+2
Assay 2	T/F	C1 x+y	C2 x+y	C3 x+y	C4 x+y	C5 x+y	R x+y
Assay 3	T/F	C1 x+y+1	C2 x+y+1	C3 x+y+1	C4 x+y+1	C5 x+y+1	R x+y+1
Assay 3	T/F	C1 x+y+2	C2 x+y+2	C3 x+y+2	C4 x+y+2	C5 x+y+2	R x+y+2
Assay 3	T/F	C1 x+y+z	C2 x+y+z	C3 x+y+z	C4 x+y+z	C5 x+y+z	R x+y+z

Table 3.2: Representative data entry table, lines 110 to 126

Assay	Mixture	Stressor_1	Stressor_2	Stressor_3	Stressor_4	Stressor_5	Response
Lead	F	0	0	0	0	0	105
Lead	F	500	0	0	0	0	98
Lead	F	1000	0	0	0	0	50
Lead	F	2000	0	0	0	0	2
Copper	F	0	0	0	0	0	98
Copper	F	0	200	0	0	0	75
Copper	F	0	400	0	0	0	60
Copper	F	0	800	0	0	0	25
Zinc	F	0	0	0	0	0	95
Zinc	F	0	0	400	0	0	96
Zinc	F	0	0	800	0	0	40
Zinc	F	0	0	1600	0	0	0
Nickel	F	0	0	0	0	0	100
Nickel	F	0	0	0	650	0	20
Nickel	F	0	0	0	1300	0	1
Nickel	F	0	0	0	2600	0	0
Cobalt	F	0	0	0	0	0	102
Cobalt	F	0	0	0	0	700	90
Cobalt	F	0	0	0	0	1400	77
Cobalt	F	0	0	0	0	2800	42
Equal Ratio	T	0	0	0	0	0	103
Equal Ratio	T	100	100	100	100	100	85
Equal Ratio	T	200	200	200	200	200	54
Equal Ratio	T	400	400	400	400	400	21

The headings for the data must remain as noted in Appendix A and Table 3.1 for the script to function properly. The names of each stressor will be placed in subsequent lines. Every Stressor_x cell requires a value, including a value of zero where no stressor is present. The Mixture column requires the user to place T or TRUE in each cell if the associated assay is a mixture, or an F or FALSE if the assay only used a single stressor. Control samples do not require a value in this column as they are indicated by the having a value of 0 in all of the Stressor_x columns. The table may be created using a spreadsheet program such as Microsoft Excel, but prior to loading into R must be saved as a comma-separated values format (.csv). Once the data is formatted as required, the name of the .csv file can be copied and pasted inside the parenthesis and quotes in line 184.

3.5.3 Required Inputs and Built-in Data Manipulation

Once the data has been loaded, the names of stressors 1 through 5 are placed into line 213 under Stressor_Names and run. Each name must be placed within the quotation marks so that it is recognized as a string by R. Further improvements of the script may remove this necessity for the input.

No inputs need to be entered for lines 215 – 367. This section of the script is used to format the data to be read as required by the optimization function. A new column named Sum_Stressor is added to Dose_Response which adds the concentrations of each stressor at each dose level. Row_Number is also added to the data so that the fitted data, determined later, can be indexed and added to the original data table. This is required as the control, single stressor, and mixture assays are separated out to simplify optimization.

Separation of the different types of assays is conducted in lines 236 – 255. The control, single stressor, and mixture assays are copied into Control_Data, Single_Data, and Mixture_Data

respectively using the subset() function. The control samples are determined by Sum_Stressor having a value of zero, and the single and mixture assays by their T or F value in the Mixture column of Dose_Response. If a T or F value has been placed in a row with a control sample it will be excluded from Single_Data and Mixture_Data due to their constraints of either a T or F value, as well as needed a greater than 0 value in Sum_Stressor.

When new tables are created using the subset() function they retain certain properties from the original data set. In this case, the three new tables "remember" the names of all original assays in the Assay column. For example, in a binary mixture study with lead, copper, and a 1:1 mixture of the two named "Equal Ratio", the Assay column of Mixture_Data would still have the levels (a list of the different values in a column) Lead, Copper, and Equal Ratio. To remove these unnecessary levels, the Assay columns in Single_Data and Mixture_Data are re-factored to themselves in lines 272 and 273. Here R reads the current values in the Assay columns of Single_Data and Mixture_Data which in my example would be Lead and Copper, and Equal Ratio respectively. It then assigns only those factors to be the levels in each data set, removing Equal Ratio from Single_Data, and Lead and Copper from Mixture_Data. Further preparation for graphing the data, if required, is done on line 288. The script reads the number of unique mixture assay names (i.e. the number of different mixture assays) using the length() function, and records that number in Num_Mixtures.

Lines 290 – 327 are intended to bypass R's tendency to order the names of different levels of a column in alphabetical order. If the stressors in Dose_Response have not been entered in alphabetical order, line 327 will tell R to read the columns in the order you have specified in Stressor_Names. Each stressor is separated into different data tables in the same fashion as Single_Data and Mixture_Data were previously. The new tables containing the data

for each stressor are noted as S1_Data through S5_Data in lines 331 – 335. The process up to this point is shown visually in Fig 3.1. The flow diagram in Fig. 3.1 and others were created using Lucidchart (Lucidchart, 2019).

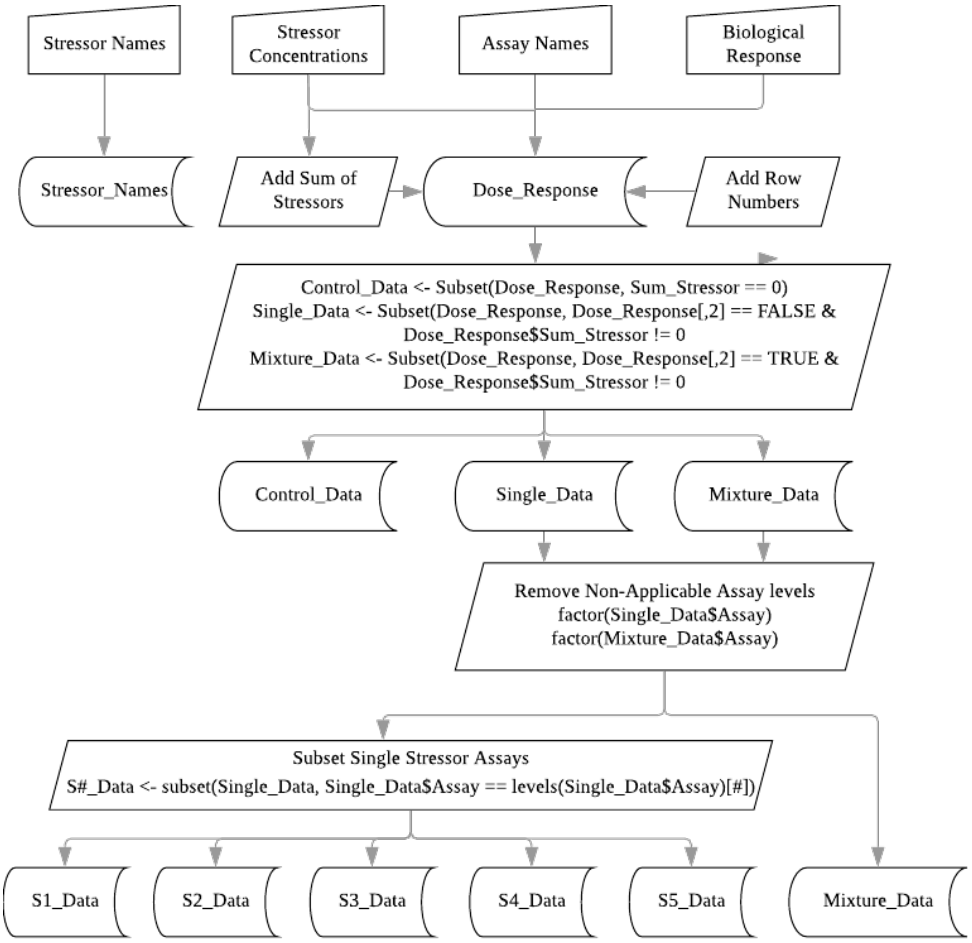

Figure 3.1: Initial data entry and organization chart

Lines 341 to 407 may be used to determine the maximum response, EC50, and β values for each stressor. The # symbol at the start of the lines where each curve is fitted needs to be deleted in order for the script to run, such as lines 359 -367. The only input required is to set the values in c(,,) to the user's initial guesses in the format c(maximum response, EC50, β). After one full stressor has been run, say Stressor 1, S1_Solved displays the results of the fit. Under the $par section the fitted maximum response, EC50, and β values are listed in order.

Data analysis begins on line 409. A function called Response_SS (lines 4285) is created which will calculate the SSE for the entire data set when given a starting point for the maximum response of the test organism, each stressor's EC50 and β values, and the a_{DL} and b_{DL} estimates for dose-level dependence. The maximum response and stressor values can be educated guesses or determined by fitting each stressor to the curve designated in Equation 2. The a_{DL} and b_{DL} variables, as designated in the Jonker et al., 2005 Excel sheet, should be designated as 0 and 1, respectively. These fitting parameters must be in the order:

1. Maximum response
2. Stressor 1 EC50
3. Stressor 2 EC50
4. Stressor 3 EC50
5. Stressor 4 EC50
6. Stressor 5 EC50
7. Stressor 1 β
8. Stressor 2 β
9. Stressor 3 β
10. Stressor 4 β
11. Stressor 5 β
12. Dose-level dependence variable a_{DL}
13. Dose-level dependence variable b_{DL}

The proper format for entering these values will be explained later, however keep in mind that the order is vital as Response_SS only reads the order of the variables and assigns them into their corresponding dose-response functions automatically.

Lines 446 – 458 shows how each variable is assigned from fit_param. fit_param is the list that R assigns your variables to when they are inputted to Response_SS. Each variable is assigned using the <<- operator, which tells R to place them in the Global Environment. This is a workaround for uniroot.all() to function properly within the function environment. Later improvements to the script may remove the need to assign variables to the Global Environment.

Beginning at line 460, Response_SS begins calculating the sum of squares according to the 13 variables provided, and shows why the control, single stressor, and mixture assays were

separated into different data tables. The differences between the organism response in Control_Data and the guessed maximum response (max2) in line 460 require only finding the differences and squaring them. S1_Data through S5_Data require using Equation 2 along with the provided EC50 and β values to find the predicted response which is then subtracted from the experimental response and squared.

The mixture responses require additional work to follow Jonker et al., 2005. Prior to fitting the mixture responses, the toxic unit contributions from each stressor need to be calculated. Toxic units 1 through 5, corresponding to Stressor_1 through Stressor_5, are determined in lines 468 to 472 using Equation 1. The relative toxicities of each stressor are noted as z1 through z5 and are calculated using Equation 6 in lines 473 – 477. Lines 478 – 483 are the expanded form of Equation 13. The inverse dose-response is calculated for each stressor concentration at each data point and summed in lines 478 – 482, which comprises the sigma notation portion of Equation 13. Line 483 is the expanded form of e^G for dose-level dependence from Equation 11.

Here uniroot.all() solves Equation 13 (line 483 subtracted from the sum of lines 478 – 482) by finding a value for *Y* that causes the left side of the equation to equal 0. On its own uniroot.all() will only solve one data point, but applying it in conjunction with adply() will make R perform the calculation for all rows of Mixture_Data. After R satisfies Equation 13 for each data point, it subtracts the fitted responses *Y* from their corresponding experimental data points. After lines 425 – 485 are ran, the script can move on to finding the sum of squares for the provided data.

Initial values to be entered into Response_SS are placed into fit_guesses on line 517. As noted before, these must be in the proper order. Unlike Stressor_Names, the values for each

variable are not placed in quotation marks. Place each variable as shown in line 507 inside the parenthesis on line 517 as shown in line 507. After running the filled line 517, run line 525 which enters the estimates from fit_guesses into the previously defined Response_SS function. The new value, Initial_SS, stores the sum of squares calculated from the variables placed into fit_guesses. This way the user can compare their original guess to the least sum of squares found in the next few steps.

The optim() function on line 543 takes the variable estimates from fit_guesses as a starting point to find the least sum of squares possible using Response_SS. This part of the script performs the same action as using Solver in the Jonker et al., 2005 spreadsheet and will take time to complete depending on the number of data points. The optimized maximum response, stressor curve parameters, dose-level dependence variables, and the sum of squares are placed into the vector Optim_param. When line 547 is ran the sum of squares from Optim_param is entered as the value of min_sum_square. The optimized variables are stored in the same order as fit_guesses inside of Optim_param.

After optimization the user can choose to graph experimental data with overlaid dose-response curves. If this is needed, run line 564 with Show_Graphs as TRUE. If not, replace TRUE with FALSE before running. If Show_Graphs is TRUE, the user should run lines 586 to 604 for R to automatically find the most square dimensions to display the response from each assay. Running line 625 creates a list of interpolated dose levels between the lowest and highest values of Sum_Stressor in Dose_Response to be later used as the x-coordinates for a smoothed curve to be displayed on the graphs.

3.5.4 Data Output

Now that the initial curve fit has been completed, the user may attempt to further optimize their data. Lines 639 to 961 constrain a single for-next loop within the R script. The user may either run line 639 only or highlight 639 – 961 inclusive to run the loop. If the previous instruction is not followed exactly the analysis will not run as R will be waiting for further lines to close the for-next loop. The "for(I in c(1:10))" in the script instructs R to create the variable *i*, starting with a value of 1, within the loop which is used for one entire run through of lines 639 – 961. If the user decides to add additional optimization the variable *i* will reach the end of the section and increase its value by 1, repeating this until the value goes over 10. The loop allows the user to continue optimization with minimal effort but can stop the process at the end of whichever optimization loop they consider sufficient.

The if(i == 1){, an if-else statement starting on line 651 and closing on line 784, is there to summarize and plot the initially guessed parameters and the first data optimization. The if statement will only run if *i* is equal to 1, therefore this section will only run on the first loop. This conditional section of the script is slightly different than the rest of the for-next loop and is explained below.

Line 656 creates a table called Fitted_Parameters to hold the EC50, β, and sum of squares values from the initial guesses and the initial optimization done previously. New data sets for individual stressors, mixtures of stressors, and control samples are created in lines 668 to 676. This is done to keep the original S1_Data, S2_Data, Mixture_Data etc. as they were at the beginning of the script while creating a space to put the fitted data points for the researcher to use later.

Another for-next loop starts on line 648 and creates a variable *j* which goes from a value of 1 to 2. A modified version of the Response_SS function runs twice, first using the initial guesses and then the first optimization parameters from their places in Fitted_Parameters. R extracts the values by selecting the *j*th column of Fitted_Parameters, 1 being the initial guesses and 2 being the first optimization. Like Response_SS, those selected values are assigned to equation variables in lines 681 to 693. These variables, along with Assay_curve_x, generate predicted responses along the entire dose response curves and are assigned as S1_curve to S5_curve. The Jonker et al., 2005 method is applied again to find responses *Y* which are stored in Mixture_curves. Using the parameters stored in Graph_Dim, a new window is opened and each single stressor curve and data point is plotted in lines 723 to 740. The mixture plots are looped along their assay names and graphed in lines 742 to 751. Depending on if the script is looking at the initial guesses ($j = 1$) or the first optimization ($j = 2$), different titles and fitted data points are added to the fit_guesses_Fitted or Optimization_1_Fitted columns in the S1_Data_DL to S5_Data_DL, Mixture_Data_DL, and Control_Data_DL datasets in lines 755 to 784.

The script then displays to the user "Optimizing Function…" on line 786 to tell the user that the script is functioning properly. After completing the next optimization on line 788 it will display "Completed Optimization", indicating that R has found another set of fitted parameters. Lines 795 to 916 add a column of values to Fitted_Parameters, append new responses *Y* to S1_Data, Mixture Data, etc., and plot the new functions in the same fashion as lines 681 to 784. Once this has been done the script will inform the user of the total number of optimizations completed (line 921) and display the entire Fitted_Parameters table so that the user can see the new EC50, β, and sum of squares for the current optimization.

A variable called “Continue_Running” is created on line 928 with no value (NA), and will be reset to “NA” every time the optimization loop is run. Lines 929 to 946 create a while{} loop which asks the user to input if they wish to run another optimization (Y) or not (N). If a Y is entered, the script will tell the user “Optimization will continue, now running Optimization X”, where X indicates the next optimization to run. If an N is entered, the while loop will break, leaving the loop. If a value other than Y or N is entered, the script will tell the user “Invalid input. Please enter either Y or N (case sensitive)” and will again ask you to enter a Y or N value. Lines 948 to 958 are run if an N is entered in the previous prompt. If this is True the original for-next loop will stop and proceed to the final steps of the script. If a Y is entered, the script returns to the top of the for-next loop and runs another optimization.

After the user has stopped optimizing their data, line 965 creates a new data set called Fitted_Response_Data. This set combines S1_Data_DL through S5_Data_DL, Mixture_Data_DL, and Control_Data_DL into a single set. Fitted_Response_Data will be the same as the original data set loaded into the script but will include the fitted response *Y* for each data point in columns designated by the number of optimizations completed in a similar manner to Table 3.1. Lines 978 and 979 may be run to save Fitted_Parameters and Fitted_Response_Data to .csv files in the same folder designated by the working directory.

3.6 Discussion

To compare the optim() function’s five applicable fit methods, a data set with five single-stressor assays and five mixture assays was generated using the Jonker et al., 2005 spreadsheet to follow Concentration Addition as per Equation 3. Three additional responses were generated for each data point with the rnorm() function. Using the ideal Concentration Addition response at each data point as a mean, three random responses were assigned using the normal distribution

and standard deviations of 2, 5, and 10. The entirety of this data set can be found in Appendix B. Four different sets of starting parameters were used to see how well optim()'s different fit methods can find to the intended parameters as shown in Fig 3.2: the parameters used to generate the data (Table 3.2), the original β values and half the EC50s (Table 3.3), the original EC50s and half of the β values (Table 3.4), and where both the EC50 and β values were halved (Table 3.5).

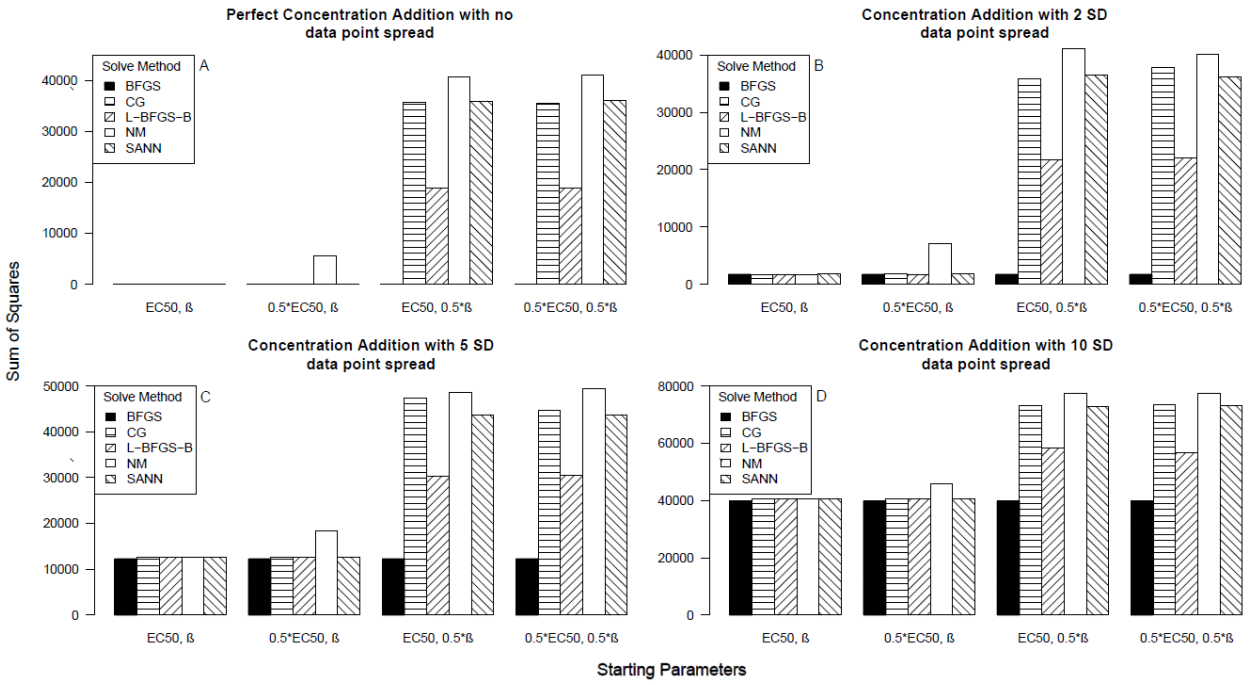

Figure 3.2: Graphical representation of the sum of squares generated when fitting parameters as per Jonker et al., 2005 for four cooked data sets. Five fitting methods from the optim() function in R were used for perfect concentration and three sets where the standard deviation of each set of 5 replicates was changed to 2, 5 and 10 standard deviations, represented by 2 SD, 5 SD, and 10 SD respectively.

Table 3.3: Differences in fitted parameters using Solver and optim()'s BFGS method and their associated sum of squares over increasing variation in a data set generated assuming perfect concentration addition. The starting parameters for each fitting were the original parameters used to generate the data set as listed in the Starting Parameters column.

Parameter	Perfect Parameters	Starting Parameters	Perfect		SD = 2		SD = 5		SD = 10	
			Excel	R	Excel	R	Excel	R	Excel	R
Max	100	100	100.0	100.0	100.0	100.0	99.3	100.1	100.5	101.0
Lead EC50	1500	1500	1500.0	1500.0	1485.4	1482.6	1421.5	1377.5	1631.0	1545.6
Copper EC50	2500	2500	2500.0	2500.0	2483.4	2494.1	2961.0	2745.9	2389.6	2008.8
Nickel EC50	700	700	700.0	700.0	688.2	685.0	709.6	690.4	674.0	629.8
Zinc EC50	4000	4000	4000.0	4000.0	3987.6	3983.8	4007.4	4006.3	3882.4	3839.4
Cobalt EC50	1000	1000	1000.0	1000.0	1012.0	1003.8	974.4	832.8	986.7	1079.3
Lead β	3.0	3.0	3.0	3.0	3.0	3.1	2.9	2.6	2.9	2.5
Copper β	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Nickel β	1.2	1.2	1.2	1.2	1.2	1.2	1.3	1.2	1.2	1.1
Zinc β	6.0	6.0	6.0	6.0	6.0	5.4	5.8	4.7	5.9	4.5
Cobalt β	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Sum of Squares			0.0	0.0	1731.6	1724.8	12501.4	12263.3	40509.0	39866.1

Table 3.4: Differences in fitted parameters using Solver and optim()'s BFGS method and their associated sum of squares over increasing variation in a data set generated assuming perfect concentration addition. The starting parameters for every fitting were the original β parameters and half of the EC50 values used to generate the data set as listed in the Starting Parameters column.

Parameter	Perfect Parameters	Starting Parameters	Perfect		SD = 2		SD = 5		SD = 10	
			Excel	R	Excel	R	Excel	R	Excel	R
Max	100	100	100.2	100.1	101.1	100.1	101.4	100.1	100.0	101.2
Lead EC50	1500	750	1489.8	1501.9	1420.4	1479.1	1309.9	1376.3	750.0	1542.7
Copper EC50	2500	1250	2502.2	2455.5	2327.3	2445.6	2364.5	2723.3	1250.0	1980.9
Nickel EC50	700	350	681.0	700.0	640.8	683.8	664.8	690.5	350.0	626.0
Zinc EC50	4000	2000	3993.2	3987.7	3941.9	3984.6	3938.0	4014.3	2000.0	3791.8
Cobalt EC50	1000	500	948.8	988.1	827.5	999.1	623.1	832.6	500.0	1044.3
Lead β	3.0	3.0	3.0	3.0	3.1	3.1	2.4	2.6	3.0	2.5
Copper β	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Nickel β	1.2	1.2	1.2	1.2	1.1	1.2	1.2	1.2	1.2	1.1
Zinc β	6.0	6.0	5.8	6.0	5.1	5.4	4.1	4.7	6.0	4.5
Cobalt β	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Sum of Squares			19.88	3.4	2002.1	1726.1	12727.5	12264.2	110816.4	39864.0

Table 3.5: Differences in fitted parameters using Solver and optim()'s BFGS method and their associated sum of squares over increasing variation in a data set generated assuming perfect concentration addition. The starting parameters for every fitting were the original EC50 parameters and half of the β values used to generate the data set as listed in the Starting Parameters column.

Parameter	Perfect Parameters	Starting Parameters	Perfect		SD = 2		SD = 5		SD = 10	
			Excel	R	Excel	R	Excel	R	Excel	R
Max	100	100	99.8	100.0	100.1	100.0	101.1	100.1	102.3	101.1
Lead EC50	1500	1500	1504.4	1500.0	1480.6	1481.4	1362.1	1377.5	1464.1	1545.1
Copper EC50	2500	2500	2525.6	2500.0	2515.9	2488.4	2624.2	2735.0	1900.6	1992.8
Nickel EC50	700	700	719.8	700.0	680.4	685.5	652.5	690.8	566.1	627.1
Zinc EC50	4000	4000	4008.6	4000.0	3980.4	3983.7	3976.4	4011.4	3765.0	3820.5
Cobalt EC50	1000	1000	991.5	1000.0	1153.1	1006.4	693.4	835.9	837.7	1076.1
Lead β	3.0	1.5	3.0	3.0	3.0	3.1	2.7	2.6	2.3	2.5
Copper β	0.7	0.35	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Nickel β	1.2	0.6	1.2	1.2	1.2	1.2	1.2	1.2	1.0	1.1
Zinc β	6.0	3	6.2	6.0	4.9	5.5	4.0	4.7	3.5	4.5
Cobalt β	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Sum of Squares			16.9	0.0	1781.0	1724.6	12476.5	12263.6	40355.0	39862.0

Table 3.6: Differences in fitted parameters using Solver and optim()'s BFGS method and their associated sum of squares over increasing variation in a data set generated assuming perfect concentration addition. The starting parameters for every fitting were half of the original EC50 and β values used to generate the data set as listed in the Starting Parameters column.

Parameter	Perfect Parameters	Starting Parameters	Perfect		SD = 2		SD = 5		SD = 10	
			Excel	R	Excel	R	Excel	R	Excel	R
Max	100	100	100.0	100.0	100.8	100.1	100.9	100.1	102.6	101.1
Lead EC50	1500	750	1498.6	1498.1	1442.9	1481.9	1380.1	1376.3	1516.5	1544.8
Copper EC50	2500	1250	2498.1	2478.5	2339.4	2462.5	2620.8	2737.6	1825.9	1981.1
Nickel EC50	700	350	698.7	700.4	665.9	684.5	676.1	690.6	618.4	626.8
Zinc EC50	4000	2000	3999.1	3993.4	3913.2	3980.4	4013.5	4012.6	3780.3	3819.5
Cobalt EC50	1000	500	985.3	994.3	796.2	1003.1	597.4	833.9	640.1	1051.3
Lead β	3.0	1.5	3.0	3.0	3.1	3.1	2.7	2.6	2.6	2.5
Copper β	0.7	0.35	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Nickel β	1.2	0.6	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.1
Zinc β	6.0	3	6.4	6.0	4.6	5.4	4.5	4.7	4.4	4.5
Cobalt β	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Sum of Squares			2.7	0.9	1907.0	1725.0	12541.7	12263.5	40550.7	39861.9

The R script was able to solve for a lower sum of squares in ever scenario tested. The R script performed increasingly better when evaluating data with higher standard deviations, however this was dependent on the solve method used. In comparison, Solver would regularly encounter errors and refuse to find a solution to the assigned problem at a standard deviation of 10. The amount of variation in a dose-response assay may affect which method could be used effectively. The optim() BFGS solve method, however, resulted in the lowest sum of squares across all of the data sets provided. Due to BFGS' apparent robustness in this situation, BFGS will be tested on data collected from laboratory experiments. While BFGS functioned well using the data in this thesis, users of these scripts are advised to try each solve method to examine their effects on dose response curve fits and the sum of squared errors.

Using a data set obtained through our lab for *F. candida* (Appendix C), the data was fitted according to the script in Appendix A and the previously described instructions. Table 3.6 shows the parameters generated using the Solver Add-in and the R script using the same initial settings for dose-level dependent concentration addition. Fig. 3.3 shows the fitted curves using the R script.

Table 3.7: Evolution of fitted parameters over 3 iterations of using the modified Jonker et al., 2005 Spreadsheet and the R code developed in this thesis. Both types of analysis began with the initial parameters listed and were further optimized using the previous fitted parameters.

Parameter	Initial Setting	Fitting 1		Fitting 2		Fitting 3	
		Excel	R	Excel	R	Excel	R
Max Response	300	307	252	307	252	307	252
Lead EC50	6500	6759	31170	6759	32390	6759	32879
Copper EC50	725	538	616	538	614	538	614
Nickel EC50	5200	5183	4319	5183	4419	5183	4334
Zinc EC50	1400	1143	1374	1143	1368	1143	1372
Cobalt EC50	2800	3329	53080	3329	53076	3329	34217
Lead β	0.10	0.10	4.48	0.10	4.18	0.10	4.08
Copper β	1.50	1.42	1.37	1.42	1.37	1.42	1.37
Nickel β	35.00	35.01	56.05	35.01	44.98	35.01	2224.91
Zinc β	19.00	18.94	2.85	18.92	2.87	18.92	2.85
Cobalt β	0.50	0.48	0.77	0.48	0.78	0.48	0.95
a_{DL}	0.00	0.00	371.05	0.00	398.61	0.00	310.43
b_{DL}	1.00	1.00	-108.47	1.00	-107.25	1.00	-92.31
Sum of Squares	$1.744*10^6$	$1.703*10^6$	$1.266*10^6$	$1.703*10^6$	$1.265*10^6$	$1.703*10^6$	$1.265*10^6$
R SS - Excel SS		-437,358		-437,440		-437,531	

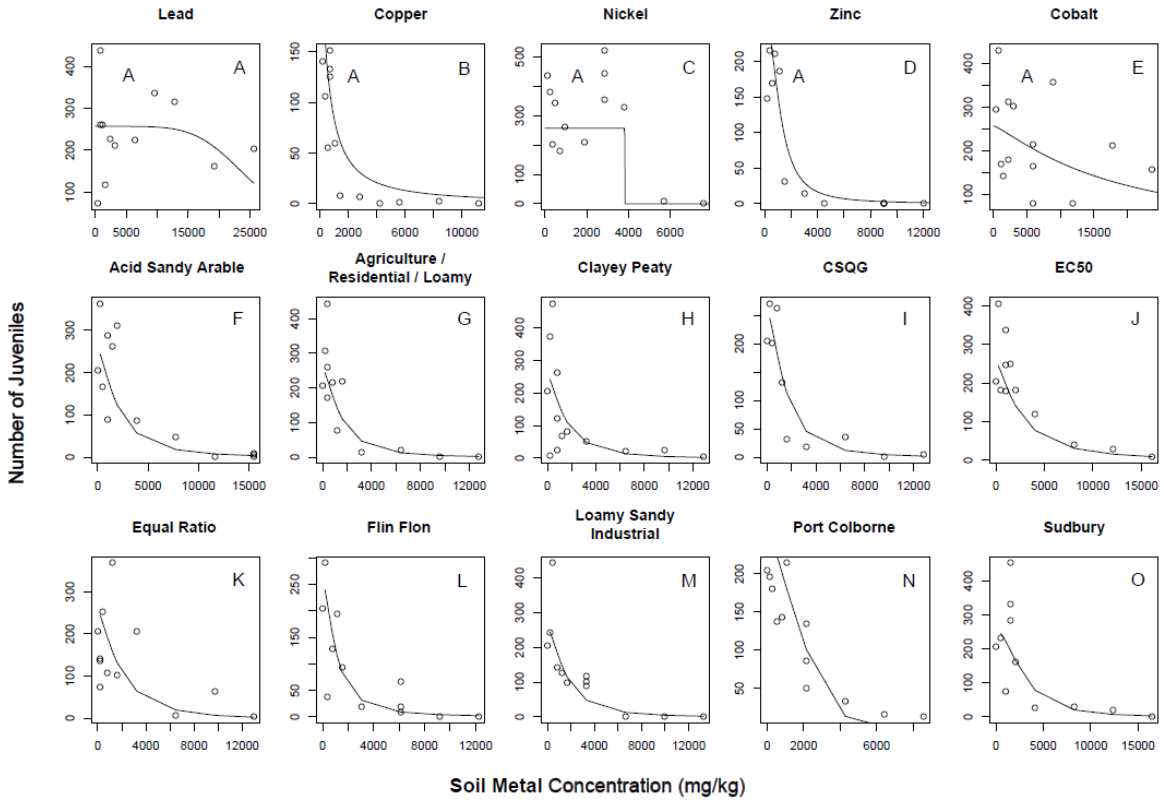

Figure 3.3: Fitted curves from Jonker et. al., 2005 R script on experimental data using the BFGS method. The top 5 graphs are single stressor data, while the bottom 10 are mixtures of lead, copper, nickel, zinc, and cobalt.

Both the Excel spreadsheet and R analysis were used three times in succession to examine how the sum of squares differentiated between the two methods. As shown in Table 3.6 the Excel sheet was unable to determine if the a_{DL} or b_{DL} had any effect. The R script was able to obtain a lower sum of squares than any of the Solver operations on the first attempt and continued to find a slightly lower sum of squares in each optimization.

3.7 Conclusion

Applying the methods from Jonker et. al., 2005 in R for five stressors appears to be more capable than the original spreadsheet in finding a range of fitted EC50, β, and CA deviation parameters. The ability of the script to display graphs and parameter values after each optimization provides another advantage over the excel sheet, which requires a separate column or sheet to store multiple fitted data curves. Rather than having Solver overwrite the parameters in each optimization, the R script will store both the parameters as well as data points it fits using those parameters.

Using this R script rather than the Excel spreadsheet also has the potential to increase productivity depending on the number of combinations of assays to be investigated. All open Excel windows become unresponsive while the Solver Add-In is running, while the R script may be ran using multiple instances of R or by creating additional Projects in RStudio.

4.0 SOIL TOXICOLOGICAL AND CHEMICAL EVALUATION OF THREE DOSING METHODS TO IMPROVE FIXED-RATIO RAY METAL MIXTURE TOXICITY ANALYSIS

4.1 Preface

While the original topic of this thesis was the evaluation of the toxicity of metals to soil invertebrates in Canadian soils, a problem with dosing the soils was encountered partway through the project. When conducting range finding tests for Collembolans and mites using leached metal nitrate dosed soils we discovered that metals were being leached from the soil at disproportionate levels. This led our research group to investigate the metal leaching phenomenon and develop a different method for dosing soils with metals. Myself, Mathieu Renaud, Kobby F. Awuah, and Olukayode Jegede worked together on this project to perform assays using *Folsomia candida, Enchytraeus crypticus, Oppia nitens,* and four microbe driven processes respectively. Kobby's data was not included in this paper, however his input to the project was invaluable. After working as a group to develop each dose method and physically dose each soil, the paper was primarily split between Mathieu and I. The actual writing of this chapter was a team effort, however I primarily focused on the Results section and Mathieu on the Discussion.

4.2 Abstract

Dosing soils with metals for invertebrate toxicity assays is usually done using metal salts. The toxic effects of salts on soil invertebrates is well known and the dosed soils are leached to return them to control conductivity levels, which also removes some metals at the same time. Leaching soils dosed with multiple metals will cause some metals to be removed from soil at a disproportionate rate from other components in the mixture. This study aimed to examine how significant the differences in metal removal during leaching were and also compare the toxicity of metal nitrate salts to that of commercial metal oxides and laboratory produced spinel group-like minerals, which do not require leaching. Three invertebrate species were exposed to four soils dosed with five mixtures of cobalt, copper, lead, nickel, and zinc using three different dose methods. Up to 60% of the total metal added as a nitrate salt was leached from the soils, with individual metal removal ranging from non-significant differences for lead and copper to 50% of the cobalt, nickel, and zinc when comparing pre- to post-leaching concentrations. Oxide and mineral doses retained their intended mixture ratios more effectively. *Folsomia candida* and *Oppia nitens* responded similarly to oxide and nitrate dosed soils, while the nitrate dosed soils reduced *Enchytraeus crypticus* reproduction compared to oxide doses. Mineral dosed soils were not toxic. The ease of use and less variable oxide dosing method shows that it is a promising alternative to dosing using metal salts.

4.3 Introduction

Soil metal contamination is a global concern caused primarily by historic as well as current mining and smelting operations. Metal accumulation and persistence in soil causes contaminated sites to remain affected long after industrial activities have ceased. As a consequence of their importance, scale and persistence metals have been extensively studied in soil ecotoxicology, mostly as single elements (Sandifer and Hopkin, 1996a, 1997; Lock and

Janssen, 2002a; Owojori and Siciliano, 2012) but also as metal mixtures (Posthuma et al., 1997; Weltje, 1998; Lock and Janssen, 2002b).

In soil ecotoxicological research, aqueous metal salt solutions are often used to dose soils (Sandifer and Hopkin, 1997; Lock et al., 2004). Salt solutions allow a high dosing precision and ease in homogenizing the metals within the soil. Despite these advantages salinity is a cause for concern when using metal salts. Salinity can in itself cause toxicity to organisms and affect the toxicity of metals (Owojori et al., 2008, 2009; Pereira et al., 2015) and the addition of salts alters the chemodynamics of metals in soils (Stevens et al., 2003). To address these concerns, soils dosed with metal salts are leached after dosing to remove salinity and hopefully assess only the toxicity of the metal. A side effect of this leaching process is metal loss in the leachate. Single metal studies can correct for this by measuring total remaining metal concentration in soil. On the other hand, metal mixture studies, especially fixed-ratio ray designs in which the ratio of each metal in the soil must remain constant, are disrupted by leaching. Leaching removes metals at different rates due to differences in metal-soil partitioning. Most previous literature on metal mixture toxicity dosed using metal salts did not leach their soils (Khalil et al., 1996; Lock and Janssen, 2002c; Baas et al., 2007) with the exception of Posthuma et al. (1997). The majority of these studies are therefore testing both metal and salt toxicity. Consequently, for metal mixture studies, alternative methods for dosing soils must be considered, such as using metal oxides or producing spinel-like minerals by annealing metal mixtures.

In this study, we assessed three different methods (metal salts, oxides and spinel-like metal minerals) for dosing four different soils with different mixtures of metals. Dosing methods will be evaluated in terms of metal retention as well as their relative toxicity to three soil invertebrate species (*Folsomia candida*, *Oppia nitens* and *Enchytraeus crypticus*) thus

accounting for different sensitivities and routes of exposure. The collembolan, *Folsomia candida*,is the most studied of the three selected test species and has been used in routine risk assessments as a standard test species for over 50 years. The main route of exposure for *F. candida* is through ingestion by means of contaminated pore water and food, but food avoidance may occur if the contaminant is detected (Fountain and Hopkin, 2005). While the standard ISO guideline for *Enchytraeus* sp. refers to the use of *E. albidus*, the enchytraeid *E. crypticus* has increased in use in recent years as a standard test species in particular because of its faster generation time and ease of use (Castro-Ferreira et al., 2012). Similarly to *F. candida*, enchytraeids are exposed through ingestion but because of their close contact with soil pore water and the lack of protective cuticle, enchytraeids are also exposed dermally and through respiration (Rombke, 2003). The mite *Oppia nitens* is a relatively new species used in soil ecotoxicological testing and has been suggested as a standard test species to represent oribatid mites, one of the most abundant groups of soil invertebrates in particular for boreal soils (Princz et al., 2010). The addition of this species also accounts for mite sensitivity for which routine testing is currently only performed on a predatory species, *Hypoaspis aculeifer* (OECD, 2008). As with most oribatid mites, adult *O. nitens* have a thick sclerotic exoskeleton and exposure is mostly through ingestion (contaminated food or water), however the lack of this exoskeleton in juvenile forms can increase the exposure routes to include dermal uptake and can affect population performance in reproduction experiments due to juvenile mortality (Princz et al., 2010; Owojori and Siciliano, 2012). In terms of sensitivity, *F. candida* have a similar sensitivity to *E. crypticus* whilst *O. nitens* is expected to be as sensitive or less (Owojori and Siciliano, 2012), due to their hard body nature, however the amount of data on this particular species is still relatively low (Pereira et al., 2015; Renaud et al., 2017; de Lima e Silva et al., 2017). Using

copper as an example, reproduction EC_{50} for each species in OECD artificial soil were 477 mg kg^{-1} for *Enchytraeus crypticus* (Posthuma et al., 1997), 700 mg kg^{-1} for *Folsomia candida* (Sandifer and Hopkin, 1996b), and 2,896 mg kg^{-1} for *Oppia nitens* (Owojori and Siciliano, 2012).

In addition to considering a variety of routes of exposure, the higher solubility of the nitrate salts could increase their availability to soil invertebrates through soil pore water compared to non-water soluble oxides or spinel-like mineral complexes (Lide, 2005). 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 compared to the less soluble oxides and spinel-like minerals. However, in terms of toxicity evidence in the literature is not always consistent, for instance Lock and Janssen (2003) found that Zinc salts, oxides and powders produced similar reproductive effects (EC_{50}) in three invertebrate species, whilst Kool et al. (2011) found that Zn salts had a much higher toxicity (lower EC_{50}) than oxides and oxide nano-particles to *F. candida* and finally Amorim and Scott-Fordsmand (2012) found that Cu salts ($CuCl_2$) were less toxic than Cu nanoparticles to *E. albidus*. While different forms of metal may influence their behavior in soil, the soil itself is also likely to affect metal retention and bioavailability. Smolders et. al. (2009) found that effective cation exchange capacity (eCEC), pH, organic carbon, and clay content were significant drivers influencing soil toxicity to soil invertebrates. Similarly, de Matos et. al (2001) determined that metal mobility and retention in soils were affected by cation exchange capacity, organic carbon, and pH (Yong and Phadungchewit, 1993; de Matos et al., 2001; Uchimiya et al., 2012).

4.4 Materials and Methods

Experiments were performed using four different Canadian soils with a range of different properties with the goal of matching soil types described in the EU reach PNEC calculator (ARCHE Consulting, 2017) (Table 4.1).

Table 4.1: Soil properties and their closest PNEC reference soil from the EU PNEC calculator

Soil	pH	eCEC (meq 100 g^{-1})	Organic C (g kg^{-1})	Clay Content (g kg^{-1})	Water Holding Capacity (ml g^{-1})	Closest PNEC Reference
3.22	3.4	33	17	45	0.29	Acid Sandy Forest
WTRS	4.6	37	25	110	0.23	Acid Sandy Arable
KUBC	5.6	33	12	24	0.20	Loamy
Elora	6.7	42	21	200	0.30	Loamy Alluvial

The four soils were dosed with five different metal mixtures using three different methods: metal nitrate salts, metal oxides and spinel-like minerals. Each soil was air dried and sieved to <2mm particle size and the water holding capacity determined by wetting upon filter paper (de Almeida et al., 2015).

4.4.1 Metal Salts

Aqueous lead, copper, nickel, zinc, and cobalt nitrate stock solutions were pipetted individually to each soil from their respected concentrated stock solutions. Distilled water was then added to each sample to reach half of the water holding capacity. Electrical conductivity of each soil was tested two weeks after dosing and compared to control levels to account for addition of anions. Soils were leached using artificial rainwater (Li et al., 2010) one pore volume at a time until conductivity reached control 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.

4.4.2 Metal Oxides

Commercially available metal oxides were ground using mortar and pestle to <20 µm. Once ground, oxides were placed on plastic weigh boats in a sealed glass container with an open beaker of nitric acid. Oxides were left with the acid vapours for 48 hours and then air dried in a fume hood for 24 hours to remove any carbonates. Dried metal oxides were weighed at the appropriate concentrations, added to dry soils and thoroughly mixed by shaking.

4.4.3 Spinel-like Metal Minerals

Spinel-like metal mineral complexes were prepared by mixing metal nitrate solutions. Using the same volumetric ratios as the metal nitrate tests, individual metal stock solutions were combined to create mixture stock solution. An iron nitrate solution was then added in a 2:1 molar ratio of iron to the sum of the five metals of interest.

To compensate for different precipitation rates and phase diagrams, one 25mL sample of each mixture was used to examine how much of each metal precipitates out of solution. Each tube was titrated to pH 7 ± 0.25 with 14.8M ammonium hydroxide. If the pH rose above 7.25, nitric acid was added to compensate. Once the correct pH was attained the tubes were shaken overnight and their pH was re-checked and adjusted as needed. The titrated solutions were centrifuged at 400 g for 30 minutes. After decanting the supernatant, the 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 (Keely and Maynor, 1963; Vratny and Gugliotta, 1963; Nikolic et al., 2006). The resulting ashes metal contents were sent for analysis at the Saskatchewan Research Council's Geo-analytical Laboratories in Saskatoon, SK to check ratio composition. Metal content in ashes was determined by digesting the samples and analysed

through ICP-AES. 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 dilute HNO_3.

Using the measured total mass and metal content of each ash and the volume of supernatant, the mass of each metal that had not been entrained in the ash could be calculated. The appropriate volume of each individual metal nitrate stock solution was added to the mixture stock solutions to compensate for the mass assumed to be left in the supernatant during precipitation. Iron nitrate was added to keep the iron:total other metals molar ratio fixed at 2:1. Each adjusted mixture stock solution was distributed in 25 mL aliquots into 50 mL centrifuge tubes. The adjusted mixture solutions were then titrated, decanted, centrifuged, roasted, and metal content analysed in the same process as previously depicted.

The amount of spinel-like 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 spinel-like minerals were 23% lead, 60% copper, and 17% nickel, in this case the mass balance for dosing each soil would be calculated as per the concentration of lead in the spinel-like material. After dosing, the soils were manually stirred to homogenize and incorporate the metals into the soil.

All soils were dosed with the previously described dosing methods with five metal mixtures of five metal elements at a dose of 4 toxic units. Toxic units were calculated from the EC50 values for *F. candida* as found in the literature for each metal element. (Table 4.2). Once soils were dosed, samples were obtained for total metal concentrations and toxicity assessment.

Table 4.2: Nominal metal mixture ratio compositions in mg kg-1 dry weight of soil at a dose of 4 toxic units and *Folsomia candida* EC50 used in estimating toxic units

Mixture	Lead (mg kg^{-1})	Copper (mg kg^{-1})	Nickel (mg kg^{-1})	Zinc (mg kg^{-1})	Cobalt (mg kg^{-1})
Port Colborne	55.6	380.9	1513	162.6	27.8
CSQG	536.2	482.6	344.7	1532	306.4
Flin Flon	202.1	618.6	9.2	2223.3	9.2
Sudbury	2314.4	160.9	297	1196.4	152.6
Clayey + Peaty	612.1	662.5	395.7	1199.2	353.6
F. candida EC50	1600[†,‡]	700[†,‡]	475[§]	750[†,‡]	1480[¶]

[†](Sandifer and Hopkin, 1997)
[‡](Sandifer and Hopkin, 1996b)
[§](Lock and Janssen, 2002b)
[¶](Lock et al., 2004)

4.4.4 Soil Invertebrates and Toxicity Tests

The toxicity of the three different dosing methods were assessed using the reproduction and survival of three different soil invertebrate species. These endpoints were determined using standard protocols for *Enchytraeus crypticus* (ISO 16387) and *Folsomia candida* (ISO 11267). *Oppia nitens* tests were conducted following the procedures performed by Princz et al. (2010). Soils were adjusted to 50% water holding capacity (WHC) prior to invertebrate testing and during incubation test vessels were maintained in the laboratory under a photoperiod of 16h: 8h light-dark. For the duration of the incubation period, test units were fed with granular yeast *(F. candida* and *O. nitens*) and rolled oats (*E. crypticus*). Water content was maintained during the test at 50% WHC by adding distilled water to maintain initial test vessel weight.

After 4 weeks of incubation for *Folsomia candida* and *Oppia nitens*, the assays were ended by extracting organisms using a heat extractor (previously tested for extraction efficiency (>90%)). The extracted organisms were counted using a binocular microscope. In the enchytraeids test, organisms were fixed in 70% ethanol and stained with Bengal red (200 to 300µL 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.

4.4.5 Metal analysis

Soil samples were collected from bulk dosed soil for chemical analysis to determine total metal concentrations. To determine total metal concentrations, 1 g of soil was added to a 60 mL Teflon digestion vessel. This soil was then digested by reverse aqua regia by adding 9 mL of nitric acid and 3 mL of sulfuric acid and swirled every 30 minutes until no bubbles were observed. The flasks were placed in an oven and digested overnight at 110°C and then filtered using Whatman 42 paper. Digested samples were analysed using ICP-AES for the metals of interest. This method was performed as described by Topper and Kotuby-Amacher (1990) and the EPA (2007). Nominal and measured total metal concentrations for each element, dose method, mixture and soil are presented in supplementary material.

4.4.6 Statistical analysis

All data analyses and data visualization were performed using R version 3.1.3 (R Development Core Team, 2008) with the Rmisc (Hope, 2013) package. Total metal and individual metal concentration differences between soils were normalized to their target dose concentration and adjusted for soil control concentrations. Results are presented as total metal retention compared to nominal concentration in each soil to demonstrate the role of soil properties in metal retention. Individual metals are presented as a percent of nominal for each element across all soils and mixtures to show specific element retention. Invertebrate reproduction was normalized to each soil's control reproduction value. Results for invertebrate reproduction are presented as average normalized response across all soils and mixtures by dosing method. Significant differences between values were calculated using a two-way ANOVA and a post hoc Tukey Test at a 95% confidence interval.

4.5 Results

As expected, acidic soils lost more total metal content during leaching (Fig 4.1). Soil 3.22, with the lowest pH of 3.7, lost 60% ($p < 0.001$) of the metals originally added. Significant metal nitrate losses were also observed in the second most acidic soil WTRS ($p = 0.002$) (pH = 4.6). In contrast, Elora and KUBC with circumneutral pH levels of 6.7 and 5.6 did not lose a significant amount of metals from leaching. There were no significant differences observed in average total metal concentration between metal oxide dosed soils and non-leached metal nitrate dosed soils ($p > 0.06$). Soil KUBC showed significant differences comparing spinel-like mineral metal concentrations to metal oxide ($p = 0.03$) and non-leached nitrate concentrations ($p = 0.04$). Spinel-like mineral metal concentration in WTRS was also lower than non-leached nitrates ($p = 0.05$). The average nominal total metal concentration was 3150 mg kg^{-1}. Non-leached metal nitrate doses and metal oxide dosing are close to this with values of 2489 mg kg^{-1} and 2255 mg kg^{-1}, respectively. The average total spinel-like metal dose was lower at 1984 mg kg^{-1} while leached metal nitrate concentrations were below 50% of nominal on average at 1438 mg kg^{-1}.

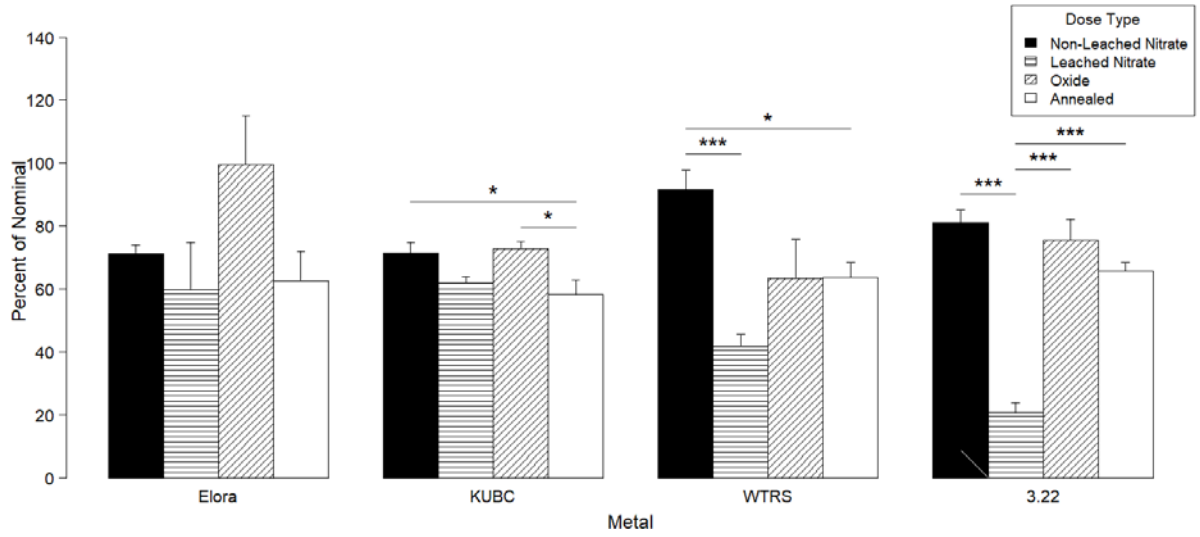

Figure 4.1: A comparison of the average measured total metal concentration in four soils dosed with a mixture of cobalt, copper, lead, nickel, and zinc as a percentage of nominal values. Three dose methods were used: leached metal nitrates (pre-leached soil concentrations also shown), powdered metal oxides, and spinel-like metal mineral ash formed from metal nitrate salts, * significant difference of $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Most of the metal loss during leaching across all soils appears to be due to only three out of the five metals tested (Fig 4.2). For example, more than 50% of initially dosed nickel ($p < 0.001$), zinc ($p < 0.001$), and cobalt ($p < 0.001$) was removed during leaching. In contrast, there were no statistically significant differences between non-leached and leached lead ($p = 0.96$) and copper ($p = 0.10$). For all five metal mixtures, metals dosed as oxides average concentrations were comparable to their non-leached nitrate counterparts. Dosing with spinel-like minerals produced similar results to dosing with oxides except for lead where significant differences are observed between oxide and spinel-like mineral dosing ($p = 0.01$). The average nominal metal concentrations for lead were 744 mg kg^{-1}, copper 461 mg kg^{-1}, nickel 512 mg kg^{-1}, zinc 1263mg kg^{-1}, and cobalt 170 mg kg^{-1}.

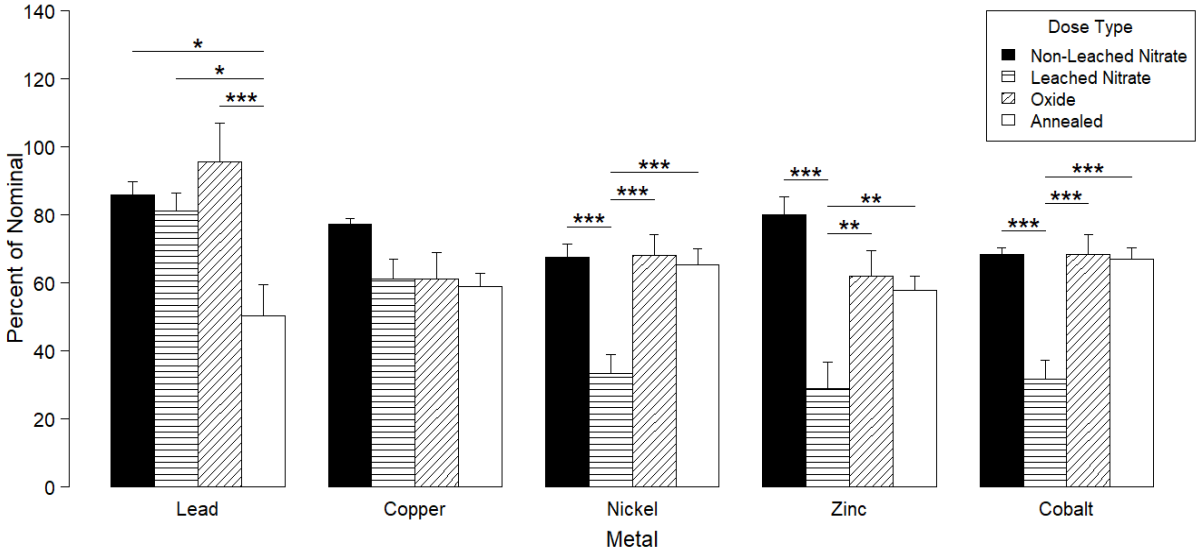

Figure 4.2: Average measured concentration of Co, Cu, Pb, Ni, and Zn separated by dose method expressed as a percentage of their nominal concentrations. These are the mean percentages calculated for each soil, * significant difference of $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Soil invertebrate reproduction changed depending on dose method (Fig 4.3). Metal nitrate and oxide dosed soils were significantly more toxic than spinel-like mineral dosed soils for all three tested species ($p < 0.001$). There were no significant differences between nitrate and

oxide dosed soils for *O. nitens* and *F. candida*, the two hard bodied invertebrates. Nitrate dosed soils were significantly more toxic than oxide soils for our soft bodied *E. crypticus* ($p < 0.001$). Data for *E. crypticus* was not collected for two of the tested soils (3.22 and WTRS) because organisms did not sufficiently reproduce in control soils. Soils 3.22 and WTRS contained less organic carbon and were more sandy than the other soils which may not provide *E. crypticus* a habitable environment. Comparing toxicity to metal retention we can observe that these are not directly linked. The spinel-like minerals had a higher overall metal retention than metal salts but the lowest toxicity. Metal oxides and salts had a similar toxicity (with the exception of *E. crypticus*) but metal concentrations in oxide dosed soils were significantly higher.

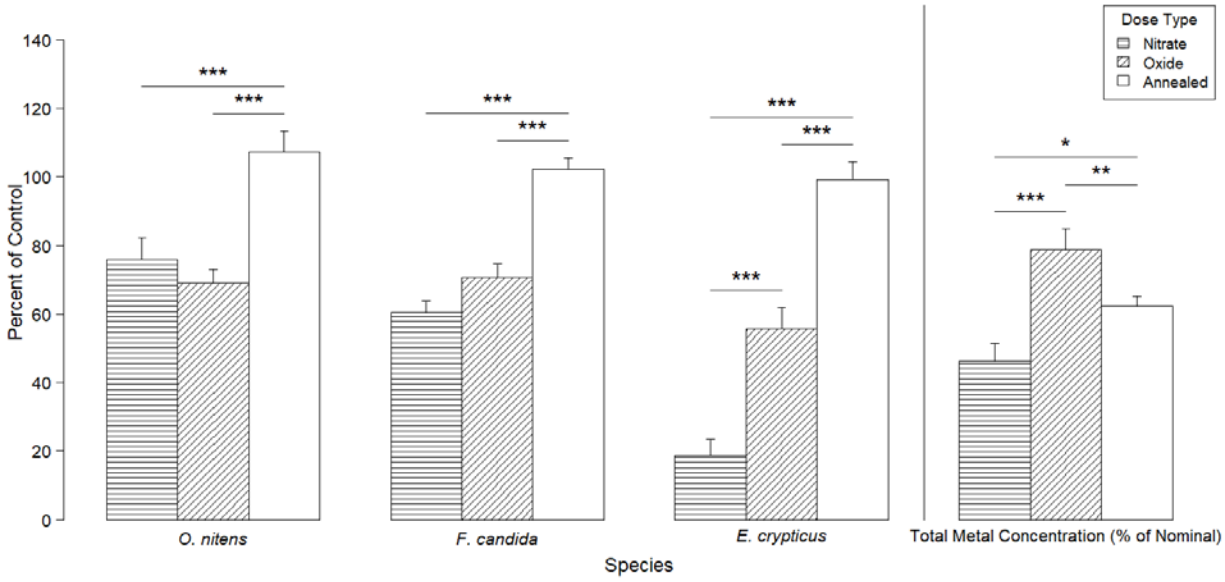

Figure 4.3: Average percent of control reproduction for all soils and mixtures for the different dosing methods compared to metal concentration as a percentage of nominal dose, * significant difference of $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4.6 Discussion

4.6.1 Methods

Leaching metal nitrate dosed soils lowers the measured metal dose compared to the intended dose. Soils with lower organic carbon content and pH (3.22 and WTRS) experienced higher overall losses. This is expected as organic matter provides adsorption sites for metals and

lower pH values increase metal solubility (Smolders et al., 2009; Houben et al., 2013; Checkai et al., 2014; Romero-Freire et al., 2016). Interestingly, in soils without a significant difference between non-leached and leached total metal concentrations (Elora and KUBC), the non-leached treatment metal concentrations were lower than expected. No soil showed significant differences in total metal concentration when comparing metal oxide to non-leached nitrate dose levels. The differences between nominal and tested concentrations are assumed to be due to errors in spiking and subsampling due to the low mass of oxides leading to a higher heterogeneity. The spinel-like mineral treatment concentrations were similar to the oxide dose method except for the Elora soil. This was unexpected as the higher mass should have reduced heterogeneity in soil and we expected a higher recovery compared to the oxide treatments. All dose methods (prior to leaching) were lower than the intended dose but were quite consistent across soils at 58 – 66% of nominal. Overall, the oxide and spinel-like mineral treatments were the most consistent across different soils whereas the leached nitrate treatment only performed adequately in soils with higher organic carbon and pH.

When testing metal mixtures in a fixed-ratio ray it is important for the relative concentrations of each metal to remain consistent (Meadows-Shropshire et al., 2004). With the exception of lead, oxide and spinel-like mineral treatments showed more consistent metal ratios than the leached nitrate treatments. While there is a disparity between tested and nominal metal concentrations across each treatment, the spinel-like and oxide treatments experienced generally consistent percentage differences from nominal concentrations across all metal elements. The nitrate leached treatments showed that metal retention was element specific. While there is no difference between the non-leached and leached lead and copper concentrations, significant losses of nickel, zinc, and cobalt were found. While this may be corrected in single metal tests

by measuring the leached concentration, it creates a problem when testing fixed ratio metal mixtures.

Considering the difference in effort and the above results, it seems prudent to dose soils using metal oxides rather than metal nitrates or spinel-like metal minerals. While no treatment reached the nominal goals, the spinel-like metal and oxide doses best retained their intended metal ratios. From a purely chemical view the oxide and spinel-like mineral treatments were comparable, but the effort required to create the spinel-like minerals lead us to recommend dosing with metal oxides when testing metal mixtures.

4.6.2 Toxicity

Metal oxides and leached nitrates caused a similar toxic effect to *F. candida* and *O. nitens*, while the leached metal nitrates were significantly more toxic to *E. crypticus*. This comparative toxicity of different metal forms has been examined previously in the literature but there is not a consensus on their toxicity (Lock and Janssen, 2003b; Kool et al., 2011; Amorim and Scott-Fordsmand, 2012).

For instance, Lock and Janssen (2003) found that *E. fetida* and *E. albidus* showed close EC_{50} values between soils dosed with zinc salts, oxides, and elemental powders. Metal oxide dosed samples were noticeably less toxic to *F. candida* where the oxide EC_{50} was 461 mg kg^{-1} compared to 271 mg kg^{-1} for zinc salts (Lock and Janssen, 2003b).

Kool, Ortiz and Van Gestel (2011) also tested different forms of zinc on the reproduction and survival of *Folsomia candida*. Their findings agreed with those shown here where oxides were less toxic than zinc chloride for both acute and chronic effects.

Finally, Amorim and Scott-Fordsmand (2012) found that copper nano particles were significantly more toxic than copper chloride salts for the survival, reproduction and avoidance

behaviour of *Enchytraeus albidus*. This study did not leach their soils after adding copper salts, therefore the comparative toxicity of the metal forms cannot directly link to this study.
Fine soil particles were observed in leachate collected from nitrate dosed soils. This could cause a change in soil texture or organic matter content. Changes in these properties can affect the toxic response of some invertebrates (Natal-da-Luz et al., 2008; Li et al., 2010; Checkai et al., 2014). The soils were leached as per the recommendations by Stevens et al. (2003). While their conclusion has been heeded by metal toxicity researchers to reduce the impact of metal salts, there does not appear to be any literature on these potential consequences of leaching these soils. Our method attempted to reduce any discrepancies in soil properties by leaching the control soils. Further research is required to determine if there are significant changes to soil texture or organic carbon contents.

We originally believed that the spinel-like minerals would be the most effective dose method as it appears to be more comparable to smelter emissions than the metal oxides or nitrates. It had the potential to reduce differences between laboratory dosed soil toxicity and that observed in field soils, however here it had no toxic effect. This method also created a thoroughly mixed ash by the nature of how it is formed, likely resulting in reduced heterogeneity compared to the low mass, individually weighed metal oxides. The absence of a toxic response could be due to the low bioavailability of the metals in their matrix with iron. Unlike traditional metal dosing methods where toxicity generally decreases over time, aging the spinel-like minerals could increase toxicity by dissolving the entrained metals into porewater (Hamilton et al., 2016). This would be another step towards mirroring smelter emissions on to soil where the deposits are broken down by rain. More research into the effect of aging on the spinel-like minerals is needed to confirm our suspicions however.

4.7 Conclusion

All dosing methods used were below nominal concentrations but dosing with metal oxides and spinel-like minerals was more effective at retaining the appropriate metal mixture ratio compared to metal nitrate treatments. Dosing with spinel-like minerals required significantly more preparation prior to dosing compared to metal oxides but did not improve metal recovery over metal oxides. In terms of toxicity, metal nitrates and metal oxide dosed soils produced similar toxic responses in *O. nitens* and *F. candida*, however the metal nitrates were at significantly lower concentrations compared to metal oxides. The effect of the metal nitrates was significantly higher to *E. crypticus*. None of the test species responded significantly to the annealed metals. Based on these results, it is recommended that metal oxides be used when testing fixed-ratio rays in metal mixture toxicity assays.

5.0 SYNTHESIS

Accurately predicting the toxic effects of large metal mixtures on soil organisms requires a robust data evaluation tool that can account for and evaluate the effect of multiple stressors on organisms as well as the effects of stressors on one another. In order to accurately predict the effects of these mixtures, the tool must be calibrated by data collected from samples that are representative of, or can be compared to, real-world conditions. While the European Union has adopted Concentration Addition as the principle method for evaluating the effects of metal mixtures (Smolders et al., 2009), mixtures of soil stressors may act synergistically, antagonistically, be dependent on the ratio of one or multiple stressors on the other(s), or have changes in toxic effects depending on the dose level (Jonker et al., 2005). The method in Jonker et al. (2005) can be expanded from their supplied binary mixture calculator to higher order mixtures. When evaluating large mixtures with the Jonker et al. (2005) Microsoft® Excel spreadsheet using the Solver Add-In (Frontline Solvers, 2015a), non-convergence issues were encountered when highly variable toxicity assay data were evaluated. In order to obtain the large-mixture data set used with the calculator, a new method of applying metals to soil was required.

Metals are usually applied to soil as salts and, more recently, subsequently leached using artificial rainwater (Li et al., 2010). The leaching process is used both to remove the anions (Cl^-, NH_3^-, etc.) inherent to the salts which both increase metal availability in soil porewater and create their own toxic effects (Stevens et al., 2003; Owojori et al., 2009; Pereira et al., 2015). The leaching process can also remove metals from soils however, which can be corrected for in single stressor or composite mixtures by testing soil metal concentration after leaching. If the post-leaching metal doses are not high enough, additional samples can be created simply by

increasing the concentration of metals prior to leaching. Other mixture assays, such as fixed-ratio rays, would require extensive dose calibration to obtain increasing dose levels that have similar ratios of metals along the entire dose-response curve. In order to address the shortcomings of these methods, this project investigates a transfer of the dose-response equations in Jonker et al. (2005) to R (R Development Core Team, 2008), as well as developing a new dose method that can more accurately create specific metal dose ratios in soil.

5.1 Principle Findings

When evaluating a data set obtained from several mixtures of five metals (Appendix C), using a modified version of the Jonker et. al (2005) spreadsheet, the Solver Add-In encountered convergence problems. The lowest sum of squares value obtained by the Solver Add-In was not consistent when using consistent starting values, indicating that there may be issues with finding local minimums. In a similar fashion to previous research (Asselman et al., 2013; Hochmuth et al., 2014), the methods in Jonker et. al (2005) was transferred to an R script (R Development Core Team, 2008) (Appendix A).

The Jonker et al., 2005 method using the Solver Add-In uses the Newtonian method to determine a predicted response *y* from a constant set of concentrations, the single stressor EC50 values, and slope modifiers (a, a_{DR}, $a_{DL,}$ b_{DR}, and b_{DL}). This portion of the analysis is completed by the Jonker et al. (2005) spreadsheet by finding the sum of squares between the single stressor responses and the predicted responses from the single stressor dose response curves, while the mixture responses are compared to the predicted mixture response using the R function uniroot.all(), which is obtained from the rootsolve package (Soetaert, 2009). The dose response parameters are then modified by the spreadsheet from their single stressor values using the Solver Add-In to find the least sum of squares between the predicted response *y* and the measured response of a data set. The R script replaces the Solver Add-In using the optim()

function which is a basic R function (R Development Core Team, 2008). The previously mentioned single stressor fits and uniroot.all() function is nested inside the optim() function, which changes the EC50 and slope parameters to determine the lowest sum of squares.

The optim() function can use the Nelder-Mead (NM), Broyden, Fletcher, Goldfarb, and Shanno (BFGS), conjugate gradients (CG), limited memory BFGS boxed (L-BFGS-B), simulated annealing (SANN), and Brent (Brent) methods (Fletcher, 1964, 1970; Nelder and Mead, 1965; Broyden, 1970a; b; Goldfarb, 1970; Shanno, 1970; Byrd et al., 1995; Lide, 2005; R Development Core Team, 2008). Brent was excluded from this study as it is typically for one-dimensional analysis. In order to compare the robustness of each optim() method, four data sets were generated using the same theoretical mixture of five stressors and their associated theoretical dose response curves. The data sets had calculated responses *y* generated at standard deviations of 0, 2, 5, and 10 in a normal distribution using the rnorm() function (R Development Core Team, 2008) (Appendix B). The generated sets were entered into the modified Jonker et al. (2005) spreadsheet using the Solver Add-In and each optim() solve method using every possible starting values of EC50, 0.5 * EC50, *b*, and 0.5**b*.

The sum of the sum of squares generated from each solve method and start parameters was calculated (Fig. 3.2) and showed that the BFGS method produced the lowest sum of squares overall. When compared to the modified Jonker et al. (2005) spreadsheet, the optim() BFGS solve method produced the lowest sum of squares for all sets of starting parameters. The improvement in lowering the sum of squares between the spreadsheet and optim() BFGS method increased at higher standard deviation data sets (Tables 3.3, 3.4, 3.5, and 3.6). These results show that the R script using the optim() BFGS method was more efficient at finding lower local minimums than the Jonker et al. (2005) spreadsheet when using a manufactured data set.

When testing against a real data set using five single metal assays and ten mixtures lead, copper, nickel, zinc and cobalt (Appendix C), the Jonker et al. (2005) spreadsheet and R script using the BFGS method were ran three times in succession to optimize the sum of squares (Table 3.6) when using a dose-level dependent response calculation. The R script obtained a sum of squares approximately 25% lower than the Excel spreadsheet on the initial optimization. Subsequent optimizations failed to produce significant improvements over the initial fits.

Further calibration of the new R script created from the methods in Jonker et al. (2005) requires a fixed-ratio ray data set to determine the expected Concentration Addition or modified Concentration Addition toxicity behaviour. Four soils (Table 4.1) were chosen to be dosed with lead, copper, nickel, zinc, and cobalt as single stressors and in five mixtures (Table 4.2) to observe their dose ratio consistency and toxic effects on *E. crypticus, F. candida, O. nitens*. These metals and their mixtures were added to the soils as aqueous metal nitrates, powdered metal oxides, and annealed powders that may represent the more typical spinel-like minerals found at contaminated sites.

While the measured total pre-leaching metal nitrate doses (Fig 4.1) are similar to that of the total measured metal oxide doses ($p > 0.05$), the lowest pH soils had approximately 50% of metals removed during leaching ($p < 0.001$, $p < 0.002$). Only the lowest soil, 3.22 (pH 3.7), showed a significant difference between total post-leaching metal nitrate doses and total metal oxide doses however ($p < 0.001$). Soil 3.22 was also the only medium to show a significant difference between total post-leaching metal nitrate doses and annealed metal doses. In almost all cases the total measured metal concentrations were 80% or lower of the nominal total metal concentrations.

A significant amount of the metals removed from the soils during leaching are attributed to the loss of three of the five applied metals (Fig 4.2). Approximate 55% of nickel ($p < 0.001$), 64% of zinc ($p < 0.001$), and 48% of cobalt ($p < 0.001$) of the pre-leaching metal nitrate doses remained in the soils after leaching, showing that it is unable to retain the required metal ratios. Consequently, the average post-leaching percent of nominal concentrations were significantly lower than metal oxide doses for nickel ($p < 0.001$), zinc ($p = 0.002$), and cobalt ($p < 0.001$). Annealed metal lead doses were significantly lower than non-leached metal nitrate ($p = 0.014$), leached metal nitrate ($p = 0.043$), and metal oxide doses ($p < 0.001$). No significant differences were observed between annealed metal and metal oxide doses for the other four metals. Similar to metal oxide doses, annealed metal doses were significantly high than the metal nitrate nickel ($p < 0.001$), zinc ($p < 0.001$), and cobalt ($p < 0.001$) doses. The measured annealed metal doses, while approximately 60% of nominal doses overall, appear to be the most consistent at retaining required dose ratios.

While the average total concentration of annealed metal doses were lower than metal oxide ($p = 0.049$) and higher than leached metal nitrate doses ($p < 0.042$), total annealed metal dosed soils do not appear to have a significant effect on soil invertebrate reproduction (Fig 4.3). While the average total metal oxide dose was significantly higher than the leached nitrate dose ($p < 0.001$), only *E. crypticus* showed a significantly different average response between leached metal nitrate and metal oxide dosed soils. Interestingly, the *E. crypticus* response was significantly higher in the case of leached metal nitrates ($p < 0.001$) even though that dose method produced significantly lower total metal concentrations in the chosen soils and mixtures.

5.2 Discussion

The results above have shown that there is room for significant improvements to the testing and analysis of metal mixture toxicity to soil invertebrates. Four issues have been addressed over the course of this research: expanding and improving the Concentration Addition deviation models over the spreadsheet provided by Jonker et al. (2005), the effect of soil type on the total post-leaching metal salt doses, differences in the proportion of metals leached from soils when applying them in a fixed ratio, and how different dosing methods affect soil invertebrate reproduction.

While the Jonker et al. (2005) methods can be expanded to any number of stressors in a mixture, the spreadsheet that was provided is limited to binary mixtures. Developing the built-in functions to include for additional mixture components requires a thorough understanding of the mathematics in Jonker et al. (2005) as well as proficiency in VBA. Creating R scripts that have the capability to perform analysis on up to five stressors as well as detailed instructions on how to perform the analysis will make the use of Jonker et al. (2005) more accessible and easier use for researchers.

Ease of use is furthered by the more sophisticated optim() function using the BFGS solve method (Broyden, 1970b; a; Fletcher, 1970; Goldfarb, 1970; Shanno, 1970; R Development Core Team, 2008) compared to the relatively aged Solver Add-In (Frontline Solvers, 2015a). Higher level mixtures and highly variable organism responses appear to be better predicted by the BFGS solve method as well. In the case of a manufactured data set the R script was able to find a lower sum of squares for every set of starting parameters and data variance. When offered laboratory data generated using ten mixtures of five metals (Appendix C) the R script also obtained a lower sum of squares. In this case, the Excel spreadsheet was unable to or did not see the need to

adjust the dose level dependent variables a_{DL} or b_{DL}, which may have been the reason for its shortcomings. Table 3.6 shows that the R script is more accurate than the Jonker et al. (2005 Excel spreadsheet, and that optim() is able to change the starting parameters by orders of magnitude without encountering errors. Lead and cobalt EC50 values increase by a power of 10, while the values of a_{DL} and b_{DL} reach two orders higher than their starting values of 0 and 1, respectively. This may increase the script's effectiveness in modelling mixtures as it is able to expand its field of reference more than the Jonker et al. (2005) spreadsheet. This potential for increased accuracy could allow researchers to better evaluate the behaviour of quality data.

The development of a new method for dosing soils with metal mixtures, especially greater than binary mixtures, is extremely important to examine the effects of one metal on another. While the initial dose of metal nitrates results in consistent dose ratios (Fig. 4.2) the leaching process appears to be significantly affected by individual metal properties. This effect may be compounded or mitigated by the effect of soil properties (Fig. 4.1) depending on the quality of soil at a site of interest. The site of interest influences the dominant species of soil organisms present which may show significantly different responses to different dose method, such as in the case of *E. crypticus* (Fig. 4.3). None of the species tested appeared to show a toxic response to the annealed metal powders.

The annealed metal doses for each metal were not significantly different from metal oxides with the exception of the lead dose ($p < 0.001$). The total metal dose appears to be fairly consistent at around 62% of nominal for both total and individual metal concentrations. Unfortunately, the dose consistency proved to be irrelevant due to the lack of a toxic effect on any of the tested organisms. Considering the effort required mix the reagents, pre-emptively precipitate, anneal, analyse, adjust, anneal, and test again with a non-significant effect on soil

organisms, the annealed metal dose method does not seem to be a viable alternative to the current metal salt or proposed metal oxide dose methods.

5.3 Future Directions

The R scripts made using the methods in Jonker et al. (2005) are fairly straightforward to use, however the requirement for each method and number of mixture components to have its own file is rather cumbersome. Some portions of the script are not compliant with typical best practices, such as assigning parameters inside Response_SS to allow uniroot.all() (Soetaert, 2009) to access them. In the future I would like to decrease the number of inputs that are required from the end user and combine all methods into one package in R.

The information obtained from these experiments has shown that reliably dosing soils with specific mixtures of metal nitrate salts is relatively difficult. Dosing soils with metal oxides show some promise compared to metal salts by eliminating the leaching process and, by extension, the non-proportional removal of certain metals. Validation of our method requires additional toxicity assay data, such as that in Appendix C, to be performed. More data is essential to predicting deviations, or lack thereof, from Concentration Addition as well as developing metal-oxide based species sensitivity distributions.

While the annealed metal compounds did not elicit a toxic response from the organisms, it is possible that this is analogous to a near-end product of metal contamination. If the powder is left in contact with soil for an extended period, it is possible that some portion of it may break down into more toxic products. Dosing a soil with the annealed metal powder and performing periodic toxicity assays could show increased toxicity over time. Even in the case of an appreciative toxic response the extensive work required to make the powders may restrict its use.

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Appendix A: Dose level dependent Concentration Addition R script using methods from Jonker et al., 2005

```
################################################################################################
#                                                                                              #
#            Fitting Single and Mixture Data for Five Stressors                                #
#              Using Methods Defined by Jonker et al., 2005                                    #
#                                                                                              #
#    Created by Mark Cousins in partial fulfillment of a M. Sc. in Soil Science                #
#      at the University of Saskatchewan, Supervised by Steven Siciliano, 2018                 #
#                                                                                              #
################################################################################################

# This script has been created to fit single and mixtures of stressors according to
# Dose-Level Dependent Concentration Addition as defined in Jonker et al., 2005.
#
# It is advised that the user read the entire script prior to attempting to analyse data.
#
# Instructions for user inputs have been denoted similar to:

################################################################################################
#######################     Script Input Instructions     ######################################
################################################################################################

# Explanations on how to fill in each input are explained just after each notation
#
# After each instruction, user input locations will be clearly defined.
#
# Make sure to read instructions before entering any information
#
# Places where the user will enter data are denoted similar to:

################################     Script Input     ##########################################

# Variable <- User defined information

################################################################################################


################################     Data Analysis    ##########################################

# Clear Workspace to avoid problems with previously defined variables, attached variables,
# and packages that may override the required packages in this script

rm(list = ls())

################################################################################################
##############      Obtaining the Required Packages for Analysis     ###########################
################################################################################################

# If you do not have the rootSolve() and/or plyr() packages installed, delete the "#"
# before each "install.packages" function.
#
# If they are installed, run both library() functions.

#####################     Installing Required Packages     ######################################

#install.packages("rootSolve")
#install.packages("plyr")
```

```
57
58  ##############################################################################################
59
60  # Load in required packages.
61
62  library(rootSolve)
63
64  # The uniroot.all function in rootSolve() is used to fit the Y value from the
65  # Jonker et al. Inverse Dose Response function in mixtures.
66  #
67  #
68
69  library(plyr)
70
71  # The adply() function in plyr() is used to apply uniroot.all() to all data points
72  # that are mixtures.
73
74  ##############################################################################################
75  ####################     How to Set the Working Directory     ######################
76  ##############################################################################################
77
78  # Set directory where your data is stored and where fitted data will be saved
79  #
80  # While Windows uses "\" to separate file paths, R uses "/".
81  #
82  # So if I copy and paste a directory, for example:
83  #
84  # C:\Users\User 1\Files\
85  #
86  # My data would be retrieved from and stored in "Files"
87  #
88  # The directory must be changed to:
89  #
90  # C:/Users/User 1/Files/
91  #
92  # Set the directory between the quotation marks in setwd("")
93  #
94  # For example, my workind directory would be:
95  #
96  # setwd("C:/Users/User 1/Files/")
97
98  ######################     Set the Working Directory     ############################
99
100 setwd("")
101
102 ##############################################################################################
103
104 ##############################################################################################
105 ##########################     Loading Your Data Set     ###########################
106 ##############################################################################################
107 #
108 # Your data must be arranged as:
109 #
110 # Assay     Mixture Stressor_1    Stressor_2     Stressor_3    Stressor_4     Stressor_5     Response
111 # Assay_1   T/F     C1_1        C2_1           C3_1          C4_1           C5_1           R_1
112 # Assay_1   T/F     C1_2        C2_2           C3_2          C4_2           C5_2           R_2
```

```
# Assay_1   T/F   C1_3      C2_3      C3_3      C4_3      C5_3      R_3
# Assay_1   T/F   C1_...    C2_...    C3_...    C4_...    C5_...    R...
# Assay_1   T/F   C1_x      C2_x      C3_x      C4_x      C5_x      R_5
# Assay_2   T/F   C1_x+1    C2_x+1    C3_x+1    C4_x+1    C5_x+1    R_x+1
# Assay_2   T/F   C1_x+2    C2_x+2    C3_x+2    C4_x+2    C5_x+2    R_x+2
# Assay_2   T/F   C1_x+3    C2_x+3    C3_x+3    C4_x+3    C5_x+3    R_x+3
# Assay_2   T/F   C1_x+...  C2_x+...  C3_x+...  C4_x+...  C5_x+...  R_x+...
# Assay_2   T/F   C1_x+y    C2_x+y    C3_x+y    C4_x+y    C5_x+y    R_x+y
# Assay_... T/F   C1_x+y+...  C2_x+y+...  C3_x+y+...  C4_x+y+...  C5_x+y+...  R_x+y+...
# Assay_n   T/F   C1_x+y+...+1  C2_x+y+...+1  C3_x+y+...+1  C4_x+y+...+1  C5_x+y+...+1  R_x+y+...+1
# Assay_n   T/F   C1_x+y+...+2  C2_x+y+...+2  C3_x+y+...+2  C4_x+y+...+2  C5_x+y+...+2  R_x+y+...+2
# Assay_n   T/F   C1_x+y+...+3  C2_x+y+...+3  C3_x+y+...+3  C4_x+y+...+3  C5_x+y+...+3  R_x+y+...+3
# Assay_n   T/F   C1_x+y+...  C2_x+...  C3_x+y+...  C4_x+y+...  C5_x+y+...  R_x+y+...
# Assay_n   T/F   C1_x+y+...+z  C2_x+y+...+z  C3_x+y+...+z  C4_x+y+...+z  C5_x+y+...+z  R_x+y+...+z
#
# Where Assay_1 and Assay_2 would be your assay name, i.e. Lead and Copper.
#
# The Mixture column contains F (FALSE) if the assay is for a single stressor, or T (TRUE) if
# the assay contains a mixture of stressors
#
# Stressor_1 to Stressor_5 are your stressors, i.e. lead, copper, toluene.
#
# For this script, they need to be named as Stressor_1 to Stressor_5.  Their actual names
# can be set later.
#
# C1_1 and other values would be the concentrations of your stressor.  The x and y values only denote
# the "length" of the data set.
#
# The R_1, R_2 etc. are the response that you observed in each test.
#
# An example data set is shown below:
#
# Assay     Mixture Stressor_1 Stressor_2 Stressor_3 Stressor_4 Stressor_5 Response
# Lead      F       0          0          0          0          0          100
# Lead      F       2          0          0          0          0          95
# Lead      F       4          0          0          0          0          75
# Lead      F       6          0          0          0          0          50
# Lead      F       8          0          0          0          0          12
# Copper    F       0          0          0          0          0          100
# Copper    F       0          2          0          0          0          90
# Copper    F       0          4          0          0          0          60
# Copper    F       0          6          0          0          0          20
# Copper    F       0          8          0          0          0          0
# Nickel    F       0          0          0          0          0          100
# Nickel    F       0          0          2          0          0          95
# Nickel    F       0          0          4          0          0          90
# Nickel    F       0          0          6          0          0          50
# Nickel    F       0          0          8          0          0          0
# Zinc      F       0          0          0          0          0          100
# Zinc      F       0          0          0          2          0          70
# Zinc      F       0          0          0          4          0          40
# Zinc      F       0          0          0          6          0          12
# Zinc      F       0          0          0          8          0          5
# Cobalt    F       0          0          0          0          0          100
# Cobalt    F       0          0          0          0          2          80
# Cobalt    F       0          0          0          0          4          62
```

```
# Cobalt      F      0        0        0        0       6         27
# Cobalt      F      0        0        0        0       8         12
# ...         ...    ...      ...      ...      ...     ...       ...
# Equal_Ratio  T      0        0        0        0       0        100
# Equal_Ratio  T      0.5      0.5      0.5      0.5     0.5       75
# Equal_Ratio  T      0.75     0.75     0.75     0.75    0.75      20
# Equal_Ratio  T      1        2        1        1       1         0
# Equal_Ratio  T      2        2        2        2       2         0

# Load in your data by replacing Test_Data.csv with the name of your data set in the quotations
#
# Data must be in .csv format

#######################     Loading Your Data     ############################

Dose_Response <- read.csv("", fileEncoding="UTF-8-BOM")

###############################################################################

###############################################################################################
#####################    Setting the Names of Your Stressors     ##################
###############################################################################################

# Now that the data is loaded, we can set the names of each stressor.
#
# For example, my example table's stressors are:
#
# Stressor_1 = Lead
# Stressor_2 = Copper
# Stressor_3 = Nickel
# Stressor_4 = Zinc
# Stressor_5 = Cobalt
#
# To set the names of the stressors, we simply make a list of these names.
#
# For example, my list would be:
#
# Stressor_Names <- c("Lead", "Copper", "Nickel", "Zinc", "Cobalt")
#
# Set the names of your stressors in Stressor_Names below by placing each in
# its applicable quotation marks:

########################     Set the Names of Stressors     ############################

Stressor_Names <- c("", "", "", "", "")

###############################################################################################

# R adds a column of total stressor content.

Dose_Response$Sum_Stressor <- Dose_Response[,3] + Dose_Response[,4] +
  Dose_Response[,5] + Dose_Response[,6] + Dose_Response[,7]

# Where Dose_Response[,x] denotes each column of concentrations of stressors.
#
# To complete the data analysis, we set a number to each data point so that it can
```

```
# be added to the original data set in the proper order

Dose_Response$Row_Number <- seq(1, nrow(Dose_Response))

# Due to how the mixture data is analysed, control samples need to be excluded
# from normal analysis.
#
# They will only be compared to the fitted maximum response value.
#
# Here we create a separate data set with only control samples

Control_Data <- subset(Dose_Response, Dose_Response$Sum_Stressor == 0)

# Now we separate Response_Data into single stressor and mixtures of stressors data sets
#
# As we already have extracted the control values to a separate set we will exclude
# those samples from these two sets.
#
# The Dose_Respose[,2] == TRUE/FALSE script checks the value in the "Mixture" column
# and extracts the FALSE rows to the Single_Data table and the TRUE rows to the
# Mixture_Data table.
#
# The Dose_Response$Sum_Stressor != 0 condition makes R only take the rows where the
# total stressor concentration is greather than 0.
#
# The "&" tells R to only take the rows that fulfill both conditions.

Single_Data <- subset(Dose_Response, Dose_Response[,2] == FALSE &
                      Dose_Response$Sum_Stressor != 0)
Mixture_Data <- subset(Dose_Response, Dose_Response[,2] == TRUE &
                       Dose_Response$Sum_Stressor != 0)

# R keeps the original levels (Lead, Copper, Equal_Ratio etc.) of each assay
# when subsetting data.
#
# For example, if I run:
#
# levels(Single_Data$Assay)
#
# R will return:
#
# "Cobalt", "Copper", "Equal_Ratio", "Lead", "Nickel", "Zinc"
#
# We need to "drop" the levels that are no longer part of each assay in each set.
#
# To do this, we re-factor() each data set

Single_Data$Assay <- factor(Single_Data$Assay)
Mixture_Data$Assay <- factor(Mixture_Data$Assay)

# For some analyses the number of Mixture assays is required.
#
# This is done using:

# length(unique(Mixture_Data$Assay))

```

```
# Where it finds how many (length()) assay names (unique()) there are in Mixture_Data$Assay
#
# This is only needed for the mixtures, as the individual stressor assays have already been
# separated into different data sets.
#
# The number of Mixture assays is stored in Num_Mixtures

Num_Mixtures <- length(unique(Mixture_Data$Assay))

################################################################################################
#################    Setting the Names and Order of Your Stressors    ###############
################################################################################################
#
# To ensure that the fitting parameters you enter are associated with the right
# single-stressor assays the "order" of the assays must be set.
#
# R orders them alphabetically by default, so we need to change that.
#
# This is to help this script, which is meant to run with any data set organized as above.
#
# For example, if I was to look at my Single_Data levels:
#
# levels(Single_Data$Assay)
#
# R returns:
#
# "Cobalt" "Copper" "Lead"   "Nickel" "Zinc"
#
# As we have already noted the order of the Stressors in Stressor_Names, this is easy.
#
#
# We apply Stressor_Names to the factors() of Single_Data$Assay:
#
# Single_Data$Assay <- factor(Single_Data$Assay, Stressor_Names)
#
# Now if I run:
#
# levels(Single_Data$Assay)
#
# R returns:
#
# "Lead" "Copper" "Nickel" "Zinc" "Cobalt"
#
# Here the Assay "levels" are ordered as we want them.

Single_Data$Assay <- factor(Single_Data$Assay, Stressor_Names)

# Now R moves each stressor assay in Single_Data to separate data sets.

S1_Data <- subset(Single_Data, Single_Data$Assay == levels(Single_Data$Assay)[1])
S2_Data <- subset(Single_Data, Single_Data$Assay == levels(Single_Data$Assay)[2])
S3_Data <- subset(Single_Data, Single_Data$Assay == levels(Single_Data$Assay)[3])
S4_Data <- subset(Single_Data, Single_Data$Assay == levels(Single_Data$Assay)[4])
S5_Data <- subset(Single_Data, Single_Data$Assay == levels(Single_Data$Assay)[5])

# Now S1_Data contains the single stressor data for Stressor_1, in my case lead,
```

```
# S2_Data contains the data for Stressor_2, copper for my data, etc.
#
# This is not required for Mixture_Data, as it reads every column anyway
#
################################################################################################
#######################    Fitting Single Stressor Curves    #######################
################################################################################################

#
# If required, you can create functions to calculate single metal and mixture responses.
#
# It may be easier to simply fit them using Excel's Solver function.
#
# Fitted EC50 and b values can be seen when running S#_Solved on its own line.  The
# variables listed under $par for each S#_Solved lists the maximum response, the EC50,
# and the b values in order as fitted by each function
#
# Initial guess values for each parameter must be entered in the c(,,) within the optim()
# function in the order c(Max Response, EC50, b).
#
# Delete the "#" at the start of each line to load them in.
#
#S1_Resp <- function (S1_Param){
#  S1_Resp_max <- S1_Param[1]
#  S1_Resp_EC <- S1_Param[2]
#  S1_Resp_b <- S1_Param[3]
#  sum((S1_Data$Response - S1_Resp_max / (1 + (S1_Data$Stressor_1 / S1_Resp_EC) ^ S1_Resp_b))^2)
#}
#
#S1_Solved <- optim(c(,,),S1_Resp)
#S1_Solved

#S2_Resp <- function (S2_Param){
#  S2_Resp_max <- S2_Param[1]
#  S2_Resp_EC <- S2_Param[2]
#  S2_Resp_b <- S2_Param[3]
#  sum((S2_Data$Response - S2_Resp_max / (1 + (S2_Data$Stressor_1 / S2_Resp_EC) ^ S2_Resp_b))^2)
#}
#
#S2_Solved <- optim(c(,,),S2_Resp)
#S2_Solved

#S3_Resp <- function (S3_Param){
#  S3_Resp_max <- S3_Param[1]
#  S3_Resp_EC <- S3_Param[2]
#  S3_Resp_b <- S3_Param[3]
#  sum((S3_Data$Response - S3_Resp_max / (1 + (S3_Data$Stressor_1 / S3_Resp_EC) ^ S3_Resp_b))^2)
#}
#
#S3_Solved <- optim(c(,,),S3_Resp)
#S3_Solved

#S4_Resp <- function (S4_Param){
#  S4_Resp_max <- S4_Param[1]
#  S4_Resp_EC <- S4_Param[2]
#  S4_Resp_b <- S4_Param[3]
```

```
#  sum((S4_Data$Response - S4_Resp_max / (1 + (S4_Data$Stressor_1 / S4_Resp_EC) ^ S4_Resp_b))^2)
#}
#
#S4_Solved <- optim(c(,,),S4_Resp)
#S4_Solved

#S5_Resp <- function (S5_Param){
#  S5_Resp_max <- S5_Param[1]
#  S5_Resp_EC <- S5_Param[2]
#  S5_Resp_b <- S5_Param[3]
#  sum((S5_Data$Response - S5_Resp_max / (1 + (S5_Data$Stressor_1 / S5_Resp_EC) ^ S5_Resp_b))^2)
#}
#
#S5_Solved <- optim(c(,,),S5_Resp)
#S5_Solved

##############################################################################################
####################    Determining Least Sum of Squares    ####################
##############################################################################################
#
# Response_SS calculates the sum of squares for an entire data set.
#
# fit_param will be filled with the parameters estimated by Excel or by guessing
# however the function may not work well with guesses.
#
# The format of running the script is Response_SS(c(Control Response, Stressor 1 EC50,
#                                    Stressor 2 EC50, Stressor 3 EC50, Stressor 4 EC50,
#                                    Stressor 5 EC50, Stressor 1 b, Stressor 2 b, Stressor 3 b,
#                                    Stressor 4 b, Stressor 5 b, a)).
#
# "a" denotes the level of synergism or antagonism

Response_SS <- function(fit_param){

 # Response_SS is assigned values from fit_param according to excel parameters or guesses
 #
 # fit_param never actually needs to be called by the user.
 #
 # Whatever input you place in Response_SS() is automatically assigned as fit_param.
 #
 # The function assigns variables using "<<-" rather than "<-" to create global variables.
 #
 # This is needed as uniroot.all() in adply() does not access the function environment.
 #
 # The uniroot is searching for an estimated Y which makes
 # concentration addition valid given the fit parameters used.
 #
 # Then it calculates the sum of squares for this estimated Y and the observed Y.
 #
 # If an alternative to assigning as global variables is found it will be added.
 #
 # Maximum Response is notated as "max2" to avoid errors with uniroot.all()

 max2 <<- fit_param[1]
 S1_EC <<- fit_param[2]
 S2_EC <<- fit_param[3]
```

```
    S3_EC <<- fit_param[4]
    S4_EC <<- fit_param[5]
    S5_EC <<- fit_param[6]
    S1_b <<- fit_param[7]
    S2_b <<- fit_param[8]
    S3_b <<- fit_param[9]
    S4_b <<- fit_param[10]
    S5_b <<- fit_param[11]
    a <<- fit_param[12]
    b <<- fit_param[13]

    sum((Control_Data$Response - max2)^2) +
      sum((S1_Data$Response - max2 / (1 + (S1_Data[,3] / S1_EC) ^ S1_b))^2) +
      sum((S2_Data$Response - max2 / (1 + (S2_Data[,4] / S2_EC) ^ S2_b))^2) +
      sum((S3_Data$Response - max2 / (1 + (S3_Data[,5] / S3_EC) ^ S3_b))^2) +
      sum((S4_Data$Response - max2 / (1 + (S4_Data[,6] / S4_EC) ^ S4_b))^2) +
      sum((S5_Data$Response - max2 / (1 + (S5_Data[,7] / S5_EC) ^ S5_b))^2) +
      sum((Mixture_Data$Response - adply(Mixture_Data, 1, summarize,
                                  solution <- uniroot.all(function (Y){
                                    TU1 <- Stressor_1/S1_EC
                                    TU2 <- Stressor_2/S2_EC
                                    TU3 <- Stressor_3/S3_EC
                                    TU4 <- Stressor_4/S4_EC
                                    TU5 <- Stressor_5/S5_EC
                                    z1 <- TU1/(TU1 + TU2 + TU3 + TU4 + TU5)
                                    z2 <- TU2/(TU1 + TU2 + TU3 + TU4 + TU5)
                                    z3 <- TU3/(TU1 + TU2 + TU3 + TU4 + TU5)
                                    z4 <- TU4/(TU1 + TU2 + TU3 + TU4 + TU5)
                                    z5 <- TU5/(TU1 + TU2 + TU3 + TU4 + TU5)
                                    Stressor_1 / (S1_EC * ((max2 - Y) / Y) ^ (1 / S1_b)) +
                                      Stressor_2 / (S2_EC * ((max2 - Y) / Y) ^ (1 / S2_b)) +
                                      Stressor_3 / (S3_EC * ((max2 - Y) / Y) ^ (1 / S3_b)) +
                                      Stressor_4 / (S4_EC * ((max2 - Y) / Y) ^ (1 / S4_b)) +
                                      Stressor_5 / (S5_EC * ((max2 - Y) / Y) ^ (1 / S5_b)) -
                                      exp((a * (1 - (b * (TU1 + TU2 + TU3 + TU4 + TU5)))) * z1 * z2 * z3 * z4 * z5)},
                                  interval = c(0,max2)))[,11])^2)
}

# Now the fitting parameters found in excel or guessed parameters should be
# assigned to fit_guesses
#
# For example, I have:
# Maximum Response (max2) = 287
# Lead EC50 (S1_EC) = 24882
# Copper EC50 (S2_EC) = 9355
# Nickel EC50 (S3_EC) = 12923
# Zinc EC50 (S4_EC) = 6054
# Cobalt EC50 (S5_EC) = 40722
# Lead b (S1_b) = 0.576
# Copper b (S2_b) = 0.418
# Nickel b (S3_b) = 2.885
# Zinc b (S4_b) = 1.192
# Cobalt b (S5_b) = 2.757
# a = 0
# b = 1
#
```

```
# So I would run:
#
# fit_guesses <- c(287, 24882, 9355, 12923, 6054, 40722, 0.576, 0.418, 2.885, 1.192, 2.757, 0, 1)
#
# Place your parameters in the order of:
#
# fit_guesses <- c(Control Response, Stressor_1 EC50, Stressor_2_EC50, Stressor_3 EC50,
#                  Stressor_4 EC50, Stressor_5 EC50, Stressor_1 b, Stressor_2 b,
#                  Stressor_3 b, Stressor_4 b, Stressor_5 b, a, b)

###############    Set your Initial Fitting Parameter Guesses    ####################

fit_guesses <- c(300,5000,10000,2000,500,1000,0.6,1,0.1,5,4,0,1)

#############################################################################################

# Now that fit_guesses has been set, we can find the sum of squares from the initial guess.
#
# We run Response_SS with fit_guesses and assign the sum of squares to Initial_SS:

Initial_SS <- Response_SS(fit_guesses)

# This gives me the sum of squares that R calculates from the parameters you
# initially guessed or excel solved for.
#
# Now the optim() function is used to make R find the parameters with the lowest sum
# of squares and store them in Optim_param.
#
# The format of this is:
#
# Optim_param <- optim(fit_guesses, Response_SS).
#
# The values R finds are stored in Optim_param$par
#
# The sum of squares with these parameters are in Optim_param$value
#
# Now run:

Optim_param <- optim(fit_guesses, Response_SS)
Optim_param$par
# Store sum of squares of minimized Juvenile_SS functions

min_sum_square <- Optim_param$value
min_sum_square
# If you wish to do multiple iterations of the data, a for-next loop has been added.
#
# Prior to running the for-next loop, tell the script if you want to view the curves
# fitted to each data set where Show_Graphs is assigned TRUE or FALSE, where TRUE will
# output the graphs every iteration and FALSE will not.
#
# For example, if I want to see the fitted curves every iteration I would type:
#
# Show_Graphs <- TRUE
#
# Show_Graphs needs a default value, so I have set it as TRUE.  If you do not want to
# see each graph, change TRUE to FALSE
```

```
561
562  ###############     Set your Initial Fitting Parameter Guesses     ####################
563
564  Show_Graphs <- TRUE
565
566  ##############################################################################################
567
568  # If Show_Graphs is TRUE, run the following script, which will determine the dimensions of
569  # the outputted graphs.
570  #
571  # This will keep them in either the most square output, or rectangular if it is ± 1 grid
572  # dimension away
573  #
574  # For example, if I have 15 assays (5 stressors and 10 mixtures), it will find the square
575  # root of 15, and round up to the nearest integer, 4.
576  #
577  # It then tests if 4 * 4 (a perfect square) is equal to the number of assays.
578  #
579  # If it is not, it will test to see if 3 * 5 equals the number of assays, which it does.
580  #
581  # The dimensions of 3x5 are then stored in Graph_Dim to be used later.
582  #
583  # If neither condition is fulfilled, Graph_Dim defaults to 4x4, which will fit the data
584  # set in any case.
585
586  if(Show_Graphs == T){
587
588    Root_of_assays <- ceiling(sqrt(5 + Num_Mixtures))
589
590    if(Root_of_assays^2 == 5 + Num_Mixtures){
591
592      Graph_Dim <- c(Root_of_assays, Root_of_assays)
593
594    }else if((Root_of_assays - 1) * (Root_of_assays + 1)  == 5 + Num_Mixtures){
595
596      Graph_Dim <- c(Root_of_assays - 1, Root_of_assays + 1)
597
598    } else{
599
600      Graph_Dim <- c(Root_of_assays, Root_of_assays)
601
602    }
603
604  }
605
606  # Next, the for-next loop is ran.  It defaults to using the parameters stored in
607  # the applicable Optim_param_# as a starting point for analysis.
608  #
609  # At the end of each optimization the data will stored in Fitted_Parameters_S# in a new column.
610  #
611  # If Show_Graphs has been assigned as TRUE, it will show the fitted curves for each stressor
612  # and mixture.
613  #
614  # After the graphs have been created, the function will ask if you want to perform another
615  # optimization.
616  #
```

```
# Answer with "Y" to continue to the next optimization, or "N" to stop the function
#
# To speed up the loop the minimum and maximum dose levels are calculated for plotting
# each fitted curve, then we create a sequence of 1000 doses between them to have a smooth
# curve.
#
# The sequence is stored in Assay_curve_x

Assay_curve_x <- seq(min(Dose_Response$Sum_Stressor), max(Dose_Response$Sum_Stressor),
                     length.out = 1000)

# Each Stressor must be ran individually.
#
# A new S#_Data, Mixture_Data, and Control_Data needs to be created to store the response
# from each optimization, which will be automatically wiped when you start over.
#
# This script may be updated in the future to allow for picking stressors and testing
# more conveniently.
#
#
# Run the following line to optimize the curves for Stressor 1 several times

for(i in c(1:10)){

  # Optimization_n is where values will be stored similar to Optim_param
  #
  # As the iterations continue, the new starting values will be taken from the previous
  # optimization_n that have been stored in Fitted_Parameters.
  #
  # As "i" increases with each iteration, Fitted_Parameters moves from column 1(i) to
  # column 10 (maximum "i" set)



  if(i == 1){

    # Fitted_Parameters_S# will start with the values from fit_guesses and Optim_param_# and continue
    # to add columns according to the number of optimizations completed.

    Fitted_Parameters <- data.frame(Fit_Guesses = numeric(14), Optimization_1 = numeric(14))

    # Assign fit_guesses and Optim_param_# (Optimization 1) parameters to Fitted_Parameters_S#

    Fitted_Parameters$Fit_Guesses[1:13] <- fit_guesses
    Fitted_Parameters$Fit_Guesses[14] <- Initial_SS

    Fitted_Parameters$Optimization_1[1:13] <- Optim_param$par
    Fitted_Parameters$Optimization_1[14] <- Optim_param$value

    # Create new data sets for Stressors, Mixtures, and Controls

    S1_Data_DL <- S1_Data
    S2_Data_DL <- S2_Data
    S3_Data_DL <- S3_Data
    S4_Data_DL <- S4_Data
    S5_Data_DL <- S5_Data
```

```
673
674       Mixture_Data_DL <- Mixture_Data
675
676       Control_Data_DL <- Control_Data
677
678       for(j in c(1:2)){
679         # An explanation of this script is found after the end of this if statement
680
681         max2 <<- Fitted_Parameters[1, j]
682         S1_EC <<- Fitted_Parameters[2, j]
683         S2_EC <<- Fitted_Parameters[3, j]
684         S3_EC <<- Fitted_Parameters[4, j]
685         S4_EC <<- Fitted_Parameters[5, j]
686         S5_EC <<- Fitted_Parameters[6, j]
687         S1_b <<- Fitted_Parameters[7, j]
688         S2_b <<- Fitted_Parameters[8, j]
689         S3_b <<- Fitted_Parameters[9, j]
690         S4_b <<- Fitted_Parameters[10, j]
691         S5_b <<- Fitted_Parameters[11, j]
692         a <<- Fitted_Parameters[12, j]
693         b <<- Fitted_Parameters[13, j]
694
695         S1_curve <- max2 / (1 + (Assay_curve_x / S1_EC) ^ S1_b)
696         S2_curve <- max2 / (1 + (Assay_curve_x / S2_EC) ^ S2_b)
697         S3_curve <- max2 / (1 + (Assay_curve_x / S3_EC) ^ S3_b)
698         S4_curve <- max2 / (1 + (Assay_curve_x / S4_EC) ^ S4_b)
699         S5_curve <- max2 / (1 + (Assay_curve_x / S5_EC) ^ S5_b)
700
701         Mixture_curves <- Mixture_Data
702
703         try(Mixture_curves$Fitted <- adply(Mixture_Data, 1, summarize,
704                                     solution <- uniroot.all(function (Y){
705                                       TU1 <- Stressor_1/S1_EC
706                                       TU2 <- Stressor_2/S2_EC
707                                       TU3 <- Stressor_3/S3_EC
708                                       TU4 <- Stressor_4/S4_EC
709                                       TU5 <- Stressor_5/S5_EC
710                                       z1 <- TU1/(TU1 + TU2 + TU3 + TU4 + TU5)
711                                       z2 <- TU2/(TU1 + TU2 + TU3 + TU4 + TU5)
712                                       z3 <- TU3/(TU1 + TU2 + TU3 + TU4 + TU5)
713                                       z4 <- TU4/(TU1 + TU2 + TU3 + TU4 + TU5)
714                                       z5 <- TU5/(TU1 + TU2 + TU3 + TU4 + TU5)
715                                       Stressor_1 / (S1_EC * ((max2 - Y) / Y) ^ (1 / S1_b)) +
716                                         Stressor_2 / (S2_EC * ((max2 - Y) / Y) ^ (1 / S2_b)) +
717                                         Stressor_3 / (S3_EC * ((max2 - Y) / Y) ^ (1 / S3_b)) +
718                                         Stressor_4 / (S4_EC * ((max2 - Y) / Y) ^ (1 / S4_b)) +
719                                         Stressor_5 / (S5_EC * ((max2 - Y) / Y) ^ (1 / S5_b)) -
720                                         exp((a * (1 - (b * (TU1 + TU2 + TU3 + TU4 + TU5)))) * z1 * z2 * z3 * z4 * z5)},
721                                       interval = c(0,max2)))[,11], silent = T)
722
723         windows(10,10)
724         par(mfrow = Graph_Dim,
725           oma = c(0,0,2,0))
726
727         plot(S1_Data$Sum_Stressor, S1_Data$Response, main = paste(Stressor_Names[1]))
728         lines(Assay_curve_x, S1_curve)
```

```
729
730         plot(S2_Data$Sum_Stressor, S2_Data$Response, main = paste(Stressor_Names[2]))
731         lines(Assay_curve_x, S2_curve)
732
733         plot(S3_Data$Sum_Stressor, S3_Data$Response, main = paste(Stressor_Names[3]))
734         lines(Assay_curve_x, S3_curve)
735
736         plot(S4_Data$Sum_Stressor, S4_Data$Response, main = paste(Stressor_Names[4]))
737         lines(Assay_curve_x, S4_curve)
738
739         plot(S5_Data$Sum_Stressor, S5_Data$Response, main = paste(Stressor_Names[5]))
740         lines(Assay_curve_x, S5_curve)
741
742         for(mixture in levels(Mixture_Data$Assay)){
743
744           mixdata <- subset(Dose_Response, Dose_Response$Assay == mixture)
745
746           mix_x <- subset(Mixture_curves, Assay == mixture)$Sum_Stressor
747           mix_y <- subset(Mixture_curves, Assay == mixture)$Fitted
748           smoothedcurve <- smooth.spline(mix_x, mix_y, spar = 0.001)
749
750           plot(mixdata$Sum_Stressor, mixdata$Response, main = mixture)
751           lines(smoothedcurve)
752
753
754         }
755         if(j == 1){
756           mtext("Plots for fit_guesses", outer = TRUE, cex = 1.5)
757
758           S1_Data_DL["fit_guesses_Fitted"] <- max2 / (1 + (S1_Data$Sum_Stressor / S1_EC) ^ S1_b)
759           S2_Data_DL["fit_guesses_Fitted"] <- max2 / (1 + (S2_Data$Sum_Stressor / S2_EC) ^ S2_b)
760           S3_Data_DL["fit_guesses_Fitted"] <- max2 / (1 + (S3_Data$Sum_Stressor / S3_EC) ^ S3_b)
761           S4_Data_DL["fit_guesses_Fitted"] <- max2 / (1 + (S4_Data$Sum_Stressor / S4_EC) ^ S4_b)
762           S5_Data_DL["fit_guesses_Fitted"] <- max2 / (1 + (S5_Data$Sum_Stressor / S5_EC) ^ S5_b)
763
764           Control_Data_DL["fit_guesses_Fitted"] <- max2
765
766           Mixture_Data_DL["fit_guesses_Fitted"] <- Mixture_curves$Fitted
767
768         } else{
769           mtext("Plots for Optimization 1", outer = TRUE, cex = 1.5)
770
771           S1_Data_DL["Optimization_1_Fitted"] <- max2 / (1 + (S1_Data$Sum_Stressor / S1_EC) ^ S1_b)
772           S2_Data_DL["Optimization_1_Fitted"] <- max2 / (1 + (S2_Data$Sum_Stressor / S2_EC) ^ S2_b)
773           S3_Data_DL["Optimization_1_Fitted"] <- max2 / (1 + (S3_Data$Sum_Stressor / S3_EC) ^ S3_b)
774           S4_Data_DL["Optimization_1_Fitted"] <- max2 / (1 + (S4_Data$Sum_Stressor / S4_EC) ^ S4_b)
775           S5_Data_DL["Optimization_1_Fitted"] <- max2 / (1 + (S5_Data$Sum_Stressor / S5_EC) ^ S5_b)
776
777           Control_Data_DL["Optimization_1_Fitted"] <- max2
778
779           Mixture_Data_DL["Optimization_1_Fitted"] <- Mixture_curves$Fitted
780         }
781
782       }
783
784     }
```

```
785
786     print("Optimizing Function...")
787
788     try(Optimization_n <- optim(Fitted_Parameters[1:13,i+1], Response_SS))
789
790     print("Completed optimization")
791
792     # Now that the function has been optimized again, the values are stored in a new column
793     # in Fitted_Parameters with the approprite number of optimizations as a column title
794
795     Fitted_Parameters[1:13, i+2] <- Optimization_n$par
796     Fitted_Parameters[14, i+2] <- Optimization_n$value
797     colnames(Fitted_Parameters)[i+2] <- paste("Optimization_", i+1, sep = "")
798
799     # To accommodate uniroot.all(), once again we have to assign global variables
800     #
801
802     max2 <<- Fitted_Parameters[1, i+2]
803     S1_EC <<- Fitted_Parameters[2, i+2]
804     S2_EC <<- Fitted_Parameters[3, i+2]
805     S3_EC <<- Fitted_Parameters[4, i+2]
806     S4_EC <<- Fitted_Parameters[5, i+2]
807     S5_EC <<- Fitted_Parameters[6, i+2]
808     S1_b <<- Fitted_Parameters[7, i+2]
809     S2_b <<- Fitted_Parameters[8, i+2]
810     S3_b <<- Fitted_Parameters[9, i+2]
811     S4_b <<- Fitted_Parameters[10, i+2]
812     S5_b <<- Fitted_Parameters[11, i+2]
813     a <<- Fitted_Parameters[12,i+2]
814     b <<- Fitted_Parameters[13,i+2]
815
816     # Then, we output the graphs of each stressor and mixture.
817     #
818     # Using Assay_curve_x, we find the corresponding fitted response for each individual Stressor
819     #
820     # The following 5 equations are simply the Sigmoid Response function using our assigned variables
821     # and Assay_curve_x
822
823     S1_curve <- max2 / (1 + (Assay_curve_x / S1_EC) ^ S1_b)
824     S2_curve <- max2 / (1 + (Assay_curve_x / S2_EC) ^ S2_b)
825     S3_curve <- max2 / (1 + (Assay_curve_x / S3_EC) ^ S3_b)
826     S4_curve <- max2 / (1 + (Assay_curve_x / S4_EC) ^ S4_b)
827     S5_curve <- max2 / (1 + (Assay_curve_x / S5_EC) ^ S5_b)
828
829     # Creating curves for each Mixture assay is more complex and needs another for-next loop
830     #
831     # A data frame called Mixture_curves is created to store fitted mixture data.
832
833     Mixture_curves <- Mixture_Data
834
835     try(Mixture_curves$Fitted <- adply(Mixture_Data, 1, summarize,
836                                 solution <- uniroot.all(function (Y){
837                                   TU1 <- Stressor_1/S1_EC
838                                   TU2 <- Stressor_2/S2_EC
839                                   TU3 <- Stressor_3/S3_EC
840                                   TU4 <- Stressor_4/S4_EC
```

```
                        TU5 <- Stressor_5/S5_EC
                        z1 <- TU1/(TU1 + TU2 + TU3 + TU4 + TU5)
                        z2 <- TU2/(TU1 + TU2 + TU3 + TU4 + TU5)
                        z3 <- TU3/(TU1 + TU2 + TU3 + TU4 + TU5)
                        z4 <- TU4/(TU1 + TU2 + TU3 + TU4 + TU5)
                        z5 <- TU5/(TU1 + TU2 + TU3 + TU4 + TU5)
                        Stressor_1 / (S1_EC * ((max2 - Y) / Y) ^ (1 / S1_b)) +
                          Stressor_2 / (S2_EC * ((max2 - Y) / Y) ^ (1 / S2_b)) +
                          Stressor_3 / (S3_EC * ((max2 - Y) / Y) ^ (1 / S3_b)) +
                          Stressor_4 / (S4_EC * ((max2 - Y) / Y) ^ (1 / S4_b)) +
                          Stressor_5 / (S5_EC * ((max2 - Y) / Y) ^ (1 / S5_b)) -
                          exp((a * (1 - (b * (TU1 + TU2 + TU3 + TU4 + TU5)))) * z1 * z2 * z3 * z4 * z5)},
                        interval = c(0,max2)))[,11], silent = T)

print("Fitting curves...")


# Add new columns to each data set, storing your fitted data from each optimization

S1_Data_DL[paste("Optim_", i+1, "_Fitted", sep = "")] <- max2 / (1 + (S1_Data$Sum_Stressor / S1_EC) ^ S1_b)
S2_Data_DL[paste("Optim_", i+1, "_Fitted", sep = "")] <- max2 / (1 + (S2_Data$Sum_Stressor / S2_EC) ^ S2_b)
S3_Data_DL[paste("Optim_", i+1, "_Fitted", sep = "")] <- max2 / (1 + (S3_Data$Sum_Stressor / S3_EC) ^ S3_b)
S4_Data_DL[paste("Optim_", i+1, "_Fitted", sep = "")] <- max2 / (1 + (S4_Data$Sum_Stressor / S4_EC) ^ S4_b)
S5_Data_DL[paste("Optim_", i+1, "_Fitted", sep = "")] <- max2 / (1 + (S5_Data$Sum_Stressor / S5_EC) ^ S5_b)

Control_Data_DL[paste("Optim_", i+1, "_Fitted", sep = "")] <- max2

Mixture_Data_DL[paste("Optim_", i+1, "_Fitted", sep = "")] <- Mixture_curves$Fitted


# Now the curves can be plotted for each Stressor and Mixture
#
# First, we bring up a new window that has been set up according to our number of assays

print("Plotting data...")

windows(10,10)
par(mfrow = Graph_Dim,
   oma = c(0,0,2,0))

# Then we do simple scatter plots for each Stressor.  First plot the experimental points

plot(S1_Data$Sum_Stressor, S1_Data$Response, main = paste(Stressor_Names[1]))

# Then add the curve we have filled.

lines(Assay_curve_x, S1_curve)

# And then do the same for the other 4 Stressors

plot(S2_Data$Sum_Stressor, S2_Data$Response, main = paste(Stressor_Names[2]))
lines(Assay_curve_x, S2_curve)

plot(S3_Data$Sum_Stressor, S3_Data$Response, main = paste(Stressor_Names[3]))
lines(Assay_curve_x, S3_curve)
```

```
plot(S4_Data$Sum_Stressor, S4_Data$Response, main = paste(Stressor_Names[4]))
lines(Assay_curve_x, S4_curve)

plot(S5_Data$Sum_Stressor, S5_Data$Response, main = paste(Stressor_Names[5]))
lines(Assay_curve_x, S5_curve)

# The mixtures need to be completed as a loop

for(mixture in levels(Mixture_Data$Assay)){

  mixdata <- subset(Dose_Response, Dose_Response$Assay == mixture)

  mix_x <- subset(Mixture_curves, Assay == mixture)$Sum_Stressor
  mix_y <- subset(Mixture_curves, Assay == mixture)$Fitted
  smoothedcurve <- smooth.spline(mix_x, mix_y, spar = 0.001)

  plot(mixdata$Sum_Stressor, mixdata$Response, main = mixture)
  lines(smoothedcurve)
 }
main = mtext(paste("Plots for Optimization ", i+1, sep = ""), outer = TRUE, cex = 1.5)

# Now that all of the plots have been created R will prompt you to ask if you
# want to continue

print(paste("Completed Optimization ", i+1, sep = ""))

cat(paste("Here are the parameters for all optimizations so far:",
          " ", sep = "\n"))

print(Fitted_Parameters)

Continue_Running <- NA
while(Continue_Running != 1 | 2){
  Continue_Running <- readline(prompt = "Do you want to continue optimizations (Y/N)?: ")
  if(Continue_Running == "Y"){

    Continue_Running <- 1
    print(paste("Optimization will continue, now running Optimization ", i+2, sep = ""))
    break

  } else if(Continue_Running == "N"){

    Continue_Running <- 2

    break

  }else{
    print("Invalid input.  Please enter either Y or N (case sensitive).")
  }
}

if(Continue_Running == 2){

  print(paste("Optimization stopped at ", i+1, " iterations.", sep = ""))

  cat(paste("Here are the parameters for all completed optimizations:",
```

```
953                 " ", sep = "\n"))
954
955       print(Fitted_Parameters)
956
957       break
958     }
959     Continue_Running <- NA
960
961   }
962
963   # Attach all of the data sets together
964
965   Fitted_Response_Data <- rbind(S1_Data_DL, S2_Data_DL, S3_Data_DL, S4_Data_DL,
966                                 S5_Data_DL, Mixture_Data_DL, Control_Data_DL)
967
968   # Save the compiled fitted parameters and responses
969
970   write.csv(Fitted_Parameters, file = "Compiled_DL_Fitted_Parameters.csv",row.names=FALSE)
971   write.csv(Fitted_Response_Data, file = "Compiled_DL_Fitted_Response_Data.csv", row.names = FALSE)
```

Appendix B: Invertebrate Reproduction Data sheet created using 0, 2, 5, and 10 SD using method in Jonker et al., 2005

Assay	Mixture	Lead	Copper	Nickel	Zinc	Cobalt	Response at SD = 0	Response at SD = 2	Response at SD = 5	Response at SD = 10
Lead	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	100.9	96.9	103.5
Lead	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	97.2	109.4	106.4
Lead	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	100.3	106.2	115.6
Lead	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	100.0	102.1	99.5
Lead	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	99.8	98.1	93.8
Lead	FALSE	75.0	0.0	0.0	0.0	0.0	100.0	97.5	97.5	87.9
Lead	FALSE	75.0	0.0	0.0	0.0	0.0	100.0	100.6	106.4	90.4
Lead	FALSE	75.0	0.0	0.0	0.0	0.0	100.0	97.0	95.3	96.1
Lead	FALSE	75.0	0.0	0.0	0.0	0.0	100.0	98.0	103.5	97.6
Lead	FALSE	75.0	0.0	0.0	0.0	0.0	100.0	101.5	99.8	96.4
Lead	FALSE	150.0	0.0	0.0	0.0	0.0	99.9	99.9	105.6	100.6
Lead	FALSE	150.0	0.0	0.0	0.0	0.0	99.9	103.2	96.9	94.3
Lead	FALSE	150.0	0.0	0.0	0.0	0.0	99.9	95.8	98.1	85.5
Lead	FALSE	150.0	0.0	0.0	0.0	0.0	99.9	98.7	105.8	106.8
Lead	FALSE	150.0	0.0	0.0	0.0	0.0	99.9	96.3	106.5	100.7
Lead	FALSE	375.0	0.0	0.0	0.0	0.0	98.5	99.2	97.3	110.1
Lead	FALSE	375.0	0.0	0.0	0.0	0.0	98.5	98.9	103.4	104.1
Lead	FALSE	375.0	0.0	0.0	0.0	0.0	98.5	101.3	97.9	96.6
Lead	FALSE	375.0	0.0	0.0	0.0	0.0	98.5	98.9	91.6	97.4
Lead	FALSE	375.0	0.0	0.0	0.0	0.0	98.5	99.8	99.3	107.6
Lead	FALSE	750.0	0.0	0.0	0.0	0.0	88.9	91.0	73.2	68.6
Lead	FALSE	750.0	0.0	0.0	0.0	0.0	88.9	86.1	83.1	90.0
Lead	FALSE	750.0	0.0	0.0	0.0	0.0	88.9	87.8	86.8	70.3
Lead	FALSE	750.0	0.0	0.0	0.0	0.0	88.9	89.4	74.4	92.4
Lead	FALSE	750.0	0.0	0.0	0.0	0.0	88.9	88.9	79.4	96.9
Lead	FALSE	1500.0	0.0	0.0	0.0	0.0	50.0	51.0	44.9	70.6
Lead	FALSE	1500.0	0.0	0.0	0.0	0.0	50.0	50.8	59.1	59.0
Lead	FALSE	1500.0	0.0	0.0	0.0	0.0	50.0	47.6	54.7	55.0
Lead	FALSE	1500.0	0.0	0.0	0.0	0.0	50.0	47.5	41.7	47.2
Lead	FALSE	1500.0	0.0	0.0	0.0	0.0	50.0	49.0	43.3	42.1
Lead	FALSE	3000.0	0.0	0.0	0.0	0.0	11.1	11.6	15.6	8.8
Lead	FALSE	3000.0	0.0	0.0	0.0	0.0	11.1	10.1	11.4	0.0
Lead	FALSE	3000.0	0.0	0.0	0.0	0.0	11.1	10.1	3.6	24.8
Lead	FALSE	3000.0	0.0	0.0	0.0	0.0	11.1	8.8	7.8	24.0
Lead	FALSE	3000.0	0.0	0.0	0.0	0.0	11.1	11.8	5.1	2.4
Lead	FALSE	6000.0	0.0	0.0	0.0	0.0	1.5	3.1	0.0	2.0
Lead	FALSE	6000.0	0.0	0.0	0.0	0.0	1.5	0.0	9.3	0.0
Lead	FALSE	6000.0	0.0	0.0	0.0	0.0	1.5	0.7	0.0	0.0
Lead	FALSE	6000.0	0.0	0.0	0.0	0.0	1.5	4.5	7.7	0.0
Lead	FALSE	6000.0	0.0	0.0	0.0	0.0	1.5	0.0	7.0	13.0

Assay	Mixture	Lead	Copper	Nickel	Zinc	Cobalt	Response at SD = 0	Response at SD = 2	Response at SD = 5	Response at SD = 10
Lead	FALSE	12000.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
Lead	FALSE	12000.0	0.0	0.0	0.0	0.0	0.2	0.0	2.6	0.0
Lead	FALSE	12000.0	0.0	0.0	0.0	0.0	0.2	0.0	2.9	0.0
Lead	FALSE	12000.0	0.0	0.0	0.0	0.0	0.2	0.0	2.7	0.0
Lead	FALSE	12000.0	0.0	0.0	0.0	0.0	0.2	0.0	3.6	0.0
Copper	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	97.3	102.4	105.0
Copper	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	101.5	105.7	93.9
Copper	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	100.8	94.7	82.9
Copper	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	101.6	103.2	102.4
Copper	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	100.9	87.2	113.7
Copper	FALSE	0.0	125.0	0.0	0.0	0.0	89.1	91.7	87.4	98.4
Copper	FALSE	0.0	125.0	0.0	0.0	0.0	89.1	85.3	87.8	78.4
Copper	FALSE	0.0	125.0	0.0	0.0	0.0	89.1	89.0	92.1	90.6
Copper	FALSE	0.0	125.0	0.0	0.0	0.0	89.1	88.8	93.0	93.3
Copper	FALSE	0.0	125.0	0.0	0.0	0.0	89.1	85.8	95.8	83.7
Copper	FALSE	0.0	250.0	0.0	0.0	0.0	83.4	84.0	79.8	75.4
Copper	FALSE	0.0	250.0	0.0	0.0	0.0	83.4	85.2	84.2	94.2
Copper	FALSE	0.0	250.0	0.0	0.0	0.0	83.4	84.0	86.5	67.8
Copper	FALSE	0.0	250.0	0.0	0.0	0.0	83.4	86.1	83.3	71.6
Copper	FALSE	0.0	250.0	0.0	0.0	0.0	83.4	83.0	82.5	91.4
Copper	FALSE	0.0	625.0	0.0	0.0	0.0	72.5	69.2	73.2	72.0
Copper	FALSE	0.0	625.0	0.0	0.0	0.0	72.5	73.6	72.1	73.0
Copper	FALSE	0.0	625.0	0.0	0.0	0.0	72.5	68.6	74.0	63.9
Copper	FALSE	0.0	625.0	0.0	0.0	0.0	72.5	74.0	64.2	70.3
Copper	FALSE	0.0	625.0	0.0	0.0	0.0	72.5	74.4	76.0	65.9
Copper	FALSE	0.0	1250.0	0.0	0.0	0.0	61.9	59.9	60.4	56.6
Copper	FALSE	0.0	1250.0	0.0	0.0	0.0	61.9	58.1	57.9	59.7
Copper	FALSE	0.0	1250.0	0.0	0.0	0.0	61.9	61.7	56.7	63.3
Copper	FALSE	0.0	1250.0	0.0	0.0	0.0	61.9	64.7	63.3	67.8
Copper	FALSE	0.0	1250.0	0.0	0.0	0.0	61.9	61.6	60.6	69.2
Copper	FALSE	0.0	2500.0	0.0	0.0	0.0	50.0	48.1	48.3	41.3
Copper	FALSE	0.0	2500.0	0.0	0.0	0.0	50.0	46.3	55.7	40.6
Copper	FALSE	0.0	2500.0	0.0	0.0	0.0	50.0	49.3	60.4	35.4
Copper	FALSE	0.0	2500.0	0.0	0.0	0.0	50.0	47.7	57.3	46.0
Copper	FALSE	0.0	2500.0	0.0	0.0	0.0	50.0	45.2	46.0	38.4
Copper	FALSE	0.0	5000.0	0.0	0.0	0.0	38.1	40.5	31.8	37.7
Copper	FALSE	0.0	5000.0	0.0	0.0	0.0	38.1	39.1	39.0	39.0
Copper	FALSE	0.0	5000.0	0.0	0.0	0.0	38.1	36.4	45.1	44.5
Copper	FALSE	0.0	5000.0	0.0	0.0	0.0	38.1	40.8	44.9	42.6
Copper	FALSE	0.0	5000.0	0.0	0.0	0.0	38.1	39.3	41.3	44.9

Assay	Mixture	Lead	Copper	Nickel	Zinc	Cobalt	Response at SD = 0	Response at SD = 2	Response at SD = 5	Response at SD = 10
Copper	FALSE	0.0	10000.0	0.0	0.0	0.0	27.5	26.6	26.4	30.7
Copper	FALSE	0.0	10000.0	0.0	0.0	0.0	27.5	28.4	36.7	20.7
Copper	FALSE	0.0	10000.0	0.0	0.0	0.0	27.5	29.9	31.9	16.3
Copper	FALSE	0.0	10000.0	0.0	0.0	0.0	27.5	27.2	25.9	0.5
Copper	FALSE	0.0	10000.0	0.0	0.0	0.0	27.5	30.3	35.1	15.4
Copper	FALSE	0.0	20000.0	0.0	0.0	0.0	18.9	22.3	13.9	28.6
Copper	FALSE	0.0	20000.0	0.0	0.0	0.0	18.9	22.8	24.3	5.7
Copper	FALSE	0.0	20000.0	0.0	0.0	0.0	18.9	18.5	22.2	21.8
Copper	FALSE	0.0	20000.0	0.0	0.0	0.0	18.9	19.6	20.4	20.5
Copper	FALSE	0.0	20000.0	0.0	0.0	0.0	18.9	18.3	17.6	11.4
Nickel	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	100.7	102.0	91.3
Nickel	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	101.3	101.1	105.6
Nickel	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	101.2	96.7	97.0
Nickel	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	98.3	94.9	92.8
Nickel	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	98.4	95.5	101.5
Nickel	FALSE	0.0	0.0	35.0	0.0	0.0	97.3	98.0	99.4	80.5
Nickel	FALSE	0.0	0.0	35.0	0.0	0.0	97.3	98.1	91.3	117.0
Nickel	FALSE	0.0	0.0	35.0	0.0	0.0	97.3	95.0	89.0	108.7
Nickel	FALSE	0.0	0.0	35.0	0.0	0.0	97.3	99.2	98.5	95.8
Nickel	FALSE	0.0	0.0	35.0	0.0	0.0	97.3	98.6	94.3	100.8
Nickel	FALSE	0.0	0.0	70.0	0.0	0.0	94.1	95.3	87.5	84.7
Nickel	FALSE	0.0	0.0	70.0	0.0	0.0	94.1	95.5	98.2	97.9
Nickel	FALSE	0.0	0.0	70.0	0.0	0.0	94.1	95.6	92.0	78.6
Nickel	FALSE	0.0	0.0	70.0	0.0	0.0	94.1	92.1	96.6	85.1
Nickel	FALSE	0.0	0.0	70.0	0.0	0.0	94.1	95.4	94.0	91.7
Nickel	FALSE	0.0	0.0	175.0	0.0	0.0	84.1	85.6	86.4	90.4
Nickel	FALSE	0.0	0.0	175.0	0.0	0.0	84.1	83.6	89.4	83.2
Nickel	FALSE	0.0	0.0	175.0	0.0	0.0	84.1	79.5	78.6	85.2
Nickel	FALSE	0.0	0.0	175.0	0.0	0.0	84.1	85.4	82.4	64.2
Nickel	FALSE	0.0	0.0	175.0	0.0	0.0	84.1	86.4	75.0	73.7
Nickel	FALSE	0.0	0.0	350.0	0.0	0.0	69.7	69.3	69.6	75.5
Nickel	FALSE	0.0	0.0	350.0	0.0	0.0	69.7	66.6	63.9	78.2
Nickel	FALSE	0.0	0.0	350.0	0.0	0.0	69.7	70.7	78.2	63.7
Nickel	FALSE	0.0	0.0	350.0	0.0	0.0	69.7	65.3	71.5	70.6
Nickel	FALSE	0.0	0.0	350.0	0.0	0.0	69.7	71.9	58.4	52.8
Nickel	FALSE	0.0	0.0	700.0	0.0	0.0	50.0	51.3	55.9	42.7
Nickel	FALSE	0.0	0.0	700.0	0.0	0.0	50.0	49.2	50.3	42.8
Nickel	FALSE	0.0	0.0	700.0	0.0	0.0	50.0	49.4	59.0	35.1
Nickel	FALSE	0.0	0.0	700.0	0.0	0.0	50.0	46.4	52.8	53.4
Nickel	FALSE	0.0	0.0	700.0	0.0	0.0	50.0	51.9	47.0	65.9

Assay	Mixture	Lead	Copper	Nickel	Zinc	Cobalt	Response at SD = 0	Response at SD = 2	Response at SD = 5	Response at SD = 10
Nickel	FALSE	0.0	0.0	1400.0	0.0	0.0	30.3	28.2	29.8	24.4
Nickel	FALSE	0.0	0.0	1400.0	0.0	0.0	30.3	29.9	34.5	20.3
Nickel	FALSE	0.0	0.0	1400.0	0.0	0.0	30.3	31.3	33.5	41.5
Nickel	FALSE	0.0	0.0	1400.0	0.0	0.0	30.3	30.8	35.2	15.9
Nickel	FALSE	0.0	0.0	1400.0	0.0	0.0	30.3	27.1	20.9	21.1
Nickel	FALSE	0.0	0.0	2800.0	0.0	0.0	15.9	14.6	18.6	8.1
Nickel	FALSE	0.0	0.0	2800.0	0.0	0.0	15.9	16.8	14.9	23.0
Nickel	FALSE	0.0	0.0	2800.0	0.0	0.0	15.9	18.4	17.0	0.0
Nickel	FALSE	0.0	0.0	2800.0	0.0	0.0	15.9	13.4	21.3	26.1
Nickel	FALSE	0.0	0.0	2800.0	0.0	0.0	15.9	16.9	0.5	10.2
Nickel	FALSE	0.0	0.0	5600.0	0.0	0.0	7.6	6.7	13.4	0.3
Nickel	FALSE	0.0	0.0	5600.0	0.0	0.0	7.6	6.8	5.4	1.9
Nickel	FALSE	0.0	0.0	5600.0	0.0	0.0	7.6	10.4	0.8	12.4
Nickel	FALSE	0.0	0.0	5600.0	0.0	0.0	7.6	8.7	10.0	0.0
Nickel	FALSE	0.0	0.0	5600.0	0.0	0.0	7.6	11.2	5.2	1.3
Zinc	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	98.3	100.1	104.1
Zinc	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	102.4	102.4	115.1
Zinc	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	98.9	102.4	114.7
Zinc	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	98.5	105.3	83.2
Zinc	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	102.4	107.1	101.4
Zinc	FALSE	0.0	0.0	0.0	200.0	0.0	100.0	101.5	105.7	96.8
Zinc	FALSE	0.0	0.0	0.0	200.0	0.0	100.0	101.7	105.8	120.8
Zinc	FALSE	0.0	0.0	0.0	200.0	0.0	100.0	98.5	104.4	111.5
Zinc	FALSE	0.0	0.0	0.0	200.0	0.0	100.0	101.4	94.6	84.8
Zinc	FALSE	0.0	0.0	0.0	200.0	0.0	100.0	100.3	101.1	104.6
Zinc	FALSE	0.0	0.0	0.0	400.0	0.0	100.0	101.8	100.5	119.9
Zinc	FALSE	0.0	0.0	0.0	400.0	0.0	100.0	99.1	92.2	103.3
Zinc	FALSE	0.0	0.0	0.0	400.0	0.0	100.0	99.7	97.7	107.2
Zinc	FALSE	0.0	0.0	0.0	400.0	0.0	100.0	98.8	87.5	100.5
Zinc	FALSE	0.0	0.0	0.0	400.0	0.0	100.0	99.0	103.8	104.3
Zinc	FALSE	0.0	0.0	0.0	1000.0	0.0	100.0	97.5	98.2	96.8
Zinc	FALSE	0.0	0.0	0.0	1000.0	0.0	100.0	96.6	99.0	90.6
Zinc	FALSE	0.0	0.0	0.0	1000.0	0.0	100.0	98.8	97.4	114.6
Zinc	FALSE	0.0	0.0	0.0	1000.0	0.0	100.0	99.1	96.4	113.8
Zinc	FALSE	0.0	0.0	0.0	1000.0	0.0	100.0	100.5	103.0	104.1
Zinc	FALSE	0.0	0.0	0.0	2000.0	0.0	98.5	97.1	92.0	97.2
Zinc	FALSE	0.0	0.0	0.0	2000.0	0.0	98.5	98.1	99.8	111.2
Zinc	FALSE	0.0	0.0	0.0	2000.0	0.0	98.5	99.2	96.1	104.7
Zinc	FALSE	0.0	0.0	0.0	2000.0	0.0	98.5	96.9	103.3	106.7
Zinc	FALSE	0.0	0.0	0.0	2000.0	0.0	98.5	97.9	98.8	87.4

Assay	Mixture	Lead	Copper	Nickel	Zinc	Cobalt	Response at SD = 0	Response at SD = 2	Response at SD = 5	Response at SD = 10
Zinc	FALSE	0.0	0.0	0.0	4000.0	0.0	50.0	49.6	43.0	44.9
Zinc	FALSE	0.0	0.0	0.0	4000.0	0.0	50.0	47.9	65.9	42.3
Zinc	FALSE	0.0	0.0	0.0	4000.0	0.0	50.0	50.2	47.0	30.4
Zinc	FALSE	0.0	0.0	0.0	4000.0	0.0	50.0	50.8	51.3	46.5
Zinc	FALSE	0.0	0.0	0.0	4000.0	0.0	50.0	48.9	42.3	48.9
Zinc	FALSE	0.0	0.0	0.0	8000.0	0.0	1.5	2.0	5.9	0.2
Zinc	FALSE	0.0	0.0	0.0	8000.0	0.0	1.5	3.4	5.9	21.5
Zinc	FALSE	0.0	0.0	0.0	8000.0	0.0	1.5	2.8	5.6	11.6
Zinc	FALSE	0.0	0.0	0.0	8000.0	0.0	1.5	2.6	11.2	15.4
Zinc	FALSE	0.0	0.0	0.0	8000.0	0.0	1.5	2.1	0.0	0.0
Zinc	FALSE	0.0	0.0	0.0	16000.0	0.0	0.0	0.0	6.8	0.0
Zinc	FALSE	0.0	0.0	0.0	16000.0	0.0	0.0	2.1	1.0	0.0
Zinc	FALSE	0.0	0.0	0.0	16000.0	0.0	0.0	0.0	0.0	3.2
Zinc	FALSE	0.0	0.0	0.0	16000.0	0.0	0.0	0.2	0.0	0.0
Zinc	FALSE	0.0	0.0	0.0	16000.0	0.0	0.0	0.0	7.1	14.1
Zinc	FALSE	0.0	0.0	0.0	32000.0	0.0	0.0	1.0	0.0	0.0
Zinc	FALSE	0.0	0.0	0.0	32000.0	0.0	0.0	3.0	8.6	13.7
Zinc	FALSE	0.0	0.0	0.0	32000.0	0.0	0.0	0.0	0.0	0.0
Zinc	FALSE	0.0	0.0	0.0	32000.0	0.0	0.0	0.0	8.5	10.5
Zinc	FALSE	0.0	0.0	0.0	32000.0	0.0	0.0	0.0	1.0	0.0
Cobalt	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	97.2	97.1	96.1
Cobalt	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	101.4	101.3	91.8
Cobalt	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	96.0	101.0	99.1
Cobalt	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	98.4	101.3	93.2
Cobalt	FALSE	0.0	0.0	0.0	0.0	0.0	100.0	103.1	98.2	105.4
Cobalt	FALSE	0.0	0.0	0.0	0.0	50.0	64.5	62.0	71.6	71.7
Cobalt	FALSE	0.0	0.0	0.0	0.0	50.0	64.5	67.5	63.3	63.6
Cobalt	FALSE	0.0	0.0	0.0	0.0	50.0	64.5	62.8	65.0	81.5
Cobalt	FALSE	0.0	0.0	0.0	0.0	50.0	64.5	67.6	62.9	78.0
Cobalt	FALSE	0.0	0.0	0.0	0.0	50.0	64.5	65.4	53.6	59.9
Cobalt	FALSE	0.0	0.0	0.0	0.0	100.0	61.3	63.2	63.3	60.3
Cobalt	FALSE	0.0	0.0	0.0	0.0	100.0	61.3	66.4	61.1	53.1
Cobalt	FALSE	0.0	0.0	0.0	0.0	100.0	61.3	58.3	71.8	70.3
Cobalt	FALSE	0.0	0.0	0.0	0.0	100.0	61.3	60.8	61.6	65.2
Cobalt	FALSE	0.0	0.0	0.0	0.0	100.0	61.3	61.5	66.9	59.6
Cobalt	FALSE	0.0	0.0	0.0	0.0	250.0	56.9	55.5	59.4	48.5
Cobalt	FALSE	0.0	0.0	0.0	0.0	250.0	56.9	57.8	57.1	58.8
Cobalt	FALSE	0.0	0.0	0.0	0.0	250.0	56.9	58.3	62.9	59.6
Cobalt	FALSE	0.0	0.0	0.0	0.0	250.0	56.9	56.1	54.5	53.0
Cobalt	FALSE	0.0	0.0	0.0	0.0	250.0	56.9	58.0	54.8	61.2

Assay	Mixture	Lead	Copper	Nickel	Zinc	Cobalt	Response at SD = 0	Response at SD = 2	Response at SD = 5	Response at SD = 10
Cobalt	FALSE	0.0	0.0	0.0	0.0	500.0	53.5	53.3	49.0	49.5
Cobalt	FALSE	0.0	0.0	0.0	0.0	500.0	53.5	55.8	52.9	47.5
Cobalt	FALSE	0.0	0.0	0.0	0.0	500.0	53.5	50.2	62.6	49.5
Cobalt	FALSE	0.0	0.0	0.0	0.0	500.0	53.5	53.6	56.3	40.0
Cobalt	FALSE	0.0	0.0	0.0	0.0	500.0	53.5	51.1	55.1	56.1
Cobalt	FALSE	0.0	0.0	0.0	0.0	1000.0	50.0	52.0	44.4	66.3
Cobalt	FALSE	0.0	0.0	0.0	0.0	1000.0	50.0	50.0	47.6	39.9
Cobalt	FALSE	0.0	0.0	0.0	0.0	1000.0	50.0	51.1	63.4	53.1
Cobalt	FALSE	0.0	0.0	0.0	0.0	1000.0	50.0	48.7	45.4	30.4
Cobalt	FALSE	0.0	0.0	0.0	0.0	1000.0	50.0	51.0	55.9	40.0
Cobalt	FALSE	0.0	0.0	0.0	0.0	2000.0	46.5	49.4	49.6	60.8
Cobalt	FALSE	0.0	0.0	0.0	0.0	2000.0	46.5	45.3	44.9	63.1
Cobalt	FALSE	0.0	0.0	0.0	0.0	2000.0	46.5	45.7	38.2	39.7
Cobalt	FALSE	0.0	0.0	0.0	0.0	2000.0	46.5	48.6	48.4	51.4
Cobalt	FALSE	0.0	0.0	0.0	0.0	2000.0	46.5	44.8	38.6	50.8
Cobalt	FALSE	0.0	0.0	0.0	0.0	4000.0	43.1	46.1	41.9	60.9
Cobalt	FALSE	0.0	0.0	0.0	0.0	4000.0	43.1	45.0	34.9	35.0
Cobalt	FALSE	0.0	0.0	0.0	0.0	4000.0	43.1	46.8	37.3	42.4
Cobalt	FALSE	0.0	0.0	0.0	0.0	4000.0	43.1	42.6	41.2	42.9
Cobalt	FALSE	0.0	0.0	0.0	0.0	4000.0	43.1	40.2	48.8	26.2
Cobalt	FALSE	0.0	0.0	0.0	0.0	8000.0	39.8	38.0	32.3	36.3
Cobalt	FALSE	0.0	0.0	0.0	0.0	8000.0	39.8	39.1	32.3	33.6
Cobalt	FALSE	0.0	0.0	0.0	0.0	8000.0	39.8	38.0	37.5	34.8
Cobalt	FALSE	0.0	0.0	0.0	0.0	8000.0	39.8	39.1	32.3	41.0
Cobalt	FALSE	0.0	0.0	0.0	0.0	8000.0	39.8	43.7	24.6	37.9
Equal	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	99.6	98.5	101.7
Equal	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	99.9	99.0	98.8
Equal	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	98.1	95.4	102.8
Equal	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	101.8	115.0	101.3
Equal	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	99.3	98.5	97.4
Equal	TRUE	13.4	13.4	13.4	13.4	13.4	70.0	70.9	70.0	95.4
Equal	TRUE	13.4	13.4	13.4	13.4	13.4	70.0	71.1	70.7	68.7
Equal	TRUE	13.4	13.4	13.4	13.4	13.4	70.0	69.7	76.6	73.2
Equal	TRUE	13.4	13.4	13.4	13.4	13.4	70.0	74.9	64.9	79.4
Equal	TRUE	13.4	13.4	13.4	13.4	13.4	70.0	72.9	70.7	64.5
Equal	TRUE	26.7	26.7	26.7	26.7	26.7	66.8	69.8	66.4	65.9
Equal	TRUE	26.7	26.7	26.7	26.7	26.7	66.8	67.6	64.9	73.0
Equal	TRUE	26.7	26.7	26.7	26.7	26.7	66.8	66.7	72.0	61.9
Equal	TRUE	26.7	26.7	26.7	26.7	26.7	66.8	66.0	58.5	60.2
Equal	TRUE	26.7	26.7	26.7	26.7	26.7	66.8	68.7	80.4	86.5

Assay	Mixture	Lead	Copper	Nickel	Zinc	Cobalt	Response at SD = 0	Response at SD = 2	Response at SD = 5	Response at SD = 10
Equal	TRUE	66.8	66.8	66.8	66.8	66.8	61.8	64.1	69.7	55.9
Equal	TRUE	66.8	66.8	66.8	66.8	66.8	61.8	65.0	52.8	59.5
Equal	TRUE	66.8	66.8	66.8	66.8	66.8	61.8	61.5	65.4	82.5
Equal	TRUE	66.8	66.8	66.8	66.8	66.8	61.8	64.2	62.7	66.1
Equal	TRUE	66.8	66.8	66.8	66.8	66.8	61.8	60.9	65.8	61.9
Equal	TRUE	133.5	133.5	133.5	133.5	133.5	57.0	55.6	60.6	58.0
Equal	TRUE	133.5	133.5	133.5	133.5	133.5	57.0	62.2	60.5	63.6
Equal	TRUE	133.5	133.5	133.5	133.5	133.5	57.0	57.0	50.8	59.7
Equal	TRUE	133.5	133.5	133.5	133.5	133.5	57.0	57.9	63.7	70.5
Equal	TRUE	133.5	133.5	133.5	133.5	133.5	57.0	56.2	64.4	48.4
Equal	TRUE	267.0	267.0	267.0	267.0	267.0	50.0	48.2	53.4	49.6
Equal	TRUE	267.0	267.0	267.0	267.0	267.0	50.0	50.4	50.7	45.0
Equal	TRUE	267.0	267.0	267.0	267.0	267.0	50.0	50.3	49.4	72.7
Equal	TRUE	267.0	267.0	267.0	267.0	267.0	50.0	50.4	55.0	53.7
Equal	TRUE	267.0	267.0	267.0	267.0	267.0	50.0	49.3	52.5	47.3
Equal	TRUE	534.0	534.0	534.0	534.0	534.0	35.7	33.4	29.2	33.5
Equal	TRUE	534.0	534.0	534.0	534.0	534.0	35.7	32.4	36.0	39.0
Equal	TRUE	534.0	534.0	534.0	534.0	534.0	35.7	37.7	25.7	33.4
Equal	TRUE	534.0	534.0	534.0	534.0	534.0	35.7	38.3	26.5	35.9
Equal	TRUE	534.0	534.0	534.0	534.0	534.0	35.7	35.9	40.2	38.2
Equal	TRUE	1068.0	1068.0	1068.0	1068.0	1068.0	15.1	12.1	6.3	28.2
Equal	TRUE	1068.0	1068.0	1068.0	1068.0	1068.0	15.1	12.4	15.6	28.9
Equal	TRUE	1068.0	1068.0	1068.0	1068.0	1068.0	15.1	18.0	13.3	13.0
Equal	TRUE	1068.0	1068.0	1068.0	1068.0	1068.0	15.1	13.7	15.3	15.7
Equal	TRUE	1068.0	1068.0	1068.0	1068.0	1068.0	15.1	18.1	17.0	18.4
Equal	TRUE	2136.0	2136.0	2136.0	2136.0	2136.0	3.7	1.1	8.5	21.6
Equal	TRUE	2136.0	2136.0	2136.0	2136.0	2136.0	3.7	0.0	3.7	10.0
Equal	TRUE	2136.0	2136.0	2136.0	2136.0	2136.0	3.7	2.9	8.8	13.7
Equal	TRUE	2136.0	2136.0	2136.0	2136.0	2136.0	3.7	1.1	5.2	13.1
Equal	TRUE	2136.0	2136.0	2136.0	2136.0	2136.0	3.7	0.2	7.4	0.0
EC50	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	98.5	101.6	105.5
EC50	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	99.2	93.5	113.3
EC50	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	100.1	96.9	89.6
EC50	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	100.4	100.0	92.8
EC50	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	100.3	105.1	98.3
EC50	TRUE	15.0	25.0	7.0	40.0	10.0	71.2	74.1	83.8	95.2
EC50	TRUE	15.0	25.0	7.0	40.0	10.0	71.2	75.9	67.8	56.9
EC50	TRUE	15.0	25.0	7.0	40.0	10.0	71.2	69.9	73.0	88.5
EC50	TRUE	15.0	25.0	7.0	40.0	10.0	71.2	73.3	72.0	63.6
EC50	TRUE	15.0	25.0	7.0	40.0	10.0	71.2	69.4	71.6	71.6

Assay	Mixture	Lead	Copper	Nickel	Zinc	Cobalt	Response at SD = 0	Response at SD = 2	Response at SD = 5	Response at SD = 10
EC50	TRUE	30.0	50.0	14.0	80.0	20.0	67.9	66.7	70.7	69.6
EC50	TRUE	30.0	50.0	14.0	80.0	20.0	67.9	66.1	64.9	55.0
EC50	TRUE	30.0	50.0	14.0	80.0	20.0	67.9	68.2	69.3	69.2
EC50	TRUE	30.0	50.0	14.0	80.0	20.0	67.9	66.9	64.2	63.3
EC50	TRUE	30.0	50.0	14.0	80.0	20.0	67.9	72.7	68.3	50.2
EC50	TRUE	75.0	125.0	35.0	200.0	50.0	62.9	63.0	60.7	74.6
EC50	TRUE	75.0	125.0	35.0	200.0	50.0	62.9	62.1	69.1	75.3
EC50	TRUE	75.0	125.0	35.0	200.0	50.0	62.9	62.8	65.9	59.9
EC50	TRUE	75.0	125.0	35.0	200.0	50.0	62.9	60.6	52.6	55.9
EC50	TRUE	75.0	125.0	35.0	200.0	50.0	62.9	59.9	64.5	72.3
EC50	TRUE	150.0	250.0	70.0	400.0	100.0	57.9	60.1	55.1	48.1
EC50	TRUE	150.0	250.0	70.0	400.0	100.0	57.9	59.5	57.6	28.7
EC50	TRUE	150.0	250.0	70.0	400.0	100.0	57.9	60.0	65.5	54.6
EC50	TRUE	150.0	250.0	70.0	400.0	100.0	57.9	58.3	60.8	54.4
EC50	TRUE	150.0	250.0	70.0	400.0	100.0	57.9	53.0	54.5	68.8
EC50	TRUE	300.0	500.0	140.0	800.0	200.0	50.0	49.5	44.1	48.0
EC50	TRUE	300.0	500.0	140.0	800.0	200.0	50.0	52.1	57.4	59.2
EC50	TRUE	300.0	500.0	140.0	800.0	200.0	50.0	50.0	44.6	39.5
EC50	TRUE	300.0	500.0	140.0	800.0	200.0	50.0	50.6	46.3	71.2
EC50	TRUE	300.0	500.0	140.0	800.0	200.0	50.0	47.1	45.7	50.5
EC50	TRUE	600.0	1000.0	280.0	1600.0	400.0	31.1	31.7	35.7	35.8
EC50	TRUE	600.0	1000.0	280.0	1600.0	400.0	31.1	32.2	44.0	27.7
EC50	TRUE	600.0	1000.0	280.0	1600.0	400.0	31.1	31.8	26.6	29.1
EC50	TRUE	600.0	1000.0	280.0	1600.0	400.0	31.1	30.4	22.6	32.4
EC50	TRUE	600.0	1000.0	280.0	1600.0	400.0	31.1	33.1	34.6	16.2
EC50	TRUE	1200.0	2000.0	560.0	3200.0	800.0	7.6	8.7	14.2	0.0
EC50	TRUE	1200.0	2000.0	560.0	3200.0	800.0	7.6	7.7	3.9	10.2
EC50	TRUE	1200.0	2000.0	560.0	3200.0	800.0	7.6	6.5	11.4	12.3
EC50	TRUE	1200.0	2000.0	560.0	3200.0	800.0	7.6	7.9	17.0	20.0
EC50	TRUE	1200.0	2000.0	560.0	3200.0	800.0	7.6	10.5	2.2	0.0
EC50	TRUE	2400.0	4000.0	1120.0	6400.0	1600.0	0.6	3.2	2.6	0.0
EC50	TRUE	2400.0	4000.0	1120.0	6400.0	1600.0	0.6	1.0	0.0	0.0
EC50	TRUE	2400.0	4000.0	1120.0	6400.0	1600.0	0.6	0.0	2.7	0.0
EC50	TRUE	2400.0	4000.0	1120.0	6400.0	1600.0	0.6	0.0	0.0	14.3
EC50	TRUE	2400.0	4000.0	1120.0	6400.0	1600.0	0.6	0.0	0.0	9.7
Atomic Weight	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	100.3	104.6	109.7
Atomic Weight	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	100.3	104.3	99.9
Atomic Weight	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	100.6	92.6	109.7

Assay	Mixture	Lead	Copper	Nickel	Zinc	Cobalt	Response at SD = 0	Response at SD = 2	Response at SD = 5	Response at SD = 10
Atomic Weight	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	101.4	102.2	99.3
Atomic Weight	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	102.8	95.9	113.6
Atomic Weight	TRUE	32.1	9.8	9.1	10.1	9.1	71.6	71.4	71.8	78.8
Atomic Weight	TRUE	32.1	9.8	9.1	10.1	9.1	71.6	71.6	64.3	62.9
Atomic Weight	TRUE	32.1	9.8	9.1	10.1	9.1	71.6	69.2	80.3	68.1
Atomic Weight	TRUE	32.1	9.8	9.1	10.1	9.1	71.6	71.0	75.4	72.9
Atomic Weight	TRUE	32.1	9.8	9.1	10.1	9.1	71.6	70.3	65.5	86.7
Atomic Weight	TRUE	64.2	19.7	18.2	20.3	18.3	68.4	72.2	73.5	66.8
Atomic Weight	TRUE	64.2	19.7	18.2	20.3	18.3	68.4	64.6	68.8	80.1
Atomic Weight	TRUE	64.2	19.7	18.2	20.3	18.3	68.4	70.1	67.2	73.7
Atomic Weight	TRUE	64.2	19.7	18.2	20.3	18.3	68.4	71.8	71.6	75.1
Atomic Weight	TRUE	64.2	19.7	18.2	20.3	18.3	68.4	68.6	68.8	61.2
Atomic Weight	TRUE	160.5	49.2	45.5	50.7	45.7	63.4	62.1	72.5	63.9
Atomic Weight	TRUE	160.5	49.2	45.5	50.7	45.7	63.4	61.4	48.9	60.9
Atomic Weight	TRUE	160.5	49.2	45.5	50.7	45.7	63.4	62.4	64.6	74.9
Atomic Weight	TRUE	160.5	49.2	45.5	50.7	45.7	63.4	66.7	58.2	83.4
Atomic Weight	TRUE	160.5	49.2	45.5	50.7	45.7	63.4	61.5	63.6	58.7
Atomic Weight	TRUE	321.1	98.5	90.9	101.3	91.3	58.4	58.4	56.7	53.7
Atomic Weight	TRUE	321.1	98.5	90.9	101.3	91.3	58.4	57.0	58.5	58.9
Atomic Weight	TRUE	321.1	98.5	90.9	101.3	91.3	58.4	55.1	61.7	60.6
Atomic Weight	TRUE	321.1	98.5	90.9	101.3	91.3	58.4	57.3	60.5	56.5
Atomic Weight	TRUE	321.1	98.5	90.9	101.3	91.3	58.4	58.8	52.1	68.8
Atomic Weight	TRUE	642.1	196.9	181.9	202.6	182.6	50.0	51.0	37.8	55.7
Atomic Weight	TRUE	642.1	196.9	181.9	202.6	182.6	50.0	46.4	50.8	53.7
Atomic Weight	TRUE	642.1	196.9	181.9	202.6	182.6	50.0	48.7	58.5	51.2
Atomic Weight	TRUE	642.1	196.9	181.9	202.6	182.6	50.0	53.4	50.7	35.9
Atomic Weight	TRUE	642.1	196.9	181.9	202.6	182.6	50.0	47.1	56.2	68.4
Atomic Weight	TRUE	1284.3	393.9	363.8	405.2	365.3	28.3	28.0	26.7	33.4
Atomic Weight	TRUE	1284.3	393.9	363.8	405.2	365.3	28.3	27.0	31.8	12.4

Assay	Mixture	Lead	Copper	Nickel	Zinc	Cobalt	Response at SD = 0	Response at SD = 2	Response at SD = 5	Response at SD = 10
Atomic Weight	TRUE	1284.3	393.9	363.8	405.2	365.3	28.3	25.4	19.1	24.7
Atomic Weight	TRUE	1284.3	393.9	363.8	405.2	365.3	28.3	29.6	17.9	42.1
Atomic Weight	TRUE	1284.3	393.9	363.8	405.2	365.3	28.3	29.3	31.0	15.6
Atomic Weight	TRUE	2568.5	787.7	727.6	810.5	730.6	7.3	9.2	6.6	8.1
Atomic Weight	TRUE	2568.5	787.7	727.6	810.5	730.6	7.3	4.5	0.0	9.7
Atomic Weight	TRUE	2568.5	787.7	727.6	810.5	730.6	7.3	7.9	8.2	0.0
Atomic Weight	TRUE	2568.5	787.7	727.6	810.5	730.6	7.3	6.0	0.0	24.6
Atomic Weight	TRUE	2568.5	787.7	727.6	810.5	730.6	7.3	6.7	3.6	8.5
Atomic Weight	TRUE	5137.0	1575.5	1455.2	1620.9	1461.1	1.1	1.9	0.0	3.4
Atomic Weight	TRUE	5137.0	1575.5	1455.2	1620.9	1461.1	1.1	2.6	0.0	3.3
Atomic Weight	TRUE	5137.0	1575.5	1455.2	1620.9	1461.1	1.1	2.2	0.0	13.4
Atomic Weight	TRUE	5137.0	1575.5	1455.2	1620.9	1461.1	1.1	4.4	0.0	16.4
Atomic Weight	TRUE	5137.0	1575.5	1455.2	1620.9	1461.1	1.1	0.0	6.8	0.0
Solubility	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	98.3	106.9	110.3
Solubility	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	94.7	102.5	98.6
Solubility	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	99.3	109.1	114.2
Solubility	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	98.3	95.9	109.6
Solubility	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	104.6	97.3	83.6
Solubility	TRUE	7.9	19.1	13.1	19.1	13.6	69.9	73.5	65.7	82.0
Solubility	TRUE	7.9	19.1	13.1	19.1	13.6	69.9	69.5	72.1	64.7
Solubility	TRUE	7.9	19.1	13.1	19.1	13.6	69.9	68.1	73.7	69.6
Solubility	TRUE	7.9	19.1	13.1	19.1	13.6	69.9	69.8	79.2	74.5
Solubility	TRUE	7.9	19.1	13.1	19.1	13.6	69.9	69.9	70.7	73.3
Solubility	TRUE	15.8	38.3	26.1	38.3	27.2	66.7	65.2	68.0	54.8
Solubility	TRUE	15.8	38.3	26.1	38.3	27.2	66.7	66.3	67.5	53.1
Solubility	TRUE	15.8	38.3	26.1	38.3	27.2	66.7	67.9	71.3	55.0
Solubility	TRUE	15.8	38.3	26.1	38.3	27.2	66.7	67.6	60.5	48.0
Solubility	TRUE	15.8	38.3	26.1	38.3	27.2	66.7	62.9	78.2	45.0
Solubility	TRUE	39.6	95.7	65.4	95.7	68.0	61.6	63.6	58.0	61.1
Solubility	TRUE	39.6	95.7	65.4	95.7	68.0	61.6	60.6	65.7	63.8
Solubility	TRUE	39.6	95.7	65.4	95.7	68.0	61.6	64.7	63.2	62.4
Solubility	TRUE	39.6	95.7	65.4	95.7	68.0	61.6	60.9	54.2	68.0
Solubility	TRUE	39.6	95.7	65.4	95.7	68.0	61.6	62.7	58.3	39.0
Solubility	TRUE	79.2	191.5	130.7	191.5	136.0	56.9	57.8	62.0	63.0
Solubility	TRUE	79.2	191.5	130.7	191.5	136.0	56.9	58.9	60.3	49.4

Assay	Mixture	Lead	Copper	Nickel	Zinc	Cobalt	Response at SD = 0	Response at SD = 2	Response at SD = 5	Response at SD = 10
Solubility	TRUE	79.2	191.5	130.7	191.5	136.0	56.9	53.8	45.5	56.0
Solubility	TRUE	79.2	191.5	130.7	191.5	136.0	56.9	56.9	53.2	57.8
Solubility	TRUE	79.2	191.5	130.7	191.5	136.0	56.9	56.1	58.6	51.6
Solubility	TRUE	158.4	382.9	261.4	382.9	272.0	50.0	51.4	46.7	47.8
Solubility	TRUE	158.4	382.9	261.4	382.9	272.0	50.0	50.6	61.2	58.1
Solubility	TRUE	158.4	382.9	261.4	382.9	272.0	50.0	53.8	55.9	53.8
Solubility	TRUE	158.4	382.9	261.4	382.9	272.0	50.0	47.6	52.0	61.7
Solubility	TRUE	158.4	382.9	261.4	382.9	272.0	50.0	49.7	48.5	61.3
Solubility	TRUE	316.9	765.8	522.9	765.8	544.0	36.7	34.2	37.4	51.7
Solubility	TRUE	316.9	765.8	522.9	765.8	544.0	36.7	34.6	44.9	40.1
Solubility	TRUE	316.9	765.8	522.9	765.8	544.0	36.7	35.6	36.7	44.8
Solubility	TRUE	316.9	765.8	522.9	765.8	544.0	36.7	36.0	40.4	25.4
Solubility	TRUE	316.9	765.8	522.9	765.8	544.0	36.7	33.7	36.7	37.0
Solubility	TRUE	633.8	1531.6	1045.7	1531.6	1088.0	16.9	15.2	6.6	10.1
Solubility	TRUE	633.8	1531.6	1045.7	1531.6	1088.0	16.9	15.5	11.9	29.4
Solubility	TRUE	633.8	1531.6	1045.7	1531.6	1088.0	16.9	15.1	18.8	6.4
Solubility	TRUE	633.8	1531.6	1045.7	1531.6	1088.0	16.9	18.1	16.7	33.8
Solubility	TRUE	633.8	1531.6	1045.7	1531.6	1088.0	16.9	18.7	24.5	7.8
Solubility	TRUE	1267.6	3063.3	2091.5	3063.3	2176.0	4.3	3.3	0.0	5.5
Solubility	TRUE	1267.6	3063.3	2091.5	3063.3	2176.0	4.3	3.1	0.0	0.0
Solubility	TRUE	1267.6	3063.3	2091.5	3063.3	2176.0	4.3	1.1	3.7	9.1
Solubility	TRUE	1267.6	3063.3	2091.5	3063.3	2176.0	4.3	4.3	1.5	7.0
Solubility	TRUE	1267.6	3063.3	2091.5	3063.3	2176.0	4.3	5.3	9.4	11.7
CaCl2 Extractable	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	102.1	95.9	93.5
CaCl2 Extractable	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	103.3	104.0	86.4
CaCl2 Extractable	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	102.7	95.1	98.8
CaCl2 Extractable	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	100.7	102.3	95.7
CaCl2 Extractable	TRUE	0.0	0.0	0.0	0.0	0.0	100.0	102.7	98.5	92.0
CaCl2 Extractable	TRUE	0.3	0.4	11.8	6.9	31.0	66.6	67.2	62.8	57.4
CaCl2 Extractable	TRUE	0.3	0.4	11.8	6.9	31.0	66.6	70.7	76.5	74.1
CaCl2 Extractable	TRUE	0.3	0.4	11.8	6.9	31.0	66.6	67.9	64.3	58.1
CaCl2 Extractable	TRUE	0.3	0.4	11.8	6.9	31.0	66.6	66.9	68.6	68.1
CaCl2 Extractable	TRUE	0.3	0.4	11.8	6.9	31.0	66.6	67.5	59.0	62.9
CaCl2 Extractable	TRUE	0.6	0.8	23.7	13.8	62.0	63.3	61.0	69.8	74.8
CaCl2 Extractable	TRUE	0.6	0.8	23.7	13.8	62.0	63.3	61.4	63.7	68.0

Assay	Mixture	Lead	Copper	Nickel	Zinc	Cobalt	Response at SD = 0	Response at SD = 2	Response at SD = 5	Response at SD = 10
CaCl2 Extractable	TRUE	0.6	0.8	23.7	13.8	62.0	63.3	59.3	61.2	66.8
CaCl2 Extractable	TRUE	0.6	0.8	23.7	13.8	62.0	63.3	63.9	70.4	83.7
CaCl2 Extractable	TRUE	0.6	0.8	23.7	13.8	62.0	63.3	61.0	63.0	53.0
CaCl2 Extractable	TRUE	1.4	2.1	59.2	34.4	155.0	58.6	57.7	61.8	56.0
CaCl2 Extractable	TRUE	1.4	2.1	59.2	34.4	155.0	58.6	57.9	63.6	69.8
CaCl2 Extractable	TRUE	1.4	2.1	59.2	34.4	155.0	58.6	58.1	52.8	72.5
CaCl2 Extractable	TRUE	1.4	2.1	59.2	34.4	155.0	58.6	57.1	54.7	59.3
CaCl2 Extractable	TRUE	1.4	2.1	59.2	34.4	155.0	58.6	55.9	53.5	46.5
CaCl2 Extractable	TRUE	2.8	4.1	118.5	68.9	310.0	54.6	55.6	55.5	71.2
CaCl2 Extractable	TRUE	2.8	4.1	118.5	68.9	310.0	54.6	60.3	55.1	65.2
CaCl2 Extractable	TRUE	2.8	4.1	118.5	68.9	310.0	54.6	56.1	57.9	60.3
CaCl2 Extractable	TRUE	2.8	4.1	118.5	68.9	310.0	54.6	56.1	52.2	43.7
CaCl2 Extractable	TRUE	2.8	4.1	118.5	68.9	310.0	54.6	55.4	51.6	72.0
CaCl2 Extractable	TRUE	5.5	8.3	237.0	137.8	620.0	50.0	51.2	45.2	50.9
CaCl2 Extractable	TRUE	5.5	8.3	237.0	137.8	620.0	50.0	46.6	57.9	20.5
CaCl2 Extractable	TRUE	5.5	8.3	237.0	137.8	620.0	50.0	49.4	50.8	67.1
CaCl2 Extractable	TRUE	5.5	8.3	237.0	137.8	620.0	50.0	49.8	43.3	48.6
CaCl2 Extractable	TRUE	5.5	8.3	237.0	137.8	620.0	50.0	51.1	50.3	47.9
CaCl2 Extractable	TRUE	11.0	16.5	474.0	275.6	1240.0	44.0	41.2	49.5	56.8
CaCl2 Extractable	TRUE	11.0	16.5	474.0	275.6	1240.0	44.0	43.7	36.1	21.9
CaCl2 Extractable	TRUE	11.0	16.5	474.0	275.6	1240.0	44.0	40.7	42.3	54.6
CaCl2 Extractable	TRUE	11.0	16.5	474.0	275.6	1240.0	44.0	45.7	45.0	30.9
CaCl2 Extractable	TRUE	11.0	16.5	474.0	275.6	1240.0	44.0	40.4	39.0	51.5
CaCl2 Extractable	TRUE	22.0	33.1	947.9	551.1	2480.1	33.8	33.2	27.4	35.2
CaCl2 Extractable	TRUE	22.0	33.1	947.9	551.1	2480.1	33.8	35.4	30.9	20.1
CaCl2 Extractable	TRUE	22.0	33.1	947.9	551.1	2480.1	33.8	33.7	40.2	44.6
CaCl2 Extractable	TRUE	22.0	33.1	947.9	551.1	2480.1	33.8	33.3	37.4	36.5
CaCl2 Extractable	TRUE	22.0	33.1	947.9	551.1	2480.1	33.8	32.9	26.9	34.5
CaCl2 Extractable	TRUE	44.1	66.1	1895.9	1102.3	4960.2	17.9	19.1	6.8	19.2

Assay	Mixture	Lead	Copper	Nickel	Zinc	Cobalt	Response at SD = 0	Response at SD = 2	Response at SD = 5	Response at SD = 10
CaCl2 Extractable	TRUE	44.1	66.1	1895.9	1102.3	4960.2	17.9	17.3	19.4	18.3
CaCl2 Extractable	TRUE	44.1	66.1	1895.9	1102.3	4960.2	17.9	14.7	21.4	22.5
CaCl2 Extractable	TRUE	44.1	66.1	1895.9	1102.3	4960.2	17.9	17.1	22.4	14.7
CaCl2 Extractable	TRUE	44.1	66.1	1895.9	1102.3	4960.2	17.9	17.4	13.1	21.9

Appendix C: Laboratory *Folsomia candida* Reproduction in Soil 3.22

Assay	Mixture	Lead	Copper	Cobalt	Nickel	Zinc	Response
Lead	F	400	0	0	0	0	72
Lead	F	0	0	0	0	0	205
Lead	F	19200	0	0	0	0	162
Lead	F	25600	0	0	0	0	204
Lead	F	2400	0	0	0	0	226
Lead	F	9600	0	0	0	0	336
Lead	F	12800	0	0	0	0	315
Lead	F	1200	0	0	0	0	260
Lead	F	800	0	0	0	0	439
Lead	F	800	0	0	0	0	261
Lead	F	800	0	0	0	0	261
Lead	F	1600	0	0	0	0	116
Lead	F	6400	0	0	0	0	223
Lead	F	3200	0	0	0	0	212
Copper	F	0	0	0	0	0	205
Copper	F	0	5600	0	0	0	1
Copper	F	0	4200	0	0	0	0
Copper	F	0	350	0	0	0	106
Copper	F	0	175	0	0	0	141
Copper	F	0	525	0	0	0	55
Copper	F	0	1400	0	0	0	8
Copper	F	0	2800	0	0	0	7
Copper	F	0	8400	0	0	0	3
Copper	F	0	700	0	0	0	152
Copper	F	0	700	0	0	0	133
Copper	F	0	700	0	0	0	126
Copper	F	0	11200	0	0	0	0
Copper	F	0	1050	0	0	0	60
Nickel	F	0	0	0	0	0	205
Nickel	F	0	0	3800	0	0	327
Nickel	F	0	0	7600	0	0	0
Nickel	F	0	0	119	0	0	436
Nickel	F	0	0	1900	0	0	209
Nickel	F	0	0	238	0	0	379
Nickel	F	0	0	950	0	0	261
Nickel	F	0	0	5700	0	0	10
Nickel	F	0	0	356	0	0	201
Nickel	F	0	0	475	0	0	342
Nickel	F	0	0	713	0	0	179
Nickel	F	0	0	2850	0	0	523
Nickel	F	0	0	2850	0	0	445
Nickel	F	0	0	2850	0	0	355

Zinc	F	0	0	0	563	0	170
Zinc	F	0	0	0	0	0	205
Zinc	F	0	0	0	12000	0	1
Zinc	F	0	0	0	750	0	211
Zinc	F	0	0	0	375	0	216
Zinc	F	0	0	0	3000	0	14
Zinc	F	0	0	0	4500	0	0
Zinc	F	0	0	0	9000	0	0
Zinc	F	0	0	0	9000	0	1
Zinc	F	0	0	0	9000	0	0
Zinc	F	0	0	0	1500	0	31
Zinc	F	0	0	0	188	0	148
Zinc	F	0	0	0	1125	0	186
Cobalt	F	0	0	0	0	1480	142
Cobalt	F	0	0	0	0	0	205
Cobalt	F	0	0	0	0	17760	212
Cobalt	F	0	0	0	0	23680	156
Cobalt	F	0	0	0	0	1110	168
Cobalt	F	0	0	0	0	8880	356
Cobalt	F	0	0	0	0	2220	312
Cobalt	F	0	0	0	0	2220	180
Cobalt	F	0	0	0	0	5920	215
Cobalt	F	0	0	0	0	5920	163
Cobalt	F	0	0	0	0	5920	80
Cobalt	F	0	0	0	0	370	295
Cobalt	F	0	0	0	0	740	431
Cobalt	F	0	0	0	0	11840	78
Cobalt	F	0	0	0	0	2960	303
CSQG	T	268	241	172	766	153	31
CSQG	T	0	0	0	0	0	205
CSQG	T	536	483	345	1532	306	18
CSQG	T	134	121	86	383	77	263
CSQG	T	1072	965	689	3064	613	35
CSQG	T	67	60	43	192	38	201
CSQG	T	1609	1448	1034	4596	919	0
CSQG	T	2145	1930	1379	6128	1226	5
CSQG	T	34	30	22	96	19	271
CSQG	T	201	181	129	575	115	131
Flin_Flon	T	0	0	0	0	0	205
Flin_Flon	T	51	155	2	556	2	129
Flin_Flon	T	13	39	1	139	1	290
Flin_Flon	T	76	232	3	834	3	195
Flin_Flon	T	606	1856	28	6670	28	0

Flin_Flon	T	25	77	1	278	1	37
Flin_Flon	T	808	2474	37	8893	37	1
Flin_Flon	T	101	309	5	1112	5	93
Flin_Flon	T	202	619	9	2223	9	19
Flin_Flon	T	404	1237	18	4447	18	9
Flin_Flon	T	404	1237	18	4447	18	19
Flin_Flon	T	404	1237	18	4447	18	67
Sudbury	T	0	0	0	0	0	205
Sudbury	T	289	20	37	150	19	232
Sudbury	T	868	60	111	449	57	283
Sudbury	T	868	60	111	449	57	452
Sudbury	T	868	60	111	449	57	331
Sudbury	T	6943	483	891	3589	458	21
Sudbury	T	4629	322	594	2393	305	31
Sudbury	T	9258	644	1188	4786	611	0
Sudbury	T	2314	161	297	1196	153	26
Sudbury	T	1157	80	149	598	76	163
Sudbury	T	579	40	74	299	38	74
Port_Colborne	T	3	24	95	10	2	196
Port_Colborne	T	0	0	0	0	0	205
Port_Colborne	T	7	48	189	20	3	180
Port_Colborne	T	223	1524	6052	651	111	13
Port_Colborne	T	56	381	1513	163	28	51
Port_Colborne	T	56	381	1513	163	28	86
Port_Colborne	T	56	381	1513	163	28	135
Port_Colborne	T	21	143	567	61	10	143
Port_Colborne	T	28	190	757	81	14	214
Port_Colborne	T	167	1143	4539	488	83	17
Port_Colborne	T	14	95	378	41	7	138
Port_Colborne	T	111	762	3026	325	56	34
EC50	T	326	148	89	148	296	339
EC50	T	326	148	89	148	296	246
EC50	T	326	148	89	148	296	178
EC50	T	0	0	0	0	0	205
EC50	T	3918	1777	1064	1777	3555	28
EC50	T	1306	592	355	592	1185	120
EC50	T	82	37	22	37	74	407
EC50	T	2612	1185	709	1185	2370	39
EC50	T	5224	2370	1419	2370	4740	7
EC50	T	163	74	44	74	148	182
EC50	T	653	296	177	296	592	181
EC50	T	490	222	133	222	444	249
Equal_Ratio	T	0	0	0	0	0	205

Equal_Ratio	T	324	324	324	324	324	103
Equal_Ratio	T	243	243	243	243	243	368
Equal_Ratio	T	1946	1946	1946	1946	1946	63
Equal_Ratio	T	81	81	81	81	81	253
Equal_Ratio	T	649	649	649	649	649	205
Equal_Ratio	T	41	41	41	41	41	141
Equal_Ratio	T	41	41	41	41	41	137
Equal_Ratio	T	41	41	41	41	41	75
Equal_Ratio	T	1297	1297	1297	1297	1297	6
Equal_Ratio	T	162	162	162	162	162	108
Equal_Ratio	T	2594	2594	2594	2594	2594	3
Clay_Peat	T	153	166	99	300	88	23
Clay_Peat	T	153	166	99	300	88	264
Clay_Peat	T	153	166	99	300	88	123
Clay_Peat	T	0	0	0	0	0	205
Clay_Peat	T	612	662	396	1199	354	50
Clay_Peat	T	306	331	198	600	177	83
Clay_Peat	T	38	41	25	75	22	9
Clay_Peat	T	38	41	25	75	22	374
Clay_Peat	T	230	248	148	450	133	68
Clay_Peat	T	77	83	49	150	44	475
Clay_Peat	T	1224	1325	791	2398	707	20
Clay_Peat	T	2448	2650	1583	4797	1415	3
Clay_Peat	T	1836	1987	1187	3598	1061	25
Ag_Res_Loamy	T	0	0	0	0	0	205
Ag_Res_Loamy	T	1679	1768	1130	4050	957	0
Ag_Res_Loamy	T	35	37	24	84	20	306
Ag_Res_Loamy	T	280	295	188	675	159	218
Ag_Res_Loamy	T	210	221	141	506	120	78
Ag_Res_Loamy	T	140	147	94	337	80	217
Ag_Res_Loamy	T	1119	1179	753	2700	638	21
Ag_Res_Loamy	T	560	589	377	1350	319	14
Ag_Res_Loamy	T	70	74	47	169	40	260
Ag_Res_Loamy	T	70	74	47	169	40	172
Ag_Res_Loamy	T	70	74	47	169	40	444
Ag_Res_Loamy	T	2238	2357	1507	5399	1276	0
Loam_Sand_Ind	T	0	0	0	0	0	205
Loam_Sand_Ind	T	2899	2057	1441	5593	1286	0
Loam_Sand_Ind	T	2174	1543	1081	4195	965	0
Loam_Sand_Ind	T	45	32	23	87	20	242
Loam_Sand_Ind	T	725	514	360	1398	322	90
Loam_Sand_Ind	T	725	514	360	1398	322	118
Loam_Sand_Ind	T	725	514	360	1398	322	100

Loam_Sand_Ind	T	181	129	90	350	80	143
Loam_Sand_Ind	T	272	193	135	524	121	126
Loam_Sand_Ind	T	362	257	180	699	161	98
Loam_Sand_Ind	T	1449	1029	721	2797	643	1
Loam_Sand_Ind	T	91	64	45	175	40	442
Acid_Sand_Ara	T	454	152	62	252	48	88
Acid_Sand_Ara	T	454	152	62	252	48	288
Acid_Sand_Ara	T	0	0	0	0	0	205
Acid_Sand_Ara	T	681	228	93	378	72	262
Acid_Sand_Ara	T	7261	2435	993	4028	772	6
Acid_Sand_Ara	T	7261	2435	993	4028	772	9
Acid_Sand_Ara	T	7261	2435	993	4028	772	2
Acid_Sand_Ara	T	5446	1827	745	3021	579	0
Acid_Sand_Ara	T	908	304	124	503	97	312
Acid_Sand_Ara	T	227	76	31	126	24	167
Acid_Sand_Ara	T	113	38	16	63	12	364
Acid_Sand_Ara	T	1815	609	248	1007	193	87
Acid_Sand_Ara	T	3631	1218	496	2014	386	48