



Combination effects of metals in soil

- acute toxicity test of bacteria with the leucine incorporation method

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Abstract

Within the field of ecotoxicology there is a concept called combination effects. There are two different approaches to combination effects, independent action (IA) and concentration addition (CA). The common assumption in both approaches is that there are no interactions between the substances within the mixture. These approaches are used for predicting the toxicity of whole mixtures and also to examine mixtures to detect potential synergistic or antagonistic effects (interactions). If a mixture does not show any synergistic or antagonistic effects, it is labelled as strict additive. In this study the CA concept was used for assessing the combination effects between the metals copper-zinc and copper-mercury in soils with different organic matter content, by measuring the bacterial growth with the leucine incorporation method. The results showed no synergistic or antagonistic effect between copper-zinc, independent of soil type. The same result was obtained from the copper-mercury experiment, which was conducted only in one soil. There was, however, a clear relationship between the toxicity of the metals in the soil (estimated as EC50) and the organic matter content. The leucine incorporation method was also compared with respiration, a more commonly used measurement within ecotoxicology.

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1. Introduction

1.1 General problem

Soil serves as a habitat for a vast range of organisms (Brady and Weil 2008). It is estimated that in 1 gram of soil there can be around 10 billion bacteria (Dunbar et al. 2002). Studies also show that 1 gram of soil can hold up to 2,000-18,000 different species of microorganisms (Dunbar et al. 2002). Their activity leads to recycling of nutrients to the benefit of both plants and other organisms. Humans also depend on these services from the soil for production of crops for food, fuel and building materials. Besides recycling nutrients, the microorganisms can detoxify organic pollutants and also decrease the mobility of some inorganic pollutants (Kirchman 2012).

Contamination of the environment from anthropogenic sources has been going on for a long period of time, probably for several thousands of years (Batty and Hallberg 2010, Persson 1999). Everything from farming to driving a car contributes to the overall contamination of the environment. Activities that have contributed heavily to the contamination by metals are mining industries. For example, there have been mining activities since the Copper and Bronze Age near the river Rio Tinto in southwest Spain, which has led to huge amounts of metal residues accumulating in the estuary (Davis Jr et al. 1999). Even though contamination and disturbance of the environment is not a new phenomenon, the general public has not always been aware of the issue. In 1963 Rachel Carson released the book "Silent Spring", which served as a wake-up call (Scragg 2005). Since then, the development of methods to evaluate chemical products has improved our ability to prevent exposure of dangerous toxicants to humans and the environment. With the development of methods to analyse chemicals and their effects, regulations have also evolved and improved greatly since then. Different forms of regulation have been implemented around the world. Sweden founded its environmental protection agency (EPA) in 1967 and implemented their first environmental law in 1969; in 1999 all environmental laws were turned into their own code (Wärneryd et al. 2002, Swedish EPA 2012a). There are directives and regulations controlling various matters, from the use of chemicals (e.g. REACH) to the implementation of best available technology (BAT).

If it is suspected that a site is contaminated, the landowner or the operator of the site is obligated by law to report it to the supervising authority (Environmental Code 2012). If the level of toxicants is higher than the background levels, the investigation will proceed with a risk assessment (Swedish EPA 2012b). The purpose is to evaluate the risk for humans and the environment and also to determine how much the risk needs to be reduced to minimize damage in the future (Swedish EPA 2009a). The assessment should include a problem description, conceptual model, an impact- and effect analysis and risk characterisation. It initially starts as a basic assessment and can become a detailed one if the situation is complicated in some way, for instance, by lack of information about guideline values in the medium or too great uncertainties about the magnitude of risk (Swedish EPA 2009a).

The level of toxicants is compared with the EPA guideline values. Different values are used depending on current or planned land use. If the land will be used for housing the guideline values are lower, so-called sensitive land use. This protection level, sensitive

land use, is defined for soil as when the ecological processes are not limited by the amount of toxicants (Swedish EPA 2009b). For industrial land use the values are higher, so-called less sensitive land use. This level is defined for soil as when the function of the soil should not be disturbed by the pollution, so that the functions still will be able to support the prospective land use (Swedish EPA 2009b). The levels of pollution should also allow animals to stay in the area for a short period of time without endangering their health (Swedish EPA 2009b). The guideline values for different toxicants in different media for sensitive and less sensitive land use are found in the first appendix of the EPA report "Riktvärden för förorenad mark" (Swedish EPA 2009b). These values are only recommendations and not legally binding (Swedish EPA 2009a). They are based on ecotoxicological data from many different sources. There are two ways of evaluating the toxicity data and determining a guideline value, by conducting a distribution test of the species sensitivity or by use of a safety factor. A value for sensitive land use for soil is based on a protection level of 75% of the species in the soil (Swedish EPA 2009b). The values for less sensitive land use are based on a lower standard, where only 50% of the species in the soil are protected. These protection levels are mainly based on the no-observed-effect-concentration (NOEC) (Swedish EPA 2009b). Concentrations where 50% of the target organisms survive are sometimes used as well, when there is a lack of data. When it comes to soil this is often the case. If there is no data from soil tests, data from aquatic toxicology tests are used instead, with some modifications.

Within a detailed risk assessment, the EPA recommends that ecotoxicological tests should be performed if, for example, there are possible combination effects of the toxicants, if they have similar effects or if there is a need to establish their bioavailability (Swedish EPA, 2009a). There are standardized ecotoxicological tests but most of them are only suited for aquatic systems. For soil, there are only a few standardized methods available. One of these is measuring respiration rate of the microorganisms (1.4.1 Respiration).

All the data that are used within risk assessments are based on experiments, where one toxicant at a time is tested, never in complex mixtures, meaning that the guideline values are not considering combination effects. To base assessments on "one chemical at a time" is problematic, because a toxicant rarely turns up alone. Without considering potential combination effects when assessing risk at a site there is a hazard of both underestimating and overestimating the risk. Hence, further research is needed within the field of complex mixtures. Another issue is the lack of data within terrestrial ecotoxicology, both of basic effect concentrations of single chemicals and of data from complex mixtures (Backhaus and Faust 2012). The lack of standardized methods to assess soil is also a problem (Swedish EPA 2009a).

This study investigated potential combination effects of metals in soil, mainly copper and zinc, using a method that measures bacterial growth as the end-point (leucine incorporation). Also a comparison of respiration and leucine incorporation was made. The role of the organic matter content in modulating the toxic response was also evaluated. The main questions posed were:

- (1) - Is leucine incorporation a more sensitive method to detect **toxicity** than respiration measurements? Is it possible to measure **combination effects** in soil using the leucine incorporation method?
- (2a) - Are there any combination effects between copper and zinc?

(2b) - Are there any combination effects between copper and mercury?

1.2 Metals

Several metals play an important part in the physiological function of plants and microorganisms, but, as with everything, too much (or too little) of an essential thing can cause more damage than good. However, some metals have no physiological role. Metals can for example bind to sulfhydryl-, phosphate and hydroxyl groups, which can lead to disruptions in both DNA and protein (Roane et al. 2009). Metal toxicity can cause decreased growth, physiological changes and inhibition of processes in the cells, with eventually lethal consequences.

Three metals were used in this study to assess potential combination effects: copper, zinc and mercury. The guideline values for soil from the Swedish EPA are presented in Table 1 below for the three metals and also the tolerable daily intake (TDI) for humans (Swedish EPA 2009b).

Table 1. Guideline values for sensitive and less sensitive land use (Swedish EPA 2009b).

Metal	Sensitive land use (mg/kg DW)	Less sensitive land use (mg/kg DW)	TDI (mg/kg bodyweight and day)
Copper (Cu)	80	200	0.5
Zinc (Zn)	250	500	0.3
Mercury (Hg)	5	10	0.23×10^{-3}

1.2.1 Copper and zinc

Both copper and zinc are essential for microorganisms, but in excess they can cause harm by binding to and destroying different molecules (Roane et al. 2009). Copper binds to both proteins and nucleic acids, while zinc mainly binds to proteins (Trevors and Cotter 1990, Hughes and Poole 1989). They have a difference in oxidation states; copper has two (Cu^{1+} , Cu^{2+}), while zinc only has one (Zn^{2+}). This difference in numbers of oxidation states gives copper different functions from zinc (Hughes and Poole 1989).

1.2.2 Mercury

Mercury, which can be present in several forms, has no known essential role in ecosystems and is classified as toxic. Because of the great toxicity of mercury, it is not allowed in Sweden to put mercury products on the market, to use them or to export them (Swedish Chemical Agency 2012). Within the European Union there is a ban that states that mercury products should not be exported, and there are also some limitations on the use of mercury products (Swedish Chemical Agency 2012).

Elemental mercury can be oxidized to mercury ions (Hg^{2+}) by bacteria (Hughes and Poole 1989). Hg^{2+} can then be transformed into the most toxic states, methyl mercury or dimethyl mercury (organic mercury). However, there is also a possibility that Hg^{2+} or organic mercury will be reduced into inorganic mercury again by bacteria that are resistant to the toxicity of mercury (Hughes and Poole 1989). Mercury can cause a lot of damage, for example inhibiting translation and transformation in DNA-synthesis or inhibiting enzyme activity (Roane et al. 2009).

1.3 Concepts of combination effects

1.3.1 Independent action, concentration addition and toxic units

Within the field of toxicology there is a concept called combination effects. It is based on the idea that it is possible to estimate the toxicity of a mixture of toxic compounds by measuring the individual toxicity of each compound (Kortenkamp et al. 2009, p.5). There are two different approaches to combination effects, independent action (IA) and concentration addition (CA). The common assumption in both concepts is that there are no interactions between the substances within the mixture. These approaches are used to predict the toxicity of whole mixtures and also to examine mixtures to detect potential synergistic or antagonistic effects (interactions). Synergism is defined as when the toxic effect of the mixture is greater than expected and antagonism is when the effect of the mixture is less than expected (Walker et al. 2006). Synergism is also sometimes called potentiation. According to Walker et al. (2006) there are some authors who make a distinction between these two words, in that synergism should only be used when a substance with a certain concentration within a mixture does not show any toxic effects when being tested alone. In this report the more wide definition of synergism will be used. If the mixture does not show any synergistic or antagonistic effects, the mixture is labelled as strict additive.

1.3.1.1 Independent action

Independent action is also known as effect addition, effect multiplication, Abbotts rule or response addition (Norwood et al. 2003, Kortenkamp et al. 2009, p.19). The IA concept is based on the assumption that the substances in a mixture have different targets and mechanisms of action (Bengtsson and Holmqvist 2008). Mechanism of action is defined as processes within the organism that occurs at exposure and is expressed as some sort of biological outcome in the organism (Kortenkamp et al. 2009, p.13). This assumption means that the different substances within a mixture act independently of each other, creating effects without any interaction with the other substances (Bengtsson and Holmqvist 2008, Kortenkamp 2007). The toxicity is calculated based on multiplying the effect caused by each individual substance after subtracting it from 1 (Norwood et al. 2003):

$$P = 1 - (1 - P_1) \cdot (1 - P_2) \cdot \dots \cdot (1 - P_n) \quad (\text{Eq. 1})$$

P is the total effect that the mixture will have. The P_n values are the effects for the individual substances within the mixture, acting alone at the same concentrations as in the mixture. In Kortenkamp et al. (2009, p.19) two equations are used to describe independent action. The first one is described above, and is used when increasing concentrations give an increasing response (Eq. 1). If increasing concentrations instead give a decreased response, the following equation should be used:

$$P = P_1 \cdot P_2 \cdot \dots \cdot P_n \quad (\text{Eq. 2})$$

1.3.1.2 Concentration addition

Concentration addition is also known as dose addition and toxic unit summation (Kortenkamp et al. 2009, p.17). It is based on the assumption that the different substances in a mixture work as dilutions of one another (Kortenkamp et al. 2009, p.17). This means that they are assumed to have the same mechanism of action. With these two assumptions it is possible to calculate an overall toxicity for a mixture without having to test all possible combinations of substances and the only information one

needs is the effect concentration (e.g. EC50) for the single substances and the concentration/fraction of them within the mixture. The formula for the calculation is shown below (Kortenkamp et al. 2009, p.17):

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

The ECx_{mix} -value is calculated by the inverse of the sum of the fractions (p_i) of all substances (n) in the mixture divided by their individual ECx_i -value. According to Kortenkamp et al. (2009, p.23) the present consensus within ecotoxicology is that the CA concept should be used as the default approach. This is partly due to the fact that CA predicts lower effect concentrations than IA, which means that CA is a more conservative model than IA (Kortenkamp et al. 2009, p.23).

1.3.1.3 Toxic units

The CA concept can also be expressed as toxic units (TU) (Bengtsson and Holmqvist 2008):

$$TU = \left(\sum_{i=1}^n \frac{c_i}{ECx_i} \right) \quad (\text{Eq. 4})$$

TU is calculated by summing the ratio of the individual concentration (c_i) divided by their individual ECx_i -values. The n stands for number of components. To be able to calculate TU for the toxicants, there is a need to calculate effect concentrations (ECx). In this study the effect concentration that is used is EC_{50} , which means the concentration that is needed to inhibit 50% of the end-point that is measured, in this case inhibition of bacterial growth, measured as leucine incorporation (Figure 1). Other ECx -values can also be calculated from the dose-response curve. A dose-response curve can look different depending on which formula that is used, but it is often a type of sigmoid curve. When using CA it is not a good idea to use a NOEL (no observed effect level) or NOEC (no observed effect concentration) in the formula because they are not representing ECx -values (Kortenkamp et al. 2009, p.25).

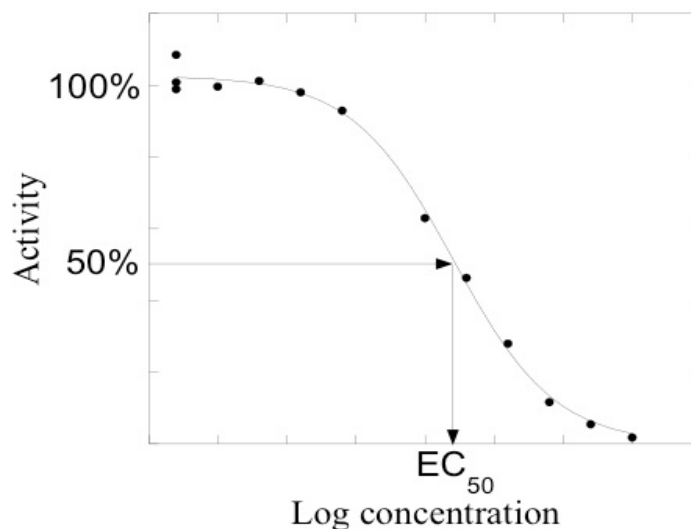


Figure 1. A dose-response curve. The EC_{50} -value is where 50% of the target organisms are affected by the toxicant.

TU can be used as an indicator for whether a mixture shows synergism, antagonism or a strictly additive effect. This is calculated by dividing the effect concentration (e.g. EC50) for a specific toxicant within a mixture with the effect concentration (e.g. EC50) for that toxicant when it is tested on its own. The different TUs in the mixture can then be added together. In theory, if a binary mixture were strict additive, the two different TU of the mixture would add up to one. If the sum of TUs are greater than one, the mixture is less than additive, i.e. antagonistic (Bengtsson and Holmqvist 2008). If the sum of TUs are less than one, the mixture is more than additive, i.e. synergistic (Bengtsson and Holmqvist 2008).

1.3.2 Isobole design

Fraser created the isobole design in 1872 (Bosgra et al. 2009). In the 1920's Loewe and Muischnek used the design when they visualised the interactions between pharmaceutical substances (Tammes 1964).

Isoboles are used to visualize binary mixtures that are expected to follow concentration addition. Effect concentrations that are derived from dose-response curves from toxicology tests can be plotted with the isobole design. Assume that there is an experiment that consists of five different concentration mixtures; one with only chemical A, one with the ratio of 75% of chemical A and 25% of chemical B, one with 50/50, one with 25% of A and 75% of B and one with only chemical B. The EC50-value for the mixture with only A is plotted on the y-axis and the EC50 for the mixture with only B is plotted on the x-axis. If the chemical A and B follow the CA concept the EC50's of the three mixtures of A and B would be on a line between A and B. If the three mixtures were situated underneath the line, it would be an indication that they are more than additive, indicating synergism (Figure 2). If they were situated above the line, it would be an indication that they are less than additive, i.e. antagonism.

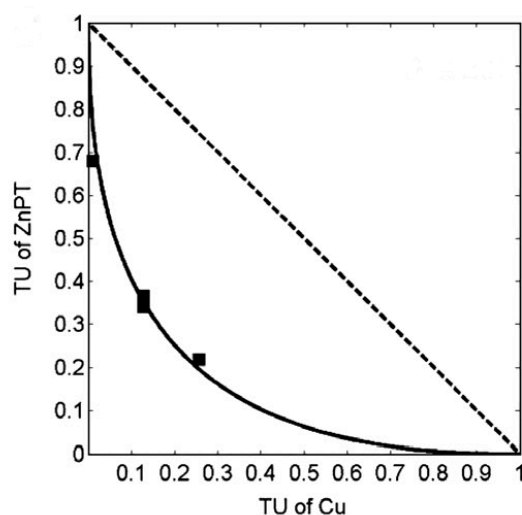


Figure 2. The graph shows an experiment with copper and zinc pyrithione visualized in an isobologram, indicating a more than additive effect, that is, synergism (from Bao et al. 2008).

Tammes (1964) describes how a dose-response relationship using more than one level of EC_x could be visualized in 3D, but also how impractical it would be because of the need to create a curved plane. This is no longer a problem thanks to the help of

computers, but still today the simple isobole design is considered to be the standard way of visualising binary mixtures (Kortenkamp et al. 2009).

1.4 Methods to measure toxicity

1.4.1 Respiration

A classic approach to study the microbial function is to measure the respiration of the soil. The respiration is a measurement of the total microbial activity and thereby provides a way of measuring the effect a metal has on general degradation processes and consequently also the effect on recycling of carbon (Hinojosa et al. 2010). Generally respiration rate is measured as CO₂ release, but can also be measured as consumed O₂. CO₂ is a more accurate way of measuring, because it is more sensitive than O₂ according to Hinojosa et al. (2010). This is due to the small amount of CO₂ that initially exists in the air compared with oxygen. One can measure several specific respiration rates; basal respiration, substrate induced respiration (SIR) and also additional microbial respiration (AMR) after substrate addition. The “normal” respiration of a soil is the basal respiration, which is measured without any additions of extra substrate. Since respiration is sensitive to nutrient limitations, temperature and moisture, it is important to have a standardized way of measuring (Sparling 1997). To reduce uncertainties of substrate limitations, it is suggested that it is better to use SIR instead of basal respiration, in order to avoid the potential limiting factor of nutrients (Hinojosa et al. 2010). To be able to measure SIR a carbon source, usually glucose, is added to the soil in excess (Anderson and Domsch 1978). Without any carbon limitations, respiration will within minutes increase to a new stable value (the SIR value); usually this stable value will not change within 10 hours. This is because the microbes cannot utilize all the carbon at this stage due to a lag period with no extra growth on glucose. After this period of stable SIR, an exponential increase in respiration occurs, indicating extra growth on glucose. AMR is then the maximum peak of respiration after the substrate has been added and shows the growth that the microbial community has had on the added substrate (Ehlers et al. 2010, Scheu 1993). By measuring respiration 24 hours after glucose addition one will mainly measure the level of AMR.

When respiration has been used as a method for examining microbial activity in contaminated soils, it has given variable results (Hinojosa et al. 2010). The contradicting results could perhaps be explained by differences between studies, such as sources of contamination, toxicants, concentrations, properties of the soil, time frame etc. (Hinojosa et al. 2010). For example, both Åkerblom et al. (2007) and Bååth et al. (1991) showed that metal addition had a negative effect on the respiration, while Bardgett and Sagar (1994) showed an increased respiration in the contaminated soil.

1.4.2 Leucine incorporation

Another way of measuring microbial function is to use the method of incorporating radioactive labelled leucine into bacteria, which is a proxy of microbial growth (Kirchman et al. 1985). Leucine is an amino acid and is used as a building block in the protein synthesis. It can be incorporated in both bacteria and fungi, but Bååth (1994) used a homogenization/centrifugation method in order to extract only bacteria from soil and thus the leucine incorporation became specific for bacteria. The leucine incorporation method has been shown to be a sensitive and reliable method to detect effects of toxicants in soil (Demoling and Bååth 2008).

The preferred method to use partly depends on the selection of target organisms. Respiration measurements show the state of the whole microbial community, while leucine incorporation method gives a view of the state of the bacterial community.

2. Method

2.1 Soils

2.1.1 Collecting and preparing

Four soils from different sites were chosen based on a visual examination and organic matter content. They were named Grassland, P2, Sand and Omberg (Table 2). Mathias Persson at Ramböll collected both P2 and Sand. P2 was taken from a shooting range near Bromölla in Blekinge and Sand was taken from a site near Rosengård in Malmö. These were checked with a portable XRF to make sure that there were no elevated levels of any metals. This was done because P2 and Sand were taken from potentially contaminated sites. Erland Bååth collected the soil named Grassland from a grass lawn near Kristianstad in Scania. The Grassland soil was taken from a site that has no records of being contaminated and was thus not checked with XRF. I collected the Omberg soil from a cultivated area, rich in peat in Östergötland, nearby the nature reserve Omberg. The Omberg soil was not checked with XRF for the same reason as the Grassland soil.

Table 2. The organic matter content of the soils ranged from 2% in the sandy soil (Sand) to 68% in the peat rich soil (Omberg).

Soil	Organic matter content	pH
Grassland	15%	6.8
P2	5%	6.2
Sand	2%	8.0
Omberg	68%	6.7

All soils were sieved through a 2,8 mm sieve and stored at 4°C until used.

2.1.1.1 XRF

A portable XRF is an X-ray fluorescence detector. The detector operates by “shooting” X-rays towards a soil sample and the energy that is emitted from the soil is then detected (Carleton College 2012). The emitted energy comes from atoms whose electrons have been ejected and replaced by other electrons from higher energy levels. Different elements are quantified by the different wavelengths that the elements emit.

2.2 Organic matter content, dry weight and pH

Two samples from each soil were weighed in crucibles and put in an oven at 90°C for four hours to estimate moisture content. Thereafter the crucibles were heated at 600°C for five hours. The organic content was then estimated as loss in ignition.

For pH measurements, 1 gram of soil was mixed with 10 ml of distillate water in plastic tubes and then shaken for 10 minutes on a multivortex at maximum effect. The soil suspension was left for 1 hour to settle before pH was measured.

2.3 Respiration

2.3.1 Method

The respiration was measured at three occasions. First the basal respiration was measured. The experiment started with the addition of 1 gram of soil into a 20 ml-vial together with 0.5 ml of the toxicant solution. The samples were sealed, shaken and incubated for 18-20 hours. The CO₂-levels were then measured with a gas chromatograph (GC) and basal respiration was calculated. The caps on the vials were then removed. A mixture of glucose and talcum was added to the soil in order to measure substrate-induced respiration (SIR) (Anderson and Domsch 1978). The samples were loosely sealed, shaken and incubated at room temperature for 30 minutes. Thereafter the caps were removed and the vials were aerated with compressed air for almost 30 seconds per sample. The aeration makes the CO₂ that is created within these 30 minutes disappear. Thereafter the vials were sealed and incubated at room temperature for another 2-3 hours. The CO₂-levels were then measured in the GC and SIR was calculated. The vials were then left to incubate for another 21-23 hours and measured once more as an indication of additional microbial respiration (AMR).

2.3.1.1 Gas chromatograph

A GC separates compounds that are volatile. The chromatograph injects gas from the sample into a column and adds a carrier gas. The gas moves through the stationary phase in the column, where the molecules are separated based on polarity and weight. Molecules are quantified using different detectors, in this case a Thermal Conductivity Detector (TCD), after passing through a 30-m capillary column (HP-5). Peaks were integrated using ChemStat software.

2.4 Leucine incorporation

2.4.1 Method

The method is based on incorporation of radioactive leucine into bacteria to measure protein synthesis (Bååth 1994, Bååth et al. 2001), which is a proxy of bacterial growth. In 1 gram of soil, 0.5 ml of toxicant solution was added and incubated at room temperature for 30 minutes. During this first incubation the bacteria is affected by the toxicant. Then 10 ml distilled water was added to the samples and shaken on a multivortex shaker for 3 minutes. The samples were centrifuged for 10 minutes at 1000 x g. From the supernatant with soil bacteria 1.5 ml was removed into microcentrifuge tubes. 2 µl of 260 nM non-radioactive leucine and 2 µl of 7.8 nM radioactive leucine (L-[4,5-³H]-leucine, Perkin Elmer, UK) were added to the supernatant and incubated at room temperature for 2 hours (Demoling and Bååth 2008). During this second incubation the bacteria incorporates the radioactive leucine into newly synthesised proteins. After the 2-hour incubation the bacteria were killed with 0.75 µl 100% trichloroacetic acid. Cleaning steps and preparation for scintillation followed Bååth et al. (2001). 1 ml of scintillator cocktail (Ultima Gold Packard) was added to each sample and measured on a liquid scintillation counter. The amount of incorporated radioactive leucine was used as a relative estimation of bacterial growth.

2.4.1.1 Liquid scintillation counter

The liquid scintillation counter detects photons that are emitted from the molecules in the scintillator cocktail. The photons are emitted because the beta-particles, which originate from the incorporated radioactive leucine in the bacteria, excite atoms in the

molecules in the cocktail. When these atoms return to their ground state, photons are emitted. There are two photomultiplier detectors in the scintillator, to avoid spurious counts. The results are presented as disintegration per minutes (DPM) after an automatic quench correction.

2.5 Experiments

2.5.1 Experimental setup

A range-finding test was conducted for each toxicant and for each soil with the leucine incorporation method. EC50-values for the toxicants were first collected from the literature to find suitable concentrations and to minimize number of samples. The tested toxicants were copper sulphate (CuSO_4), zinc sulphate (ZnSO_4) and mercuric chloride (HgCl_2). These toxicants were tested in the form of solutions. Cu, Zn and Hg will be used as abbreviations for Cu^{2+} , Zn^{2+} and Hg^{2+} from here on. Approximately eight concentrations were tested for each toxicant in each soil. A new range-finding test was conducted if the result from the tests did not show a proper dose-response curve.

Based on the range-finding test, a 2-fold dilution series of Cu was produced for the comparison of leucine incorporation and respiration. Different binary mixtures of the three toxicants were also created to be able to measure combination effects with the leucine incorporation method. The tested mixtures contained Cu-Zn and Cu-Hg and the setup of the concentrations was similar for both mixtures. The number of concentrations within the series differed between the tests, but all series followed a 2-fold dilution. The following is an example of the setup of concentrations for Cu-Zn in the Grassland soil.

Five dilution series were set up, where the first series only contained Cu, the second had a ratio of 75% Cu and 25% Zn, the third had a ratio of 50/50, the fourth had a ratio of 25% Cu and 75% Zn and the fifth had only Zn (Figure 3). The concentration setup contained 14 samples in each dilution series. For all the dilution series there was a 2-fold dilution between each sample (Figure 3).

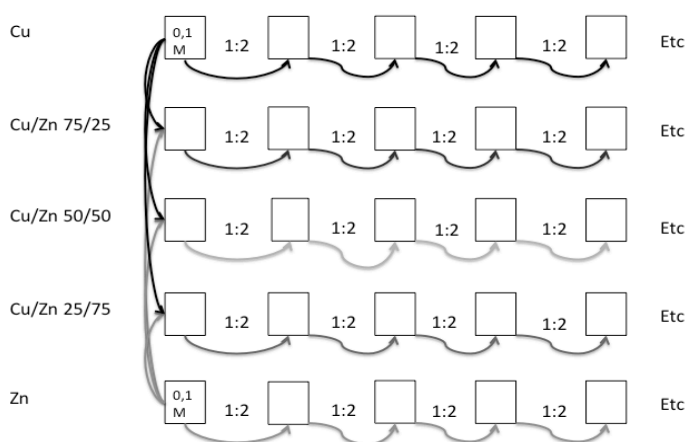


Figure 3. An example of the concentration setup for the five dilution series from the experiment with Cu and Zn. The first sample in the dilution series with only Cu was 0.1 M. The Cu dilution series was diluted 2-fold with distilled water, which the arrows with the “1:2” indicate. The same procedure was conducted for the dilution series of Zn. The highest concentration of both Cu and Zn (0.1 M) was also used for mixing the three binary mixtures, indicated by the vertical arrows, according to their respective proportions. The three binary mixtures were diluted in the same way as the Cu and Zn series.

2.5.2 Individual experiments

2.5.2.1 Comparison of respiration and leucine incorporation

Respiration was tested on the Grassland soil with the toxicant copper sulphate (CuSO_4). A dilution series of the CuSO_4 was created, containing 8 samples and 1 control (only water), where the highest addition was 0.5 ml of 0.1 M CuSO_4 . This concentration is equivalent to 4.6 g Cu/kg dry weight (DW) soil. The respiration rate ($\mu\text{g CO}_2/\text{g soil} \times \text{hour}$) for the three different measurements (basal, SIR and after 21-23 h) was plotted against the concentration. The leucine incorporation was tested on the Grassland soil as well, with a 2-fold dilution series with a starting value of 0.1 M (4.6 g Cu/kg DW).

2.5.2.2 Comparison of incubation time of leucine incorporation

Two experiments were conducted with the leucine incorporation method to compare the incubation time of leucine. A dilution series of CuSO_4 was created and added in 12 soil samples from the Grassland soil. The maximum concentration that was added was 0.5 ml of 0.1 M of the CuSO_4 solution. The first experiment was conducted as previously stated (2.4.1 Method) with an incubation time with leucine of 2 hours. The incubation time with leucine was extended to 18 hours in the second experiment and had in addition to the 12 samples with Cu also 2 controls, where only water had been added.

2.5.2.3 Toxicity of the combination of copper and zinc

The experiment with Cu and Zn was conducted on all four soils to examine combination effects. The tested toxicant solutions were copper sulphate (CuSO_4) and zinc sulphate (ZnSO_4) and the dilutions series was based on the range-finding tests.

2.5.2.3.1 Grassland

This was a full-scale experiment testing the interactions between Cu and Zn, with a concentration setup as previously mentioned (Figure 3), containing a total of 14 samples in each series. The highest concentration that was added was 0.5 ml of 0.1 M of both Cu and Zn, equivalent to 4.6 g Cu/kg DW soil and 4.7 g Zn/kg DW soil.

2.5.2.3.2 P2

P2 was tested with a full-scale concentration setup, containing 14 samples in each series, using up to 70 samples in total. Based on implausible results from the first run, a second experiment was conducted on the P2 soil, but this time the experiment was only carried out on three dilution series, one with only Cu, one with only Zn and the 50/50 series. In the P2-soil the highest concentration 0.1 M of Cu and of Zn was equivalent to 3.8 g Cu/kg DW soil and 3.9 g Zn/kg DW soil, respectively.

2.5.2.3.3 Sand

The Sand soil was studied once, with the same concentration setup as Grassland and P2, containing 14 samples in each series, adding up to a total of 70 samples. 3.5 g Cu/kg DW soil and 3.6 g Zn/kg DW soil was added as the highest concentrations.

2.5.2.3.4 Omberg

The experiment with the Omberg soil, with its high organic matter content, was conducted a bit differently. The experiment still followed the method (2.4.1 Method) and the series still contained 14 samples, adding up to 70 samples in total. The only difference was the dilution series. Based on the range-finding test, the highest concentration that was added was 0.5 ml of 0.8 M of both Cu and Zn. The 0.8 M of Cu and of Zn was equivalent to 67 g Cu/kg DW soil and 69 g Zn/kg DW soil.

2.5.2.4 Toxicity of the combination of copper and mercury

The experiment with Cu and Hg was only conducted on the Grassland soil. 0.5 ml of 0.1 M was the highest concentration that was added of the Cu solution and 0.5 ml of 1 mM (0.15 g Hg/kg DW soil) was the highest concentration of the HgCl₂ solution. Each concentration series contained 16 samples, adding up to a total of 80 samples.

2.6 Calculations

The effect concentration of 50% (EC50) was chosen to evaluate potential combination effects. For the leucine incorporation this meant that EC50 is when 50% of the protein synthesis (which is proportional to bacterial growth) in the bacteria community is inhibited. The EC50 for respiration is when the respiration is reduced by 50%.

The result from the leucine incorporation and respiration experiments was plotted in the program MATLAB (R2011a). To create the dose-response curves and to be able to calculate EC50-values, the following sigmoid equation was used:

$$m_3 / (1 + e^{m_2 * (x - m_1)}) \quad (\text{Eq. 5})$$

m_3 is the maximum value of bacterial growth or respiration with no toxic effect, m_2 is the slope of the dose-response curve and m_1 is the EC50-value, expressed as concentration in a logarithmic scale. The dose-response curves are shown in Appendix 1. EC50-values for the mixtures of Cu-Zn and Cu-Hg are shown in Appendix 2. TU for the individual and the whole mixtures were calculated based on the EC50-values and plotted in isobolograms, to visualise potential combination effects. In addition the TUs were plotted in a graph with 95% confidence interval (95%CI). This made it possible to evaluate if there was any synergistic or antagonistic effect, based on the concentration addition model. Overlapping 95%CI indicates that the mixture is strict additive, not synergistic or antagonistic. The 95%CI values were calculated in MATLAB at the same time as the dose-response curves were created.

3. Results

3.1 Comparison of respiration and leucine incorporation

The respiration measurements showed that the basal respiration was highest in the samples with the highest amounts of Cu, almost 2 times higher than the respiration from the control (Figure 4). The SIR respiration followed no clear trend (Figure 4), while the measurement after 21-23 hours (mostly indicating AMR) gave a clear dose-response curve to the toxicant (Figure 4, Figure 5). The respiration after 21-23 hours thus showed the opposite result from the basal respiration, the respiration in the control was almost 4 times higher than in the sample with 0.1 M CuSO₄.

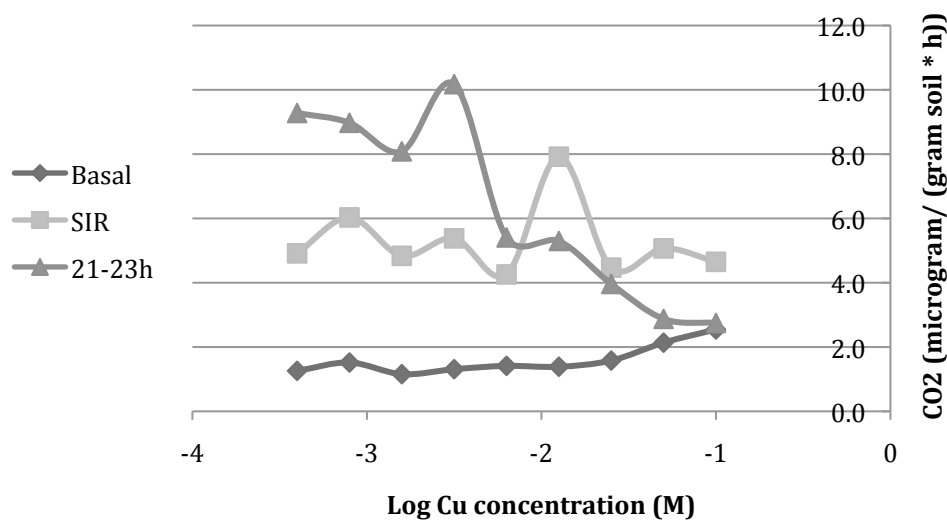


Figure 4. The three respiration measurements of Grassland-soil with different copper additions. The highest amount of toxicant that was added was 0.5 ml of 0.1 M CuSO₄. The concentration was then diluted 2-fold.

However, when comparing with the leucine incorporation, the respiration measure after 21-23 hours was inferior. Not only did the respiration not decrease to almost zero at the highest Cu addition, which was the case for leucine incorporation (Figure 5), the EC50-value was also slightly higher than for the leucine incorporation test (Table 3).

Table 3. EC50-values for the Grassland-soil from the respiration after 21-23 h (indicating AMR) and leucine incorporation.

Toxicant	Respiration	Leucine incorporation
Cu (mM)	16	13
Cu (mg/kg DW)	750	590

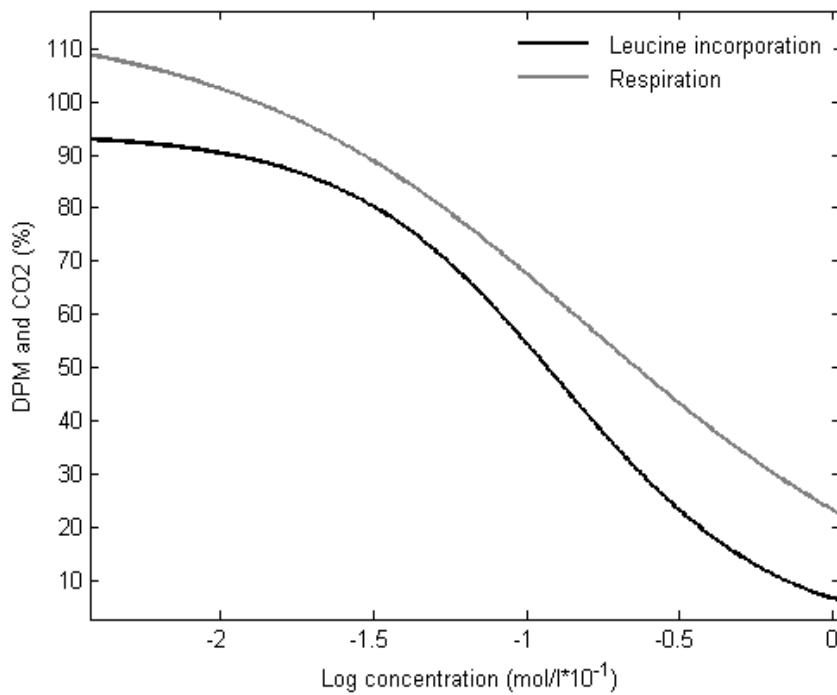


Figure 5. Comparison of Cu inhibition on respiration after 21-23 h (indicating AMR) and leucine incorporation.

3.2 Comparison of incubation times of leucine incorporation

Two different incubation times were tested for the incorporation of leucine, 2 hours and 18 hours, in the Grassland soil (Figure 6). The tested toxicant was CuSO_4 .

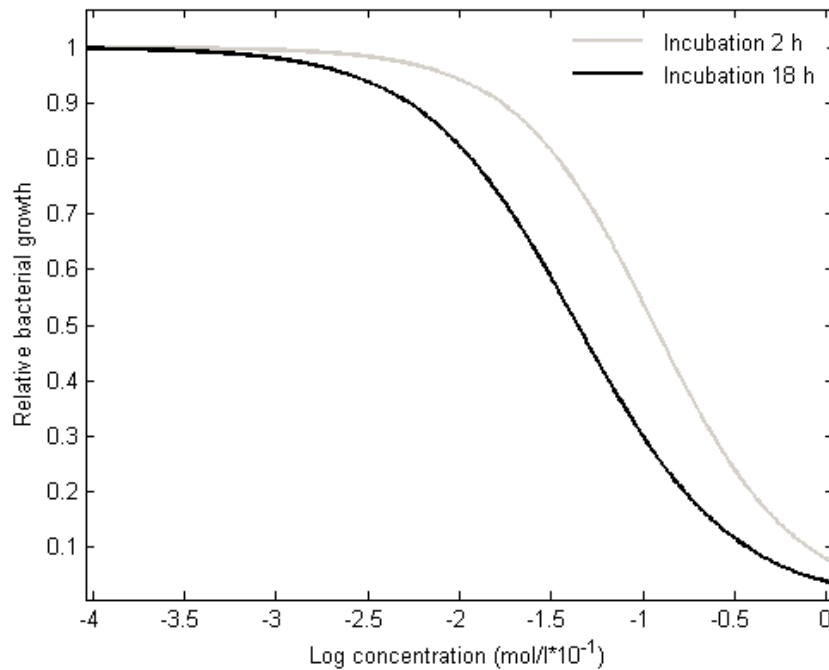


Figure 6. Dose-response curves for Cu toxicity of two different incubation times of leucine.

The EC50-value for the 2-hour incubation was 530 mg Cu/kg DW soil, while the 18-hour incubation gave the EC50-value of 200 mg Cu/kg DW soil. Since both methods gave adequate dose-response curve, the shorter period was used throughout the study.

3.3 Toxicity of the combination of copper and zinc

3.3.1 Grassland

The TU for the different toxicants were summed for a total TU for the mixture (Table 4). The TU-values were also plotted in an isobologram (Figure 7), suggesting that the mixture toxicity followed the straight line indicating strict additive effect, without any antagonistic or synergistic effects.

Table 4. TU for the different substances within the mixtures including the sum for the mixtures.

Mixtures	TU (Cu)	TU (Zn)	Sum TU (Cu+Zn)
Only Cu	1	0	1
Cu:Zn 75:25	0.57	0.18	0.76
Cu:Zn 50:50	0.45	0.43	0.88
Cu:Zn 25:75	0.29	0.83	1.1
Only Zn	0	1	1

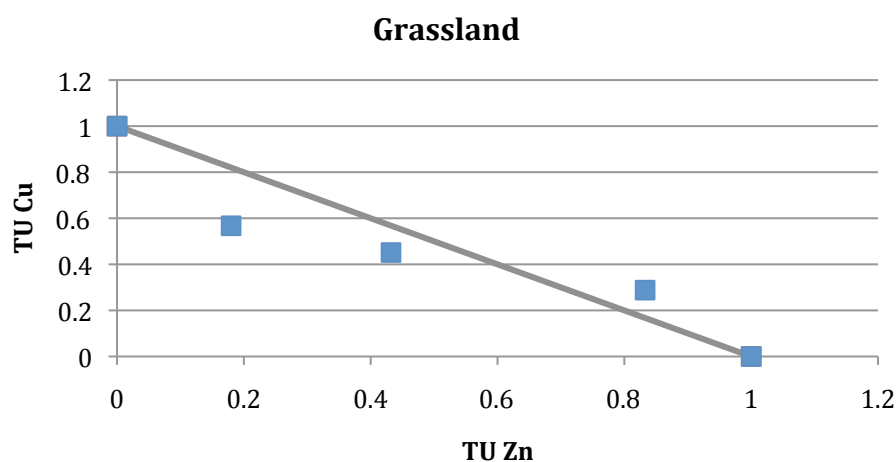


Figure 7. Isobologram with TU of Cu and Zn.

The calculated TUs were also plotted with their 95% confidence interval (95%CI) (Figure 8). If the 95%CI of the three binary mixtures overlaps the 95%CI of the two solutions with only Cu and Zn, there will be an indication of statistical difference, i.e. indicating synergism or antagonism. However, the 95%CI of the binary mixtures overlapped with both Cu and Zn, indicating no synergistic or antagonistic effects of Cu and Zn in the Grassland soil. It was strict additive following the CA concept.

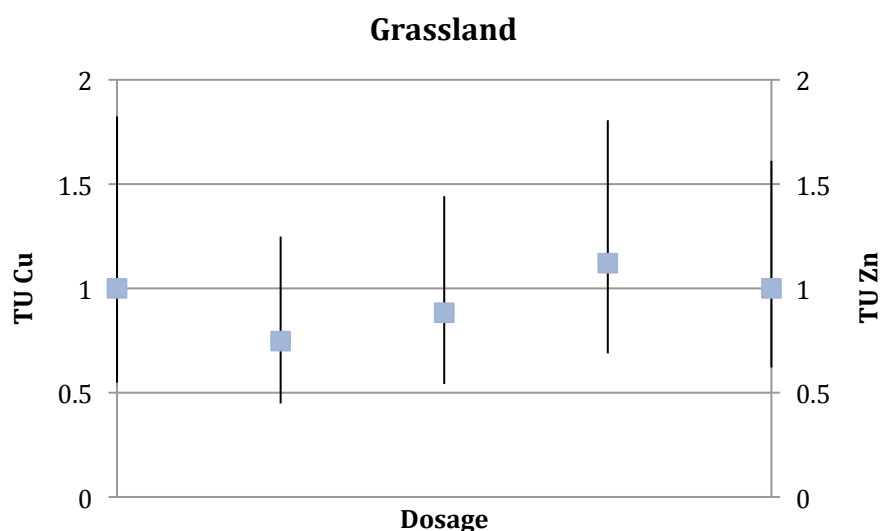


Figure 8. TU with 95%CI for the five different dilution series of Cu and Zn in the Grassland soil.

3.3.2 P2

There was one mixture (Cu:Zn 50:50) that did not follow a proper dose-response curve (Appendix 1). The experiment was therefore repeated to be able to evaluate if something went wrong the first time. By comparing the EC50-values of Cu from the first and second experiment (Appendix 2), it is clear that there was a large difference, the first EC50-value was almost half of the second. EC50 for Zn, on the other hand, was similar in both experiments. Even though the results from the first experiment seemed to be a bit out of order, they are described below.

3.3.2.1 First experiment on the P2 soil

The isobologram, obtained from the TU-values (Table 5, Figure 9), suggested no interactions. It was not possible to calculate the whole 95%CI for the 50/50 mixture, only the lower part (Figure 10). This was due to the odd result. There were no synergistic or antagonistic effects, only strict additive effect between Cu and Zn in the P2 soil, based on the overlapped 95%CIs.

Table 5. TU for the different substances within the mixture and also the sum of the TU.

Mixtures	TU (Cu)	TU (Zn)	Sum TU (Cu+Zn)
Only Cu	1	0	1
Cu:Zn 75:25	1.15	0.19	1.34
Cu:Zn 50:50	0.88	0.45	1.33
Cu:Zn 25:75	0.31	0.47	0.78
Only Zn	0	1	1

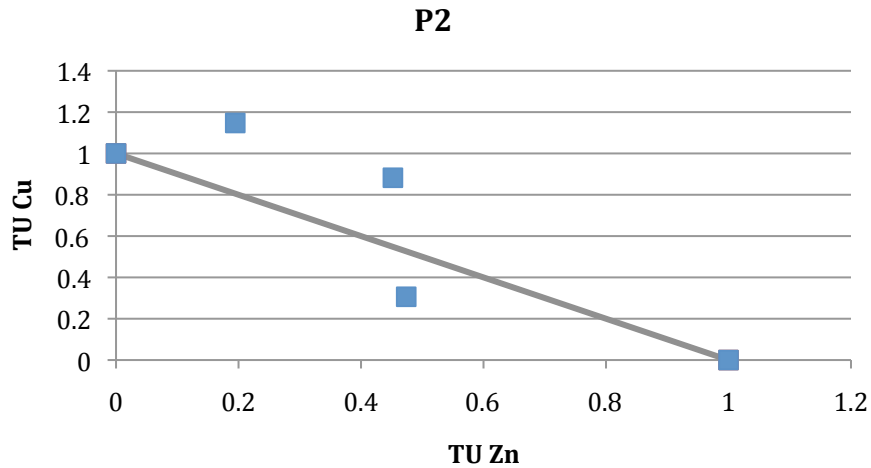


Figure 9. Isobologram with TU of Cu and Zn.

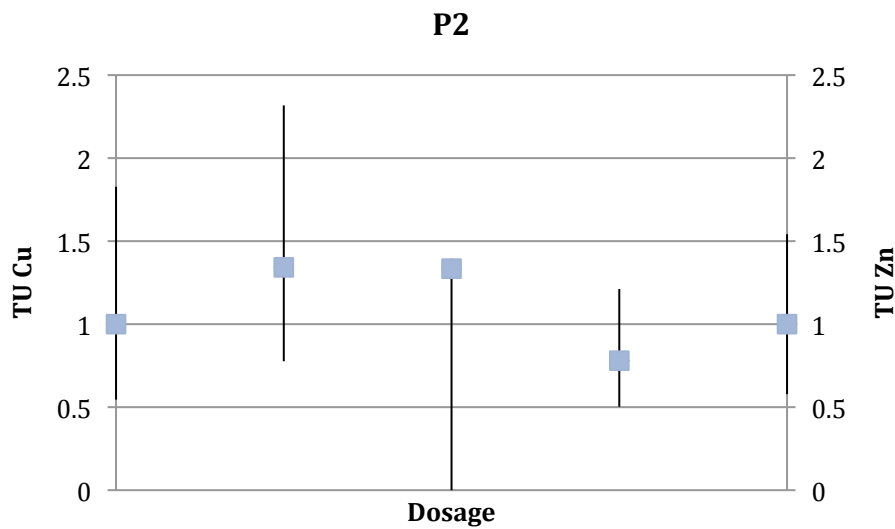


Figure 10. TU with 95%CI for the five different dilution series of Cu and Zn in the P2 soil.

3.3.2.2 Second experiment on the P2 soil

The EC50-value from the first experiment with the P2 soil was 7 mM for Cu, 13 mM for Zn and 6 mM for the 50/50 mixture. The EC50-value for the second experiment was 13 mM for Cu, 15 mM for Zn and 4 mM for the 50/50 mixture.

The TU for the Cu and Zn mixtures in the second experiment were summed and plotted in an isobologram (Table 6, Figure 11). The TU of the binary mixture with 50/50 was situated underneath the line, indicating synergism. However, the TUs were also plotted in a graph with 95% CIs (Figure 12), which showed that the mixture of Cu and Zn was strict additive.

Table 6. TU for the different substances in the mixtures and also the sum of the TU.

Mixtures	TU (Cu)	TU (Zn)	Sum TU (Cu+Zn)
Only Cu	1	0	1
Cu:Zn 50:50	0.31	0.28	0.60
Only Zn	0	1	1

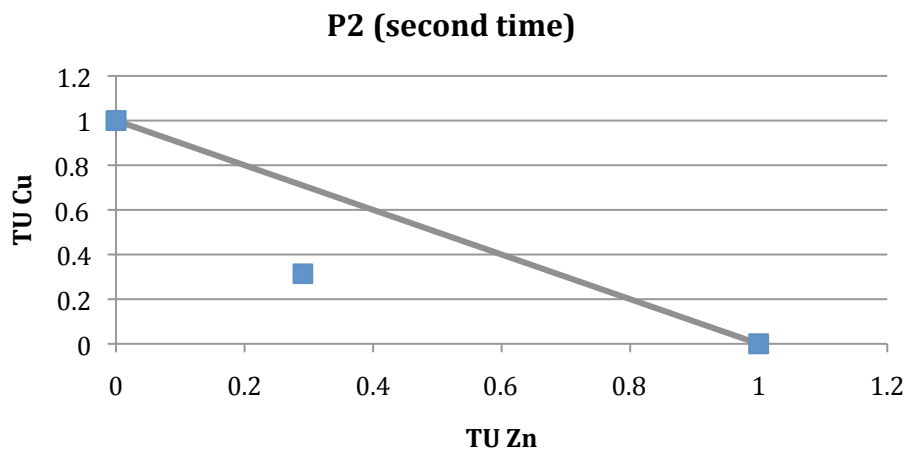


Figure 11. Isobologram with TU of Cu and Zn.

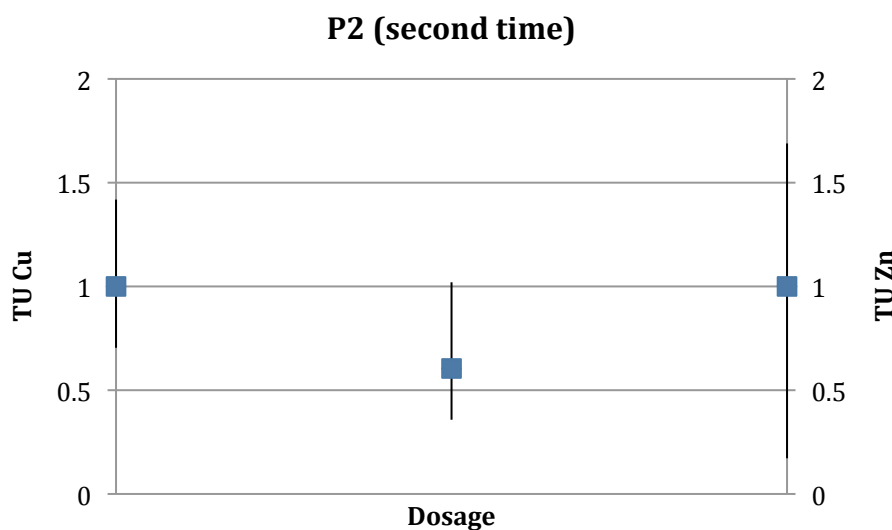


Figure 12. TU with 95%CI for the three different dilution series of Cu and Zn in the P2 soil (second time).

3.3.3 Sand

The TU was calculated, added together and plotted in an isobologram (Table 7, Figure 13). The result showed that Cu and Zn was strict additive in the Sand soil (Figure 14), since the 95%CIs overlapped.

Table 7. TU for the different substances in the different mixtures and the sum of TU.

Mixtures	TU (Cu)	TU (Zn)	Sum TU (Cu+Zn)
Only Cu	1	0	1
Cu:Zn 75:25	0.85	0.15	0.99
Cu:Zn 50:50	0.80	0.42	1.2
Cu:Zn 25:75	0.62	0.98	1.6
Only Zn	0	1	1

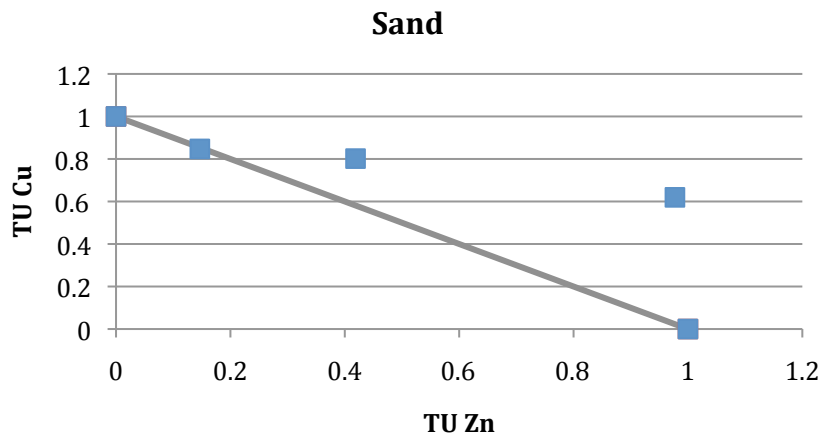


Figure 13. Isobologram with TU of Cu and Zn.

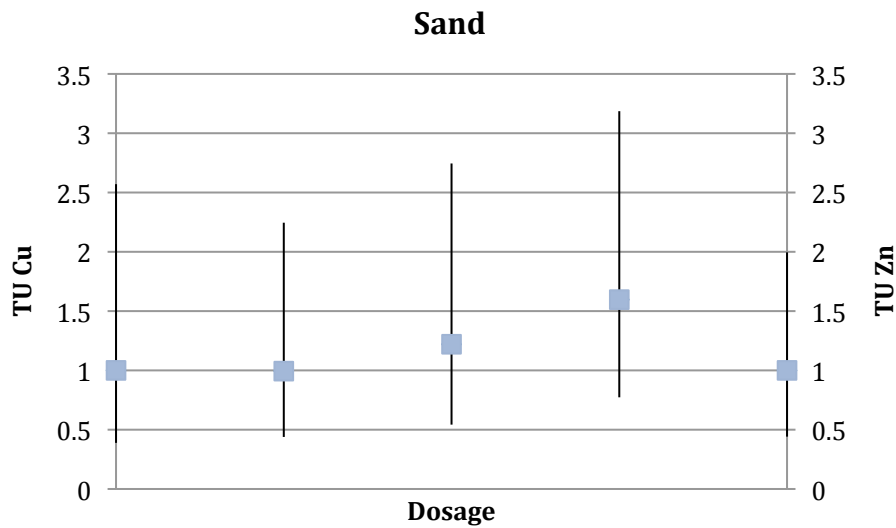


Figure 14. TU with 95%CI for the five different dilution series of Cu and Zn in the Sand soil.

3.3.4 Omberg

TU for the mixtures were calculated and plotted in an isobologram (Table 8, Figure 15). The TUs for the three binary mixtures were all situated underneath the line, indicating synergism. However, the 95%CI of all three binary mixtures of Cu and Zn overlapped and thereby clearly showed no synergistic or antagonistic effects (Figure 16).

Table 8. TU for the different substances in the mixtures and also the sum of TU.

Mixtures	TU (Cu)	TU (Zn)	Sum TU (Cu+Zn)
Only Cu	1	0	1
Cu:Zn 75:25	0.56	0.16	0.72
Cu:Zn 50:50	0.51	0.35	0.87
Cu:Zn 25:75	0.29	0.48	0.77
Only Zn	0	1	1

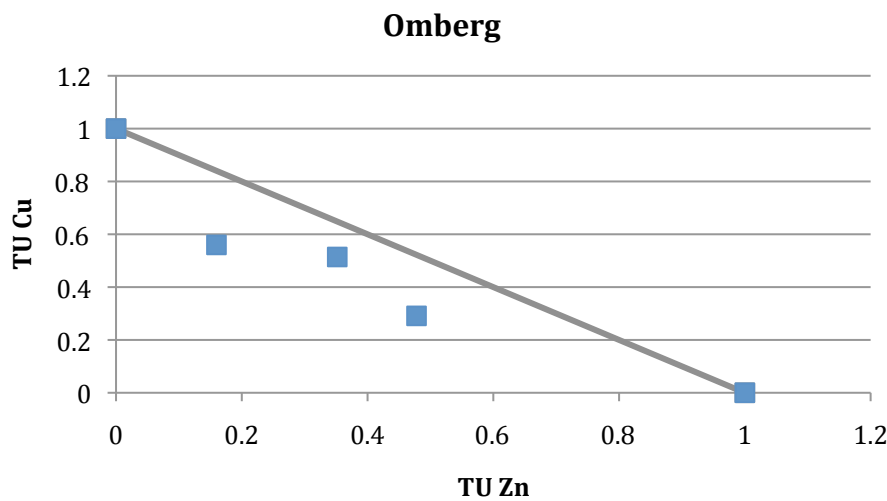


Figure 15. Isobologram with TU of Cu and Zn.

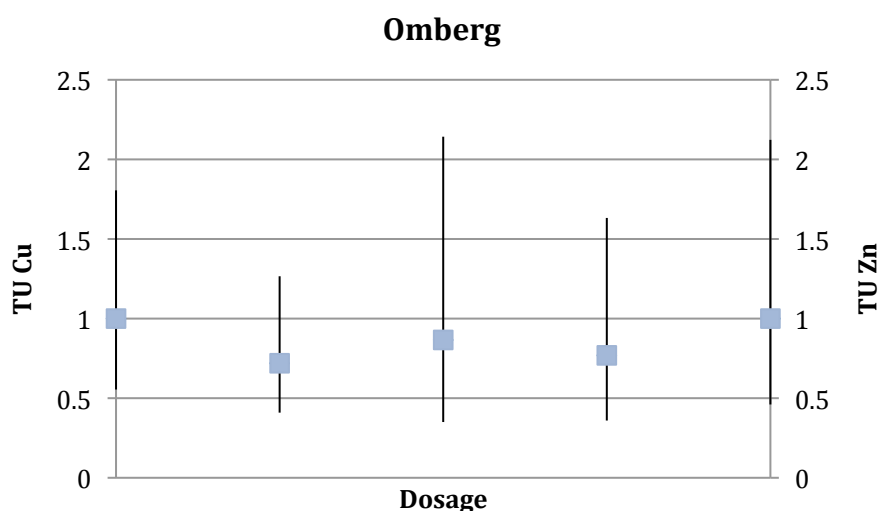


Figure 16. TU with 95%CI for the five different dilution series of Cu and Zn in the Omberg-soil.

3.4 Toxicity of the combination of copper and mercury

The dose-response curve for the five dilutions series with Cu and Hg are shown in Appendix 1. The individual TUs for the different mixtures were added together, creating sum of TUs (Table 9). The TUs was plotted in an isobologram, where all three binary mixtures were below the line, indicating synergism (Figure 17). However, Figure 18 shows that there were no synergistic or antagonistic effects, based on the overlapping 95% CIs of the binary mixtures and the mixtures with only Cu and Zn.

Table 9. TU for the Cu and Hg in the different mixtures and also the sum of TU.

Mixtures	TU (Cu)	TU (Hg)	Sum TU (Cu+Hg)
Only Cu	1	0	1
Cu:Hg 75:25	0.29	0.32	0.61
Cu:Hg 50:50	0.17	0,58	0.75
Cu:Hg 25:75	0.078	0.78	0.86
Only Hg	0	1	1

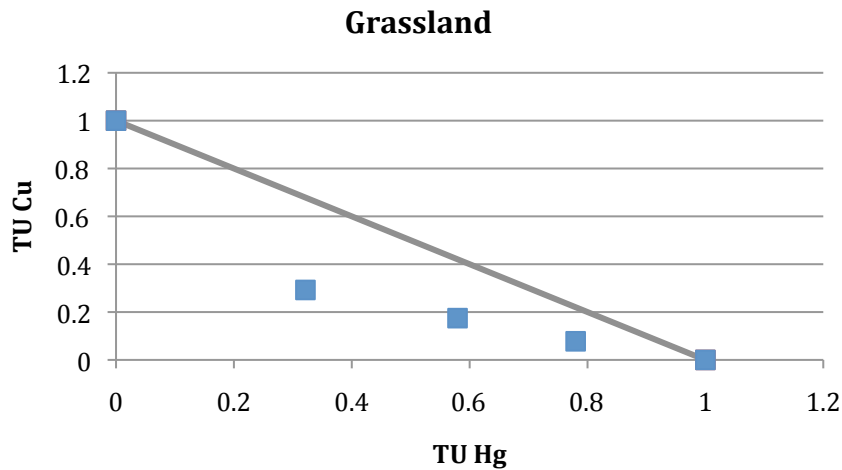


Figure 17. Isobologram with TU of Cu and Hg.

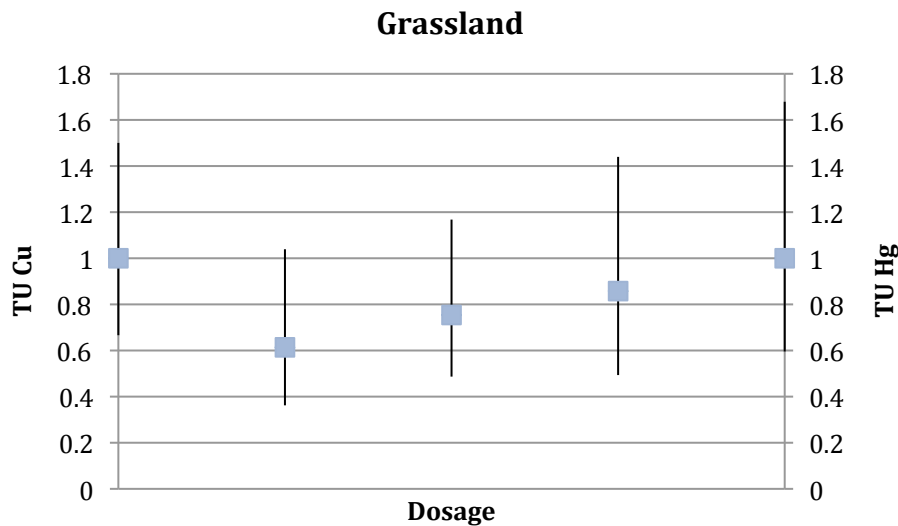


Figure 18. TU with 95%CI for the five different dilution series of Cu and Hg in the Grassland soil.

3.5 The relationship between organic matter content and metal toxicity

All four soils were tested with Cu and Zn separately. With increasing organic matter content the EC50-values of both Cu and Zn increased ($p < 0.05$ for both, Figures 19 and 20). In all of these soils, the EC50-value of Cu was lower than the EC50-value of Zn (Table 10). In the soil with 2% organic matter (Sand), the EC50-value of Cu was almost half of that for Zn. In the soil with 68% organic matter content (Omberg), the EC50-value for Zn was almost 1.5 times higher than Cu. There was no statistically significant relationship between the EC50-values for Cu or Zn and the pH ($p > 0.05$).

Table 10. The guideline values for less sensitive land use from the Swedish EPA (2009b), which should provide a 50% protection level of all soil living species. Also the EC50-values from the leucine incorporation measurement from the four soils, with different organic matter content.

Metals	Less sensitive land use	Grassland	P2	P2 (second time)	Sand	Omberg
Cu (mg/kg DW)	200	540	260	510	75	4800
Zn (mg/kg DW)	500	580	520	570	150	7200
Hg (mg/kg DW)	10	7.0				

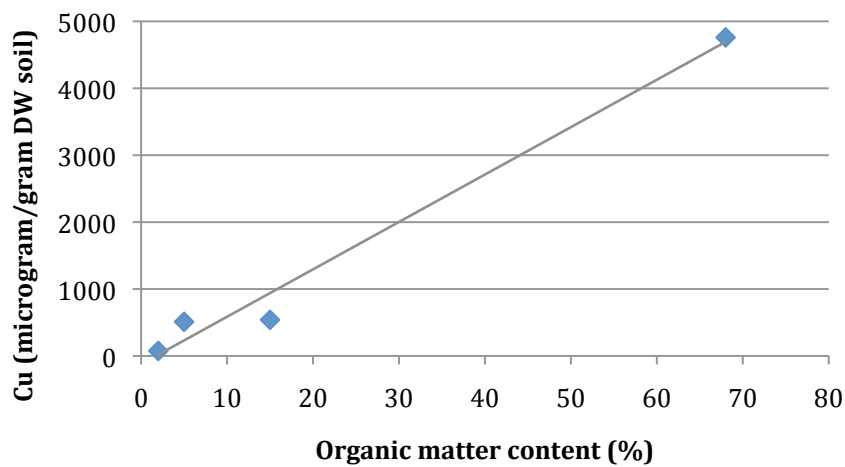


Figure 19. EC50-values for Cu in the four different soils plotted against the soil organic matter content.

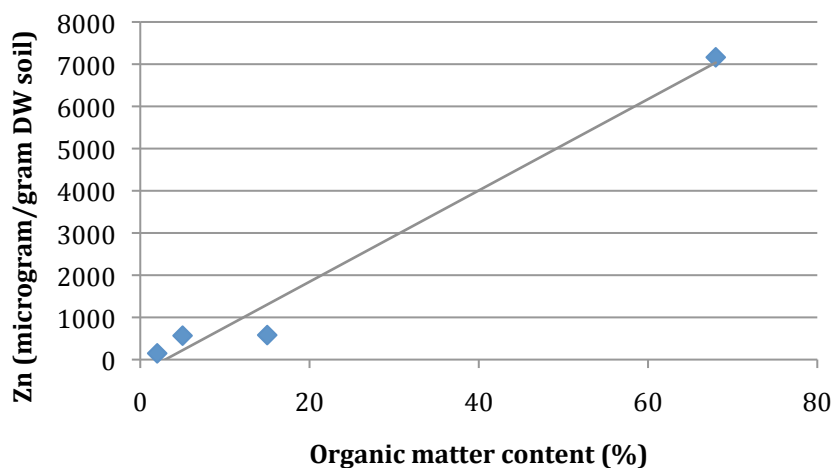


Figure 20. EC50-values for Zn in the four different soils plotted against the soil organic matter content.

4. Discussion

4.1 Combination effects of metals

The results from this study indicate that Cu and Zn are strictly additive, independent of soil type, based on the CA concept. The same results were obtained for Cu and Hg, but they were only tested in one soil type. As previously stated, not much ecotoxicological research has been done in terrestrial systems, especially not with the present method (leucine incorporation), which means that it is difficult to compare results. However, there exist a few studies on combination effects that employed different methodologies. For example, Fulladosa et al. (2005) showed combination effects between several metal mixtures using the luminescent bacteria *Vibrio fischeri* and the CA concept. The end-point was reduction of light from the bacteria. They concluded that there was a strict additive effect between cobalt(Co)-lead(Pb), Cu-cadmium(Cd) and Cu-Zn, a synergistic effect between Co-Cu and Zn-Pb, an antagonistic effect between Co-Cd, Cd-Zn, Cd-Pb and Cu-Pb. My results were similar with a strict additive effect between Cu-Zn for soil bacterial growth compared with Fulladosa et al. (2005).

Fulladosa et al. (2005) stated that the results of combination effects appeared to be dependent on soil properties, physiological factors, concentrations of the metals etc. Chaperon and Sauvé (2008) also discussed that the physicochemical parameters of the environment and different target organisms could affect the result and be the reason for the huge variation between studies. An et al. (2004), who studied combination effects between metal mixtures on cucumbers, wrote that physiological parameters of the soil and the experiment setup likely made Cu more available to the plant than the other metals (Cd and Pb). Also, Jonker et al. (2004) wrote about the properties of the soil and its effect on the combination effects. They investigated the sorption of certain metals in the soil and the relationship to the combined effects of the mixtures of metals. They could not find any clear, general relationship. However, they stated that the synergistic effects that showed up between Cu and Zn could be explained by decreased sorption due to interactions between the metals. Since there are several different parameters affecting toxicity and the combination effects in the soil, the demand for information about the soil parameters and the experimental setup increases to be able to consider which parameters are affecting the toxicity the most. However, my results showed that there was no difference in combination effects for Cu-Zn between the four soils, despite the high variation in organic matter content.

According to Backhaus and Faust (2012) it is still uncommon with studies in terrestrial systems. There is more research within aquatic systems. In an article by Norwood et al. (2003), where experiments of different metal mixtures were summarized, all studies were from aquatic systems. They were able to compare 134 tests of binary metal mixtures. Of these, 47 % showed a less than additive effect, 34 % were more than additive and only 19 % strictly additive. There were 21 tests with the binary mixture of Cu-Zn. Of these, 11 were less than, 1 was strict and 9 were more than additive. There were no binary mixtures with Cu and Hg but four tests with Hg and Zn. Of the four tests, two were found to be less than additive and two more than additive. Based on these results it seems Cu and Zn are more likely to be synergistic or antagonistic than strict additive in aquatic environments. However, it is hard to evaluate the results from Norwood et al. (2003) due to several factors. For example, the information on which organisms or which media that were tested in which mixture was not possible to

retrieve from the article. In a review by Chapman (2008) about metal mixtures, it is stated that predictions of combination effects are difficult to make, even if it is possible to consider the physiological parameters in the surroundings that may affect the toxicity, due to the interactions between metals and other substances. To be able to actually make a sound prediction of the interactions Chapman (2008) recommends site-specific testing.

To assess combination effects by conducting full-scale experiments for all possible mixtures, even with the time-efficient method of leucine incorporation, would take extreme amounts of time. The CA and IA concepts are used to overcome this problem. It is possible to evaluate mixtures toxicity with these equations, and the only values that are needed are the EC_x-values for each of the substances and their individual concentration/fraction in the mixture. It is also possible to use the CA concept with its TUs in another way, compared with determining synergism and antagonism. The TUs can determine critical levels of a mixture without full-scale toxicity tests. For example, the strict additive effect of Cu and Zn observed in my study, means that a soil with a mixture of 100 mg Cu/kg DW soil and 200 mg Zn/kg DW soil, that has to be risk assessed to make sure that 50% of a certain specie can cope and function, can be assessed simply by summing the concentrations in TUs. The concentrations should be divided by their own representative EC₅₀-values, creating TUs, which then can be summed. If the sum of TUs is less than 1, the level is not considered to cause critical damage, otherwise the soil has to be remediated. The use of TUs to predict the toxicity of a mixture has been done for a long period of time. Sprague (1970) described how TUs could be used when studying toxic effect of mixtures on fish, but in that study the end-point was death (lethal concentration, LC₅₀). However, there is a risk of overestimating the toxicity, if very low concentrations are summed. Sprague (1970) gave an example of what would happen if all substances with very low concentrations in the oceans would be divide by their LC₅₀ and summed. It could lead to the wrong conclusion that the ocean would be too toxic for the fish to survive.

Backhaus and Faust (2012) described a model where both CA and IA should be used to assess chemical mixtures, to reduce the risk of over- or underestimating the toxicity. However, the model is only functional if the mixture follows the concept of having no interactions between the substances within the mixture, thus not having any synergistic or antagonistic effects. If there would be mixtures with these effects they would need to be handled individually, with a case-by-case approach. However, as Backhaus and Faust (2012) stated, it is not common that synergism or antagonism occurs, which also were the results from my study. To use both CA and IA together or to compare them gives a better prediction of the toxicity of the mixture, giving a “prediction window” as Kortenkamp et al. (2009, p.107-108) stated. Kortenkamp et al. (2009, p.87) also stated that the predictive power of the CA concept is high, but when it comes to metal mixtures the predictions and the observed results seem to deviate more often. I did not know the exact mode of action for the three metals in bacteria at the start of this study, which led to the use of CA, which is commonly used as default model. In hindsight, the IA concept should have been used in parallel, to improve the evaluation of the combination effects.

The assessment of combination effects is further complicated by the fact that several metals are essential to organisms (Backhaus and Faust 2012, Kortenkamp et al. 2009, p.89-90). Chaperon and Sauvé (2008) tested combination effects of Cu, Cd and Pb on soil enzymes, and concluded that the mixtures of the metals indicated antagonism, using

both CA and IA. They showed that the activity of free ions of Cu and Pb in a mixture decreased compared to the individual tests of each individual toxicant. These results also indicated that the enzymes were stimulated by the combination of Cu and Pb. To use the IA concept on a mixture that stimulates the target is a problematic issue, because of the assumption that there only is an effect-scale of 0%-100%. If the effect increases over 100 % the IA concept becomes inapplicable (Backhaus et al. 2004, Backhaus et al. 2011). Also the CA concept can be problematic to apply, due to the assumption that the substances act only as dilutions of each other, implying that they should have similar dose-response curves (Kortenkamp et al. 2009, p.89-90). According to Kortenkamp et al. (2009), the dose-response curves need to be similar, though it does not matter if they are not parallel. In my case the dose-response curves were very similar, which made it possible to use the CA concept (Appendix 1). However, there are some who believe that if the curves are not parallel the CA concept will not be applicable (Bosgra et al. 2009). Kortenkamp et al. (2009, p.18) discusses this belief, and states that the CA equation does not assume or demand this.

The use of the combination effect concept is not commonly used in risk assessment of contaminated sites, even if the Swedish EPA states that it is an aspect to consider. The values that the metal concentrations in the field are compared with are based, as previously stated, on experiments using the “one chemical at a time” approach. With the knowledge that the toxicity of different chemicals often are additive, this approach is foolish. My study showed that mixing half of the needed amount to reach EC50 of both Zn and Cu would give the same effect as a solution with only Zn or Cu would give. Assessing a mixture of 10 substances with low individual concentrations, below guideline values, and stating that the mixture will not do any substantial harm, would probably be an incorrect assumption. There are several studies that have shown that mixtures with low concentrations of the components exert a higher effect than expected from the individual substances (Walter et al. 2002, Faust et al. 2003). For example, Faust et al. (2003) mixed 16 substances with the individual concentration equal to EC1 (1% inhibition) and the whole mixture had an inhibition of around 18%.

4.2 Influence of the organic matter content on the EC50-values

My study showed that organic matter content is an important parameter that has to be considered when assessing risk in connection to metal toxicity, because increased organic matter content in the soils increased the EC50-values of both Cu and Zn (Figures 19 and 20). This does not mean that the bacteria were more tolerant in the soils with high organic matter content, but organic matter will affect availability. Organic matter content can bind metals in two ways, by chemical bonding (complex formation) and by electrostatic bonding (cation exchange) (Babich and Stotzky 1980), making the metals less available to the bacteria.

The EC50-value for the Sand soil with 2% organic matter was 75 mg Cu/kg DW soil (Table 10), while the EC50 for P2 (5% organic matter content) was around 500 mg Cu/kg DW soil, almost the same as in the Grassland soil (15% organic matter content). It seemed a threshold existed between soils with 2% and 5% organic matter. However, one aspect that I did not examine fully was the clay content. Clay can also bind metals by electrostatic bonds as organic matter does. However, I found it rather unlikely that the clay content would be the only explanation to the different values between Sand and P2, and the similar values for P2 and Grassland. None of the three soils seemed to have that high clay content; it was almost impossible to roll them in to rolls, which is an easy test

for indicating high clay content. To increase the certainty of the clay content, a proper test should, however, be performed. It would also be interesting to conduct tests on soils that have an organic matter content ranging from 15% to 68%, to evaluate (and validate) if there is a linear relationship between the EC50-values and the organic matter content.

When calculating guideline values for a risk assessment an organic matter content of 2% is normally used (Swedish EPA 2009a). Comparing the Sand soil (2% organic matter content) with the guideline values showed that the EC50-values for both Cu and Zn were lower than the guideline values (Table 10). However, the guideline values for less sensitive land use are protecting 50% of all species in the soil, not only bacteria. Using the respiration measurements instead may give a broader view of the soil, because it includes both bacteria and fungi. However, even if the relationship between the respiration measurements and leucine incorporation from the Grassland soil were extrapolated to the Sand soil, it would still suggest that the EC50-values for Cu and Zn would be lower than the guideline values. Using the guideline values for less sensitive land use would therefore probably reduce the amount of and the diversity of bacteria in the soil.

However, there is no way of knowing if the guideline values really sustain the ecological function and protect 50% of all species, as recommended by the Swedish EPA, based on this study. The guideline values are based on data from several different sources, for example the American EPA, the Dutch National Institute for Public Health and Environment and the Canadian Council of Ministers of the Environment (Swedish EPA 2009b). To compare an acute, short-term test to guideline values that hopefully are based on well-grounded data is not recommended. Still, there are a lot of studies that conduct short-term, acute tests in laboratories that tries to relate their results to regulations and levels of toxicants that occur in the environment (Giller et al. 1998). Thus, the only clear conclusion that was possible to make considering the EC50-values was that the organic matter content is an important factor to assess, and that more test in soils with low organic matter and different clay content are needed.

4.3 Ecotoxicological methods

4.3.1 Design of ecotoxicological experiments

A parameter to strive for is to conduct ecotoxicological tests on a higher level than single species tests, for example on a community or ecosystem level (Backhaus and Faust 2012, Korthals et al. 2000). For example, Korthals et al. (2000) were able to study nematode communities and populations in a long-term experiment with Cu and Zn. They stated that they saw additive and less than additive effects. To know the effects of certain substances on a community level is more valuable information than on an individual level, because of the impact it may have on an ecosystem level. This is one of the strengths of the leucine incorporation method; it examines growth of the whole bacterial community, i.e. not on a single specie level.

Ecotoxicological studies of metals can be generalised to three categories: (1) laboratory studies, (2) field studies and (3) environmental monitoring (Giller et al. 1998). There are both advantages and drawbacks to all of the above-mentioned categories. In this study only laboratory studies were conducted and it is also the most applied form, but the ability to give a realistic picture of the real environment may be low (Giller et al. 1998). Short-term, acute toxicity test are not recommended in general, because of the low

resemblance to real life situations. However, this often occurs in a laboratory, toxicants are added in form of solutions, often at one occasion and has extreme end-points such as death. This study was partly conducted in that manner, adding toxicants in solution at one occasion, but the end-point was not death, instead it was inhibition of growth, which should be a more sensitive end-point.

A chronic test is conducted over a whole lifespan of the test organism measuring less extreme end-points, e.g. reproduction or growth (Swedish EPA 2009a). There are also sub-chronic tests, which are shorter than the chronic and examine less extreme effects than acute tests, e.g. behavioral changes or enzyme activity (Swedish EPA 2009a). One should always strive to conduct tests that are over a longer period of time, because of the higher resemblance to real events. However, an acute, short-term test is more time and money efficient. In this study it is probably not incorrect to use an acute toxicity test, due to the main objective to investigate synergistic and antagonistic effects of Cu-Zn and Cu-Hg in soil. The benefits with leucine incorporation are, as previously stated, that it measures on a community level and also that conducting the test over a short period of time results in clear dose-response patterns. If the test would be conducted over a much longer period of time, clear dose-response relationship would probably not be detected due to interactions between and among bacteria and fungi, where the latter group could replace bacteria (Rajapaksha et al. 2004). Furthermore, with time after the metal addition, bacterial growth will recover, due to emerging tolerant communities. This may even result in metal polluted soils having higher growth than the controls, due to the input of substrate from microorganisms killed by the metals (Díaz-Raviña and Bååth 1992). Without dose-response curves it would be impossible to calculate effect concentrations and thereby it would be unfeasible to use the CA or IA concept.

4.3.2 Respiration and leucine incorporation

The only respiration measurement that showed a negative effect of metal concentration, in the meaning that the respiration decreased, was the measurement after 21-23 hours, indicating AMR (Figure 4). The basal respiration showed increased values in the samples with highest concentration of Cu addition, while SIR showed no clear trend. Basal respiration has been found to be less sensitive than measurements of growth rate (Bååth 1992). The basal respiration probably increased because of the deaths of some microorganisms, due to metal toxicity, leading to degradation and increased amount of substrate for the surviving organisms. Respiration after 21-23 hours after the substrate had been added appeared to be a stable measurement without lacking in sensitivity. For example, Witter et al. (2000) used the increased respiration rate after SIR, similar to my 21-23 hour measurement, to investigate metal tolerance by inhibition of the respiration rate.

Respiration is a standardised ecotoxicological test and can be conducted both as short-term and long-term tests. For example, OECD (2000) standardization of substrate-induced respiration measurements for long-term effects is conducted over a 28-day period, which will allow growth on the added glucose and thus resembles my 21-23 hour measurement. However, I only used a short-term substrate-induced respiration measurement, which made it possible to compare the leucine incorporation and respiration measurements. There are not many studies where both respiration and leucine incorporation tests have been applied, but there are studies where both respiration and thymidine incorporation (another growth rate measurement) have been used and compared. Witter et al. (2000) used thymidine incorporation and respiration

after glucose addition to study metal tolerance and showed that the two dose-response curves were rather similar, even though thymidine incorporation was measured for a much shorter period of time.

The comparison of leucine incorporation and respiration showed that it is possible to use leucine incorporation as an ecotoxicological method similar to or even better than standardized respiration measurements. The results showed that respiration after 21-23 hours gave a less sensitive value than leucine incorporation, not giving total inhibition even at the highest Cu concentration (Figure 5). This could be because enzymes remain active, even though the microorganism dies in the soil, still producing CO₂. Another reason could be that respiration measurements target all microorganisms, while leucine incorporation only focuses on bacteria (Bååth 1992). This may be a reason for the higher sensitivity of the leucine incorporation method, since bacteria are regarded as more metal sensitive than fungi (Rajapaksha et al. 2004).

The extended incubation time of leucine incorporation to 18 hours gave a significantly lower EC₅₀-value than the normal incubation time of 2 hours. This result could indicate that the bacteria were more sensitive than the 2-hour incubation implied. However, this was probably not the case; instead the lower EC₅₀-value was most likely due to the increased growth rate in the samples with low Cu concentration. Bacteria have a lag period, where the growth rate stays constant before it can utilize substrate released during the centrifugation-homogenization step and growth increases exponentially (Rousk and Bååth 2011). Studies have shown that leucine incorporation (and thymidine incorporation) has a constant incorporation rate for at least 4 hours after adding the substances, in room temperature, and even up to 24 hours at 5°C (Rousk and Bååth 2011, Bååth 1992). Thus, the increased growth rates started somewhere between the 2-hour and 18-hour incubation time of leucine. This results in a higher incorporation of leucine in the samples where there is no inhibition of protein synthesis, while the incorporation of leucine in the samples that are heavily affected by the toxicants does not grow at all, staying on the same level of incorporation or having a much longer lag period with subsequently little extra growth (Figure 21). These changes will result in that the inhibition of 50% of the bacterial growth will shift to a lower EC₅₀-value of Cu.

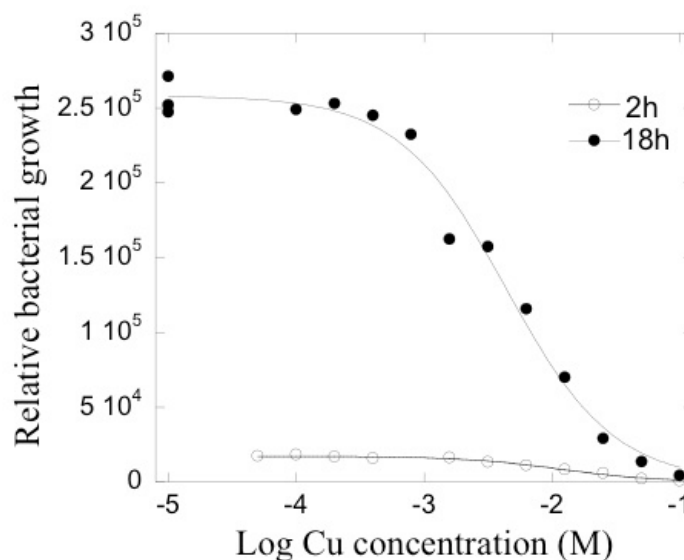


Figure 21. The dose-response curves for Cu with the two different incubation times (2 hours and 18 hours). The EC₅₀-value will shift from a higher Cu concentration to a lower when the incubation time increases to 18 hours. This is due to the increased growth in samples that are not affected by the toxicant, while the samples that are heavily affected will not grow and therefore incorporate less leucine, resulting in a 50% inhibition at a lower concentration of Cu.

4.4 Concluding remarks and future studies

The conclusions of this study are:

- (1) Leucine incorporation can be used as a method to measure metal toxicity in soils as well as to study combination effects of toxicants.
- (2) Cu-Zn and Cu-Hg show strict additive effect, independent of soil type, according to the CA concept.
- (3) Increased organic matter content increased the EC50-value of both Cu and Zn. The organic matter content in the soil is thereby an important parameter when it comes to toxicity of metals in soil and should be considered in risk assessments of contaminated sites.

It would be useful to extend the number of soils, including soils with both less organic matter content than 2% and also soils ranging from 15% to 68%, making it possible to determine a clearer relationship between the organic matter and toxicity of the metals. The increased number of soils with various amounts of organic matter would also help to confirm the observation that the organic matter content seems to have no influence on the combination effects of the metals. An improvement of the experiment would be to thoroughly investigate other properties of the soil, to focus on one parameter at a time and alter it, to observe if the strict additive effect would change. It would also be an improvement if the toxicant mixture could be added in pulses instead of only at one occasion, to investigate if the relationship between the metals would alter. That would also make the experiment a bit more comparable to reality.

Another factor to consider is the statement from Jonker et al. (2004), that they observed a synergistic effect among the higher concentrations of metals. It would be interesting to evaluate combination effects on the whole range of the dose-response curve, to validate or reject that statement.

Most ecotoxicological tests are conducted in water, probably because it is easier to observe the effect that the contaminants have, usually at lower effects levels than in soils. A contributing factor may be that it is easier to conduct experiments in water, as one eliminates the difficulty of considering the soil matrix. However, we have to remember that fungi and bacteria are the main decomposers in the soil, providing nutrients to other organisms and plants. Hence, it is important to evaluate the soil. It seems hard to find only one method for evaluating soil that covers all aspects, which makes it important to use different methods. I believe that the leucine incorporation method is an applicable method to evaluate soil, giving a good overview over the bacteria community and its response to different treatments. The method can also be used to evaluate tolerance of the bacteria for different substances and parameters in the surroundings. If the method could be standardized, I think it would be a great complement to respiration measurements. This would, however, require future testing of the method. For example, the incubation time of both the toxicant and the leucine would have to be altered to test and observe if or how the results would change.

There is a clear need for more research in the field of mixture toxicity in terrestrial systems. The guideline values that we use today to protect future generations and the environment do not even consider combination effects. More research has to be conducted, to aid the authorities, so they can create more proper legislation.

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Appendix 1.

The dose-response curves for the Cu-Zn (Figure A-E) and Cu-Hg (Figure F) experiment. All concentrations are plotted with their highest concentration as log concentration 0, for further explanation see the text for each figure.

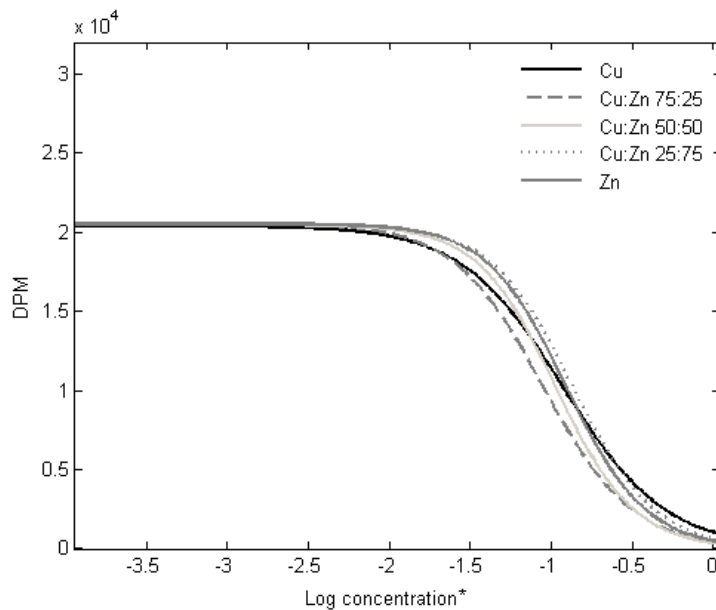


Figure A. The dose-response curves for the five different dilution series with Cu and Zn in the Grassland soil. *The concentration is plotted in log, where 0.1 M is plotted as 0, both for Cu and Zn. In the mixtures of Cu:Zn 75:25, Cu start off value (log concentration 0) is 0.075 M and Zn start off value (log concentration 0) is 0.025 M. For Cu:Zn 50:50 it is 0.05 M for both copper and zinc. For Cu:Zn 25:75 it is 0.025 M for Cu and 0.075 M of Zn.

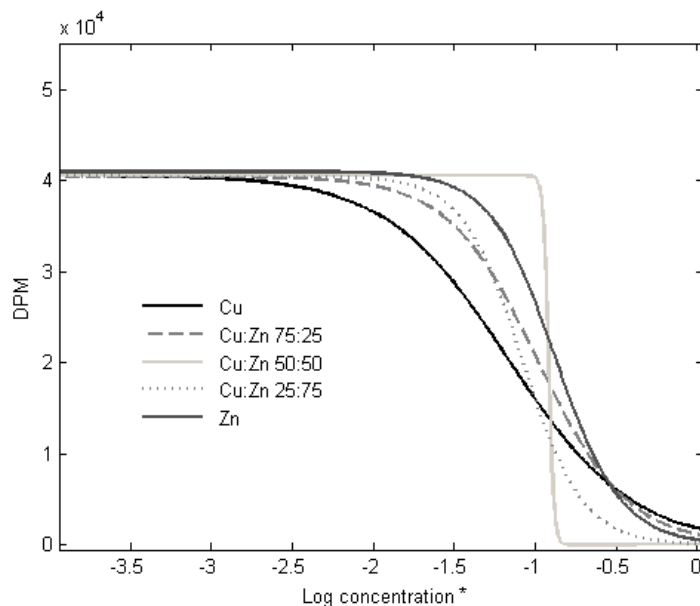


Figure B. The dose-response curves for the five different dilution series with Cu and Zn in the P2 soil. *The concentration is plotted in log, where 0.1 M is plotted as 0, both for Cu and Zn. In the mixtures of Cu:Zn 75:25, Cu start off value (log concentration 0) is 0.075 M and Zn start off value (log concentration 0) is 0.025 M. For Cu:Zn 50:50 it is 0.05 M for both copper and zinc. For Cu:Zn 25:75 it is 0.025 M for Cu and 0.075 M of Zn.

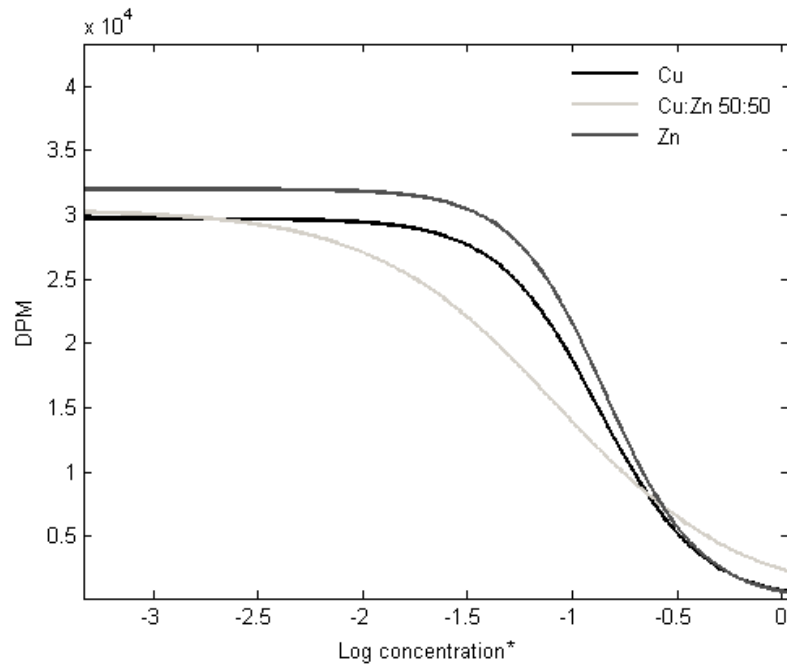


Figure C. Dose-response curves for the three different dilution series with Cu and Zn in the P2 soil, the second experiment. *The concentration is plotted in log, where 0.1 M is plotted as 0, both for Cu and Zn. For Cu:Zn 50:50 it is 0.05 M for both Cu and Zn.

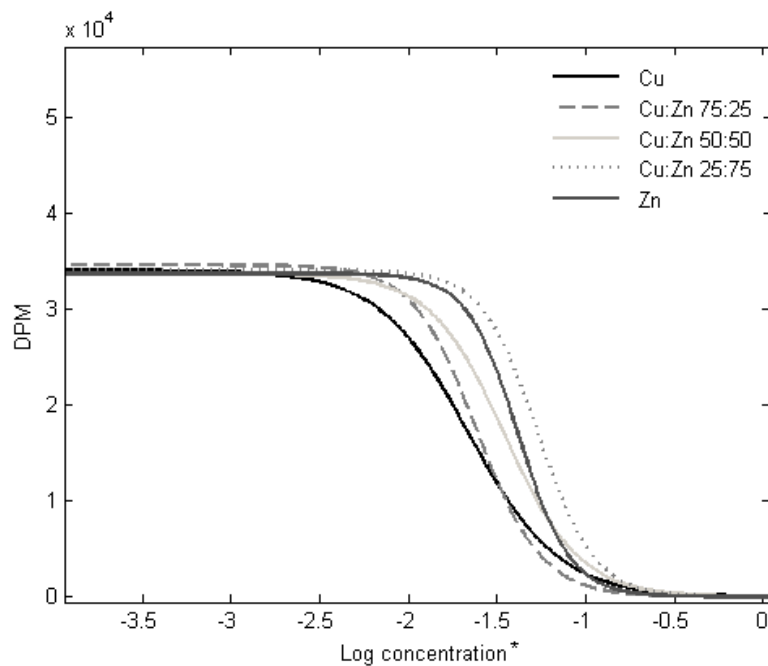


Figure D. Dose-response curves for the five different dilution series with Cu and Zn in the Sand soil. *The concentration is plotted in log, where 0.1 M is plotted as 0, both for Cu and Zn. In the mixtures of Cu:Zn 75:25, Cu start off value (log concentration 0) is 0.075 M and Zn start off value (log concentration 0) is 0.025 M. For Cu:Zn 50:50 it is 0.05 M for both Cu and Zn. For Cu:Zn 25:75 it is 0.025 M for Cu and 0.075 M of Zn.

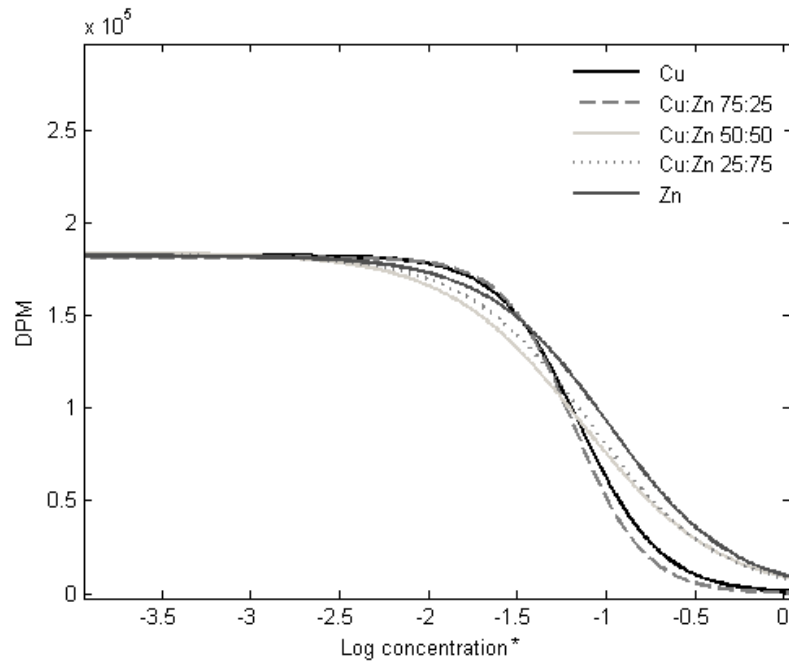


Figure E. Dose-response curves for the five different dilution series with Cu and Zn in the Omberg soil. *The concentration is plotted in log, where 0.8 M is plotted as 0, both for Cu and Zn. In the mixtures of Cu:Zn 75:25, Cu start off value (log concentration 0) is 0.6 M and Zn start off value (log concentration 0) is 0.2 M. For Cu:Zn 50:50 it is 0.4 M for both Cu and Zn. For Cu:Zn 25:75 it is 0.2 M for Cu and 0.6 M of Zn.

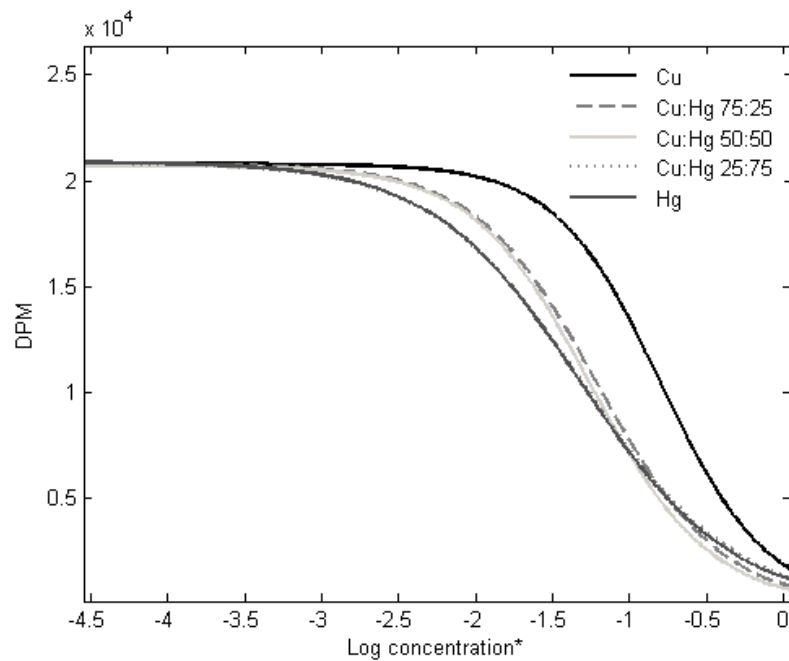


Figure F. Dose-response curves for the five different dilutions series of Cu and Hg in the Grassland soil. *The concentration is plotted in log, where 0.1 M is plotted as 0 for Cu and 1 mM is 0 for Hg. In the mixtures of Cu:Hg 75:25, Cu start off value (log concentration 0) is 0.075 M and Hg start off value (log concentration 0) is 0.25 mM. For Cu:Hg 50:50 it is 0.05 M for Cu and 0.5 mM for Hg. For Cu:Hg 25:75 it is 0.025 M for Cu and 0.75 mM of Hg.

Appendix 2.

The EC50-values for each metal in each soil in the different mixtures. The Cu values in the Grassland soil are from the Cu-Zn experiment. All amounts are expressed in microgram metal per kilogram dry weight soil.

Mixture	Metal	Grassland (mg/kg DW soil)	P2 (mg/kg DW soil)	P2 (second time) (mg/kg DW soil)	Sand (mg/kg DW soil)	Omberg (mg/kg DW soil)
100 %	Cu	540	261	510	75.3	4760
	Zn	581	524	567	148	7160
	Hg	6.99				
75 %	Cu	306	299		63.8	2660
	Zn	484	248		145	3420
	Hg	5.45				
50 %	Cu	244	230	160	60.3	2450
	Zn	251	237	165	62.2	2520
	Hg	4.05				
25 %	Cu	156	79.9		46,6	1390
	Zn	105	102		21.8	1140
	Hg	2.25				



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