|  |
| --- |
| Wageningen University & Research |
| The determination of bioavailable copper and zinc |
| An evaluation of the calcium chloride extraction    MSc thesis research proposal  SOQ-81336 |

|  |
| --- |
| Author: Frank van Raffe  Supervisors: Rob Comans, Elise van Eynde, Susan Klinkert  Chairgroup: Soil chemistry and chemical soil quality  Date: 9th of October, 2018 |

Contents

[1 Introduction 1](#_Toc534899587)

[1.1 The effect of calcium concentration 1](#_Toc534899588)

[1.1.1 The effect of calcium on binding of copper and zinc to soil solid surfaces 2](#_Toc534899589)

[1.1.2 The effect of calcium on the DOM concentration 2](#_Toc534899590)

[1.1.3 Relative importance of the effects 3](#_Toc534899591)

[1.2 The effect of the solid-solution ratio 3](#_Toc534899592)

[1.3 The effect of equilibration time 4](#_Toc534899593)

[2 Aim 4](#_Toc534899594)

[3 Research questions 4](#_Toc534899595)

[4 Hypotheses 4](#_Toc534899596)

[5 Methodology 6](#_Toc534899597)

[5.1 Sample selection 6](#_Toc534899598)

[5.2 Acid/base fractionation 6](#_Toc534899599)

[5.3 Soil extractions 6](#_Toc534899600)

[5.3.1 The effect of equilibration time 7](#_Toc534899601)

[5.3.2 The effect of calcium concentration 7](#_Toc534899602)

[5.3.3 The effect of the solid-solution ratio 8](#_Toc534899603)

[5.4 Dissolved organic carbon fractionation 8](#_Toc534899604)

[5.5 Modelling 8](#_Toc534899605)

[6 Appendix 9](#_Toc534899606)

[7 Planning 9](#_Toc534899607)

[8 Budget 9](#_Toc534899608)

[8.1 Sample preparation 10](#_Toc534899609)

[8.2 Shaking time experiment 10](#_Toc534899610)

[8.3 Calcium experiment 10](#_Toc534899611)

[8.4 SSR experiment 10](#_Toc534899612)

[8.5 Total 10](#_Toc534899613)

[9 References 11](#_Toc534899614)

# Introduction

Copper (Cu) and zinc (Zn) are two micronutrients which are essential for plant growth, limiting plant development when present in suboptimal concentrations. This growth limitation has been shown to be widespread, and have economic consequences on a global scale. Due to these consequences, assuring crops take up enough Cu and Zn is of great importance, which may done through the application of fertiliser. However, when crops are exposed to too high concentrations, toxic effects may occur, which also reduce a plants’ growth [1, 2]. It is therefore important to know how much Cu and Zn is available to plants for uptake, in order to prevent any growth limitation.

As plants take up Cu and Zn from the soil, knowledge about the speciation of the micronutrients is important for predicting the plant uptake. The total Cu and Zn concentrations in soils are irrelevant for estimating this uptake; more important are the reactive and bioavailable fractions. The reactive fraction refers to the Cu and Zn which is readily adsorbed to soil surfaces and may potentially become available through desorption. It thereby replenishes the soil solution, regulating plant uptake in the long term. This fraction is commonly measured by a 0.43 M HNO3 extraction [3]. The bioavailable fraction is the concentration of Cu and Zn present in soil solution. It is directly available to plants and regulates plant uptake on a short timescale [4]. Soil extractions have been developed which mimic soil solution conditions, allowing for the measurement of the bioavailable concentration. As of yet there is no universal method for determining bioavailability, making comparison of results difficult. Hence, there is an ongoing discussion about which method is most suitable for this purpose [5-9].

One method used to determine the bioavailable concentration is the CaCl2 extraction, which uses a 0.01 M CaCl2 solution to extract a soil. This extraction is commonly used to measure bioavailable Cu and Zn, as it is considered a good representation of the soil solution [5, 10-13]. The measured concentrations often relate to the actual plant uptake and can be used to predict toxicity or deficiency of Cu and Zn within plants [14-16]. The standard protocol of a CaCl2 extraction is a shaking time of 2 hours, a solid-solution ratio (SSR) of 1:10, and an extraction solution concentration of 0.01 M CaCl2. Modifying these conditions may result in differences in the measured Cu and Zn concentration, and may more accurately represent soil solution conditions. These changes may therefore allow for more accurate predictions of the bioavailable Cu and Zn concentrations, and as a result, plant uptake and plant nutrient conditions.

## The effect of calcium concentration

Altering the CaCl2 concentration, and consequently the Ca2+ concentration, has an important effect on the measured Cu and Zn concentrations. The effect is not straightforward, however, as both an increase and a decrease in measured concentrations has been observed with an increase in the Ca2+ concentration. Two counteracting mechanisms are hypothesized to cause this effect: the effect of Ca2+ on the binding of Cu and Zn to soil solid surfaces, and the effect of Ca2+ on the dissolved organic matter (DOM) concentration.

### The effect of calcium on binding of copper and zinc to soil solid surfaces

When Cu and Zn bind to soil solid surfaces, they are removed from soil solution and no longer bioavailable. A change in Cu and Zn binding due to variations in the Ca2+ concentration would therefore affect the bioavailable concentration. Changes in the Ca2+ concentration may alter the binding of Cu and Zn either through changes in competition for binding sites or through changes in ionic strength.

As cations compete for binding sites, an increase in the Ca2+ concentration will decrease the binding of Cu and Zn, thereby increasing the concentration of bioavailable Cu and Zn [17-25]. This competition is dependent on the binding affinity of the ions, where ions with a higher affinity will be relatively less affected by competition. Because Cu has a higher affinity for OM than Zn, the competition effect is more important for Zn [24, 25].

Increasing the Ca2+ concentration results in an increased ionic strength. It has been found that an increase in ionic strength will cause an increase in the negative charge of soil particles [26-28]. This increased negative charge would increase electrostatic interactions with cations, suggesting an increase in binding of Cu and Zn. On the contrary, studies have found that increases in ionic strength correspond to a decrease in cation binding, which may be explained by a suppression of electrostatic effects with increased ionic strength [29-32].

### The effect of calcium on the DOM concentration

Soil organic matter (SOM) is one of the main soil solid surface to which micronutrients such as Cu and Zn bind [33]. Humic substances, and in particular humic and fulvic acids, are the most reactive fraction of OM, and hence most relevant for the binding of ions such as Cu and Zn. Humic substances can be further subdivided based on solubility in humin, humic acid, fulvic acid, hydrophilic acids and hydrophobic neutrals [34]. Dissolved organic matter is the OM fraction which is able to pass a 0.45 µm filter, and consists of fulvic acid, humic acid and other hydrophilic compounds [35]. It is considered part of the soil solution, and as a result, DOM-metal complexes are considered part of the bioavailable fraction. If DOM is removed from the soil solution, this decreases the bioavailable concentration of Cu and Zn. Altering the Ca2+ concentration may influence the DOM concentration and its fractions through changes in coagulation rate and changes in the binding rate of OM with oxides [35-48].

Coagulation of organic matter causes the particles to flock together and increase in size, thereby reducing their solubility. Hence, an increase in coagulation due to increasing the Ca2+ concentration would result in a decrease in the bioavailable concentrations of Cu and Zn. As coagulation will occur more easily for the more insoluble humic acids, coagulation may also affect the composition of the DOM. Increasing the Ca2+ concentration will result in a larger salt concentration, which has been found to increase coagulation [35-37]. Furthermore, calcium allows for bridging between carboxylic groups, further instigating coagulation [38-40]. Consequently, calcium increases the coagulation rates and therefore decreases the measured Cu and Zn concentration in solution. This calcium induced coagulation was found to occur within a critical calcium concentration range of 1 to 8 mM, depending on pH and OM composition [35, 38, 41, 42].

Besides coagulation, binding of OM to oxides results in removal of DOM, and hence complexed Cu and Zn from soil solution [43-48]. The binding rate is dependent on the OM composition and on electrostatic processes [46-48]. The effect of calcium in this process is twofold. Firstly, Ca2+ increases the positive charge of soil particles, instigating an increased adsorption of the negatively charged OM [43, 44]. Secondly, Ca2+ functions as a bridge between OM and negatively charged soil particles, further increasing OM binding [45]. As such, a higher Ca2+ decreases the measured bioavailable Cu and Zn. Hence, when considering the mechanisms of DOM loss, extracting at a higher CaCl2 concentration results in a decrease in measured Cu and Zn.

### Relative importance of the effects

Whether an altered Ca2+ concentration results in an increase or a decrease of Cu and Zn concentrations in soil solution is dependent on the importance of DOM for Cu and Zn speciation in solution, and on the metal loading of the soil. When DOM becomes more important, for instance when it is present in large concentrations, more metals will be complexed with the DOM and lost from soil solution when coagulation occurs or when the DOM binds to oxides. This effect is exacerbated when the DOM contains more hydrophobic compounds, as these are more insoluble and therefore more easily lost. Consequently, for soils in which DOM plays an important role, an increase in calcium concentration is expected to result in a decrease of Cu and Zn concentrations in solution. On the contrary, at higher metal loadings more metals will be bound to low affinity sites. Particles bound to these low affinity sites are more easily desorbed, increasing the competition effect of Ca2+ and resulting in an increase in Cu and Zn concentrations in solution with an increase in calcium concentration. The effect of calcium will also differ between Cu and Zn. As Cu has a larger affinity for OM, the effect of Ca on OM loss will be larger for Cu than for Zn, whereas the opposite is found for the effect on competition for binding sites. As such, Cu is expected to show a larger decrease than Zn with an increase in calcium concentration.

## The effect of the solid-solution ratio

Altering the solid-solution ratio may have a considerable impact on the measured Cu and Zn concentrations. An increase in SSR has been found to decrease pH and increase DOC [47, 49]. Following this, the measured bioavailable Cu and Zn are expected to increase with a higher SSR. However, the metal-DOC ratio was found to decrease with increasing SSR, indicating a lower metal binding capacity of DOC at higher SSR. As this effect is solely observed for the CaCl2 extraction, and not for the water extraction, an explanation may be the competition of calcium [47]. However, competition of calcium is expected to decrease with increasing SSR, as the amount of calcium per soil unit decreases. An increase in SSR may also affect the DOC composition, resulting in the observed decreased metal binding capacity. Whilst this reduces the effectiveness of the enhanced DOC concentrations in mobilising Cu and Zn, heavy metals were still found to be more mobile at higher SSR [50].

## The effect of equilibration time

Equilibration time may also play a part in the amount of bioavailable Cu and Zn measured. In order to understand the processes taking place, a system is generally assumed in equilibrium. Thus, measurements should ideally be performed under equilibrium conditions, which require a certain shaking time. Similar extraction techniques to the CaCl2 extraction, such as the EDTA and DTPA extraction, found a significant increase in heavy metal concentrations with shaking time, up until equilibrium was reached [5, 51, 52]. It is unclear whether the same effect will occur for a CaCl2 extraction, however. Besides, the previously mentioned mechanisms may occur at different timescales, making the effect of equilibration time less straightforward.

# Aim

Whilst there is knowledge on the above-mentioned effects of varying different factors within the CaCl2 extraction, the precise mechanisms remain unknown. As Ca2+ is abundant in natural soils, understanding these precise mechanisms may aid in understanding the availability of Cu and Zn in soils. Besides, furthering knowledge about the CaCl2 extraction may contribute towards the discussion about a universal method for determining bioavailable concentrations. The aim of the present research is therefore to further increase our understanding of the underlying mechanisms of the CaCl2 extraction under different soil conditions.

# Research questions

Following the aim, the following research questions will be investigated:

1. What is the effect of the calcium concentration of a CaCl2 extraction on the measured Cu and Zn concentrations for different soil conditions?
2. What is the effect of the solid-solution ratio of a CaCl2 extraction on the measured Cu and Zn concentrations for different soil conditions?
3. What is the effect of the shaking time of a CaCl2 extraction on the measured Cu and Zn concentrations for different soil conditions?
4. What are the underlying mechanisms of the CaCl2 extraction, and what is their relative importance under different soil conditions?

# Hypotheses

1. It is expected that an increase in calcium concentration corresponds to a decrease in the DOC concentration, and a shift in DOC composition towards a more hydrophilic nature. The effect of the calcium concentration on Cu and Zn is expected to be variable and different for the two metals, and is dependent on whether the effect of binding or the effect of DOC loss is expected to be more important. Higher DOC concentrations and lower metal loadings are expected to cause DOC loss to become more important, resulting in a decrease of Cu and Zn with increase calcium concentrations. This will become even more important when the DOC contains more HA. Besides, DOC loss will be more important for Cu, due to its higher affinity to organic matter than Zn. With lower DOC concentrations and higher metal loadings, the effect of calcium on binding is expected to be more important, which results in an increase in concentrations with an increased calcium concentration.
2. It is expected that and increase in SSR corresponds to an increase in concentrations, which will be independent of soil conditions
3. It is expected that all concentrations will increase with shaking time, up until equilibrium has been established, after which concentrations will remain constant. This will be independent of soil conditions.
4. It is expected that the mechanisms of the CaCl2 extraction are mainly governed by competition for binding sites and the solubility of DOC. The relative importance of the mechanisms will largely depend on the soil conditions. It is expected that the importance of DOC solubility will become more important when a soil contains more OM which is of a more hydrophobic nature.

# Methodology

## Sample selection

For the experiment, 5 different samples are chosen: 2 samples of temperate soils, 2 samples of tropical soils, and only 1 compost, due to a lack of available compost material. These samples are chosen based on a range of environmental properties, such as OC content, oxide content, pH, and metal loading, which allows for a more thorough investigation of the mechanisms. Additional data on these samples can be found in table 1.

Table 1. Additional data for the samples used in the experiment.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Sample | Sample type | CaCl2 - pH | HNO3-Zn | HNO3-Cu | OC | Fe-Amox | Al-Amox |
|  |  | - | mg/kg | mg/kg | g/kg | mg/kg | mg/kg |
| M16 | Tropical | 4.15 | 1.2 | 4 | 14 | 2085 | 1709 |
| R1 | Tropical | 4.91 | 0.9 | 5.1 | 32 | 3671 | 3667 |
| Marsep235 | Compost | 5.22 | 25.4 | 5 | 450 | 490 | 290 |
| SETOC765 | European | - | 140 | 36.6 | 28 | - | - |
| ISE958 | European | 4.38 | 16.4 | 7.29 | 14.3 | - | - |

## Acid/base fractionation

For three samples, data is already known regarding the OM fractions. Acid/base fractionation will be performed on the remaining two samples. For this fractionation, 4 g of sample is extracted with 35 mL of 0.1 M HCl. The attained solution is acidified to a pH of 1 using 1 M HCl, and then filled to an end volume of 40 mL using 0.1 M HCl. The solution is then shaken for 1 hour and subsequently centrifuged for 20 minutes at 3750 RPM. The supernatant is decanted and filtered over a 0.45 µm filtered and shipped to CBLB for DOC analysis. The pellet, which has remained in the tube after decanting, is extracted with 35 mL of 0.1 M NaOH, adjusted to a pH of 12 using 1 M NaOH, and further filled to a total volume of 40 mL using 0.1 M NaOH solution. This solution is shaken overnight, and then centrifuged for 20 minutes at 3750 RPM, before being decanted and filtered over a 0.45 µm filter. The filtered solution is once more shipped to the CBLB.

After this procedure, 5 mL of filtered acid solution is combined with 5 mL of filtered base solution and 40 mL UPW in a 50 ml centrifuge tube. The solution is shaken and can be used for further OC fractionation, as is outlined below in its separate section.

## Soil extractions

Soils are extracted according to Houba et al. [13]. Initially 8 g of a sample is weighed and then extracted with 80 mL of 10-2 M CaCl2 solution into 150 mL centrifuge tubes, corresponding to a solid-solution ratio of 1:10. The compost samples require more liquid, and as such 14 g of compost is extracted with 140 mL of CaCl2 solution. The acquired solution is shaken in an horizontal shaker for 2 hours, and consequently measured for pH using a glass electrode. Following this, the solution is centrifuged at 3000 rpm for 10 minutes after which the supernatant of the different tubes is combined and filtered over a 0.45 µm filter. Part of the supernatant is filtered into four separate 10 mL tubes, which are given to the CBLB for further analysis. The glass tubes are analysed using different machines, and therefore require separate tubes. Measurements will be done for Cu and Zn, Ca, PO4, and total DOC concentrations using the Inductively Coupled Plasma – Mass spectrometer (ICP-MS), Inductively Coupled Plasma - Optical Emission Spectrometer (ICP-OES), Segmented Flow Analyser – Phosphorus (SFA-P), and Segmented Flow Analyser – Total Organic Carbon (SFA-TOC) respectively. The remainder of the supernatant is filtered into a 50 mL centrifuge tube and further processed during OC fractionation, which is outlined in further detail down below.

The above-mentioned procedure will be used in 3 different experiments, in each of which a certain factor is varied. The precise details of these experiments are outlined below. Unless stated otherwise, the earlier mentioned values are used. Important to note is that each time a new solution is created, two blanks are added to the experiment, which undergo the same treatment as outlined above. This is done to correct for potential contamination.

### The effect of equilibration time

In order to get the most precise measurement of the bioavailable concentrations under environmental conditions, the system should be in equilibrium. The standard procedure for a CaCl2 extraction is a shaking time of 2 hours, however this may be too little time for equilibrium to be established. To investigate the significance of an established equilibrium, soil extractions will be performed as described previously. However, the shaking time in the end-over-end shaker will be varied, with shaking times of 2 hours, 1 day, 2 days, and 1 week. As the equilibrium time may differ for different Ca2+ concentrations, the CaCl2 concentration will also be varied, using the following concentrations: 10-1 M, 10-2 M, 10-3 M. This will be performed for 1 sample of each origin (1 European soil, 1 tropical soil, and 1 compost). All samples will be analysed for Cu, Zn, Ca, PO4 and total DOC, however no OC fractionation will be conducted for this experiment. As such, the extractions will be conducted with 4 g of soil and 40 mL of CaCl2 solution, as less volume is needed for analysis.

### The effect of calcium concentration

In order to test the precise effects of calcium concentration on the availability of Cu and Zn, and the concentration of DOC, all soil samples are extracted using different CaCl2 concentrations. As the purpose of a CaCl2 extraction is to mimic soil conditions, as well as to investigate the tipping point of DOC coagulation, the following CaCl2 concentrations are used for extraction: 10-1 M, 10-2 M, 10-3 M. Besides, extractions will be performed for two shaking times: 2 hours, and until equilibrium has been reached, which is determined during the shaking time experiment. 8 g of samples will be extracted for the European and tropical soils, and 14 g of compost will be extracted. All samples will be analysed for Cu, Zn, Ca, PO4, and OC fractionation will be conducted to determine the concentration of the different OC fractions.

### The effect of the solid-solution ratio

To estimate the effect of SSR, extractions will be performed as mentioned previously, however the solid-solution ratios are varied. The ratios which are investigated are 1:2, 1:10, and 1:20. As 1:10 is already done in the calcium concentration experiment, only 1:2 and 1:20 are conducted for this experiment, which correspond to 40 g and 4 g of soil added to 80 mL of CaCl2 solution respectively. However, as composts behave as a sponge, a SSR of 1:2 is not feasible. Therefore, the compost sample is extracted using a SSR 1:20, which corresponds to 7 g of compost added to 140 mL of CaCl2 solution. All samples will be analysed for Cu, Zn, Ca, PO4 and total DOC. OC fractionation is conducted for 1 sample of each origin.

## Dissolved organic carbon fractionation

DOC will be fractionated according to Comans and van Zomeren [53]. Initially, the supernatant, which was separated at the end of the soil extractions, is acidified to a pH of 1 using 6 M HCl under constant stirring, in order to precipitate humic acid (HA). This solution is left overnight, to allow for precipitation, and the following day the solution is centrifuged for 10 minutes at 3750 RPM and the supernatant is separated from the newly formed HA pellet. The pellet is dissolved in 15 mL of 0.1M KOH solution, shaken for 10 minutes and adjusted to a pH of 11, before being send to the CBLB laboratory for total organic carbon (TOC) analysis to determine the HA concentration. The separated supernatant is filtered over a 0.45 μm filter, acidified to a pH between 2.5 and 4, and send for TOC analysis, in order to determine the concentration of fulvic acids (FA), hydrophobic neutrals (HON), and hydrophilic acids (Hy) combined. Afterwards, aliquots of the filtered solution are transferred to polycarbonate containers and XAD-8 resin is added. This solution is shaken for 1 hour, to allow for equilibration, and afterwards the solution is filtered, acidified to a pH between 2.5 and 4, and sent for TOC analysis to determine the Hy concentration. FA is desorbed from the resin using 0.1 M KOH, acidified to a pH between 2.5 and 4, and sent for TOC analysis, which will be done three times to ensure a high recovery rate of the FA. Finally, the HON concentration is considered the difference between the measured FA and the adsorbed FA + HON concentration.

## Modelling

Modelling of the experimental data is done within the multi-speciation environment of Orchestra [54]. As different soil particles are present, different models are required to describe the sorption behaviour of the ions. Sorption to clays is described using the Donnan model, sorption to organic matter is described using the NICA-Donnan model, and sorption to oxides is described using the CD-MUSIC model. Standard parameters acquired from literature will be used to describe the behaviour within the system [55, 56]. Besides the standard parameters, input is required on the specific conditions within the systems. Therefore, pH, specific surface area of oxides, clay content, total concentrations of Cu, Zn, Ca, PO4, and DOC, as well as the concentrations of the different DOC fractions will serve as further input for the model. In order to validate the model output, the concentrations of Cu and Zn in solution given by the model will be compared to the found concentrations within the different experiments.

# Appendix

The methods used for the proposed research are:

* CaCl2 extractions
* Organic carbon fractionation

# Planning

# Budget

As the analysis of the samples will be performed by the CBLB, there is a certain cost associated to this research. The costs for each analysis are:

* Measurement of Cu and Zn concentrations: €19.90,-
* Measurement of the Ca concentration: €10.90,-
* Measurement of PO4 concentration: €12.10,-
* Measurement of total DOC: €12.10,-
* OC fractionation: €60.50,-
* Acid/base fractionation: €72.60,-

As the amount of samples per experiment differ, I will summarize the precise costs of each experiment separately.

## Sample preparation

Two of the six chosen samples will be fractionated using acid/base fractionation. This corresponds to a total cost of €145.20.

## Shaking time experiment

For the shaking time experiment, the extraction will be performed for 3 samples and for 4 different shaking times, leading to a total of 12 samples. These samples will all be analysed for Cu, Zn, Ca, PO4, and total DOC, corresponding to a total cost of €55,- per sample. In total, this experiment costs €660,-.

## Calcium experiment

For the calcium experiment, all samples are analysed for 3 different calcium concentrations and 2 shaking times. All samples will undergo OC fractionation, and all samples except 6 will be analysed for Cu, Zn, Ca, and PO4, as 6 of these samples have already been analysed during the shaking time experiment. This leads to a total cost of 30 \* €60.5 + 24 \* €42.9 = €2844.60 for the calcium experiment.

## SSR experiment

During the SSR experiment, all samples will be extracted for two different ratios. These will all be analysed for Cu, Zn, Ca, total DOC, and PO4, and three of the samples will undergo OC fractionation. Correspondingly, the total cost for the SSR experiment is 10 \* €55 + 5 \* €60.5 = €913,-.

## Total

Combining all analyses costs together results in a total cost of €4562.80 for the entire experiment.

# References

1. Alloway, B.J., *Zinc in soils and crop nutrition*. 2004, International Zinc Association Brussels.

2. Gupta, U.C., W. Kening, and S. Liang, *Micronutrients in soils, crops, and livestock.* Earth Science Frontiers, 2008. **15**(5): p. 110-125.

3. Groenenberg, J.E., et al., *Evaluation of the single dilute (0.43 M) nitric acid extraction to determine geochemically reactive elements in soil.* Environmental science & technology, 2017. **51**(4): p. 2246-2253.

4. Ma, L.Q. and G.N. Rao, *Chemical fractionation of cadmium, copper, nickel, and zinc in contaminated soils.* Journal of Environmental Quality, 1997. **26**(1): p. 259-264.

5. Feng, M.-H., et al., *A comparison of the rhizosphere-based method with DTPA, EDTA, CaCl2, and NaNO3 extraction methods for prediction of bioavailability of metals in soil to barley.* Environmental Pollution, 2005. **137**(2): p. 231-240.

6. Feng, M.-H., et al., *Comparison of a rhizosphere-based method with other one-step extraction methods for assessing the bioavailability of soil metals to wheat.* Chemosphere, 2005. **59**(7): p. 939-949.

7. Sauvé, S., et al., *Speciation and complexation of cadmium in extracted soil solutions.* Environmental Science & Technology, 2000. **34**(2): p. 291-296.

8. Soriano-Disla, J.M., et al., *Evaluation of single chemical extractants for the prediction of heavy metal uptake by barley in soils amended with polluted sewage sludge.* Plant and soil, 2010. **327**(1-2): p. 303-314.

9. Soriano-Disla, J.M., et al., *Evaluation of different extraction methods for the assessment of heavy metal bioavailability in various soils.* Water, Air, & Soil Pollution, 2010. **213**(1-4): p. 471-483.

10. Pueyo, M., J. Lopez-Sanchez, and G. Rauret, *Assessment of CaCl2, NaNO3 and NH4NO3 extraction procedures for the study of Cd, Cu, Pb and Zn extractability in contaminated soils.* Analytica chimica acta, 2004. **504**(2): p. 217-226.

11. Tyler, L.D. and M.B. McBRIDE, *Mobility and extractability of cadmium, copper, nickel, and zinc in organic and mineral soil columns.* Soil Science, 1982. **134**(3): p. 198-205.

12. McBride, M.B., M. Pitiranggon, and B. Kim, *A comparison of tests for extractable copper and zinc in metal-spiked and field-contaminated soil.* Soil science, 2009. **174**(8): p. 439-444.

13. Houba, V.J.G., et al., *Soil analysis procedures using 0.01 M calcium chloride as extraction reagent.* Communications in Soil Science and Plant Analysis, 2000. **31**(9-10): p. 1299-1396.

14. Brun, L., et al., *Relationships between extractable copper, soil properties and copper uptake by wild plants in vineyard soils.* Environmental pollution, 1998. **102**(2-3): p. 151-161.

15. Zhang, M.-K., Z.-Y. Liu, and H. Wang, *Use of Single Extraction Methods to Predict Bioavailability of Heavy Metals in Polluted Soils to Rice.* Communications in Soil Science and Plant Analysis, 2010. **41**(7): p. 820-831.

16. Menzies, N.W., M.J. Donn, and P.M. Kopittke, *Evaluation of extractants for estimation of the phytoavailable trace metals in soils.* Environmental Pollution, 2007. **145**(1): p. 121-130.

17. Atanassova, I., *Competitive effect of copper, zinc, cadmium and nickel on ion adsorption and desorption by soil clays.* Water, Air, and Soil Pollution, 1999. **113**(1-4): p. 115-125.

18. Cerqueira, B., et al., *The influence of soil properties on the individual and competitive sorption and desorption of Cu and Cd.* Geoderma, 2011. **162**(1-2): p. 20-26.

19. Escrig, I. and I. Morell, *Effect of calcium on the soil adsorption of cadmium and zinc in some Spanish sandy soils.* Water, Air, and Soil Pollution, 1998. **105**(3-4): p. 507-520.

20. Iglesias, A., et al., *Analysis of copper and calcium–fulvic acid complexation and competition effects.* Water research, 2003. **37**(15): p. 3749-3755.

21. Martınez, C. and H. Motto, *Solubility of lead, zinc and copper added to mineral soils.* Environmental Pollution, 2000. **107**(1): p. 153-158.

22. Milne, C., et al., *Analysis of proton binding by a peat humic acid using a simple electrostatic model.* Geochimica et Cosmochimica Acta, 1995. **59**(6): p. 1101-1112.

23. Pulford, I., *Mechanisms controlling zinc solubility in soils.* European Journal of Soil Science, 1986. **37**(3): p. 427-438.

24. Zhu, B. and A. Alva, *Differential adsorption of trace metals by soils as influenced by exchangeable cations and ionic strength.* Soil Science, 1993. **155**(1): p. 61-66.

25. Bradl, H.B., *Adsorption of heavy metal ions on soils and soils constituents.* Journal of colloid and Interface Science, 2004. **277**(1): p. 1-18.

26. Christensen, J.B., et al., *Proton binding by groundwater fulvic acids of different age, origins, and structure modeled with the model V and NICA− Donnan model.* Environmental science & technology, 1998. **32**(21): p. 3346-3355.

27. Christl, I. and R. Kretzschmar, *Relating ion binding by fulvic and humic acids to chemical composition and molecular size. 1. Proton binding.* Environmental science & technology, 2001. **35**(12): p. 2505-2511.

28. Robertson, A. and J. Leckie, *Acid/base, copper binding, and Cu2+/H+ exchange properties of a soil humic acid, an experimental and modeling study.* Environmental science & technology, 1999. **33**(5): p. 786-795.

29. Acosta, J., et al., *Salinity increases mobility of heavy metals in soils.* Chemosphere, 2011. **85**(8): p. 1318-1324.

30. Christl, I., et al., *Effect of humic and fulvic acid concentrations and ionic strength on copper and lead binding.* Environmental science & technology, 2005. **39**(14): p. 5319-5326.

31. Kinniburgh, D.G., et al., *Metal Ion Binding by Humic Acid:  Application of the NICA-Donnan Model.* Environmental Science & Technology, 1996. **30**(5): p. 1687-1698.

32. Naidu, R., et al., *Ionic‐strength and pH effects on the sorption of cadmium and the surface charge of soils.* European journal of soil science, 1994. **45**(4): p. 419-429.

33. Spark, K., J. Wells, and B. Johnson, *The interaction of a humic acid with heavy metals.* Soil Research, 1997. **35**(1): p. 89-102.

34. Aiken, G.R., *Humic substances in soil, sediment, and water: geochemistry, isolation, and characterization.* 1985.

35. Weng, L., E. Temminghoff, and W. Van Riemsdijk, *Interpretation of humic acid coagulation and soluble soil organic matter using a calculated electrostatic potential.* European journal of soil science, 2002. **53**(4): p. 575-588.

36. Weng, L., et al., *Transport of humic and fulvic acids in relation to metal mobility in a copper-contaminated acid sandy soil.* Environmental science & technology, 2002. **36**(8): p. 1699-1704.

37. Wall, N.A. and G.R. Choppin, *Humic acids coagulation: influence of divalent cations.* Applied Geochemistry, 2003. **18**(10): p. 1573-1582.

38. Kloster, N., et al., *Aggregation kinetics of humic acids in the presence of calcium ions.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2013. **427**: p. 76-82.

39. Tipping, E. and M. Ohnstad, *Aggregation of aquatic humic substances.* Chemical Geology, 1984. **44**(4): p. 349-357.

40. Tipping, E., *Cation binding by humic substances*. Vol. 12. 2002: Cambridge University Press.

41. Oste, L.A., E.J.M. Temminghoff, and W.H.V. Riemsdijk, *Solid-solution Partitioning of Organic Matter in Soils as Influenced by an Increase in pH or Ca Concentration.* Environmental Science & Technology, 2002. **36**(2): p. 208-214.

42. Römkens, P.F.A.M. and J. Dolfing, *Effect of Ca on the Solubility and Molecular Size Distribution of DOC and Cu Binding in Soil Solution Samples.* Environmental Science & Technology, 1998. **32**(3): p. 363-369.

43. Bolan, N.S., et al., *Surface Charge and Solute Interactions in Soils*, in *Advances in Agronomy*, D.L. Sparks, Editor. 1999, Academic Press. p. 87-140.

44. Harding, I.S., N. Rashid, and K.A. Hing, *Surface charge and the effect of excess calcium ions on the hydroxyapatite surface.* Biomaterials, 2005. **26**(34): p. 6818-6826.

45. Römkens, P.F., J. Bril, and W. Salomons, *Interaction between Ca2+ and dissolved organic carbon: implications for metal mobilization.* Applied Geochemistry, 1996. **11**(1-2): p. 109-115.

46. Weng, et al., *Adsorption of Humic Substances on Goethite:  Comparison between Humic Acids and Fulvic Acids.* Environmental Science & Technology, 2006. **40**(24): p. 7494-7500.

47. Fest, E.P.M.J., et al., *Partitioning of organic matter and heavy metals in a sandy soil: Effects of extracting solution, solid to liquid ratio and pH.* Geoderma, 2008. **146**(1): p. 66-74.

48. Weng, L., W.H. Van Riemsdijk, and T. Hiemstra, *Cu2+ and Ca2+ adsorption to goethite in the presence of fulvic acids.* Geochimica et Cosmochimica Acta, 2008. **72**(24): p. 5857-5870.

49. Kaiser, K., M. Kaupenjohann, and W. Zech, *Sorption of dissolved organic carbon in soils: effects of soil sample storage, soil-to-solution ratio, and temperature.* Geoderma, 2001. **99**(3): p. 317-328.

50. Yin, Y., et al., *The importance of organic matter distribution and extract soil:solution ratio on the desorption of heavy metals from soils.* Science of The Total Environment, 2002. **287**(1): p. 107-119.

51. Pickering, W.F. and L.M. Shuman, *Selective Chemical Extraction of Soil Components and Bound Metal Species.* C R C Critical Reviews in Analytical Chemistry, 1981. **12**(4): p. 233-266.

52. Lo, I.M. and X. Yang, *EDTA extraction of heavy metals from different soil fractions and synthetic soils.* Water, Air, and Soil Pollution, 1999. **109**(1-4): p. 219-236.

53. van Zomeren, A. and R.N. Comans, *Measurement of humic and fulvic acid concentrations and dissolution properties by a rapid batch procedure.* Environmental science & technology, 2007. **41**(19): p. 6755-6761.

54. C L Meeussen, J., *ORCHESTRA: An object-oriented framework for implementing chemical equilibrium models*. Vol. 37. 2003. 1175-82.

55. Milne, C.J., D.G. Kinniburgh, and E. Tipping, *Generic NICA-Donnan model parameters for proton binding by humic substances.* Environmental Science & Technology, 2001. **35**(10): p. 2049-2059.

56. Milne, C.J., et al., *Generic NICA− Donnan model parameters for metal-ion binding by humic substances.* Environmental Science & Technology, 2003. **37**(5): p. 958-971.