# Iron fertilization with FeEDDHA

## The fate and effectiveness of FeEDDHA chelates in soil-plant systems

**Thesis committee**

**Thesis supervisor:**
Prof. Dr. W.H. van Riemsdijk
Professor of Soil Chemistry and Chemical Soil Quality, Wageningen University

**Thesis co-supervisor:**
Dr. Ir. E.J.M. Temminghoff
Assistent professor at the Sub-department of Soil Quality, Wageningen University

**Other members:**
Prof. Dr. H.P. van Leeuwen
University of Geneva, Switzerland/Wageningen University, The Netherlands
Prof. Dr. J.J. Lucena
Autónoma University of Madrid, Spain
Prof. Dr. S.M. Krämer
University of Vienna, Austria
Prof. Dr. P.C. de Ruiter
Wageningen University, The Netherlands

This research was conducted under the auspices of the Graduate School SENSE (Socio-Economic and Natural Science of the Environment)

# Iron fertilization with FeEDDHA

## The fate and effectiveness of FeEDDHA chelates in soil-plant systems

**W.D.C. Schenkeveld**

**Thesis**
Submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 19 November 2010
at 11 a.m. in the Aula

Iron fertilization with FeEDDHA. The fate and effectiveness of FeEDDHA chelates in soil-plant systems.
Walter D.C. Schenkeveld

PhD thesis, Wageningen University, Wageningen, NL (2010)
With references, with summaries in Dutch and English

ISBN 978-90-8585-798-3

*Solve et coagula*
*(Dissolve and coagulate)*

Primary dictum of alchemy

“B'asarah ma'amarot nivra ha-olam”
*trans.* “With ten utterances was the world created.”

*Talmud* “Pirkey Aboth” c. 5, *Mish*. I

# Abstract

Fe deficiency chlorosis is a nutritional disorder in plants that reduces crop yields both quantitatively and qualitatively, and causes large economic losses. It occurs world-wide, predominantly in plants grown on calcareous soils, as a result of a limited bioavailability of Fe, related to the poor solubility of Fe at high soil-pH (7 - 8.5). Fe fertilizers based on FeEDDHA (iron ethylene diamine-N,N'-bis(hydroxy phenyl acetic acid)) chelates are among the most efficient in preventing and remedying Fe deficiency in soil-grown plants. FeEDDHA fertilizers comprise a mixture of FeEDDHA components which can be divided into racemic o,o-FeEDDHA, meso o,o-FeEDDHA, o,p-FeEDDHA and rest-FeEDDHA. Both the composition of FeEDDHA fertilizers and the properties of the FeEDDHA components differ considerably. In this thesis the fate and effectiveness of FeEDDHA chelates in soil-plant systems were examined, to facilitate a more efficient use of FeEDDHA fertilizer.

First, FeEDDHA components were screened with regard to their reactivity towards soil reactive compounds. Upon interaction with soils, the o,o-FeEDDHA isomers remained mostly in solution, while o,p-FeEDDHA and rest-FeEDDHA were largely removed. The dominant reactive soil compound controlling the remaining concentration differed per FeEDDHA component.
FeEDDHA-facilitated Fe uptake by plants was examined in three pot trials studies with soybean grown on calcareous soils. In the first pot trial, the effectiveness of FeEDDHA components was examined by applying FeEDDHA treatments of equal Fe dosage, but differing in composition. On soils, in which plants of the blank treatment became chlorotic, Fe uptake proved a function of the Fe concentration in soil solution, which in turn was a function of the amount of o,o-FeEDDHA applied. In a second pot trial, FeEDDHA-facilitated Fe uptake was examined in relation to the concentration behaviour of the FeEDDHA components. After an initial concentration drop due to adsorption, the soil solution concentration of both racemic and meso o,o-FeEDDHA gradually declined. For meso o,o-FeEDDHA the decline was exponential and largely unrelated to Fe uptake by plants; for racemic o,o-FeEDDHA the decline was slower and largely related to Fe uptake, which was highest in the progressed vegetative stage and the reproductive growth-stage. In a third pot trial the effectiveness of FeEDDHA components was assessed in relation to the moment of application; at t=0 and in the aforementioned two growth stages. o,p-FeEDDHA application did not significantly increase Fe uptake in any growth stage. Both racemic and meso o,o-FeEDDHA did contribute to Fe uptake, approximately to the same extent. The moment of application significantly affected yield and residual pore water concentration of the FeEDDHA components, but not Fe uptake, demonstrating the elevated Fe uptake efficiency of chlorotic plants.

Processes potentially causing a (plant-independent) gradual decline in FeEDDHA component concentrations were further examined. The biodegradability of EDDHA chelates was examined in a soil incubation experiment. It was concluded that biodegradation does not significantly compromise the performance of FeEDDHA components. The potential impact of Cu displacing Fe from o,o-FeEDDHA was explored by means of mechanistic multi-surface

modeling and batch interaction experiments with soil and goethite suspensions. Model predictions indicate that, under equilibrium conditions, a fraction of the o,o-EDDHA ligands in soil solution can be chelated to Cu, in particular of meso o,o-EDDHA. Batch experiments demonstrated that o,o-CuEDDHA has a high affinity for the soil solid phase, which greatly enhances the potential impact from Cu competition. The displacement reaction could be reproduced in goethite suspensions, proving it is not kinetically inhibited. The effect of soil parameters on the rate at which the displacement reaction decreases the FeEDDHA solution concentration, was examined in goethite suspensions. The rate of decline depends on the available reactive surface area as well as on the FeEDDHA and Cu loading. Soil factors decreasing FeEDDHA adsorption (high ionic strength, adsorption of organic matter onto the reactive surface, monovalent instead of divalent cations in the electrolyte, etc) decrease the rate of decline in FeEDDHA concentration. For meso o,o-FeEDDHA, the rate equation of the displacement reaction could be simplified and solved for soil conditions, to an exponential decay function in meso o,o-FeEDDHA concentration – corresponding with observations in the second pot trial.

The hypothetical shuttle mechanism, in which the ligand is "recycled" by chelating and mobilizing Fe from the soil after delivering Fe at the root surface, was considered for EDDHA ligands. In batch experiments, it was demonstrated that, if the efficiency of the mechanism is determined by metal availability in the bulk soil, it is heavily compromised by complexation of competing cations, in particular Cu. Experimental support for the shuttle mechanism was found in data from the pot trial experiments, demonstrating specific metal mobilization upon FeEDDHA-facilitated Fe uptake by plants.

In conclusion a conceptual model for the behaviour of FeEDDHA components in soil-plant systems was composed. The essence of the model consists of three processes: 1) FeEDDHA adsorption, 2) Fe displacement from FeEDDHA by Cu on a soil reactive surface, followed by release of CuEDDHA into soil solution, and 3) re-adsorption of CuEDDHA. The effectiveness of FeEDDHA components in soil application is largely determined by their ability to remain in solution. A limited affinity for the soil solid phase and a high (relative) affinity for Fe proved essential in this respect. Clay content, Fe(hydr)oxide content and Cu content were identified as soil characteristics substantially compromising the effectiveness of FeEDDHA components.

# Contents

“I long to learn the things that are,
and comprehend their nature,
and know God.
This is, I said, what I desire to hear.”

Excerpt from:
*Corpus Hermeticum I: “Pimander”*
(translation by G.R.S. Mead (1863 - 1933))

# Chapter 1

General introduction

## Problem outline

Iron (Fe) is an essential micronutrient for plants, humans and other animals. An adequate uptake of Fe is needed to ensure proper growth and development, as well as good health of organisms (Marschner, 1995; Vasconcelos and Grusak, 2007). When provided with insufficient quantities of Fe, organisms will suffer from Fe deficiency symptoms.

Fe deficiency is a worldwide problem in crop production, affecting yield both qualitatively and quantitatively (Mortvedt, 1991); plants do not reach their full growth potential, and the nutritional value is compromised, leading to economic losses and limitations in crop selection (Chaney, 1984). In extreme cases, Fe deficiency may result in complete crop failure (Chen and Barak, 1982). The list of plant species affected is vast and includes apple, citrus, grapevine, peanut, soybean, sorghum and dryland rice (Marschner, 1995).

Fe deficiency is typically found in crops grown on calcareous or alkaline soils, in arid and semi-arid regions of the world; these soils cover over 30% of the earths' land surface (Figure 1.1) (Alvarez-Fernandez, et al., 2006; Chen and Barak, 1982; Hansen, et al., 2006; Mortvedt, 1991). Fe is abundantly present in all soils including calcareous ones; in mineral soils the average Fe content amounts approximately 2% (20,000 μg/g) (Marschner, 1995; Mengel and Kirkby, 2001). Most agricultural crops require less than 0.5 μg/g in the plough layer (Lindsay, 1974). The occurrence of Fe deficiency in plants grown on calcareous soils, despite the excessive soil-Fe pool, is caused by the limited bioavailability of Fe in such soils.

Soil application of synthetic Fe chelates, principally FeEDDHA (and derivatives) increases the bioavailability of Fe and is very successful in treating Fe deficiency (Chen and Barak, 1982). However, because treatment with this type of Fe fertilizers is expensive, they are only applied

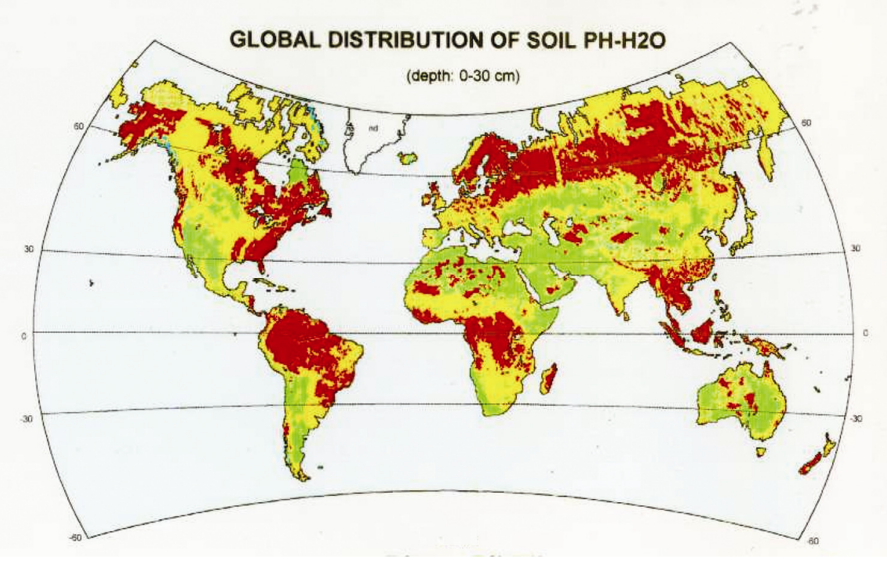



**Figure 1.1:** Global pH-map of the top soil (0-30 cm); red indicates pH < 5.5; yellow indicates 5.5 < pH < 7.3; green indicates pH > 7.3. Calcareous soils are to be found in the green areas. Source: ISRIC, 1995, derived from the WISE- database.

to high value crops (Hansen, et al., 2006; Lucena, 2006). A more efficient application of more effective FeEDDHA fertilizer products would reduce the costs of FeEDDHA treatment. As a result, FeEDDHA application might also become feasible for more crops. To realize a more efficient use of FeEDDHA fertilizer, the fate and effectiveness of FeEDDHA chelates in the soil-plant system need to be better understood. This thesis aims to contribute to this understanding.

## Fe in plants

**Functions**

The Fe content of green plant tissue is in the order of 50-100 mg per kg dry weight (Mengel and Kirkby, 2001). Therefore, Fe is among the most prevalent micronutrients in plants (Bauer and Hell, 2006). Fe is involved in a large number of metabolic processes, including photosynthesis, cell respiration, etc. In green leaves approximately 80% of the Fe is localized in the chloroplasts, regardless of the Fe nutritional status. The tendency of Fe to form chelates and its ability to undergo reversible oxidation-reduction reactions are two important characteristics underlying its numerous physiological effects (Marschner, 1995; Mengel and Kirkby, 2001).

Two important groups of Fe containing proteins can be distinguished: heme-proteins and iron-sulfur proteins. Heme-proteins contain Fe-porphyrin as a prosthetic group and include the cytochromes, catalase and peroxidases and leghemoglobin. In iron-sulfur proteins Fe is coordinated to the thiol group of cysteine, to inorganic sulfur, or to both; this group of proteins includes ferredoxin, isoenzymes of superoxide dismutase and aconitase (Marschner, 1995).

Fe also plays a catalytic role in the biosynthesis of chlorophyll in several reaction steps; it is not incorporated into the chlorophyll structure itself. The rate of δ-aminolevulinic acid (ALA) formation, the common precursor of chlorophyll and heme synthesis, is controlled by Fe (Pushnik and Miller, 1989). Fe is also required for the formation of protoporphyrinogen from coproporphyrinogen (Machold and Stephan, 1969), and for the formation of protochlorophylide from Mg-protoporphyrin (Spiller, et al., 1982).

**Uptake and translocation**

To ensure adequate Fe acquisition from soil and avoid excess Fe in the cells, the uptake, distribution and storage of Fe are tightly regulated in plants. Plants face two major challenges in this respect. First, the solubility of Fe is limited within the physiological pH range, but Fe precipitation inside plant cells needs to be prevented. Secondly, Fe needs to be shielded to prevent it from contributing to oxidative stress (Hell and Stephan, 2003). In particular free Fe can generate hydroxyl radicals through Fenton reactions (Halliwell and Gutteridge, 1986). Hydroxyl radicals can damage cell components including DNA and proteins. The required regulation is accomplished through intricate chelation mechanisms (Hell and Stephan, 2003).

By most plants, Fe is preferentially taken up in ferrous form. Therefore Fe(III) arriving at the root surface, first needs to be reduced by a membrane-bound ferric chelate reductase before

transport into the cytoplasm (Chaney, et al., 1972; Robinson, et al., 1999; Romheld and Marschner, 1983). In grasses however, Fe(III) uptake is of major importance (Romheld and Marschner, 1986).

The transport of Fe inside the plant is presumably largely managed with three transporter compounds (Hell and Stephan, 2003):

- Nicotianamine (NA), which forms ferrous complexes and is the principal transporter of Fe inside cells as well as between cells through symplastic transport (a.o. radial transport).
- Citrate, which forms ferric complexes and is the principal transporter in the xylem (from the roots upwards), as well as in the leaf apoplast.
- Iron transport protein (ITP), which forms ferric complexes and is the principal transporter in the phloem (from the leaves to the non-photosynthetic plant parts including roots, seeds and buds).

Fe storage takes place in phytoferritin, an Fe storage protein located in plastids, with a capacity for storing up to 4,500 Fe atoms in a central cavity (Harrison and Arosio, 1996), and as Fe precipitate in the apoplasmic space between cell wall and plasma membrane, and possibly in the vacuoles (Briat, et al., 2006).

**Fe deficiency**

*Symptoms*

Fe deficiency in plants typically causes chlorosis of leaf tissue because of inadequate chlorophyll synthesis; the leaves become pale green to yellow, often with darker coloured veins. In case of severe chlorosis, leaves can also become necrotic (Figure 1.2).

Chlorophyll is imbedded in thylakoid membranes inside the chloroplast. The Fe requirements for the functional and structural integrity of thylakoid membranes are high. Therefore, in Fe deficient leaves, the content of chlorophyll containing thylakoid membrane is low (Alvarez-Fernandez, et al., 2006; Marschner, 1995). The rate of photosynthesis decreases per unit leaf but not per unit chlorophyll, indicating that the photosynthetic apparatus remains intact (Terry, 1980). Due to the reduction in photosynthetic capacity, carbon fixation by plants also becomes reduced, leading to slower growth rates and yield losses (Figure 1.2) (Alvarez-Fernandez, et al., 2006).

Fe chlorosis develops most strongly in young leaves, because growing plant parts (also fruits, buds and storage organs) have incomplete xylem structures. As a result, Fe is not directly transported from the roots to these sites with the highest demand, but remobilized from older plant parts and secondarily transported through the phloem (Grusak, et al., 1999; Zhang, et al., 1995). It has been observed that chlorotic leaves can have comparable or even higher Fe contents than green leaves (the “chlorosis paradox”). This phenomenon has been attributed to

**Figure 1.2:** Examples of Fe deficiency symptoms in plants.

*Upper left:* chlorotic gerbera with characteristic darker veins;

*Upper right*: necrosis in the leaves of chlorotic soybean plants;

*Below:* reduced growth in chlorotic soybean plants.

impaired expansion growth, leading to diminished dilution of the high Fe concentration in young leaves (Römheld, 2000).

Fe deficiency also causes morphological changes in the roots: inhibition of root elongation, increase in diameter of apical rootzone, abundant root hair formation (Romheld and Marschner, 1981) and formation of rhizodermal transfer cells.

*Causes*

The occurrence of Fe deficiency in plants grown on calcareous soils is caused by a limited bioavailability of Fe. Two related soil characteristics are principally responsible for this low Fe availability: 1) the relatively high pH in calcareous soils (7 - 8.5) (Figure 1.1), and 2) the presence of a bicarbonate pH-buffer in soil solution (Boxma, 1972; Chaney, 1984; Lucena, 2000; Marschner, 1995; Mengel, et al., 1984; Mengel and Kirkby, 2001).

In order for soil-Fe to be taken up, it needs to be transported through soil solution to the root surface. The solubility of soil Fe(hydr)oxides is a function of pH and the type of Fe(hydr)oxide. The concentration of inorganic Fe species in solution reaches a minimum around pH 7.5 - 8.5: in the order of $10^{-10}$ M (Figure 1.3); the free $Fe^{3+}$ concentration is around $10^{-21}$ M (Lindsay and Schwab, 1982). For optimal growth, plants require an Fe concentration in soil solution in the order of $10^{-6}$ to $10^{-5}$ M (Marschner, 1995). Complexation by dissolved organic substances, like humic acids, fulvic acids and siderophores can increase the total Fe concentrations in soil solution by orders of magnitude in comparison to the inorganic Fe concentration (O'Conner G.A., et al., 1971), but not always sufficiently to prevent Fe deficiency.

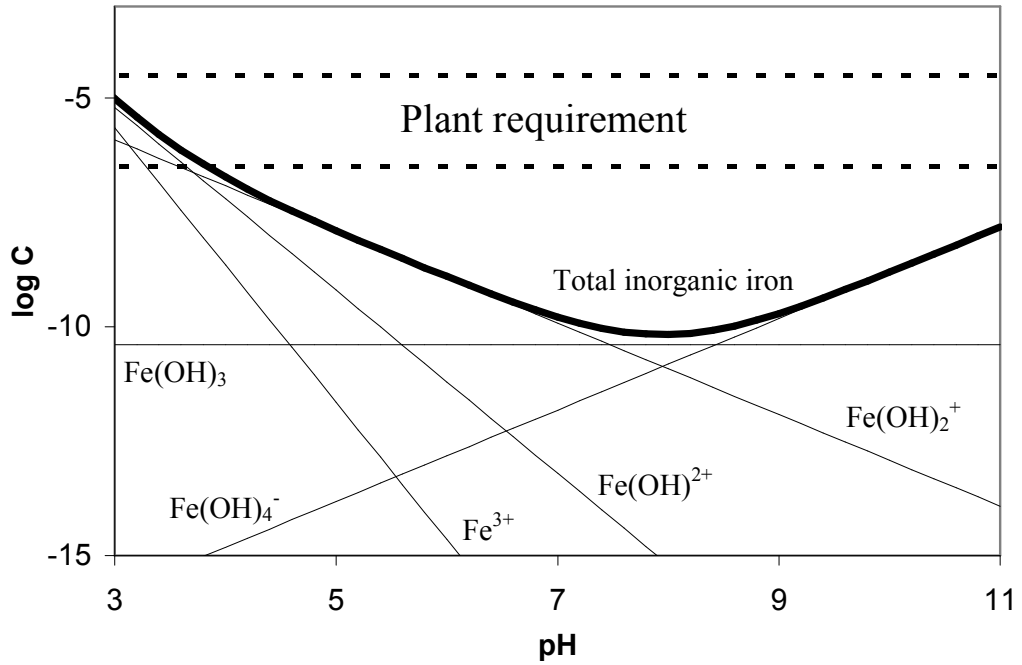



**Figure 1.3:** Hydrolysis species of Fe(3+) in equilibrium with soil-Fe ($pK_{sol}$ = 39.3; I = 0.03 M), after Lindsay (1979).

The bicarbonate pH-buffer prevents plants from adapting the rhizosphere pH and causes impairment of Fe deficiency stress response mechanisms (except in grasses). Although the pH-buffer capacity of calcareous soils is largely determined by the lime content, the dissolution of carbonate minerals is relatively slow in comparison to bicarbonate diffusion.

Therefore, on the short term, the bicarbonate concentration in soil solution is more important for maintaining a high rhizosphere pH (Lucena, 2000). In addition to the role of bicarbonate as pH-buffer in soil solution, there has been much debate on bicarbonate uptake leading to Fe immobilization inside plants (Gruber and Kosegarten, 2002; Mengel, 1994; Nikolic and Romheld, 2002; Römheld, 2000).

A large number of additional factors can affect the bioavailability of Fe in soil (Morris, et al., 1990), including:

- Indigenous soil factors – e.g. content, mineralogy and crystallinity of soil Fe(hydr)oxide (particularly important for grasses), organic matter content, content and reactivity of the $CaCO_3$ phase
- Environmental factors – e.g soil water content, compaction, gas-phase composition and rooting volume
- Nutritional factors – e.g. $NH_4/NO_3$ and Mn/Fe balance, and P and K fertilization
- Microbial factors

*Fe stress response mechanisms*

Two plant strategies for Fe acquisition from soil under Fe deficiency stress have been distinguished (Marschner and Romheld, 1994; Romheld and Marschner, 1986). Strategy I is observed in dicotylodonous and non-graminaceous monocotylodous plants and includes enhanced proton release, enhanced Fe(III) reduction at the plasma membrane, and enhanced release of reductors/chelators (mainly phenolics). When effective, these responses enhance the solubilization of soil Fe and the transport of Fe into the plant.

Strategy II is observed in graminaceous plants and involves the release of phytosiderophores – plant produced complexing agents, which bind and solubilize Fe from soil. Uptake of the Fe(III)phytosiderophore complex is mediated by a specific transporter in the plasma membrane of the root cells.

**Prevention and remediation of Fe deficiency**

When Fe stress response mechanisms of plants prove inadequate, techniques to prevent or remedy Fe deficiency need to be applied to avoid yield losses. Breeding and genetically modifying plants for a more efficient Fe uptake mechanism is a promising approach. Developing new cultivars should however be done carefully and requires much time. Once crops are in the field, application of Fe fertilizer is the most certain and efficient treatment to ensure that plants do not suffer from Fe deficiency.

Fe fertilizers can be administered through trunk injection, foliar application, and soil application. Trunk injection is expensive and only suitable for trees. Foliar application does not provide full control of Fe chlorosis, but can be useful as complementary technique next to soil application (Alvarez-Fernandez, et al., 2004). Soil application is the most common technique to manage Fe deficiency in soil grown crops (Lucena, 2006). The technique is based on increasing the Fe concentration in soil solution. On calcareous soils, soil application of Fe

fertilizers based on organic Fe salts, Fe complexes of lignosulfonates, citrates, gluconates, and synthetic Fe chelates of limited stability (e.g. FeEDTA, FeDTPA and FeHEDTA) has limited or no result, because these fertilizers are not able to maintain Fe in soil solution. Only Fe chelates of higher stability (FeEDDHA and derivatives, with phenolic functional groups) are effective and provide the most efficient treatment to control Fe deficiency (Lucena, 2006).

## FeEDDHA based Fe fertilizer

**Chelates**

Chelates comprise a metal ion bound to a chelating agent. Chelating agents are bi- or multidentate complexing agents i.e. ligands with more than one functional group. The term chelate is derived from the Greek αί Χηλαί (~ hai chelai), which means crustacean's claw, referring to the ligand clamping the metal ion, and was first used by Morgan and Drew (1920). Due to their ability to form stable, water-soluble complexes with di- and trivalent cations, chelating agents act as moderators of the solubility, mobility, bioavailability and reactivity of the chelated metal ion (Bucheli-Witschel and Egli, 2001).

The complexation constant is a thermodynamic measure for the stability of a chelate. For a complexation reaction in which metal ion and chelating agent react in a stoichiometric ration of 1, the complexation constant can be described as:

$$Ch^{m-} + M^{n+} \leftrightarrows MCh^{(m-n)-} \qquad K_{st} = \frac{(MCh^{(m-n)-})}{(Ch^{m-})(M^{n+})} \tag{1}$$

In soil systems containing multiple metals, the extent to which these metals are chelated under equilibrium conditions depends on both complexation constants and metal activities (equation (1)).

**FeEDDHA**

FeEDDHA is the iron(3+) complex of the chelating agent EDDHA, which is an acronym for ethylene diamine di(hydroxy phenyl acetic acid). EDDHA is also referred to as EHPG (ethylenebis-(hydroxy phenyl glycine)). This chelating agent was first synthesized by Kroll, introduced in 1955, but only fully described in 1957 (Kroll, 1957; Kroll, et al., 1957; Wallace, 1966). FeEDDHA was quickly recognized as very effective in correcting Fe chlorosis under soil conditions, also in comparison to other chelating agents (Wallace, et al., 1955; Wallace, 1962). The $Fe^{3+}$ ion is bound by 2 carboxylate groups, 2 phenolate groups and 2 secondary amine groups in an octahedral complex of high stability with an intense red colour at neutral pH. The FeEDDHA complex owes its high stability in comparison to FeEDTA or FeDTPA complexes to the Fe-O (phenolate) bonds. On account of this high stability, EDDHA has also been evaluated as a model compound for the binding site in the Fe transport protein serum

transferrin (Gaber, et al., 1974; Patch, et al., 1983). The primary application of FeEDDHA is however as a fertilizer.

**Synthesis and stereochemistry**

Kroll (1957) first synthesized the EDDHA ligand through addition of hydrogen cyanide to the Schiff base from salicylaldehyde and ethylenediamine (the salen ligand), and subsequent acidic hydrolysis of the dinitrile in hydrochloric acid (a Strecker reaction). Because the application of hydrogen cyanide on an industrial scale requires extensive safety measures and is therefore costly, alternative pathways for the synthesis of EDDHA were sought and found by Petree et al. (1978) and Julien and Aymard (1989). The current synthesis pathway for manufacturing EDDHA on an industrial scale is a Mannich-like reaction between phenol, ethylenediamine and glyoxylic acid. This reaction produces a mixture of 1) positional isomers, 2) diastereomers and 3) polycondensates, because 1) the reaction pathway allows for aromatic substitution in (o) ortho and (p) para position, 2) two chiral centers are introduced into the molecule leading to (R,R); (R,S); (S,R) and (S,S) configurations, and 3) undesired addition reactions take place between reactants and half products. The composition of the mixture of reaction products can be steered. After the reaction is terminated, an Fe salt is added to the reaction products to form Fe chelates

Commercial FeEDDHA formulations can be operationally divided into 4 groups of compounds:

1) racemic o,o-FeEDDHA (Figure 1.4a); referring to the (R,R) and (S,S) configurations of o,o-FeEDDHA (iron (3+) ethylene diamine-N,N'-bis(2-hydroxy phenyl acetic acid) complex). These configurations are mirror images, but identical in most physical and chemical properties, including binding strength.
2) meso o,o-FeEDDHA (Figure 1.4b); referring to the (S,R) = (R,S) configuration of o,o-FeEDDHA. Due to the internal mirror plane of the chelate, the (S,R) and (R,S) configurations are identical.
3) o,p-FeEDDHA (Figure 1.4c); referring to the 4 configurations of o,p-FeEDDHA (iron (3+) ethylene diamine-N-(2-hydroxy phenyl acetic acid)-N'-(4-hydroxy phenyl acetic acid) complex). The o,p-FeEDDHA configurations are not identical in physical and chemical properties.
4) rest-FeEDDHA; referring to the 3 configurations of p,p-FeEDDHA (iron (3+) ethylene diamine-N,N'-bis(4-hydroxy phenyl acetic acid) and a variety of polycondensates and half products. An example of a polycondensate is depicted in Figure 1.4d.

These 4 groups will be referred to as the FeEDDHA components, and the corresponding groups of EDDHA ligands as the EDDHA components. In commercial FeEDDHA formulations, the sum of the racemic and meso o,o-FeEDDHA content is referred to as the o,o-FeEDDHA content of the product. Generally racemic and meso o,o-FeEDDHA are synthesized in a ratio close to 1

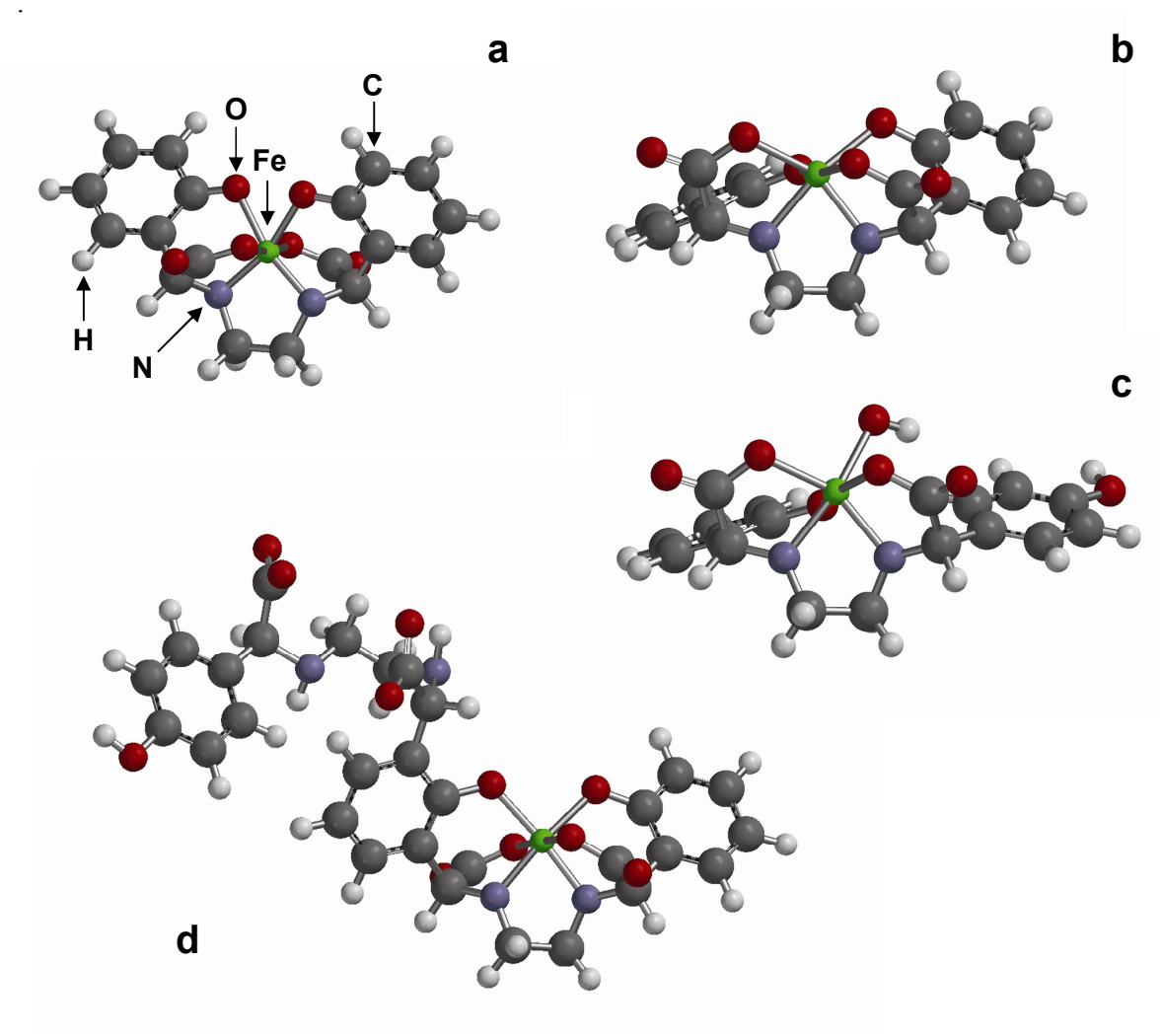



**Figure 1.4:** Spatial structures of the FeEDDHA components **a)** racemic o,o-FeEDDHA; **b)** meso o,o-FeEDDHA; **c)** o,p-FeEDDHA with $OH^-$ on the coordination complex; and **d)** rest-FeEDDHA (one possible polycondensate).

Racemic and meso o,o-FeEDDHA are diastereomers; the chelated Fe is bound by the same functional groups, but the geometry of the chelate differs: in racemic o,o-FeEDDHA, both phenolic rings are in equatorial position, while in meso o,o-FeEDDHA one phenolic ring is in equatorial and the other in axial position (Figure 1.4a and 1.4b). Due to the difference in geometry the amount of strain on the bonds with Fe differs, which is reflected in a higher complexation constant for racemic o,o-FeEDDHA.

The position of the hydroxyl group on the phenolic ring affects the complexation constant of FeEDDHA components more strongly than strain: in para-position the hydroxyl group is sterically inhibited from contributing to binding Fe. As a consequence, o,o-EDDHA binds Fe

more strongly than o,p-EDDHA (see Table 1.1), which in turn binds Fe more strongly than p,p-EDDHA. Rest-FeEDDHA is a very heterogeneous group, comprising of compounds that vary in molecular weight, number of functional groups, etc, and hence also in complexation constant.

**Table 1.1:** Complexation constants of FeEDDHA components.

| Component | Log K |
|---|---|
| racemic o,o-FeEDDHA | 35.86[b] |
| meso o,o-FeEDDHA | 34.15[b] |
| o,p-FeEDDHA | 28.72[a] |

[a] Yunta et al. (2003b); [b] Yunta et al. (2003a) (I = 0.1 M (NaCl)).

**The market and regulation of FeEDDHA products**

The market size for products based on FeEDDHA or related phenolic aminocarboxylate Fe chelates (e.g. FeEDDHMA, FeEDDHSA), is approximately 10,000 tonnes per year, corresponding with a market value of around 60 million Euros. It is linked to areas of high soil-pH, in particular the Mediterranean area and the Middle East. The FeEDDHA market has oligopolistic features with three main producers Ciba Geigy, Syngenta and AkzoNobel. However, in recent years the market has been increasingly shifting towards monopolistic competition. Due to the lack of barriers, new producers (a.o. Lab Jaer, Valagro and Tradecorp.) have entered. The market has many consumers that perceive there are differences between competitors' products besides the price, namely the composition of the product in terms of FeEDDHA components. This difference in composition offers producers a certain degree of control over the price of their FeEDDHA products.

From the variability in composition of FeEDDHA products, and the difference in fertilizer value of the FeEDDHA components arose the need to ensure the quality of commercial FeEDDHA formulations. Several tests and methodologies have been developed to asses the quality of FeEDDHA products (Cantera, et al., 2002; Garcia-Marco, et al., 2003; Lucena, et al., 1992a; b). At present the quality of FeEDDHA products is guarded in the European Fertilizer Law (Regulation (EC) No. 2003/2003; amendment (EC) No. 162/2007) through the following parameters: (1) soluble Fe content of the product, (2) percentage of Fe chelated, and (3) percentages of Fe chelated by respectively o,o-EDDHA and o,p-EDDHA. Data on these parameters have to be indicated on the product label. FeEDDHA products should comprise at least 5 weight percent of water-soluble Fe, of which at least 80 percent should be chelated, and at least 50 percent should be chelated to either o,o- or o,p-EDDHA. To be included on the product label, there is a threshold value for both o,o- and o,p-EDDHA of 1 weight percent of chelated Fe.

**Analysis of FeEDDHA products**

In order to quantify the composition of FeEDDHA products, both for product information and law enforcement purposes, suitable protocols for analysis had to be developed. Much effort has been invested in the synthesis, separation, identification and quantification of FeEDDHA components (Alvarez-Fernandez, et al., 2007; Bailey, et al., 1981; Barak and Chen, 1987; Cremonini, et al., 2001; Garcia-Marco, et al., 2006b; Gomez-Gallego, et al., 2002; Hernandez-Apaolaza, et al., 1997; Hernandez-Apaolaza, et al., 2006; Hill-Cottingham, 1962). The method that is currently used for quantitative analysis is the high performance liquid chromatography (HPLC) method laid down by the European Committee for Standardization (CEN. EN 13368-2:2007). This method is almost identical to the ion-pair HPLC method developed by Lucena et al (1996).

**Characterization of EDDHA components**

The properties of EDDHA components have been intensively examined, both in view of understanding and predicting their behaviour in agricultural systems and in evaluating their aptness as a model compound for transferrins. Several studies have been dedicated to determining protonation and metal-complexation constants of EDDHA components (Ahrland, et al., 1990; Anderegg and L'Eplattenier, 1964; Bannochie and Martell, 1989; Frost, et al., 1958; Yunta, et al., 2003a; Yunta, et al., 2003b). Furthermore, the crystal structure (Bailey, et al., 1976; Bailey, et al., 1981; Riley, et al., 1983), the spin structure (Ainscough, et al., 1980; Gomez-Gallego, et al., 2006), redox properties (Gomez-Gallego, et al., 2005b; Schroder, 1964), and spectroscopic properties (Patch, et al., 1982; 1983) of EDDHA complexes have been examined. FeEDDHA complexes owe their characteristic deep red colour to the absorption of visible light with a maximum around $\lambda$ = 480 nm, typical for the Fe-phenolate bond.

**Application of FeEDDHA fertilizer**

FeEDDHA is used as Fe fertilizer both on soil and in hydroponic systems. Fe fertilizers need to meet different requirements for these two types of systems in order to be effective, because the conditions in these systems differ considerably. This study only considers FeEDDHA in soil application.

As mentioned, FeEDDHA is applied to soil to increase the solubility of Fe, thereby enhancing its bioavailability through an increase in diffusive flux of Fe to the root. When introduced into soil-plant systems, FeEDDHA components participate in and are exposed to various physical, chemical and biological processes affecting their soil solution concentration. These processes include adsorption, cation competition, biological and photochemical degradation, leaching, complex dissociation, precipitation on the soil surface, Fe transfer to the plant and chelation of metals from the soil by the corresponding EDDHA components. If these processes have a substantial negative impact on the concentration, the effectiveness of FeEDDHA components becomes compromised or even negligible.

Considerable effort has been invested in better understanding several of the aforementioned processes. The interaction between FeEDDHA components and soil and soil constituents has

been examined (Alvarez-Fernandez, et al., 2002; Cantera, et al., 2002; Garcia-Marco, et al., 2006a; Hernandez-Apaolaza, et al., 2006; Hernandez-Apaolaza and Lucena, 2001), and so has Fe uptake from FeEDDHA components (Cerdan, et al., 2006; Garcia-Marco, et al., 2006a; Hernandez-Apaolaza, et al., 2006; Lucena and Chaney, 2006; 2007; Rojas, et al., 2008), leaching of FeEDDHA components (Cesco, et al., 2000; Lucena, et al., 2005), mobilization of Fe from Fe oxides by EDDHA ligands (Perez-Sanz and Lucena, 1995) and photochemical degradation of FeEDDHA components (Gomez-Gallego, et al., 2005a).
In the aforementioned studies, these processes were generally considered separately and under experimental conditions quite remote from those observed in soil-plant systems. A more integral approach is required for determining the actual impact of individual processes on the effectiveness of FeEDDHA components in soil application. In this approach, soil solution concentrations of FeEDDHA components and Fe uptake by plants should serve as principal touchstones for effectiveness, and should be considered in the same system.

## Objective and approach

The effectiveness of FeEDDHA products in mending Fe chlorosis has been proven in the 1950s and is undisputed. However, nowadays, the composition of these products in terms of FeEDDHA components varies greatly. The extent to which individual FeEDDHA components contribute to supplying plants with Fe is unclear, in particular in soil-plant systems.
An efficient use of FeEDDHA fertilizer, implying maximizing the benefits in terms of crop yield and Fe uptake by plants, while minimizing the applied FeEDDHA dosage, is desirable both for the applier in view of cost efficiency, and from an environmental perspective to minimize the input of synthetic chemicals into the environment. In practical terms efficient FeEDDHA application translates into applying the right fertilizer (right composition) at the right moment in the right quantity. This requires an advanced understanding of the processes affecting the residence time of FeEDDHA components in soil solution as well as of the effectiveness of the individual FeEDDHA components in supplying plants with Fe. This understanding has been lacking.
The objective of this study was to establish the processes determining the fate of FeEDDHA components in soil-plant systems, and to relate these processes to the characteristics of FeEDDHA components, determining their effectiveness as Fe fertilizer.

To achieve this objective, the following tools were used:

- Pot trials – to examine FeEDDHA-facilitated Fe uptake by plants.
- Soil interaction experiments – to examine the speciation of EDDHA ligands in soil systems.
- Interaction experiments in model systems – to examine the interaction between EDDHA species and specific soil reactive compounds and competing cations.
- Mechanistic multi-surface modeling – to predict the equilibrium speciation of EDDHA ligands under soil conditions.

This study has been approached as follows:
First the constituents of FeEDDHA fertilizers were screened with regard to their reactivity towards soil reactive compounds and their effectiveness in supplying soil-grown plants with Fe. Then, the behaviour of FeEDDHA components in soil plant-systems was studied as a function of time and related to Fe uptake by plants. After that, the effectiveness of FeEDDHA components was individually assessed. Subsequently, the contribution of individual processes to the observed behaviour of FeEDDHA components in soil-plant system was examined. Finally, based on the gathered experimental data, a conceptual model was composed, for qualitatively describing and mechanistically explaining the fate and effectiveness of FeEDDHA components in soil-plant systems.

## Outline

**Chapter 2** considers the behaviour of FeEDDHA components and EDDHA ligands upon interaction with eight soils as a function of time, and relates the observed behaviour to soil properties. Interaction was examined in a batch experiment, involving FeEDDHA treatments consisting of mixtures of FeEDDHA components and EDDHA ligands.
**Chapter 3** investigates the relation between the composition of soil-applied FeEDDHA treatments and Fe uptake by plants in order to determine which FeEDDHA components are effective in providing plants with Fe and preventing Fe deficiency chlorosis. The effectiveness of the FeEDDHA components was examined in a pot trial experiment with soybean involving eight soils.
**Chapter 4** reports a pot trial experiment in which soybean plants grown on a calcareous soil received FeEDDHA treatments differing in composition and Fe dosage. The pore water concentration of FeEDDHA components was examined as a function of time and related to Fe uptake by the plants.
**Chapter 5** assesses the effectiveness of FeEDDHA components in supplying soil-grown plants with Fe, in relation to the growth stage in which FeEDDHA is applied. The assessment included the quantitatively most important FeEDDHA components: the isomers racemic o,o-FeEDDHA, meso o,o-FeEDDHA and o,p-FeEDDHA and was done by means of a pot trial study with soybean plants grown on a calcareous soil.
**Chapter 6** examines the potential impact of biodegradation on the soil solution concentrations of EDDHA chelates upon administration of an FeEDDHA treatment to a calcareous soil. The examination was done by means of a soil-incubation study involving sterilized and non-sterilized treatments and including two conditioning regimes.
**Chapter 7** explores the potential impact of cation competition from Cu on the performance of o,o-FeEDDHA isomers in application on calcareous soils. Through mechanistic multi-surface modeling the thermodynamic basis for Fe displacement from FeEDDHA by Cu was examined. In a soil interaction experiment the affinity of o,o-CuEDDHA for the soil solid phase was studied. And also, potential kinetic inhibition of the displacement reaction was investigated in goethite suspensions containing FeEDDHA and Cu.

**Chapter 8** addresses the kinetics of the reaction in which Fe is displaced from FeEDDHA by Cu. In a study with goethite suspensions, the influence of soil properties like Cu content, available Fe(hydr)oxide surface, etc on the displacement rate was examined. For the displacement reaction with meso o,o-FeEDDHA the rate equation was derived and the kinetic parameters determined. In conclusion, the rate equation was solved for soil conditions.
**Chapter 9** deals with a hypothesized mechanism of action for Fe chelates applied as fertilizer, in which the chelating agent partakes in a cyclic process of delivering Fe at the root surface and mobilizing Fe from soil. The performance of this "shuttle mechanism" was evaluated for FeEDDHA chelates. The effectiveness of EDDHA ligands at specifically chelating and mobilizing Fe from soils of low Fe availability was tested in batch experiments. Furthermore, data collected in the pot trials reported in Chapter 3 and 4 were examined for experimental support for the shuttle mechanism with FeEDDHA.
**Chapter 10** provides a conceptual model for the behaviour of FeEDDHA components in soil-plant systems, based on a synthesis of the research presented in the previous chapters, supplemented with additional experimental data. Within the framework of this model, the effectiveness of the FeEDDHA components is considered, as well as how this effectiveness is affected by soil parameters. The chapter concludes with practical implications and limitations of the presented research, and recommendations for further research.

"… serving the deep red elixir."

Excerpt from the lyrics to:
*"Into the Faculty of Wonderful Secrets"*
by Tartaros / Joachim Rygg

# Chapter 2

## The behaviour of EDDHA components in soils as influenced by soil properties

W.D.C. Schenkeveld, A.M. Reichwein, E.J.M. Temminghoff, and W.H. van Riemsdijk

*Adapted from:* Plant and Soil (2007), 290(1/2): 85-102

## Abstract

FeEDDHA products are applied to correct Fe chlorosis in plants and consist of a mixture of EDDHA components chelated to Fe. In this study such mixtures have been divided into four (groups of) components: racemic o,o-EDDHA, meso o,o-EDDHA, o,p-EDDHA and rest-EDDHA. The physical and chemical properties of these components differ and consequently, so does their ability to deliver Fe to plants. To come to a soil-specific Fe fertilization recommendation, the behaviour of the EDDHA components in the soil needs to be understood. This behaviour has been examined in a soil interaction experiment as a function of time, and it has been related to soil properties.

The FeEDDHA component fractions remaining in solution can be ranked: racemic o,o-FeEDDHA > meso o,o-FeEDDHA > rest-FeEDDHA > o,p-FeEDDHA, regardless of soil properties. The o,o-EDDHA isomers largely determine the Fe concentration in soil solution. Although rest-EDDHA also consists of compounds that chelate Fe more strongly than meso o,o-EDDHA, the latter is on average better capable of keeping Fe in solution upon interaction with soil. The principal adsorption surface differs per FeEDDHA component; for racemic o,o-FeEDDHA it is organic matter, for meso o,o-FeEDDHA, Fe(hydr)oxides, and for o,p-FeEDDHA, clay minerals. Cu and Al are important competing cations. Cu forms soluble complexes with o,p-EDDHA, and Al with meso o,o-EDDHA not chelated to Fe. Al is likely to affect the effectiveness of a potential shuttle effect. The tendency of o,p-FeEDDHA and rest-FeEDDHA to be removed from solution, makes these components less effective as Fe fertilizer in soil application, in particular on clay soils.

# Introduction

Fe is an essential micronutrient for its role in the formation of chlorophyll and in various enzymatic processes (Marschner, 1995). Although generally present in the soil in sufficient quantities, Fe is not always sufficiently available to plants. A low bioavailability of Fe may lead to Fe chlorosis in crops. Fe chlorosis is a nutritional disorder characterized by a significant decrease of chlorophyll in the leaves. It reduces crop quality and depresses crop yields and hence causes economic losses. In particular in alkaline and calcareous soils, the bioavailability of Fe can be severely limited (Chaney, 1984; Mortvedt, 1991). This mainly results from the low solubility of Fe(hydr)oxides at high pH (Lindsay, 1979), and from the elevated bicarbonate concentration in the soil solution (Boxma, 1972; Mengel, et al., 1984; Shi, et al., 1993). Bicarbonate either impairs Fe uptake mechanisms of the plant (Marschner, 1995; Venkatraju and Marschner, 1981) or inactivates Fe in the leaf apoplast (Mengel, 1994). Most alkaline and calcareous soils are found in areas of the reference soil groups calcisols and solonchaks which cover nearly 10 million $km^2$ of the earth's surface (FAO/AGL, 2000).

The most common practice to overcome Fe deficiency in plants is the application of synthetic Fe chelates (Chen and Barak, 1982). FeEDDHA (iron (3+) ethylene diamine-N,N'-bis(hydroxy phenylacetic acid)) is among the most effective Fe fertilizers on neutral and alkaline soils (Lucena, et al., 1992b; Papastylianou, 1990; Reed, et al., 1988). Its agronomical performance has been intensively studied since the 1950s (Hill-Cottingham and Lloyd-Jones, 1958; Kroll, 1957; Wallace, et al., 1955). Several studies have been dedicated to the determination and the quantification of FeEDDHA (Barak and Chen, 1987; Hernandez-Apaolaza, et al., 1997; Lucena, et al., 1996).

The synthesis pathway applied for manufacturing commercial FeEDDHA formulations is a Mannich-like reaction between phenol, ethylene diamine and glyoxylic acid (Julien and Aymard, 1989; Petree, et al., 1978). The reaction produces a mixture of (1) positional isomers, (2) diastereomers and (3) polycondensates. This is because: (1) the reaction pathway allows for aromatic substitution in both ortho (o) and para (p) position, (2) two chiral centres are introduced into the molecule, leading to (R,R), (R,S), (S,R) and (S,S) enantiomers and (3) undesired addition reactions take place between reactants and half products (Cremonini, et al., 2001).

Throughout this paper, the EDDHA synthesis products are divided into four groups, labelled as follows: (1) racemic o,o-EDDHA (referring to the (R,R) and (S,S) o,o-EDDHA (ethylene diamine-N,N'-bis(2-hydroxy phenyl acetic acid)) enantiomers; the enantiomers are mirror images, differing in the direction they deviate polarized light, but identical in binding strength), (2) meso o,o-EDDHA (referring to the (R,S) = (S,R) enantiomer; due to the internal mirror plane of the molecule, the (R,S) and (S,R) configurations are identical (Bailey, et al., 1981; Hill-Cottingham, 1962; Ryskievich and Boka, 1962)), (3) o,p-EDDHA (referring to the four o,p-EDDHA (ethylene diamine-N-(2-hydroxy phenyl acetic acid)-N'-(4-hydroxy phenyl acetic acid)) enantiomers) and (4) rest-EDDHA (referring to the 3 p,p-EDDHA (ethylene diamine-N,N'-bis(4-hydroxy phenyl acetic acid) enantiomers and a variety of polycondensates

and half-products (Cremonini, et al., 2001; Hernandez-Apaolaza, et al., 2006)). In general these four groups are referred to as EDDHA components.

Because the physical and chemical properties of these EDDHA components differ, so does their ability to bind Fe and deliver it to the plant. Binding strength parameters such as protonation constants and complexation constants for Fe and several other metals have been determined for the most important EDDHA components (Ahrland, et al., 1990; Bannochie and Martell, 1989; Frost, et al., 1958; Yunta, et al., 2003a; Yunta, et al., 2003b).
The compositions of commercially available FeEDDHA formulations differ, and therefore the need for a quality parameter arose. Several parameters have been proposed (Hernandez-Apaolaza, et al., 1995; Lucena, et al., 1992a; b). At present, the quality aspect is assured in the European Fertilizer Law (Regulation (EC) No. 2003/2003; amendment (EC) No. 162/2007) through three parameters: (1) soluble Fe content of the product, (2) percentage of Fe chelated, and (3) percentages of Fe chelated by respectively o,o-EDDHA and o,p-EDDHA. The o,o-EDDHA isomers are supposedly the strongest chelating agents present in FeEDDHA products (Table 1.1) (Hernandez-Apaolaza, et al., 2006; Yunta, et al., 2003a; Yunta, et al., 2003c). Commercial FeEDDHA formulations tend to contain significant amounts of EDDHA components other than o,o-EDDHA that are not chelated to Fe.
The characteristics of an Fe chelate that determine its effectiveness in agronomic practice are: (1) its ability to remain in solution, (2) its susceptibility to competition from other metal ions, (3) its ability to deliver Fe to the plant, and (4) its selectivity to pick up Fe from the soil, either after having delivered an Fe ion to the plant (shuttle effect) or upon initial contact with the soil (Lucena, 2003). In the case of soil application of Fe chelates, at least three out of four of these features are co-determined by the characteristics of the soil.

To improve the understanding on the interaction of FeEDDHA components with soil and soil constituents, several studies have been done (Alvarez-Fernandez, et al., 1997; Alvarez-Fernandez, et al., 2002; Cantera, et al., 2002; Garcia-Marco, et al., 2006; Garcia-Mina, et al., 2003; Hernandez-Apaolaza, et al., 2006; Hernandez-Apaolaza and Lucena, 2001; Siebner-Freibach, et al., 2004). Soil organic matter (acid peat) and Fe(hydr)oxides (ferrihydrite) have been identified as the most reactive, and calcium carbonate and clay (Ca-montmorillonite) as less reactive soil constituents with respect to FeEDDHA sorption (Alvarez-Fernandez, et al., 1997; Alvarez-Fernandez, et al., 2002). Meso o,o-FeEDDHA was found to be more susceptible to sorption than racemic o,o-FeEDDHA (Alvarez-Fernandez, et al., 2002; Hernandez-Apaolaza and Lucena, 2001). The speciation of certain metals other than Fe is also affected from soil application of dissolved FeEDDHA products: Cu and Mn have been reported to go into solution. (Alvarez-Fernandez, et al., 1997; Alvarez-Fernandez, et al., 2002; de Kreij, 1998; Gil-Ortiz and Bautista-Carrascosa, 2004). Whether this is the result of Fe displacement or of complexation by chelating compounds initially not chelating Fe has not been cleared up. o,p-EDDHA not chelated to Fe has been reported to dissolve Cu from soils (Garcia-Marco, et al., 2006).

Up until now, interaction studies with EDDHA components have mainly focused on isolated and synthesized soil constituents, while actual soils have been largely approached as black boxes. In this study a novel approach was followed, in which the influence of reactive soil constituents and competing cations on EDDHA component behaviour was examined within the soil system itself. An understanding of this issue is crucial to come to an adequate, soil specific Fe fertilization recommendation.

The aim of this research was (1) to examine EDDHA component behaviour upon interaction with soils as a function of time, and (2) to relate this behaviour to soil properties. More specifically, an attempt was made to pinpoint per component which reactive surfaces and competing cations are dominant in determining their behaviour upon interaction with soil. Because o,p-EDDHA and rest-EDDHA are present in commercial formulations both chelated to and non-chelated to Fe, this distinction was also included in this study.
An experiment was done in which a number of soils were allowed to interact with a number of FeEDDHA solutions. The FeEDDHA solutions differed in (1) EDDHA component composition, primarily the o,o-EDDHA content, and (2) the degree to which the chelating capacity of the EDDHA solutions was saturated with Fe. The aqueous phase was examined as a function of time.

# Materials and Methods

**Soils**
Soils were collected from seven sites, located in Italy (Bologna), Spain (Xeraco and Santomera), Saudi Arabia (Nadec and Hofuf) and the Netherlands (Droevendaal and Herveld). The soils are named after the location of collection. The sites were selected so that there were ranges in soil properties and constituents reported to interact with FeEDDHA components (Alvarez-Fernandez, et al., 1997). Four clay soils and four sandy soils were included. At all sites the top layer (0 – 20 cm) was sampled. From one site (Xeraco, Spain), soil from both the top layer and the layer directly underneath (20 - 40 cm) was collected separately. The top layer is relatively rich in organic material. In crops grown at the sites in Spain, Italy and Saudi Arabia, Fe chlorosis was manifest. The two Dutch sites were included to extend the ranges of potentially relevant soil characteristics. Pre-treatment consisted of drying (40 °C) and sieving (2 mm). The chemical and textural properties of the soils were analysed, the prime results of which are presented in Table 2.1.

**Experimental solutions**
Seven FeEDDHA solutions and a blank were used in the interaction experiment. The FeEDDHA solutions were prepared from three sodium-EDDHA stock solutions and solid o,o-$H_4$EDDHA (99% pure). The stock solutions were synthesized through the aforementioned Mannich-like reaction (Petree, et al., 1978) and differed in o,o-EDDHA-content: approximately 20%, 40% and 60% on an ethylene diamine input basis. Out of each stock

**Table 2.1:** Soil characteristics.

| Soil (origin) | Country | Soil class. | pH-$CaCl_2$[a] | SOC[b] | Clay[c] | $CaCO_3$[d] | CEC[e] | 0.01 M $CaCl_2$ DOC[f] | Oxalate-extraction[g] Fe | Oxalate-extraction[g] Al | DTPA-extraction[h] Fe | Mn | Cu | Zn | Co | Ni |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | (g $kg^{-1}$) | (g $kg^{-1}$) | (g $kg^{-1}$) | (cmol eq $kg^{-1}$) | (mg $kg^{-1}$) | (g $kg^{-1}$) | (g $kg^{-1}$) | (mg $kg^{-1}$) | | | | | |
| **Bologna** | IT | entisol | 7.9 | 8.7 | 230 | 140 | 17 | 53 | 1.54 | 0.46 | 18 | 8.6 | 2.8 | 0.6 | 0.0 | 0.7 |
| **Xeraco top** | ES | entisol | 7.8 | 43.7 | 100 | 420 | 33 | 234 | 1.68 | 0.78 | 82 | 3.8 | 1.1 | 7.1 | 0.0 | 0.5 |
| **Xeraco lower** | ES | entisol | 7.8 | 13.7 | 360 | 150 | 30 | 78 | 0.90 | 1.74 | 10.5 | 5.3 | 3.0 | 6.7 | 0.0 | 0.2 |
| **Santomera** | ES | entisol | 8.0 | 5.4 | 260 | 520 | 10.3 | 30 | 0.30 | 0.44 | 3.5 | 4.6 | 4.1 | 0.9 | 0.0 | 0.2 |
| **Nadec** | SA | aridisol | 8.1 | 8.7 | 70 | 140 | 5.5 | 93 | 0.13 | 0.18 | 2.1 | 5.7 | 0.1 | 0.5 | 0.0 | 0.1 |
| **Hofuf** | SA | aridisol | 7.9 | 7.1 | 40 | 60 | 3.5 | 53 | 0.19 | 0.08 | 6.7 | 3.8 | 2.3 | 5.0 | 0.0 | 0.1 |
| **Droevendaal** | NL | spodosol | 6.5 | 15.2 | 40 | 0 | 3.3 | 54 | 1.68 | 1.40 | 60 | 1.5 | 1.1 | 1.8 | 0.0 | 0.1 |
| **Herveld** | NL | spodosol | 7.2 | 15.2 | 260 | 30 | 24 | 139 | 2.33 | 0.68 | 31 | 21.5 | 5.3 | 10.8 | 0.0 | 0.8 |

[a] ISO/DIS 10390 Soil Quality – Determination of pH
[b] Walinga et al. (1992)
[c] Houba et al. (1997)
[d] ISO 10693, Soil Quality – Determination of carbonate content, volumetric method
[e] ISO/DIS 11260 Soil Quality – Determination of cation exchange capacity and base saturation – method using barium chloride solution
[f] Houba et al. (2000)
[g] Schwertmann (1964)
[h] Linday and Norvell (1978) and Quevauvillier et al. (1996)

solution, two experimental solutions were prepared through the addition of different amounts of $FeCl_3*6H_2O$. To the first solution an amount of Fe was added, equal to the molar equivalent of o,o-EDDHA. This solution was given the P-suffix for "Partly chelating Fe". To the second solution an amount of Fe was added, 5% in excess based on a 1:1 stoichiometry between Fe and ethylene diamine. This solution was given the F-suffix for "Fully chelating Fe". The pH was raised to 7 (± 0.5) and the solutions were left over-night in the dark in order for excess Fe to precipitate as (hydr)oxides. The experimental solution from solid o,o-$H_4$EDDHA was prepared as described by Alvarez-Fernandez et al. (2002). The following day, the solutions were filtered through a 0.45 μm nitrocellulose micropore filter (Schleicher & Schuell, ref-no: 10401114). After filtration the solutions were further diluted. The final experimental solutions had a total chelating capacity in between an equivalent of 12 and 15 mg $l^{-1}$ Fe (see Figure 2.1b). The solutions were named after their o,o-EDDHA content (o,o20%; o,o40%, o,o60% and o,o100%) and their degree of chelation (P or F).
In order to impose ionic strength, $CaCl_2$ was added, so that the final solutions including the blank had a 0.01M $CaCl_2$ concentration. The composition of the experimental solutions was analysed by ICP-AES, ICP-MS and HPLC analysis at t=0 and at the different sampling moments, as described under sampling and measurement. Prior to analysis the samples were filtered through a 0.45 μm cellulose acetate micro pore filter (Schleicher & Schuell, ref no: 10462650). To avoid contamination, the preparation of experimental solutions and dilution of samples for measurement was done with analytical grade chemicals and ultra pure water.

**Soil-FeEDDHA interaction studies**

The selected soils were allowed to interact with the experimental solutions in a soil-solution ratio of 1:1 (w/v) for respectively 1, 2, 4 and 6 weeks in 50 ml polypropylene test tubes (Greiner bio-one, Cat No 210296). The tubes were placed in an end-over-end shaker, rotating at 18 rpm in absence of light. Room temperature was kept at 20 (± 1) °C. To avoid drastic changes in redox conditions throughout the experiment, the tubes were taken out of the shaker, opened for 30 minutes every three to four days. The experiment was carried out in triplicates. Control treatments with the experimental solutions without soil were included for t=0 and the four interaction times.

**Sampling and measurement**

After interaction, the samples were centrifuged for 15 minutes at 3000 rpm. The pH and EC of the supernatant were measured. Subsequently the supernatant was filtered over a 0.45 μm cellulose acetate micro pore filter (Schleicher & Schuell, ref no: 10462650). The filtrate was further analyzed. Fe, Ca and Mg concentrations were measured by ICP-AES (Varian, Vista Pro); Cu, Al, Mn, Zn, Ni and Co concentrations were measured by ICP-MS (Perkin Elmer, ELAN 6000. The samples were acidified with nitric acid before analysis. FeEDDHA components were separated through high-performance liquid chromatography (HPLC). HPLC was performed on a Waters HPLC system consisting of a Waters 600E Multisolvent Delivery System, an Alltech online degasser, a Waters 717plus Autosampler, a Waters 2487 Dual Wavelength Absorbance Detector and Millennium32 software. A Waters Spherisorb ODS2

cartridge, $d_p$ = 5 μm, 250x4.6 mm with guard column was used. The injection volume was 20 μl and the flow rate was 1 mL $min^{-1}$. Detection was done at 277 nm. The mobile phase was prepared by mixing 915 ml of a filtered formate buffer (0.015 M sodium formate adjusted to pH = 3.0 with HCl) with 85 ml of acetonitrile. The concentrations of Fe chelated by racemic o,o-EDDHA, meso o,o-EDDHA and o,p-EDDHA were determined using an external calibration method. The Fe concentration chelated by rest-EDDHA was calculated by subtracting the Fe concentrations chelated by the other three components and the Fe concentration in the blank from the total Fe concentration as measured by ICP-AES.

## Results and Discussion

**Experimental solutions**

Figure 2.1a depicts the HPLC chromatogram of the 60%o,oF experimental solution. It illustrates that the peaks were clearly separated. The two o,p-EDDHA peaks were not calibrated separately, but combined. In chromatograms of soil interaction samples a drift in elution time was observed but the peaks remained readily identifiable. Peaks resulting from dissolved organic carbon (DOC) did not interfere disturbingly for integrating the FeEDDHA component peaks. From this and similar chromatograms, the composition of the experimental solutions was determined.

In Figure 2.1b the Fe concentrations of the experimental solutions are presented, specified per FeEDDHA component. All experimental solutions with the F-suffix were expected to have Fe concentrations of 15 mg $l^{-1}$ Fe. A deviation was observed, increasing with decreasing o,o-EDDHA content. This observation might be explained from the following 3 factors: First, the Fe addition was based on a 1:1 stoichiometry between Fe and ethylene diamine. However, polycondensates may contain more than one ethylene diamine group, but may not be able to bind equally more Fe. Solutions containing more polycondensates will therefore have an over-all stoichiometric ratio of ethylene diamine to Fe, further from 1. Secondly, the relatively large Fe chelating polycondensates might be more susceptible to sorption to Fe(hydr)oxides formed from excess Fe. The Fe(hydr)oxides and adsorbed complexes are removed from the experimental solutions through filtration. Thirdly, the addition of $CaCl_2$ might lead to precipitation and competition effects. Where o,o- and o,p-FeEDDHA concentrations are hardly affected by Ca, rest-FeEDDHA concentrations might be.

In the experimental solutions with the P-suffix, all o,o-EDDHA was expected to chelate Fe, due to its high complexation constants (Table 1.1). The compositions in Figure 2.1b however show, that not all meso-o,o-EDDHA is chelating Fe and a significant amount of Fe is being chelated by rest-EDDHA. This is most evident in the 20%o,oP solution. Since p,p-EDDHA binds Fe much less strongly than meso o,o-EDDHA, polycondensates must be responsible for chelating Fe. This demonstrates that there are polycondensates in commercial FeEDDHA formulations that form Fe complexes of higher chemical stability than meso o,o-FeEDDHA. Hernandez-Apaolaza et al. (2006) recently found that rest-FeEDDHA complexes remain in

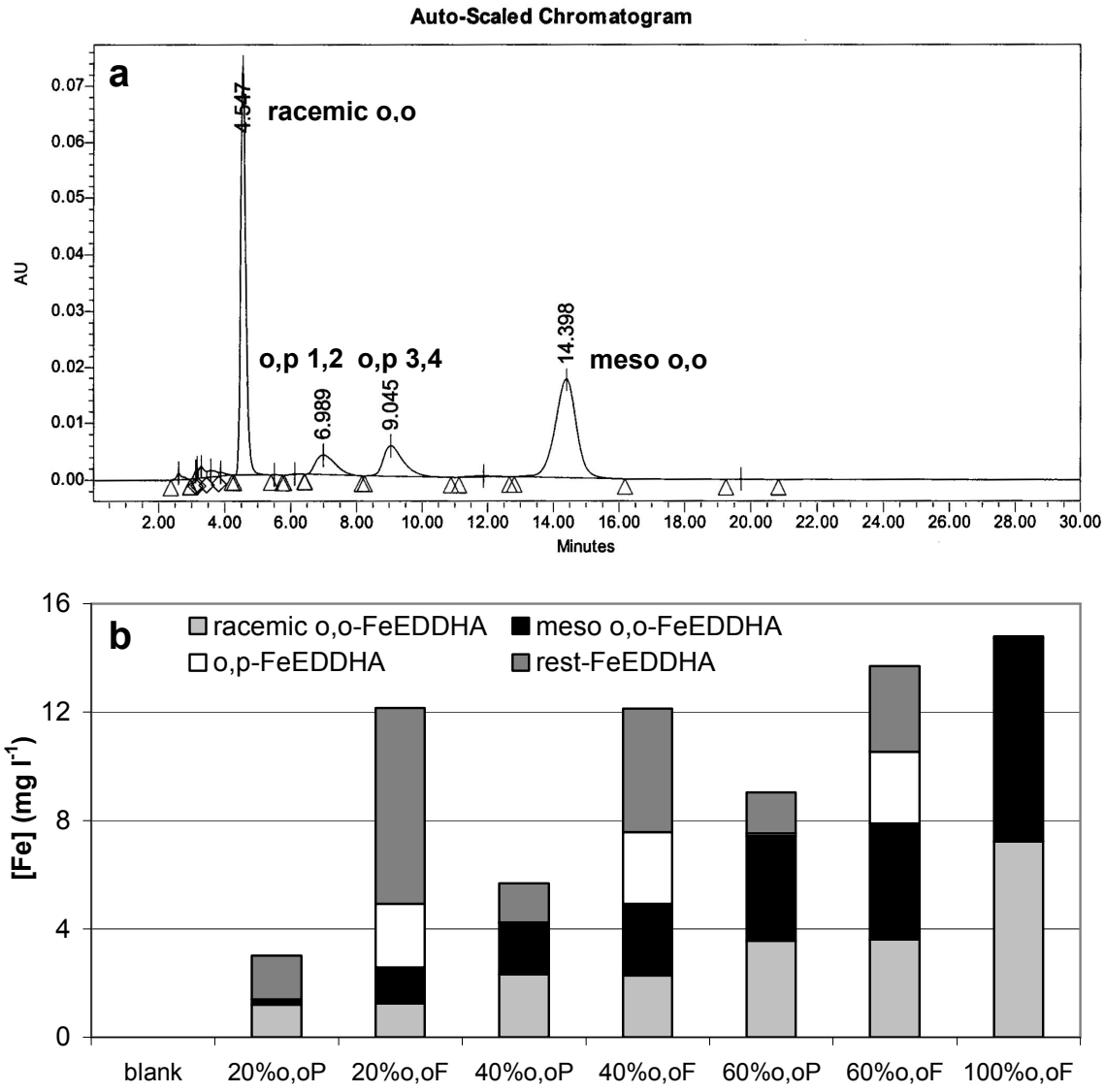



**Figure 2.1: a)** The HPLC chromatogram for the 60%o,oF experimental solution. o,p-FeEDDHA elutes in two sets of two enantiomers o,p1,2 and o,p3,4; **b)** Composition of the experimental solutions before interaction with soil. The concentrations of the FeEDDHA components are expressed in terms of Fe concentration chelated by a particular component. All solutions had a 0.01 M $CaCl_2$ background and pH 6-7.

solution over a smaller pH range than o,o-FeEDDHA and concluded from that that rest-FeEDDHA complexes are less stable. The findings from this study contradict this conclusion. Possibly, these polycondensates have racemic o,o-EDDHA resembling segments enabling them to bind Fe this strong. The higher chemical stability of such complexes compared to meso o,o-FeEDDHA does not necessarily imply a higher stability in solution in

the presence of reactive surfaces. The stability of the various EDDHA components in solution upon interaction with soil will be discussed further on.

**Soil-EDDHA-interaction**

The results from the interaction experiment are presented and discussed in the following four sections: 1) Fe and FeEDDHA component concentrations as a function of time; 2) Reactive surfaces, dealing with the relation between FeEDDHA component concentrations and the contents of reactive surface compounds in the soil; 3) Degree of chelation, dealing with the difference in Fe and FeEDDHA component concentrations between F- and P-treatments; and 4) Competing cations, dealing with the effect of EDDHA treatments on the concentrations of competing cations.

*Fe and FeEDDHA component concentrations as a function of time*

**Fe –** The observed changes in Fe concentration over time for the different treatments were similar for all soils. The effects of treatment are illustrated for the Bologna soil in Figure 2.2a. (Results for Santomera soil, Nadec soil and Hofuf soil are presented in the Appendix; Figure A2.1). The measured Fe concentrations can be fully attributed to the addition of EDDHA, because the Fe concentration in the blank-treatment was below the detection limit of the ICP-AES (11 $\mu g\ l^{-1}$ Fe).

The Fe concentrations in the F-treatments decreased strongly within the first week and were relatively constant afterwards, whereas the Fe concentrations in the P-treatments were relatively constant from the beginning onward. After one week, the differences in Fe concentration between the P- and F-treatment of the same o,o-EDDHA content had become small relative to the differences between the treatments with different o,o-EDDHA contents. Hence, the o,o-EDDHA content of the experimental solution largely determines how much Fe remains in solution during interaction.

The initial decrease in Fe concentration in the F-treatments is caused by EDDHA components, which form Fe complexes in plain solutions, but either adsorb, precipitate or have Fe displaced by a competing cation when interacting with soil. Cantera et al. (2002) and Garcia-Mina et al. (2003) found a similar strong decrease in Fe concentration as observed in the F-treatments for a number of FeEDDHA products, already within the first day.

Except in two sandy soils (Hofuf and Droevendaal), the EDDHA components in the P-treatments that initially do not chelate Fe, do not establish a net increase in Fe concentration upon interaction with soils. To determine whether Fe is in fact chelated by the same EDDHA components before and after soil interaction requires HPLC analyses.

**FeEDDHA components -** The overall Fe concentration is the resultant of the contributions of the individual FeEDDHA components. In Figure 2.2b the fractions of these FeEDDHA components remaining in solution ($[FeEDDHA]_{t=t}/[FeEDDHA]_{t=0}$) are plotted as a function of time for the 60%o,oF treatment interacting with Bologna soil (Results for Santomera soil, Nadec soil and Hofuf soil are presented in the Appendix; Figure A2.2.) After one week,

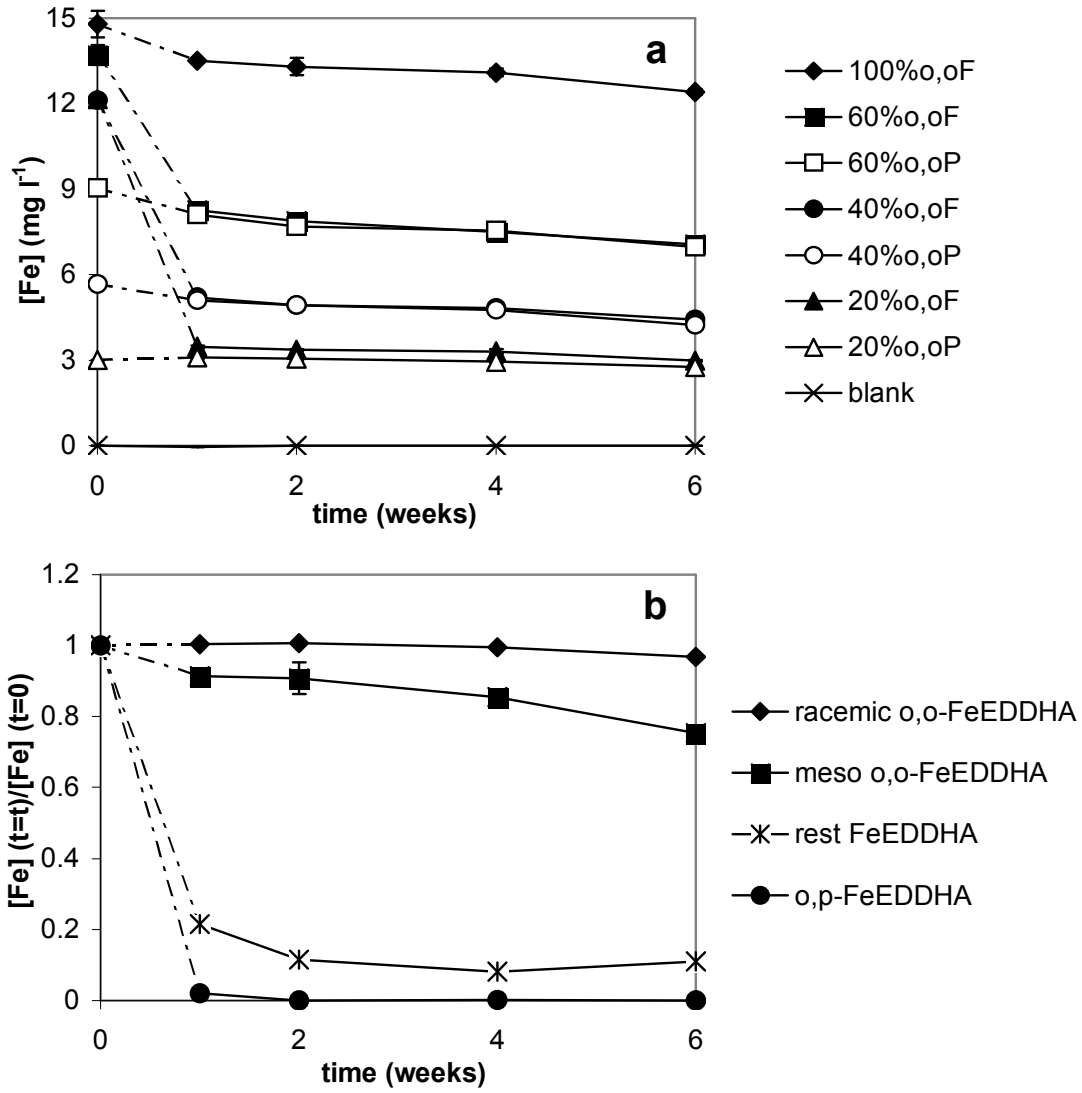



**Figure 2.2: a)** Fe concentration as a function of time upon interaction of the experimental solutions with Bologna soil. Concentrations at t=0 are prior to soil interaction; **b)** Fraction of the FeEDDHA components remaining in solution as a function of time upon interaction of the 60%o,oF treatment with Bologna soil. The lines in between t=0 and t=1 week are dashed, because the data do not show how quickly the decrease in concentration took place within the first week. Error bars indicate standard deviations.

o,p-FeEDDHA had disappeared from solution almost completely and the rest-FeEDDHA concentration had strongly decreased (a remaining fraction of 0.21). Both o,o-FeEDDHA isomers remained in solution to a much larger extent. In accordance with other adsorption and soil interaction studies, the racemic o,o-FeEDDHA concentration is most constant, hardly displaying any decrease (Alvarez-Fernandez, et al., 2002; Cantera, et al., 2002; Hernandez-

Apaolaza and Lucena, 2001). The meso o,o-FeEDDHA concentration showed some initial decrease in concentration. From one to six weeks of soil interaction, the FeEDDHA component concentrations remained largely constant, except for meso o,o-FeEDDHA. Its remaining fraction declined from 0.91 to 0.75. Similar observations were found for the other soils.

After 6 weeks of interaction with the different soils, the remaining fractions of the FeEDDHA components in the 60%o,oF treatment ranged from 0.85 to 1.04 for racemic o,o-FeEDDHA, from 0.58 to 0.94 for meso o,o-FeEDDHA, from 0.11 to 0.42 for rest-FeEDDHA and from 0 to 0.16 for o,p-FeEDDHA. For all F-treatments, similar trends in remaining fractions were found. The remaining fractions can be ranked as follows for all soils: racemic o,o-FeEDDHA > meso o,o-FeEDDHA > rest-FeEDDHA > o,p-FeEDDHA. It should be added that rest-EDDHA consists of a variety of compounds, some of which are able to maintain Fe in solution better than o,p-EDDHA, while others are not. The composition of the rest-EDDHA fraction differs between treatments with different o,o-content. The average chain length of the polycondensates increases with increasing rest-EDDHA fraction in the experimental solutions. This complicates the comparison of the rest-FeEDDHA concentration between treatments with different o,o-contents.

In the P-treatments an increase in meso o,o-FeEDDHA concentration and a decrease in rest-FeEDDHA concentration were found, compared to the experimental solutions. The increase in meso o,o-FeEDDHA was largest in the 20%o,oP-treatment, amounting 0.45 to 1.00 mg $l^{-1}$ Fe for the different soils. Apparently the stability of the meso o,o-FeEDDHA complex is lower, but its ability to keep Fe in solution during interaction with soil is larger. Whether meso o,o-EDDHA chelated native Fe or Fe initial chelated by rest-FeEDDHA remains unclear.

*Reactive surfaces*

The chemical properties and sorption behaviour of the FeEDDHA components differ. Therefore it was expected that different soil constituents might play a dominant role in their sorption behaviour. Relations were studied between the contents of reactive soil constituents and FeEDDHA component concentrations.

**Racemic o,o FeEDDHA –** In Figure 2.3, the racemic o,o-FeEDDHA concentrations are shown as a function of time for the 60%o,oF treatment interaction with all soils. The concentrations hardly decrease over time. This complicates the interpretation of which soil characteristics affect the racemic o,o-FeEDDHA concentration. The soil that stands out, in that relatively much of racemic o,o-FeEDDHA is removed from solution is the Xeraco top soil (Figure 2.3). The distinctive feature of this soil is its relatively high organic matter content (Table 2.1). Alvarez-Fernandez et al. (1997; 2002) and Hernandez-Apaolaza and Lucena (2001) pointed out that organic matter might be an important reactive soil constituent with respect to o,o-FeEDDHA sorption. However, the overall sorption of racemic o,o-FeEDDHA remains small; approximately 15% after 6 weeks at an organic matter content of 9%.

At pH 7-8 organic matter is substantially negatively charged. As a consequence, there is an electrostatic repulsion between the deprotonated carboxylate groups of soil organic mater and the negatively charged racemic o,o-FeEDDHA complexes. Adsorption to organic matter might be established through bridging mechanisms with di- or trivalent cations. Such mechanisms have been reported for linking functional groups within and between DOC molecules. Van der Waals interaction between the uncharged areas of organic matter and the aromatic rings of the complex might also play a role (Stevenson, 1994).

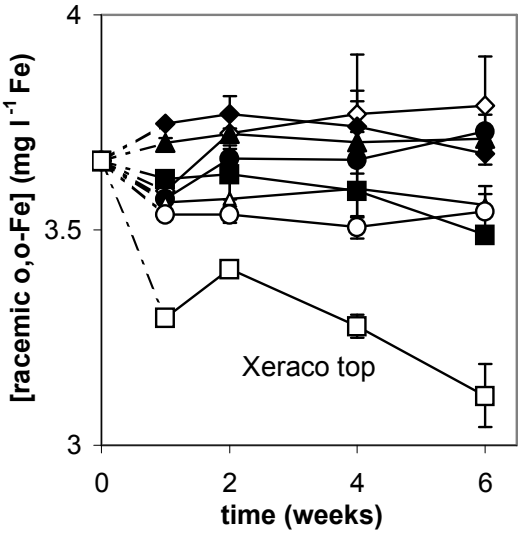



**Figure 2.3:** Fe concentration chelated by racemic o,o-EDDHA as a function of time upon interaction of the 60%o,oF treatment with all soils. Concentrations at t=0 are prior to soil interaction. Error bars indicate standard deviations. Open symbols represent sandy soils, closed symbols represent clay soils.

**Meso o,o-FeEDDHA –** The extent to which meso o,o-FeEDDHA is removed from solution appears to be related to the amount of reactive Fe (oxalate extractable Fe) present in the soil (Figure 2.4). This suggests adsorption of meso o,o-FeEDDHA to reactive Fe(hydr)oxides. The data point for Droevendaal soil does not match the trend line. An explanation for this might be sought in the deviating pH of the soil.

The potential relevance of Fe(hydr)oxides with respect to FeEDDHA sorption in soils has already been outlined by Alvarez-Fernandez et al. (1997; 2002) and Hernandez-Apaolaza and Lucena (2001). Fe(hydr)oxides have positively charged surface groups and a net positively charged surface at pH values below the pristine point of zero charge (7.9 - 8.2 for hydrous ferric oxide (HFO) (Dzombak and Morel, 1990); 9.2 - 9.3 for goethite (Filius, et al., 1997)). The electrostatic attraction between the negatively charged meso o,o-FeEDDHA complex and a positively charged Fe(hydr)oxide surface will enhance sorption.

Why adsorption of meso o,o-FeDDHA to Fe(hydr)oxides is stronger than of racemic o,o-FeEDDHA is not clear. Both complexes have the same charge. More adsorption of the

isomer with the lower stability constant could indicate the breaking of chemical bonds to establish sorption (Hernandez-Apaolaza and Lucena, 2001).

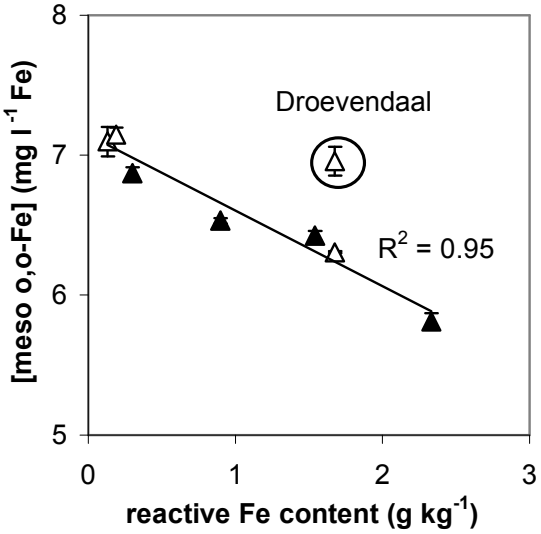



**Figure 2.4:** Fe concentration chelated by meso o,o-EDDHA after 4 weeks of interaction between the 100%o,oF treatment and all soil, as a function of the reactive Fe content of the soils. Standard deviations fall within the data points. Open symbols represent sandy soils, closed symbols represent clay soils. The encircled data point was not included for calculating the linear relation.

**o,p-FeEDDHA** – In Figure 2.5, the o,p-FeEDDHA concentrations after one week of interaction between the 60%o,oF treatment and all soils are plotted against the contents of reactive constituents of the respective soils. No relations were found with reactive Fe (Figure 2.5a), soil organic carbon (Figure 2.5b) and $CaCO_3$ (Figure 2.5c). However, a relation between o,p-FeEDDHA concentration and clay content is evident: an increase in clay content leads to a decrease in o,p-FeEDDHA concentration in solution (Figure 2.5d). An effect of soil texture on Fe concentration upon interaction of commercial FeEDDHA formulations with soil has been reported by Gil-Ortiz and Bautista-Carrascosa (2004). o,p-FeEDDHA is always present in commercial formulations (Garcia-Marco, et al., 2006) and its tendency to adsorb to clay minerals can explain Gil-Ortiz and Bautista-Carrascosa's observation.

Adsorption of o,p-FeEDDHA to clay minerals is counter-intuitive because both have an overall negative charge and the two repel each other. A bridging mechanism with Ca has been suggested to bind FeEDDHA complexes to clay surfaces (Wallace and Wallace, 1992). A similar mechanism has been demonstrated for the adsorption of DOC to clay (Muneer and Oades, 1989). The sixth position on the coordination complex of Fe chelated by o,p-EDDHA is not occupied by a phenolic hydroxyl group of the chelating agent, but, depending on pH, by either a separate hydroxide anion or a water molecule (Yunta, et al., 2003a) (Figure 1.4c). This hydroxide anion or water molecule may play a central role in the adsorption behaviour,

because it is displaced relatively easily and may hence facilitate binding to a negatively charged surface group on a clay-edge or it may act as a bridge between complex and surface. Another explanation for the enhanced removal of o,p-FeEDDHA in clay soils could be that these soils generally contain more competing cations like Cu that might replace Fe from the o,p-FeEDDHA complex. Competition effects will be further discussed in a following section. In this study, the soil with the highest organic matter content, Xeraco top soil, sorbed most racemic o,o-FeEDDHA, but did preserve a remaining o,p-FeEDDHA fraction of 0.05 over 6 weeks, while in clay soils all o,p-FeEDDHA was removed from solution. There are several possible explanations for this. First, organic matter may be less specific in its affinity for the

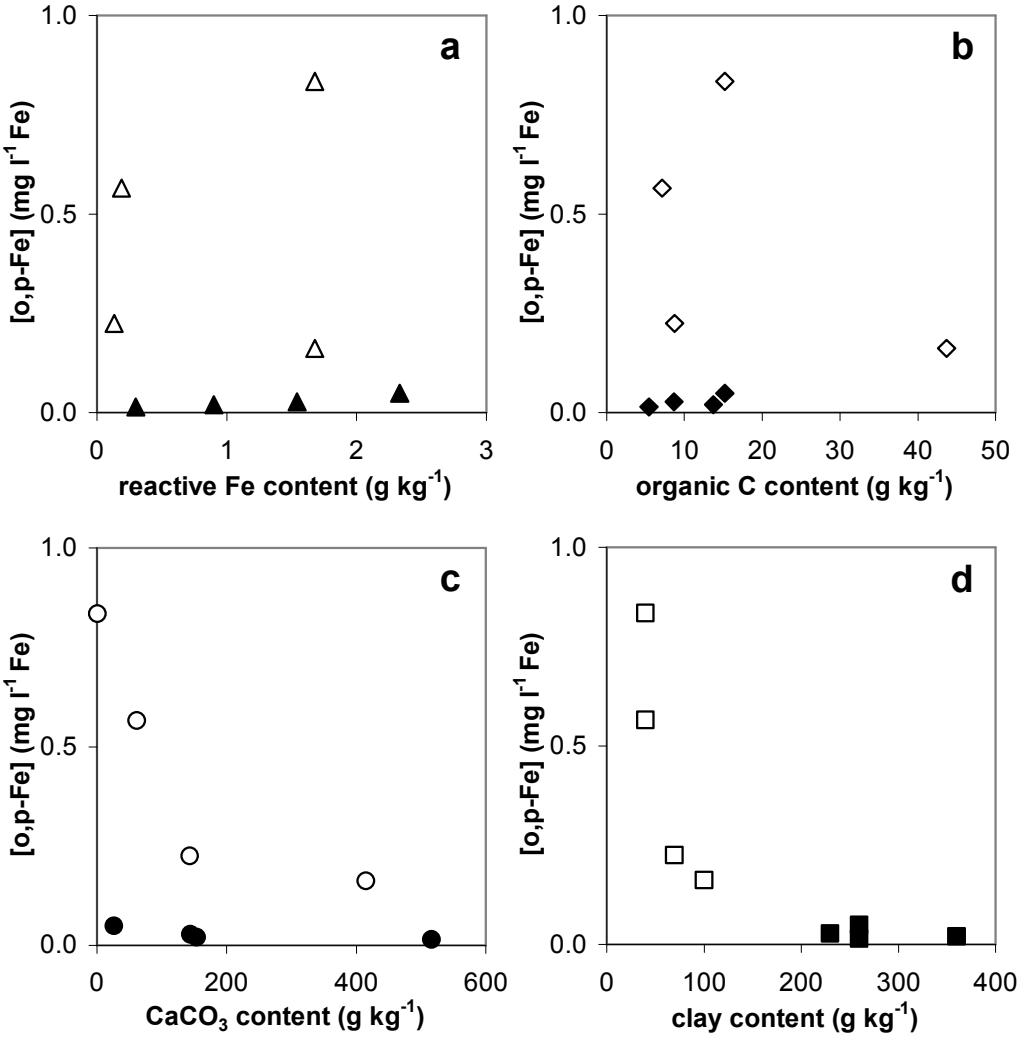



**Figure 2.5:** Fe concentration chelated by o,p-EDDHA as a function of the reactive soil constituent contents of the soils - **a)** reactive Fe; **b)** organic carbon; **c)** calcium carbonate; and **d)** clay. Open symbols represent sandy soils, closed symbols represent clay soils.

different FeEDDHA components, because sorption may be partly based on the interaction between the non-polar sections of both soil organic matter molecules and the EDDHA complexes. Secondly, organic matter is a source of DOC. DOC might act as a competitor for adsorption sites on clay minerals, which are shown to provide the key adsorption surface for o,p-FeEDDHA.
In literature the role of organic matter with respect to sorption of FeEDDHA seems to be overestimated. Alvarez-Fernandez et al.(1997; 2002), and Hernandez-Apaolaza and Lucena (2001) have shown substantial degrees of sorption by peat over a wide pH range; this however is not representative for the soils where FeEDDHA products are actually applied, which have a much lower organic matter content.

**Rest-FeEDDHA** – Due to the varying composition of the rest-FeEDDHA fraction among the experimental solutions, unambiguous comments on the adsorption behaviour of rest-FeEDDHA are not possible. For all treatments containing rest-FeEDDHA, the largest fraction remained in solution upon interaction with soils with the least clay: Droevendaal and Hofuf (data not shown), again supporting Gil-Ortiz's and Bautista-Carrascosa's observations with respect to soil texture (2004).

*Degree of chelation*
Figure 2.2a shows that differences in Fe concentration were found between the P- and F-treatments of the same EDDHA component composition. This was most evident for the 20%o,o treatments. For all EDDHA component compositions, the Fe concentrations in the P-treatments were lower. The differences in Fe concentration varied per soil and after one week they ranged from 0.18 to 1.07 mg $l^{-1}$ Fe for Xeraco top soil and Nadec soil respectively. For Nadec soil the difference of 1.07 mg $l^{-1}$ Fe amounted to little over a third of the Fe concentration of the P-treatment after interaction (2.98 mg $l^{-1}$ Fe). The most obvious explanation for the difference in Fe concentration is the following: in the P-treatments EDDHA molecules are not able to pick up Fe, either from the soil or from adsorbed FeEDDHA complexes, to such an extent that the same Fe concentration is reached as in the corresponding F-treatments. The difference in Fe concentration between the P- and F-treatments should then be a function of the Fe availability of the soils. Figure 2.6a, in which the difference in Fe concentration is presented as a function of the Fe availability parameter diethylene triamine penta acetic acid (DTPA)-extractable Fe (DTPA-Fe) confirms this relation: with increasing DTPA-Fe, the difference in measured Fe concentration between the P- and F-treatment becomes smaller. At low DTPA-Fe, the relative decrease is largest and the slope becomes gradually less steep with increasing DTPA-Fe.
The differences in Fe concentration have been examined in terms of EDDHA components. For Nadec, Bologna and Xeraco top soil (encircled in Figure 2.6a), these differences are specified in Figure 2.6b. Generally, the largest contribution is from meso o,o-FeEDDHA, followed by rest-FeEDDHA .This large contribution of meso o,o-FeEDDHA is remarkable in view of its high complexation constant (Table 1.1). The amount of DTPA-Fe suffices in all soils to have all meso o,o-EDDHA in the P-treatment chelate Fe. Apparently a lasting kinetic effect is

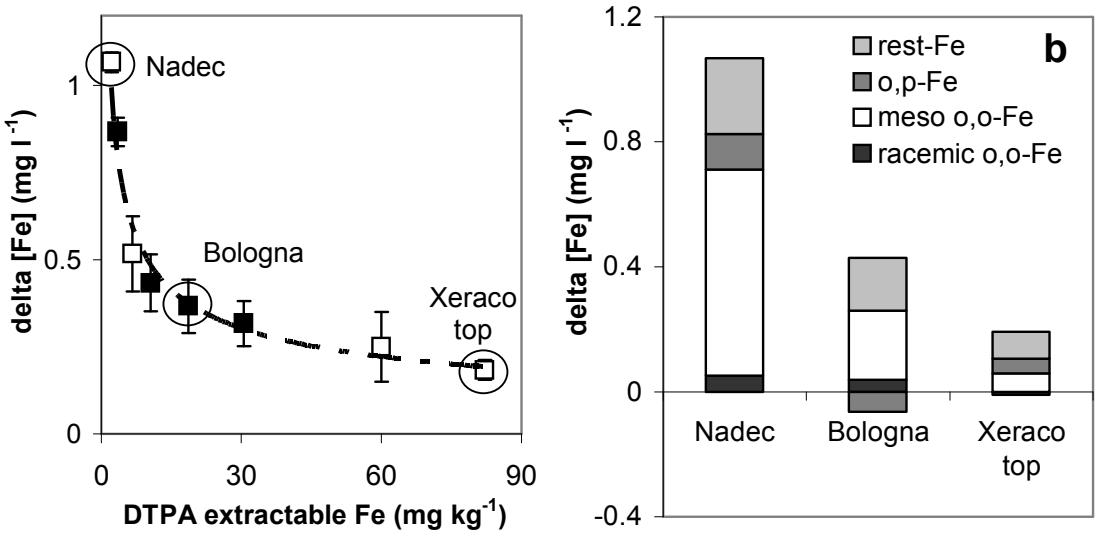



**Figure 2.6: a)** Difference in Fe concentration between the fully (F) and partly (P) chelating 20%o,o treatments after 1 week of interaction with soils, as a function of the DTPA-extractable Fe content of the soils. Open symbols represent sandy soils, closed symbols represent clay soils. Error bars indicate standard deviations; **b)** Difference in Fe concentration between the fully (F) and partly (P) chelating 20%o,o treatments after 1 week, specified per FeEDDHA component.

limiting the ability of meso o,o-EDDHA to chelate Fe from the soil. With commercial FeEDDHA formulations this is not a direct concern, because all o,o-EDDHA tends to be chelated to Fe. When however Fe has been delivered to the plant and EDDHA returns into solution to chelate a new Fe-ion (the shuttle effect), it becomes of relevance.

*Competing cations*

The effects of EDDHA-treatments on Cu and Al concentrations are discussed here in more detail. Findings on other cations are briefly commented on afterwards.

**Cu** - In several studies, elevated Cu concentrations were found due to the addition of commercial FeEDDHA formulations. Addition of an o,o-FeEDDHA standard solution however, hardly led to an increase in Cu concentration (Alvarez-Fernandez, et al., 1997; Alvarez-Fernandez, et al., 2002). Therefore one of the other components must be responsible for the increase in Cu concentration. In Figure 2.7, the Cu concentrations are presented as a function of time for all treatments interacting with Santomera soil. Hardly any increase in Cu concentration was observed for the 100%o,oF treatment, compared to the blank treatment (up to 0.06 mg $l^{-1}$ Cu). Increased Cu concentrations, up to approximately 1.9 mg $l^{-1}$ Cu after 1 week, were observed in all other treatments. The data shown in Figure 2.7 have 3 important implications.

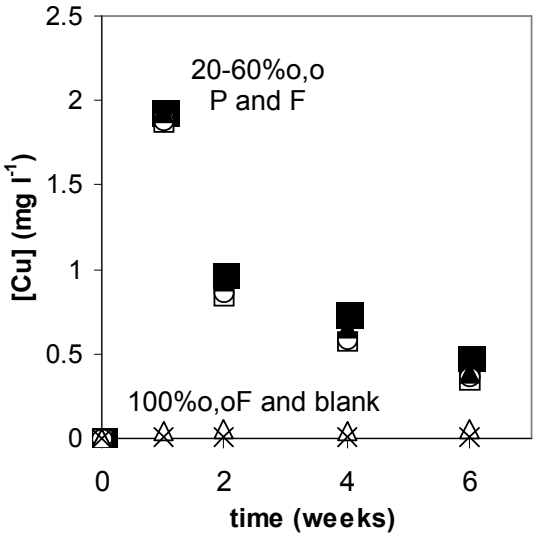



**Figure 2.7:** Cu concentration as a function of time upon interaction of all treatments with Santomera soil. Concentrations at t=0 are prior to soil interaction.

First, it is irrelevant if the EDDHA component responsible for Cu solubilization initially chelates Fe or not; there is hardly any difference in Cu concentration between corresponding P- and F-treatments interacting with soil. Because Cu concentrations are about equal for P- and F-treatments, there is no lag time and the displacement of Fe from the FeEDDHA complex can not be the time determining step in Cu solubilization.

Secondly, o,p-EDDHA determines the extent of Cu solubilization. The ability of o,p-EDDHA to dissolve Cu from the soil has been reported by Garcia-Marco et al. (2006). For the six treatments in this study containing o,p-EDDHA (20%o,o; 40%o,o and 60%o,o; both F and P), o,p-EDDHA was the only EDDHA component of which the initial concentrations were approximately the same (Figure 2.1b). The Cu concentrations after interaction with soil were also approximately the same for these treatments, for all four interaction times (Figure 2.7). A similar effect of treatment was found for all eight soils. The Cu concentrations varied between the soils ranging from 0.06 to 1.93 mg $l^{-1}$ Cu after one week, amongst others due to differences in Cu availability. The treatments containing o,p-EDDHA gave equal Cu concentrations, regardless if DTPA-extractable Cu exceeded the amount of o,p-EDDHA added (in the case of Santomera and Herveld; Cu-DTPA amounted 4.13 and 5.30 mg $kg^{-1}$ Cu respectively), or not (in the case of the remaining soils). This advocates that Cu is not just preferentially, but exclusively dissolved through complexation by o,p-EDDHA.

Thirdly, Cu concentrations decrease over time for all treatments containing o,p-EDDHA, in all soils except for Hofuf soil. Adsorption of o,p-CuEDDHA seems the most likely cause, because no other trace metal concentrations increase and competition with Ca and Mg is unlikely. The fact that Hofuf soil has low reactive surface contents, and in particular the lowest

clay content, supports the adsorption hypothesis, but e.g. degradation can not be excluded. However, ion adsorption equilibria are generally reached within a few days in the soil and in between week 4 and 6, Cu concentrations still decrease by 25 to 70% for the soils other than Hofuf and Droevendaal. Parallel occurring processes like Cu complexation/solubilization, and slow adsorption kinetics may extend the time required to reach equilibrium. Further research is needed to resolve the adsorption and exchange mechanism of metal-EDDHA complexes.

**Al** – Al had not yet been reported as a competing cation, potentially affecting the performance of FeEDDHA products. The data in Figure 2.8a show the Al concentrations as a function of time for all treatments interacting with Nadec soil. The Al concentrations of the F-treatments do not differ from those in the blank treatment. The Al concentrations in the P-treatments are however higher, increasing with increasing concentration of EDDHA components not chelating Fe. Contrary to other competing cations, the Al concentrations are constant over time, at least up until and including 4 weeks of interaction. In week 6, an increase in Al concentration was observed. Similar results were observed for the other soils. These results imply that Al does not displace Fe from FeEDDHA complexes. However, when EDDHA, not chelated to Fe, is brought into contact with soil, it can form AlEDDHA complexes. In turn, Fe does not displace Al from AlEDDHA complexes.
The observed trends in Al concentrations exclude racemic o,o-EDDHA and o,p-EDDHA as Al complexing EDDHA component. Further examination of the data shows that meso o,o-EDDHA is the dominant EDDHA component chelating Al, for two reasons. First, when considering the 20%o,o P- and F-treatments for all soils, the differences in Al concentration correlate better with the differences in meso o,o-FeEDDHA concentration (-0.95) than with the differences in rest-FeEDDHA concentration (-0.57). Secondly, the differences in rest-FeEDDHA concentration between the 20%o,o P- and F-treatment are not for all soils large enough to account for the increase in Al concentration, whereas the differences in meso o,o-FeEDDHA are. Complexation of Al is to a large extent able to explain the differences in Fe concentration observed in the *degree of chelation* section.
In this study it was found that meso o,o-EDDHA is able to dissolve both Fe and Al from the soil. As a consequence Fe and Al compete for meso o,o-EDDHA. Because neither drives off the other from the complex within the time span examined, a relation between relative availabilities and concentrations is expected. Relative availability can be expressed as a ratio of two availability parameters. The oxalate extractable fractions of Al and Fe were used as availability parameters. The expected relation was found and is displayed in Figure 2.8b: an increased relative availability of Al results in higher Al concentrations in solution. Hence, in soil application Al complexation may cause the alleged shuttle effect to become partly impeded. The impeding effect will be stronger with increasing relative availability of Al.

**Other cations -** The remaining trace metals measured (Zn, Mn, Ni and Co) were generally present in solution in lower concentrations than Al and Cu. When elevated concentrations were found as a result of EDDHA-treatments, this effect diminished over time, like in the case of Cu, except in Hofuf soil for Ni and Co. Elevated Zn concentrations were only found in

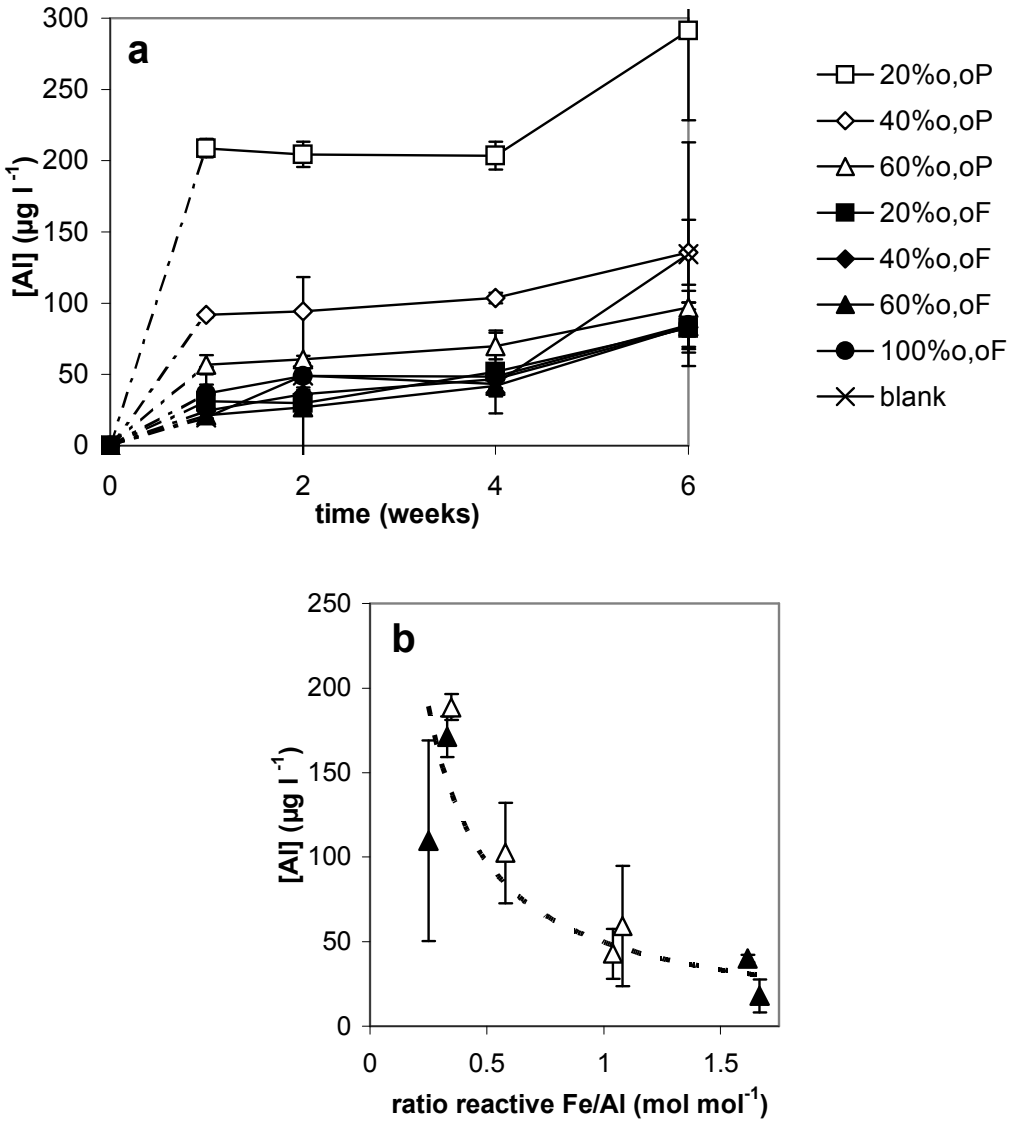



**Figure 2.8: a)** Al concentration as a function of time upon interaction of all treatments with Nadec soil. Concentrations at t=0 are prior to soil interaction. The lines in between t=0 and t=1 week are dashed, because the data do not show how quickly the increase in Al concentration took place within the first week; **b)** Al concentration after 1 week of interaction between the 20%o,oP treatment and all soils, corrected for the Al concentration in the blank treatment, as a function of the ratio between the reactive Fe and the reactive Al content of the soils. Open symbols represent sandy soils, closed symbols represent clay soils. Error bars indicate standard deviations.

Hofuf soil (up to 160 µg $l^{-1}$ Zn after 1 week) and elevated Mn concentration were found in 4 soils (up to 80 µg $l^{-1}$ Mn after 1 week). Elevated Co and Ni concentrations were found in all soils (up to 52 µg $l^{-1}$ Co and 150 µg $l^{-1}$ Ni respectively). Co and Ni were generally present in

higher concentrations than Zn and Mn as a result of the EDDHA treatments. Even if only to a small extent (5.3 μg $l^{-1}$ Co), unlike Al, Ni, Zn and Mn, Co was able to displace Fe from o,o-FeEDDHA in the 100%o,oF treatment.

# Conclusion

The results from this study have shown that regardless of soil properties, the o,o-fraction of the FeEDDHA treatment largely determines how much Fe remains in solution upon interaction with soil. The fractions of the EDDHA components that remain in solution can be ranked as follows: racemic o,o-FeEDDHA > meso o,o-FeEDDHA > rest-FeEDDHA > o,p-FeEDDHA. FeEDDHA component concentrations were relatively constant from week 1 to 6 of the interaction experiment. Although rest-EDDHA also consists of compounds that chelate Fe more strongly than meso o,o-EDDHA, the latter is on average better capable of keeping Fe in solution upon interaction with soil. The principal adsorption surface in the soil differs per FeEDDHA component. For racemic o,o-FeEDDHA it is organic matter, for meso o,o-FeEDDHA, Fe(hydr)oxides, and for o,p-FeEDDHA, clay minerals. For rest-FeEDDHA no single surface has been identified, but the clay fraction seems to be of relevance.

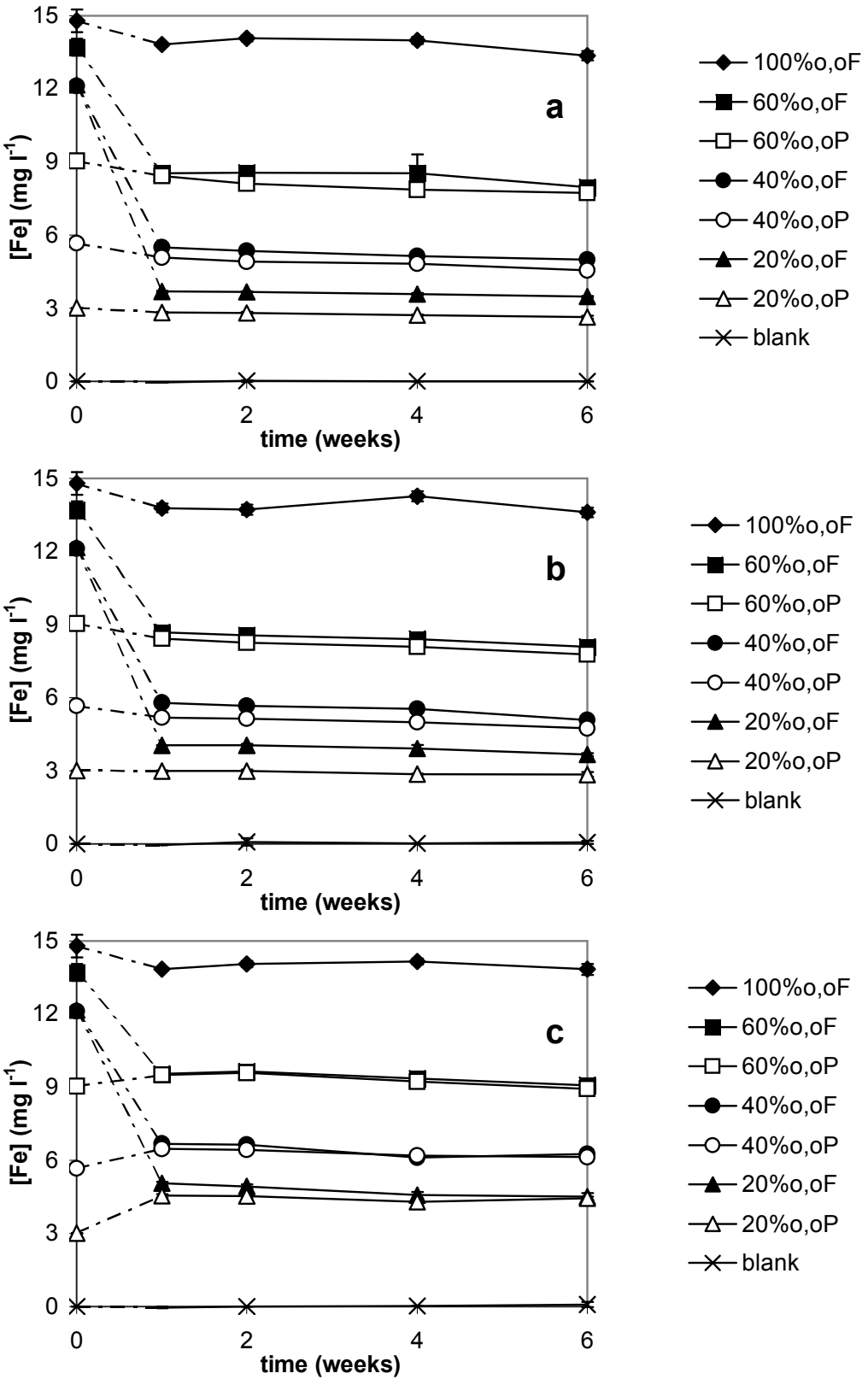
The difference in Fe concentration between corresponding Partly and Fully chelating treatments interacting with soil, is dependent on the Fe availability of the soil. This difference can largely be attributed to a difference in meso-FeEDDHA and to a lesser extent rest-FeEDDHA concentration. When meso o,o-EDDHA, not chelated to Fe, is brought into contact with soil, it will not exclusively form complexes with Fe but also with Al. The fact that meso o,o-EDDHA can chelate Al from the soil, while Fe does not displace Al from the EDDHA-complex, has implications for the effectiveness of a possible shuttle mechanism. Concentrations of other chelated trace elements (Cu, Zn, Mn, Ni and Co) generally decreased during the experiment. In terms of concentration, Cu is by far the most important competing cation. Cu is practically exclusively chelated by o,p-EDDHA. Cu was dissolved, regardless if o,p-EDDHA was initially chelating Fe or not.

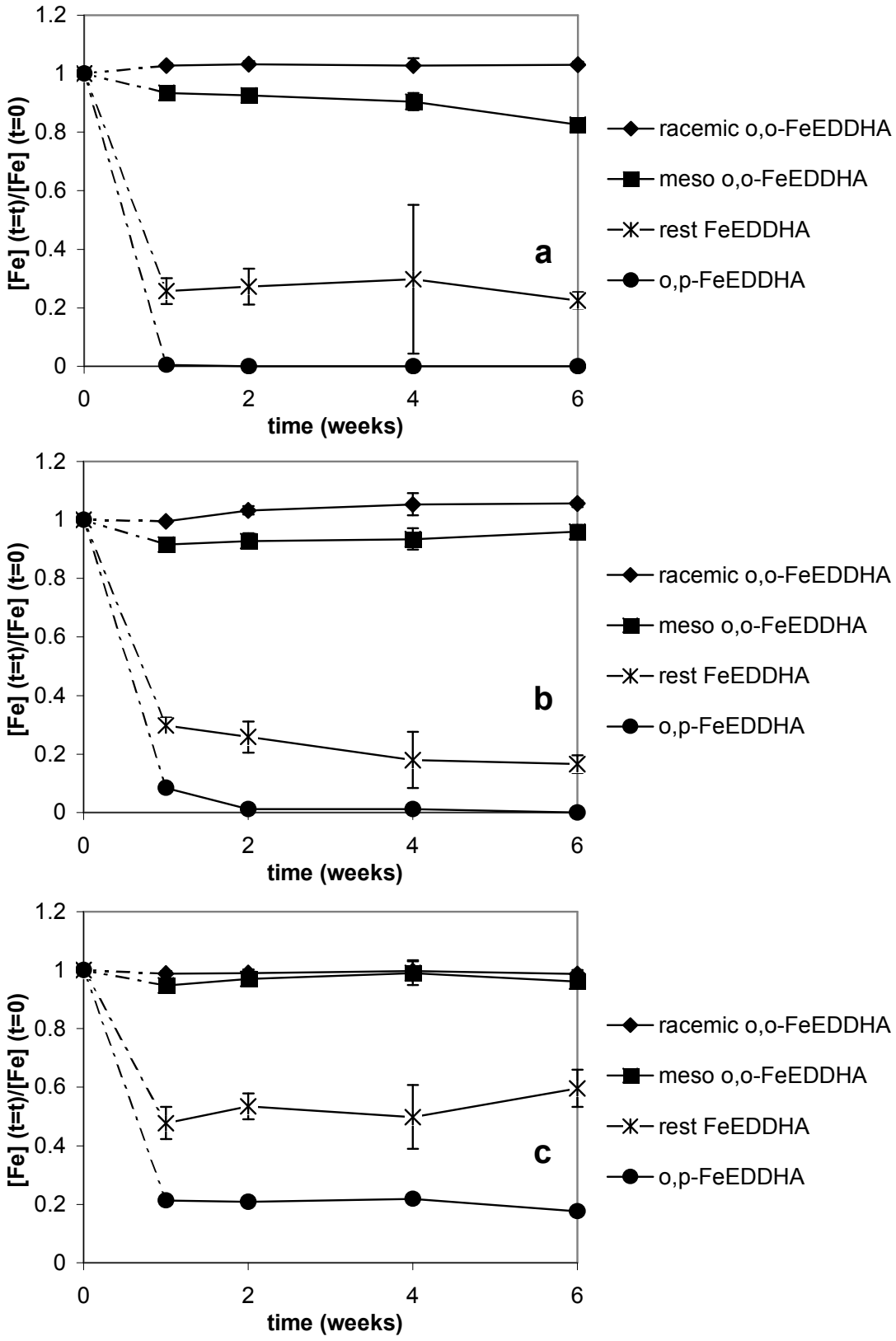
The tendency of o,p-FeEDDHA and rest-FeEDDHA to be removed from solution and the tendency of o,p-FeEDDHA to exchange Fe for Cu, make these FeEDDHA components less effective as Fe fertilizer in soil application, in particular on clay soils. The agronomic value of the different FeEDDHA components however depends on more factors than their ability to preserve Fe in solution. Additional studies involving plants are needed to comment on this.

# Appendix

**Figure A2.1** ***(p. 51)*****:** Fe concentration as a function of time for all treatments interacting with **a)** Santomera soil, **b)** Nadec soil, and **c)** Hofuf soil. Concentrations at t=0 are prior to soil interaction. The lines in between the initial concentration and the concentration after 1 week are dashed, because the data do not show how quickly the decrease in concentration took place within the first week. Error bars indicate standard deviations.

**Figure A2.2** ***(p. 52)*****:** Fractions of the FeEDDHA isomers remaining in solution as a function of time for the 60%o,oF treatment interacting with **a)** Santomera soil, **b)** Nadec soil, and **c)** Hofuf soil. The lines in between t=0 and t=1 week are dashed, because the data do not show how quickly the decrease in concentration took place within the first week. Error bars indicate standard deviations.


a
b
c
[Fe] (mg l-1)
time (weeks)
0
2
4
6
0
3
6
9
12
15
100%o,oF
60%o,oF
60%o,oP
40%o,oF
40%o,oP
20%o,oF
20%o,oP
blank


1.2
1
0.8
0.6
0.4
0.2
0
[Fe] (t=t)/[Fe] (t=0)
0
2
4
6
time (weeks)
a
racemic o,o-FeEDDHA
meso o,o-FeEDDHA
rest FeEDDHA
o,p-FeEDDHA
b
c

"Water, water everywhere,
and all the boards did shrink;
water, water everywhere,
nor any drop to drink."

Excerpt from:
*"The Rime of the Ancient Mariner"*
S.T. Coleridge (1772 - 1834)

# Chapter 3

## The effectiveness of soil-applied FeEDDHA treatments in preventing iron chlorosis in soybean as a function of the o,o-FeEDDHA content

W.D.C. Schenkeveld, R. Dijcker, A.M. Reichwein, E.J.M. Temminghoff, and W.H. van Riemsdijk

Plant and Soil (2008), 303(1/2): 161-176

## Abstract

The application of FeEDDHA products is the most common practice to prevent and remedy Fe chlorosis in crops grown on calcareous soils. These products consist of a mixture of EDDHA components chelated to Fe. In this study such mixtures have been divided into four (groups of) components: racemic o,o-EDDHA, meso o,o-EDDHA, o,p-EDDHA and rest-EDDHA. Because the physical and chemical properties of these components differ, so does their effectiveness in delivering Fe to the plant. This effectiveness has not yet been examined in soil application, but needs to be understood to come to an adequate Fe fertilization recommendation. In this study the influence of composition of FeEDDHA treatments on Fe uptake by soybean plants (*Glycine max* (L.) Merr. cv. Mycogen 5072) grown on calcareous soils was examined in two pot trials involving eight soils. The FeEDDHA treatments were equal in Fe dose but differed in o,o-FeEDDHA content, and were applied prior to the set in of chlorosis.

The o,o-FeEDDHA content largely determined the Fe concentration in the pore water. In turn, in soils that induced chlorosis, the Fe concentration in the pore water determined the Fe uptake. The relationship between Fe concentration and Fe uptake is non-linear: initially Fe uptake increases strongly with increasing Fe concentration, but the slope flattens and a plateau is reached. FeEDDHA treatments increased both yield (up to 30%) and Fe content of the plant tissue (up to 50%). From FeEDDHA products with a higher o,o-FeEDDHA content, a smaller Fe dose is required to obtain the same results in terms of yield and Fe nutritional value.

# Introduction

Fe deficiency chlorosis is a nutritional disorder characterized by a significant decrease of chlorophyll in the leaves, often observed in plants grown on alkaline and calcareous soils. It decreases crop yield both quantitatively and qualitatively, resulting in economic losses (Chaney, 1984; Mortvedt, 1991). A large number of soil factors, including high soil moisture content, poor aeration, extreme temperatures, high phosphate content have been reported to induce or enhance Fe chlorosis (Wallace and Lunt, 1960). High pH and elevated bicarbonate concentrations are generally found to be the most critical factors (Boxma, 1972; Mengel, et al., 1984; Shi, et al., 1993). The low solubility of Fe(hydr)oxides at high pH leads to a low bioavailability of Fe in soil solution (Lindsay, 1979) and bicarbonate either impairs Fe uptake (Marschner, 1995; Venkatraju and Marschner, 1981) or inactivates Fe in the leaf apoplast (Mengel, 1994).

Synthetic Fe chelates are applied to avoid or to mend Fe chlorosis. These chelates increase Fe solubility and function as a transporter through solution to the plant. FeEDDHA (iron (3+) ethylene diamine-N,N'-bis(hydroxy phenyl acetic acid)) is among the most effective synthetic Fe chelates under neutral and alkaline soil conditions (Lucena, et al., 1992; Papastylianou, 1990; Reed, et al., 1988). Commercial FeEDDHA formulations consist of a mixture of positional isomers, diastereomers and polycondensates. In this study such mixtures have been divided into 4 groups: 1) racemic o,o-FeEDDHA (iron (3+) (R,R) and (S,S) ethylene diamine-N,N'-bis(2-hydroxy phenyl acetic acid) complexes), 2) meso o,o-FeEDDHA (iron (3+) (R,S) = (S,R) ethylene diamine-N,N'-bis(2-hydroxy phenyl acetic acid) complex), 3) o,p-FeEDDHA (iron (3+) ethylene diamine-N-(2-hydroxy phenyl acetic acid)-N'-(4-hydroxy phenyl acetic acid) complexes), and 4) rest-FeEDDHA (largely consisting of polycondensates). In general these four groups are referred to as FeEDDHA components. The chemical properties of these FeEDDHA components, including their protonation and complexation constants differ strongly (Ahrland, et al., 1990; Bannochie and Martell, 1989; Frost, et al., 1958; Yunta, et al., 2003a; Yunta, et al., 2003b). As a result, so does their ability to preserve Fe in solution and deliver it to the plant.

The composition of commercial FeEDDHA formulations on the market varies largely (Garcia-Marco, et al., 2003). Therefore product quality needs to be guarded. At present the quality aspect is assured in the European fertilizer law (Regulation (EC) No. 2003/2003; amendment (EC) No. 162/2007) through the following parameters: (1) soluble Fe content of the product, (2) percentage of Fe chelated, and (3) percentages of Fe chelated by o,o-EDDHA and o,p-EDDHA.
In view of the large variation in composition of FeEDDHA products, it is crucial to have an understanding of the effectiveness of the individual FeEDDHA components in order to come to an adequate Fe fertilization recommendation. This effectiveness is determined by the following characteristics: 1) the ability to remain in solution, 2) the susceptibility to competition from other metal ions, 3) the ability to deliver Fe to the plant, and 4) the

selectivity of the corresponding EDDHA component to pick up Fe from the soil (Lucena, 2003).
The first two characteristics have been addressed in a number of interaction studies with soil and soil constituents (Alvarez-Fernandez, et al., 1997; Alvarez-Fernandez, et al., 2002; Garcia-Marco, et al., 2006; Hernandez-Apaolaza, et al., 2006; Hernandez-Apaolaza and Lucena, 2001; Schenkeveld, et al., 2007). These studies have shown that the o,o-FeEDDHA isomers are best capable to preserve Fe in soil solution. In particular in clay soils, o,p-FeEDDHA and rest-FeEDDHA are largely removed from solution. The reactive soil compounds that determine the adsorption behaviour of the FeEDDHA isomers are: soil organic matter, Fe(hydr)oxides and clay minerals. The soil compound dominating the adsorption behaviour differs per FeEDDHA component. With regards to competition, Cu is the principal competing cation affecting the performance of commercial FeEDDHA formulations in alkaline soils, particularly reducing the effectiveness of o,p-FeEDDHA due to displacement of Fe by Cu.
The third characteristic, the ability of FeEDDHA to deliver Fe to the plant, has been studied since the 1950s, both in nutrient solution and in soil-plant systems. Often this was done in comparative studies with other Fe fertilizers (Alvarez-Fernandez, et al., 2005; Hernandez-Apaolaza, et al., 1995; Reed, et al., 1988; Wallace, et al., 1955; Wallace and Wallace, 1983). The effectiveness of the individual FeEDDHA components in delivering Fe to the plant has however received little attention up until now. A number of recent studies have addressed this issue for plants grown in nutrient solutions. It was found that in hydroponics o,p-FeEDDHA offers a more effective remedy to Fe chlorosis in soybean, than o,o-FeEDDHA (Garcia-Marco, et al., 2006), and that both are more effective than synthesis byproducts (referred to as rest-FeEDDHA in this study) (Hernandez-Apaolaza, et al., 2006). Furthermore, meso o,o-FeEDDHA has been claimed to be more effective in mending chlorosis in strategy 1 plants than racemic o,o-FeEDDHA (Cerdan, et al., 2006).

The effectiveness of FeEDDHA components regarding Fe uptake by plants grown on soil has not previously been examined. Because adsorption and cation competition are more dominant processes in soil systems than in nutrient solutions, the order of effectiveness of FeEDDHA components may well be different. In order to relate to agricultural practice and to European regulation, the effectiveness of FeEDDHA treatments in soil application has been addressed in this study by applying mixtures of FeEDDHA components, rather than isolated FeEDDHA components. The aim of this research was: 1) to examine the influence of composition of FeEDDHA treatments on Fe uptake by plants grown on calcareous soils, and 2) to gain insight in the effectiveness of the individual FeEDDHA components as Fe fertilizer in soil application.
A pot trial involving eight soils was conducted in which soybean plants were offered Fe through FeEDDHA treatments prior to the set in of chlorosis. The treatments were similar in Fe concentration, but differed in composition of EDDHA components chelating the Fe. In particular the o,o-FeEDDHA content (i.e. the amount of Fe chelated by the two o,o-EDDHA components) varied among the treatments.

# Materials and methods

**Soils**

Soils were collected from seven sites, located in Italy (Bologna), Spain (Xeraco and Santomera), Saudi Arabia (Nadec and Hofuf) and the Netherlands (Droevendaal and Herveld). The soils are named after the location of collection. The sites were selected so that there were ranges in soil properties and constituents reported to interact with FeEDDHA components (Alvarez-Fernandez, et al., 1997). Four clay soils and four sandy soils were included. At all sites, the top layer (0 – 20 cm) was sampled. From one site (Xeraco, Spain), soil material from both the top layer (Xeraco T) and the layer directly underneath (20 - 40 cm) (Xeraco L) were sampled separately. The top layer is relatively rich in organic material. In crops grown at the sites in Spain, Italy and Saudi Arabia, Fe chlorosis was manifest. The two Dutch sites were included as reference soils. Pre-treatment consisted of air drying and sieving (1 cm). Relevant soil characteristics are presented in Table 3.1.

**FeEDDHA solutions**

The FeEDDHA solutions were prepared from three sodium-EDDHA stock solutions and solid o,o-$H_4$EDDHA (99% pure). The stock solutions were synthesized through a Mannich-like reaction between phenol, ethylene diamine and glyoxylic acid (Petree, et al., 1978), and differed in o,o-EDDHA content (i,e. the sum of racemic and meso o,o-EDDHA content): approximately 16%, 34% and 49% on an ethylene diamine input basis. The experimental solutions were prepared as described by Alvarez-Fernandez et al. (2002). Fe was added as $FeCl_3.6H_2O$ in a 5% excess based on a 1:1 stoichiometry between Fe and ethylene diamine. The pH was raised to 7 (± 0.5) and the solutions were left over-night in the dark, in order for excess Fe to precipitate as hydroxides. The following day, the solutions were filtered over a 0.45 μm nitro cellulose micro pore filter (Schleicher & Schuell, ref-no: 10401114) and further diluted for application in the pot trial. The composition of the experimental solutions was analysed through combined ICP-AES, ICP-MS and HPLC analysis at time t=0.

**Pot trial**

Two pot experiments were done simultaneously from March until May 2005. In pot experiment 1 with a runtime of eight weeks, six treatments were included for all soils (Table 3.2): a treatment with plants without FeEDDHA addition (blank); four treatments with plants and FeEDDHA (16%o,o; 34%o,o; 49%o,o and 99%o,o with plants); and an FeEDDHA treatment without plants (34%o,o without plants). The FeEDDHA treatments were similar in Fe concentration but differed in composition in terms of FeEDDHA components.

In pot experiment 2, with a run-time of three weeks, only the Santomera soil was used. The same treatments were applied as in pot trial 1 except for the 34%o,o treatments with and without plants.

**Table 3.1:** Soil characteristics.

| Soil (origin) | Country | Region | Soil classification | Water holding capacity (g kg$^{-1}$) | SOC[a] (g kg$^{-1}$) | Clay[b] (g kg$^{-1}$) | $CaCO_3$[c] (g kg$^{-1}$) | Oxalate[d] Fe (g kg$^{-1}$) | DTPA-extraction[e] Fe (mg kg$^{-1}$) | DTPA-extraction[e] Mn (mg kg$^{-1}$) | Blank treatment pH-pore water | Blank treatment DOC pore water (mg l$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Santomera** | ES | Murcia | entisol | 320 | 5.4 | 260 | 520 | 0.30 | 3.5 | 4.57 | 7.8 | 37 |
| **Xeraco L** | ES | Valencia | entisol | 350 | 13.7 | 360 | 150 | 0.90 | 10.5 | 5.33 | 7.7 | 36 |
| **Bologna** | IT | Bologna | entisol | 560 | 8.7 | 230 | 140 | 1.54 | 18.6 | 8.63 | 8.0 | 39 |
| **Nadec** | SA | Near Persian Gulf | aridisol | 190 | 8.7 | 70 | 140 | 0.13 | 2.1 | 5.67 | 7.5 | 82 |
| **Hofuf** | SA | Near Persian Gulf | aridisol | 180 | 7.1 | 40 | 60 | 0.19 | 6.7 | 3.80 | 7.3 | 153 |
| **Xeraco T** | ES | Valencia | entisol | 330 | 43.7 | 100 | 420 | 1.68 | 82.2 | 3.77 | 7.6 | 138 |
| **Droevendaal** | NL | Gelderland | spodosol | 260 | 15.2 | 40 | 0 | 1.68 | 60.0 | 1.50 | 5.1 | 84 |
| **Herveld** | NL | Gelderland | spodosol | 360 | 15.2 | 260 | 30 | 2.33 | 30.5 | 21.5 | 6.0 | 150 |

[a] Walinga et al. (1992)
[b] Houba et al. (1997)
[c] ISO 10693, Soil Quality – Determination of carbonate content, volumetric method
[d] Schwertmann (1964)
[e] Linday and Norvell (1978) and Quevauvillier et al. (1996)

**Table 3.2:** Composition of the FeEDDHA treatments expressed as pore water concentrations at t=0.

| Treatment | racemic o,o-FeEDDHA (mg $l^{-1}$ Fe) | meso o,o-FeEDDHA (mg $l^{-1}$ Fe) | o,p-FeEDDHA (mg $l^{-1}$ Fe) | rest-FeEDDHA (mg $l^{-1}$ Fe) | total Fe (mg $l^{-1}$ Fe) |
|---|---|---|---|---|---|
| blank with plants | 0 | 0 | 0 | 0 | 0 |
| 16%o,o with plants | 0.58 (8%) | 0.61 (8%) | 1.15 (16%) | 5.02 (68%) | 7.36 |
| 34%o,o with plants | 1.07 (16%) | 1.24 (18%) | 1.26 (19%) | 3.14 (47%) | 6.71 |
| 34%o,o without plants | 1.07 (16%) | 1.24 (18%) | 1.26 (19%) | 3.14 (47%) | 6.71 |
| 49%o,o with plants | 1.69 (22%) | 2.03 (27%) | 1.31 (18%) | 2.50 (33%) | 7.53 |
| 99%o,o with plants | 3.44 (48%) | 3.64 (51%) | 0.00 (0%) | 0.10 (1%) | 7.18 |

The pot experiments were carried out in a greenhouse with 7 liter Mitscherlich pots in triplicates. Pots, bottom plates and related materials were cleaned with 0.01 M HCl prior to usage. The inside of the pots was covered with polyethylene sacks with tiny holes, allowing for aeration. 6 kg of soil was thoroughly and successively mixed with a number of nutrient solutions. Per pot 40 mmol $NH_4NO_3$, 25 mmol $K_2HPO_4$, 20 mmol $CaCl_2$, 10 mmol $MgSO_4$, 0.5 mmol $H_3BO_3$ and 3.75 μmol $(NH_4)_6Mo_7O_{24}$ was added. In the treatments including FeEDDHA addition, an amount of FeEDDHA solution was added, corresponding to a soil solution concentration at t=0 of 7 mg $l^{-1}$ Fe (i.e. 0.13 mM Fe). The moisture content was made up to 50% of the water holding capacity of the individual soils (Table 3.1) with demineralized water. After the nutrients had been added, the soil was put into the pot.

The plant species grown in the pot trial was soybean (*Glycine max* (L.) Merr.). There is much experience with soybean in Fe chlorosis research; in nutrient solutions, in pot cultures and in the field (Garcia-Marco, et al., 2006; Goos, et al., 2004; Goos and Johnson, 2000; Heitholt, et al., 2003; Wallace and Cha, 1986). Seeds of the Fe chlorosis susceptible cultivar Mycogen 5072 were germinated on quartz sand with demineralised water. After five days eight seedlings were transferred to each pot, which had been filled with soil one day prior to the transfer. The pots were positioned on tables, grouped per soil, and were rotated on a daily basis. Every day the pots received an amount of demineralized water equal to the weight loss due to evapo-transpiration. At later growth stages the plant weight was compensated for in this respect. Until mid April, additional light was given with 400 Watt HPI lamps for 16 hours per day. The lamps were hanging 1 m above the pots. The temperature in the greenhouse was kept above 20 °C.

Micronutrients other than Fe were administered through foliar application, to ascertain that no deficiencies other than Fe deficiency would arise. The foliar spray consisted of 1.7 mM B; 0.20 mM Cu; 0.82 mM Mn; 0.27 mM Zn and 0.04 mM Mo. Cu, Zn and Mn were applied as dissolved EDTA salts, B as sodium tetraborate and Mo as ammonium molybdate. 1 ml $l^{-1}$ Agral was added as a wetting agent. Spraying was done on a weekly basis starting 3 weeks after plant transfer to the pots. The week before harvest, foliar spraying was omitted. After four weeks in pot experiment 1, the pots with Nadec and Hofuf soil were thinned out to five and four plants respectively, because of severe differences in plant development due to salt stress.

**Sampling and measurement**

*SPAD-measurement*

SPAD-indices give an indication of the relative chlorophyll content of leaves and are a widely used tool in the research on Fe chlorosis (Alvarez-Fernandez, et al., 2005; Alvarez Fernandez, et al., 2004; Banuls, et al., 2003). SPAD-measurements were done with a Minolta–502 SPAD-meter every two weeks including the day before harves,t to compare the chlorophyll content of leaves from different treatments. Per pot, SPAD-indices were measured for two youngest leaves and for two leaves from the second youngest trifoliate of every second plant. Measurement was done at the middle section of the leaf, midway between the central vein and the leaf edge. If a leaflet was necrotic or too small to analyze, no value was recorded. The SPAD-indices for youngest and second youngest trifoliate were averaged separately per pot.
A calibration experiment was done in which chlorophyll a + b was extracted with acetone from fresh leaves of plants three weeks of age. A linear relation between chlorophyll content and SPAD-index was found within the range of SPAD-indices measured (SPAD-indices ranged from 20 to 37; n = 4; $R^2 = 0.98$; Calibration data are presented in the Appendix).

*Yield*

At harvest, 16 of the youngest trifoliate leaves per pot were collected separately. The (remaining) shoots were cut off right above the soil surface. A 1 kg mixed subsample was taken from the soil, from which roots were collected manually. The soil subsample was stored overnight at 4 °C. The roots from the remaining soil were collected by rinsing out over a 1 mm sieve. Plant parts (youngest leaves, shoot and roots) were washed with demineralized water and dried at 70 °C. After 48 hours, the plant parts were weighed (dry weight).

*Mineral analysis of plant tissue*

The mineral contents of the plant parts were determined through microwave digestion with nitric acid, fluoric acid and hydrogen peroxide (Novozamsky, et al., 1996). Al, Cu, Fe, Mn and Zn concentrations were measured by ICP-AES (Varian, Vista Pro).

*Pore water analyses*

Pore water was collected from the soil subsamples by centrifugation at 7,443 *g* (7,000 rpm) for 15 minutes (Sorvall RC 5C plus) in Delrin (polyacetal) cylindrical 2 compartment containers, the day after harvest. The centrifugate was led from the soil containing compartment (about 150 $cm^3$) over a 0.45 μm nitro cellulose micro pore filter (Schleicher & Schuell, ref-no: 10401114) into a soil solution collection compartment (about 40 $cm^3$). Electro conductivity (EC) and pH were measured directly after collection. Dissolved organic carbon (DOC) concentrations were determined by subtracting the inorganic carbon from the total carbon as measured by the Shimadzu 5050A in accordance with EN1484. Fe, Ca and Mg concentrations were measured by ICP-AES (Varian, Vista Pro); Cu, Al, Mn, Zn, Ni and Co concentrations were measured by ICP-MS (Perkin Elmer, ELAN 6000). Samples were acidified with nitric acid before ICP-measurement.

FeEDDHA component concentrations were determined after separation through high-performance liquid chromatography (HPLC) as described in Schenkeveld et al. (2007). The Fe concentration chelated by rest-EDDHA was calculated by subtracting the Fe concentrations chelated by the other three FeEDDHA components and the Fe concentration in the blank treatment from the total Fe concentration as measured by ICP-AES.
To avoid contamination, preparation of the experimental solutions and dilution of samples for measurement was done with analytical grade chemicals and ultra pure water.

# Results and Discussion

**Chlorosis**

Chlorosis was established through comparison of SPAD-indices of the youngest leaves between the treatment without FeEDDHA addition (blank treatment) and the treatment with the highest SPAD-index (99%o,o treatment), for each soil individually. If the ratio of the two deviated from 1 ($\alpha = 0.05$), the blank was labeled chlorotic. In Table 3.3, the ratios for the SPAD-measurements after 2 weeks are presented. Chlorosis was found in plants grown on Santomera, Xeraco L, Bologna and Droevendaal soil. No chlorosis was expected on the reference soil from Droevendaal. Compared to other soils the initial plant growth was very rapid on the Droevendaal soil, which may have caused some temporal Fe shortage in the blank treatment. Although chlorosis was observed on more soils in the field, a direct comparison between field observations and pot trial data is not sound because root densities in pots are generally much higher than in the field, resulting in increased rhizosphere effects, which may enhance Fe availability (Marschner, et al., 1989). Furthermore, bicarbonate-rich or brackish water is generally used for irrigation in the field instead of demineralized water. The use of demineralized water causes a temporal drop in pH and bicarbonate concentration, thereby complicating the induction of chlorosis. Also the exposure to environmental stress factors is much higher in the field, making plants more susceptible to chlorosis (Morris, et al., 1990).

**Table 3.3:** SPAD-index ratios of the youngest leaves after 2 weeks. Standard deviations are indicated between parentheses.

| Soil | SPAD-index ratio* |
|---|---|
| Santomera | 0.71 (0.02) |
| Xeraco L | 0.87 (0.05) |
| Bologna | 0.93 (0.02) |
| Nadec | 0.96 (0.06) |
| Hofuf | 0.94 (0.06) |
| Xeraco T | 1.01 (0.03) |
| Droevendaal | 0.95 (0.02) |
| Herveld | 0.97 (0.04) |

* SPAD-index ratio is calculated: SPAD-index of the blank treatment divided by SPAD-index of the 99%o,o treatment.

In Figure 3.1 the degree of chlorosis in the blanks is presented as a function of time for Santomera soil, Xeraco L soil and Bologna soil. The data show that the degree of chlorosis diminishes over time until eventually SPAD-indices do no longer significantly differ. On Bologna soil, chlorosis was only observed in the measurement after 2 weeks, on Xeraco L soil up until and including 4 weeks, and on Santomera soil up until and including 6 weeks. The disappearance of chlorosis might result from an increased ability to take up soil native Fe through an ongoing development of the plant's roots system.

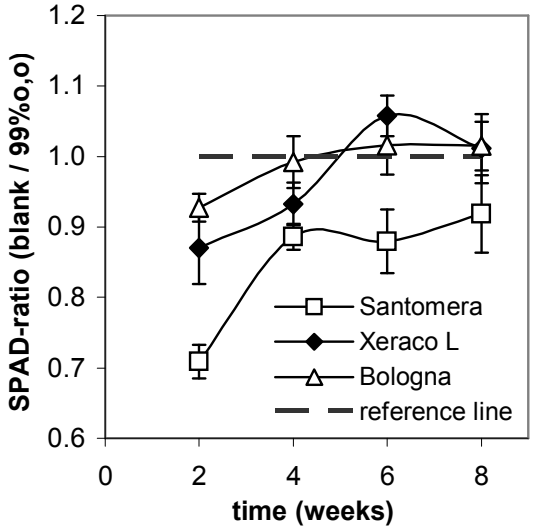



**Figure 3.1:** Development of chlorosis in soybean over time for the three soils on which chlorosis was induced. Error bars indicate standard deviations.

An attempt was made to link the occurrence of chlorosis to soil characteristics (see Table 3.1). From the soils on which chlorosis was observed in the field, all clay soils also induced chlorosis in pot experiment 1, while all sandy soils did not. This observation is not in agreement with the study by Morris et al. (1990) who found a positive correlation between clay content and chlorophyll concentration in soybeans grown on calcareous soils. A second distinctive feature is the DOC concentration in the pore water of the blank treatment, which was much lower for the soils on which the plants turned chlorotic (36 - 39 mg $l^{-1}$ C), than for the soils on which the plants did not (82 - 153 mg $l^{-1}$ C). Alike EDDHA, DOC may act as a carrier for Fe through soil solution and deliver Fe to the plant (Cesco, et al., 2002; Cesco, et al., 2000). If the DOC concentration is low, the Fe flux towards the plant may become insufficient to meet the requirements of the crop (Pandeya, et al., 1998). Furthermore, DOC has been reported to enhance root growth and induce changes in the root plasma membrane in favour of ion uptake (Canellas, et al., 2002; Pinton, et al., 1997). Both factors could also contribute to increased Fe uptake at higher DOC concentrations. Fe availability parameters (diethylene triamine penta acetic acid (DTPA)- and oxalate extractable Fe) do not offer a

primary explanation for the incidence of chlorosis in the blank treatments. However, for the clay soils which did induce chlorosis, both severity and duration of chlorosis increased with decreasing DTPA- and oxalate extractable Fe.

**The interaction between FeEDDHA components and soil**

Through the 34%o,o treatment without plants the interaction between FeEDDHA components and soil was examined without the interference of plant responses. The Fe concentrations measured in the pore water of this treatment after 8 weeks are presented in Table 3.4 for all soils. To demonstrate that almost all Fe is present as FeEDDHA complexes (except in Droevendaal soil), the Fe concentrations in the pore water of the blank treatment with plant are included as well. Even in absence of plants the Fe concentration in the pore water had strongly declined after 8 weeks, from an initial 6.74 mg $l^{-1}$ Fe to 0.50 – 1.09 mg $l^{-1}$ Fe; a reduction of 84 to 92%.

**Table 3.4:** Fe concentrations in the pore water after 8 weeks for the blank treatment with plants and the 34%o,o treatments with and without plants. Standard deviations are indicated between parentheses.

| Soil | Blank with plant [Fe] (mg $l^{-1}$ Fe) | 34%o,o without plant [Fe] (mg $l^{-1}$ Fe) | 34%o,o with plant [Fe] (mg $l^{-1}$ Fe) |
|---|---|---|---|
| Santomera | 0.00 (0.00) | 0.70 (0.00) | 0.40 (0.05) |
| Xeraco L | 0.00 (0.00) | 0.58 (0.08) | 0.25 (0.00) |
| Bologna | 0.02 (0.03) | 1.08 (0.23) | 0.62 (0.08) |
| Nadec | 0.00 (0.00) | 0.73 (0.06) | 0.60 (0.00) |
| Hofuf | 0.00 (0.00) | 0.87 (0.12) | 0.83 (0.15) |
| Xeraco T | 0.05 (0.00) | 0.50 (0.09) | 0.65 (0.05) |
| Droevendaal | 0.18 (0.03) | 0.58 (0.06) | 0.70 (0.05) |
| Herveld | 0.07 (0.03) | 0.62 (0.26) | 0.52 (0.03) |

In Table 3.5 the relative contributions of the different FeEDDHA components to the total dissolved Fe concentration are presented, both before and after 8 weeks of interaction with soil. In general, interaction with soil causes a strong increase in racemic o,o-FeEDDHA fraction in the pore water. The meso o,o-FeEDDHA fraction may either increase or decrease, depending on the soil. o,p-FeEDDHA is practically removed from solution resulting in negligible relative contributions in all soils. The relative contribution of rest-FeEDDHA decreases in all soils, the extent being strongly soil dependant. With exception of the two Dutch soils, the sum of racemic and meso o,o-FeEDDHA accounts for at least 75% of the Fe in solution after 8 weeks.

In the last column, the remaining fractions of the FeEDDHA components in soil solution after soil interaction are listed. The remaining fraction of an FeEDDHA component is calculated by dividing its concentration at t=t by its concentration at t=0. The sequence in remaining fractions is identical to the one found in a batch interaction experiment: racemic

o,o-FeEDDHA > meso o,o-FeEDDHA > rest-FeEDDHA > o,p-FeDDHA (Schenkeveld, et al., 2007). The remaining fractions in the pot experiment are however much lower than in the batch experiment. This can be explained from the higher soil-solution ratios in the pots, resulting in larger reactive surface areas per unit solution and thus leading to larger FeEDDHA component fractions adsorbing. Furthermore, it was observed that the soil surface had become red-stained, bearing witness to FeEDDHA surface precipitation due to water transport from lower parts of the pot to the surface with concentration through evaporation. This phenomenon contributes to a decrease in remaining fractions and was also observed in FeEDDHA treatments with plants. No significant relations between FeEDDHA component concentrations in the pore water and soil reactive surfaces were found. This probably results from the differences in soil-solution ratio between the soils.

**Table 3.5:** FeEDDHA component fractions in the 34%o,o treatment without plants, at t=0 and at t=8 weeks.

| FeEDDHA component | Fraction of total dissolved Fe $[Fe]_{component}/[Fe]_{total}$ | | Remaining fraction $[Fe]_{t=8\ weeks}/[Fe]_{t=0}$ |
|---|---|---|---|
| | at t=0 | at t=8 weeks | |
| racemic o,o-FeEDDHA | 0.16 | 0.46 – 0.75 | 0.25 – 0.55 |
| meso o,o-FeEDDHA | 0.18 | 0.01 – 0.37 | 0.01 – 0.26 |
| o,p-FeEDDHA | 0.19 | 0 – 0.04 | 0.00 – 0.03 |
| rest-FeEDDHA | 0.47 | 0 – 0.30 | 0.00 – 0.06 |

**FeEDDHA treatments with plants**

*Fe and FeEDDHA components in the pore water*

Also in the treatments with plants, the Fe concentration in solution is largely determined by the o,o-FeEDDHA isomers. Linear relations were found between Fe concentration in the pore water after 8 weeks and o,o-FeEDDHA content of the treatments, as exemplified for the Santomera soil in Figure 3.2. The figure shows that, for this soil, total Fe in solution is basically the sum of Fe chelated by racemic o,o-EDDHA and meso o,o-EDDHA. The concentrations of both isomers increase linearly with increasing o,o-FeEDDHA content of the treatment. As a consequence, the concentration ratio of racemic o,o-FeEDDHA and meso o,o-FeEDDHA is approximately the same for all treatments, ranging from 3.0 to 3.9.

The 34%o,o treatments with, and without plants were compared with respect to Fe concentrations in the pore water after 8 weeks (see Table 3.4). The impact of plant processes, including Fe uptake, on the Fe concentration in soil solution is relatively small compared to the overall decrease in Fe concentration since t=0. A significantly lower Fe concentration ($\alpha = 0.05$) in the treatment with plants was only found for Santomera, Xeraco L, Bologna and Nadec soil. For the other soils, the Fe concentrations did not significantly differ. The soils with a lower Fe concentration in the treatment with plants are the same soils on which

chlorosis in the blank treatment was observed (Figure 3.1), with exception of the Nadec soil. This suggests a relation between consumption of FeEDDHA and Fe deficiency in plants. Although no chlorosis was observed in the blank treatment on Nadec soil, in addition to Santomera and Xeraco L soil, it was the only soil on which the blank treatment had a lower yield than the 99%o,o treatment ($\alpha$ = 0,05; data not shown). Reduced growth as an Fe deficiency symptom prior to the manifestation of chlorosis has previously been reported by Gruber and Kosegarten (2002). So despite the absence of chlorosis, the plants of the blank treatment on Nadec soil may still have been Fe deficient, resulting in FeEDDHA consumption. With regard to soil characteristics, Nadec is the soil next in line after Santomera, Xeraco L and Bologna with respect to DOC concentration in the pore water of the blank treatments and it has an Fe availability (DTPA/oxalate extractable Fe) comparable to Santomera soil. It is however not a clay soil.

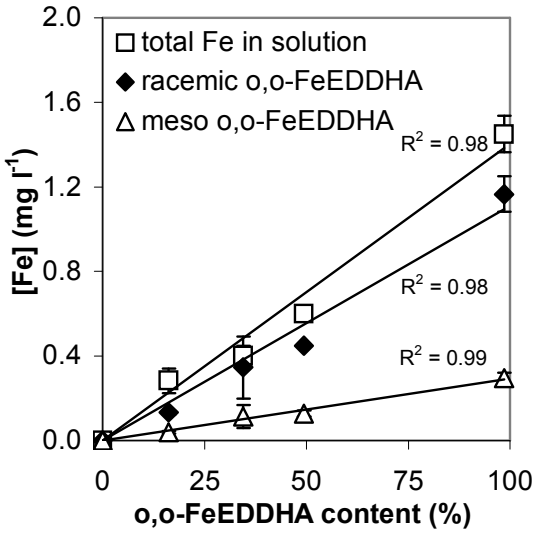



**Figure 3.2:** Total Fe-concentration, racemic o,o-FeEDDHA concentration and meso o,o-FeEDDHA concentration in the pore water of Santomera soil after 8 weeks as a function of the o,o-FeEDDHA content of the treatments. Error bars indicate standard deviations.

The observed differences in Fe concentration between the 34%o,o treatments with, and without plants may be explained in several ways. First, FeEDDHA complexes may be taken up by the plant as a whole, as reported by Bienfait et al (2004) for plants grown on substrate. When taken up, the chelating agent EDDHA cannot participate in a shuttle mechanism; EDDHA cannot readily move back into soil solution to form a new FeEDDHA complex, and hence the Fe concentration in soil solution decreases. If this would be the only mechanism through which FeEDDHA delivers Fe to the plant, the observed relation between FeEDDHA consumption and Fe deficiency would imply that plants only utilize Fe chelated by EDDHA if otherwise they will grow Fe deficient.

Secondly, plants may take up Fe from the FeEDDHA complex, for instance through an Fe reduction and chelate splitting mechanism (Chaney, et al., 1972; Marschner, et al., 1989). The chelating agent EDDHA may move back into solution, but not be able to form a new FeEDDHA complex due to degradation of the chelating agent or a combined effect of a low Fe availability and a high availability of competing cations like Cu and Al (Schenkeveld, et al., 2007). This also results in a decrease in Fe concentration in the pore water.
If however, the bioavailability of Fe in the soil is high, plants may take up Fe from FeEDDHA, without the Fe concentration in the pore water decreasing, due to an effective shuttle mechanism. In other words: the effectiveness of a possible shuttle mechanism is intrinsically limited by the low Fe availability of the soils on which there is a need to apply FeEDDHA. Further study is required to determine if these two Fe uptake mechanisms co-exist in soil-plant systems and to determine which one is dominant.

*SPAD-indices and Fe content*

The influence of the composition of FeEDDHA treatments on the degree of chlorosis is illustrated best when chlorosis in the blank treatment is most severe. Data from the 3-week experiment (experiment 2) were used for this purpose. Figure 3.3a displays the relation between SPAD-index of the youngest leaves and Fe concentration in the pore water. The shape of the curve is typical for a dose-response relation involving a micronutrient up to the point of inversion (Marschner, 1995): at low Fe concentrations the SPAD-index increases strongly with increasing Fe concentration, but the slope flattens and a plateau is reached. As demonstrated in Figure 3.2 the Fe concentration in the pore water is a linear function of the o,o-FeEDDHA content of the treatment. So until the plateau is reached, a treatment with a higher o,o-FeEDDHA content and an equal amount of Fe leads plants to develop a lesser degree of chlorosis.
Because Fe catalyzes chlorophyll synthesis, and a SPAD-index is an indicator for chlorophyll content, a relation between SPAD-index and Fe content of the green plant parts was expected. This relation was verified with the results from the microwave digestion of the youngest leaves. A linear relation between SPAD-index and Fe content was found as depicted in Figure 3.3b. Hence, Fe content and SPAD-index of the youngest leaves relate to Fe concentration in the pore water similarly. Because SPAD-indices are also dependent on several other factors like e.g. leaf thickness, they are only suitable for comparing the Fe content of leaves from plants of the same species, of the same age and grown under the same conditions. Therefore the relation found in Figure 3.3b does not have a general validity. When after 8 weeks chlorosis had disappeared from the plants grown on Santomera soil, the Fe content of the youngest leaves did no longer differ among the treatments and consequently no relation with Fe concentration in the pore water was found anymore (Figure 3.4a). However, the aforementioned dose-response relation was observed between Fe content of the shoot after 8 weeks and Fe concentration in the pore water (Figure 3.4b). So even though the plants in the blank treatment managed to overgrow chlorosis, a memory effect remained in terms of Fe content in the shoot. Apparently, an enhanced Fe uptake results in facilitating the youngest leaves with sufficient Fe rather than in raising the Fe content of older leaves. The effect was

substantial: the Fe content of the shoot was twice as high for the 99%o,o treatment as for the blank treatment (respectively 60 and 31 mg kg(dw)$^{-1}$ Fe); the 16%o,o treatment already increased the Fe content of the shoot by half as much as the 99%o,o treatment.

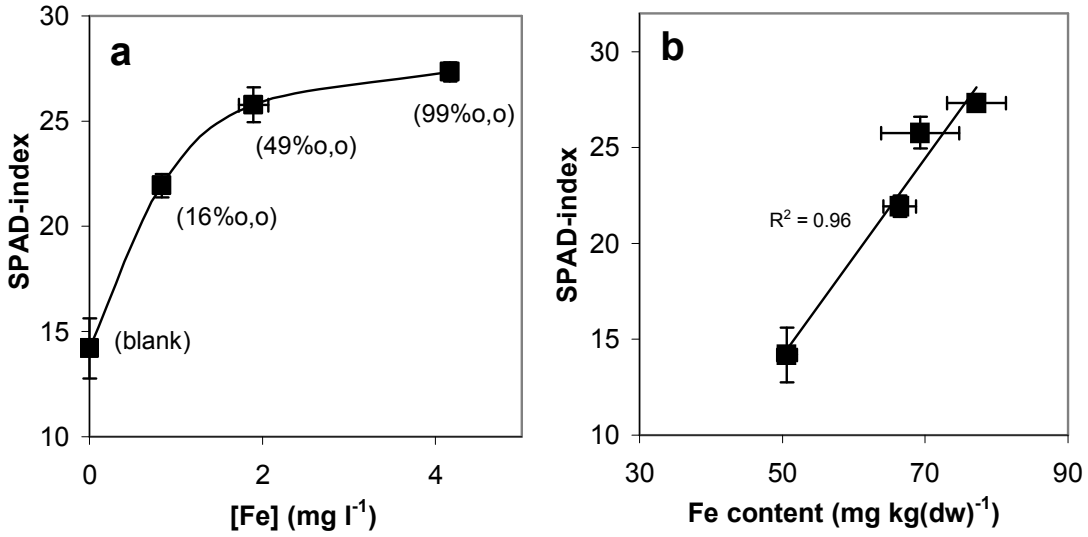



**Figure 3.3: a)** SPAD-index of the youngest leaves of soybean plants grown on Santomera soil as a function of Fe concentration in the pore water after 3 weeks; **b)** Relation between SPAD-index and Fe content in the youngest leaves of soybean plants grown on Santomera soil after 3 weeks. Error bars indicate standard deviations.

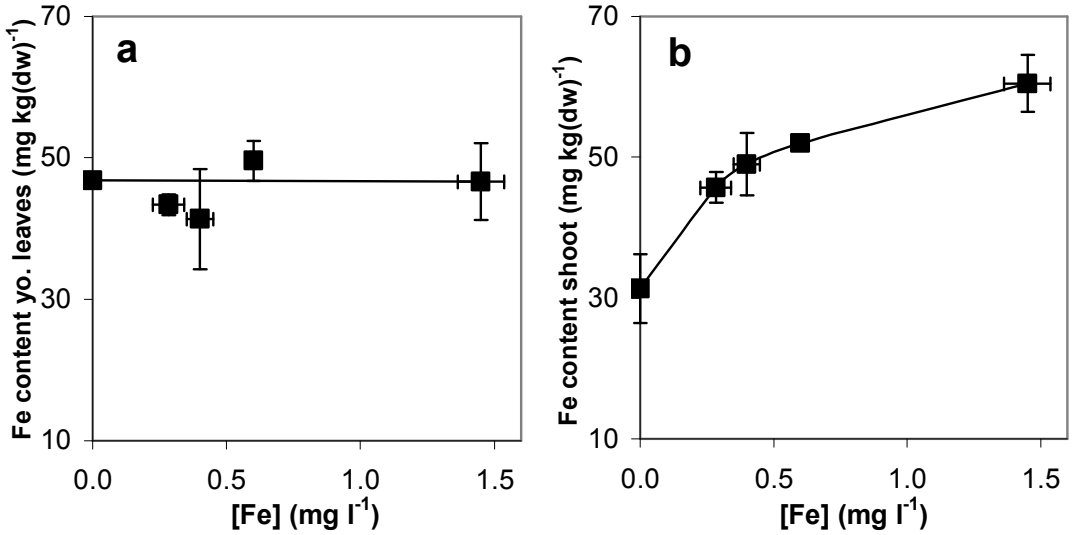



**Figure 3.4:** Fe content of **a)** the youngest leaves, and **b)** the shoot of soybean plants grown on Santomera soil as a function of Fe concentration in the pore water after 8 weeks. Error bars indicate standard deviations.

*Yield*

Chlorophyll is a plant's biomass producing unit and a reduction in chlorophyll content as a result of Fe deficiency was expected to lead to a reduction in yield. This effect was indeed observed and is illustrated in Figure 3.5a for the soils on which plants became chlorotic. The figure shows that, alike SPAD-index of the youngest leaves after 3 weeks (Figure 3.3a) and Fe content of the shoot after 8 weeks (Figure 3.4b), yield after 8 weeks increases with increasing Fe concentration in the pore water until a plateau is reached. The 99%o,o treatment yielded approximately 30% more biomass than the blank treatment on Santomera soil (29 and 22 g(dw) per pot respectively), and approximately 20% more on Xeraco L soil (47 and 38 g(dw) per pot respectively). For Bologna soil the duration of chlorosis was too short to result in a lasting effect in terms of yield. In general, the overall biomass production on Xeraco L and Bologna soil was higher than on Santomera soil. This is due to soil specifics not further considered here. The yield of the roots of plants grown on Santomera and Xeraco L soil was linearly related to the yield of the shoots ($R^2$ = 0.98 and 0.97 respectively; data not shown).

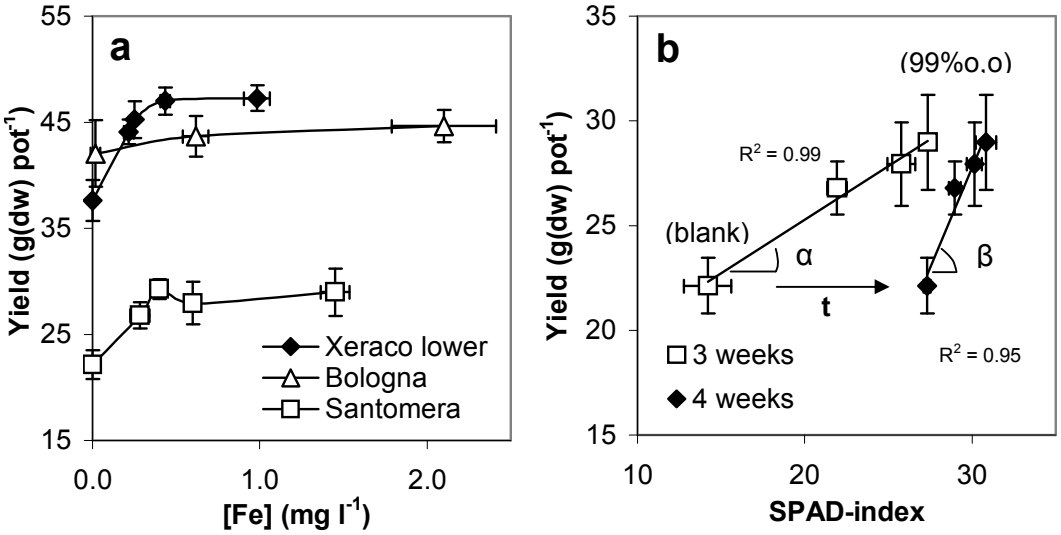



**Figure 3.5: a)** Yield (shoot including youngest leaves) after 8 weeks as a function of Fe concentration in the pore water, for soybean plants grown on Xeraco L, Bologna and Santomera soil; **b)** Yield (shoot including youngest leaves) after 8 weeks as a function of SPAD-index after 3 and 4 weeks for soybean plants grown on Santomera soil. Error bars indicate standard deviations.

The largest differences in yield between the treatments (after 8 weeks) lag behind the largest differences in chlorophyll content of the youngest leaves (after 3 weeks; yield data after 3 weeks are not presented). This is because produced biomass is an integral of photosynthesis intensity over time. So differences in biomass yield will grow until photosynthesis intensity is equal among the treatments. Photosynthesis intensity is in turn amongst others a function of chlorophyll content. In Figure 3.5b the yield on Santomera soil after 8 weeks is presented as a

function of SPAD-index after 3 weeks (pot experiment 2) and after 4 weeks (pot experiment 1). The observed linear relations imply, that SPAD-indices are also good relative predictors for (final) yield in crops grown under equal conditions. The slope of the linear trend line becomes steeper ($\beta > \alpha$) over time, until there are no longer differences in SPAD-index among the treatments.

*Fe uptake*

Fe uptake is the product of yield and Fe content summarized for the different plant parts and corrected for the Fe initially present in the seeds. Because both yield and Fe content display similar trends in response to the FeEDDHA treatments, this response becomes even more pronounced for Fe uptake.

In calculating Fe uptake the contribution of Fe in the roots was left out of consideration due to its probable overestimation and the large standard deviations within the treatments. This probably resulted from different amounts of apoplastic Fe and different extents of contamination of the roots with soil material (Strasser, et al., 1999). The latter factor is supported by the good correlation between the contents of Fe and the non-essential Al as determined in the root material ($R^2$ = 0.92 and 0.77 for Santomera and Xeraco L respectively). The Fe initially present in the soybean seeds (0.14 ± 0.02 mg Fe per pot) could not be specifically attributed to either roots or shoot and was not corrected for.

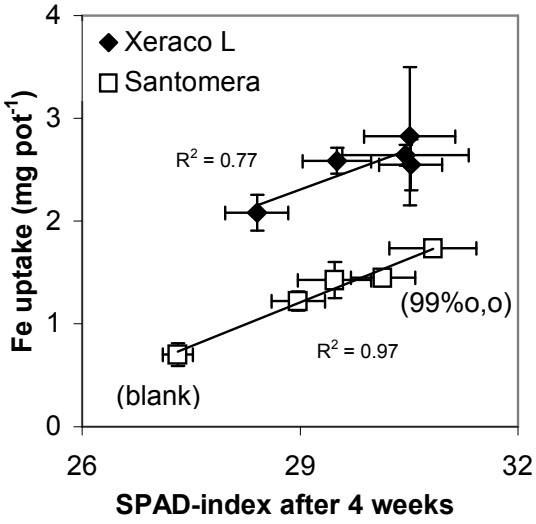



**Figure 3.6:** Fe uptake by soybean plants (shoots including youngest leaves) grown on Xeraco L and Santomera soil after 8 weeks as a function of SPAD-index after 4 weeks. Error bars indicate standard deviations.

For plants grown on Santomera soil, Fe uptake was 2.5 times higher in the 99%o,o treatment than in the blank treatment (1.74 and 0.70 mg Fe per pot respectively). The difference in Fe content of the shoot (31 to 60 mg Fe $kg(dw)^{-1}$) contributed more to the difference in Fe uptake

than the difference in yield (22 to 29 g(dw) per pot). The 16%o,o treatment already increased Fe uptake by half as much as the 99%o,o treatment. Additional Fe uptake and translocation to the shoot (including the youngest leaves), as a result of application of FeEDDHA treatments to Santomera soil, account for 7 to 15% of the Fe applied with the treatment. This corresponds with 15 to 44% of the Fe that was offered as o,o-FeEDDHA. Stress responses in the blank treatment may have resulted in a higher non-FeEDDHA mediated uptake of soil native Fe in the blank than in the FeEDDHA treatments. So the actual Fe uptake due to FeEDDHA may be higher than the additional Fe uptake.
An attempt was made to relate Fe uptake to SPAD-indices for both Santomera and Xeraco L soil. SPAD-indices after 2, 4, 6 and 8 weeks were tried, as well as different averages. The SPAD-indices after 4 weeks gave the best linear relationships with Fe uptake, as shown in Figure 3.6. The $R^2$ for Santomera soil (0.97) is much closer to 1 than for Xeraco L soil (0.77). This is probably partly due to the lesser intensity of chlorosis in the blank and the smaller range in SPAD indices for Xeraco L soil. These specific linear relationships cannot be extrapolated to data outside this experiment. The similarity in slope is based on coincidence; Xeraco L soil combines higher yields with a smaller difference in Fe content between the treatments, compared to Santomera.

*Effect on Mn uptake*
Since the 1950s, soil application of FeEDDHA is known to negatively affect Mn uptake in a large number of plant species including soybean (Ghasemi Fasaei, et al., 2003; Heenan and Campbell, 1983; Holmes and Brown, 1955; Moraghan, 1979). For this reason a possible effect of composition of FeEDDHA treatments on Mn uptake has been examined in this study. The effect of FeEDDHA application on the Mn concentration in soil solution is small but significant ($\alpha = 0.05$): an increase from 10.8 to 13.2 mg $l^{-1}$ Mn with increasing o,o-FeEDDHA content. However, in above-soil plant tissue the Mn content decreased exponentially with increasing o,o-FeEDDHA content of the FeEDDHA treatments (Figure 3.7a). It declined by a factor 4.6 (from 406 to 88 mg kg(dw)$^{-1}$ Mn) in the youngest leaves, and by a factor 3.5 (from 188 to 54 mg kg(dw)$^{-1}$ Mn) in the remaining shoot. Despite this strong decrease, the plants did not grow Mn deficient. Mn deficiency in soybean occurs at Mn contents in the shoot of around 10 to 20 mg kg(dw)$^{-1}$ Mn (Adams, et al., 2000; Reuter, et al., 1997). If the dose of o,o-FeEDDHA would be further increased or Mn fertilization through foliar spraying would be omitted, Mn deficiency could become a serious concern. The Mn content of the roots was lower than of the other plant parts (22 to 44 mg kg(dw)$^{-1}$ Mn) and did not display the same exponential trend with o,o-FeEDDHA content of the treatment. Only the 99%o,o treatment had a significantly lower Mn content in the roots than the other treatments.
Mn uptake ranged from 1.69 mg Mn in the 99%o,o treatment to 4.44 mg Mn in the blank treatment. Only 0.75 mg Mn per pot had been applied through foliar application, assuming a 100% efficient use of foliar spray and an equal dose for all pots. Hence differences in Mn uptake should be primarily explained from root uptake.
The decrease in Mn uptake with increasing o,o-FeEDDHA content can not be attributed to a decreased Mn concentration in soil solution. If Mn and Fe uptake are regulated by the same

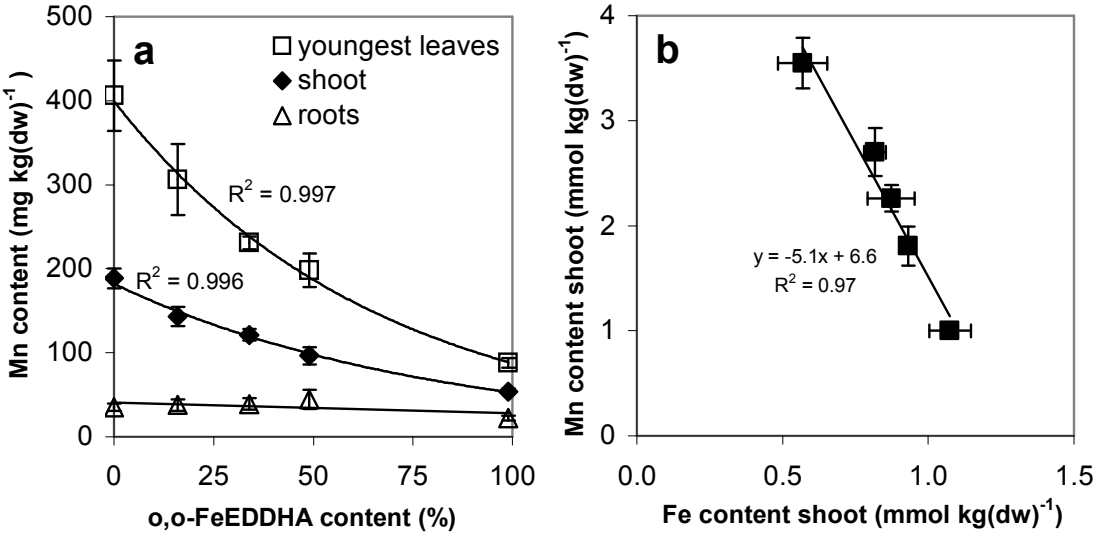



**Figure 3.7: a)** Mn content of the youngest leaves, shoot and roots of soybean plants grown on Santomera soil, after 8 weeks as a function of the o,o-FeEDDHA content of the treatment; **b)** The relation between Mn and Fe content of the shoot of soybean plants grown on Santomera soil after 8 weeks. Error bars indicate standard deviations.

uptake sites at the root surface, FeEDDHA treatments may increase the competition with Fe for these sites. This competition may involve the FeEDDHA complex at the uptake site, in case *i)* Fe uptake takes place either through a reduction and chelate splitting mechanism (Chaney, et al., 1972; Marschner, et al., 1989), or *ii)* through uptake of the complex as a whole (Bienfait, et al., 2004). Or *iii)* FeEDDHA complexes may serve as a replenishing pool in solution, preventing $Fe^{3+}$ depletion in the rhizosphere due to Fe uptake by the plant and slow dissolution kinetics of Fe hydroxides. In this case, the FeEDDHA complex needs to be sufficiently labile to increase Fe activity in the rhizosphere. Additional research is needed on the mechanisms underlying Fe uptake and Fe-Mn competition.

The decrease in Mn content of the shoot is not equal, but proportional to the increase in Fe content of the shoot (Figure 3.7b); the slope of the curve indicates that an increase in Fe content leads to a 5 times larger decrease in Mn content. This proportionality probably primarily results from difference in uptake rate between Fe and Mn.

# Conclusion

In this pot trial study, Fe chlorosis was induced in soybean plants on 3 out of 6 calcareous soils facing Fe chlorosis problems under field conditions. Chlorosis was only induced on the clay soils, which had lower DOC concentrations in the pore water (36 to 39 mg $l^{-1}$ C) than the

sandy soils (82 to 153 mg $l^{-1}$ C). The severity and duration of chlorosis correlated with Fe availability parameters (DTPA- and oxalate extractable Fe).

In calcareous soils, the o,o-FeEDDHA content of the FeEDDHA treatment largely determined how much Fe remained in solution. o,o-FeEDDHA content and Fe concentration were linearly related. The sequence in remaining fractions of the FeEDDHA components corresponded to previous findings in batch experiment: racemic o,o-FeEDDHA > meso o,o-FeEDDHA > rest-FeEDDHA > o,p-FeEDDHA. In corresponding FeEDDHA treatments with and without plants, differences in Fe concentration in the pore water were only found for soils on which plants from the blank treatment exhibited Fe deficiency symptoms.

In soils on which chlorosis was induced, the Fe concentration in soil solution determined Fe uptake by the plants. The relationship between Fe concentration and Fe uptake is non-linear: initially Fe uptake increases strongly with increasing Fe concentration, but the slope flattens and a plateau is reached.

Although chlorosis in the youngest leaves had disappeared after 8 weeks on all soils on which it had been induced, its consequences remained in terms of reduced yield (up to 24% on Santomera soil), reduced Fe content in the shoot (up to 47% on Santomera soil) and reduced Fe uptake to the shoot (up to 60% on Santomera soil).

Despite the fact that Mn concentrations in the pore water were hardly affected and in spite of foliar application of Mn, Mn uptake decreased exponentially with the o,o-FeEDDHA content of the FeEDDHA treatments. In the shoot, the decrease in Mn content was proportional to the increase in Fe content. In case of excessive FeEDDHA application or the omission of Mn fertilization, Mn deficiency may arise.

The evident increase in both yield and Fe nutritional value of plants confirms the usefulness of FeEDDHA application on calcareous soils. When FeEDDHA is applied prior to the set in of chlorosis, the Fe concentration in the pore water proved to be the key parameter determining the treatment's effectiveness. This parameter is largely determined by the Fe dose and the o,o-FeEDDHA content of the treatment. Hence, from a formulation with a higher o,o-FeEDDHA content, a smaller Fe dose suffices to obtain the same results in terms of yield and Fe nutritional value.

# Appendix

### Calibration of the SPAD-meter

*Extraction protocol*

An extraction solution was prepared by mixing acetone (99%) and 0.05 M $K_2CO_3$ solution in a volumetric ratio of 9:1. The solution was stored in a refrigerator. 1.00 g of fresh plant material was transferred to a homogenization vessel. 10 ml of the cooled extraction solution (T = 4 ± 1 °C) was added to the plant material. The suspension was homogenized for one minute and subsequently the homogenate was filtered over a glass filter by vacuum filtration. The filtrate was made up to 100 ml with extraction solution. The chlorophyll concentration was determined by spectrophotometry. The sum of the chlorophyll a and b concentration was determined by measuring the extinction at 652 nm. The extraction is based on the method described by Bruinsma (1963).

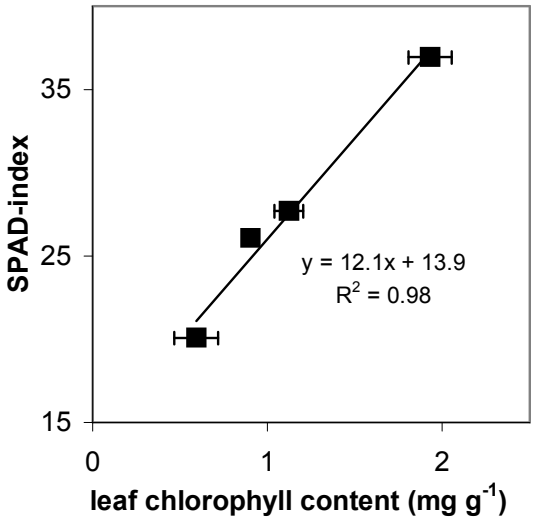



**Figure A3.1:** Relation between chlorophyll content and SPAD-index of the youngest leaves of soybean plants.

“Recipe ferrum!”
*trans.* “Receive the iron!”

Roman expression, urging not to show
mercy to a defeated opponent in combat.

# Chapter 4

## FeEDDHA-facilitated Fe uptake in relation to the behaviour of FeEDDHA components in the soil-plant system as a function of time and dosage

W.D.C. Schenkeveld, E.J.M. Temminghoff, A.M. Reichwein, and W.H. van Riemsdijk

Plant and Soil (2010), 332(1/2): 68-85

## Abstract

FeEDDHA products are widely used to prevent and remedy Fe chlorosis in crops grown on calcareous soils. These products consist of a mixture of FeEDDHA components: racemic o,o-FeEDDHA, meso o,o-FeEDDHA, o,p-FeEDDHA and rest-FeEDDHA. The FeEDDHA components differ in physical and chemical properties, and as a consequence also in effectiveness as Fe fertilizer. In order to efficiently match dose, frequency and moment of FeEDDHA application with the Fe requirements of plants, it is important to understand the behaviour of the FeEDDHA components in the soil-plant system as a function of time and dosage, and to relate this behaviour to Fe uptake by plants. These issues have been examined in a pot trial study with soybean plants (*Glycine max* (L.) Merr. cv Mycogen 5072) grown on calcareous soil from Santomera, Spain. Four FeEDDHA treatments (two compositions, two dosages) were applied prior to the set in of chlorosis. Leaching of FeEDDHA components was prevented. Plant and soil were sampled every week, for six weeks. From one week onward the Fe concentration in the pore water was largely gouverned by racemic and meso o,o-FeEDDHA. The concentration behaviour of the o,o-FeEDDHA isomers underwent two stages: a strong decline within the first week resulting from linear adsorption, and a gradual decline from one week onward. For meso o,o-FeEDDHA, unlike racemic o,o-FeDDHA, the gradual decline could be mathematically well described with an exponential decay function. Soybean plants mainly took up Fe in the progressed vegetative stage ($3^{rd}$ and $4^{th}$ week) and in the reproductive stage, when the pods were being filled with seeds ($6^{th}$ week). Fe uptake and removal of racemic o,o-FeEDDHA from the soil system displayed a similar time-trend, whereas the removal of meso o,o-FeEDDHA had a plant-independent character. This indicates the removal of racemic o,o-FeEDDHA was to a larger extent plant-related.

# Introduction

Fe deficiency chlorosis is a nutritional disorder characterized by a significant decrease of chlorophyll in the leaves, often observed in plants grown on alkaline and calcareous soils. It decreases crop yield both quantitatively and qualitatively, resulting in economic losses (Chaney, 1984; Mortvedt, 1991). Elevated bicarbonate concentrations and high pH have been identified as the main soil conditions explaining the incidence of Fe chlorosis (Boxma, 1972; Mengel, et al., 1984; Shi, et al., 1993). Under such conditions, the solubility of Fe(hydr)oxides is low (Lindsay, 1979) and Fe uptake mechanisms become impaired (Marschner, 1995; Venkatraju and Marschner, 1981), or Fe becomes inactivated inside the leaf's apoplast (Mengel, 1994).

The application of synthetic Fe chelates is the most common practice to mend or to prevent Fe chlorosis. These chelates increase the solubility of Fe and function as a transporter through solution to the plant. FeEDDHA (iron (3+) ethylene diamine-N,N'-bis(hydroxy phenylacetic acid)) is among the most effective synthetic Fe chelates under neutral and alkaline soil conditions (Lucena, et al., 1992; Reed, et al., 1988; Wallace, et al., 1955). Commercial FeEDDHA formulations consist of a mixture of positional isomers, diastereomers and polycondensates. Such mixtures can be divided into 4 groups of FeEDDHA components: 1) racemic o,o-FeEDDHA (iron (3+) (R,R) and (S,S) ethylene diamine-N,N'-bis(2-hydroxy phenyl acetic acid) complexes), 2) meso o,o-FeEDDHA (iron (3+) (R,S) = (S,R) ethylene diamine-N,N'-bis(2-hydroxy phenyl acetic acid) complex), 3) o,p-FeEDDHA (iron (3+) ethylene diamine-N-(2-hydroxy phenyl acetic acid)-N'-(4-hydroxy phenyl acetic acid) complexes), and 4) rest-FeEDDHA (largely consisting of polycondensates). The physical and chemical properties of these FeEDDHA components differ strongly (Ahrland, et al., 1990; Bannochie and Martell, 1989; Frost, et al., 1958; Gomez-Gallego, et al., 2005; Gomez Gallego, et al., 2006; Yunta, et al., 2003a; Yunta, et al., 2003b), and as a consequence, so does their ability to preserve Fe in solution and deliver it to the plant.
The effectiveness of the individual FeEDDHA components is determined by the following characteristics: 1) their ability to remain in solution, 2) their susceptibility to cation competition and biodegradation, 3) their ability to transfer Fe to the plant, and 4) the ability of the corresponding EDDHA component to selectively chelate Fe from the soil (Lucena, 2003).
The first two characteristics have been addressed in a number of interaction studies with soil (Alvarez-Fernandez, et al., 1997; Alvarez-Fernandez, et al., 2002; Garcia-Marco, et al., 2006; Hernandez-Apaolaza, et al., 2006; Schenkeveld, et al., 2007). These studies show that racemic o,o-FeEDDHA is superior in maintaining Fe in solution, followed by meso o,o-FeEDDHA, while rest-FeEDDHA and particularly o,p-FeEDDHA are largely removed from solution.

With respect to Fe transfer to the plant, studies with hydroponic systems show that in such systems o,p-FeEDDHA is more effective in mending Fe chlorosis in soybean than o,o-FeEDDHA (Garcia-Marco, et al., 2006) and that both are more effective than synthesis by-products (referred to as rest-FeEDDHA in this study) (Hernandez-Apaolaza, et al., 2006).

Furthermore, meso o,o-FeEDDHA has been claimed to be more effective than racemic o,o-FeEDDHA (Cerdan, et al., 2006).
Still little is known about the performance of FeEDDHA components in the soil-plant system. Recently, Schenkeveld et al.(2008) reported a pot trial study with soybean and FeEDDHA administration prior to the set in of chlorosis. The amount of o,o-FeEDDHA (i.e. the sum of racemic an meso o,o-FeEDDHA) largely determined the effectiveness of the treatment in terms of Fe uptake. The superiority of o,o-FeEDDHA over o,p-FeEDDHA in delivering Fe to soil-grown crops was confirmed by Rojas et al.(2008). Furthermore, Schenkeveld et al. (2008) observed that the concentration of the o,o-FeEDDHA isomers decreased considerably throughout the experiment, and that Fe uptake by the plants could not account for the loss of o,o-FeEDDHA.

Thus far, Fe dynamics in the soil-plant system with FeEDDHA addition have not yet been examined. Both FeEDDHA component concentrations in the pore water and Fe requirements of the plant vary over time. An understanding of these time dependencies is essential to determine dose, frequency and moment of FeEDDHA application to soil grown crops, for optimizing yield and crop quality, while minimizing the use of FeEDDHA. The aim of this study was 1) to examine the pore water concentration of FeEDDHA components in a soil-plant system as a function of time and dosage, and 2) to relate the observed concentration behaviour to FeEDDHA-facilitated Fe uptake by plants.
For this purpose a pot trial study was set up, with soybean grown on a calcareous soil from Spain. Four FeEDDHA treatments (two compositions, two Fe dosages) were administered prior to the set in of chlorosis. Leaching of FeEDDHA components from the root-zone, occurring under field conditions as a result of excessive irrigation or atmospheric precipitation (Rombola and Tagliavini, 2006), was prevented. Harvesting was done destructively on a weekly basis.

# Material and Methods

**Soil**

Calcareous soil was collected from the top soil layer (0 – 20 cm) at a site located in Santomera (Murcia, Spain). Relevant soil characteristics are presented in Table 4.1. Santomera soil is a clay soil with a lutum fraction of 260 g $kg^{-1}$ and a $CaCO_3$ content of 520 g $kg^{-1}$, common for calcareous soils from that area. The pH of the soil is 8.0 (pH-$CaCl_2$). The soil organic carbon (SOC) content is low (0.5%), and the dissolved organic carbon (DOC) concentration equals 30 mg $l^{-1}$ (0.01 M $CaCl_2$). Fe availability parameters are low: the oxalate extractable ('reactive') Fe content amounts 0.30 g $kg^{-1}$ Fe, and the diethylene triamine penta acetic acid (DTPA) extractable content amounts 3.5 mg $kg^{-1}$ Fe. Plants grown on Santomera soil became chlorotic, both under field conditions and in a previous pot trial (Schenkeveld, et al., 2008). Pre-treatment consisted of air drying and sieving (1 cm).

**Table 4.1:** Soil characteristics.

| | | Extraction | | |
|---|---|---|---|---|
| **Origin/Name** | Santomera | **$CaCl_2$ (0.01 M)[g]** | **DOC (mg $l^{-1}$)** | 30 |
| **Region** | Murcia | **Oxalate[h]** | **Fe (g $kg^{-1}$)** | 0.30 |
| **Country** | Spain | **DTPA[i]** | **Fe (mg $kg^{-1}$)** | 3.5 |
| **Soil classification** | entisol | | **Mn (mg $kg^{-1}$)** | 4.6 |
| **Water holding capacity (g $kg^{-1}$)** | 320 | | **Cu (mg $kg^{-1}$)** | 4.1 |
| **pH-$CaCl_2$[a]** | 8.0 | | **Zn (mg $kg^{-1}$)** | 0.9 |
| **Electro conductivity (mS $m^{-1}$)[b]** | 23 | **$HNO_3$ (0.43 M)[j]** | **Fe (mg $kg^{-1}$)** | 494 |
| **SOC (g $kg^{-1}$)[c]** | 5.4 | | **Mn (mg $kg^{-1}$)** | 179 |
| **Clay (g $kg^{-1}$)[d]** | 260 | | **Cu (mg $kg^{-1}$)** | 10 |
| **$CaCO_3$ (g $kg^{-1}$)[e]** | 520 | | **Zn (mg $kg^{-1}$)** | 5 |
| **CEC (cmol $kg^{-1}$)[f]** | 10.3 | | | |

[a] ISO/DIS 10390 Soil Quality – Determination of pH
[b] ISO/DIS 11265 Soil Quality – Determination of the specific electric conductivity
[c] Walinga et al. (1992)
[d] Houba et al. (1997)
[e] ISO 10693, Soil Quality – Determination of carbonate content, volumetric method
[f] ISO/DIS 11260 Soil Quality – Determination of cation exchange capacity and base saturation – method using barium chloride solution
[g] Houba et al. (2000)
[h] Schwertmann (1964)
[i] Lindsay and Norvell (1978) and Quevauvillier et al. (1996)
[j] Tipping et al. (2003). Fest el al. (2005)

**FeEDDHA solutions**

FeEDDHA solutions were prepared from a sodium-EDDHA stock solution and solid o,o-$H_4$EDDHA (99% pure). The sodium-EDDHA stock solution was synthesized through a Mannich-like reaction (patent - Petree, et al., 1978). Prior to Fe addition, the solid o,o-$H_4$EDDHA was dissolved by adding sufficient 1 M NaOH. Fe was added as $FeCl_3$*$6H_2O$ in a 5% excess based on a 1:1 stoichiometry between Fe and ethylene diamine. The pH was raised to 7 (± 0.5) and the solutions were left over-night in the dark to allow excess Fe to precipitate as hydroxides. The following day, the solutions were filtered over a 0.45 µm nitro cellulose micro pore filter (Schleicher & Schuell, ref-no: 10401114) and further diluted for application in the pot trial. The composition of the FeEDDHA solutions was analysed by combined ICP and HPLC analysis at t=0.

**Pot trial**

A pot trial with a runtime of six weeks was done from late August until mid October 2005. The main experiment consisted of five treatments with plants: a blank and four FeEDDHA treatments. The FeEDDHA treatments varied in amount of chelated Fe and in FeEDDHA component composition (Table 4.2). In this way, the effect of dosage of the FeEDDHA components on their concentration behaviour in soil solution, and on Fe uptake by plants could be examined. Labels of the treatments indicate the combined percentage of Fe chelated by racemic and meso o,o-EDDHA (30 or 100%), and the Fe dose applied (L or H). The L(ow)

dose corresponds to ≈ 4 mg $l^{-1}$ Fe (0.07 mM) and the H(igh) dose to ≈ 40 mg $l^{-1}$ Fe (0.7 mM) in the pore water at t=0 (see Table 4.2). FeEDDHA was applied once, at the start of the trail. The blank and L-treatments were harvested (destructively) every week, the H-treatments every second week. The L-treatments were harvested more frequently, because at 4 mg $l^{-1}$ Fe, the composition of the FeEDDHA treatment was certain to affect Fe uptake; at 40 mg $l^{-1}$ Fe this was questionable (Schenkeveld, et al., 2008). The experiment was carried out in triplicates, comprising 72 pots in total.
To examine the influence of plants on the concentration of the FeEDDHA components in soil solution, a second experiment was carried out with the blank and the L-treatments, both with and without plants. Harvest was after 6 weeks only. This experiment was done in duplicates.

**Table 4.2:** Composition of the FeEDDHA treatments expressed as pore water concentrations at t=0.

| Treatment | racemic o,o-FeEDDHA (mg $l^{-1}$ Fe) | meso o,o-FeEDDHA (mg $l^{-1}$ Fe) | o,p-FeEDDHA (mg $l^{-1}$ Fe) | rest-FeEDDHA (mg $l^{-1}$ Fe) | totaal Fe (mg $l^{-1}$ Fe) |
|---|---|---|---|---|---|
| blank | 0 | 0 | 0 | 0 | 0 |
| 30%o,oL | 0.60 (14%) | 0.68 (16%) | 0.79 (19%) | 2.18 (51%) | 4.25 |
| 100%o,oL | 1.93 (48%) | 2.00 (50%) | 0 | 0.05 (1%) | 3.98 |
| 30%o,oH | 5.97 (14%) | 6.75 (16%) | 7.94 (19%) | 21.8 (51%) | 42.5 |
| 100%o,oH | 19.3 (48%) | 20.0 (50%) | 0 | 0.55 (1%) | 39.8 |

The pot experiment was executed in a greenhouse with 7 liter Mitscherlich pots. Pots, bottom plates and related materials were cleaned with 0.01 M HCl prior to usage. The inside of the pots were covered with polyethylene sacks with tiny holes allowing for aeration. The pots contained six kg of soil, thoroughly and successively mixed with a number of nutrient solutions. Per pot 40 mmol $NH_4NO_3$, 25 mmol $K_2HPO_4$, 20 mmol $CaCl_2$, 10 mmol $MgSO_4$, 0.5 mmol $H_3BO_3$ and 3.75 μmol $(NH_4)_6Mo_7O_{24}$ were added. Additionally, the L- and H-treatments received respectively 0.069 and 0.69 mmol $pot^{-1}$ Fe as FeEDDHA solution. Based on previous trials with Santomera soil, no micronutrient deficiencies other than Fe deficiency were expected in the blank. Therefore Cu, Mn and Zn fertilization was omitted. The moisture content was made up to 50% of the water holding capacity with demineralized water.

Seeds of the Fe chlorosis susceptible soybean (*Glycine max* (L.) Merr.) cultivar Mycogen 5072 were germinated on quartz sand with demineralised water. After five days eight seedlings were transferred to each pot, which had been filled with soil one day prior to the transfer. The pots from the main experiment were rotated on a daily basis. Every day the pots received an amount of demineralized water equal to the weight loss due to evapo-transpiration. At later growth stages the plant weight was compensated for in this respect. Evapo-transpiration did not exceed 260 ml $pot^{-1}$ $day^{-1}$ (i.e. 27% of the soil water content). The temperature in the greenhouse was kept above 20 °C. The time-span between sunrise and sunset decreased from 14 to 11 hours, approximately. No supplementary light was provided to assist plant-growth.

**Sampling and measurement**

*SPAD-measurement*

SPAD-measurements were done three times per week with a Minolta–502 SPAD-meter to compare the chlorophyll content of leaves among treatments. Measurements started 6 days after the transfer of the seedlings to the pots, when the leaves had grown sufficiently large. Per pot, SPAD-indices were measured for two youngest leaves and two leaves from the second youngest trifoliate of every second plant. Measurement was done at the middle section of the leaf, midway between the central vein and the leaf edge. If a leaflet was necrotic or too small to analyze, no value was recorded. SPAD-indices for the youngest and second youngest trifoliate were averaged separately per pot. The plants harvested after 6 weeks were monitored throughout the experiment. SPAD-measurements on plants harvested earlier, started respectively one and two weeks before harvest for the L- and H-treatments. To ascertain representitativeness, the SPAD-values of plants harvested after 6 weeks were compared, per treatment, to the SPAD-values of plants harvested earlier. Chlorosis was operationally established as a significant difference ($\alpha = 0.05$) in SPAD-indices of the youngest leaves between the blank and the treatment with the highest SPAD-indices. The size of the difference in SPAD-value has been interpreted as a measure for the severity of chlorosis.

*Harvest*

At each harvesting round, 16 of the youngest trifoliate leaves were separately collected per pot, with exception of the first harvesting round when plants were still too small. The (remaining) shoots were cut off right above the soil surface. Prior to harvest, the moisture content of the soil had been restored to 50% of the water holding capacity. After a few hours of equilibration time, a 1 kg mixed subsample was taken from the soil, from which roots were collected manually. The soil subsample was stored overnight at 4 °C. The remaining roots were collected by rinsing out the soil over a 1 mm sieve. Plant parts (youngest leaves, shoot and roots) were washed with demineralized water and dried at 70 °C. After 48 hours, the plant parts were weighed (dry weight).

*Mineral analysis of plant tissue*

The mineral contents of the plant parts were determined through microwave digestion with nitric acid, fluoric acid and hydrogen peroxide (Novozamsky, et al., 1996). Al, Co, Cu, Fe, Mn, Ni and Zn concentrations were measured by ICP-AES (Varian, Vista Pro).

*Pore water collection and analysis*

A fraction of the pore water was collected from the soil subsamples by centrifugation at 7,443 *g* (7,000 rpm) for 15 minutes (Sorvall RC 5C plus) in Delrin (polyacetal) cylindrical 2 compartment containers the day after harvest. The centrifugate was led from the soil containing compartment (approximately 150 $cm^3$) over a 0.45 μm nitro cellulose micro pore filter (Schleicher & Schuell, ref-no: 10401114) into a soil solution collection compartment

(approximately 40 $cm^3$). Per unit soil, the amount of water collected did not exceed evapotranspiration; therefore the collected pore water was assumed available to plants.
pH was measured directly after pore water collection. Fe, Ca and Mg concentrations were measured by ICP-AES (Varian, Vista Pro); Cu, Al, Mn, Zn, Ni and Co concentrations were measured by ICP-MS (Perkin Elmer, ELAN 6000). The samples were acidified with nitric acid before ICP-measurement. FeEDDHA component concentrations were determined after separation through high-performance liquid chromatography (HPLC) as described by Schenkeveld et al. (2007). The Fe concentration chelated by rest-EDDHA was calculated by subtracting the Fe concentrations related to the other FeEDDHA components and the Fe concentration in the blank treatment from the total Fe concentration measured by ICP-AES. To avoid contamination, the preparation of the experimental solutions and dilution of samples for measurement were done with analytical grade chemicals and ultra pure water.

**Statistical analysis**
Statistical analyses were performed with the program SPSS 12.0. Differences between variables were determined by applying the one-way ANOVA procedure with a Tukey post-hoc test ($\alpha = 0.05$). The growth rates of the shoot were compared by analysis of the slopes of linear regression lines ($\alpha = 0.05$).

# Results

**Fe and FeEDDHA component concentrations in soil solution**
The Fe concentration in soil solution of the blank treatment was below detection limit throughout the experiment (data not shown). This implies that the Fe concentrations in the treatments with FeEDDHA addition entirely resulted from FeEDDHA components. In Figure 4.1, the Fe and FeEDDHA component concentrations are presented as a function of time for the 30%o,oL treatment. During the first week, o,p-FeEDDHA and rest-FeEDDHA were removed from solution practically entirely, resulting in a drop in Fe concentration from 4.25 mg $l^{-1}$ Fe at t=0 to 0.81 mg $l^{-1}$ Fe after 1 week (Figure 4.1a). From week 1 onward, the Fe concentration was largely determined by the sum of the racemic and the meso o,o-FeEDDHA concentration (> 92%) (Figure 4.1b).
The concentration behaviour of the o,o-FeEDDHA isomers can be subdivided into two stages: a rapid decline within the first week, and a gradual decline from one week onward. Within the first week, the meso o,o-FeEDDHA concentration decreased more strongly (≈ 54%) than the racemic o,o-FeEDDHA concentration (≈ 28%). From one week onward, the decline in meso o,o-FeEDDHA concentration remained faster, resulting in an increasing relative contribution of racemic o,o-FeEDDHA to the total Fe concentration in soil solution.
Adsorption is proposed as the process causing the strong decline in concentration within the first week; FeEDDHA components are known to adsorb to soil reactive surfaces (Hernandez-Apaolaza and Lucena, 2001; Schenkeveld, et al., 2007), and adsorption (pseudo-)equilibrium

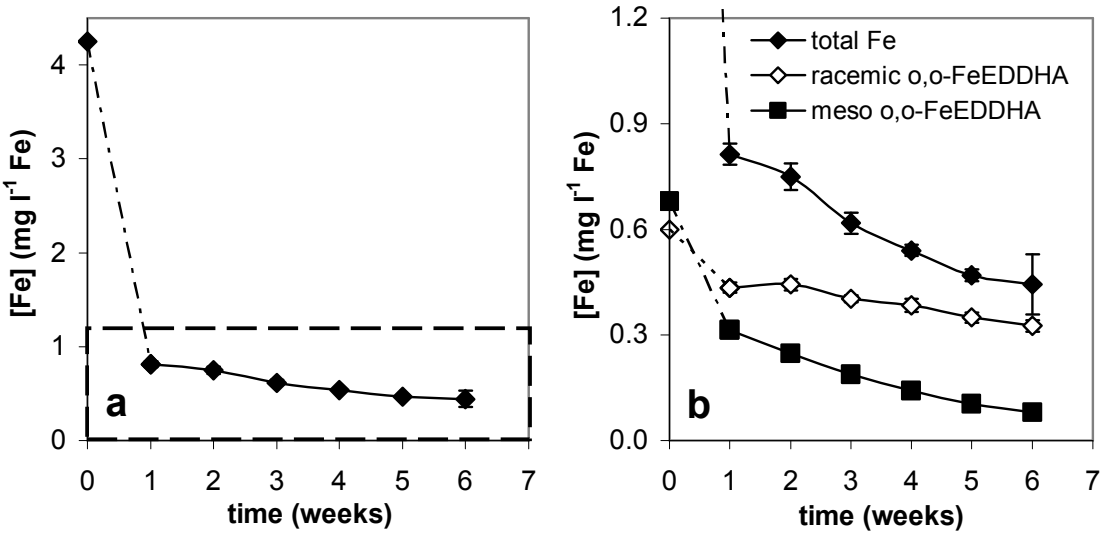



**Figure 4.1: a)** Fe concentration in the pore water of Santomera soil as a function of time for the 30%o,oL treatment; **b)** Enlargement of the indicated area from Figure 1a; Total Fe, racemic o,o-FeEDDHA and meso o,o-FeEDDHA concentrations in the pore water of Santomera soil as a function of time for the 30%o,oL treatment. Error bars indicate standard deviations.

of ionic compounds interacting with soil is generally reached within hours to days rather than weeks. Moreover, the larger decline in concentration of meso o,o-FeEDDHA compared to racemic o,o-FeEDDHA corresponds with the stronger tendency of meso o,o-FeEDDHA to adsorb (Alvarez-Fernandez, et al., 1997; Alvarez-Fernandez, et al., 2002; Hernandez-Apaolaza and Lucena, 2001; Schenkeveld, et al., 2007). Potential causes for the gradual decline in concentration of racemic and meso o,o-FeEDDHA after the first week are discussed further on in relation to Fe uptake by plants.

In both L-treatments, the gradual decline in meso o,o-FeEDDHA concentration was continuous from one week onward, while for racemic o,o-FeEDDHA there was no decline during the second week, and the gradual decline only set in after two weeks. In Figure 4.2a, the meso o,o-FeEDDHA concentration data are presented on a logarithmic scale as a function of time for all FeEDDHA treatments. The gradual decline in meso o,o-FeEDDHA concentration can be accurately described with an exponential decay function[1]:

$$C_t = A_0 \cdot e^{-\lambda t} \qquad (1)$$

in which $C_t$ is the meso o,o-FeEDDHA concentration in the pore water at t=t; $A_0$ is a fitted meso o,o-FeEDDHA concentration at t=0, disregarding the concentration drop caused by

[1] The terms “exponential decay” and “decay constant” are used in a mathematical sense as opposed to “exponential growth” and “growth constant”; as such these terms do not address the cause for the decline and bear no direct reference to “decomposition” or “biodegradation”.

adsorption and the moment the gradual decline set in; λ is the decay constant; and t is the time passed since FeEDDHA application. For all treatments $R^2$ of the exponential fit was 0.99 or higher.

A similar procedure was followed for the racemic o,o-FeEDDHA concentration data from 2 weeks onward (Figure 4.2b). The data from the L-treatments could also be fitted reasonably well with an exponential equation ($R^2$ = 0.99); for the H-treatments, the fits were poor ($R^2$ = 0.83 and 0.23).

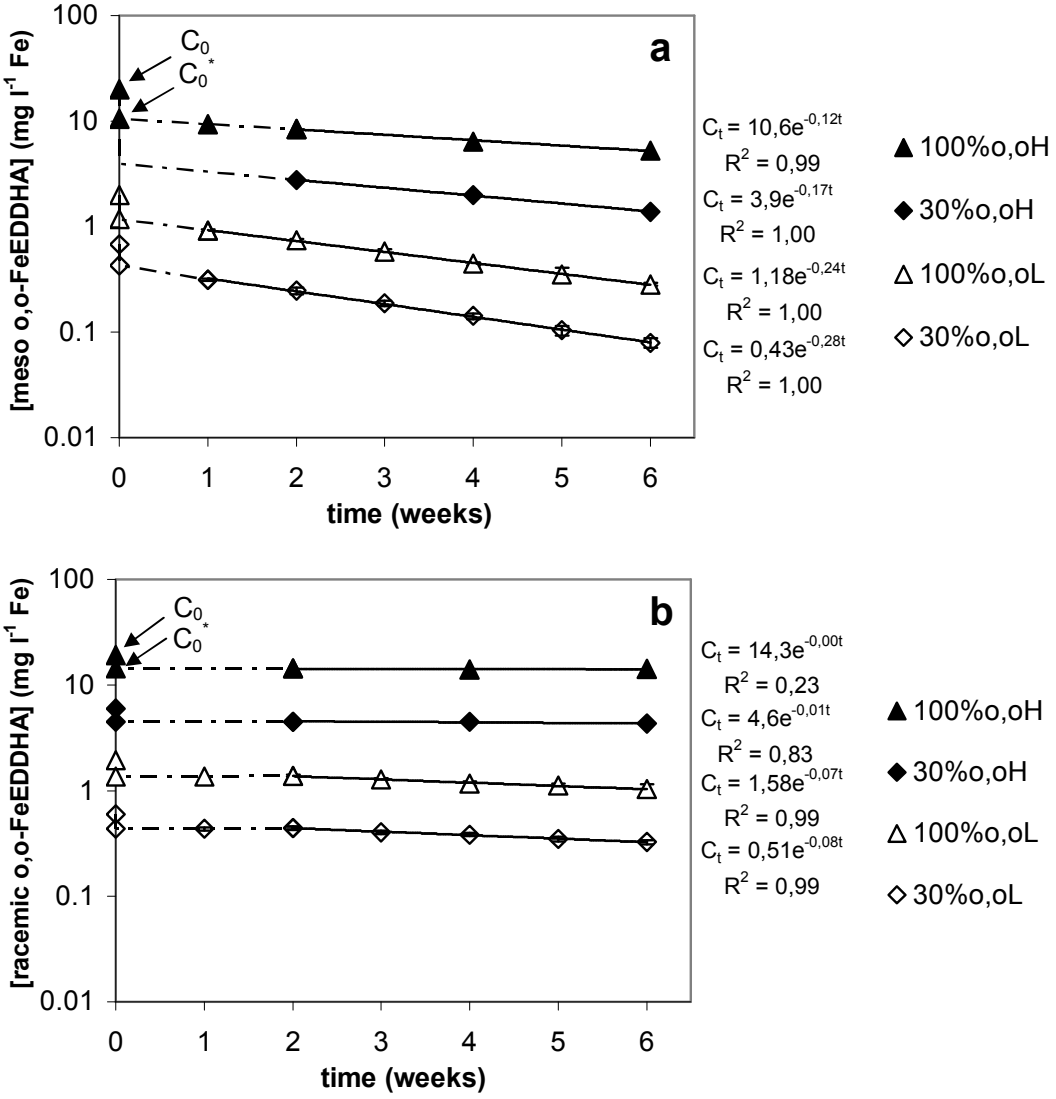



**Figure 4.2:** Concentration of **a)** meso o,o-FeEDDHA and **b)** racemic o,o-FeEDDHA in the pore water of Santomera soil as a function of time for all FeEDDHA treatments. The concentrations are presented on a logarithmic scale. Error bars indicate standard deviations. The interrupted lines represent trend extrapolations in between the start of the experiment and the first moment of harvest. $C_0$ indicates the concentration applied with the treatment, $C_0^*$ indicates the extrapolated concentration corresponding to adsorption equilibrium at $t \approx 0$.

Under the assumption that adsorption equilibrium was reached before the impact of the process causing the gradual decline became substantial, the amounts of racemic and meso o,o-FeEDDHA adsorbed in the first stage ($Q_0$) can be determined through extrapolation of the concentration trends to t=0. $Q_0$ can then be calculated from:

$$Q_0 = \frac{(C_0 - C_0^*)}{SSR} \qquad (2)$$

in which $C_0$ is the initial pore water concentration applied with the treatment; $C_0^*$ is the pore water concentration at adsorption equilibrium, determined through extrapolation to t=0; and SSR is the soil-solution ratio. For meso o,o-FeEDDHA, the intercepts ($C_0^*$) were determined through extrapolation of the exponential fits (as indicated by the dashed lines in Figure 4.2a), and equal $A_0$ (equation (1)). For racemic o,o-FeEDDHA, $C_0^*$ was assumed to equal the concentration after 1 and 2 weeks, since the gradual decline only set in after 2 weeks (indicated in Figure 4.2b). $C_0$ and $C_0^*$ are indicated on the Y-axis of Figure 4.2a and b for all treatments. For meso o,o-FeEDDHA, $C_0$ is consequently a factor 1.6 - 1.9 higher than $C_0^*$; for racemic o,o-FeEDDHA consequently a factor 1.3 - 1.4.

With the extrapolated pore water concentrations at adsorption equilibrium ($C_0^*$) and the corresponding adsorbed amounts ($Q_0$) at t≈0, adsorption isotherms for racemic and meso o,o-FeEDDHA to Santomera soil were derived (Figure 4.3a). The adsorption isotherms are linear in shape and can be described with:

$$Q = K \cdot C \qquad (3)$$

in which Q is the adsorbed amount by the soil, K is an effective affinity parameter of Santomera soil for the o,o-FeEDDHA isomers, and C is the equilibrium concentration in soil solution. The slope of the meso o,o-FeEDDHA isotherm (0.139) is a factor 2.5 steeper than the slope of the racemic o,o-FeEDDHA isotherm (0.055). Because the isotherms only comprise 4 data points, a double logarithmic transformation of the data was carried out to check for over-representation of the data point with the highest concentration (see Figure A4.1 in the Appendix). Also the transformed data could be fitted linearly with $R^2 = 1.00$ and tangents of 1.10 and 0.95 for meso and racemic o,o-FeEDDHA respectively, implying the assumption of linear adsorption isotherms is reasonable.

The decay constant in equation (1) increases with decreasing meso o,o-FeEDDHA concentration applied with the treatment (Figure 4.3b). This dependency is remarkable because it implies that neither the pore water concentration, nor the linearly dependent amount adsorbed determines the rate of decline, like in standard first order reaction kinetics, at which the exponential fits hint (Figure 4.2a). The relation between decay constant and concentration applied can be accurately described ($R^2 = 0.99$) with the following equation:

$$\lambda = b + c \cdot \ln C_0 \qquad (4)$$

in which b and c are fitting constants.

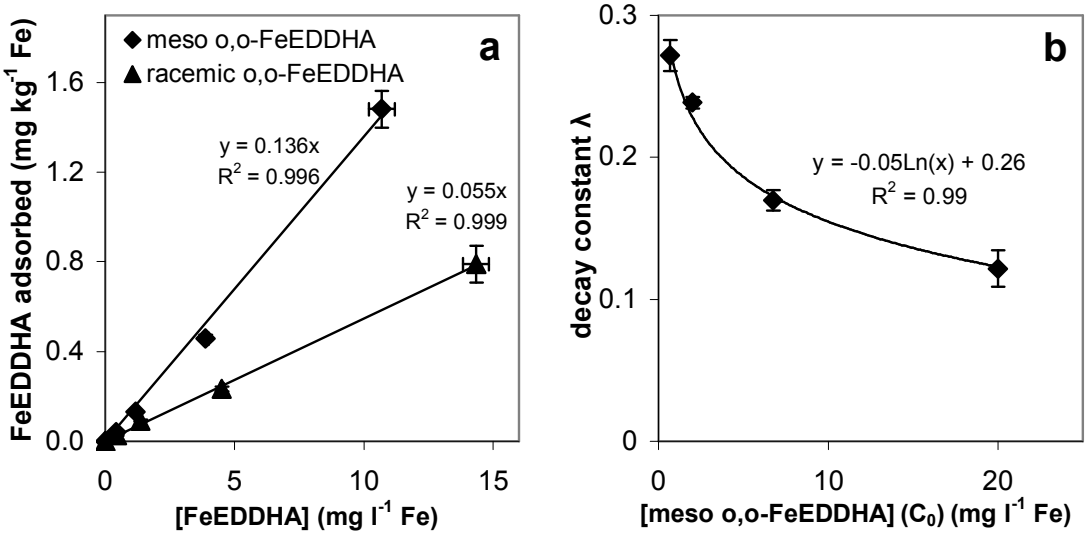



**Figure 4.3: a)** Derived adsorption isotherms for racemic and meso o,o-FeEDDHA to Santomera soil; **b)** The decay constant (from equation (1)), as a function of the meso o,o-FeEDDHA concentration applied with the treatment. Error bars indicate standard deviations.

With the linear adsorption isotherm (equation (3)) and the relation between the decay constant and the applied concentration (equation (4)), the parameters needed to describe the exponential decrease in meso o,o-FeEDDHA concentration in the Santomera soil as a function of time from one week onward (equation (1)), can be calculated for any applied meso o,o-FeEDDHA concentration in between 0.68 and 20 mg $l^{-1}$ Fe.

The results from the second experiment show that, after 6 weeks, the racemic as well as the meso o,o-FeEDDHA concentration were significantly higher in treatments without plants than in corresponding treatments with plants (Table 4.3). However, in agreement with the results from Schenkeveld et al. (2008), the effect of plants on the concentration is relatively small.

**Table 4.3:** Comparison of FeEDDHA component concentrations after 6 weeks for the 30%o,oL and 100%o,oL treatment with and without plants. Standard deviations are indicated between parentheses.

| Treatment | Plants | racemic o,o-FeEDDHA (mg $l^{-1}$ Fe) | meso o,o-FeEDDHA (mg $l^{-1}$ Fe) | o,p-FeEDDHA (mg $l^{-1}$ Fe) | rest-FeEDDHA (mg $l^{-1}$ Fe) |
|---|---|---|---|---|---|
| 30%o,oL | - | 0.35 (0.01) | 0.07 (0.00) | b.d.* | b.d. |
| | + | 0.30 (0.00) | 0.05 (0.00) | b.d. | b.d. |
| 100%o,oL | - | 1.18 (0.00) | 0.25 (0.01) | b.d. | b.d. |
| | + | 0.97 (0.00) | 0.17 (0.02) | b.d. | b.d. |

* b.d. = below determination limit.

Furthermore, the concentration profiles as a function of time were similar in shape in systems with and without plants (see Figure 4.1, and Figure A4.2 in the Appendix), in particular for meso o,o-FeEDDHA. Hence, non-plant related processes, made a large contribution to the gradual decline in concentration of the o,o-FeEDDHA isomers. The amount of racemic and meso o,o-FeEDDHA removed from soil solution as a result of Fe uptake cannot be calculated from the difference in concentration between the treatments with and without plants, because the kinetics of Fe uptake and other processes affecting the o,o-FeEDDHA isomer concentrations are mutually affected; they have at least one reactant (the o,o-FeEDDHA isomer) and possibly also reaction products in common.

**Chlorosis and SPAD-indices**

SPAD-indices of the youngest leaves of plants harvested after 6 weeks are presented as a function of time in Figure 4.4a for the blank, the 30%o,oL and the 30%o,oH treatment. The general trend shows a strong decline in SPAD-index after 8 days and a more or less gradual increase from 2 weeks onward. The plants of the blank treatment became chlorotic after 8 days and remained chlorotic until the end of the experiment. It required 17 days before the SPAD-indices of the youngest leaves of the 30%o,oL treatment differed from those of the blank treatment ($\alpha = 0.05$). In Figure 4.4b the differences in SPAD-indices relative to the blank are presented for all FeEDDHA treatments as a function of time. Because this figure merely serves to illustrate the trends, error bars have been omitted. Chlorosis was most severe after 3 weeks

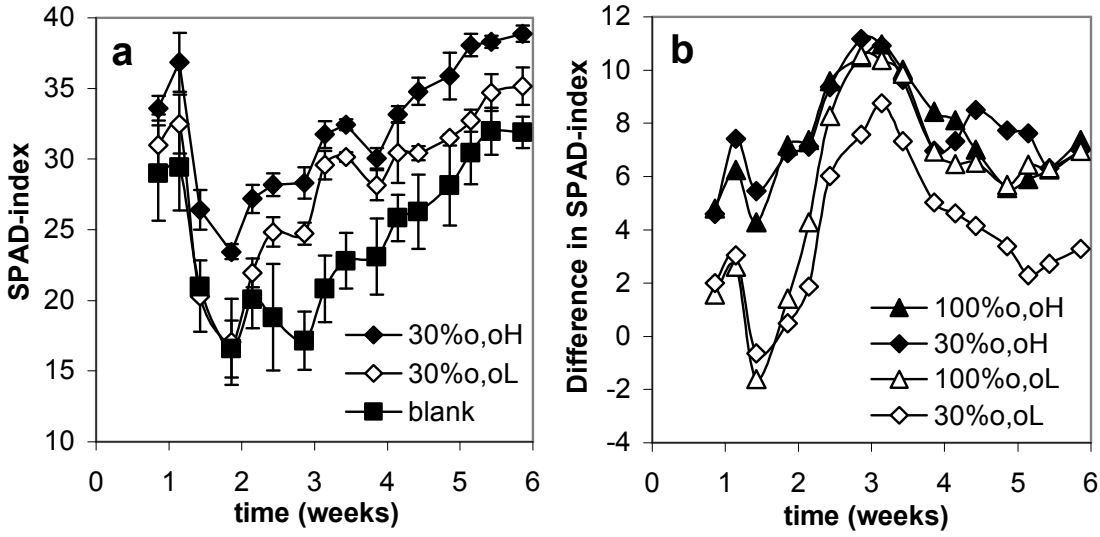



**Figure 4.4: a)** SPAD-indices of the youngest leaves of soybean plants grown on Santomera soil as a function of time for the blank, 30%o,oL and 30%o,oH treatment. Error bars indicate standard deviations; **b)** Difference in SPAD-index of the youngest leaves of soybean plants grown on Santomera soil between the blank treatment and the FeEDDHA treatments as a function of time. Error bars have been omitted.

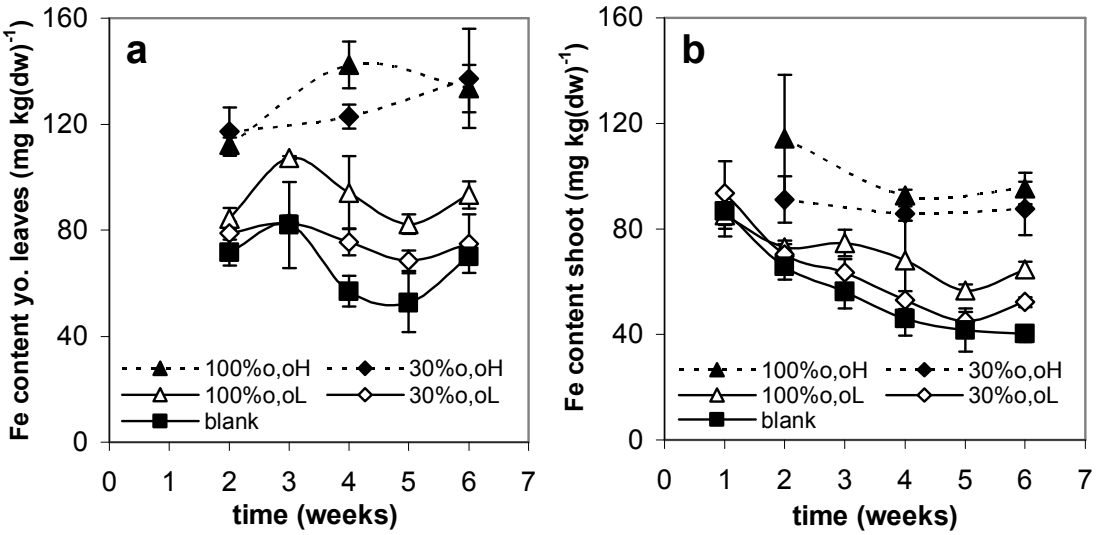



**Figure 4.5:** Fe content of **a)** the youngest leaves, and **b)** the shoot of soybean plants grown on Santomera soil as a function time for all treatments. Error bars indicate standard deviations.

(up to a difference of 11 SPAD-units), corresponding with observations from a previous pot trial (Schenkeveld, et al., 2008). Initially, the youngest leaves of the H-treatments had higher SPAD-indices than those of the L-treatments, while the SPAD-indices of the L-treatments hardly differed from those of the blank. After 2 weeks, the difference in SPAD-indices between the blank and the L-treatments rapidly increased. SPAD-indices of the 100%o,oL treatment reached the same level as those of the H-treatments after about 3 weeks and remained similar until the end of the experiment. So, after 3 weeks no additional cosmetic effects were obtained from increasing the o,o-FeEDDHA dose beyond the level of the 100%o,oL treatment. The SPAD-indices of 30%o,oL treatment remained lower than those of the H-treatments, but higher than those of the blank.

**Fe content**

The influence of FeEDDHA treatments on the Fe content was examined separately for the youngest leaves and the shoot (see Figure 4.5). The Fe content of the roots was not further considered, due to its overestimation resulting from contamination of the roots with soil material - as confirmed by the linear relation between the Fe and Al content of the roots ($R^2 = 0.93$).

The Fe content of the youngest leaves (53 to 143 mg $kg^{-1}$ Fe) was at all times higher than the corresponding Fe content of the shoot (40 to 114 mg $kg^{-1}$ Fe) and in 75% of the cases significantly different ($\alpha = 0.05$). For the blank treatment, differences were only significant for 2 out of 5 sampling moments. No lasting significant increase in Fe content of the youngest leaves was observed, except in the 100%o,oH treatment (from 112 to 133 mg $kg^{-1}$ Fe).

The Fe content of the shoot declined over time in both the blank and the 30%o,oL treatment. This decrease indicates a dilution effect resulting from a higher relative increase in biomass dry weight than in accumulated Fe in the shoot. In the 100%o,oL treatment the Fe content of the shoot was only significantly lower after 5 weeks, while in the H-treatments no differences were observed among sampling moments. So, a minimum amount of o,o-FeEDDHA needed to be applied to maintain the Fe content of the shoot.
Throughout the trial, the Fe content of the youngest leaves and the shoot of the blank were lower than in all other treatments. Compared to the 30%o,oL treatment the differences were at no stage significant, compared to the 100%o,oL treatment the differences were significant from 3 weeks onward with exception of the shoot after 4 weeks. The H-treatments had significantly higher Fe contents than both the blank and the 30%o,oL treatment throughout the trial, with exception of the shoot of the 30%o,oH treatment after 2 weeks. No significant differences in Fe content were found between the H-treatments, despite the substantially higher Fe concentration in the pore water of the 100%o,oH treatment; apparently a plateau had been reached.

**Yield**

The biomass of the shoot (including youngest leaves) increased linearly from 2 weeks onward for all treatments ($R^2$ = 1.00), as illustrated in Figure 4.6a through the blank, the 30%o,oL and the 30%o,oH treatment. The total dry weight yield displayed a similar trend (data not shown). After 6 weeks the difference in shoot biomass between the blank (lowest yield) and the 30%o,oH treatment (highest yield) amounted 3.1 g per pot; an increase of 27% due to FeEDDHA application. The growth rates (corresponding to the slopes of the regression lines

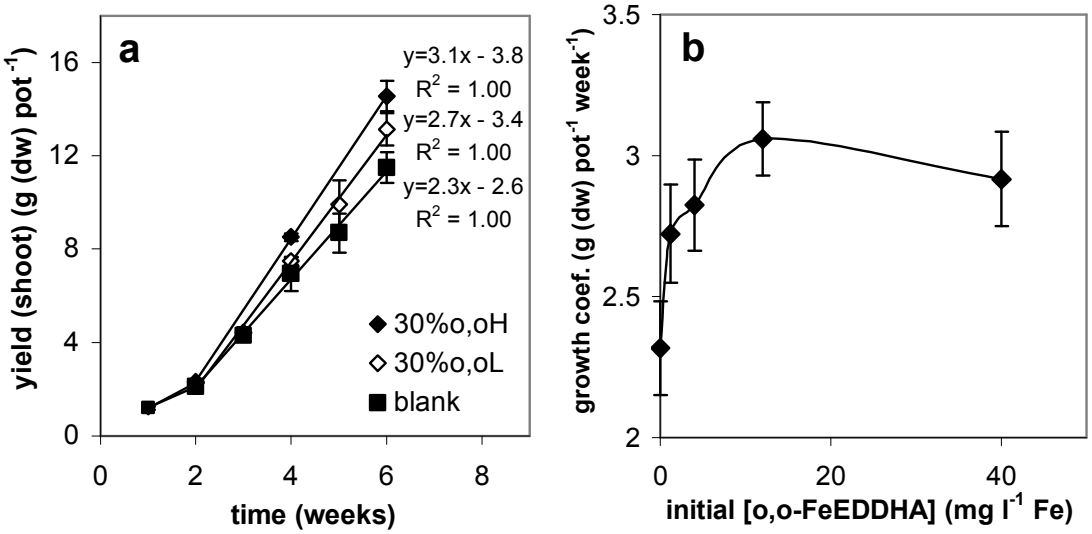



**Figure 4.6: a)** Shoot dry weight yield per pot of soybean plants grown on Santomera soil as a function of time for the blank, the 30%o,oL and the 30%o,oH treatment; **b)** Relation between the growth coefficient of the shoot of soybean plants grown on Santomera soil and the initial o,o-FeEDDHA concentration of the treatment. Error bars indicate standard errors.

in Figure 4.6a) increased from 2.32 to 3.06 g (dw) $pot^{-1}$ $week^{-1}$ (an increase of 32%) and depended on the amount o,o-FeEDDHA applied with the treatment, as shown in Figure 4.6b. With increasing o,o-FeEDDHA dosage, the growth rate initially increased strongly but the slope flattened. A plateau was reached and the growth rate corresponding to the 100%o,oH treatment, although not statistically different from that of that 30%o,oH treatment, may hint at the set-in of a decline in yield as a result of excessive FeEDDHA application. This is supported by the observation that, in particular at harvest after 6 weeks, parts of the roots of the 100%o,oH treatment were coloured black and seemed necrotic.

**Fe uptake**

Cumulative Fe uptake was calculated as the product of shoot dry weight yield and Fe content of the shoot (including youngest leaves). The Fe initially present in the soybean seeds (0.14 ± 0.02 mg Fe per pot) was not corrected for. The combination of a linear time-trend for yield and a non-linear one for Fe content results in a non linear cumulative Fe uptake function, as illustrated by the blank and the L-treatments in Figure 4.7a. All cumulative Fe uptake curves continuously increase throughout the experiment; implying that decreases in Fe content were always overcompensated by increases in yield.

From 2 weeks onward, cumulative Fe uptake increased with increasing o,o-FeEDDHA concentration applied with the treatment, as illustrated in Figure 4.7b for 2, 4 and 6 weeks. After 2 weeks, cumulative Fe uptake ranged from 0.14 to 0.26 mg $pot^{-1}$ Fe, after 4 weeks from 0.32 to 0.78 mg $pot^{-1}$ Fe and after 6 weeks from 0.48 to 1.33 mg $pot^{-1}$ Fe. The slope at the lower-end of the curve grows steeper with time, indicating FeEDDHA continues to enhance Fe

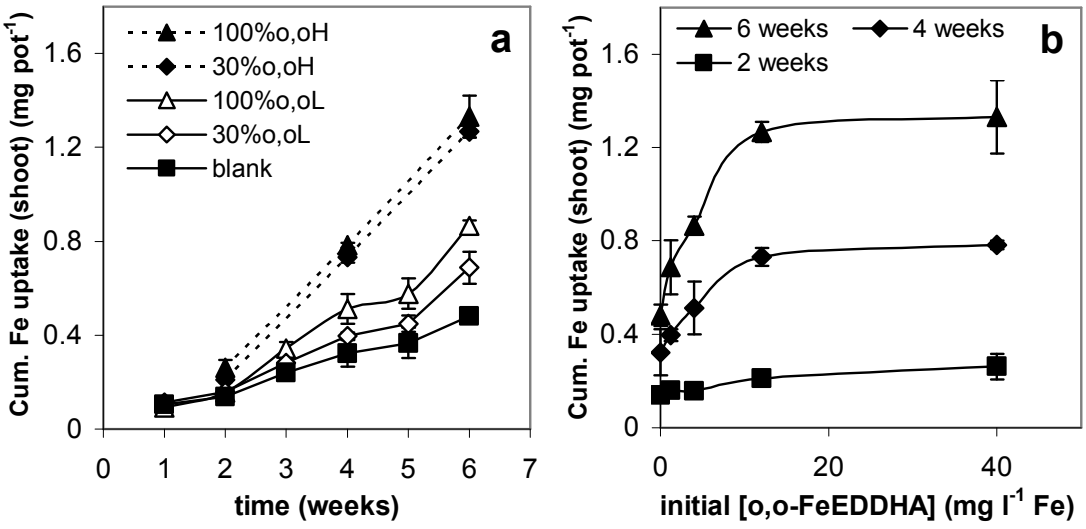
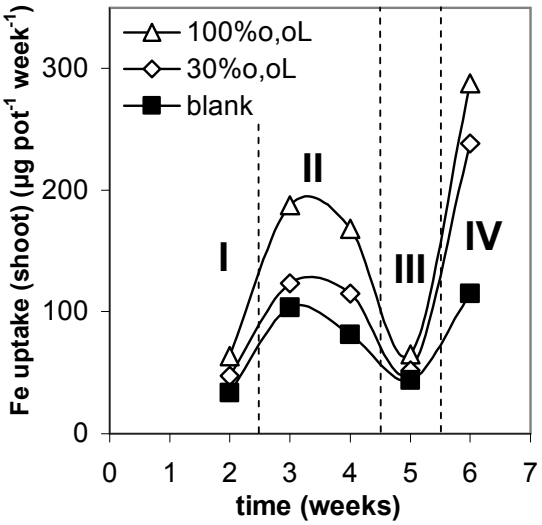



**Figure 4.7: a)** Cumulative Fe uptake (shoot) per pot as a function of time for all treatments. Error bars indicate standard errors; **b)** Cumulative Fe uptake (shoot) per pot after 2, 4 and 6 weeks as a function of the initial o,o-FeEDDHA concentration. Error bars indicate standard deviations.




I = Early vegetative stage (initial chlorosis);
II = Progressed vegetative stage (maximum chlorosis);
III = Transfer from vegetative to reproductive stage (flowering and pod formation);
IV = Progressed reproductive stage (pod filling)

**Figure 4.8:** Fe uptake (shoot) per pot, per week by soybean plants grown on Santomera soil as a function of time, for the blank, the 30%o,oL and the 100%o,oL treatment. Error bars have been omitted.

uptake throughout the experiment. Cumulative Fe uptake does not differ significantly between the 30%o,oH and the 100%o,oH treatment at any sampling moment. This implies Fe uptake is maximized in the 30%o,oH treatment and application of additional o,o-FeEDDHA is superfluous.

The (additional) Fe uptake per week has been calculated as the difference in cumulative Fe uptake between two consecutive data points of the same treatment and is presented as a function of time in Figure 4.8 for the blank and the L-treatments. The Fe uptake indicated at 2 weeks is in fact the Fe uptake during the 2$^{nd}$ week, and so on. Because the figure only serves to illustrate the trends, error bars have been omitted.

For all sampling moments the sequence in Fe uptake per week was identical: blank < 30%o,oL < 100%o,oL. Fe uptake per week displayed the same time trend in all three treatments. During the 2$^{nd}$ week Fe uptake was relatively low. The small Fe requirements at this stage are probably related to the small size of the plants during the early vegetative stage and the utilization of Fe present in the seeds. Fe uptake increased during the 3$^{rd}$ and the 4$^{th}$ week. As the vegetative stage progressed, the plants grew bigger and Fe from the seeds became increasingly insufficient, resulting in an increased Fe demand. In the blank treatment chlorosis was most severe at this stage (Figure 4.4b). In the course of the 4$^{th}$ and during the 5$^{th}$ week, the plants flowered and pods were formed, indicating the shift from the vegetative to the reproductive stage. The plants hardly grew in size anymore, leading to small Fe requirements during the 5$^{th}$ week. During the 6$^{th}$ week the seed formation inside the pods progressed and Fe uptake increased strongly again, in order to provide the seeds with sufficient Fe (Grusak, 1995). The Fe requirements in the reproductive stage were larger than in any preceding week.

**Fe uptake in relation to removal of o,o-FeEDDHA isomers from the soil system**

Finally, the relation between FeEDDHA-facilitated Fe uptake to the shoot, and the amount of racemic and meso o,o-FeEDDHA removed from the soil system (solid and solution phase combined) was examined as a function of time. The removed amounts of racemic and meso o,o-FeEDDHA were calculated from the decrease in soil solution concentration under the assumption adsorption equilibrium was preserved and could be described by the derived adsorption isotherms (Figure 4.3a). In Figure 4.9a the amounts of racemic, meso and total o,o-FeEDDHA removed from the soil system per week, are presented for the 100%o,oL treatment. The amount of meso o,o-FeEDDHA removed per week was larger than the amount of racemic o,o-FeEDDHA, throughout the experiment. Racemic o,o-FeEDDHA however, appears to have a more pronounced influence on the shape of the total o,o-FeEDDHA removal-curve.

In Figure 4.9b FeEDDHA-facilitated Fe uptake is presented as a function of time for the 100%o,oL treatment. Two uptake scenarios have been included: 1) a maximum scenario, in which all Fe uptake was assumed FeEDDHA-facilitated, and 2) a minimum scenario, in which only the difference in Fe uptake between the 100%o,oL and the blank treatment was assumed FeEDDHA-facilitated. In both scenarios, FeEDDHA-facilitated Fe uptake was highest in the growth stages that Fe requirements were largest (3$^{rd}$, 4$^{th}$ and 6$^{th}$ week). The curve representing the amount of racemic o,o-FeEDDHA removed from the soil system per week shows a strong similarity in shape to the Fe uptake curves, and up until the 6$^{th}$ week, the amount of racemic o,o-FeEDDHA removed was in range with Fe uptake. This indicates that the removal of

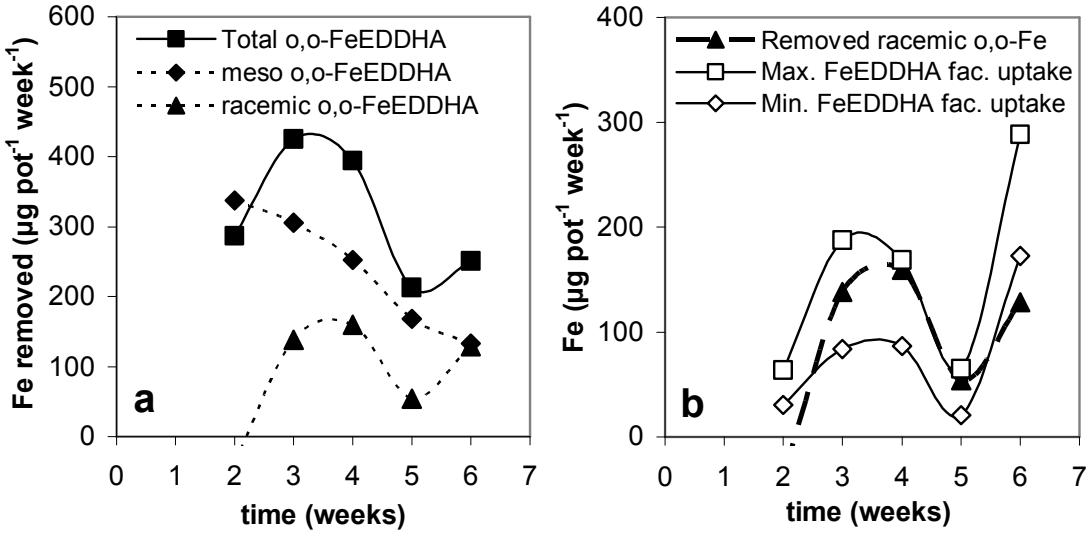



**Figure 4.9: a)** Amounts of total, racemic and meso o,o-FeEDDHA removed from the soil system per pot, per week for the 100%o,oL treatment; **b)** Minimum and maximum FeEDDHA-facilitated Fe uptake (shoot) per pot, per week by soybean plants grown on Santomera soil as a function of time, and the amount of racemic o,o-FeEDDHA removed from the soil system per pot per week, both for the 100%o,oL treatment. Error bars have been omitted.

racemic o,o-FeEDDHA from the soil-system was to a larger extent plant-determined than the removal of meso o,o-FeEDDHA. The fact that the gradual decline in racemic o,o-FeEDDHA concentration, only started after 2 weeks, when the plants developed a strong need for Fe, further supports this reasoning.

## Discussion

For an efficient use of FeEDDHA fertilizer in soil application, dosage and moment of FeEDDHA application should be matched with the Fe requirements of the plant. In order to do so, the fate of FeEDDHA components in the soil-plant system and the plant's demand for (FeEDDHA-facilitated) Fe need to be understood as a function of time. This study presents important first insights in both these issues.

The concentration behaviour of the o,o-FeEDDHA isomers, which almost completely governed the Fe concentration in the pore water from one week onward, could be subdivided into two stages: a rapid decline in concentration within the first week, and a gradual decline from one week onward. The rapid decline has been attributed to adsorption, the extent of which could be described with linear adsorption isotherms, for both o,o-FeEDDHA isomers. For meso o,o-FeEDDHA, the gradual decline was accurately described with an exponential decay function, in which the decay constant is a logarithmic function of the concentration applied; for racemic o,o-FeEDDHA a similar description was inadequate.
The linear shape of the adsorption isotherms does not correspond with the shapes of adsorption isotherms determined by Hernandez-Apaolaza and Lucena (2001) for racemic and meso o,o-FeEDDHA to several soil constituents. For this reason, the adsorption behaviour of racemic and meso o,o-FeEDDHA to Santomera soil was further examined in a separate batch experiment, presented in the Appendix. This experiment also resulted in linear adsorption isotherms (see Figure A3 in the Appendix). However, the slopes of corresponding isotherms from batch and pot experiment differ considerably; in the pot experiment the slopes are approximately a factor 4 and 6 higher for racemic and meso o,o-FeEDDHA respectively. This difference in adsorption behaviour may result from the difference in soil-solution ratio or the difference in ionic strength of the solution phase. Results from an incubation experiment at field capacity by Cantera et al. (2002) support this. FeEDDHA was recovered from a calcareous soil comparable to Santomera soil through extraction with distilled water instead of through direct centrifugation. This lowered the soil-solution ratio (SSR = 0.83) and ionic strength. The FeEDDHA recovery and corresponding adsorbed fraction strongly differed from those in the pot experiment (SSR = 6), but were approximately equally large as in the batch experiment with Santomera soil (SSR = 1). The effect of ionic strength and soil-solution ratio on FeEDDHA adsorption need to be further examined.
Evapo-transpiration caused daily fluctuations in the pore water concentration of the FeEDDHA components, on top of the trends described. Near the end of the trial, the relative increase may have amounted 35% at maximum, before replenishment with demineralized water. The actual fluctuations were probably smaller due to the mitigating effect of adsorption.

Preventing leaching may have caused the FeEDDHA component concentrations to decline slower than under field conditions. Studies with soil columns have demonstrated that FeEDDHA components are susceptible to leaching (Cesco, et al., 2000; Lucena, et al., 2005). The actual impact of leaching will however depend on the water balance in the field. The effects of leaching on FeEDDHA component concentrations and Fe uptake in the soil-plant system need to be further examined.
The impact of plants on the racemic and meso o,o-FeEDDHA concentration after 6 weeks was relatively small, but significant. Plants may enhance the gradual decline through several processes. First, uptake of the FeEDDHA complex as a whole (Bienfait, et al., 2004) may play a role. Secondly, after Fe reduction and chelate splitting at the root surface, and subsequent Fe uptake (Chaney, et al., 1972), the chelating agent o,o-EDDHA may move back into soil solution and be degraded or form a complex with a strongly binding competing cation like Cu or Al instead of with Fe (Schenkeveld, et al., 2007). Thirdly, plants enhance evapo-transpiration which may increase FeEDDHA surface precipitation resulting from water transport from lower parts of the pot to the soil surface.
The gradual decline of in particular meso o,o-FeEDDHA, is however mostly caused by non-plant related processes. Leaching is excluded as a sink, because the pots were closed at the bottom end. Precipitation at the soil surface may decrease the o,o-FeEDDHA isomer concentrations, but if this process were dominant, the most mobile isomer i.e. racemic o,o-FeEDDHA would be most affected, which was not the case. FeEDDHA consumption by microorganisms (e.g. through biodegradation of the chelating agent), chemical degradation (e.g. related to local anaerobic conditions in the soil), or slow adsorption onto, or absorption into soil particles may (partly) account for the loss of o,o-FeEDDHA. Furthermore, a slow displacement of Fe from FeEDDHA complexes by a competing cation could decrease the o,o-FeEDDHA concentrations. However, no corresponding increases in concentration of competing cations like Cu, Al or Co were observed (data not shown). Further research is needed to clear up the processes underlying the plant-independent decrease in concentration of the o,o-FeEDDHA isomers.

Time trends in SPAD-index and Fe content of the plants did not correspond: SPAD-indices more or less continuously increased from two weeks onward in all treatments (Figure 4.4a), while the Fe content of the leaves only significantly increased in the 100%o,oH treatment (Figure 4.5a); the Fe content of the shoot even decreased unless a certain dose of o,o-FeEDDHA was applied (Figure 4.5b). This confirms the notion that SPAD-indices are useful for comparing the Fe status of plants among treatments, but give no absolute indication of the Fe content of a leaf. The plant's stage of development and growth conditions also affect SPAD-indices through parameters like e.g. leaf-thickness.
The soybean plants mainly took up Fe from FeEDDHA in the progressed vegetative stage ($3^{rd}$ and $4^{th}$ week) and in the reproductive stage, when the pods were being filled with seeds ($6^{th}$ week). Fe deficiency in the reproductive stage may be overlooked, because Fe storage in seeds does not reflect in a visible parameter like leaf colour; SPAD-indices did not change much during the $6^{th}$ week (Figure 4.4a). A good allocation of Fe to the seeds is however vital

for a high Fe nutritional value in the plant parts suitable for consumption, and for the viability of the next generation of plants; Fe deficient plants are likely to generate offspring that will be more susceptible to Fe deficiency than non Fe-deficient plants (Grusak, 1994).

Fe uptake and removal of racemic o,o-FeEDDHA from the soil system display a similar time-trend, whereas the removal of meso o,o-FeEDDHA had a plant-independent character. This indicates the removal of racemic o,o-FeEDDHA is to a larger extent plant-related and suggests that racemic o,o-FeEDDHA might be more effective in supplying soil-grown plants with Fe than meso o,o-FeEDDHA. The effectiveness of the individual isomers in soil application needs to be further examined.

# Appendix

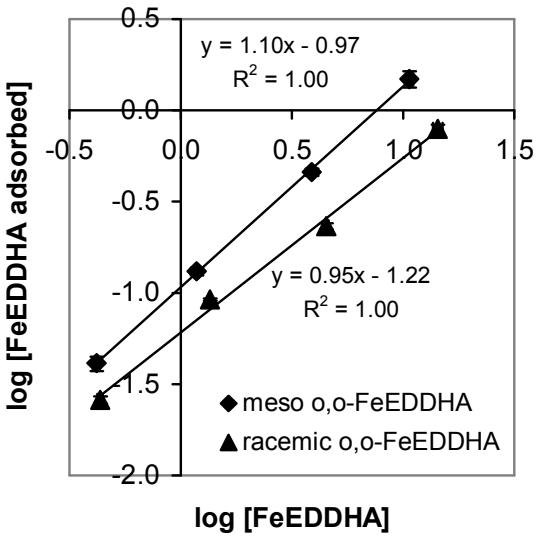



**Figure A4.1:** Log-log transformed derived adsorption isotherms of racemic and meso o,o-FeEDDHA to Santomera soil.

**Pore water concentrations of FeEDDHA components as a function of time**

Pots filled with Santomera soil without plants, prepared as described under materials and methods, were given the blank and L-treatments and were placed on a table in the greenhouse. The trial was conducted in duplicates. The pots were rotated and were supplied with demineralized water to compensate for evaporation, both on a daily basis. FeEDDHA component concentrations were monitored for 6 weeks by in situ sampling with rhizon pore water samplers (rhizons) (SMS MOM, Rhizosphere Research Products, Wageningen, The Netherlands). The rhizons consist of a cylindrical polyethersulfone (PES) membrane (diameter: 2.5 mm; length: 10 cm; pore size: < 0.2µm), which is connected to a PVC/PE tube. Before use, the rhizons were cleaned with 0.14 M $HNO_3$ and ultra pure water, and rinsed with 1 mM $NaNO_3$. Afterwards they were stored in a 1 mM $NaNO_3$ solution until use. The rhizons were incorporated in the soil when the pots were filled, with the membrane placed horizontally at a height of approximately 10 cm from the bottom plate. The PVC/PE tube was led upwards and the ending was connected to the rim of the pot. Sampling was done twice per week by imposing a vacuum on the inside of the rhizon with a 10 ml syringe with luer lock (SS*10LZ1, Terumo) for maximally 16 hours. To rinse the rhizons, the first ml sampled was put back into the sand column. At most 6 ml of pore water were sampled per rhizon per sampling session. During sampling the PVC/PE tube and the syringe were covered to avoid exposure of the sample to light. FeEDDHA component concentrations were determined as described in Schenkeveld et al. (2007). The racemic and meso o,o-FeEDDHA concentrations measured in the pore water of the 100%o,oL treatment are presented in Figure A4.2.

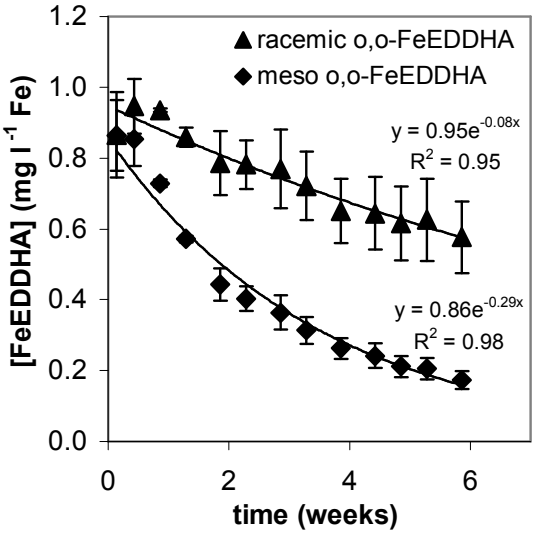



**Figure A4.2:** Racemic and meso o,o-FeEDDHA concentrations measured in the pore water of Santomera soil as a function of time for the 100%o,oL treatment without plants. Sampling was executed in situ. Error bars indicate standard deviations.

**Batch experiment for determining adsorption isotherms to Santomera soil**

Racemic o,o-$H_4$EDDHA (purity: 100%) and meso o,o-$H_4$EDDHA (purity: 99.5%) were obtained by separation as described in Bannochie and Martell (1989) and Bailey et al. (1981), starting from solid o,o-$H_4$EDDHA, also used for the preparation of the 100%o,o treatments. The racemic and meso o,o-FeEDDHA solutions were prepared in the same way as the 100%o,o FeEDDHA solutions as described under materials and methods. Santomera soil was allowed to interact with racemic and meso o,o-FeEDDHA solutions with concentrations of 0.2; 0.5; 2; 5; 10; 20; 50 and 100 mg $l^{-1}$ Fe in a soil-solution ratio of 1:1 (w/v) for 3 days in 50 ml polypropylene test tubes (Greiner bio-one, Cat No 210296). The tubes were placed in an end-over-end shaker, rotating at 18 rpm in absence of light. Room temperature was kept at 20 (± 1) °C. The experiment was carried out in duplicates. Control treatments with the different experimental solutions without soil were included. After interaction, the samples were centrifuged for 15 minutes at 3000 rpm. The pH and EC of the supernatant were measured. Subsequently the supernatant was filtered through a 0.45 µm cellulose acetate micro pore filter (Schleicher & Schuell, ref no: 10462650). The filtrate was further analyzed. o,o-FeEDDHA isomer concentrations were determined as described in Schenkeveld et al. (2007). The adsorption isotherms are presented in Figure A4.2.

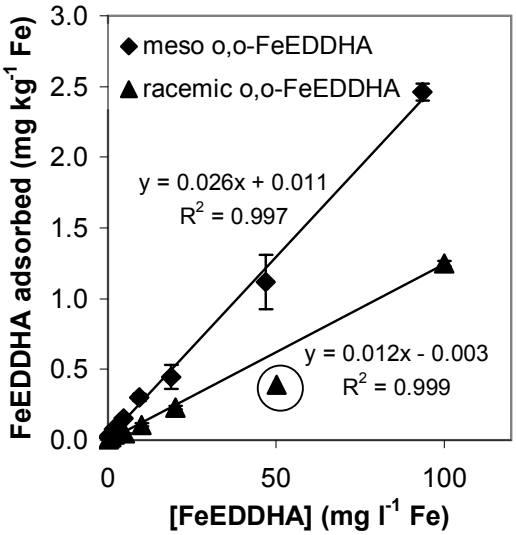



**Figure A4.3:** Adsorption isotherms of racemic and meso o,o-FeEDDHA to Santomera soil as determined in a batch shaking experiment. Encircled data point was left out of the regression.

“Parsin, mene, tekel, upharsin.”
*trans.* “Divisions, counted, weighed and divisions.”

Adapted from:
*Daniel 5:1–31;*
“Mene, mene, tekel, upharsin.”
*interpr.* “Weighed and found wanting.”

# Chapter 5

## The performance of soil-applied FeEDDHA isomers in delivering Fe to soybean plants in relation to the moment of application

W.D.C. Schenkeveld, A.M. Reichwein, M. Bugter, E.J.M. Temminghoff, and W.H. van Riemsdijk

Journal of Agricultural and Food Chemistry *(accepted)*

## Abstract

FeEDDHA (iron (3+) ethylenediamine-N,N'-bis(hydroxy phenyl acetic acid) products are commonly applied to mend and prevent Fe deficiency chlorosis in soil-grown crops. Plants mainly take up Fe in the progressed vegetative and in the reproductive stage. In this study it was examined which of the principal constituents of FeEDDHA products (the isomers racemic o,o-FeEDDHA, meso o,o-FeEDDHA and o,p-FeEDDHA), most effectively meets the Fe requirements of soybean plants (*Glycine max* (L.) Merr.), grown on calcareous soil, in the aforementioned growth stages. FeEDDHA isomers were applied once, separately or in mixtures, either at t=0, in the progressed vegetative stage or in the reproductive stage. O,p-FeEDDHA did not significantly contribute to Fe uptake in either growth stage. Both racemic and meso o,o-FeEDDHA were effective in supplying plants with Fe, approximately to the same extent. Moment of application had a significant effect on yield and FeEDDHA pore water concentrations at harvest, but not on Fe uptake. For optimizing yield while minimizing FeEDDHA dosage, FeEDDHA is best applied to soybean plants prior to the set in of chorosis.

# Introduction

Fe deficiency chlorosis is a common nutrient deficiency, occurring worldwide. It is characterized by a significant decrease in the chlorophyll content of the leaves, and results in diminished yield and crop quality (Chaney, 1984; Mortvedt, 1991). Chlorosis is found mainly on alkaline and calcareous soils. The high pH and elevated bicarbonate concentrations in these soils (Boxma, 1972) result in a limited bioavailability of Fe, due to the low solubility of Fe(hydr)oxides (Lindsay, 1979), the impairment of the Fe uptake mechanism (Venkatraju and Marschner, 1981), and the inactivation of Fe in the leaf apoplast (Mengel, 1994) under such conditions.

The application of synthetic Fe chelates is the most common practice for mending and preventing iron chlorosis. FeEDDHA (iron (3+) ethylene diamine-N,N'-bis(hydroxy phenyl acetic acid)) is among the most effective synthetic Fe chelates under neutral and alkaline soil conditions (Lucena, et al., 1992; Reed, et al., 1988). Commercial FeEDDHA formulations consist of a mixture of positional isomers, diastereomers and polycondensates. The three quantitatively most important compounds are: (1) racemic o,o-FeEDDHA (iron (3+) (R,R) and (S,S) ethylene diamine-N,N'-bis(2-hydroxy phenyl acetic acid) complexes), (2) meso o,o-FeEDDHA (iron (3+) (R,S) = (S,R) ethylene diamine-N,N'-bis(2-hydroxy phenyl acetic acid) complex ), and (3) o,p-EDDHA (iron (3+) ethylene diamine-N-(2-hydroxy phenyl acetic acid)-N'-(4-hydroxy phenyl acetic acid) complexes). These compounds will be addressed as FeEDDHA isomers in this study. The physical and chemical properties of the FeEDDHA isomers differ (Bannochie and Martell, 1989; Gomez Gallego, et al., 2005; Yunta, et al., 2003a; Yunta, et al., 2003b), and as a consequence, so does their ability to preserve Fe in solution and deliver it to the plant. Because the isomeric composition varies strongly among commercial FeEDDHA formulations on the market (Garcia-Marco, et al., 2003), the need for quality assurance arose. This issue has been addressed in the European Fertilizer Law (Regulation (EC) No. 2003/2003; amendment (EC) No. 162/2007), through the following parameters: (1) soluble Fe content of the product, (2) percentage of Fe chelated, and (3) percentages of Fe chelated by the o,o-EDDHA and o,p-EDDHA isomers.

In recent years, considerable effort has been made to assess the effectiveness of the individual Fe chelate compounds present in FeEDDHA products. The ability of FeEDDHA isomers to deliver Fe to plants has been examined in several studies with hydroponic systems. Garcia-Marco et al. (2006) concluded that o,p-FeEDDHA offers a more effective remedy to Fe chlorosis in soybean than o,o-FeEDDHA. This conclusion is in line with the findings of Lucena and Chaney (2006; 2007) that less stable Fe chelates are more effective in supplying cucumber plants with Fe, as long as they manage to maintain Fe chelated. Contradicting findings by Rojas et al. (2008) are probably related to the absence of EDTA in the nutrient solution, resulting in an incremental displacement of Fe from o,p-FeEDDHA by Cu upon replenishment with nutrient solution. Moreover, both o,o-FeEDDHA and o,p-FeEDDHA were found more effective than synthesis byproducts (polycondensates) (Hernandez-Apaolaza, et

al., 2006), and meso o,o-FeEDDHA has been claimed to be more effective in mending chlorosis in strategy 1 plants than racemic o,o-FeEDDHA (Cerdan, et al., 2006).
The effectiveness of FeEDDHA isomers in soil application has received little attention so far. Schenkeveld et al (2008) concluded from a pot trial study with soybean that the amount of o,o-FeEDDHA in FeEDDHA treatments, administered prior to the set-in of chlorosis, determines the Fe uptake of plants that would otherwise become chlorotic. Rojas et al (2008) confirmed the superiority of o,o-FeEDDHA over o,p-FeEDDHA in delivering Fe, in repeated soil application to chlorotic plants. Ryskievich and Boka (1962) assessed the effectiveness of separated racemic and meso o,o-FeEDDHA in soil application and concluded both were effective in providing bean plants with Fe. Presumably, the applied FeEDDHA dosage was however so high that Fe uptake reached an optimum for both racemic and meso o,o-FeEDDHA (Schenkeveld, et al., 2008), preventing a potential difference in effectiveness from being uncovered.

For an efficient use of FeEDDHA fertilizer in soil application, knowledge is required on when plants have need for FeEDDHA, and on what FeEDDHA isomers most adequately serve the plant's Fe demand at that particular growth stage. It was recently found that soil grown soybean mainly takes up Fe from FeEDDHA when the Fe demand is highest: in the progressed vegetative stage and in the reproductive stage (Schenkeveld, et al., 2010a). The efficiency of individual FeEDDHA isomers in supplying soil-grown plants with Fe at these particular growth stages had not previously been addressed, and has been examined in this study. For the sake of comparison, FeEDDHA application prior to the set-in of chlorosis has also been included. As part of this study, racemic and meso o,o-FeEDDHA have been separately re-assessed with regard to their effectiveness in soil application, at considerably lower dosages in comparison to the study by Ryskievich and Boka (1962).
A pot trial study was conducted with soybean plants grown on a calcareous soil from Spain, involving 6 FeEDDHA treatments (separated FeEDDHA isomers, as well as characterized FeEDDHA isomer mixtures) applied, at the 3 aforementioned growth stages.

# Material and Methods

**Soil**

Calcareous clay soil was collected from the top soil layer (0 – 20 cm) at a site located in Santomera (Murcia, Spain). Lutum fraction (260 g $kg^{-1}$) and $CaCO_3$ content (520 g $kg^{-1}$) are common for calcareous soils from that area. The pH of the soil is 8.0 (pH-$CaCl_2$). The soil organic carbon (SOC) content is low (5 g $kg^{-1}$), and the dissolved organic carbon (DOC) concentration equals 30 mg $l^{-1}$ (0.01 M $CaCl_2$). Fe availability parameters are low: the oxalate extractable ('reactive') Fe content amounts 0.30 g $kg^{-1}$ Fe, and the diethylene triamine penta acetic acid (DTPA) extractable content amounts 3.5 mg $kg^{-1}$ Fe. Plants grown on this soil became chlorotic, both under field conditions and in previous pot trials (Schenkeveld, et al.,

2008; Schenkeveld, et al., 2010a). Pre-treatment consisted of air drying and sieving (1 cm). Additional relevant soil characteristics are presented in Table 5.1.

**Table 5.1:** Soil characteristics.

| | | Extraction | | |
|---|---|---|---|---|
| **Origin/Name** | Santomera | **$CaCl_2$ (0.01 M)[g]** | **DOC (mg $l^{-1}$)** | 30 |
| **Region** | Murcia | **Oxalate[h]** | **Fe (g $kg^{-1}$)** | 0.30 |
| **Country** | Spain | | **Al (g $kg^{-1}$)** | 0.44 |
| **Soil classification** | entisol | **DTPA[i]** | **Fe (mg $kg^{-1}$)** | 3.5 |
| **Water holding capacity (g $kg^{-1}$)** | 320 | | **Mn (mg $kg^{-1}$)** | 4.6 |
| **pH-$CaCl_2$[a]** | 8.0 | | **Cu (mg $kg^{-1}$)** | 4.1 |
| **Electro conductivity (mS $m^{-1}$)[b]** | 23 | | **Zn (mg $kg^{-1}$)** | 0.9 |
| **SOC (g $kg^{-1}$)[c]** | 5.4 | **$HNO_3$ (0.43 M)[j]** | **Fe (mg $kg^{-1}$)** | 494 |
| **Clay (g $kg^{-1}$)[d]** | 260 | | **Mn (mg $kg^{-1}$)** | 179 |
| **$CaCO_3$ (g $kg^{-1}$)[e]** | 520 | | **Cu (mg $kg^{-1}$)** | 10 |
| **CEC (cmol $kg^{-1}$)[f]** | 10.3 | | **Zn (mg $kg^{-1}$)** | 5 |

[a] ISO/DIS 10390 Soil Quality – Determination of pH
[b] ISO/DIS 11265 Soil Quality – Determination of the specific electric conductivity
[c] Walinga et al. (1992)
[d] Houba et al. (1997)
[e] ISO 10693, Soil Quality – Determination of carbonate content, volumetric method
[f] ISO/DIS 11260 Soil Quality – Determination of cation exchange capacity and base saturation – method using barium chloride solution
[g] Houba et al. (2000)
[h] Schwertmann (1964)
[i] Lindsay and Norvell (1978) and Quevauvillier et al. (1996)
[j] Tipping et al. (2003) and Fest et al. (2005)

**FeEDDHA solutions**

FeEDDHA solutions were prepared from racemic o,o-$H_4$EDDHA (purity: 100%), meso o,o-$H_4$EDDHA (purity: 99.5%), o,p-$H_4$EDDHA (purity: 90%) and a mixture of racemic and meso o,o-$H_4$EDDHA (in a ratio close to 1; purity: 99%). Racemic and meso o,o-$H_4$EDDHA were obtained by separation of the o,o-$H_4$EDDHA mixture, as described in Bannochie and Martell (1989) and Bailey et al. (1981).

Solid $H_4$EDDHA was dissolved by adding sufficient 1 M NaOH. Fe was added as $FeCl_3$*$6H_2O$ in a 2% excess based on a 1:1 stoichiometry between metal and EDDHA ligand (chelating capacity of impurities was corrected for). The pH was raised to 7 (± 0.5) and the solutions were left over-night in the dark in order for excess Fe to precipitate as hydroxides. The following day, the solutions were filtered over a 0.45 μm nitro cellulose micro pore filter (Schleicher & Schuell, ref-no: 10401114) and further diluted for application in the pot trial. The composition of the experimental solutions was analysed through combined ICP and HPLC analysis at time t=0.

**Pot trial**

A pot trial with a runtime of 8 weeks was done from mid August until mid October 2006. The experiment involved a blank treatment and 6 FeEDDHA treatments: *o,p*; *meso o,o*; *racemic o,o*; *o,o-mix low*; *o,o mix-low + o,p*; and *o,o-mix high*. In the first 4 FeEDDHA treatments, an Fe dose corresponding to a pore water concentration of around 0.6 mg $l^{-1}$ Fe (i.e. 11 μM) was applied, in the latter 2, an Fe dose corresponding to around 1.8 mg $l^{-1}$ Fe (i.e. 32 μM). The treatments with 1.8 mg $l^{-1}$ Fe were included to ascertain that Fe uptake had not yet reached its maximum in the treatments with 0.6 mg $l^{-1}$ Fe. This is a precondition for a sound comparison of the effectiveness of the FeEDDHA isomers. The composition of the treatments is presented in Table 5.2. The mixed treatments have been included to examine potential synergetic effects. Except for the blank treatments, all pots received an FeEDDHA treatment once; either at t=0, at the start of the experiment, a day after the transfer of the seedlings to the pots; at t=3 weeks, in the middle of the vegetative stage; or at t=6 weeks, at the beginning of the reproductive stage when the pods started to fill. Not all FeEDDHA treatments were administered at all three moments; moments of application are also indicated per treatment in Table 5.2. The o,p-FeEDDHA treatment has been omitted at t=0, because of its short lifetime in a soil environment and the lack of Fe deficiency at this growth stage. Treatments are named after the FeEDDHA treatment administered and the moment of application. The experiment was carried out in triplicates, which were distributed over three tables in accordance with a randomized complete block design.

**Table 5.2**: Treatment overview. The composition of the treatments is expressed in terms of pore water concentration prior to interaction with soil.

| Treatment | Composition | | | Moment of application | | |
|---|---|---|---|---|---|---|
| | racemic o,o-FeEDDHA (mg $l^{-1}$ Fe) | meso o,o-FeEDDHA (mg $l^{-1}$ Fe) | o,p-FeEDDHA (mg $l^{-1}$ Fe) | t=0 | t=3 weeks | t=6 weeks |
| blank | | | | | | |
| o,p | | | 0.53 | | x | x |
| meso o,o | | 0.56 | | x | x | x |
| racemic o,o | 0.58 | | | x | x | x |
| o,o-mix low | 0.29 | 0.31 | | | x | |
| o,o-mix low + o,p | 0.29 | 0.31 | 1.06 | | x | |
| o,o-mix high | 0.87 | 0.93 | | x | x | |

The pot experiment was executed in a greenhouse with 7 liter Mitscherlich pots containing 5 kg of soil at 50% of the waterholding capacity. Each pot received 35 mmol $NH_4NO_3$, 20 mmol $K_2HPO_4$, 17.5 mmol $CaCl_2$, 10 mmol $MgSO_4$, 0.5 mmol $H_3BO_3$ and 5 μmol $(NH_4)_6Mo_7O_{24}$. All FeEDDHA treatments were applied through a sand column with a diameter of around

6 cm, that was positioned in the centre of the soil surface and went about 10 cm deep into the soil. After FeEDDHA addition, the column was flushed with demineralized water.
After 5 days of germination, 8 soybean (*Glycine max* (L.) Merr.) seedlings of the Fe chlorosis susceptible cultivar Mycogen 5072 were transferred to each pot. Preparation of the pot trial, germination of the seeds, foliar fertilization with micronutrients other than Fe, and plant care were performed as described in Schenkeveld et al. (2008).

**Sampling and measurement**
SPAD-measurements were done three times per week on leaves from the youngest and the second youngest trifoliate with a Minolta–502 SPAD-meter, as described in Schenkeveld et al. (2008). At harvest, the shoots were cut off right above the soil surface. A 1 kg mixed subsample was taken from the soil, from which roots were collected manually. The soil subsample was stored at 4 °C until further use. The shoots were washed with demineralized water and dried at 70 °C. After 48 hours, the shoots were weighed (dry weight). The mineral contents of the shoots were determined through microwave digestion with nitric acid, fluoric acid and hydrogen peroxide (Novozamsky, et al., 1996). Cu, Fe, Mn and Ni concentrations were measured on ICP-AES (Varian, Vista Pro).
Pore water was collected by centrifugation of the soil subsample at 7,443 *g* (7,000 rpm) for 15 minutes as described in Schenkeveld et al 2008. pH was measured directly after collection. Fe, Ca and Mg concentrations were measured by ICP-AES (Varian, Vista Pro); Cu, Al, Mn, Zn, Ni and Co concentrations were measured by ICP-MS (Perkin Elmer, ELAN 6000). The samples were acidified with nitric acid before ICP-measurement. FeEDDHA isomer concentrations were determined after separation through high-performance liquid chromatography (HPLC) as described in Schenkeveld et al. (2007). The limits of quantification (LOQ) were respectively 2 µg $l^{-1}$ Fe for racemic o,o-FeEDDHA, 5 µg $l^{-1}$ Fe for meso o,o-FeEDDHA, and 40 µg $l^{-1}$ Fe for o,p-FeEDDHA (20 µg $l^{-1}$ Fe for each o,p-FeEDDHA peak). To avoid contamination, the preparation of the experimental solutions and dilution of samples for measurement were done with analytical grade chemicals and ultra pure water.

**Statistical analysis**
Statistical analyses were done with the program SPSS 12.0. Homogeneity of the data was tested with the Levene's test ($\alpha = 0.05$). A data transformation was executed in case data proved non-homogenous. Differences among treatments were determined by applying the univariate general linear model procedure with a Tukey post-hoc test ($\alpha = 0.05$). Block effects from the tables were accounted for by including table as a random factor.

# Results and Discussion

**Chlorosis and SPAD-indices**

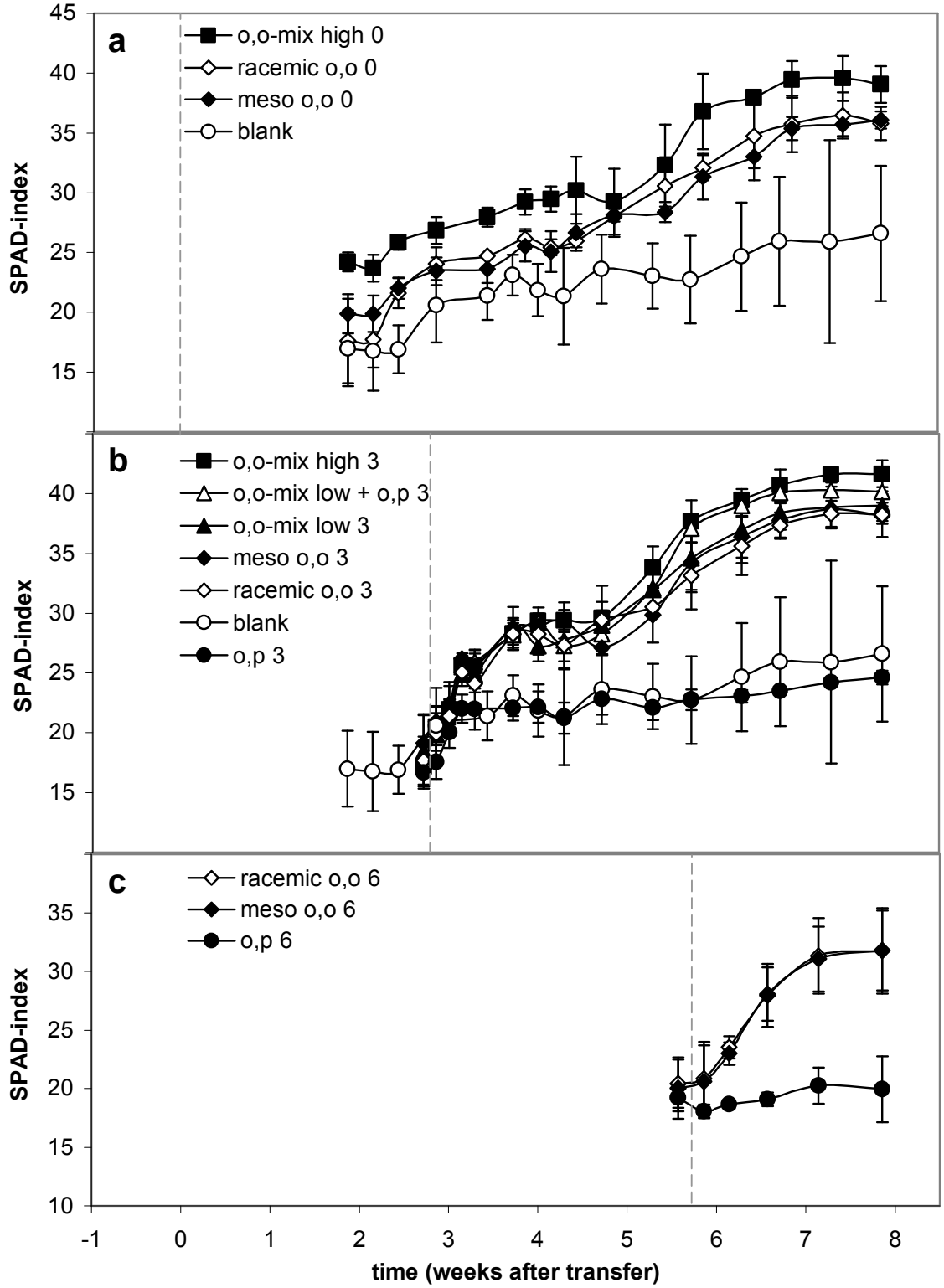
SPAD-measurements were done to monitor and compare the treatments with regard to Fe status of the plants. In Figure 5.1, the SPAD-indices of the youngest leaves are presented as a function of time for all treatments. The treatments have been clustered per moment of FeEDDHA application. The moments of application are indicated by the dashed vertical lines. The plants receiving treatment at t=3 and t=6 weeks were chlorotic at the moment of FeEDDHA application, with SPAD-indices ranging from 16.7 to 20.4. These SPAD-indices were significantly lower than the SPAD-indices of plants that had received an (o,o-)FeEDDHA application at t=0.

Throughout the experiment, the SPAD-indices of neither *o,p*-treatment significantly exceeded those of the blank treatment. Market-claims of a fast re-greening effect of o,p-FeEDDHA were not confirmed in either *o,p*-treatment. From 3 weeks onward, the SPAD-indices of the blank and the *o,p*-treatments remained more or less constant, while in previous pot trials, SPAD-indices of the blank had gradually increased or chlorosis had even entirely been overgrown (Schenkeveld, et al., 2008; Schenkeveld, et al., 2010a).

All treatments involving o,o-FeEDDHA isomers, either separated or in a mixture, resulted in an increase in SPAD-indices relative to the blank. In general, the time-trends in SPAD-indices of o,o-FeEDDHA treatments, that were applied at the same moment, were similar. For both application at t=0 and t=3 weeks, SPAD-indices were highest for the *o,o-mix high* treatment.

For all corresponding treatments applied at t=0 and t=3 weeks, the SPAD-indices of the treatment applied at t=3 weeks eventually surpassed those of the treatment applied at t=0, and remained higher for the rest of the experiment. SPAD-indices were analyzed by means of the GLM procedure, from the moment the SPAD-index of the treatment applied at t=3 weeks exceeded the value of the corresponding treatment applied at t=0, until the end of the experiment (approximately the final 4 weeks). Although differences in SPAD-index did not exceed 4 SPAD-units, the effect of moment of application proved significant ($\alpha = 0.05$) for all corresponding treatments. o,o-FeEDDHA application at t=6 weeks did lead to an increase in SPAD-indices, but to a lesser extent than o,o-FeEDDHA application at t=0 or t=3 weeks. Although at t=6 weeks the reproductive stage was already progressing and the pods were filling, the plants apparently did not exclusively invest in securing the Fe demand of their offspring, but also still allocated Fe to the leaves for chlorophyll synthesis.

**Figure 5.1** ***(p. 111)*****:** SPAD-indices of the youngest leaves of soybean plants grown on Santomera soils as a function of time for all treatments. FeEDDHA treatments were applied at (approximately) t=0 **(a)**; t=3 weeks **(b)**; and t=6 weeks **(c)**. Dashed lines indicate the moment of FeEDDHA application. Error bars indicate standard deviations. The blank treatment has been omitted as a reference in the third cluster (c) because the large standard deviations in the blank treatment obscure the trends in the other treatments.


a
o,o-mix high 0
racemic o,o 0
meso o,o 0
blank
b
o,o-mix high 3
o,o-mix low + o,p 3
o,o-mix low 3
meso o,o 3
racemic o,o 3
blank
o,p 3
c
racemic o,o 6
meso o,o 6
o,p 6
SPAD-index
time (weeks after transfer)

**Fe and FeEDDHA isomer concentrations in the pore water**

The Fe and FeEDDHA isomer concentrations measured in the pore water after harvest are presented in Table 5.3, grouped per FeEDDHA treatment. O,p-FeEDDHA was not detected in any of the samples, and has been omitted from Table 5.3. In none of the treatments, including those receiving FeEDDHA application only 2 weeks before harvest, the remaining FeEDDHA concentration accounted for more than 24% of the dose applied. For each moment of application separately, racemic o,o-FeEDDHA remained in soil solution to a larger extent than meso o,o-FeEDDHA. This corresponds with previous observations (Schenkeveld, et al., 2008; Schenkeveld, et al., 2010a).

**Table 5.3:** Fe, racemic o,o-FeEDDHA and meso o,o-FeEDDHA concentrations in the pore water of Santomera soil at harvest for all treatments. Standard deviations are indicated between parentheses.

| Treatment | Total Fe ($\mu g\ l^{-1}$ Fe) | | racemic o,o-FeEDDHA ($\mu g\ l^{-1}$ Fe) | | meso o,o-FeEDDHA ($\mu g\ l^{-1}$ Fe) | |
|---|---|---|---|---|---|---|
| blank | 7 (3) | a* | -- | | -- | |
| o,p 3 | 17 (8) | a | -- | | -- | |
| o,p 6 | 20 (13) | a | -- | | -- | |
| meso o,o 0 | 62 (33) | a | -- | | 20 (3) | bc |
| meso o,o 3 | 28 (3) | a | -- | | 12 (1) | ab |
| meso o,o 6 | 55 (5) | a | -- | | 47 (4) | d |
| racemic o,o 0 | 150 (13) | b | 133 (16) | b | -- | |
| racemic o,o 3 | 63 (6) | a | 54 (7) | a | -- | |
| racemic o,o 6 | 147 (25) | b | 145 (26) | b | -- | |
| o,o-mix low 3 | 40 (15) | a | 27 (5) | a | 6 (3) | a |
| o,o-mix low + o,p 3 | 57 (6) | a | 33 (5) | a | 11 (1) | ab |
| o,o-mix high 0 | 313 (86) | c | 212 (17) | c | 42 (7) | d |
| o,o-mix high 3 | 168 (15) | b | 133 (9) | b | 29 (5) | c |

* Letters indicate the significantly different groups as identified by the Tukey post-hoc test, including all FeEDDHA treatments and all moments of application.

For corresponding FeEDDHA treatments, administered at t=0 and t=3 weeks, the FeEDDHA isomer concentrations were consequently lower for the treatments applied at t=3 weeks (Table 5.3). This is remarkable, for despite the shorter residence time in the soil-plant system, a larger portion of the FeEDDHA had been removed from soil solution. The principal difference in conditions between FeEDDHA application at t=0 and t=3 weeks, is that the plants receiving treatment at t=3 weeks were chlorotic at that stage, while the plants receiving treatment at t=0 never grew Fe deficient to this extent. This suggests that the enhanced FeEDDHA consumption in treatments administered at t=3 weeks is related to an Fe deficiency stress response mechanism of the plants. When Strategy I plants, i.c. soybean, become Fe deficient, the enzymatic ferric chelate reductase (FCR) system at the root surface is

up-regulated (Marschner and Romheld, 1994; Robinson, et al., 1999). Thus, the efficiency with which chelated Fe is reduced, detached from the chelating agent and taken up by the plant increases. Provided that the efficiency of the corresponding EDDHA ligand in complexing and solubilizing Fe from the soil is limited, the FeEDDHA isomer concentration in soil solution will decrease more swiftly and strongly in the presence of Fe deficient plants than with plants which are not Fe deficient. Other stress response mechanisms like the excretion of protons and phenolic compounds (Marschner, et al., 1986) alter rhizosphere conditions like pH, availability of competing cations and bacterial activity, and may lead to enhanced cation competition or biodegradation. Contrary to an increase in reduction capacity, such processes only result in a decreased FeEDDHA concentration in soil solution, and not in increased Fe uptake, relative to the blank treatment. In order to discriminate, these findings will be further discussed in relation to Fe uptake.

Comparison of corresponding treatments applied at t=0 and t=6 weeks (Table 5.3) shows that racemic o,o-FeEDDHA concentrations at harvest (133 and 145 µg $l^{-1}$ Fe respectively) were equal, while meso o,o-FeEDDHA concentrations (20 and 47 µg $l^{-1}$ Fe respectively) differed only little, relative to the concentration applied (560 µg $l^{-1}$ Fe; Table 5.2). In this study, the impact of stress response mechanisms on FeEDDHA concentrations exceeded the impact of 3 weeks of residence time in the soil-plant system for meso o,o-FeEDDHA and equaled the impact of 6 weeks of residence time for racemic o,o-FeEDDHA (Table 5.3).

Comparison of the concentrations of both o,o-FeEDDHA isomers between the *o,o-mix low 3* and the *o,o-mix low + o,p 3* treatment showed that, for pots from the same table, both racemic and meso o,o-FeEDDHA concentration were always higher in the *o,o- mix low + o,p 3* treatment. As indicated in Table 5.3, the concentrations were however not identified as different in the overall Tukey post-hoc test. Since the treatments were identical, except for the additional o,p-FeEDDHA in the *o,o-mix low + o,p 3* treatment, the concentrations of both o,o-FeEDDHA isomers could also be compared with a simple t-tests. Then the concentration of meso o,o-FeEDDHA proved significantly higher in the *o,o-mix low + o,p 3* treatment ($p = 0.04$), while the concentration of the racemic o,o-FeEDDHA isomer was not ($p = 0.20$). Despite the fact that the effect is small, the observation implies that o,p-FeEDDHA is involved in a "guarding mechanism" which somewhat diminishes the decrease in meso o,o-FeEDDHA concentration. Nonetheless, comparison of the *o,o-mix low + o,p 3* and the *o,o-mix high 3* treatment with regard to the remaining Fe concentration in soil solution shows that the beneficial effect from o,p-FeEDDHA on the meso o,o-FeEDDHA concentration cannot compete with simply applying more meso o,o-FeEDDHA instead.

**Yield**

The dry weight yield ranged from 16.7 to 22.3 g $pot^{-1}$ (see Table 5.4). The Tukey post-hoc test indicated that differences in yield were neither significant between different FeEDDHA treatments applied at the same moment, nor between corresponding FeEDDHA treatments applied at different moments. An overall effect of moment of application was examined by comparing the yields from different moments of application collectively instead of per treatment. Only corresponding FeEDDHA treatments were included in the comparison. The

overall effect of moment of application was significant (p = 0.022) and, for corresponding treatments, application at t=6 weeks yielded less biomass than application at t=3 weeks (p = 0.013).

**Table 5.4:** Dry weight yield and Fe content of the shoot of soybean plants grown on Santomera soil for all treatments. Standard deviations are indicated between parentheses.

| Treatment | Dry weight yield (g pot$^{-1}$) | | Fe content (mg kg$^{-1}$) | |
|---|---|---|---|---|
| blank | 19.0 (1.4) | abc* | 38.6 (5.7) | a |
| meso o,o 0 | 21.1 (0.9) | bc | 59.2 (6.5) | cd |
| racemic o,o 0 | 20.0 (1.7) | abc | 57.6 (5.6) | c |
| o,o-mix high 0 | 21.7 (1.6) | bc | 62.2 (2.8) | cd |
| o,p 3 | 19.3 (1.2) | abc | 41.4 (1.4) | ab |
| meso o,o 3 | 21.5 (2.1) | bc | 51.6 (4.0) | bc |
| racemic o,o 3 | 22.2 (0.4) | c | 57.5 (1.8) | c |
| o,o-mix low 3 | 22.1 (1.7) | bc | 50.1 (2.7) | abc |
| o,o-mix low + o,p 3 | 22.2 (1.7) | bc | 52.7 (2.7) | bc |
| o,o-mix high 3 | 21.6 (1,0) | bc | 70.8 (3.8) | d |
| o,p 6 | 16.7 (0.8) | a | 37.9 (2.4) | a |
| meso o,o 6 | 18.9 (3.2) | ab | 58.8 (6.7) | cd |
| racemic o,o 6 | 19.8 (0.7) | abc | 55.2 (2.3) | c |

* Letters indicate the significantly different groups as identified by the Tukey post-hoc test, including all FeEDDHA treatments and all moments of application.

**Fe content shoot**

After 8 weeks of growth, the Fe content of the shoot ranged from 37.9 to 70.8 mg kg$^{-1}$ Fe (see Table 5.4). The Fe contents of the blank (38.6 mg kg$^{-1}$ Fe) and the *o,p*-treatments (37.9 and 41.4 mg kg$^{-1}$ Fe) did not significantly differ, but were all significantly lower than the Fe contents of all treatments involving o,o-FeEDDHA with exception of the *o,o-mix low 3* treatment (50.1 mg kg$^{-1}$ Fe). Plants grown with the *o,o-mix high* treatments had the highest Fe contents (62.2 to 70.8 mg kg$^{-1}$ Fe). For none of the moments of application, significant differences in Fe content between the *racemic o,o* and the *meso o,o* treatment were found. Only at t=3 weeks significant differences between the treatments involving o,o-FeEDDHA were found: the *o,o-mix high* treatment had a significantly higher Fe content than the other treatments. For corresponding FeEDDHA treatments, the Tukey post-hoc test did not identify significant differences in Fe content between the moments of application.

**Fe uptake**

Fe uptake was calculated as the product of shoot dry weight and Fe content of the shoot. Due to previous experience with contamination of the roots with soil material, the roots were left out of consideration. As shown in Figure 5.3, Fe uptake ranged from 0.63 to 1.52 mg Fe per pot. No significant differences in Fe uptake were observed between the blank, the *o,p 3* and

the *o,p 6* treatment. So, the plants did not benefit from o,p-FeEDDHA, even under conditions in which the Fe uptake efficiency had increased as a result of Fe deficiency stress mechanisms (Marschner and Romheld, 1994); neither in the vegetative stage, nor in the reproductive stage. Regarding the vegetative stage, this finding is in agreement with findings from Rojas et al. (2008). O,p-FeEDDHA is known to largely adsorb to soil reactive surfaces and exchange Fe for Cu (Schenkeveld, et al., 2007). Apparently, fast adsorption and cation competition kinetics heavily outweighed the preferential Fe transfer by o,p-FeEDDHA compared to o,o-FeEDDHA, demonstrated in hydroponics systems (Garcia-Marco, et al., 2006).
By comparing Fe uptake in the *o,o-mix low 3* and the *o,o-mix low + o,p 3* treatment, it was examined if the presence of o,p-FeEDDHA might enhance the performance of o,o-FeEDDHA, through the aforementioned "guarding mechanism". However, no significant difference in Fe uptake was found, although the o,p-FeEDDHA dose in the *o,o-mix low + o,p 3* treatment was twice as high as in the *o,p 3* and *o,p 6* treatments. The higher Fe uptake in the *o,o mix high 3* treatment confirms that distinction between the *o,o-mix low* and *o,o-mix low + o,p 3* treatments is not compromised by Fe saturation of the plants, and indicates that substituting o,p-FeEDDHA from the *o,o-mix low + o,p 3* treatment for o,o-FeEDDHA significantly increases the effectiveness of the treatment.

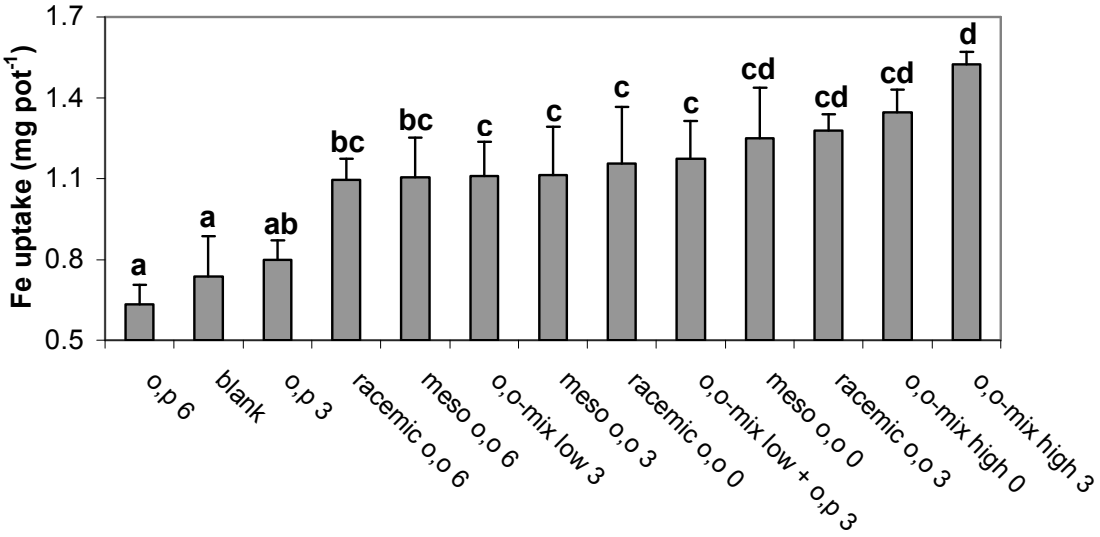



**Figure 5.3:** Fe uptake by soybean plants grown on Santomera soil for all FeEDDHA treatments. Error bars indicate standard deviations. Letters indicate the significantly different groups as identified by the Tukey post-hoc test including all FeEDDHA treatments and all moments of application.

Fe uptake was highest in the *o,o-mix high* treatments (1.35 (t=0) and 1.52 (t=3) mg Fe), but not significantly different from all other treatments. An overall effect of FeEDDHA treatment was examined by comparing Fe uptake from different FeEDDHA treatments for all corresponding moments of application combined. Overall, the *o,o-mix high* treatments led to a significantly higher Fe uptake than the *racemic o,o* treatments ($p = 0.030$) and the *meso o,o*

treatments ($p = 0.012$). This is in agreement with the finding by Schenkeveld et al. (2008) that a higher dose of o,o-FeEDDHA results in a higher Fe uptake.
Fe uptake in *racemic o,o* and *meso o,o* treatments was in all cases significantly higher than in the blank. This testifies that both racemic and meso o,o-FeEDDHA are effective in soil-applied Fe fertilization, in accordance with the study by Ryskievich and Boka (1962). Fe uptake in the *racemic o,o* treatments was not significantly different from Fe uptake in the *meso o,o* treatments, neither overall ($p = 0.73$), nor for any of the moments of application separately. The lack of significant difference in Fe uptake, while Fe uptake was not maximal, demonstrates that the effectiveness of racemic and meso o,o-FeEDDHA in soil application is comparable. This does not correspond with the conclusions from hydroponics studies (Cerdan, et al., 2006; Lucena and Chaney, 2006; 2007), in which meso o,o-FeEDDHA was found to be more effective than racemic o,o-FeEDDHA. In studies in which racemic and meso o,o-FeEDDHA were applied as a mixture to Fe deficient plants, the decrease in meso o,o-FeEDDHA concentration was considerably stronger than in racemic o,o-FeEDDHA concentration (Cerdan, et al., 2006; Hill-Cottingham and Lloyd-Jones, 1965). This stronger decrease may however in part be explained by a redistribution of Fe over the available o,o-EDDHA ligands, upon Fe transfer from racemic o,o-FeEDDHA to the plant; the larger stability of the racemic o,o-FeEDDHA complex favours Fe complexation by racemic o,o-EDDHA (Bannochie and Martell, 1989).
A possible explanation why, in the soil-plant system, contrary to hydroponics systems (Lucena and Chaney, 2006; 2007), Fe uptake was not larger in *meso o,o* treatments than in *racemic o,o* treatments, involves the lower Fe concentration in soil solution in the *meso o,o* treatments, due to the larger degree of meso o,o-FeEDDHA adsorption to soil reactive surfaces, and the faster non-plant-related decline in concentration (Schenkeveld, et al., 2010a). The effect of preferential Fe uptake from meso o,o-FeEDDHA may thus have been undone by the lower soil solution concentration. Schenkeveld et al. (2010a) even suggested that racemic o,o-FeEDDHA might be more effective in supplying soil-grown plants with Fe than meso o,o-FeEDDHA, because the removal of racemic o,o-FeEDDHA from the soil-plant system proved to a larger extent plant-related. The present study does however not support this suggestion.

The effect of moment of application on Fe uptake was not significant; neither for FeEDDHA treatments individually, nor for corresponding treatments collectively. This seems counter-intuitive, because plants that receive a treatment containing o,o-FeEDDHA at a later stage, have less time to benefit from it until harvest. Still, the difference in Fe uptake between the *blank* treatment on the one hand and the *racemic o,o 6* and *meso o,o 6* treatments on the other, amounted 0.36 mg Fe per pot, and was built up in merely 2 weeks. 0.36 mg Fe per pot corresponds to 50% of the total Fe uptake in the blank treatment after 8 weeks. Two factors may help to explain the lack of significant difference in Fe uptake between the moments of application. First, as mentioned earlier, when FeEDDHA treatments were applied after 3 or 6 weeks, the plants were Fe deficient and stress response mechanisms facilitated a faster, more efficient Fe uptake (Marschner and Romheld, 1994). Secondly, by applying the same

FeEDDHA dose at a later stage, losses related to residence in the soil were smaller and the FeEDDHA concentrations in soil solution were highest when plants were actually Fe deficient. Although no quantitative effect on Fe uptake was found, it seems likely the moment of application will affect the Fe distribution over the plant (leaves versus seeds) (Grusak, 1994).

The mechanism of Fe uptake has not been specifically considered in this study. Although Fe reduction and chelate splitting is considered the prevailing Fe uptake mechanism (Chaney, et al., 1972; Robinson, et al., 1999), uptake of the racemic and meso o,o-FeEDDHA complex as a whole has been demonstrated in several plant studies on substrate and in plain nutrient solution (Bienfait, et al., 2004; Orera, et al., 2009) In these studies racemic and meso o,o-FeEDDHA were taken up to approximately to the same extent. Recently, also uptake of the o,p-FeEDDHA complex was demonstrated (Orera, et al., 2009). If uptake of the FeEDDHA complex as a whole also occurs in soil-grown plants, and if so, to what extent it accounts for overall Fe uptake, needs to be further examined.

## Practical implications

In this study o,p-FeEDDHA did not significantly contribute to Fe uptake of Fe deficient, soil-grown plants, regardless whether it was applied in the vegetative or in the reproductive stage, as a single substance or in a mixture. The acclaimed short-term effectiveness in soil application (Garcia-Marco, et al., 2006) was rejected. Soil contamination with Cu had been identified to cause limited stability of o,p-FeEDDHA, thereby reducing its effectiveness (Garcia-Marco, et al., 2006; Lucena, et al., 2005; Yunta, et al., 2003a). The Santomera soil is however not Cu-contaminated[2], so the lack of Cu contamination is no guarantee for o,p-FeEDDHA functionality. In general, it seems questionable if o,p-FeEDDHA in commercial FeEDDHA formulations has any added value as Fe fertilizer in soil application; chances for positive results seem highest in sandy soils with a low Cu content (Schenkeveld, et al., 2007).

Both racemic and meso o,o-FeEDDHA isomer proved effective in supplying soil-grown plants with Fe, approximately to the same extent. The outcomes of this study offer no incentive for altering the ratio between racemic and meso o,o-FeEDDHA in commercial FeEDDHA formulations for soil application, or for discriminating between the o,o-FeEDDHA isomers on product labels.

The moment of application had no significant effect on Fe uptake by soybean plants, but did have a significant effect on yield and on the FeEDDHA isomer concentrations in the pore water at harvest. o,o-FeEDDHA application in the reproductive stage resulted in diminished yield in comparison to earlier application. o,o-FeEDDHA application in the vegetative stage, after the set in of chlorosis, resulted in lower FeEDDHA isomer concentrations in comparison

[2] The Cu content of Santomera soil is below the Dutch soil-specific background value “AW-2000” (35 mg $kg^{-1}$ Cu), indicating that, with respect to Cu, the soil is not contaminated and suitable for any application in accordance with current regulation (Besluit Bodemkwaliteit 2008/Administrative Order on Soil Quality 2008).

to corresponding treatments applied earlier or later. Thus, in view of an efficient use of Fe fertilizer, FeEDDHA application prior to the set in of chlorosis is recommendable; for obtaining similar results, less FeEDDHA is required than in application after the set in of chlorosis, and yield loss related to FeEDDHA application in the reproductive stage is avoided. The distribution of Fe over the plant has not been examined in this study; FeEDDHA application in a later growth stage might be favorable for the allocation of Fe to edible plant parts, e.g. the fruits or the seeds.

In conclusion, since the amount of o,o-FeEDDHA applied determined the effectiveness of the treatment, regardless of the moment of application, the conclusion that FeEDDHA formulations with a higher o,o-content can be applied at a lower dose (Schenkeveld, et al., 2008), is not limited to application prior to the set in of chlorosis but remains valid throughout the growing season.

“Do not wait to strike until the iron is hot,
but make it hot by striking.”

By W.B. Sprague

# Chapter 6

## The biodegradability of EDDHA chelates under calcareous soil conditions

W.D.C. Schenkeveld, E. Hoffland, A.M. Reichwein, E.J.M. Temminghoff, and W.H. van Riemsdijk

## Abstract

FeEDDHA (iron (3+) ethylenediamine-N,N'-bis(hydroxy phenyl acetic acid) products are commonly applied to mend or prevent Fe chlorosis in plants. In soil application, racemic and meso o,o-FeEDDHA constitute the effective components, while o,p-FeEDDHA tends to adsorb or react to o,p-CuEDDHA. Upon interaction with soil, a plant-independent, gradual decline in soil solution concentration of meso o,o-FeEDDHA and o,p-CuEDDHA had been observed. The aim of this study was to examine to what extent biodegradation contributes to this gradual decline. A 4-week incubation experiment was done with calcareous soil receiving an FeEDDHA treatment. The experiment involved 3 sterility regimes, 2 conditioning regimes and 3 time steps.

Soil solution concentrations of meso o,o-FeEDDHA and o,p-CuEDDHA gradually declined in all sterility regimes. Biodegradation did not significantly contribute to the decline in concentration of any EDDHA chelate, except for the marginally formed CoEDDHA. The rate of the process causing the decline was higher at higher temperature and in soil not exposed to gamma irradiation. This study offers no evidence that the effectiveness of soil-applied FeEDDHA fertilizers is compromised by biodegradation.

# Introduction

Synthetic aminocarboxylate chelating agents consist of a group of chemicals, which have a strong ability to form stable, water soluble complexes with di- and trivalent cations. These chemicals, with NTA (nitrilo tri acetic acid), EDTA (ethylene diamine tetra acetic acid) and DTPA (diethylene triamine penta acetic acid) being their most well-known representatives, are widely utilized in industrial, nutritional, medical, domestic and agricultural applications to prevent the formation of precipitates, to prevent reactions catalyzed by metal ions, to remove toxic metal ions and to increase metal bioavailability (Bucheli-Witschel and Egli, 2001).
Many micronutrient fertilizers for Fe, Zn, Cu and Mn are based on aminocarboxylate chelates; the chelating agents enhance metal solubility and transport the metal ions through solution to the plant root. FeEDDHA (iron (3+) ethylene diamine-N,N'-bis(hydroxy phenyl acetic acid)) is among the most effective fertilizers to prevent and remedy Fe deficiency in plants under neutral and alkaline soil conditions (Lucena, 2003; Reed, et al., 1988). The current world market for FeEDDHA products is in the order of 10 thousand metric tons per year, the majority of which is sold in the Mediterranean countries and the Middle East. FeEDDHA products are manufactured through a Mannich-like reaction between ethylenediamine, glyoxylic acid and phenol, and subsequent Fe addition (Petree, et al., 1978). The Mannich-like reaction produces a mixture of positional isomers, diastereomers and polycondensates. The quantitatively most important components in FeEDDHA products are: 1) racemic o,o-FeEDDHA (iron (3+) (R,R) and (S,S) ethylene diamine-N,N'-bis(2-hydroxy phenyl acetic acid) complexes), 2) meso o,o-FeEDDHA (iron (3+) (R,S) = (S,R) ethylene diamine-N,N'-bis(2-hydroxy phenyl acetic acid) complex) and 3) o,p-FeEDDHA (iron (3+) ethylene diamine-N-(2-hydroxy phenyl acetic acid)-N'-(4-hydroxy phenyl acetic acid) complexes). The remaining components, mainly consisting of polycondensates are labelled 4) rest-FeEDDHA. The behaviour and effectiveness of these FeEDDHA components has been extensively studied in soil systems, hydroponics systems with plants, and soil-plant systems (Alvarez-Fernandez, et al., 2002; Garcia-Marco, et al., 2006; Garcia-Mina, et al., 2003; Hernandez-Apaolaza, et al., 2006; Lucena and Chaney, 2007; Schenkeveld, et al., 2008; Schenkeveld, et al., 2007). It has been shown that upon interaction with soil, o,p-FeEDDHA and rest-FeEDDHA are quickly removed from soil solution almost completely, while racemic and meso o,o-FeEDDHA remain in solution to a much larger extent. O,p-EDDHA rapidly mobilizes Cu from the soil by exchanging Fe for Cu and forming a soluble o,p-CuEDDHA complex. Subsequently, the soil solution concentrations of particularly meso o,o-FeEDDHA and o,p-CuEDDHA gradually declined over time. This decline cannot be attributed to plant uptake (Schenkeveld, et al., 2007; Schenkeveld, et al., 2010a) and it is yet unclear which soil process is responsible. The biodegradation of the EDDHA chelates has been identified as a possible cause, but has not been previously examined. In the present study this issue has been addressed.

The biodegradability of various other aminocarboxylate chelates has been intensively studied in view of environmental persistence, particularly observed in certain EDTA chelates (Tandy, et al., 2004). Therefore, a potentially high risk of (heavy) metal mobilization and subsequent leaching to the groundwater (Wenzel, et al., 2003; Wu, et al., 2004) arises when chelating

agents like EDTA are used in soil applications at high doses, e.g. in soil remediation techniques (Nowack, et al., 2006; Römkens, et al., 2002). To reduce this risk, the application of alternative aminocarboxylate chelating agents, like [S,S]-EDDS (ethylene diamine disuccinic acid), which form biodegradable chelates (Tandy, et al., 2006; Van Devivere, et al., 2001), is being examined.
Recently, a readily biodegradable chelate has also been considered for Fe fertilization: FeIDSA (iron (3+) imino disuccinic acid) (Villen, et al., 2007). In soil application of chelate-based fertilizers there is however a trade-off between risk of leaching and the time span the fertilizer remains effective in soil solution. Due to its biodegradability, its low stability (< FeEDTA) and its relatively high affinity for reactive surfaces, the residence time of FeIDSA in soil solution is strongly compromised. Therefore the effectiveness of FeIDSA in soil application is small in comparison to o,o-FeEDDHA, and its potential limited.

Both from an agricultural and an environmental perspective it is important to further the understanding on the fate of EDDHA chelates in the soil. This understanding is required to improve estimates on the time span FeEDDHA components remain effective as fertilizer and to assess potential risks to the environment of leaching of heavy metal after mobilization by EDDHA components (e.g. o,p-CuEDDHA). The aim of this study was to examine to what extent biodegradation contributes to the observed gradual decline in EDDHA chelate concentrations upon interaction with soil. For this purpose an incubation study was done with FeEDDHA application to sterilized and non-sterilized calcareous soil.

## Material and Methods

**Soil**
Calcareous soil was collected from the top soil layer (0 – 20 cm) at a site located in Santomera (Spain). Plants grown on this soil became chlorotic, both under field conditions and in pot trials (Schenkeveld, et al., 2008; Schenkeveld, et al., 2010a). Pre-treatment consisted of air drying and sieving (1 cm). Relevant soil characteristics are presented in Table 6.1.

**FeEDDHA solution**
The FeEDDHA solution was prepared from a sodium-EDDHA stock solution. Fe was added as $FeCl_3*6H_2O$ in a 5% excess based on a 1:1 stoichiometry between Fe and ethylene diamine. The pH was raised to 7 (± 0.5) and the solution was left over-night in the dark in order for excess Fe to precipitate as hydroxides. The following day, the solution was filtered over a 0.45 μm nitro cellulose micro pore filter (Schleicher & Schuell, ref-no: 10401114) and further diluted for application in the incubation experiment. The composition of the FeEDDHA solution was analyzed by combined ICP and HPLC analysis at time t=0.

**Table 6.1:** Soil characteristics.

| | | Extraction | | |
|---|---|---|---|---|
| **Origin/Name** | Santomera | **Oxalate**[g] | **Fe (g $kg^{-1}$)** | 0.30 |
| **Region** | Murcia | | | |
| **Country** | Spain | **DTPA**[h] | **Fe (mg $kg^{-1}$)** | 3.5 |
| **Soil classification** | entisol | | **Cu (mg $kg^{-1}$)** | 4.1 |
| **Water holding capacity (g $kg^{-1}$)** | 320 | | **Co (mg $kg^{-1}$)** | b.d. |
| **pH-$CaCl_2$**[a] | 8.0 | | | |
| **Electro conductivity (mS $m^{-1}$)**[b] | 23 | **$HNO_3$ (0.43 M)**[i] | **Fe (mg $kg^{-1}$)** | 494 |
| **SOC (g $kg^{-1}$)**[c] | 5.4 | | **Cu (mg $kg^{-1}$)** | 10 |
| **Clay (g $kg^{-1}$)**[d] | 260 | | **Co (mg $kg^{-1}$)** | 3 |
| **$CaCO_3$ (g $kg^{-1}$)**[e] | 520 | | | |
| **CEC (cmol $kg^{-1}$)**[f] | 10.3 | | | |

[a] ISO/DIS 10390 Soil Quality – Determination of pH
[b] ISO/DIS 11265 Soil Quality – Determination of the specific electric conductivity
[c] Walinga et al. (1992)
[d] Houba et al. (1997)
[e] ISO 10693, Soil Quality – Determination of carbonate content, volumetric method
[f] ISO/DIS 11260 Soil Quality – Determination of cation exchange capacity and base saturation – method using barium chloride solution
[g] Schwertmann (1964)
[h] Lindsay and Norvell (1978) and Quevauvillier et al. (1996)
[i] Tipping et al. (2003) and Fest et al. (2005)

**Table 6.2:** FeEDDHA component concentrations in soil solution at t=0 and t=1 day. Standard deviations are indicated in between parentheses. The concentrations at t=1 day are averaged for all FeEDDHA treatments.

| FeEDDHA component | Concentration at t=0 (mg $l^{-1}$ Fe) | Fraction of Fe chelated at t=0 | Concentration at t=1 day (mg $l^{-1}$ Fe) | Remaining fraction ($[FeEDDHA_{t=1}]$ / $[FeEDDHA_{t=0}]$) |
|---|---|---|---|---|
| racemic o,o-FeEDDHA | 1.57 (0.01) | 0.22 | 1.19 (0.06) | 0.76 |
| meso o,o-FEDDHA | 1.91 (0.02) | 0.27 | 1.27 (0.06) | 0.67 |
| o,p-FeEDDHA | 1.22 (0.01) | 0.17 | b.d. | 0.00 |
| rest-FeEDDHA | 2.49 (0.04) | 0.35 | 0.07 (0.03) | 0.03 |
| total Fe | 7.18 (0.09) | | 2.53 (0.14) | 0.35 |

b.d.: below detection limit.

### Incubation experiment

A soil incubation experiment with a runtime of 4 weeks was executed to examine the effect of biodegradation on the concentration of EDDHA-chelates in soil solution after administration of an FeEDDHA treatment. The composition of the FeEDDHA treatment is listed in Table 6.2. The experiment started in late June 2006 and had a randomized complete block design. The variables included were: FeEDDHA addition (with or without FeEDDHA application),

interaction time (1, 14 or 28 days), sterility (*sterile*, sterilized and *reinoculated*, or *non-sterilized*) and conditioning (*conditioned* room or *greenhouse*). The reinoculated treatments were included to rule out side effects from the sterilization process on the concentration of EDDHA-chelates. Two conditioning regimes were included to examine the potential effect of temperature on the biodegradation rate. A greenhouse was preferred over a second controlled regime, to relate more closely to the conditions of the pot trial study in which the dynamics in EDDHA-chelate concentration were first observed. Soil sampling was done destructively and the experiment was carried out in duplicates.

One liter poly ethylene (PE) pots were filled with 0.5 kg of soil, to which 3.3 mmol $NH_4NO_3$, 2.1 mmol $K_2HPO_4$, 1.7 mmol $CaCl_2$, 0.83 mmol $MgSO_4$, 42 μmol $H_3BO_3$ and 0.31 μmol $(NH_4)_6Mo_7O_{24}$ had been added by thoroughly and successively mixing through nutrient solutions. Subsequently, the top openings of the pots were covered with aluminum foil to prevent microbial invasion and to minimize evaporation. The foil-covered pots involved in the sterile, and the sterilized and reinoculated treatments were gamma-sterilized[3] in 2 batches, both receiving over 25 kGy of radiation.

FeEDDHA treatments were applied after gamma sterilization of the soil to avoid breakdown of the chelates due to irradiation. Prior to administration, the FeEDDHA solution was filter-sterilized over a 0.2 μm micro pore filter (Schleicher and Schuell FP30/0.2 CA-S). The FeEDDHA solution was applied to and mixed through the soil in a laminar flow cupboard under sterile conditions. All materials used were either sterilely packaged prior to usage, or sterilized by flame or in an autoclave. The overall FeEDDHA dose applied equalled a pore water concentration of 7.18 mg $l^{-1}$ Fe; the composition of the FeEDDHA treatment is specified in Table 6.2.

Reinoculation was done by mixing through 5 ml of a soil extract obtained by adding ultra pure water to Santomera soil in a ratio of 10:1, leaving it for a few hours and filtering off the soil over a cheesecloth. After addition of the FeEDDHA solution and the soil extract for reinoculation, the moisture content in all pots equalled 50% of the water holding capacity. In accordance with the treatment, the pots were positioned in the conditioned room or in the greenhouse. The temperature in the conditioned room was kept at 20 (± 1) °C. The average maximum day temperature in the greenhouse amounted 30 °C and the average overall temperature 25 °C; at all time, the temperature in the greenhouse was minimally 20 °C. In the vicinity of the pots, 100% water saturation of the air was preserved. The pots were weighed at t=0 and before sampling; evaporation never exceeded 2% of the water added and consequently did not substantially affect pore water concentrations.

**Soil colonization**

At harvest, a selection of pots was sampled under sterile conditions to check if the sterile treatments had remained sterile throughout the experiment and if the reinoculated treatments had been colonized. The selection included all sterile treatments harvested after 2 and 4 weeks and all reinoculated treatments harvested after 4 weeks. From each pot, three soil samples of

[3] Gamma sterilization was carried out by a specialized company Isotron Nederland B.V., located in Ede (Gld); the Netherlands

0.5 ± 0.2 g were taken. The samples were vigorously shaken for 30 seconds in glass tubes containing 5 ml 0.01 M $MgSO_4$ and glass beads. Serial dilutions were plated on King's medium B (King, et al., 1954). Colonies were counted after 48 hours.

**Pore water collection and analyses**

Pore water was collected by centrifugation at 7,443 *g* (7,000 rpm) for 15 minutes the day after soil sampling (Schenkeveld, et al., 2008). pH was measured directly after pore water collection. In the soil solution samples from t=28 days, DOC (dissolved organic carbon) concentrations were measured to examine for concentration effects from gamma irradiation, because DOC potentially affects the speciation of EDDHA chelates as a competitor for cations and reactive surface sites. DOC concentrations were measured with a segmented flow analyzer (SFA) (Skalar, SK12) by persulphate and tetraborate oxidation under ultraviolet light and infrared detection. Fe, Ca and Mg concentrations were measured by ICP-AES (Varian, Vista Pro); Cu, Al, Mn, Zn, Ni and Co concentrations were measured by ICP-MS (Perkin Elmer, ELAN 6000). The samples were acidified with nitric acid before ICP-measurement. The concentrations of metals chelated to EDDHA components were calculated by subtracting the metal concentrations of corresponding treatments with and without FeEDDHA addition.
FeEDDHA component concentrations were determined after separation through high-performance liquid chromatography (HPLC) (Schenkeveld, et al., 2007). To avoid contamination, the preparation of the experimental solutions and dilution of samples for measurement were done with analytical grade chemicals and ultra pure water.

**Statistical analysis**

Statistical analysis of the data was performed using SPSS 12.0. The homogeneity of the data was tested with the Levene's test ($\alpha = 0.05$). A data transformation was executed in case data proved non-homogenous. Differences among treatments were determined by applying the multivariate general linear model procedure with a Tukey post-hoc test ($\alpha = 0.05$).

## Results and Discussion

**Sterilization**

Colonization data confirm that the sterile treatments had remained sterile throughout the experiment (Table 6.3). Reinoculation had been successful and at the moments of sampling, the number of colony forming units (cfu's) in reinoculated treatments was lower but within one order of magnitude of the non-sterilized treatments. After 4 weeks, the number of cfu's in the reinoculated treatments was significantly higher in the conditioned room than in the greenhouse. This effect was larger in the treatments that received FeEDDHA. Furthermore, the number of cfu's appears to be constant over time in the non-sterilized treatments, but variable in the reinoculated treatments, indicating bioequilibrium had not yet been restored two weeks after reinoculation.

**Table 6.3:** Colonization of Santomera soil in sterilized, sterilized and reinoculated, and non-sterilized treatments. Standard deviations are indicated in between parentheses.

| FeEDDHA treatment | Conditioning | Sterilized ($10^5$ cfu $g^{-1}$) | | Reinoculated ($10^5$ cfu $g^{-1}$) | | Non-sterilized ($10^5$ cfu $g^{-1}$) | |
|---|---|---|---|---|---|---|---|
| | | 2 weeks | 4 weeks | 2 weeks | 4 weeks | 2 weeks | 4 weeks |
| - | c. room | <0.001 | <0.001 | 14.82 | 6.01 (0.54) **b** | 19.07 | 18.51 |
| - | greenhouse | <0.001 | <0.001 | n.d. | 3.03 (0.28) **c** | n.d | n.d |
| + | c. room | <0.001 | <0.001 | n.d. | 11.6 (0.33) **a** | n.d | 19.90 |
| + | greenhouse | <0.001 | <0.001 | n.d | 2.37 (0.09) **c** | n.d | n.d. |

n.d.: not determined.

Gamma irradiation induces chemical degradation of organic material, resulting in increased DOC (dissolved organic carbon) concentrations in the treatments that have been gamma sterilized (Powlson and Jenkinson, 1976). This is illustrated in Figure 6.1 for the treatments without FeEDDHA. DOC represents the main source of energy-rich substrates for microorganisms (Jandl and Sollins, 1997; Neff and Asner, 2001). As a result of microbial activity, the DOC concentration in the reinoculated treatments declined over a time span of 4 weeks to one third of the DOC concentration in sterile treatments.

For the sterile treatments, the effect of conditioning on DOC concentration is significant ($p = 0.033$). The lower concentration in the conditioned treatment cannot be attributed to biodegradation. The oxidation of irradiated organic material may have been enhanced by the higher temperatures in the greenhouse, resulting in more hydrophilic compounds and hence a higher DOC concentration. The higher temperature in the greenhouse may also have resulted

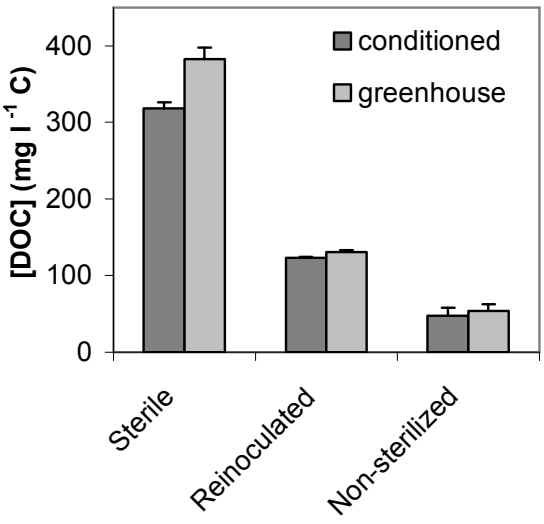



**Figure 6.1:** DOC concentrations after 4 weeks in the treatments without FeEDDHA addition. Error bars indicate standard deviations.

in a shift in adsorption equilibrium of DOC in favour of the solution phase (Kaiser, et al., 2001). Although soil from both the conditioned and the greenhouse treatments had been stored over-night under equal conditions prior to pore water collection, a difference in DOC concentration may well have remained because reinstallment of the DOC adsorption equilibrium is a slow process, requiring over 24 hours (Jardine, et al., 1992).

**Fe and FeEDDHA component concentrations**

For all treatments without FeEDDHA, the Fe concentration in soil solution was below detection limit throughout the experiment (data not presented). This implies that Fe concentrations in treatments with FeEDDHA exclusively result from FeEDDHA complexes. The Fe concentration strongly decreased in all FeEDDHA treatments within the first 24 hours, on average from 7.18 to 2.53 mg $l^{-1}$ Fe (Table 6.2). O,p-FeEDDHA and rest-FeEDDHA were almost completely removed from soil solution and have not been further considered in the statistical data analysis. The concentrations of racemic and meso o,o-FeEDDHA decreased by respectively 24 and 33 %, and largely determined the remaining Fe concentration in soil solution. Adsorption has been identified as the principal process underlying the strong initial decline in FeEDDHA component concentrations (Schenkeveld, et al., 2010a). For o,p-FeEDDHA adsorption is complemented with cation competition from Cu (Garcia-Marco, et al., 2006; Schenkeveld, et al., 2007). The small standard deviations in FeEDDHA component concentrations after 24 hours (Table 6.2) indicate the processes responsible for the fast initial decline are hardly affected by the sterility of the soil. Biodegradation is therefore excluded as a substantial contributor to this fast decline.

After the fast initial decline, the Fe concentration in soil solution of all FeEDDHA treatments gradually decreased further, from 2.53 ± 0.08 (means ± s.d.) mg $l^{-1}$ Fe after 1 day, to 1.60 ± 0.30 mg $l^{-1}$ Fe after 4 weeks (Figure 6.2a). This decrease mainly results from a decline in meso o,o-FeEDDHA concentration from 1.27 ± 0.06 to 0.48 ± 0.23 mg $l^{-1}$ Fe (Figure 6.2c), which can be described with an exponential decay function (Schenkeveld, et al., 2010a). The increase in standard deviation results from differences in rate of decline among the treatments. The racemic o,o-FeEDDHA concentration remained relatively constant, only decreasing from 1.19 ± 0.06 to 1.13 ± 0.08 mg $l^{-1}$ Fe (Figure 6.2b). The ANOVA confirms that incubation time had a significant effect on the Fe and meso o,o-FeEDDHA concentration ($p = 0.000$), but not on the racemic o,o-FeEDDHA concentration ($p = 0.1$; Table 6.4a). Specifically for the non-sterilized treatments, the effect of incubation time on the racemic o,o-FeEDDHA concentration was significant ($p = 0.002$). Contrary to meso o,o-FeEDDHA, the decrease in racemic o,o-FeEDDHA concentration in these treatments was larger in the second 2 weeks of the experiment. The lack of a significant effect of incubation time on the racemic o,o-FeEDDHA concentration in the sterilized treatments indicates that adsorption equilibrium had been reached within the first 24 hours.

The meso o,o-FeEDDHA concentration was significantly affected by both conditioning and sterility of the treatment ($p = 0.000$; Table 6.4a). All FeEDDHA treatments in the greenhouse had significantly lower meso o,o-FeEDDHA concentrations than the treatments in the

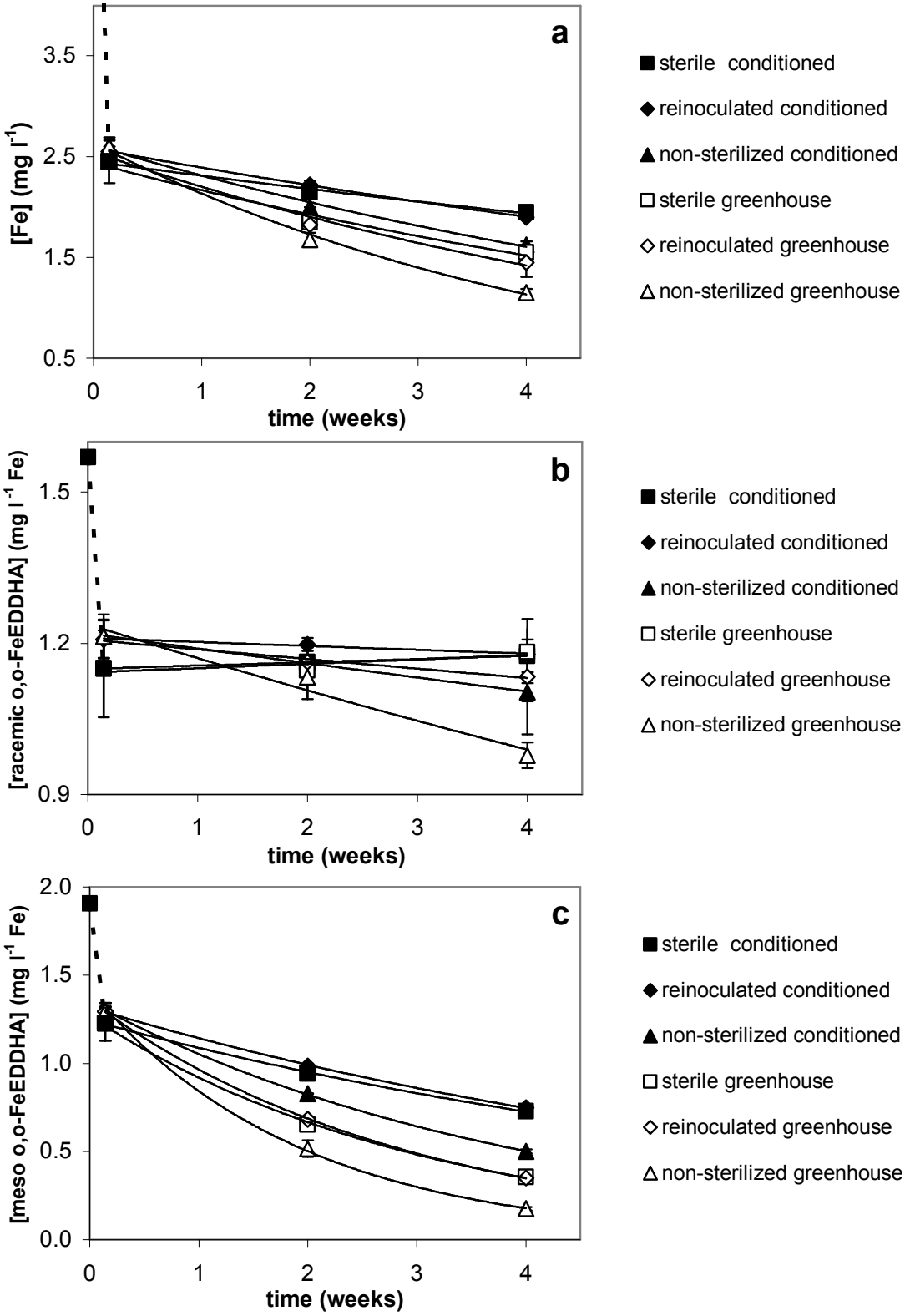

a
[Fe] (mg l-1)
3.5
2.5
1.5
0.5
0
1
2
3
4
time (weeks)
sterile conditioned
reinoculated conditioned
non-sterilized conditioned
sterile greenhouse
reinoculated greenhouse
non-sterilized greenhouse
b
[racemic o,o-FeEDDHA] (mg l-1 Fe)
1.5
1.2
0.9
0
1
2
3
4
time (weeks)
sterile conditioned
reinoculated conditioned
non-sterilized conditioned
sterile greenhouse
reinoculated greenhouse
non-sterilized greenhouse
c
[meso o,o-FeEDDHA] (mg l-1 Fe)
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
time (weeks)
sterile conditioned
reinoculated conditioned
non-sterilized conditioned
sterile greenhouse
reinoculated greenhouse
non-sterilized greenhouse

**Figure 6.2** ***(p. 130)*****: a)** Total Fe; **b)** racemic o,o-FeEDDHA; and **c)** meso o,o-FeEDDHA concentration in the pore water of Santomera soil as a function of time for all treatments with FeEDDHA addition. Error bars indicate standard deviations.

conditioned room, both after 2 and after 4 weeks (Figure 6.2c). For both greenhouse and conditioned room separately, the meso o,o-FeEDDHA concentration in the non-sterilized treatments was lower than in the sterile and the reinoculated treatments, also both after 2 and after 4 weeks (Figure 6.2c). The post-hoc test confirms this difference is significant ($p = 0.000$) (Table 6.4b), but does not distinguish between sterile and reinoculated ($p = 0.08$). All observed effects in the meso o,o-FeEDDHA concentration reflect in the Fe concentration, yet less significantly (Table 6.4a). The racemic o,o-FeEDDHA concentration was neither significantly affected by conditioning ($p = 0.06$), nor by sterility ($p = 0.07$).
The interaction term sterility*time is significant for the concentrations of meso o,o-FeEDDHA ($p = 0{,}000$), Fe ($p = 0.002$), and racemic o,o-FeEDDHA ($p = 0.049$) (Table 6.4a), implying that the rate of decline in concentration is affected by sterility. In Figure 6.2c this is illustrated

**Table 6.4:** Results of the statistical analysis on the effect of time, sterility and conditioning on the total Fe and several EDDHA chelate concentrations: **(a)** P-values of the ANOVA. Interactions without P-values < 0.05 have not been included into the model (reduced model); **(b)** P-values of the post-hoc test on sterility - reduced model; and **(c)** P-values of the post- hoc test on incubation time - reduced model.

| **(a)** | **Total Fe** | **Fe-rac** | **Fe-meso** | **CuEDDHA** | **CoEDDHA** |
|---|---|---|---|---|---|
| Time (T) | 0.000 | 0.098 | 0.000 | 0.000 | 0.000 |
| Sterile (S) | 0.003 | 0.068 | 0.000 | 0.000 | 0.000 |
| Conditioning (C) | 0.000 | 0.064 | 0.000 | 0.000 | 0.243 |
| T*S | 0.002 | 0.049 | 0.000 | 0.005 | 0.001 |
| S*C | 0.711 | 0.404 | 0.737 | 0.423 | 0.009 |

| **(b)** | **Total Fe** | **Fe-meso** | **CuEDDHA** | **CoEDDHA** |
|---|---|---|---|---|
| Sterile - reinoculated | 0.897 | 0.078 | 0.764 | 0.000 |
| Sterile – non-sterilized | 0.008 | 0.000 | 0.000 | 0.000 |
| Reinoculated – non-sterilized | 0.003 | 0.000 | 0.000 | 0.000 |

| **(c)** | **Total Fe** | **Fe-meso** | **CuEDDHA** | **CoEDDHA** |
|---|---|---|---|---|
| 1 day – 14 days | 0.000 | 0.000 | 0.000 | 0.000 |
| 1 day – 28 days | 0.000 | 0.000 | 0.000 | 0.004 |
| 14 days – 28 days | 0.000 | 0.000 | 0.000 | 0.000 |

from the concentration difference in meso o,o-FeEDDHA between the sterile and the non sterilized treatments of the same conditioning regime, which increases as a function of time. The interaction term sterility*conditioning is not significant for the concentration of either Fe ($p = 0.711$), racemic o,o-FeEDDHA ($p = 0.404$) or meso o,o-FeEDDHA ($p = 0.737$). This implies the effect of conditioning on the rate of decline is similar for all sterility treatments. This is illustrated from the exponential fits of the meso o,o-FeEDDHA concentration data as a function of time (Table 6.5). The ratios of the exponential terms of treatments of corresponding sterility and different conditioning are approximately the same.

**Table 6.5:** Exponential fits of the meso o,o-FeEDDHA concentration data from 1 day onward for all FeEDDHA treatments; t is expressed in weeks after FeEDDHA application.

| Sterility | Conditioning | Exponential fit | $R^2$ | Ratio of the exponents |
|---|---|---|---|---|
| Sterile | greenhouse | $1.27e^{-0.321t}$ | 0.999 | 2,4 |
| | conditioned room | $1.24e^{-0.135t}$ | 0.999 | |
| Reinoculated | greenhouse | $1.36e^{-0.340t}$ | 1.000 | 2.4 |
| | conditioned room | $1.32e^{-0.142t}$ | 0.999 | |
| Non-sterilized | greenhouse | $1.41e^{-0.518t}$ | 0.999 | 2.1 |
| | conditioned room | $1.35e^{-0.247t}$ | 1.000 | |

Biodegradation can be excluded as a single cause for the decline in meso o,o-FeEDDHA concentration, because a gradual decline was observed in all treatments, including the sterile ones. The fact that under both conditioning regimes, the meso o,o-FeEDDHA concentration declined faster in the non-sterilized treatment than in the sterile treatment suggests at least a contribution from biodegradation. However, the results from the ANOVA offer two principal objections. First, if the concentration difference between corresponding sterile and non-sterilized treatment were due to biodegradation, an effect of biodegradation would also be expected in the reinoculated treatments; microbial activity was confirmed and the number of cfu's was in the same range as in the non-sterilized treatments. However, the post hoc test on sterility (Table 6.4b) indicates that the meso o,o-FeEDDHA concentrations of corresponding sterile and reinoculated treatments did not significantly differ ($p = 0.08$). Secondly, an impact of biodegradation on the meso o,o-FeEDDHA concentration was expected to be temperature dependent, because microbial activity in the soil is a function of temperature, reaching its optimum between 25 and 30 °C (Pietikainen, et al., 2005). Therefore a larger temperature effect would be expected in the non-sterile treatments. However, for all sterility treatments, the temperature dependency of the process causing the gradual decline was similar, as shown by the approximately equal ratios of the exponents of the exponential fits (Table 6.5). Based on these objections, it was concluded that biodegradation did not substantially contribute to the gradual decline in meso o,o-FeEDDHA concentration. Including the reinoculated treatments in the experimental setup proved essential for drawing this conclusion.

The observed differences in rate of decline (Figure 6.2c) indicate that the major process responsible for the gradual decline in meso o,o-FeEDDHA is affected by temperature and gamma irradiation. The faster decline in meso o,o-FeEDDHA concentration in the greenhouse, where temperatures up to 17 °C higher than in the conditioned room were registered, obeys the general rule that the speed of processes and reactions increases with increasing temperature. Possible processes involved include sorption, cation competition and oxidation of the chelating agent. Photodecomposition and precipitation on the soil surface were excluded, because the soil was not exposed to light and evaporation was negligible. The more constant concentration of racemic o,o-FeEDDHA in comparison to meso o,o-FeEDDHA might be explained from the higher stability constant of the former, potentially making it less susceptible to the aforementioned processes.
Sorption processes tend to be relatively fast, reaching equilibrium within hours to days rather than weeks. Slow sorption e.g. in relation to organic matter can however not be excluded. Jorda et al. (1992) studied the effect of temperature on the sorption kinetics of FeEDDHA to a calcareous soil in 2-day batch experiments and also observed a faster decline in concentration at higher temperature. The study does however not offer any argumentation why the observed decline in concentration should be related to sorption and not some other process. Moreover, it does not distinguish between the individual FeEDDHA components; only the total Fe concentration in soil solution was considered.
Reactions involved in cation competition are known to potentially have slow reaction kinetics, in particular when it concerns double-exchange reactions and both metal ions are chelated prior to exchange. Also the involvement of trivalent cations in the exchange may contribute to a low reaction rate (Morel and Hering, 1993). However, in relation to the decline in meso o,o-FeEDDHA concentration, no increase in concentration of any competing cation has been observed (see paragraph on competing cations). This implies, that either cation competition was not causing the decline, a competing cation has been overlooked, or the newly formed chelate has a high affinity for the solid phase.
The oxidation of the chelating agent EDDHA under soil conditions has hardly been examined. For other chelating agents including EDTA several pathways are known, involving Mn or Mn-oxides (Nowack, 2002).

The cause for the decreased rate of decline in meso o,o-FeEDDHA concentration in treatments with irradiated soil is unclear, but is probably related to the radiation-induced degradation of the SOM (soil organic matter). It was beyond the scope of this research to extensively examine the effect of irradiation on the size and quality of the organic matter pools in the soil. The larger amount of DOC in irradiated treatments (Figure 6.1) was expected to increase the competition with meso o,o-FeEDDHA for reactive surfaces sites on clay minerals and Fe(hydr)oxides, and to decrease meso o,o-FeEDDHA adsorption. However, the approximately equally large decline in meso o,o-FeEDDHA concentration in irradiated and non-sterilized treatments within the first 24 hours rebuts that adsorption was substantially affected.

**Competing cations**

Significant chelate-related increases in concentration were only observed for Cu and Co. The concentration behaviour of other cations (data not shown) is not further discussed.

*Copper*

In treatments without FeEDDHA addition, the Cu and DOC concentrations were linearly related (Figure 6.3), indicating that basically all Cu in soil solution was bound to DOC.

In treatments with FeEDDHA addition, a mobilization of Cu was observed within the first day (Figure 6.4a). This Cu mobilization is almost entirely caused by the formation of the soluble o,p-CuEDDHA complex, resulting from the displacement of Fe from the o,p-FeEDDHA complex by Cu from the soil (Schenkeveld, et al., 2007). After 1 day, the CuEDDHA concentrations in the treatments with FeEDDHA addition were approximately equally large, ranging from 797 to 861 μg $l^{-1}$ Cu. Subsequently, the concentrations declined over time, approaching equilibrium after 4 weeks. The ANOVA and post-hoc tests indicate the o,p-CuEDDHA concentration is significantly affected by the same factors as the meso o,o-FeEDDHA concentration (Table 6.4a). Along the same line of reasoning as for meso o,o-FeEDDHA, it is concluded that the concentration of o,p-CuEDDHA had not been affected by biodegradation. In comparison to meso o,o-FeEDDHA, the decline in o,p-CuEDDHA concentration went faster and the impact of conditioning on the rate of decline was smaller relative to sterility. Besides the rate of decline, also the chemical equilibrium appears to be substantially affected: more o,p-CuEDDHA remained in solution in sterilized treatments and in the conditioned room. The process underlying the decline in o,p-CuEDDHA remains unclear. There is no experimental evidence that hints at further displacement of Cu by another competing cation.

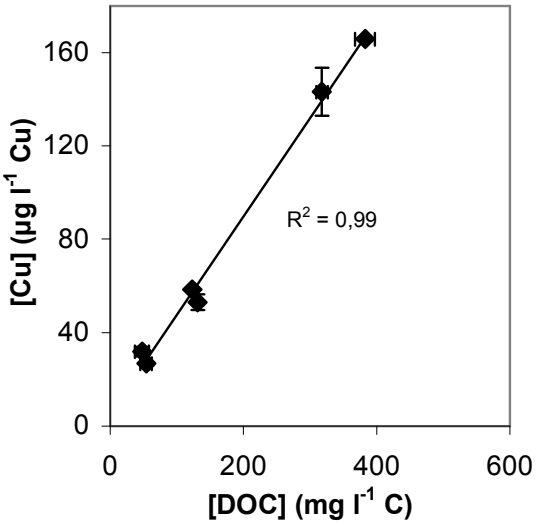



**Figure 6.3:** Relation between Cu and DOC concentration in the pore water of Santomera soil for treatments without FeEDDHA addition. Error bars indicate standard deviations.

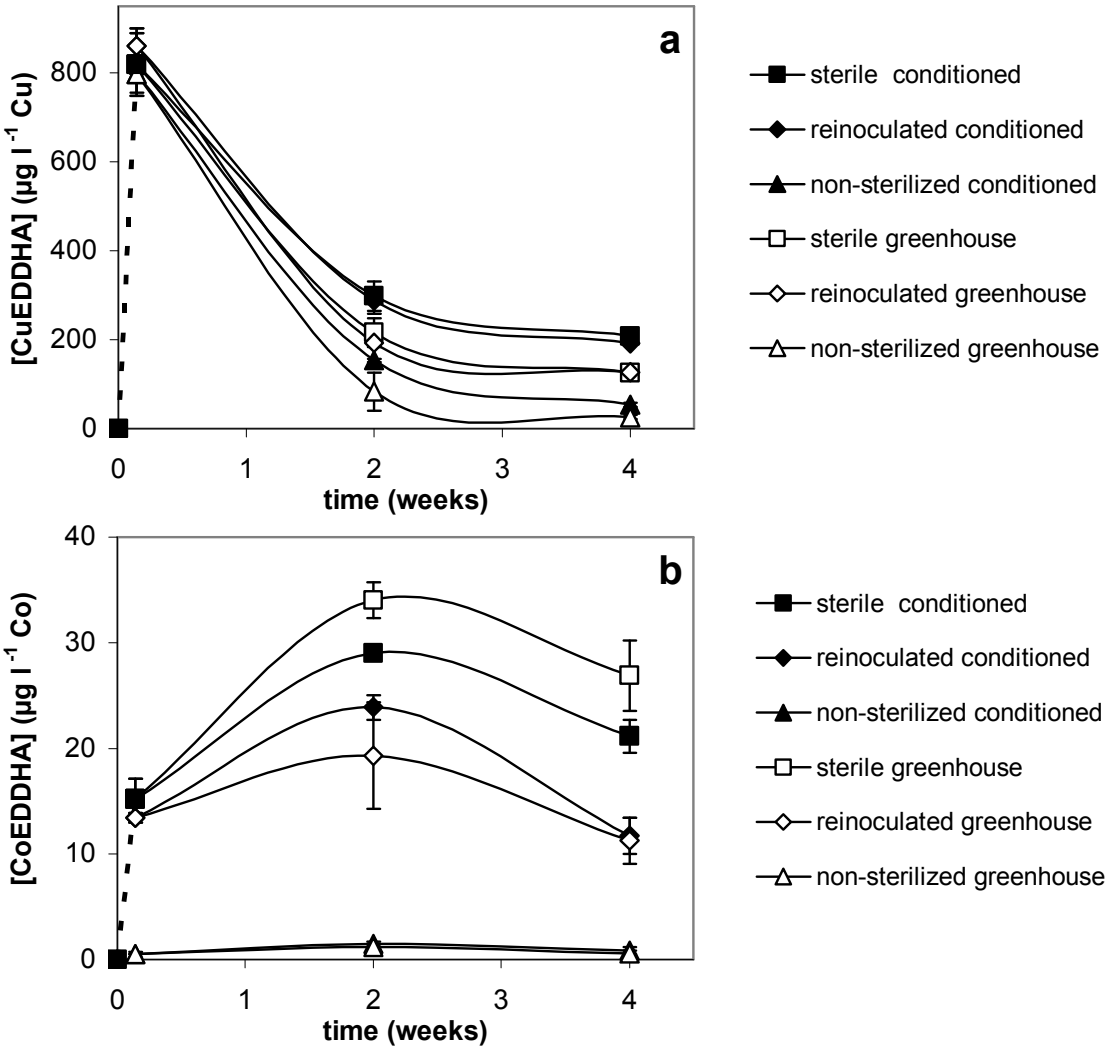



**Figure 6.4: a)** CuEDDHA and **b)** CoEDDHA concentration in the pore water of Santomera soil as a function of time for all treatments with FeEDDHA addition. Error bars indicate standard deviations.

*Cobalt*

Unlike Cu, Co concentrations in the treatments without FeEDDHA addition were not related to the DOC concentration. In the treatments with FeEDDHA addition, CoEDDHA concentrations ranged from 0.50 and 34 µg $l^{-1}$ Co (Figure 6.4b), which is orders of magnitude lower than CuEDDHA and FeEDDHA concentrations. No specific EDDHA component could be appointed in relation to Co solubilization. The results from the ANOVA show that CoEDDHA concentrations were significantly affected by time and sterility ($p = 0.000$). Also two significant interaction terms were found, namely: sterility*conditioning ($p = 0.009$) and

sterility*time (p = 0.001) (Table 6.4a). In all treatments with FeEDDHA addition, CoEDDHA concentrations were highest after 2 weeks. Gamma irradiation significantly increased the impact of Co as a competing cation (Figure 6.4b): 11.3 to 34.2 μg $l^{-}$ Co chelated to EDDHA in the sterilized treatments versus 0.50 to 1.47 μg $l^{-}$ Co in the non-sterilized treatments. The reason for this is unclear. After 2 and 4 weeks the CoEDDHA concentrations in the sterile treatments were significantly higher than in the reinoculated treatments (p = 0.000; Figure 6.3b). So, microbial activity had a negative effect on the CoEDDHA concentration. It remains unclear if CoEDDHA complexes simply formed to a lesser extent in the reinoculated treatments, or if they were removed from soil solution more quickly. Studies on CoEDTA indicate that the valence of the complexed Co strongly affects the behaviour of the complex in the soil system. Co(III)EDTA is more chemically inert and has a much higher stability than Co(II)EDTA, and is therefore less susceptible to adsorption and cation competition reactions. Under soil conditions, the valence of the complexed Co is affected by oxidation, catalyzed by Mn(IV)oxides (Jardine and Taylor, 1995) and bacterial reduction (Caccavo, et al., 1994; Gorby, et al., 1998). Assuming the same processes affect CoEDDHA, the higher concentration in the sterile treatments may be explained from the lack of bacterial reduction of the more stable and mobile Co(III)EDDHA.

## Practical implications

This study offers no evidence that the effectiveness of soil-applied FeEDDHA fertilizers is compromised by biodegradation; FeEDDHA component concentrations in soil solution were not significantly affected by microbial activity. The gradual decline in pore water concentration of particularly meso o,o-FeEDDHA was caused by another, yet undetermined soil process. The rate of decline is temperature dependent. The faster decline at higher temperatures signifies that in warm areas or warm seasons larger or more frequent FeEDDHA application is necessary to maintain a certain Fe concentration in soil solution, even disregarding plant uptake.

Upon FeEDDHA application, the heavy metals Cu and Co can be mobilized from the soil matrix through the formation of soluble CuEDDHA and CoEDDHA complexes, as a result of cation competition. In this study, CuEDDHA concentrations were not significantly affected by microbial activity, but CoEDDHA concentrations were.

The risks of leaching EDDHA chelates should be controlled through an efficient application regime. The observed gradual decline in meso o,o-FeEDDHA and CuEDDHA concentration in soil solution imply the risk of leaching grows smaller over time. Because Cu is particularly solubilized as o,p-CuEDDHA, the risk of Cu leaching can be specifically reduced by applying FeEDDHA fertilizer containing little o,p-FeEDDHA. The residence time of o,p-FeEDDHA in soil solution is very short, and its added value as Fe fertilizer in soil application is debatable to begin with. Additional research is needed to clear up the processes that affect the residence time of EDDHA chelates in soil solution, as well as the underlying mechanisms.

“Nothing is true, everything is permitted.”

Allegedly by Hassan-i-Sabbah (1050s - 1124)
(first leader of the Nizârî Ismâ'îlîs at Alamut)

# Chapter 7

## Evaluation of the potential impact of Cu competition on the performance of o,o-FeEDDHA in soil application

W.D.C. Schenkeveld, L.P. Weng, E.J.M. Temminghoff, A.M. Reichwein, and W.H. van Riemsdijk

## Abstract

FeEDDHA products are commonly applied as Fe fertilizer to plants grown on calcareous soil and comprise a mixture of FeEDDHA components. The isomers racemic and meso o,o-FeEDDHA determine the effectiveness of soil-applied FeEDDHA treatments. In pot and incubation trials, a gradual decline in pore water concentration of in particular meso o,o-FeEDDHA has been observed, not related to plant uptake or biodegradation. In this study the potential impact of cation competition from Cu on o,o-FeEDDHA concentrations under calcareous soil conditions has been evaluated through modelling and batch experiments.

Predictions with mechanistic multi-surface models show there is a thermodynamic basis for assuming that under equilibrium conditions a certain fraction of o,o-EDDHA ligands in soil solution can be chelated to Cu, in particular of meso o,o-EDDHA. The high affinity of o,o-CuEDDHA for the soil solid phase, demonstrated in a batch interaction experiment, greatly increases the potential impact of Cu competition; for a given quantity of o,o-CuEDDHA in soil solution, a much larger quantity of o,o-CuEDDHA is adsorbed to the solid phase. Finally, the displacement of Fe from o,o-FeEDDHA by Cu was reproduced in a single-surface goethite suspension, demonstrating that the displacement reaction is not kinetically inhibited. With these results, the boundary conditions are met for explaining the observed gradual decline in meso o,o-FeEDDHA concentration with Cu competition.

# Introduction

The environmental fate of aminocarboxylate chelating agents (e.g. ethylene diamine tetra acetic acid (EDTA) and diethylene triamine penta acetic acid (DTPA)) has received considerable attention, in particular in view of the environmental persistence of certain aminocarboxylate chelates (Tandy, et al., 2004), and the risk of mobilizing and leaching heavy metals to the groundwater (Wenzel, et al., 2003; Wu, et al., 2004). Aminocarboxylate chelating agents are amongst others widely applied in micronutrient fertilizers, for their outstanding ability to form soluble complexes with di- and trivalent cations. FeEDDHA (iron (3+) ethylene diamine-N,N'-bis(hydroxy phenyl acetic acid)) is among the most effective fertilizers in preventing and remedying Fe deficiency chlorosis in plants grown on alkaline and calcareous soils (Wallace, et al., 1955). The current world market for FeEDDHA products is in the order of 10 thousand metric tons per year, the majority of which is sold in the Mediterranean countries and the Middle East.

FeEDDHA products are produced through a Mannich-like reaction (Petree, et al., 1978), resulting in a mixture of positional isomers, diastereomers and polycondensates. The quantitatively most important components are the isomers: 1) racemic o,o-FeEDDHA (iron (3+) (R,R) and (S,S) ethylene diamine-N,N'-bis(2-hydroxy phenyl acetic acid) complexes), 2) meso o,o-FeEDDHA (iron (3+) (R,S) = (S,R) ethylene diamine-N,N'-bis (2-hydroxy phenyl acetic acid) complex) and 3) o,p-FeEDDHA (iron (3+) ethylene diamine-N-(2-hydroxy phenyl acetic acid)-N'-(4-hydroxy phenyl acetic acid) complexes). The physical and chemical properties of the isomers differ strongly, and so do their behaviour in the soil and their performance in delivering Fe to plants. The effectiveness of FeEDDHA treatments in soil application is determined by the amount of o,o-FeEDDHA (racemic and meso o,o-FeEDDHA combined) (Schenkeveld, et al., 2008). The environmental fate of the o,o-FeEDDHA isomers is however still poorly understood.

In a recent pot trial study by Schenkeveld et al (2010a), a gradual, exponential decline in meso o,o-FeEDDHA concentration was observed, which was not caused by plant related processes or biodegradation (Schenkeveld, et al., 2010c). Cation competition has been suggested as a possible cause. Cation displacement reactions commonly occur in soil and surface water with synthetic aminocarboxylate chelating agent released into the environment (Nowack, 2002).

Studies examining cation competition under soil conditions in relation to FeEDDHA have mainly focused on Cu (Garcia-Marco, et al., 2006; Yunta, et al., 2003a); EDDHA isomers have a high affinity for Cu (Bannochie and Martell, 1989; Yunta, et al., 2003a; Yunta, et al., 2003b), the Cu content of soils is generally sufficiently high to potentially compromise the effectiveness of FeEDDHA treatments, and formation of o,p-CuEDDHA has been demonstrated upon interaction of o,p-FeEDDHA with soil (Garcia-Marco, et al., 2006; Schenkeveld, et al., 2007).

Thus far, soil applied o,o-FeEDDHA was assumed not to be affected by competition from Cu. This is supported by results from model calculations by Yunta et al. (2003a). Moreover, contrary to o,p-FeEDDHA, the interaction of o,o-FeEDDHA with soil does not lead to a strong increase in Cu concentration in soil solution (Alvarez-Fernandez, et al., 2002;

Schenkeveld, et al., 2007) Nonetheless, an impact of Cu on the concentration of o,o-FeEDDHA isomers in soil solution cannot be excluded for the following reasons: 1) Cation displacement reactions from aminocarboxylate chelating agents often have slow reaction kinetics (Nowack, 2002), which fits the observed gradual nature of the decline in meso o,o-FeEDDHA. 2) o,o-CuEDDHA may have a high affinity for soil reactive surfaces causing it to adsorb rather than to increase the Cu concentration in soil solution. 3) The predictive value of the model calculations by Yunta et al. (2003a) is compromised by over-simplification, in particular regarding the description of Cu speciation in the soil through a single solid phase binding constant.

It is essential to further the understanding of processes (potentially) compromising the effectiveness of FeEDDHA in soil application for improving soil-specific Fe fertilizing strategies and for developing alternative Fe fertilizers. The aim of this study was to re-evaluate the potential impact of competition from Cu on the effectiveness of soil-applied o,o-FeEDDHA isomers. Re-evaluation of Cu competition was addressed in 3 stages: 1) the thermodynamic basis underlying the potential threat of Cu, displacing Fe from o,o-FeEDDHA isomers was examined through mechanistic multi-surface modelling, 2) the behaviour of o,o-CuEDDHA was examined upon interaction with soil, and 3) cation competition from Cu was examined in single-surface goethite suspension systems.

## Model description

The distribution of Cu over soil reactive surfaces was modelled for 8 actual soils (6 calcareous soils and 2 reference soils). Addition of EDDHA isomers to these soil systems was simulated and the resulting Cu and EDDHA isomer speciation were examined. Validation of the 8 soil system models was done through comparison of the free Cu concentrations in simulations without EDDHA addition with the corresponding free Cu concentrations in $CaCl_2$ extracts.

The computer program ECOSAT (Keizer and Van Riemsdijk, 1994) was used for chemical speciation calculations. Complexation and protonation constants of the EDDHA isomers were taken from Yunta et al. (2003a; 2003b), and corrected to I = 0 with the Davies equation. Cu speciation in soil systems was modelled using a multi-surface approach, in which soil is considered as a set of independent reactive surfaces (Weng, et al., 2001). By combining surface complexation models and a model describing the aquatic chemistry, the Cu speciation in the solid and the soil solution phase can be well described (Dijkstra, et al., 2004; Fest, et al., 2005; Weng, et al., 2001). Binding to solid and dissolved organic matter (respectively SOM and DOM) was described with the Non-Ideal consistent Competitive Adsorption (NICA)-Donnan model (Kinniburgh, et al., 1999). SOM and DOM were assumed to consist for 50% of organic carbon. Humic acid (HA) was used as a model analogue for SOM. The maximum binding capacity ($Q_{max}$) of SOM was assumed to be one third of the binding capacity of HA (5.7 mol $kg^{-1}$ eq) (Weng, et al., 2001). DOM was modelled comprising 30% HA, 30% fulvic

acid (FA) and 40% inert material (Weng, et al., 2002). Generic NICA-Donnan parameters for metal and proton binding to HA and FA were taken from Milne et al. (2001; 2003).
Binding to crystalline Fe(hydr)oxide surfaces was described with the Charge Distribution Multi-Site Complexation (CD-MUSIC) model (Hiemstra and Van Riemsdijk, 1996; 1999) using goethite as model analogue. Binding to amorphous Fe(hydr)oxide surfaces was described with the two site Diffuse Double Layer (DDL) model (Dzombak and Morel, 1990) using hydrous ferric oxide (HFO) as model analogue. For both models, parameters describing metal and proton binding were taken from Weng et al. (2001).
Binding to Al- and Mn(hydr)oxide surfaces as well as to clay minerals was disregarded. Non-specific adsorption to clay and organic matter were not included, because the model describing Donnan-phase adsorption, as implemented at present in ECOSAT, proved inadequate at high soil solution ratios. It was tested that the effect of excluding non-specific adsorption of Cu on Cu speciation in the soil solid phase and on the free Cu concentration was negligible.

The reactive Cu content of the soils was determined with a 0.43 M $HNO_3$ extraction (Fest, et al., 2005; Tipping, et al., 2003); the amorphous Fe(hydr)oxide content with an ammonium-oxalate extraction (Schwertmann, 1964), and the crystalline Fe(hydr)oxide content was determined from the difference in Fe extracted between a dithionite-citrate (Holmgren, 1967), and an ammonium-oxalate extraction. The free Cu concentration in 0.01 M $CaCl_2$ extracts was calculated from the measured pH, and the dissolved organic carbon (DOC) and total Cu concentrations using the NICA-Donnan model (Weng, et al., 2002).
Soil conditions were modelled largely in accordance with the conditions from the pot trial presented in Schenkeveld et al. (2008). Soil-solution ratios corresponded to 50% of the water holding capacity of the soils. Ionic strength was fixed at 0.1 M; 30 mM $CaCl_2$ was used as background electrolyte. pH-$CaCl_2$ was used as input for the pH. Fe activity was imposed by the solubility of Fe(hydr)oxides; the solubility product of $Fe(OH)_3$ was set at $10^{-40}$. The effects of EDDHA addition to soil, up to a pore water concentration of 0.1 mM, were simulated separately per EDDHA isomer. Competition effects from metals other than Fe, Cu and Ca were not taken into account. Adsorption of EDDHA ligands and complexes was not included into the model. Data on soil characteristics, used as model input, are presented in Table 7.1.

# Experimental section

### Materials

*Soils* – 8 soil samples were collected from seven sites, located in Italy (Bologna), Spain (Xeraco and Santomera), Saudi Arabia (Nadec and Hofuf) and the Netherlands (Droevendaal and Herveld) and are described in more detail in Schenkeveld et. al (2007). Pre-treatment consisted of drying (40 °C) and sieving (2 mm). Relevant soil characteristics are presented in Table 7.1.

**Table 7.1:** Soil characteristics.

| Name (origin) | Country | Soil class. | Water holding capacity ($g\ kg^{-1}$) | pH-$CaCl_2$[a] | SOM[b] ($g\ kg^{-1}$) | Clay[c] ($g\ kg^{-1}$) | $CaCO_3$[d] ($g\ kg^{-1}$) | 0.01 M $CaCl_2$ | | Dithionite[g] | Oxalate[h] | 0.43 M $HNO_3$[i] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | DOC[f] ($mg\ kg^{-1}$) | Cu ($\mu g\ kg^{-1}$) | Crystalline Fe(hydr)-oxide ($g\ kg^{-1}$) | Amorphous Fe(hydr)-oxide ($g\ kg^{-1}$) | Cu ($mg\ kg^{-1}$) |
| **Santomera** | ES | entisol | 320 | 8.0 | 10.8 | 260 | 520 | 55 | 62 | 8.1 | 0.57 | 10.1 |
| **Xeraco L** | ES | entisol | 350 | 7.7 | 27.4 | 360 | 150 | 108 | 54 | 21.3 | 1.72 | 12.6 |
| **Bologna** | IT | entisol | 560 | 7.8 | 17.4 | 230 | 140 | 107 | 75 | 10.1 | 2.95 | 9.1 |
| **Herveld** | NL | spodosol | 360 | 7.0 | 30.3 | 260 | 30 | 177 | 137 | 10.6 | 4.46 | 15.1 |
| **Xeraco T** | ES | entisol | 330 | 7.6 | 87.4 | 100 | 420 | 281 | 59 | 4.5 | 3.21 | 4.8 |
| **Nadec** | SA | aridisol | 190 | 8.0 | 17.5 | 70 | 140 | 101 | 15 | 2.2 | 0.25 | 0.8 |
| **Hofuf** | SA | aridisol | 180 | 7.8 | 14.2 | 40 | 60 | 78 | 127 | 0.7 | 0.36 | 6.3 |
| **Droevendaal** | NL | spodosol | 260 | 5.3 | 30.3 | 40 | 0 | 108 | 44 | 1.3 | 3.21 | 4.5 |

[a] ISO/DIS 10390 Soil Quality – Determination of pH

[b] Walinga et al. (1992)

[c] Houba et al. (1997)

[d] ISO 10693, Soil Quality – Determination of carbonate content, volumetric method

[e] ISO/DIS 11260 Soil Quality – Determination of cation exchange capacity and base saturation – method using barium chloride solution

[f] Houba et al. (2000)

[g] Holmgren (1967); figures have been corrected for the Fe extracted with oxalate

[h] Schwertmann (1964)

[i] Tipping et al. (2003) and Fest et al. (2005)

*Goethite* - A stock goethite suspension was prepared from $Fe(NO_3)_3$ according to Atkinson et al. (1967) as described in more detail by Hiemstra et al. (1989). The BET($N_2$) specific surface area amounted 98.6 $m^2$ $g^{-1}$ and EC was below 10 µS.
*Reagent solutions* - Racemic o,o-$H_4$EDDHA (purity: 100%) and meso o,o-$H_4$EDDHA (purity: 99.5%) were obtained by separation of an o,o-$H_4$EDDHA mixture (99% pure; 49% racemic o,o-EDDHA, 51% meso o,o-EDDHA), as described in Bannochie and Martell (1989) and Bailey et al. (1981). EDDHA solutions were prepared by dissolving racemic and meso o,o-$H_4$EDDHA (either separated or as a mixture) by adding sufficient 1 M NaOH. Metal-EDDHA solutions were prepared by adding dissolved metal chloride salt to EDDHA solution in a 2% excess based on a 1:1 stoichiometry between metal and EDDHA. The pH was raised to 7 (± 0.5). FeEDDHA solutions were left over-night in the dark in order for excess Fe to precipitate as hydroxides. The following day, FeEDDHA solutions were filtered over a 0.45 µm nitro cellulose micro pore filter (Schleicher & Schuell, ref-no: 10401114).
$CO_2$-free 0.1 M NaOH solution was prepared from Titrasol. MOPS-buffer was prepared by dissolving solid MOPS (3-(N-Morpholino)-propanesulfonic acid) in pre-boiled ultra pure water, and was made to pH by adding 0.1 M NaOH solution. MOPS does not complex transition metals nor does it interfere in spectrophotometric measurement at wavelengths above 250 nm (Yu, et al., 1997). Both MOPS- and NaOH solution were stored in a desiccator to avoid $CO_2$ contamination. Stock and experimental solution were prepared from analytical grade chemicals and ultra pure water. The composition of experimental solutions was examined at t=0 and at the end of the experiment.

**Experiments**
*Soil interaction experiment* – The interaction between o,o-CuEDDHA and the aforementioned 8 soils was examined in a 1-week 1:1 (w/v) interaction experiment in triplicates. The experiment involved 2 treatments: 1) a blank treatment, and 2) a 142 µM o,o-CuEDDHA solution (70 µM racemic o,o-CuEDDHA and 72 µM meso o,o-CuEDDHA; in total 9 mg $l^{-1}$ Cu). In both treatments ionic strength was imposed with 0.01 M $CaCl_2$.
The soil interaction experiment was executed in 50 ml polypropylene test tubes (Greiner bio-one, Cat No 210296). The tubes were placed in an end-over-end shaker, rotating at 18 rpm in absence of light. Room temperature was kept at 20 (± 1) °C. After interaction, the samples were centrifuged for 10 minutes at 3000 rpm. The pH and EC of the supernatant were measured. The supernatant was filtered over a 0.45 µm cellulose acetate micro pore filter (Schleicher & Schuell, ref no: 10462650) and the filtrate was further analyzed by ICP and HPLC.
*Goethite suspension experiment* - EDDHA speciation was examined as a function of time in a 10 g $l^{-1}$ goethite suspension containing 90 µM Cu, 90 µM "fresh" Fe[4] and 90 µM EDDHA. EDDHA was introduced into suspension as either: 1) FeEDDHA, 2) CuEDDHA or 3) EDDHA-ligand. Depending on the scenario, metals were introduced into suspension, either as chelate or as dissolved metal chloride salt. Fe addition in scenario 2 and 3 was done to harmonize the solubility of the Fe(hydr)oxide phase for all experiments in relation to the

[4] "fresh" refers to newly introduced Fe, (initially) not included into the goethite crystal matrix.

(potential) fresh precipitation of displaced Fe from FeEDDHA in scenario 1. pH was fixed at 7 and I = 0.1 M. The experiments were separately conducted for racemic and meso o,o-EDDHA and executed in duplicates.

NaCl, and when required, $CuCl_2$ and $FeCl_3$ solutions were added to a portion of the goethite stock solution. pH was lowered to 5.5. The suspensions were purged overnight with washed (1 M $H_2SO_4$/1 M NaOH), moist $N_2$ gas to remove $CO_2$. The following day, the pH was raised to around 7 with 0.1M NaOH and a MOPS buffer was added (0.005 M; pH 7 ± 0.05). Suspensions were made to volume with pre-boiled water. 0.9 mM FeEDDHA, CuEDDHA or EDDHA solution, purged with washed, moist $N_2$ gas for 30 minutes prior to addition, was added to the goethite suspension in a 100 ml polytethylene bottle under $N_2$-atmosphere in a ratio 1:9 (v/v). The moment of addition was recorded as t=0. The suspension was stirred and divided over 10 gas-tight 23.6 ml low-density polyethylene bottles (Rietra, et al., 2001), which had been flushed with $N_2$; 5 ml of goethite suspension was added to each bottle. The bottles were placed in an end-over-end shaker, rotating at 24 rpm in absence of light. Room temperature was kept at 20 (± 1) °C. Sampling was done destructively after 0.5; 1.25; 2; 4; 8; 24; 48; 96; 192 and 336 hours. The samples were centrifuged for 10 minutes at 10,000 rpm. The supernatant was filtered over a 0.45 μm cellulose acetate micro pore filter (Schleicher & Schuell, ref no: 10462650). The filtrate was further analysed by UV-VIS spectrophotometry.

**Analysis**

Al, Co, Cu, Mn, Ni and Zn concentrations were measured by ICP-MS (Perkin Elmer, ELAN 6000), Ca, Mg and Fe concentrations by ICP-AES (Varian, Vista Pro). Samples were acidified with nitric acid before ICP-measurement.

In soil extracts, racemic and meso o,o-FeEDDHA concentrations were determined after separation through high-performance liquid chromatography (HPLC) as described in Schenkeveld et al. (2007). The concentrations of other metal-EDDHA chelates (racemic and meso o,o-EDDHA combined) were calculated from the difference in metal concentration between the treatment involving EDDHA and the blank treatment.

EDDHA and metal-EDDHA concentrations in the supernatant of goethite suspensions were determined through spectrophotometry with a double beam spectrophotometer (Unicam Helios α) using disposable UltraVette cuvettes (BrandTech Scientific), with a 10 mm light path. Local absorption maxima of EDDHA compounds were determined by scanning from 250 to 900 nm (Table A7.1 in the Appendix). An external calibration method was used to determine the concentrations of the EDDHA compounds. Absorbance was measured at wavelengths corresponding to absorption maxima of all EDDHA compounds that could be present in the sample. Measured absorbances were considered linear combinations of the absorbances of the individual EDDHA compounds. EDDHA compound concentrations were calculated from the measured absorbances by a smallest sum of squares method.

# Results and Discussion

**Modelling Cu and EDDHA speciation in soil systems**

Prior to the soil system simulations, factors affecting EDDHA and Cu speciation in soil solution were individually assessed. Results are presented in the Appendix and are briefly summarized below.

The extent to which EDDHA isomers chelate Fe and Cu is largely determined by pH, Fe and Cu activity and the specific affinity of the EDDHA isomers for Fe and Cu (Figure A7.1 in the Appendix). In soil systems, Fe activity is mainly governed by pH and solubility of the Fe(hydr)oxide phase. Because a soil specific Fe(hydr)oxide solubility is not easily determined, but its impact on EDDHA speciation can be considerable, it is an important source of uncertainty in the soil system models. Cu activity mainly depends on pH, Cu content and the contents of soil reactive surfaces (Figure A7.2 in the Appendix). The sequence in susceptibility of the EDDHA isomers to Cu competition based on the relative affinity for Cu in comparison to Fe is: o,p-EDDHA >> meso o,o-EDDHA > racemic o,o-EDDHA (Figure A7.1a in the Appendix). Complexation of Cu instead of Fe is favoured by a high pH, a high soil Cu content, low contents of soil reactive surfaces binding Cu, and a low solubility of the Fe(hydr)oxide phase.

A summary of results from the soil system simulations is presented in Table 7.2. Results are clustered per soil and include free Fe and Cu concentration, EDDHA speciation upon addition of EDDHA isomer, and Cu distribution over the soil reactive surfaces.

$pFe^{3+}$ ranges from 12.9 to 21.0. Because $pFe^{3+}$ is imposed by the solubility of the Fe(hydr)oxide phase, it is a mere function of pH, and unaffected by EDDHA addition. $pCu^{2+}$ ranges from 9.3 to 15.0. EDDHA addition (0.1 mM) does not affect $pCu^{2+}$ by more than 0.3 unit, except in the Nadec soil (0.9 unit). This soil has by far the lowest $HNO_3$-extractable Cu content, and becomes almost 50% Cu-depleted upon o,p-EDDHA addition. The fact that Fe and Cu activity are generally hardly affected by EDDHA speciation, implies that the concentration ratio in which FeEDDHA and CuEDDHA occur in soil solution is barely affected by not including specific adsorption of EDDHA complexes into the models.

In all soils, most Cu is adsorbed to SOM; without EDDHA addition, SOM accounts for 98.0 -100% of the adsorbed Cu in sandy soils and for 59.4 - 92.7% in clay soils. Goethite substantially contributes to Cu adsorption in clay soils (6.2 - 40.4%). The contribution of HFO to Cu adsorption is almost negligible (0 - 1.0%). In sandy soils, Cu chelated by EDDHA originates almost entirely from SOM; only in the Hofuf soil, goethite modestly contributes (4.9%). In clay soils, both SOM (33.8 - 83.0%) and goethite (14.6 - 65.8%) substantially contribute. The relative decrease in adsorbed Cu content as a result of EDDHA addition is larger for goethite than for SOM in all soils. The fraction of soil-Cu that becomes chelated upon EDDHA addition ranges from 0 to 2.5% for racemic o,o-EDDHA, from 0 to 8.1% for meso o.o-EDDHA and from 6.5 to 51.3% for o,p-EDDHA.

**Table 7.2:** EDDHA and Cu speciation in the modelled soil systems.

| Soil | Treatment | Free metal conc. | | EDDHA speciation | | Cu distribution | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $pFe^{3+}$ | $pCu^{2+}$ | FeEDDHA (µM) | CuEDDHA (µM) | Cu SOM | Cu goethite | Cu HFO | CuEDDHA |
| Santomera (ES) | blank | 21.0 | 10.9 | - | - | 59.4% | 40.4% | 0.2% | 0.0% |
| (clay) | racemic o,o | 21.0 | 10.9 | 74.9 | 25.1 | 58.6% | 38.7% | 0.2% | 2.5% |
| | meso o,o | 21.0 | 10.9 | 27.7 | 72.3 | 56.9% | 35.6% | 0.2% | 7.2% |
| | o,p | 21.0 | 11.0 | 0.0 | 100.0 | 55.9% | 33.9% | 0.2% | 10.0% |
| Xeraco L (ES) | blank | 20.1 | 11.0 | - | - | 81.6% | 18.1% | 0.2% | 0.0% |
| (clay) | racemic o,o | 20.1 | 11.0 | 95.4 | 4.6 | 81.4% | 18.0% | 0.2% | 0.4% |
| | meso o,o | 20.1 | 11.0 | 68.0 | 32.0 | 79.9% | 17.0% | 0.2% | 2.8% |
| | o,p | 20.1 | 11.1 | 0.0 | 100.0 | 76.2% | 14.8% | 0.2% | 8.8% |
| Bologna (IT) | blank | 20.4 | 11.0 | - | - | 80.5% | 18.8% | 0.7% | 0.0% |
| (clay) | racemic o,o | 20.4 | 11.0 | 92.2 | 7.8 | 79.6% | 18.2% | 0.7% | 1.5% |
| | meso o,o | 20.4 | 11.1 | 58.6 | 41.4 | 75.6% | 15.7% | 0.6% | 8.1% |
| | o,p | 20.4 | 11.2 | 0.0 | 100.0 | 68.3% | 11.6% | 0.4% | 19.6% |
| Herveld (NL) | blank | 18.0 | 9.8 | - | - | 92.7% | 6.2% | 1.0% | 0.0% |
| (clay) | racemic o,o | 18.0 | 9.8 | 97.8 | 2.2 | 92.6% | 6.2% | 1.0% | 0.2% |
| | meso o,o | 18.0 | 9.8 | 78.2 | 21.8 | 91.3% | 6.0% | 1.0% | 1.7% |
| | o,p | 18.0 | 9.9 | 0.0 | 100.0 | 86.4% | 5.1% | 0.9% | 7.6% |
| Xeraco T (ES) | blank | 19.8 | 13.3 | - | - | 100.0% | 0.0% | 0.0% | 0.0% |
| (sand) | racemic o,o | 19.8 | 13.3 | 100.0 | 0.0 | 100.0% | 0.0% | 0.0% | 0.0% |
| | meso o,o | 19.8 | 13.3 | 99.8 | 0.2 | 99.9% | 0.0% | 0.0% | 0.0% |
| | o,p | 19.8 | 13.6 | 10.1 | 89.3 | 80.6% | 0.0% | 0.0% | 19.4% |
| Nadec (SA) | blank | 21.0 | 14.1 | - | - | 99.9% | 0.1% | 0.0% | 0.0% |
| (sand) | racemic o,o | 21.0 | 14.1 | 100.0 | 0.0 | 99.9% | 0.1% | 0.0% | 0.0% |
| | meso o,o | 21.0 | 14.1 | 99.8 | 0.2 | 99.8% | 0.1% | 0.0% | 0.1% |
| | o,p | 21.0 | 15.0 | 31.2 | 66.7 | 48.7% | 0.0% | 0.0% | 51.3% |
| Hofuf (SA) | blank | 20.4 | 11.0 | - | - | 98.0% | 1.9% | 0.1% | 0.0% |
| (sand) | racemic o,o | 20.4 | 11.0 | 91.7 | 8.3 | 97.3% | 1.8% | 0.1% | 0.7% |
| | meso o,o | 20.4 | 11.0 | 55.6 | 44.4 | 94.2% | 1.7% | 0.1% | 4.0% |
| | o,p | 20.4 | 11.1 | 0.0 | 100.0 | 89.5% | 1.5% | 0.1% | 8.9% |
| Droevendaal (NL) | blank | 12.9 | 9.3 | - | - | 100.0% | 0.0% | 0.0% | 0.0% |
| (sand) | racemic o,o | 12.9 | 9.3 | 100.0 | 0.0 | 100.0% | 0.0% | 0.0% | 0.0% |
| | meso o,o | 12.9 | 9.3 | 99.9 | 0.1 | 99.9% | 0.0% | 0.0% | 0.0% |
| | o,p | 12.9 | 9.4 | 62.2 | 35.6 | 93.5% | 0.0% | 0.0% | 6.5% |

All EDDHA isomers chelate Cu to some degree when introduced into soil systems. For the o,o-EDDHA isomers this particularly concerns the clay soils and Hofuf soil: 2.2 - 25.1% of racemic o,o-EDDHA and 21.8 - 72.3% of meso o,o-EDDHA is chelated to Cu. For the remaining sandy soils, less than 1% of racemic and meso o,o-EDDHA is chelated to Cu. In Droevendaal soil this is due to the lower pH, favouring Fe complexation, and in Nadec and Xeraco T soil, due to the very low Cu activity resulting from respectively a low soil Cu content and a relatively high SOM content. O,p-EDDHA is exclusively chelated to Cu in all clay soils and for 35.6 to 100% in sandy soils. In the sandy soils other than Hofuf, a small fraction of o,p-EDDHA occurs as mere ligand, not chelated to Fe or Cu.

*Model validation and discussion*

$pCu^{2+}$ values, calculated through multi-surface modelling, were compared with $pCu^{2+}$ values, calculated from the pH, and the total Cu and DOC concentration in 0.01 M $CaCl_2$-extracts. Corresponding $pCu^{2+}$ values were within 1 order of magnitude for all soils, except for Xeraco L soil (1.3 units; Figure 7.1). Conformity was higher for sandy soils, with a maximum deviation of 0.2 units. The range of $pCu^{2+}$ determined in sandy soils (9.3 - 14.1) supplements the range (4.9 - 7.9), successfully determined through multi-surface modelling by Weng et al. (2001).

For clay soils, $pCu^{2+}$ predicted by multi-surface modelling is structurally lower (0.8 - 1.3 units); the soil with the highest clay content having the largest deviation. The lack of a surface complexation model describing specific Cu binding to clay edges may contribute to the consequent overestimation of Cu activity in clay soils. EDDHA speciation was re-calculated for the clay soils using the (calculated) free Cu concentrations in the $CaCl_2$-extracts (Table A7.2 in the Appendix): 0.2 - 4.3% of racemic o,o-EDDHA, 2.5 - 27.4% of meso o,o-EDDHA and 99.6 – 100% of o,p-EDDHA is chelated to Cu.

The assumed solubility of the Fe(hydr)oxide phase is conservative with respect to Cu competition; $pK_{sol}$ = 40 is only 0.7 units above the average solubility of soil-Fe (Lindsay, 1979). In principle, the most soluble Fe(hydr)oxide phase determines Fe solubility. However, in highly weathered soils where crystalline Fe(III)oxides predominate, hematite ($pK_{sol}$ = 41.9) and goethite ($pK_{sol}$ = 42.0) are expected to control $Fe^{3+}$ solubility (Lindsay, 1988). Crystalline Fe(hydr)oxide is dominant in all modeled soil systems except Droevendaal soil. In Santomera, Xeraco L and Nadec soil, the crystalline Fe(hydr)oxide content exceeds the amorphous Fe(hydr)oxide content by an order of magnitude (Table 7.1); in particular for these soils $pK_{sol}$ probably exceeds 40. A decrease in solubility of Fe(hydr)oxide favours Cu complexation (see Figure A7.1b in the Appendix). Due to the uncertainty in solubility of the Fe(hydr)oxide phase, the model outcomes regarding o,o-EDDHA speciation should be interpreted as affirmation of the potential impact of competition from Cu on the concentration of o,o-FeEDDHA, in particular of the meso isomer, rather than as exact predictions of the concentration ratios between o,o-FeEDDHA and o,o-CuEDDHA.

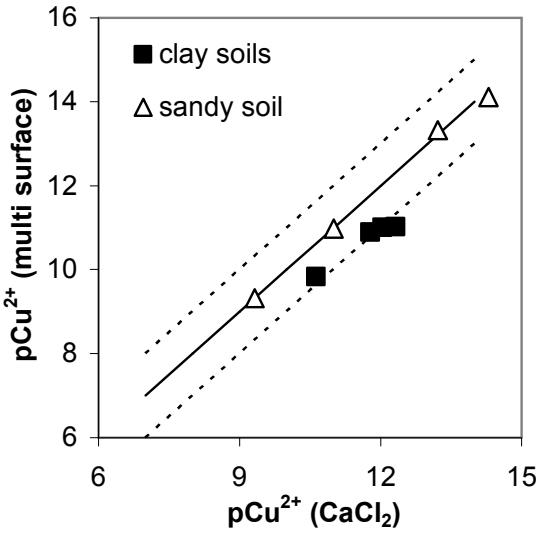



**Figure 7.1:** Comparison of free Cu concentrations, predicted through multi-surface modelling, and calculated from the pH, and the total Cu and DOC concentration in $CaCl_2$-extracts. The solid line represents the 1:1 line; the dashed lines represent the ± 1 log unit interval.

In a model study by Yunta et al. (2003a), no effect from Cu competition on o,o-FeEDDHA concentration was predicted under soil conditions within the pH range 7 - 8. Yunta et al. described Cu adsorption to soil as a precipitation equilibrium of Cu(hydr)oxide, with $pK_{sol}$ = 25.2, which is six log units above the actual $pK_{sol}$ of Cu(hydr)oxide: 19.3 (Lindsay, 1979)). This approach does not take into account soil-Cu or reactive surface contents and may lead to a strong underestimation of the Cu activity in soil solution. Furthermore, Yunta et al. assumed a higher solubility for the Fe(hydr)oxide phase ($pK_{sol}$ = 39.3; an average value for soils, reported by Lindsay (1979)), than in the present study ($pK_{sol}$ = 40), thereby favouring complexation of Fe. Without determining the free $Fe^{3+}$ concentration, the assumption for $pK_{sol}$ remains arbitrary. Because of the generally high ratio between crystalline and amorphous Fe(hydr)oxide content in the simulated calcareous soils (Table 7.1), a $pK_{sol}$ somewhat above average seemed more appropriate.

In describing o,p-EDDHA speciation in "normal calcareous soils", Yunta et al. accounted for (limited) soil-Cu availability by imposing a maximum o,p-CuEDDHA concentration ($10^{-5}$ M). The assumption underlying this maximum was that Cu concentrations in soil solution only become as high as $10^{-4}$ M in Cu-contaminated soils. This assumption is correct under "normal" conditions, but erroneous when chelating agents are added to the soil. None of the soils simulated in this study is Cu contaminated according to Dutch law or even exceeds the former remediation target value (Circulaire bodemsanering 2006 & 2009). Still, in most of these soils, all o,p-EDDHA is predicted to be chelated to Cu, up to a concentration of $10^{-4}$ M (Table 7.2). In conclusion, Yunta et al. potentially underestimate the impact of Cu competition as a result of their modelling approach and assumptions.

**Interaction experiment of o,o-CuEDDHA with soils**

The impact of Cu competition on o,o-FeEDDHA concentration in soil solution does not only depend on the solution equilibrium, as discussed in the previous section, but also on the extent to which o,o-CuEDDHA adsorbs to the solid phase (Figure 7.2). Therefore, adsorption of o,o-CuEDDHA to eight soils was examined. In seven out of eight soils over 80%, and in five soils over 95% of the added o,o-CuEDDHA was removed from soil solution within one week (Figure 7.3). Only in 3 soils o,o-CuEDDHA was replaced by o,o-FeEDDHA for more than 15%, including the 2 Dutch reference soils with a lower pH (Table 7.1). o,o-FeEDDHA adsorption accounted for a few percent of the removed o,o-CuEDDHA, at most (Schenkeveld, et al., 2010a). The removal of o,o-CuEDDHA in calcareous soils should therefore be largely attributed to adsorption, indicating a high affinity of o,o-CuEDDHA for the solid phase in comparison to o,o-FeEDDHA. O,o-CuEDDHA adsorption in calcareous soils is positively related to crystalline Fe(hydr)oxide and clay content. Fe displaced more Cu from meso o,o-CuEDDHA than from racemic o,o-CuEDDHA, indicating that equilibrium had not yet been reached (Figure A7.1a in the Appendix), and that displacement of Cu from meso o,o-CuEDDHA is kinetically favoured.

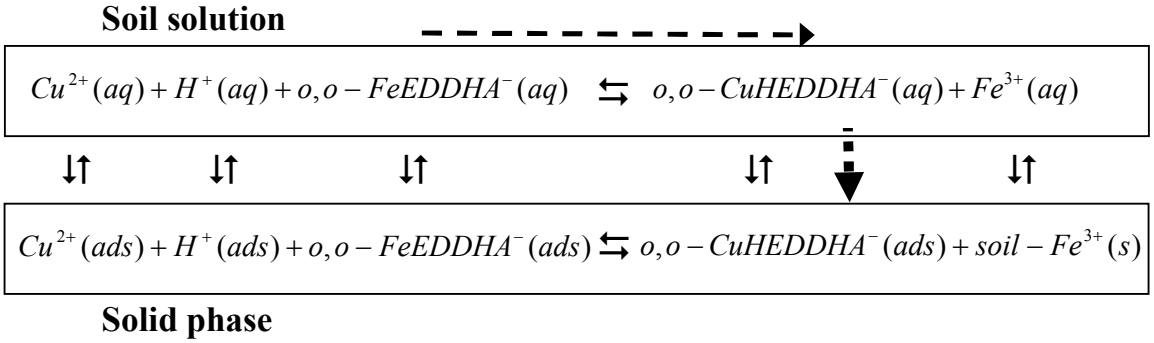



**Figure 7.2:** Chemical equilibria involved with Cu competition affecting the o,o-FeEDDHA concentration in soil solution. The dashed darts indicate that the impact of Cu competition also depends on the extent to which o,o-CuEDDHA adsorbs to the solid phase. Around pH 7, o,o-CuEDDHA chelates are mainly singly protonated. Hydroxylation of metals in the solid phase has been omitted.

The tendency of o,o-CuEDDHA to adsorb strongly increases the potential impact of Cu competition on the o,o-FeEDDHA concentration in soil solution. The ratio between the o,o-FeEDDHA and o,o-CuEDDHA concentration in soil solution is determined by Fe and Cu activity and pH (Figure 7.2; Figure A7.1b in the Appendix) and remains constant as long as these factors remain approximately constant (Table 7.2). Removal of o,o-CuEDDHA from solution will lead to a re-establishment of the solution equilibrium, which in turn will lead to more o,o-CuEDDHA adsorption, etc. Even if o,o-CuEDDHA accounts for a relatively small fraction in soil solution, the high affinity of CuEDDHA for the solid phase multiplies the

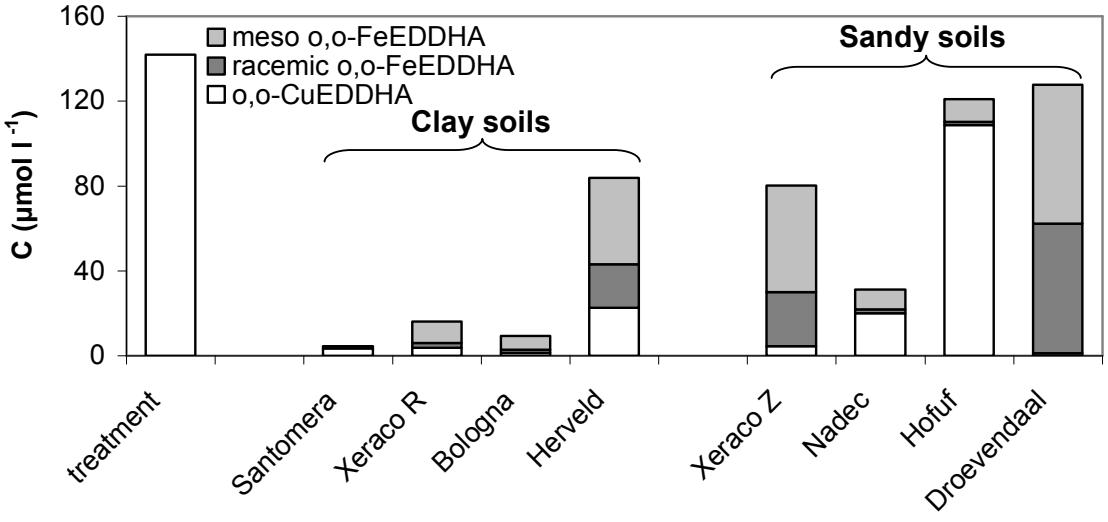



**Figure 7.3:** Measured o,o-FeEDDHA and o,o-CuEDDHA concentrations in soil solution upon interaction of o,o-CuEDDHA with 6 calcareous soils and 2 reference soils for 7 days.

overall effect of Cu competition on the o,o-FeEDDHA concentration, resulting in a much further o,o-FeEDDHA depletion than indicated by the presented model predictions.
In pot trials or under field conditions, the soil-solution ratio is even much higher, and more reactive surface area is available per unit pore water than in this soil interaction experiment. This will further shift the o,o-CuEDDHA adsorption equilibrium towards adsorption and may explain how Cu competition can affect o,o-FeEDDHA concentration without a substantial increase in Cu concentration in soil solution (Schenkeveld, et al., 2010a).

**Goethite suspension experiment**

Upon introduction into a goethite suspension containing Cu, both racemic and meso o,o-FeEDDHA concentrations decreased, and the concentrations of the corresponding o,o-CuEDDHA isomers increased (Figure 7.4). This indicates that displacement of Fe from o,o-FeEDDHA by Cu is not kinetically inhibited and can be reproduced in a well-defined system with a single adsorption surface, governing Fe and Cu activity, on a reasonable timescale. Introduction of o,o-CuEDDHA into a goethite suspension led to a partial displacement of Cu by Fe. Introduction of o,o-EDDHA into a goethite suspension containing Cu resulted in chelation and solubilization of both Fe and Cu (Figure 7.4). Meso o,o-FeEDDHA and both o,o-CuEDDHA isomers underwent an initial concentration drop upon introduction into suspension, as a result of adsorption to goethite. Adsorption was relatively fast in comparison to the displacement reaction.
Rates of displacement reactions involving meso o,o-EDDHA were higher than of corresponding reactions involving racemic o,o-EDDHA. This corresponds with the faster

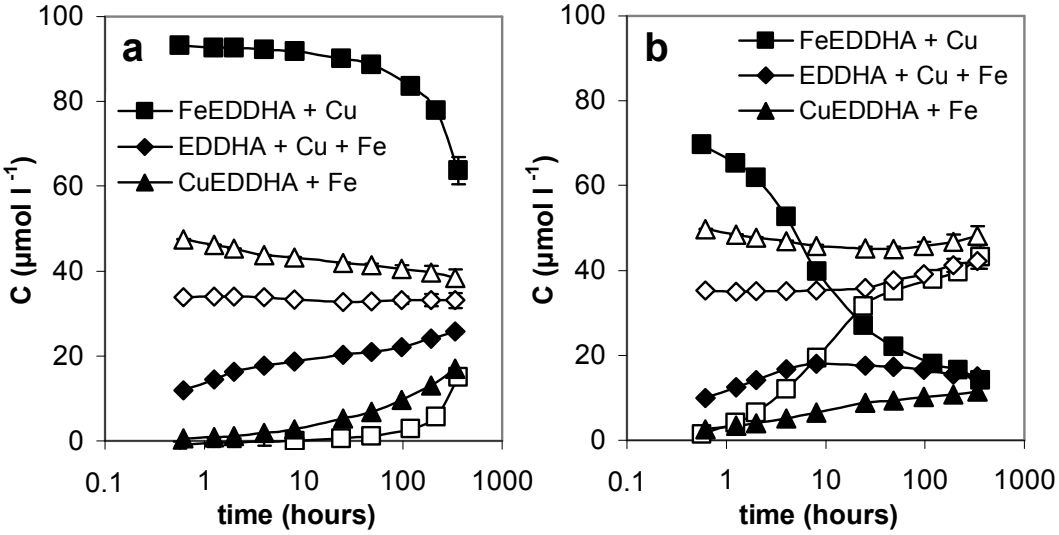



**Figure 7.4: a)** racemic o,o-EDDHA, and **b)** meso o,o-EDDHA speciation as a function of time in a 10 g $l^{-1}$ goethite suspensions with 90 µM EDDHA, 90 µM Cu, and 90 µM additional Fe; I = 0.1 M, pH = 7. Names of the treatments refer to EDDHA speciation (FeEDDHA, CuEDDHA or EDDHA) at t=0. Open symbols represent o,o-CuEDDHA concentrations, closed symbols represent o,o-FeEDDHA concentrations. Time is presented on a logarithmic scale. Error bars indicate standard deviations.

decline in meso o,o-FeEDDHA concentration in soil solution observed in pot and incubation studies (Schenkeveld, et al., 2010a; Schenkeveld, et al., 2010c); the rates of decline in goethite suspension were however much higher than in soil. Contrary to most soil systems in the soil interaction experiment, a substantial fraction of CuEDDHA (≈ 50%) did not adsorb. After 336 hours (i.e. 2 weeks), meso o,o-FeEDDHA (≈ 13.5 µM) and meso o,o-CuEDDHA (≈ 44.5 µM) concentrations from the three treatments had converged, implying (pseudo-) equilibrium was attained; for racemic o,o-EDDHA, this was not yet the case.

$pCu^{2+}$, $pFe^{3+}$ and $pK_{sol}$ (goethite) were calculated from the (pseudo-)equilibrium concentrations in the suspensions containing meso o,o-EDDHA. $pCu^{2+}$ was calculated with the CD-MUSIC model, from the amount of Cu adsorbed to goethite. By mass balance, the amount of adsorbed Cu equaled the total amount of meso o,o-FeEDDHA in suspension[5]. The latter was estimated by means of an adsorption isotherm (Figure A7.3 in the Appendix); 25% of the meso o,o-FeEDDHA was adsorbed to goethite. Subsequently, $pFe^{3+}$ was calculated by simulating the solution equilibrium in ECOSAT, and $pK_{sol}$ (goethite) was calculated from the

[5] $[EDDHA]_T = [CuEDDHA]_T + [FeEDDHA]_T$ ; $[Cu]_T = [CuEDDHA]_T + [Cu(ads)]$

$[EDDHA]_T = [Cu]_T = 90$ µM ; ergo $[Cu(ads)] = [FeEDDHA]_T$

[Cu(aq)] and [EDDHA] can be neglected

$pFe^{3+}$ and pH. $pCu^{2+}$ equaled 9.8, $pFe^{3+}$ equaled 18.6 and $pK_{sol}$(goethite) equaled 41.0. $pCu^{2+}$ and $pFe^{3+}$ values are in range with the values from the modeled soil systems (Table 7.2). The solubility of goethite was higher than reported in literature ($pK_{sol}$ = 42.0; Lindsay, 1988), presumably as a result of Fe addition to the suspension.
The treatment receiving meso o,o-EDDHA ligand reached (pseudo-)equilibrium most quickly. This observation is of interest in view of the shuttle mechanism of Fe chelates in soil-plant systems (Lindsay and Schwab, 1982), and suggests that by taking up Fe from o,o-FeEDDHA, which leads to a release of EDDHA ligand into soil solution, plants may enhance the rate at which FeEDDHA is replaced by CuEDDHA.

# Implications

This study has shown that there is a thermodynamic basis for assuming that cation competition from Cu can affect o,o-FeEDDHA concentrations in calcareous soils, in particular of the meso o,o-FeEDDHA isomer. The high affinity of o,o-CuEDDHA for the solid phase enhances the potential impact of Cu competition on the performance of FeEDDHA in soil application, while the increase in Cu concentration in soil solution upon displacement of Fe from o,o-FeEDDHA remains small. The displacement of Fe from FeEDDHA by Cu could be reproduced in a goethite suspension system, indicating the displacement is not inhibited by slow kinetics.
With these results, the boundary conditions are met for explaining the gradual decrease in meso o,o-FeEDDHA concentration, observed in pot trial and incubation studies (Schenkeveld, et al., 2010a; Schenkeveld, et al., 2010c) with Cu competition. Given the extent of the observed decline in these studies, the successful application of (meso) o,o-FeEDDHA in certain soils may be largely based on slow kinetics involved with Cu displacing Fe from o,o-FeEDDHA. Additional experimental research is needed to establish Cu competition related to o,o-FeEDDHA in soil systems and to examine which soil factors affect the rate of the displacement reaction.

# Appendix

**Effect of Cu activity, pH and Fe(hydr)oxide solubility on the preferential chelation of Fe and Cu by EDDHA isomers**

In a basic system with pH 7, an ionic strength of 0.1 M and a $K_{sol}(Fe(OH)_3)$ of $10^{-40}$, Fe accounts for over 99.4% of the metals chelated to any EDDHA isomers if Cu activity is below $10^{-16}$ (Figure A7.1a)[6]. If Cu activity increases above $10^{-16}$, EDDHA isomers successively start to chelate Cu to a substantial degree, and if Cu activity exceeds $10^{-6}$, all EDDHA isomers are for at least 99.8% chelated to Cu (Figure A7.1a). The sequence in relative affinity for Cu (compared to Fe) is: o,p-EDDHA >> meso o,o-EDDHA > racemic o,o-EDDHA. This sequence indicates the relative susceptibility of the corresponding FeEDDHA isomers to competition from Cu. A decrease in solubility of the Fe(hydr)oxide phase and an increase in pH lead to a decrease in Fe activity. For a fixed Cu activity this causes a shift in favour of CuEDDHA as illustrated for meso o,o-EDDHA in Figure A7.1b.

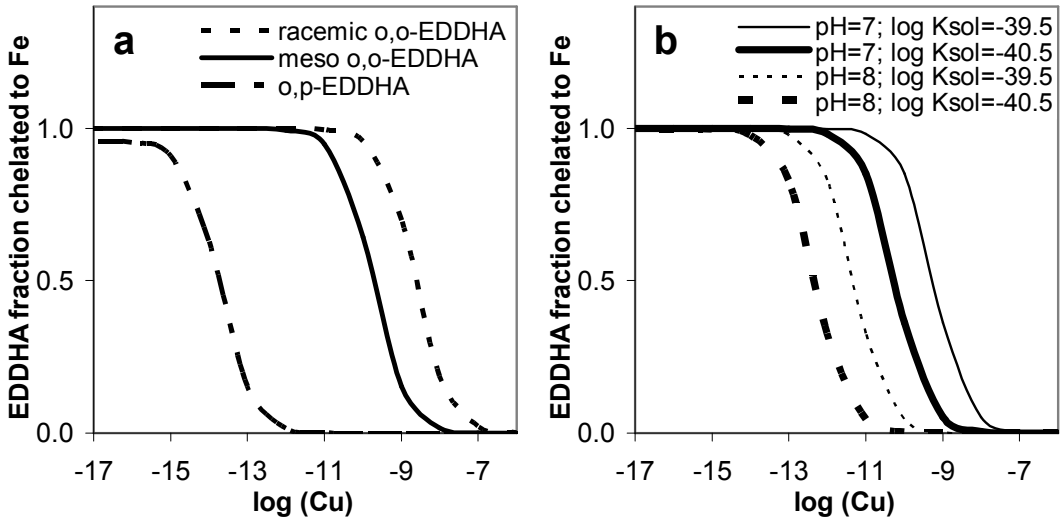



**Figure A7.1: a)** Calculated fraction of EDDHA isomers chelated to Fe as a function of Cu activity at pH 7, I = 0.1 M and $K_{sol}(Fe(OH)_3) = 10^{-40}$. The fraction not chelated tot Fe is chelated to Cu, except for o,p-EDDHA: at low Cu activity ($<10^{-12}$), a small fraction of o,p-EDDHA is present as mere ligand (4% at maximum, at Cu activity $10^{-17}$ ); **b)** Calculated fraction of meso o,o-EDDHA chelated to Fe as a function of Cu activity for I = 0.1 M, pH 7 and pH 8 and $K_{sol}(Fe(OH)_3) = 10^{-39.5}$ and $K_{sol}(Fe(OH)_3) = 10^{-40.5}$. The fraction not chelated tot Fe is chelated to Cu.

[6] At low Cu activity ($<10^{-12}$), a fraction of o,p-EDDHA (max 4%) is present as mere ligand. The complexation constant for o,p-FeEDDHA ($10^{28.7}$; I = 0.1 M) is relatively low in comparison to the complexation constants for racemic o,o-FeEDDHA isomers ($10^{35.9}$ ) and meso o,o-FeEDDHA ($10^{34.2}$) and Fe complexation by o,p-EDDHA is limited by the Fe(hydr)oxide solubility.

**Cu binding by reactive soil compounds**

Reactive soil compounds display different Cu adsorption behaviour. Their affinity for Cu is a function of Cu activity and pH, and can be expressed as a surface accumulation factor (log[Cu(ads) $g^{-1}$/(Cu)] ) (Figure A7.2a). The accumulation factor increases with decreasing Cu activity; for SOM this increase is linear throughout the examined range of Cu activities, while for Fe(hydr)oxides a maximum is reached. The horizontal parts of the Fe(hydr)oxide curves represent linear adsorption, implying impact of adsorbed Cu on the surface charge is negligible. At high Cu activity Fe(hydr)oxide surfaces become saturated with Cu, leading to a decrease in accumulation factor. The absence of a horizontal area in the SOM curves is due to the heterogeneity in binding strength of the reactive surface sites. At low Cu activity (< $10^{-12}$), the sequence in accumulation factor is SOM > goethite > HFO, implying SOM can bind Cu most strongly. At high Cu activity (> $10^{-6.5}$), the sequence is reversed, implying HFO has the highest complexation capacity per unit mass. For a fixed Cu activity, an increase in pH leads to a larger Cu accumulation on reactive surfaces. At higher pH, there is less competition from protons for reactive surface sites, and electrostatic repulsion between Cu and Fe(hydr)oxide surfaces decreases because Fe(hydr)oxide surfaces become less positively charged (pristine point of zero charge (PPZC) of HFO = 8.1 (Dzombak and Morel, 1990); PPZC of goethite = 9.3 (Filius, et al., 1997)). The effect is smaller at high Cu activity, and wears off almost entirely for Fe(hydr)oxides due to Cu saturation.

In Figure A7.2b the distribution of Cu over reactive surfaces is presented as a function of pH for 2 model systems comprising 1g $l^{-1}$ HFO, 1g $l^{-1}$ goethite, 1g $l^{-1}$ SOM, and respectively 5 and 250 µM Cu. With 5 µM Cu, SOM is the dominant adsorption surface for the examined pH range (5 - 9), while with 250 µM Cu SOM is the dominant adsorption surface from

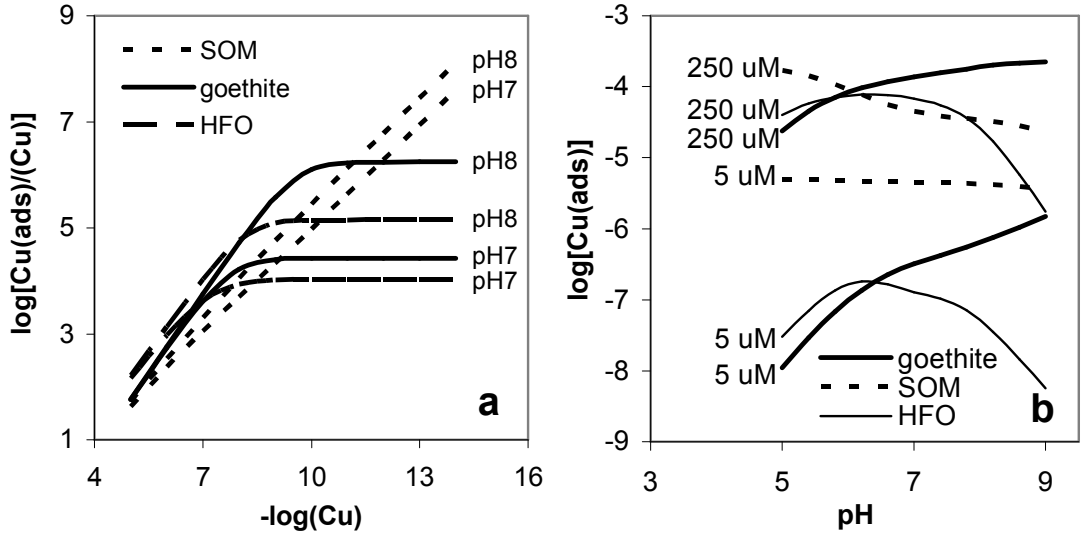



**Figure A7.2: a)** Accumulation of Cu on reactive surfaces per gram of reactive soil compound, relative to the Cu activity, as a function of Cu activity for pH 7 and pH 8; I = 0.1 M; **b)** Distribution of fixed amounts of Cu (5 and 250 µM Cu) over 1 g $l^{-1}$ goethite, SOM and HFO as a function of pH. I = 0.1 M.

pH 5 - 6, and goethite from pH 6 upward. HFO plays a subordinate role in Cu adsorption in both systems. In actual soil systems, the distribution of Cu over reactive surfaces can be substantially determined by the contents of the reactive soil materials present in the soil, besides by their affinity for Cu.

**Adsorption isotherm for meso o,o-FeEDDHA to goethite**

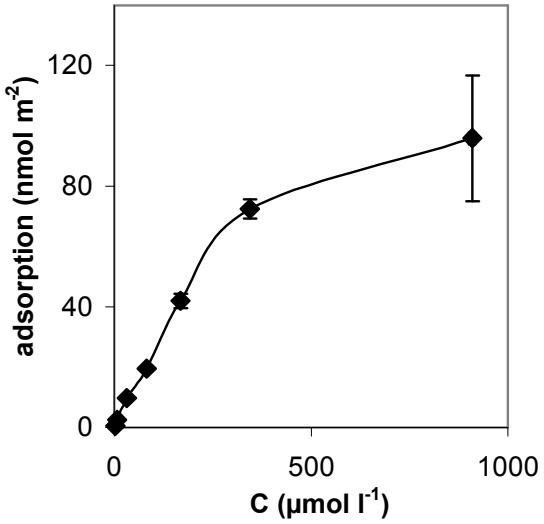



**Figure A7.3:** Adsorption isotherm of meso o,o-FeEDDHA to goethite at pH 7; 0.1 M NaCl.

## Calibration of spectrophotometer

**Table A7.1a:** $\lambda_{max}$-values (nm) for EDDHA ligands and metal-EDDHA complexes (0.1 M NaCl).

| Ligand/complex | racemic o,o-EDDHA | meso o,o-EDDHA |
|---|---|---|
| EDDHA | 275 | 275 |
| FeEDDHA | 280, 477 | 280, 487 |
| CuEDDHA | 277, 639* | 273, 646* |

* Absorbance at the indicated $\lambda_{max}$ values was to low to use for determining EDDHA compound concentrations.

**Table A7.1b:** Extinction coefficients (ε) for the relevant wavelengths at 20 °C (0.1 M NaCl).

| Ligand/complex | $10^6$ $M^{-1}$ $cm^{-1}$ | | | |
|---|---|---|---|---|
| *Wavelength (nm)* | **275** | **277** | **280** | **477** |
| racemic o,o-FeEDDHA | 9.7 | 10.3 | 10.7 | 4.8 |
| racemic o,o-CuEDDHA | 9.6 | 9.7 | 9.3 | < 0.1 |
| racemic o,o-EDDHA | 4.6 | 4.5 | 4.1 | < 0.1 |
| *Wavelength (nm)* | **273** | **275** | **280** | **487** |
| meso o,o-FeEDDHA | 9.2 | 9.8 | 10.6 | 4.5 |
| meso o,o-CuEDDHA | 9.1 | 9.1 | 8.6 | < 0.1 |
| meso o,o-EDDHA | 4.9 | 5.0 | 4.4 | < 0.1 |

## Re-modelling EDDHA-speciation

**Table A7.2:** Re-modelling EDDHA-speciation in clay soils, based on the free Cu concentration calculated from the total Cu concentration measured in 0.01 M $CaCl_2$-extracts.

| Soil | rac o,o-FeEDDHA (µM) | rac o,o-CuEDDHA (µM) | meso o,o-FeEDDHA (µM) | meso o,o-CuEDDHA (µM) | o,p-FeEDDHA (µM) | o,p-CuEDDHA (µM) |
|---|---|---|---|---|---|---|
| Santomera | 95.7 | 4.3 | 72.6 | 27.4 | 0.0 | 100.0 |
| Xeraco T | 99.8 | 0.2 | 97.5 | 2.5 | 0.4 | 99.6 |
| Bologna | 99.2 | 0.8 | 92.8 | 7.2 | 0.1 | 99.9 |
| Herveld | 99.6 | 0.4 | 95.6 | 4.4 | 0.2 | 99.8 |

“Omnia vincit amor, sed festinat lente.”
*trans.* “Love conquers all, but makes haste slowly.”

Conjunction of:
“Omnia vincit amor, …”
*Eclogae 10.69* by Vergil (70 - 19 BC)
and,
“Festina lente!”
motto of emperor Augustus (63 BC - 14 AD).

# Chapter 8

## The effect of soil parameters on the kinetics of the displacement of Fe from FeEDDHA chelates by Cu

W.D.C. Schenkeveld, A.M. Reichwein, E.J.M. Temminghoff, and W.H. van Riemsdijk

## Abstract

In soil application, o,o-FeEDDHA is the active ingredient of FeEDDHA chelate based Fe fertilizers. The effectiveness of o,o-FeEDDHA is potentially compromised by the displacement of Fe from FeEDDHA by Cu. The actual impact of Cu competition is co-determined by the rate at which it causes the o,o-FeEDDHA solution concentration to decline, and hence by the kinetics of the displacement reaction. In this study the influence of soil parameters on the displacement kinetics have been examined in goethite suspensions.

The displacement reaction predominantly takes place on the reactive surface rather than in solution. The rate at which o,o-FeEDDHA concentration declined, depended on the available reactive surface area, the Cu loading and the FeEDDHA loading. Soil factors reducing FeEDDHA adsorption (high ionic strength, humic acid adsorption onto the goethite surface and monovalent instead of divalent cations in the electrolyte) decreased the displacement rate. For meso o,o-FeEDDHA the displacement rate equation was derived, which is first order in FeEDDHA loading and half order in Cu loading. For soil conditions the equation can be simplified to an exponential decay function in meso o,o-FeEDDHA solution concentration.

# Introduction

Aminocarboxylate chelating agents are widely applied for their ability to form stable and water soluble complexes with di- and trivalent metal ions (Bucheli-Witschel and Egli, 2001). Their environmental fate has received considerable attention (Nowack, 2002), because these ligands can enhance mobilization and migration of toxic heavy metals from soils and sediments (Nowack, et al., 1996; Nowack and Sigg, 1996). An additional concern is the environmental persistence of certain of these synthetic chelating agents, e.g. EDTA, resulting from a poor biodegradability (Bucheli-Witschel and Egli, 2001).

The aminocarboxylate chelate FeEDDHA (iron ethylene diamine-N,N'-bis(hydroxy phenyl acetic acid)) is commonly used as Fe fertilizer and is considered among the most efficient in preventing and remedying Fe deficiency chlorosis in crops grown on calcareous soils (Lucena, 2003; Reed, et al., 1988). Commercially available FeEDDHA products consist of a mixture of FeEDDHA components. The quantitatively most important components are the isomers racemic o,o-FeEDDHA (iron (3+) (R,R) and (S,S) ethylene diamine-N,N'-bis(2-hydroxy phenyl acetic acid) complexes), meso o,o-FeEDDHA (iron (3+) (R,S) = (S,R) ethylene diamine-N,N'-bis(2-hydroxy phenyl acetic acid) complex) and o,p-FeEDDHA (iron (3+) ethylene diamine-N-(2-hydroxy phenyl acetic acid)-N'-(4-hydroxy phenyl acetic acid) complexes). Physical and chemical properties of the isomers differ and, so does their effectiveness in delivering Fe to plants. In the soil application studies reported thus far, o,o-FeEDDHA isomers determined the effectiveness of FeEDDHA treatments (Rojas, et al., 2008; Schenkeveld, et al., 2008; Schenkeveld, et al., 2010b); o,p-FeEDDHA proved ineffective due to its strong tendency to adsorb to reactive soil compounds and to have its Fe displaced by Cu (Garcia-Marco, et al., 2006; Schenkeveld, et al., 2007).

In pot and incubation trial studies, a gradual exponential decline in soil solution concentration of meso o,o-FeEDDHA has been observed, which was not related to biodegradation, leaching or plant uptake (Schenkeveld, et al., 2010a; Schenkeveld, et al., 2010c). Recently, Schenkeveld et al. (2010d) demonstrated through multi-surface modeling that there is a thermodynamic basis for assuming that competition from Cu can affect o,o-FeEDDHA concentrations under calcareous soil conditions, in particular of the meso o,o-FeEDDHA isomer (Equation 1).

$$meso\ o,o-FeEDDHA^{-1} + Cu^{2+} \leftrightarrows\ meso\ o,o-CuH_nEDDHA^{-(2-n)} + Fe(OH)_3 + (3-n)H^+ \quad (1)$$

Model outcomes are supported by observed Fe displacement from o,o-FeEDDHA by Cu in goethite suspension systems (Schenkeveld, et al., 2010d). These findings suggest that competition from Cu could be an important factor limiting the effectiveness of FeEDDHA application in soil-plant systems. Still, the actual impact of Cu competition will be co-determined by the rate of the displacement reaction (equation 1) under soil conditions. The kinetics and reaction mechanism of metal displacement from EDDHA chelates have, however, hardly been addressed yet.

For other aminocarboxylate chelating agents, in particular EDTA, displacement kinetics have been intensively studied (Hering and Morel, 1988; 1989; Nowack, 2002). Displacement

reactions with EDTA have been reported to occur both in solution (Hering and Morel, 1989) and on reactive surfaces (Nowack and Sigg, 1997), and the kinetics strongly depend on the metal initially chelated (Nowack, et al., 2001; Nowack and Sigg, 1997; Xue, et al., 1995). When displacement occurs at a reactive surface, EDTA chelates need to first adsorb. Adsorption behaviour of EDTA chelates is anionic and strongly depends on the chelated metal ion (Nowack, et al., 1996; Nowack and Sigg, 1996). Competing cations may either be adsorbed to or incorporated in the surface. Rate limiting steps identified for metal displacement on reactive surfaces are: dissociation of the metal-EDTA complex, and detachment of metal from the solid phase (Nowack, et al., 2001; Nowack and Sigg, 1997). The newly formed EDTA-chelate will redistribute over the solid and solution phase. Given the structural resemblance between EDTA and EDDHA, similarities in cation displacement behaviour are to be expected.

A better understanding of the kinetics involved with the displacement of Fe from FeEDDHA by Cu is essential for improving soil-specific estimates on the time span FeEDDHA components remain effective as fertilizer in soil application. The aim of this study was to identify the factors determining the displacement rate under calcareous soil conditions and to establish the influence of a selection of soil parameters on the displacement kinetics. For his purpose, a series of batch experiments were done. Goethite suspensions were used instead of actual soils, because model systems are more suitable for assessing the influence of (soil) parameters separately.

# Material and Methods

### Materials

*Goethite* - A stock goethite suspension was prepared from $Fe(NO_3)_3$ according to Atkinson et al. (1967) as described in more detail by Hiemstra et al. (1989). The BET($N_2$) specific surface area amounted 98.6 $m^2\ g^{-1}$ and EC was below 10 μS.

*Reagent solutions* – EDDHA solutions were prepared by dissolving racemic o,o-$H_4$EDDHA (purity: 100%), meso o,o-$H_4$EDDHA (purity: 99.5%) and o,p-EDDHA (purity: 90%) in sufficient 1 M NaOH. Racemic and meso o,o-$H_4$EDDHA were obtained by separation of an o,o-$H_4$EDDHA mixture (99% pure; 49% racemic o,o-EDDHA, 51% meso o,o-EDDHA), as described in Bannochie and Martell (1989) and Bailey et al. (1981). Metal-EDDHA solutions were prepared by adding dissolved metal chloride salt of Cu(II) and Fe(III) to EDDHA solutions in a 2% excess based on a 1:1 stoichiometry between metal and EDDHA ligand; chelating capacity of impurities was corrected for. pH was raised to 7 (± 0.5). FeEDDHA solutions were left over-night in the dark in order for excess Fe to precipitate as hydroxides. The following day, FeEDDHA solutions were filtered over a 0.45 μm nitro cellulose micro pore filter (Schleicher & Schuell, ref-no: 10401114).

$CO_2$-free 0.1 M NaOH solution was prepared from Titrasol. MOPS-buffer was prepared by dissolving solid MOPS (3-(N-Morpholino)-propanesulfonic acid) in pre-boiled ultra pure water and was made to pH with 0.1 M NaOH solution. MOPS does not complex transition

metals nor does it interfere in spectrophotometric measurement at wavelengths above 250 nm (Yu, et al., 1997). Both MOPS- and NaOH solution were stored in a desiccator to avoid $CO_2$ contamination. Humic acid (HA) solutions were prepared by dissolving purified humic acid from Tongbergsven forest, near Oisterwijk, the Netherlands (Temminghoff, et al., 1997) with 0.1 M NaOH solution in pre-boiled water under $N_2$ atmosphere. Stock and experimental solution were prepared from analytical grade chemicals and ultra pure water.

**Experimental section**

EDDHA speciation in solution was examined as a function of time upon addition of FeEDDHA chelates to a goethite suspension. The parameters varied to examine their effect on the reaction rate of the Fe displacement reaction were: goethite suspension density (2 and 10 g $l^{-1}$), Cu concentration in suspension (0, 90 and 450 μM), background electrolyte (0.01 M NaCl, 0.1 M NaCl and 0.033 M $CaCl_2$), humic acid concentration in suspension (0, 0.3 and 1.0 g $l^{-1}$), and EDDHA isomer (racemic o,o-EDDHA, meso o,o-EDDHA and o,p-EDDHA). Goethite suspension density was varied to examine the influence of available (soil reactive) Fe(hydr)oxide surface; Cu concentration was varied to examine the influence of Cu availability (in the soil); background electrolyte was varied to examine the influence of ionic strength and the valence of the dominant cation in (soil) solution; HA concentration in suspension was varied to examine the influence of adsorbed humic substances onto soil Fe(hydr)oxides. Temperature (20 ± 1° C), pH (7 ± 0.05) and total amount of EDDHA ligand in suspension (90 μM) were kept constant. The experiments were executed in duplicates.

Background electrolyte and, when required, $CuCl_2$ solution were added to a portion of the goethite stock solution in polyethylene bottles. pH was lowered to 5.5 and the suspension was purged overnight with washed (1 M $H_2SO_4$/1 M NaOH), moist $N_2$ gas to remove $CO_2$. The following day, pH was raised to around 7 with 0.1 M NaOH and MOPS buffer (0.005 M; pH 7 ± 0.05) was added. Suspensions were made to volume with pre-boiled water. In experiments involving HA, MOPS buffer was omitted; HA served as pH buffer and was added to suspension after overnight purging. pH was raised to and maintained at 7 with 0.1 M NaOH, while the suspension equilibrated for 72 hours (Weng, et al., 2006) under continuous purging with $N_2$. Dissolved organic carbon (DOC) concentration in solution was determined after the suspension had been made to volume; in all cases, over 98% of the HA was adsorbed to goethite.

0.9 mM metal-EDDHA solutions, purged with $N_2$ for 30 minutes prior to addition, were added to goethite suspensions in 100 ml polytethylene bottles under $N_2$-atmosphere in a 1:9 (v/v) ratio. The moment of addition was recorded as t=0. Suspensions were stirred and divided over 10 gas-tight 23.6 ml low-density polyethylene bottles (Rietra, et al., 2001), which had been flushed with $N_2$; 5 ml of goethite suspension was added to each bottle. The bottles were placed in an end-over-end shaker, rotating at 24 rpm in absence of light. Sampling was done destructively after approximately 0.5; 1.25; 2; 4; 8; 24; 48; 96; 192 and 336 hours. Samples were centrifuged for 10 minutes at 10,000 rpm. The supernatant was filtered over a 0.45 μm cellulose acetate micro pore filter (Schleicher & Schuell, ref no: 10462650). The filtrate was further analysed by UV-VIS spectrophotometry.

**Analyses**

EDDHA and metal-EDDHA concentrations in the supernatant of goethite suspensions were determined through spectrophotometry with a double beam spectrophotometer (Unicam Helios α) using disposable UltraVette cuvettes (BrandTech Scientific), with a 10 mm light path. Local absorption maxima were determined for all relevant EDDHA compounds by scanning from 250 to 900 nm (Table A8.1a in the Appendix). An external calibration method was used to determine the concentrations of the EDDHA compounds (absorption coefficients are presented in Table A8.1b in the Appendix). Absorbance was measured at wavelengths corresponding to absorption maxima of all EDDHA compounds that could be present in the sample. Measured absorbances were considered linear combinations of the absorbances of the individual EDDHA compounds. EDDHA compound concentrations were calculated from the measured absorbances by a smallest sum of squares method. The contribution of dissolved HA in the supernatant to light absorbance never exceeded 15% in the UV and was negligible in the visible light. Absorbance by HA was corrected for in calculating the EDDHA compound concentrations. To samples from experiments involving HA, MOPS buffer was added prior to measurement, to reinstall pH 7 after centrifugation and to obtain a similar matrix as in the treatments without HA.

DOC concentrations were measured with a segmented flow analyzer (SFA) (Skalar, SK12) by oxidation with persulphate and tetraborate and UV and IR-detection. Cu concentrations were measured by ICP-MS (Perkin Elmer, ELAN 6000). ICP-MS samples were acidified with nitric acid before measurement.

**Modelling**

The computer program ECOSAT (Keizer and Van Riemsdijk, 1994) was used for chemical speciation calculations. Cu speciation in goethite suspensions was modeled using a multi-surface approach, in which goethite and HA are treated as independent reactive surfaces (i.e. linear additivity) (Weng, et al., 2001). Cu binding to goethite was described with the Charge Distribution Multi-Site Complexation (CD-MUSIC) model (Hiemstra and Van Riemsdijk, 1996; 1999). Parameters describing metal and proton binding were taken from Weng et al. (2001). Specific and non-specific Cu binding to HA was described with the Non-Ideal consistent Competitive Adsorption (NICA)-Donnan model (Kinniburgh, et al., 1999). Generic NICA-Donnan parameters for metal and proton binding to HA and FA were taken from Milne et al. (2001; 2003). Adsorption of HA to goethite has not been included into the models.

## Results and Discussion

**Adsorption**

To distinguish between adsorption and cation displacement kinetics, FeEDDHA adsorption to goethite was first examined as a function of time in absence of Cu. In goethite suspensions with NaCl as background electrolyte, FeEDDHA isomer concentrations remained

approximately constant after an initial drop within the first 0.5 hour (Figure 8.1a). So, on the time-scale considered, adsorption (near-)equilibrium was reached practically instantaneously. The observed sequence in degree of FeEDDHA isomer adsorption: o,p-FeEDDHA[7] > meso o,o-FeEDDHA > racemic o,o-FeEDDHA corresponds with previous findings (Hernandez-Apaolaza and Lucena, 2001; Schenkeveld, et al., 2007). A decrease in ionic strength of the solution phase from 0.1 M to 0.01 M led to a substantial (relative) increase in adsorption of the o,o-FeEDDHA isomers (illustrated most clearly by meso o,o-FeEDDHA; Figure 8.1a). This indicates an important role for electrostatic interaction in the adsorption mechanism, and suggests weak, possibly outer-sphere complexation. A small temporary overshoot in meso o,o-FeEDDHA adsorption was observed at I = 0.01 M. Furthermore, Fe activity imposed by goethite proved too low to maintain Fe chelated by o,p-EDDHA, leading to complex dissociation (Figure 8.1a). o,p-EDDHA ligand was gradually released into solution, at a rate much slower than at which adsorption equilibrium was attained. o,p-FeEDDHA concentration remained almost unaffected.

In goethite suspensions containing adsorbed HA, or with $CaCl_2$ as background electrolyte instead of NaCl, FeEDDHA adsorption was comparably swift, as illustrated for meso o,o-FeEDDHA in Figure 8.1b. In certain treatments (0.033 M $CaCl_2$ and both 0.3 g $l^{-1}$ HA treatments) meso o,o-FeEDDHA concentration gradually decreased somewhat after the initial concentration drop. The reason for this is unclear. However, as will be demonstrated, this rate of decline is negligible in comparison to cation displacement.

FeEDDHA adsorption decreased with increasing HA loading on the goethite surface. HA may compete for reactive surface sites and negatively affects the apparent surface charge of goethite (Weng, et al., 2008a; b); adsorption of the negatively charged HA decreases the electrostatic potential in the vicinity of the goethite surface (Saito, et al., 2004), inducing a smaller electrostatic attraction or even repulsion between the anionic FeEDDHA complex and the goethite surface.

For corresponding treatments with NaCl and $CaCl_2$, similar in ionic strength, o,o-FeEDDHA adsorption was consistently higher in the $CaCl_2$ treatment. Ca has a higher affinity for goethite surfaces than Na, and increases the electrostatic potential near the goethite surface through the formation of surface complexes (Rahnemaie, et al., 2006). This leads to a larger electrostatic attraction between FeEDDHA chelate and surface, which enhances adsorption. At high pH, Weng et al. (2005) also demonstrated an increase in electrostatic potential near the goethite surface in ternary systems with goethite and (adsorbed) fulvic acid (FA) as a result of Ca adsorption. Although the effect-size may differ, a comparable effect from Ca should be expected in systems including HA instead of FA. Electrostatic effects of Ca in ternary systems with goethite and HA need to be further examined.

[7] o,p-FeEDDHA concentrations are overestimated by spectrophotometric measurement. Impurities in the o,p-EDDHA standard, forming Fe chelates with lower affinity for the goethite surface (e.g. the o,o-FeEDDHA isomers) absorb light in the same range of wavelengths as o,p-FeEDDHA. However, measurement of the o,p-FeEDDHA concentration after separation by HPLC as described in Schenkeveld et al (2007), confirms the trends in o,p-FeEDDHA concentration displayed in Figure 1a (data not shown).

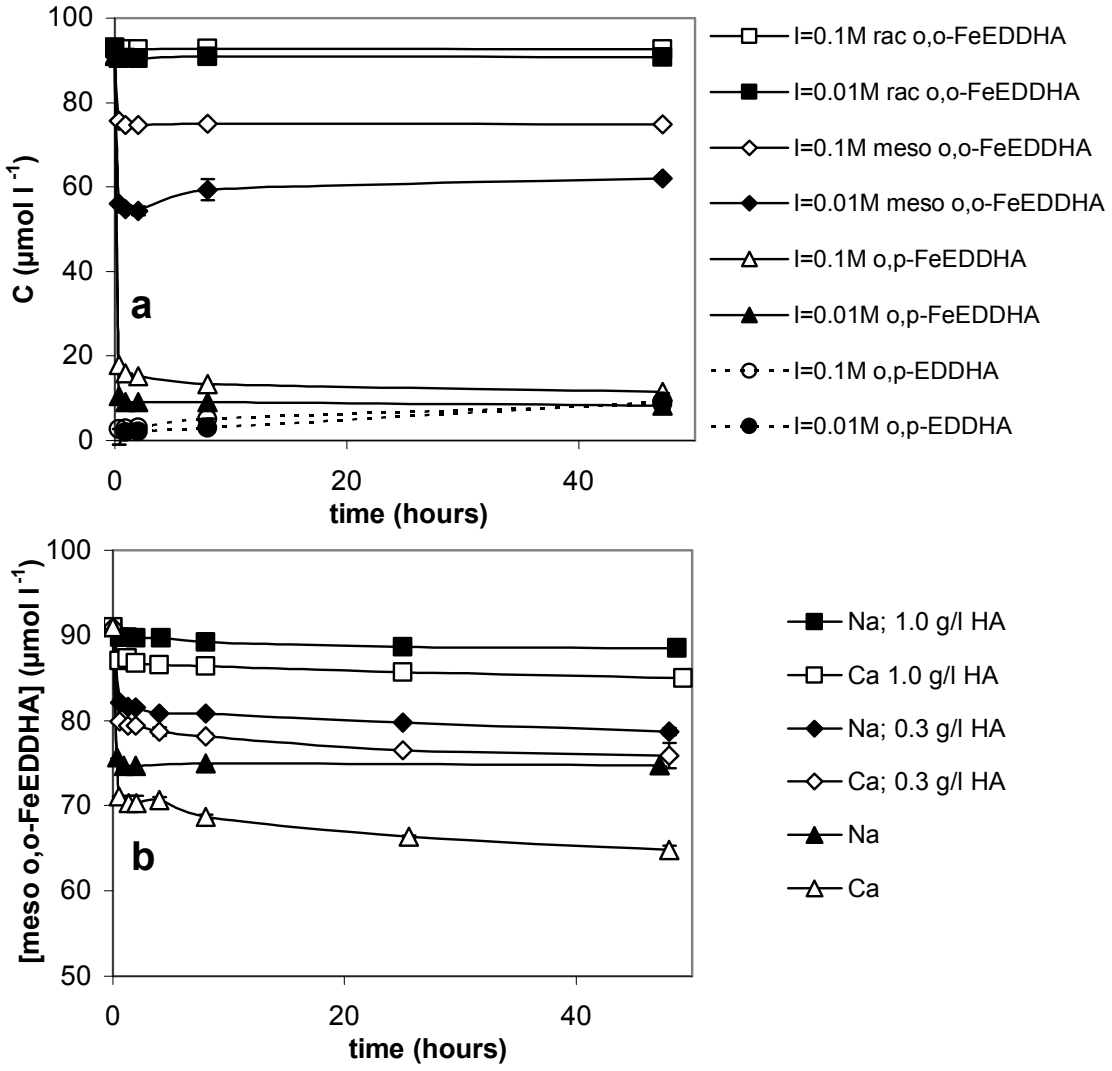



**Figure 8.1: a)** FeEDDHA isomer concentrations as a function of time in 10 g $l^{-1}$ goethite suspensions differing in ionic strength (NaCl) upon addition of 90 µM FeEDDHA; **b)** meso o,o-FeEDDHA concentration as a function of time in 10 g $l^{-1}$ goethite suspensions differing in background electrolyte (NaCl and $CaCl_2$; I = 0.1 M), and humic acid loading (0, 0.3 and 1.0 g $l^{-1}$). Error bars indicate standard deviations.

**Fe displacement rate**

As a starting point, Fe displacement and the soil parameters potentially affecting the displacement rate have been approached on a macroscopic level. The displacement rate has been interpreted in terms of change in amount of FeEDDHA in suspension per unit time. The impact of the selected parameters on the displacement rate is discussed consecutively, below.

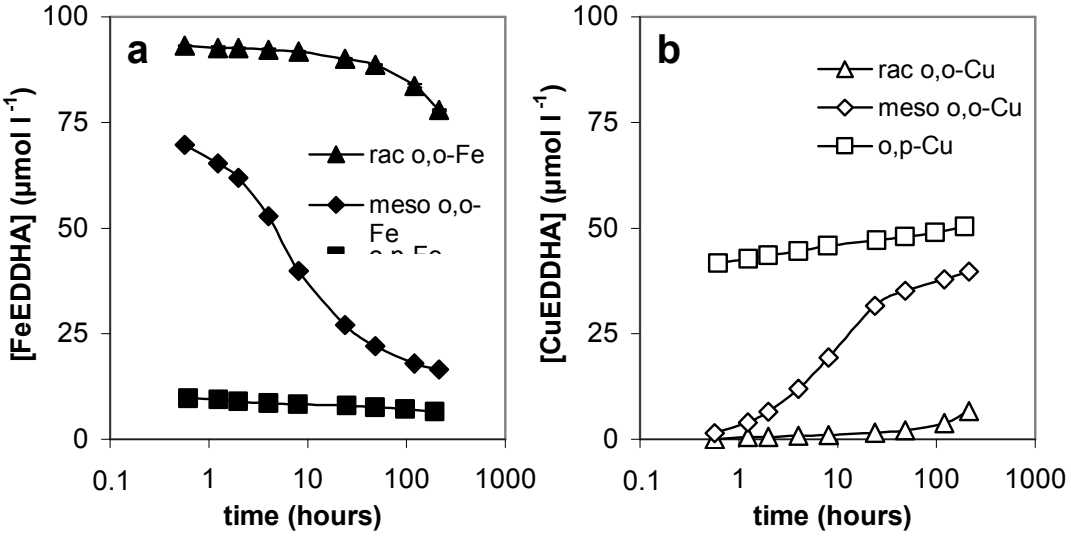



**Figure 8.2: a)** FeEDDHA and **b)** CuEDDHA concentrations as a function of time in 10 g $l^{-1}$ goethite suspensions (0.1 M NaCl) with 90 µM Cu upon addition of 90 µM FeEDDHA. Error bars indicate standard deviations.

*FeEDDHA isomer*

A strong effect of FeEDDHA isomer on the displacement rate by Cu was observed upon introduction of 90 µM FeEDDHA into a goethite suspension (10 g $l^{-1}$; 0.1 M NaCl), containing 90 µM Cu[8]: o,p-FeEDDHA was removed from solution almost instantaneously (Figure 8.2a), and the corresponding o,p-CuEDDHA concentration increased strongly within the first 0.5 hour and only moderately afterwards (Figure 8.2b). The decrease in meso o,o-FeEDDHA, and corresponding increase in meso o,o-CuEDDHA, were more gradual, while the decrease in racemic o,o-FeEDDHA and corresponding increase in racemic o,o-CuEDDHA were by far slowest. This order in rate of decline in FeEDDHA isomer concentration corresponds with observations upon interaction with soil (Schenkeveld, et al., 2010a). In soil studies, however, no comparable increase in o,o-CuEDDHA concentration in soil solution was observed, and the increase in o,p-CuEDDHA was temporary. The rate of decline in FeEDDHA concentration increased with 1) increasing relative affinity of the EDDHA ligand for Cu (compared to Fe) (Schenkeveld, et al., 2010d), 2) decreasing stability of the FeEDDHA complex (Yunta et al. 2003 a en b), and 3) increasing affinity of the FeEDDHA complex for the goethite surface (Figure 8.1a). The impact of FeEDDHA adsorption on Fe displacement is considered in more detail further on.

[8] $pCu_{free}$ prior to FeEDDHA addition was calculated in ECOSAT, and ranged from 8.3 to 9.6 for the goethite suspensions included in this study - see Table 8.1.

*Reactive surface area*

By varying the goethite suspension density (2 and 10 g $l^{-1}$), the effect of reactive surface area on the displacement rate of Fe from FeEDDHA by Cu was examined. For treatments, equal in Cu dose (90 μM) the initial drop in FeEDDHA concentration was larger in the treatment with a higher suspension density (10 g $l^{-1}$), as illustrated for meso o,o-FeEDDHA (Figure 8.3a); a larger available reactive surface area leads to more adsorption. However, during the first 8 hours, the difference in meso o,o-FeEDDHA concentration continued to increase, and the meso o,o-CuEDDHA concentration increased faster in the 10 g $l^{-1}$ goethite treatment (Figure 8.3a), despite the higher degree of meso o,o-CuEDDHA adsorption. This indicates that the displacement rate was higher in the treatment with a higher suspension density. Because Cu loading and hence Cu activity were lower in this treatment, Cu activity did not govern the displacement rate. Furthermore, the solution concentrations of both reactants (Cu and meso o,o-FeEDDHA) were lower in the goethite suspension with a higher displacement rate. This indicates that the displacement reaction predominantly took place on the goethite surface rather than in solution.

After 8 hours the difference in meso o,o-FeEDDHA concentration started to decrease, and the meso o,o-CuEDDHA concentration in the 2 g $l^{-1}$ goethite-treatment even exceeded the concentration in the 10 g $l^{-1}$ goethite-treatment after approximately 30 hours. From a thermodynamic perspective, this is the result of differences in equilibrium concentrations between the goethite suspensions; the lesser degree of adsorption and the higher Cu loading (and Cu activity) for a given amount of reacted meso o,o-FeEDDHA favour the meso o,o-CuEDDHA concentration in the goethite suspension with a lower suspension density. As a result of the slower reaction kinetics, related to the smaller available reactive surface area, it merely requires more time to reach equilibrium. After 9 days, equilibrium had not yet been attained.

*Copper availability*

The Cu loading on the goethite surface positively affected the displacement rate, as illustrated for meso o,o-FeEDDHA introduced into goethite suspensions of equal suspension density and different amounts of adsorbed Cu (90 and 450 μM) (Figure 8.3b). Initial meso o,o-FeEDDHA adsorption was comparable, and during the early stages of the experiment, when the inverse displacement reaction (eq (1)) was of relatively little importance, both the decrease in meso o,o-FeEDDHA and the increase in meso o,o-CuEDDHA concentration were faster in the treatment with the higher Cu loading.

Assuming adsorption equilibrium was reached instantaneously, only the reaction to the right of equation (1) took place at t=0, because there was no meso o,o-CuEDDHA yet. The rate of this reaction was determined by fitting the meso o,o-FeEDDHA solution concentration data as a function of time from t=0.5 to t=8 hours with a second order polynomial equation and extrapolating to t=0 ($R^2 \geq 0.995$). Subsequently, the displacement rate at t=0 was calculated by dividing the tangent of the extrapolated fitting curve at t=0 by the FeEDDHA fraction in solution at adsorption equilibrium at t=0. The obtained displacement rate is expressed as a

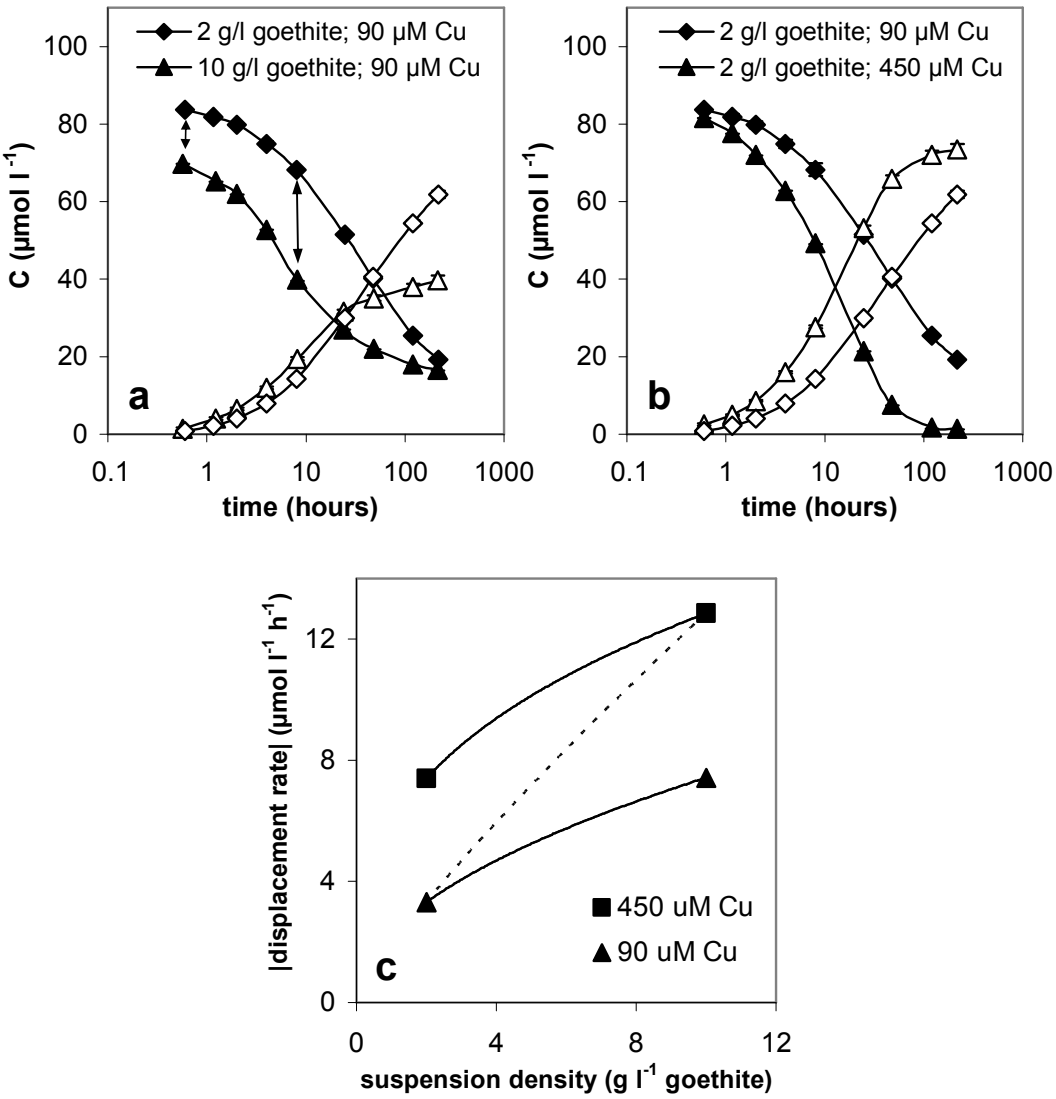



**Figure 8.3:** Concentration of meso o,o-FeEDDHA (closed symbols) and meso o,o-CuEDDHA (open symbols) as a function of time, in goethite suspensions differing in **a)** suspension density and **b)** amount of adsorbed Cu. Error bars indicate standard deviations; and **c)** the absolute displacement rate at t≈0 expressed as change in solution concentration per unit time, as a function of suspension density for goethite suspensions with 90 and 450 µM Cu. Solid curves represent fits to a power function for treatments of equal amount of Cu in suspension, the dashed curve represents a fit to a power function for treatments of equal Cu loading.

decrease in solution concentration per unit time, but accounts for removal of meso o,o-FeEDDHA from both solution and goethite surface, assuming (linear) adsorption equilibrium was preserved.
The calculated reaction rates confirm a pronounced effect from both soil factors reactive surface area and Cu availability on the displacement rate (Figure 8.3c). For a given suspension density, a fivefold increase in Cu loading approximately doubled the reaction rate. For a given amount of adsorbed Cu, a fivefold increase in suspension density also approximately doubled the reaction rate, despite the fivefold decrease in Cu loading. Hence for a given Cu loading, a fivefold increase in suspension density led to a fourfold increase in displacement rate, which corresponds with a fourfold increase in meso o,o-FeEDDHA adsorption, assuming adsorption is linear.

*Ionic strength*
Decreased ionic strength positively affected the displacement rate, as illustrated for meso o,o-FeEDDHA introduced into goethite suspensions of equal suspension density and equal Cu loading (Figure 8.4a). Up to 8 hours, meso o,o-FeEDDHA concentration declined faster, and the corresponding meso o,o-CuEDDHA concentration increased faster, in the treatment with a lower ionic strength (0.01 M NaCl). Initial FeEDDHA adsorption, and hence the FeEDDHA loading were higher in this treatment (Figure 8.1a and 8.4a). After 8 hours the difference in meso o,o-FeEDDHA concentration started to decrease, and after approximately 120 hours the meso o,o-CuEDDHA concentration in the 0.1 M NaCl treatment exceeded the concentration in the 0.01 M NaCl treatment. The higher eventual meso o,o-CuEDDHA concentration in the 0.1 M NaCl treatment is the net result of a lesser degree of adsorption and a shift in equilibrium of the displacement reaction (equation 1).
Three factors affecting the displacement rate of Fe from FeEDDHA by Cu have been identified: available reactive surface area, Cu loading, and FeEDDHA loading. The intrinsic reaction rate of the displacement reaction is however standardized per unit reactive surface area, leaving only Cu loading and Fe loading as rate determining factors. In Figure 8.4b, the intrinsic reaction rate a $t \approx 0$ is presented as a function of meso o,o-FeEDDHA loading. 4 levels of Cu loading have been distinguished. For each Cu loading larger than zero, the intrinsic displacement rate increased with increasing FeEDDHA loading. And, for treatments of comparable FeEDDHA loading, the intrinsic displacement rate increased with increasing Cu loading.
Moreover, Cu loading positively affected FeEDDHA loading. The effect was more pronounced for treatments with ionic strength 0.01 M (indicated with open symbols), than for treatments with ionic strength 0.1 M (indicated with closed symbols). It was examined if meso o,o-FeEDDHA adsorption predominantly increased due to an increase in electrostatic potential near the goethite surface (1-plane) resulting from Cu adsorption, or due to chemical binding to adsorbed Cu. The contribution of the electrostatic effect was established through the increase in Boltzmann factor[9] ($B^Z$) near the goethite surface (1-plane).

[9] The Boltzmann factor is a surface accumulation factor for charged particles near a charged surface.

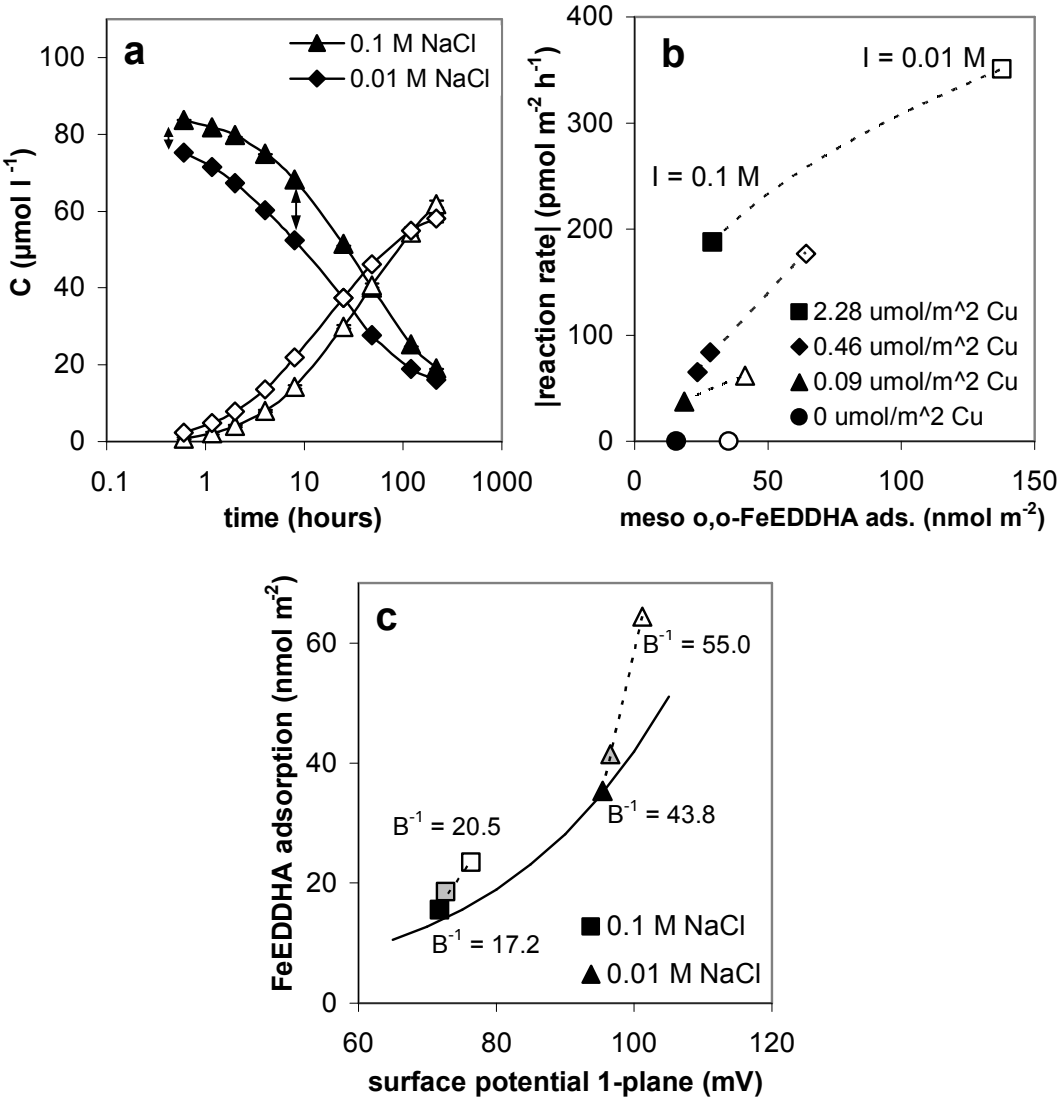



**Figure 8.4: a)** Concentration of meso o,o-FeEDDHA (closed symbols) and meso o,o-CuEDDHA (open symbols) as a function of time in 2 g $l^{-1}$ goethite suspensions with 90 µM adsorbed Cu, differing in ionic strength. Error bars indicate standard deviations; **b)** the absolute intrinsic displacement rate at t≈0 as a function of FeEDDHA surface loading; open symbols represent experiments with 0.01 M NaCl, closed symbols with 0.1 M NaCl. Dashed curves represent fits to a power function for treatments of equal Cu loading; and **c)** meso o,o-FeEDDHA surface loading at t≈0 as a function of the surface potential in the 1-plane before FeEDDHA adsorption. Black symbols represent 0 µmol $m^{-2}$ Cu; grey symbols represent 0.09 µmol $m^{-2}$ Cu; white symbols represent 0.46 µmol $m^{-2}$ Cu. Per ionic strength, Boltzmann factors for FeEDDHA are indicated for the two extreme treatments. The solid curve represents the predicted meso o,o-FeEDDHA adsorption based on the Boltzmann factor in the 1-plane, calibrated on FeEDDHA adsorption data from the 0.01 M NaCl; 0 µmol $m^{-2}$ Cu treatment. Dashed lines represent linear fits of FeEDDHA adsorption with increasing Cu loading for a given background electrolyte.

With the CD-MUSIC model, the surface potential in the 1-plane (ψ) was calculated for goethite suspensions with and without Cu, prior to FeEDDHA addition. From the calculated surface potentials, Boltzmann factors for meso o,o-FeEDDHA (with a charge (Z) of -1) were calculated, using equation 2:

$$B = e^{\frac{-F\psi}{RT}} \qquad (2)$$

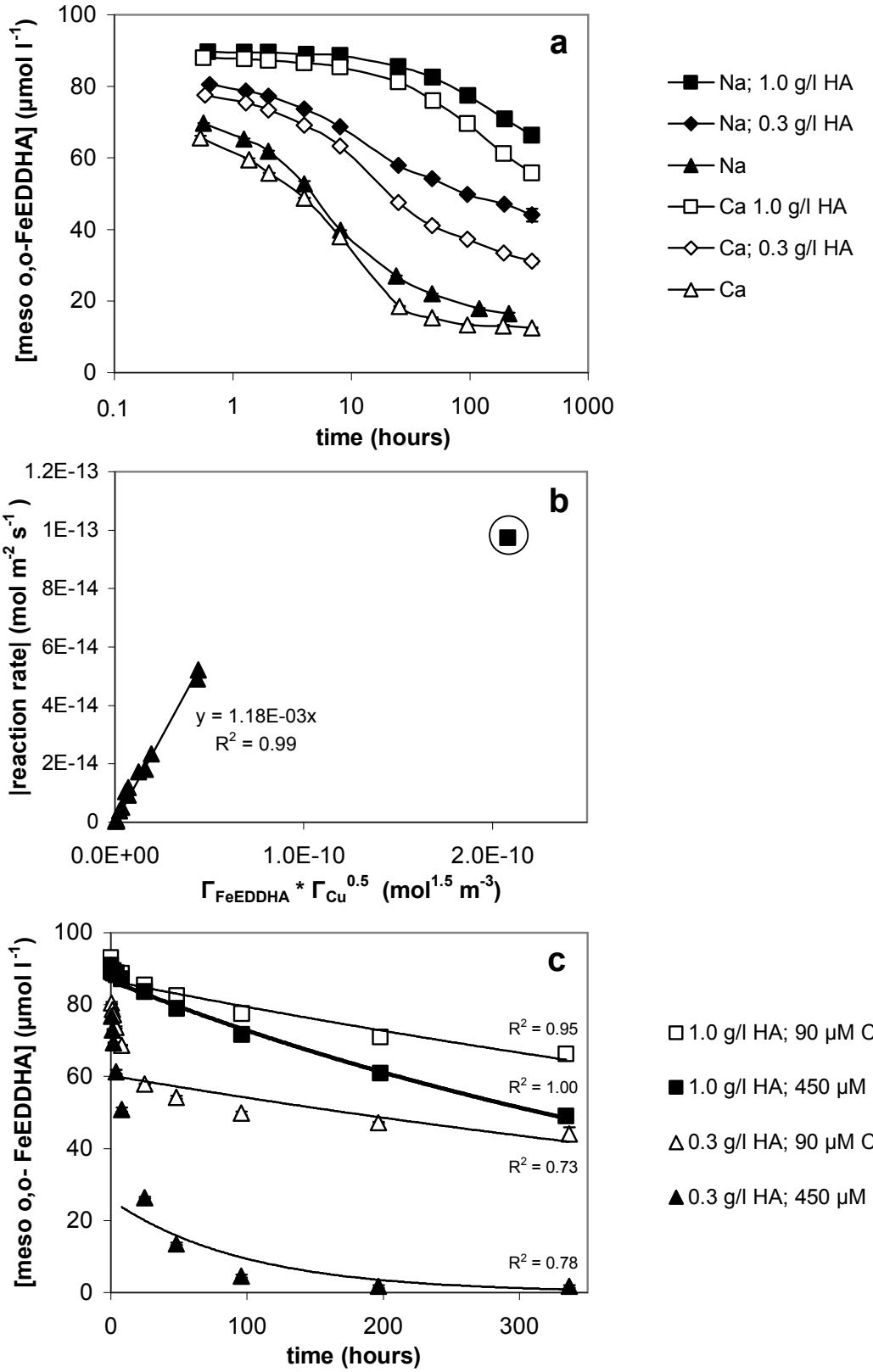
In Figure 8.4c, measured meso o,o-FeEDDHA adsorption is presented as a function of the calculated surface potential, for goethite suspensions, differing in background electrolyte (0.01 M and 0.1 M NaCl) and Cu loading (0, 0.09 and 0.46 μmol $m^{-2}$ Cu). Boltzmann factors have been indicated for treatments with 0 and 0.46 μmol $m^{-2}$ Cu. The solid curve represents the (simulated) effect of surface potential on meso o,o-FeEDDHA adsorption to goethite, calibrated on adsorption data from the 0.01 M NaCl; 0 μmol $m^{-2}$ Cu treatment. Adsorption in the 0.1 M NaCl; 0 μmol $m^{-2}$ Cu treatment is predicted fairly accurately. The relative increase in meso o,o-FeEDDHA adsorption due to adsorbed Cu (51 - 82%) is however much larger than predicted from the increase in Boltzmann factor (20 - 26%). This suggests that adsorbed Cu increased FeEDDHA adsorption mainly through chemical bonding: the formation of a ternary surface complex between the goethite surface, Cu and FeEDDHA, rather than through an electrostatic effect. Spectroscopic data to verify this suggestion are currently lacking.

.

*Background electrolyte and HA*

The effect of HA adsorbed onto the goethite surface (0, 0.3 and 1.0 g $l^{-1}$) and of the background electrolyte (NaCl and $CaCl_2$) on the displacement rate has been examined in 10 g $l^{-1}$ goethite suspensions containing 90 μM Cu with I = 0.1 M. The rate of decline in meso o,o-FeEDDHA concentration decreased with increasing amount of adsorbed HA, and was higher in treatments with $CaCl_2$ than in corresponding treatments with NaCl (Figure 8.5a). These observations can be interpreted in relation to the factors identified to determine the intrinsic displacement rate. FeEDDHA adsorption and consequently FeEDDHA loading were negatively affected by HA adsorbed onto the goethite surface and positively by Ca in the electrolyte (see section on adsorption). The effect of HA and background electrolyte on Cu loading on the goethite surface was examined by chemical speciation modeling with

**Figure 8.5** ***(p. 175)*****: a**) Meso o,o-FeEDDHA concentration as a function of time in 10 g $l^{-1}$ goethite suspensions with 90 μM Cu, differing in background electrolyte (NaCl and $CaCl_2$; I = 0.1 M) and HA loading (0, 0.3 and 1 g $l^{-1}$). Error bars indicate standard deviations; **b)** absolute intrinsic displacement rate at t≈0 as a function of the surface loading product of meso o,o-FeEDDHA and Cu. In suspensions containing HA, the Cu loading on goethite was calculated with the NICA-Donnan and the CD-MUSIC model assuming linear additivity; and **c)** exponential fits of meso o,o-FeEDDHA concentration data as a function of time from 8 hours onward in 10 g $l^{-1}$ goethite suspensions differing in HA loading (0.3 and 1 g $l^{-1}$) and Cu loading (90 and 450 μM). Error bars indicate standard deviations.


a
[meso o,o-FeEDDHA] (µmol l-1)
time (hours)
Na; 1.0 g/l HA
Na; 0.3 g/l HA
Na
Ca 1.0 g/l HA
Ca; 0.3 g/l HA
Ca
b
|reaction rate| (mol m-2 s-1)
y = 1.18E-03x
R2 = 0.99
ΓFeEDDHA * ΓCu0.5 (mol1.5 m-3)
c
[meso o,o- FeEDDHA] (µmol l-1)
R2 = 0.95
R2 = 1.00
R2 = 0.73
R2 = 0.78
time (hours)
1.0 g/l HA; 90 µM Cu
1.0 g/l HA; 450 µM Cu
0.3 g/l HA; 90 µM Cu
0.3 g/l HA; 450 µM Cu

ECOSAT, assuming linear additivity of the CD-MUSIC and NICA-Donnan model (Weng, et al., 2001). Cu distributes over the available reactive surfaces, and with the introduction of HA, a fraction of Cu adsorbed to HA, thereby negatively affecting the Cu loading on the goethite surface. Addition of 1.0 g $l^{-1}$ HA, reduced the Cu loading on the goethite surface from 90 to 27 nmol $m^{-2}$ (Table 8.1). The effect that Cu, adsorbed to goethite, increased FeEDDHA adsorption (previous section and Figure 8.4c) decreased with increasing HA adsorption (data not shown); less Cu adsorbed to goethite implied fewer possibilities to form ternary surface complexes. Furthermore, in suspensions containing HA, Ca positively affected the Cu loading on the goethite surface in comparison to Na; Ca adsorption attributes positive charge to both the Donnan-gel of HA and the 1-plane of the goethite surface, making Cu adsorption to both surfaces less favourable and increasing the free Cu concentration. An increase in free Cu concentration causes a shift in Cu adsorption from HA to goethite (Table 8.1).

**Table 8.1:** Model predictions for the free Cu concentration and the Cu loading on the goethite surface in 10 g $l^{-1}$ goethite suspensions, differing in background electrolyte, amount of Cu and amount of HA; I = 0.1 M.

| Treatment | $pCu_{free}$ | Cu loading goethite (nmol $m^{-2}$) |
|---|---|---|
| Na; 90 µM Cu | 9.07 | 90 |
| Ca; 90 µM Cu | 9.01 | 90 |
| Na; 450 µM Cu | 8.33 | 450 |
| Na; 90 µM Cu + 0.3 g $l^{-1}$ HA | 9.23 | 63 |
| Ca; 90 µM Cu + 0.3 g $l^{-1}$ HA | 9.07 | 79 |
| Na; 450 µM Cu + 0.3 g $l^{-1}$ HA | 8.40 | 387 |
| Na; 90 µM Cu + 1.0 g $l^{-1}$ HA | 9.60 | 27 |
| Ca; 90 µM Cu + 1.0 g $l^{-1}$ HA | 9.21 | 58 |
| Na; 450 µM Cu + 1.0 g $l^{-1}$ HA | 8.58 | 267 |

*Rate equation*

Given the very low Fe concentrations in solution ($pFe_{free} \approx 21$), it is reasonable to assume that the inverse displacement reaction (Fe displacing Cu from CuEDDHA) also occurs on the goethite surface. The intrinsic reaction rate of Fe displacement from FeEDDHA by Cu can then be described with rate equation 3a:

$$-r_{FeEDDHA} = k_1 \cdot \Gamma_{FeEDDHA}^{\alpha} \cdot \Gamma_{Cu}^{\beta} - k_2 \cdot \Gamma_{CuEDDHA}^{\gamma} \cdot \Gamma_{Fe}^{\varepsilon} \qquad (3a)$$

in which $-r_{FeEDDHA}$ is the intrinsic displacement rate; $k_1$ and $k_2$ are the rate constants, $\Gamma_{FeEDDHA}$, $\Gamma_{Cu}$, $\Gamma_{CuEDDHA}$ and $\Gamma_{Fe}$ are respectively the FeEDDHA, Cu, CuEDDHA and Fe loadings on the goethite surface, and $\alpha$, $\beta$, $\gamma$ and $\varepsilon$ represent the reaction orders of the respective reactants.

Including Fe loading in the rate equation is arbitrary; Fe displaced from FeEDDHA will probably precipitate as hydroxide and become part of the Fe(hydr)oxide matrix either as hydrous ferric oxide (HFO) or as goethite.
When introducing FeEDDHA into a goethite suspension containing Cu and assuming instant adsorption equilibrium, the second term of the rate equation equals zero, because the CuEDDHA loading is still zero. Equation 3a can then be simplified to equation 3b:

$$-r_{FeEDDHA} = k_1 \cdot \Gamma_{FeEDDHA}^{\alpha} \cdot \Gamma_{Cu}^{\beta} \qquad (3b)$$

in which $\Gamma_{Cu}$ was imposed with the treatment, $\Gamma_{FeEDDHA}$ could be determined from the initial drop in FeEDDHA concentration and $r_{FeEDDHA}$ could be determined from the slope of the FeEDDHA concentration curve as a function of time, at $t \approx 0$. For meso o,o-FeEDDHA the parameters $k_1$, $\alpha$ and $\beta$ were fit to the $\Gamma_{Cu}$, $\Gamma_{FeEDDHA}$ and $r_{FeEDDHA}$ data collected. For treatments including HA, $\Gamma_{Cu}$ refers to Cu loading on the goethite surface as simulated in ECOSAT (see previous section). Results of the fitting exercise are presented in Figure 8.5b. The quality of the fit is good ($R^2 = 0.99$; $n = 13$) and the simplest solution indicates the displacement of Fe from FeEDDHA by Cu is first order in $\Gamma_{FeEDDHA}$ ($\alpha = 1$) and half-order in $\Gamma_{Cu}$ ($\beta = 0.5$). The overall order of the reaction is 1.5 and rate constant $k_1$ equals $1.2\ 10^{-3}$ m $s^{-1}$ $mol^{-0.5}$ ($T = 293K$; $P = 1$ atm). The higher reaction order for $\Gamma_{FeEDDHA}$ compared to $\Gamma_{Cu}$ is possibly related to a higher mobility of FeEDDHA over the goethite surface due to its lower tendency to adsorb. The encircled data point (Figure 8.5b), which corresponds with the treatment with the highest FeEDDHA loading (0.01 M NaCl, 2 g $l^{-1}$ goethite, 450 μM Cu) has not been included into the fit. Based on the fit, a higher reaction rate would be expected for the given FeEDDHA and Cu loading in this treatment. Apparently, at FeEDDHA loadings this high, the reaction order in $\Gamma_{FeEDDHA}$ decreases, possibly as a result of effects related to steric hindrance.
Parameters related to the inverse displacement reaction ($k_2$, $\gamma$, $\varepsilon$; equation 3a) are commonly derived from equilibrium conditions when the rates of both displacement reaction are equal. This proved impossible, because in most experiments equilibrium had not yet been reached upon termination. Furthermore, meso o,o-FeEDDHA and meso o,o-CuEDDHA loadings at equilibrium could not be derived sufficiently accurately. A more detailed understanding of the adsorption behaviour of EDDHA components and how this behaviour is affected by the simultaneous adsorption of other ions would aid deriving kinetic parameters of surface displacement reactions as examined in this study.

*Soil conditions*
In soil and soil-plant systems an exponential decline in meso o,o-FeEDDHA concentration in the pore water has been observed (Schenkeveld, et al., 2010a; Schenkeveld, et al., 2010c). An exponential decay function for FeEDDHA concentration can be derived from rate equation 3a under the following three constraints:
1) Concentrations are sufficiently remote from equilibrium, that the part of the rate equation regarding the inverse displacement reaction (the $k_2$ term) can be neglected. This leads to the simplification of equation 3a to equation 3b (with $\alpha = 1$ and $\beta = 0.5$).

2) Cu loading remains approximately constant. In soil application FeEDDHA is generally applied in such quantities that Fe displacement by Cu will have a limited effect on the Cu loading of soil reactive surfaces. This allows for further simplification from equation 3b to equation 3c, in which the Cu loading is incorporated in the conditional (soil specific) rate constant $k_1^{\dagger}$:

$$-r_{FeEDDHA} = k_1^{\dagger} \cdot \Gamma_{FeEDDHA} \tag{3c}$$

3) FeEDDHA adsorption to goethite can be considered linear and adsorption equilibrium is preserved. Equation 3c can then be rewritten to equation 3d, in which adsorption coefficient $K_d$ is incorporated in the conditional rate constant $k_1^{\dagger\dagger}$:

$$-\frac{\delta[FeEDDHA]}{\delta t} = k_1^{\dagger\dagger} \cdot [FeEDDHA] \tag{3d}$$

which can be integrated to an exponential decay function for the FeEDDHA concentration in soil solution (equation 3e):

$$[FeEDDHA]_t = [FeEDDHA]_0 e^{-k_1^{\dagger\dagger} \cdot t} \tag{3e}$$

Figure 8.5c illustrates that an exponential decline in meso o,o-FeEDDHA solution concentration was only observed in the goethite suspension for which the aforementioned constraints for simplification of the rate equation were sufficiently met: the treatment with 1 g $l^{-1}$ HA and 450 μM Cu (0.1 M NaCl; 10 g $l^{-1}$ goethite). 1) As a result of the high HA loading on the goethite surface (mimicking the effect of humic substances adsorbed onto soil (hydr)oxide surfaces), meso o,o-FeEDDHA adsorption, and consequently, the displacement rate of Fe from meso o,o-FeEDDHA by Cu was sufficiently low for surface loadings to remain remote from equilibrium throughout the experiment. 2) The amount of Cu in suspension largely exceeded the amount of meso o,o-FeEDDHA added, so that the impact of displacement on the Cu loading remained marginal. 3) The meso o,o-FeEDDHA concentration range was sufficiently small to consider meso o,o-FeEDDHA adsorption linear.

# Practical implications

Although the results presented have focused on Fe displacement by Cu from meso o,o-FeEDDHA, comparable trends regarding the factors affecting the displacement rate have been observed for racemic o,o-FeEDDHA and o,p-FeEDDHA (data not shown).

Results from this study help to better understand the fate of FeEDDHA components in soil and soil-plant systems and contribute to a better understanding of metal displacement reactions from chelates in the soil, in general. The reaction in which Fe is displaced from FeEDDHA by Cu takes place on a (soil) reactive surface rather than in (soil) solution. Therefore, adsorption and cation competition should not be considered two separate threats to the performance of FeEDDHA components: factors that increase adsorption also increase the rate of cation

competition reactions. The effectiveness of the o,o-FeEDDHA isomers in soil application relies on slow displacement kinetics related to a limited affinity for soil reactive surfaces. A high Fe(hydr)oxide and Cu content, along with soil factors enhancing adsorption (a low ionic strength, predominantly divalent cations on the CEC and a low content of humic substances) may substantially compromise the time-span FeEDDHA components remain effective as fertilizer in soil application. For an efficient use of Fe fertilizer, the aforementioned soil parameters should be considered for determining dosage and frequency of FeEDDHA application.

For developing new (Fe)chelate-based fertilizers for soil application, the risk of leaching related to a low affinity for reactive soil surfaces should be carefully weighed against a tendency to adsorb with the potential risk of Fe being displaced by a competing cation. With regard to the latter, a high relative affinity for binding Fe in comparison to other metals seems recommendable.

# Appendix

## Calibration of spectrophotometer

**Table A8.1a:** $\lambda_{max}$-values (nm) for EDDHA ligands and metal-EDDHA complexes.

| Ligand/complex | racemic o,o-EDDHA* | meso o,o-EDDHA | o,p-EDDHA |
|---|---|---|---|
| EDDHA | 275 | 275 | 274 |
| FeEDDHA | 280, 477 | 280, 487 | 274, 463 |
| CuEDDHA | 277, 639** | 273, 646** | 271, n.d. |

*: $\lambda_{max}$-values were separately determined for al matrices, but coincided.
** Absorbance at the indicated $\lambda_{max}$ values was to low to use for determining EDDHA compound concentrations.
n.d. = not determined.

**Table A8.1b:** Extinction coefficients (ε) for the relevant wavelengths at 20 °C.

| Ligand/complex | Matrices | $10^6$ $M^{-1}$ $cm^{-1}$ | | | |
|---|---|---|---|---|---|
| *Wavelength (nm)* | | **275** | **277** | **280** | **477** |
| racemic o,o-FeEDDHA | 0.01M & 0.1M NaCl; 0.033M $CaCl_2$ | 9.7 | 10.3 | 10.7 | 4.8 |
| racemic o,o-CuEDDHA | 0.01M & 0.1M NaCl | 9.6 | 9.7 | 9.3 | < 0.1 |
| | 0.033M $CaCl_2$ | 8.7 | 8.8 | 8.6 | < 0.1 |
| racemic o,o-EDDHA | 0.01M & 0.1M NaCl; 0.033M $CaCl_2$ | 4.6 | 4.5 | 4.1 | < 0.1 |
| *Wavelength (nm)* | | **273** | **275** | **280** | **487** |
| meso o,o-FeEDDHA | 0.01M & 0.1M NaCl; 0.033M $CaCl_2$ | 9.2 | 9.8 | 10.6 | 4.5 |
| meso o,o-CuEDDHA | 0.01M & 0.1M NaCl; 0.033M $CaCl_2$ | 9.1 | 9.1 | 8.6 | < 0.1 |
| meso o,o-EDDHA | 0.01M & 0.1M NaCl; 0.033M $CaCl_2$ | 4.9 | 5.0 | 4.4 | < 0.1 |
| *Wavelength (nm)* | | **271** | **274** | **275** | **463** |
| o,p-FeEDDHA | 0.01M & 0.1M NaCl; 0.033M $CaCl_2$ | 8.8 | 9.0 | 9.0 | 2.1 |
| o,p-CuEDDHA | 0.01M & 0.1M NaCl | 10.2 | 10.0 | 9.9 | 0.1 |
| | 0.033M $CaCl_2$ | 10.1 | 9.9 | 9.7 | 0.1 |
| o,p-EDDHA | 0.01M & 0.1M NaCl; 0.033M $CaCl_2$ | 4.0 | 4.4 | 4.4 | < 0.1 |

“Von des Steines craft, der Fênîs
verbrinnet, daz er ze aschen wirt:
diu asche im aber leben birt.”

*trans*.
“By virtue of the Stone,
the Phoenix is burnt to ashes,
in which he is reborn.”

*Parzival - V.469, 8-10*
by Wolfram von Eschenbach (ca. 1170 - 1220)

# Chapter 9

## Considerations on the shuttle mechanism of FeEDDHA chelates at the soil-root interface in case of Fe deficiency

W.D.C. Schenkeveld, A.M. Reichwein, E.J.M. Temminghoff, and W.H. van Riemsdijk

## Abstract

A mechanism of action for the performance of Fe chelates as soil-applied fertilizer has been hypothesized by Lindsay and Schwab (1982), in which the ligand participates in a cyclic process of delivering Fe at the root surface and mobilizing Fe from the soil. This "shuttle mechanism" seems appealing in view of fertilizer efficiency, but little is known about its performance. FeEDDHA is a commonly used Fe fertilizer on calcareous soil. In this study, the performance of the shuttle mechanism has been examined for FeEDDHA chelates in batch and pot trial experiments. The specificity of EDDHA ligands for chelating Fe from soils of low Fe availability is limited. If the efficiency of the shuttle mechanism is largely controlled by metal availability in the bulk soil, it is heavily compromised by complexation of competing cations: Al, Mn and particularly Cu. Experimental support for a shuttle mechanism in soil-plant systems with FeEDDHA was found in specific metal mobilization upon FeEDDHA-facilitated Fe uptake. The mobilized metals originated at least in part from the root surface instead of the soil.

# Introduction

Fe deficiency chlorosis is a common nutrient disorder in plants grown on calcareous soils. It is characterized by a significant decrease in chlorophyll content in the leaves and decreases yield both quantitatively and qualitatively (Chaney, 1984; Mortvedt, 1991). The limited bioavailability of Fe in calcareous soils arises from the poor solubility of Fe(hydr)oxides at high soil-pH (7 - 8.5), and the presence of a bicarbonate buffer (Boxma, 1972; Lindsay, 1979). The most common practice to prevent and remedy Fe chlorosis is through the application of synthetic Fe chelates, which can greatly increase the Fe concentration in soil solution. FeEDDHA (iron (3+) ethylene diamine-N,N'-bis(hydroxy phenyl acetic acid)) is among the most effective chelates in soil application (Lucena, 2003; Reed, et al., 1988).
Commercially available FeEDDHA products consist of a mixture of FeEDDHA components. The quantitatively most important components are the isomers racemic o,o-FeEDDHA (iron (3+) (R,R) and (S,S) ethylene diamine-N,N'-bis(2-hydroxy phenyl acetic acid) complexes), meso o,o-FeEDDHA (iron (3+) (R,S) = (S,R) ethylene diamine-N,N'-bis(2-hydroxy phenyl acetic acid) complex) and o,p-FeEDDHA (iron (3+) ethylene diamine-N-(2-hydroxy phenyl acetic acid)-N'-(4-hydroxy phenyl acetic acid) complexes). Physical and chemical properties of these isomers differ and, as a consequence, so does their effectiveness in delivering Fe to the plant.

Lindsay and Schwab (1982) have proposed a mechanism of action for the performance of Fe chelates in soils, in which the chelating agent, i.c. EDDHA, ideally functions as a shuttle transporter for Fe between soil particle and plant root (Figure 9.1). This mechanism was later referred to as the "shuttle effect" (Lucena, 2003). Driving force behind the mechanism are the concentration gradients arising from chelate splitting, Fe uptake and chelation of soil-Fe, which induce diffusive transport. The applicability of the shuttle mechanism concept to the performance of FeEDDHA has been experimentally supported by the ability of EDDHA ligands to mobilize Fe from synthetic Fe oxides and soil (Garcia-Marco, et al., 2006; Perez-Sanz and Lucena, 1995), as well as from an adsorbed Fe siderophore (ferrioxamine B) (Siebner-Freibach, et al., 2003). In the latter study, the Fe was actually subsequently transported and transferred to plants grown in hydroponic culture.
Although the concept of recycling the chelating agent seems particularly appealing in view of fertilizer efficiency, hardly anything is known, still, about the actual performance of the shuttle mechanism in soil-plant systems. In accordance with Lindsay and Schwab's concept, the effectiveness of the shuttle is determined by the rates at which diffusive transport takes place, EDDHA ligands mobilize soil-Fe and plants take up Fe from FeEDDHA chelates. The efficiency of the shuttle will however be compromised by processes occurring in the soil (Figure 9.1). FeEDDHA components may leach out of the root zone as a result of excessive irrigation or atmospheric precipitation (Rombola and Tagliavini, 2006), or be transported upwards due to evapo-transpiration and precipitate on the soil surface (Schenkeveld, et al., 2008). Reactive soil constituents adsorb FeEDDHA components (Garcia-Marco, et al., 2006; Hernandez-Apaolaza, et al., 2006; Hernandez-Apaolaza and Lucena, 2001) and competing cations like Cu can displace Fe from the FeEDDHA chelate (Schenkeveld, et al., 2010d;

Schenkeveld, et al., 2010e). Biodegradation of EDDHA ligands presents a potential sink, but needs to be further examined; biodegradation did not affect FeEDDHA chelate concentrations in a soil incubation study (Schenkeveld, et al., 2010c). Although Fe uptake from FeEDDHA is generally preceded by chelate splitting, Bienfait et al. (2004) found FeEDDHA inside plants grown on substrate with nutrient solution, suggesting passive uptake of the FeEDDHA chelate as a whole. Finally, EDDHA ligands do not exclusively chelate Fe upon interaction with soil, but also competing cation like Cu and Al (Garcia-Marco, et al., 2006; Schenkeveld, et al., 2007).

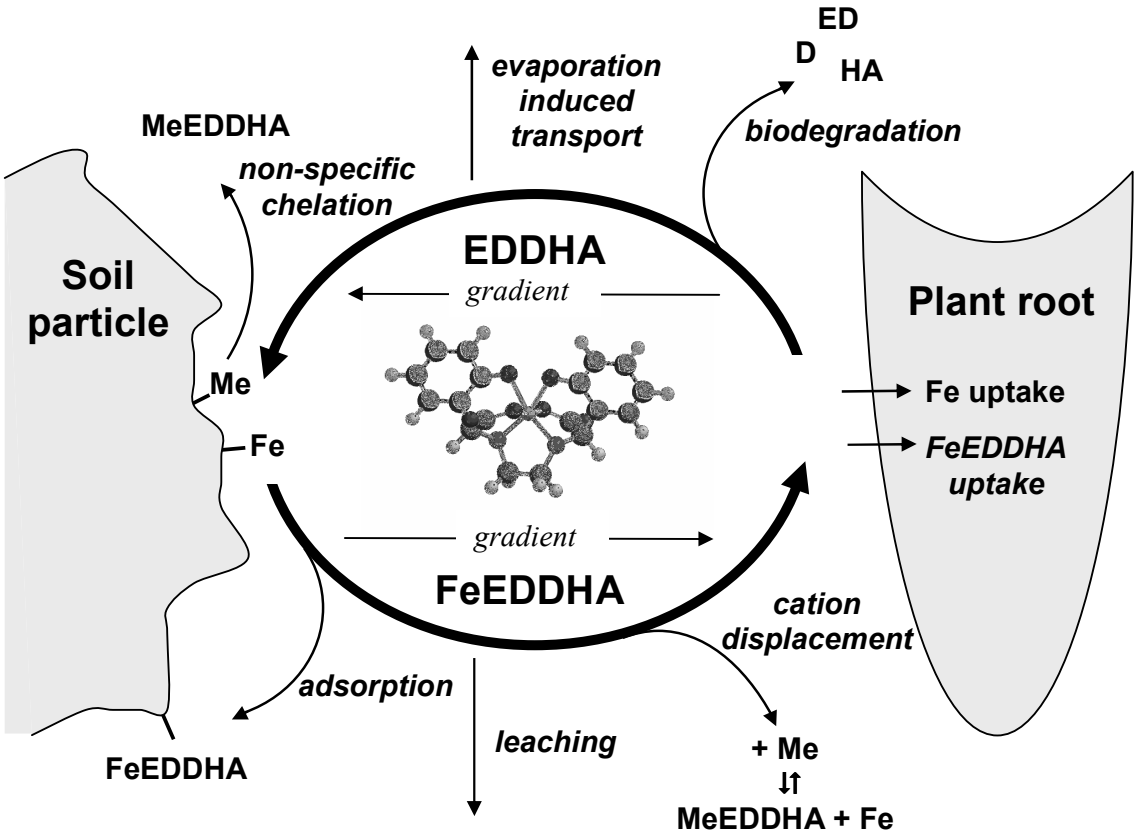



**Figure 9.1:** Shuttle mechanism for EDDHA ligands in soil solution as proposed by Lindsay and Schwab, 1982. FeEDDHA is transported through the rhizosphere by diffusive transport; chelate splitting occurs; Fe is taken up and further allocated inside the plant; EDDHA ligands are transported from the root surface along the concentration gradient; EDDHA ligands chelate and solubilize Fe from soil particles. Potential sinks for the EDDHA ligand are indicated in italic.
Chelate splitting can be preceded by Fe reduction (Chaney, et al., 1972) at ferric chelate reductase (FCR) sites (Robinson, et al., 1999) – this step has not been included in the figure. FeEDDHA facilitated Fe uptake, as illustrated with the shuttle mechanism, does not necessarily exclude "conventional Fe uptake" without synthetic chelates; conventional Fe uptake has not been included in the figure.

The extent to which EDDHA ligands are capable of specifically mobilizing Fe from target soils is presumably a key factor in determining the success of the shuttle mechanism, but has hardly been touched upon. Moreover, the validity of the mechanism as proposed by Lindsay and Schwab (1982) has not yet been experimentally demonstrated for EDDHA ligands in soil-plant systems. The aim of the present study was twofold. First, the specificity of EDDHA ligands for chelating Fe from soil was examined in a series of soil interaction experiments involving 4 calcareous soils and 3 separated EDDHA ligands. Secondly, experimental support was sought for the shuttle mechanism actually taking place in soil-plant systems with FeEDDHA. This was done by means of data from two previously reported pot trial studies, in which FeEDDHA facilitated Fe uptake by soybean plants grown on calcareous soil was established (Schenkeveld, et al., 2008; Schenkeveld, et al., 2010a). Emphasis has been on the o,o-EDDHA ligands, because thus far only racemic and meso o,o-FeEDDHA have been reported to facilitate soil grown plants with Fe (Rojas, et al., 2008; Schenkeveld, et al., 2010b), which is a prerequisite for the shuttle mechanism.

# Material and Methods

**Soils**

Calcareous soils (two clay soils, two sandy soils) were collected from sites, located in Spain (Santomera and Xeraco L) and Saudi Arabia (Nadec and Hofuf). At all sites, the top layer (0 – 20 cm) was sampled, with exception of the Xeraco site (20 - 40 cm). The soils were selected for their low Fe availability (diethylene triamine penta acetic acid (DTPA)-extractable Fe < 11 mg $kg^{-1}$). Fe chlorosis was manifest in crops grown at all sites, and could be reproduced in pot experiments on the Spanish soils (Schenkeveld, et al., 2008; Schenkeveld, et al., 2010a) Pre-treatment consisted of air drying and sieving (mesh size: 1 cm (plant experiments); 0.2 cm (batch experiments)). Relevant soil characteristics are presented in Table 9.1.

**Experimental solutions**

Racemic and meso o,o-$H_4$EDDHA were obtained by separation of an o,o-$H_4$EDDHA mixture (99% pure; 49% racemic o,o-EDDHA, 51% meso o,o-EDDHA), as described by Bannochie and Martell (1989) and Bailey et al. (1981). EDDHA solutions were prepared by dissolving racemic o,o-$H_4$EDDHA (purity: 100%), meso o,o-$H_4$EDDHA (purity: 99.5%) and o,p-$H_4$EDDHA (purity: 90%) in sufficient 1 M NaOH. pH was set to 7 (± 0.5). 0.01 M $CaCl_2$ was used as background electrolyte.

**Batch experiments**

Metal mobilization upon interaction of separated EDDHA isomers with soil was examined as a function of: interaction time (batch experiment 1), concentration of the chelating agent added (batch experiment 2), and interaction time with periodical EDDHA addition (batch

**Table 9.1:** Soil characteristics.

| Soil (origin) | Country | Region | Soil class. | pH-$CaCl_2$[a] | SOC[b] | Clay[c] | $CaCO_3$[d] | Oxalate[e] | | 0.01 M $CaCl_2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Al | Fe | DOC[f] |
| | | | | | (g $kg^{-1}$) | (g $kg^{-1}$) | (g $kg^{-1}$) | (g $kg^{-1}$) | (g $kg^{-1}$) | (mg $kg^{-1}$) |
| **Santomera** | Spain | Murcia | entisol | 8.0 | 5.4 | 260 | 520 | 0.44 | 0.30 | 55 |
| **Xeraco L** | Spain | Valencia | entisol | 7.7 | 13.7 | 360 | 150 | 1.74 | 0.90 | 108 |
| **Nadec** | Saudi Arabia | Near Persian Gulf | aridisol | 8.0 | 8.7 | 70 | 140 | 0.18 | 0.13 | 101 |
| **Hofuf** | Saudi Arabia | Near Persian Gulf | aridisol | 7.8 | 7.1 | 40 | 60 | 0.08 | 0.19 | 78 |

| Soil (origin) | DTPA-extractable[g] | | | | | 0.43 M $HNO_3$-extractable[h] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fe | Cu | Mn | Ni | Zn | Al | Fe | Co | Cu | Mn | Ni | Zn |
| | (mg $kg^{-1}$) | (mg $kg^{-1}$) | (mg $kg^{-1}$) | (mg $kg^{-1}$) | (mg $kg^{-1}$) | (g $kg^{-1}$) | (g $kg^{-1}$) | (mg $kg^{-1}$) | (mg $kg^{-1}$) | (mg $kg^{-1}$) | (mg $kg^{-1}$) | (mg $kg^{-1}$) |
| **Santomera** | 3.5 | 4.1 | 4.6 | 0.20 | 0.90 | 0.70 | 0.49 | 2.6 | 10.1 | 180 | 3.7 | 4.5 |
| **Xeraco L** | 10.5 | 3.0 | 5.3 | 0.20 | 6.7 | 2.1 | 0.46 | 2.8 | 12.6 | 240 | 3.5 | 24 |
| **Nadec** | 2.1 | 0.13 | 5.7 | 0.13 | 0.47 | 0.32 | 0.18 | 0.70 | 0.77 | 50 | 1.9 | 2.3 |
| **Hofuf** | 6.7 | 2.3 | 3.8 | 0.10 | 5.0 | 0.16 | 0.17 | 0.34 | 6.3 | 41 | 1.1 | 24 |

[a] ISO/DIS 10390 Soil Quality – Measurement of pH
[b] Walinga et al. (1992)
[c] Houba et al. (1997)
[d] ISO 10693, Soil Quality – Determination of carbonate content, volumetric method
[e] Schwertmann (1964)
[f] Houba et al. (2000)
[g] Linday and Norvell (1978) and Quevauvillier et al. (1996)
[h] Tipping et al. (2003) and Fest el al. (2005)

experiment 3). Batch experiment 3 was done to mimic the gradual release of EDDHA in soil-plant systems, resulting from Fe uptake from FeEDDHA by plants. All batch experiments were executed in duplicates.
In batch experiment 1, all four soils interacted with 89.5 µM solutions of all three EDDHA isomers, for respectively 1, 8, 24, 48, 96 and 168 hours.
In batch experiment 2, all four soils interacted for 24 hours with solutions of all three EDDHA isomers, at the following concentrations: 1.8; 3.6; 7.2; 14.4; 28.6 and 57.3 µM.
In batch experiment 3, Santomera soil interacted with racemic and meso o,o-EDDHA. After 2 days of equilibration with 0.01 M $CaCl_2$ solution, EDDHA concentration was raised by 1.8 µM three times per week, for 5 weeks, through addition of aliquots of EDDHA solution. Metal concentrations were monitored throughout the experiment.

Soils interacted with EDDHA solutions in a 1:1 (w/v) soil-solution ratio, in 50 ml polypropylene test tubes (Greiner bio-one, Cat No 210296) (batch experiment 1 and 2) and 0.5 l polyethylene shaking bottles (batch experiment 3). Tubes and bottles were placed in an end-over-end shaker, rotating at 18 rpm in absence of light. Room temperature was kept at 20 (± 1) °C. Blank treatments without EDDHA were included in all batch experiments. After interaction, the samples from batch experiment 1 and 2 were centrifuged for 15 minutes at 3000 rpm. pH and EC of the supernatant were measured.
In batch experiment 3, sampling directly preceded each EDDHA addition. Sample and aliquot of EDDHA solution were equal in volume, to preserve a constant total volume. Before sampling, pH and redox potential were measured in suspension; neither changed substantially during the experiment. The solid phase was allowed to precipitate and a sample was drawn from the supernatant accounting for 1% of the solution volume. Subsequently, the shaking bottle was stirred up and placed back in the end-over-end shaker. After termination of batch experiment 3, the soil was centrifuged for 15 minutes at 3,000 rpm and extracted for 48 hours with 20 mM $K_{1.5}H_{1.5}PO_4$ (1:1, 1:5, 1:25 and 1:100 (w/v)), to gain insight in the amounts of metal-EDDHA complexes adsorbed. $PO_4$ was used as extractant because it has a high affinity for Fe(hydr)oxide surfaces and strongly competes with other adsorbed species for reactive surface sites. A mixture of $KH_2PO_4$ en $K_2HPO_4$ was applied to avoid strong pH effects from the extractant. Extractions were done analogue to batch experiment 1 and 2. All samples and extracts were filtered over 0.45 µm cellulose acetate micro pore filters (Schleicher & Schuell, ref no: 10462650). The filtrates were further analyzed.

**Plant experiments**
Metal concentrations in soil solution from two previously reported pot trial studies, in which FeEDDHA facilitated Fe uptake had been established, were examined in view of the shuttle mechanism. Details regarding the preparation of the pot trials, the experimental solutions and plant care are presented in Schenkeveld et al. (2008; 2010a).
In plant experiment 1, metal concentrations in the pore water of Santomera soil were monitored for 6 weeks using rhizon pore water samplers (rhizons) (SMS MOM, Rhizosphere

Research Products, Wageningen, The Netherlands). The pot trial included a blank and 2 FeEDDHA treatments, applied to pots both with and without soybean plants. FeEDDHA treatments were equal in Fe dose (4 mg $l^{-1}$ Fe (72 μM)), but differed in o,o-FeEDDHA content (i.e. the sum of racemic and meso o,o-FeEDDHA): 30% and 100%. Rhizons consist of a cylindrical polyethersulfone (PES) membrane (diameter: 2.5 mm; length: 10 cm; pore size: < 0.2 μm) connected to a PVC/PE tube. Before use, the rhizons were cleaned with 0.14 M $HNO_3$ and ultra pure water, rinsed with 1 mM $NaNO_3$ and stored in a 1 mM $NaNO_3$ solution. The rhizons were incorporated in the soil when the pots were filled, with the membrane placed horizontally at a height of approximately 10 cm above the bottom plate. The ending of the PVC/PE tube was connected to the rim of the pot. Sampling was done twice per week by imposing a vacuum on the inside of the rhizon with a 10 ml syringe with luer lock (SS*10LZ1, Terumo) for 16 hours at most. The first ml sampled was used for rinsing and led back into the pot. The sample size did not exceed 6 ml and exposure of the sample to light was prevented. Experiment 1 was executed in duplicates.
In plant experiment 2, soybean plants grown on calcareous soil from Santomera were offered FeEDDHA treatments, equal in Fe dose (7 mg $l^{-1}$ Fe (0.13 mM Fe)) but differing in o,o-FeEDDHA content. Two control treatments were included: 1) with plants, without FeEDDHA addition, 2) without plants with FeEDDHA addition. Eight weeks after transfer of the seedlings to the pots, the plants were harvested. Collection and analysis of roots and pore water are described in Schenkeveld et al. (2008). Additionally, after collection and rinsing with demineralized water, the roots were extracted with 150 ml 10 mM $Na_2H_2EDTA$ (ethylene diamine tetra acetic acid) solution by manual shaking for one minute. The amount of metals extracted represents a measure for the amount of metals adsorbed onto the root surface (Bates, et al., 1982; Hassler, et al., 2004; Kalis, et al., 2007; Kalis, et al., 2006). Extracts were filtered over a 0.45 μm cellulose acetate micro pore filter (Schleicher & Schuell, ref no: 10462650). The filtrate was further analyzed. Experiment 2 was conducted in triplicates.

**Chemical analyses**
Cu, Al, Mn, Zn, Ni and Co concentrations were measured by ICP-MS (Perkin Elmer, ELAN 6000). Samples were acidified with nitric acid before ICP-measurement, with exception of the EDTA extracts. FeEDDHA isomer concentrations were determined after separation through high-performance liquid chromatography (HPLC) as described in Schenkeveld et al. (2007). The concentrations of metals other than Fe, chelated to EDDHA components were calculated as the difference between the metal concentration in the EDDHA treatment and the blank.

**Statistical analysis**
Statistical analysis of the data was performed using SPSS 12.0. Homogeneity of the data was tested with the Levene's test ($\alpha = 0.05$). Differences among treatments were determined by applying the multivariate general linear model procedure with a Tukey post-hoc test ($\alpha = 0.05$).

## Results and Discussion

**Batch experiment 1**

Metal mobilization was examined as a function of time upon interaction of EDDHA ligands with 4 calcareous soils. For Santomera soil, soil solution concentrations of the quantitatively most important cations (Al, Cu, Fe and Mn) are presented in Figure 9.2; for the 3 other soils, the results are presented in the Appendix (Figure A9.1 - A9.3). The kinetics of metal mobilization from soil by EDDHA ligands are fast: after 1 hour, mobilized metals accounted for 90 - 92% of the racemic o,o-EDDHA, 83 - 88% of the meso o,o-EDDHA, and 36 - 67% of the o,p-EDDHA added. This implies the average residence time of EDDHA ligand in soil solution is short.

Metal mobilization by racemic and meso o,o-EDDHA is fairly similar, while metal mobilization by o,p-EDDHA differs distinctively. In all soils o,p-EDDHA mobilized more Cu and less Fe than o,o-EDDHA ligands. Furthermore, contrary to o,o-EDDHA, o,p-EDDHA hardly mobilized Al or Mn from any soil. To a substantial degree, the different mobilization behaviour results from the fact that phenolic hydroxyl groups in para position are sterically inhibited from contributing to chelation. The lack of a second phenolic hydroxyl group binding the metal considerably affects the relative affinity of o,p-EDDHA for metals in comparison to o,o-EDDHA.

The concentrations of Fe and Al mobilized by o,o-EDDHA remained approximately constant over time, while the concentrations of Cu and Mn decreased and eventually approached zero, except in the Hofuf soil. The fact that Fe and Al concentrations remained unaffected by the decrease in Cu and Mn concentration suggests o,o-CuEDDHA and o,o-MnEDDHA complexes adsorbed and no substantial degree of complex dissociation or cation displacement reaction took place; Fe availability (DTPA-extractable Fe) did not limit further Fe mobilization. The strong tendency of o,o-CuEDDHA to adsorb to soil has been previously reported by Schenkeveld et al. (2010d) and the relatively slow decline in Cu and Mn concentration in the Hofuf soil, which has the lowest content of reactive soil compounds, further supports the involvement of adsorption.

O,o-CuEDDHA is removed from solution more quickly than o,p-CuEDDHA and thus displays a stronger tendency to adsorb, despite its higher complexation constant. In view of adsorption, it is remarkable that the decrease in CuEDDHA concentration did not set in immediately, but, in the case of Santomera soil, only after 2 days. The adsorption mechanisms of metal-EDDHA complexes to soil need to be further examined and are presumably affected by the geometry of the metal-EDDHA coordination complexes: FeEDDHA has a mildly distorted octahedral structure, resulting from heterogeneity in coordinating groups (Bailey, et al., 1981); AlEDDHA has an octahedral structure (Rajan, et al., 1981), presumably also mildly distorted; Mn(III)EDDHA has a more severely distorted octahedral structure with elongated metal-ligand bonds along the z-axis (a Jahn-Teller effect) (Bihari, et al., 2002); and CuEDDHA has the most severe (Jahn-Teller) octahedral distortion (Riley, et al., 1983), in solution presumably

resulting in a square planar structure, in which the carboxylic groups are detached at high pH and the (protonated) phenolate groups at low pH (Frost, et al., 1958). Functional groups which are detached or participate in relatively weak elongated bonds may bind to soil reactive surface groups more easily, and positions on octahedral coordination complexes not occupied by, or bonded relatively weakly to EDDHA ligands may facilitate ligand exchange reactions, with e.g. soil organic matter.

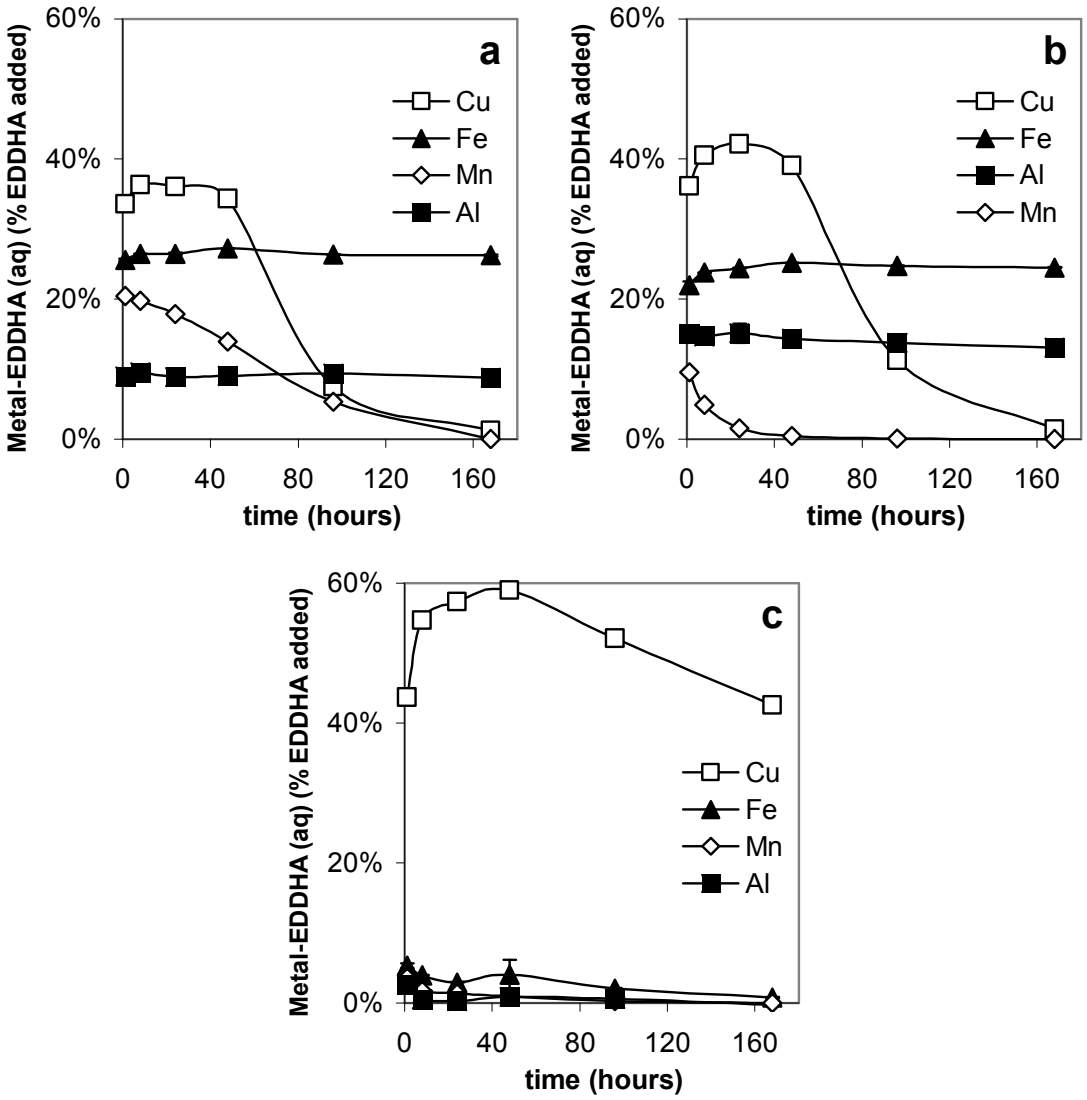



**Figure 9.2:** Percentage of **a)** racemic o,o- EDDHA; **b)** meso o,o-EDDHA; and **c)** o,p-EDDHA added, chelated to Fe, Cu, Mn and Al in solution upon interaction with Santomera soil as a function of time. Error bars indicate standard deviations.

**Batch experiment 2**

Because the Fe uptake flux at plant roots is relatively small and complexation kinetics are fast, the EDDHA concentration near the roots resulting from Fe uptake from FeEDDHA will be near-zero. In batch experiment 1 the concentration administered was orders of magnitude higher, and mobilization of certain metals can then become limited by decreasing metal availability on the soil reactive surfaces. In batch experiment 2, metal mobilization from soil was examined as a function of the administered EDDHA concentration to address metal-EDDHA speciation resulting from (infinitesimally) low EDDHA doses. Interaction time was set at 24 hours, because, on average, overall metal mobilization was highest at that moment. For Santomera soil, mobilized Al, Cu, Fe and Mn concentrations are presented in Figure 9.3. For the other soils, the results are presented in the Appendix (Figure A9.4 – A9.6).

In Santomera soil, metal concentrations increased approximately linearly with increasing EDDHA concentration added; for the other soils this linear relation was not always observed. Deviation from the linear increase was most pronounced for racemic and meso o,o-EDDHA interacting with Hofuf soil (Figure A9.6): at low EDDHA concentrations mainly Cu was mobilized, at high EDDHA concentrations, as a result of decreased Cu availability due to extraction, mainly Fe.

Metal concentration data were fit and the fitting curves extrapolated to $[EDDHA]_{added} = 0$. The tangent of a fitting curve at $[EDDHA]_{added} = 0$ corresponds to the fraction of the EDDHA ligand, mobilizing a specific metal upon addition of an infinitesimally low EDDHA concentration. All tangents are presented in Table 9.2, as percentages instead of fractions. Chelate adsorption by the soil is the most plausible explanation for the fact that the sum of percentages (per EDDHA ligand, per soil) remained mostly below 100%. Upon addition of (infinitesimally) low EDDHA ligand concentrations, only 20.8 – 47.8% of racemic o,o-EDDHA, 20.5 – 52.0% of meso o,o-EDDHA, and 0 – 14.6% of o,p-EDDHA would mobilize Fe from soil. This implies that the efficiency of a potential shuttle mechanism would be heavily compromised by the limited specificity of EDDHA ligands for mobilizing Fe from soils with a low Fe availability. In general, Cu is the principal competing metal; 9.2 – 70.1% of racemic o,o-EDDHA, 15.4 – 69.4% of meso o,o-EDDHA and 14.3 – 89.5% of o,p-EDDHA would mobilize Cu. Over 90% of the mobilized metal by o,p-EDDHA would be Cu, except in the Nadec soil. Although the relative affinity of racemic and meso o,o-EDDHA for Fe and Cu differ (Schenkeveld, et al., 2010d), the extent to which they mobilize Fe and Cu are similar, except in the Nadec soil. Therefore metal mobilization must at least in part be governed by kinetic rather than thermodynamic principles.

The deviant metal mobilization from Nadec soil is related to the fact that racemic o,o-EDDHA mobilized more Mn than meso o,o-EDDHA, which strongly manifested due to the very low Fe and Cu availability of Nadec soil (Table 9.1 and 9.2). Upon chelation under aerobic conditions, Mn(II) (from the soil) is oxidized to Mn(III) (Patch, et al., 1982). The difference in Mn mobilization by racemic and meso o,o-EDDHA is probably (co-)related to the difference in oxidation rate. In plain solution, it was observed that Mn oxidation, which is accompanied

by a change in colour of the complex from pale pink to brown, is considerably faster for racemic o,o-MnEDDHA. For ligands like EDDHA, primarily consisting of hard Lewis bases, complexation constants are generally higher for the trivalent than for the divalent species of a metal. The faster formation of a more stable o,o-Mn(III)EDDHA chelate is advantageous in competition with other metals. Redox behaviour and stability of MnEDDHA complexes need to be further examined, but were outside the scope of this study.

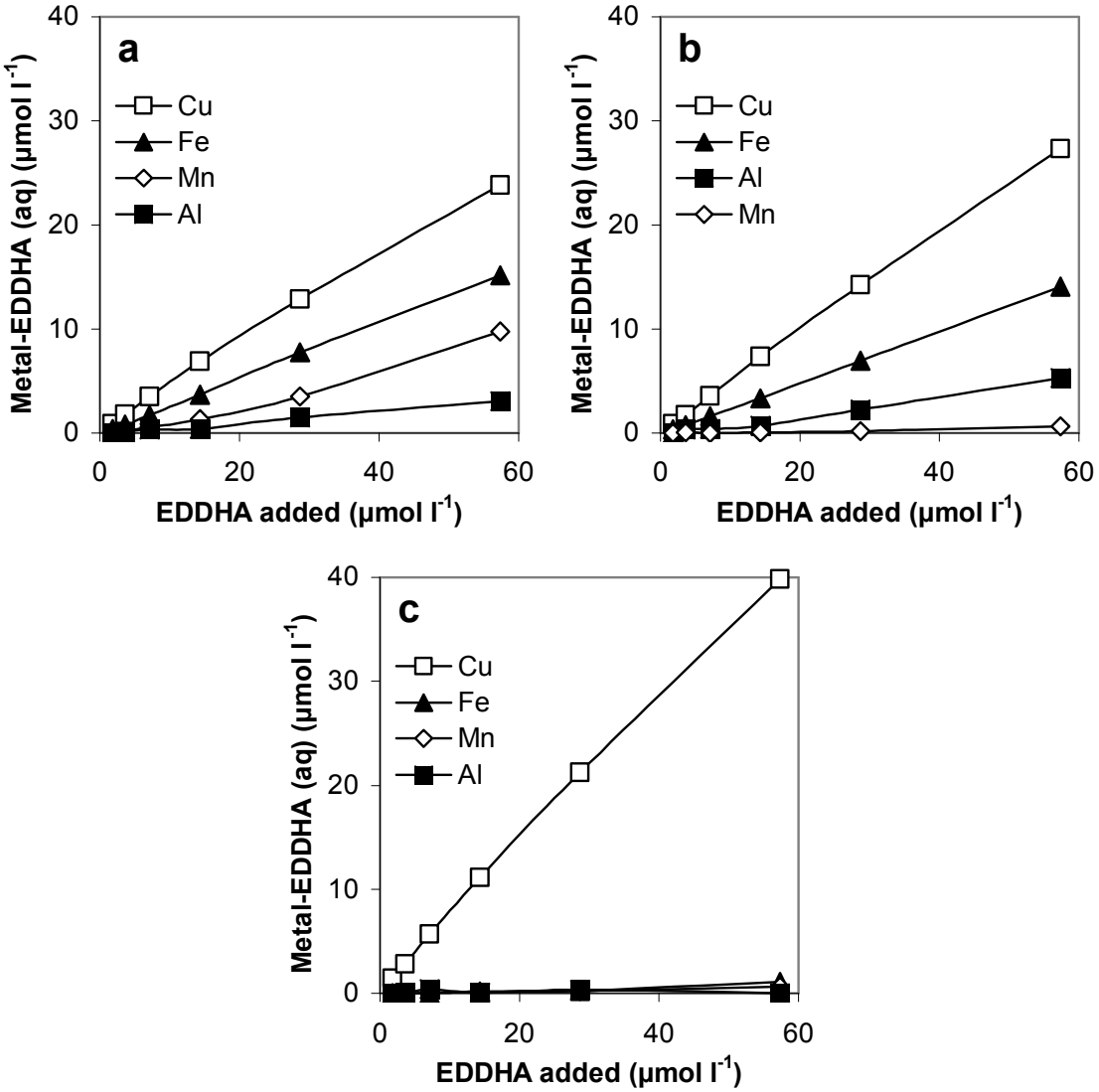



**Figure 9.3:** Metal-EDDHA concentrations in solution after 24 hours of interaction between EDDHA isomers and Santomera soil as a function of the concentration of **a)** racemic o,o-EDDHA; **b)** meso o,o-EDDHA; and **c)** o,p-EDDHA added. Error bars indicate standard deviations.

**Table 9.2:** Metal mobilization by infinitesimally small amounts of EDDHA ligands interacting with 4 calcareous soils. Metal mobilization is expressed as a percentage of the amount of EDDHA ligand added, chelating a specific metal in soil solution. Percentages were determined from the tangents of concentration curves at $[EDDHA]_{added} = 0$ (See Figure 9.3).

| | Santomera | Xeraco L | Nadec | Hofuf |
|---|---|---|---|---|
| **racemic o,o-EDDHA** | | | | |
| Al | 5.5% | n.d. | 9.3% | 0.4% |
| Co | 0.1% | 0.1% | 2.3% | 0.4% |
| Cu | 49.4% | 24.9% | 9.2% | 70.1% |
| Ni | 0.4% | 0.2% | 0.7% | 0.1% |
| Mn | 5.4% | 1.7% | 60.7% | 6.6% |
| Fe | 26.8% | 47.8% | 20.8% | 25.0% |
| total | 87.6% | 74.8% | 102.9% | 102.5% |
| **meso o,o-EDDHA** | | | | |
| Al | 5.6% | n.d. | 24.4% | 2.9% |
| Co | 0.2% | 0.2% | 5.0% | 0.5% |
| Cu | 52.5% | 23.3% | 15.4% | 69.4% |
| Ni | 0.1% | 0.0% | 1.4% | 0.1% |
| Mn | 0.1% | 0.0% | 12.5% | 0.8% |
| Fe | 24.8% | 52.0% | 44.8% | 20.5% |
| total | 83.3% | 75.6% | 103.5% | 94.1% |
| **o,p-EDDHA** | | | | |
| Al | n.d. | n.d. | n.d. | n.d. |
| Co | 0.1% | 0.1% | 4.2% | 0.3% |
| Cu | 79.0% | 58.3% | 14.3% | 89.5% |
| Ni | 0.0% | 0.2% | 3.3% | 0.5% |
| Mn | 0.7% | 0.1% | 6.1% | 1.2% |
| Fe | 0.0% | 2.1% | 14.6% | 3.1% |
| total | 79.7% | 60.9% | 42.4% | 94.5% |

n.d.: not determined – concentration data could not be properly fit for deriving the tangent.

**Batch experiment 3**

The gradual release of racemic and meso o,o-EDDHA at plant roots resulting from FeEDDHA facilitated Fe uptake was mimicked by periodical addition of small amounts of o,o-EDDHA to Santomera soil. Over a period of 36 days, EDDHA was added in 15 aliquots to a total concentration of 27 μM. Less than 2% of the EDDHA added was removed by periodical sampling. Fe and Cu concentration data are presented in Figure 9.4.

The FeEDDHA concentration data presents 3 points of attention. First, the slope of the concentration curve near t=0 indicates that both o,o-FeEDDHA isomers accounted for only 10 - 12% of the EDDHA added. This is a factor 2 - 2.5 lower than derived with the tangent in

batch experiment 2 (Table 9.2). Contrary to batch experiment 2, the soil had been allowed to equilibrate 2 days with background electrolyte solution prior to the first EDDHA addition. Apparently, equilibration negatively affected the relative availability of Fe compared to other metals. Secondly, o,o-FeEDDHA concentrations did not increase linearly with the amount of o,o-EDDHA added, as would be expected from batch experiment 2 (Figure 9.3), but the slope flattened (Figure 9.4a). And thirdly, meso o,o-FeEDDHA concentrations deviated more strongly from the expected linear increase than racemic o,o-FeEDDHA concentrations.

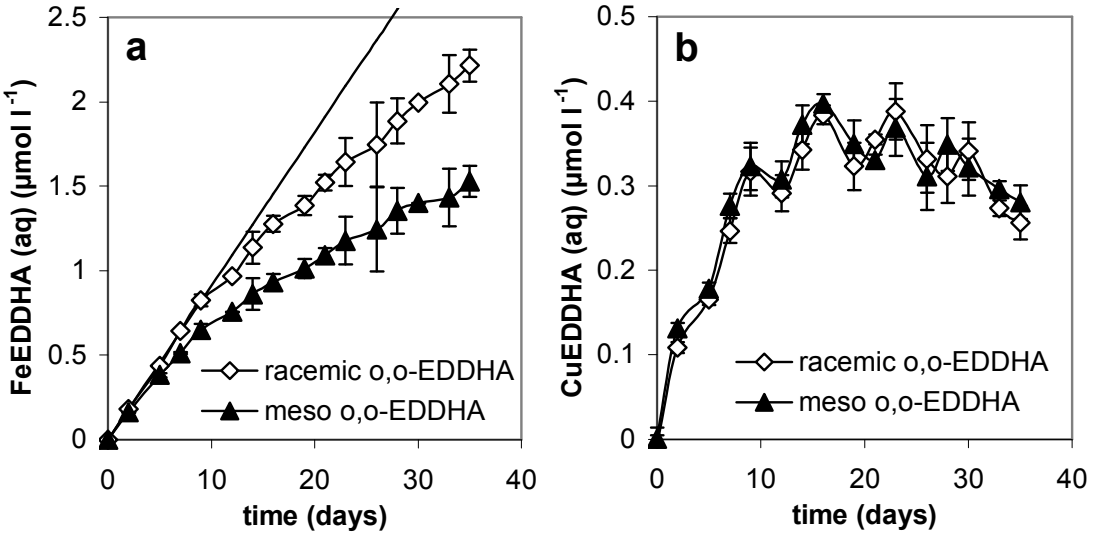



**Figure 9.4: a)** o,o-FeEDDHA, and **b)** o,o-CuEDDHA concentration as a function of time. After each sampling session EDDHA was added, raising the concentration by 1.8 µmol $l^{-1}$. The straight line in 9.4a represents the o,o-FeEDDHA concentration as a function of time if the increase had continued at the rate of t≈0. Because Fe availability does not become limiting for Fe mobilization as FeEDDHA, deviation from the straight line must be caused by a second process decreasing the FeEDDHA concentration. Therefore, the straight line represents the cumulative gross Fe mobilization.

An ongoing decrease in relative Fe availability, affecting Fe mobilization by meso o,o-EDDHA more strongly than by racemic o,o-EDDHA, seems improbable. Instead, the involvement of a second process, causing a decrease in o,o-FeEDDHA concentration, simultaneous to the increase due to addition of o,o-EDDHA, is more likely. Assuming the relative availability of metals remained constant after equilibration and an equal amount of Fe was mobilized with each o,o-EDDHA addition, the rate of decrease was proportional to the o,o-FeEDDHA concentration, which suggests the second process caused an exponential decline. An exponential decline in concentration of o,o-FeEDDHA interacting with Santomera soil has been previously reported (Schenkeveld, et al., 2010a; Schenkeveld, et al., 2010c). Recent studies suggest this decline is caused by displacement of Fe from o,o-FeEDDHA by Cu (Schenkeveld, et al., 2010d; Schenkeveld, et al., 2010e). Results from the present study support this, demonstrating the high relative affinity of o,o-EDDHA for Cu, and the tendency

of o,o-CuEDDHA to be removed from soil solution, inducing an ongoing reinstallment of the solution equilibria involving EDDHA species and causing the o,o-FeEDDHA concentrations to decline. Meso o,o-FeEDDHA is more susceptible to Fe displacement by Cu than racemic o,o-FeEDDHA (Schenkeveld, et al., 2010d; Schenkeveld, et al., 2010e), which corresponds with the stronger deviation from linear increase in meso o,o-FeEDDHA concentration. No substantial decrease in o,o-FeEDDHA concentrations was however observed in batch experiment 1. This probably results from a combination of factors: the shorter interaction time (without previous equilibration), a (partial) depletion of available Cu due to the much higher o,o-EDDHA dose which caused a decrease in Cu activity, and the lower relative rate of decline at (considerably) higher initial FeEDDHA concentrations (Schenkeveld, et al., 2010a).

The o,o-CuEDDHA concentration curves are typical for repetitive dosing with elimination: initially the concentration increased, but the slope flattened and a plateau was reached relatively quickly (Figure 9.4b). A saw-tooth concentration pattern underlies the measured Cu concentrations; upon o,o-EDDHA addition, Cu was mobilized from the soil and o,o-CuEDDHA concentration increased rapidly, after which it gradually decreased again due to adsorption, until new o,o-EDDHA was added again. The racemic and meso o,o-CuEDDHA concentration curves are identical, because the corresponding o,o-EDDHA ligands mobilize Cu to the same extent (Table 9.2), and adsorption kinetics of racemic and meso o,o-CuEDDHA are similar (Figure 9.2). The slopes of the concentration curves near t=0 indicate that o,o-CuEDDHA accounted for only 6% of the o,o-EDDHA added, i.e. much less than the 49 - 53% derived with the tangent in batch experiment 2 (Table 9.2). In batch experiment 1, a strong increase in o,o-CuEDDHA adsorption was observed after 2 days. The 2-day equilibration time in batch experiment 3, probably resulted in more favourable conditions for o,o-CuEDDHA adsorption already upon the first addition of EDDHA ligand, resulting in less o,o-CuEDDHA mobilization. The overall EDDHA fraction mobilizing metals was much lower than in batch experiment 2. After 2 days of interaction it equaled around 21% for both o,o-EDDHA isomers, compared to 83 - 88% in batch experiment 2. The fraction further decreased throughout the experiment.
Statistical analyses on metal concentrations in the 1:1, 1:5 and 1:25 $PO_4$-extracts (Table 9.3) indicate that only Cu concentrations were significantly elevated in the racemic and meso o,o-EDDHA treatments in comparison to the blank; approximately to the same extent, as would be expected from the CuEDDHA concentrations in soil solution. FeEDDHA concentrations in the extracts were below determination limit and could not be measured. The increase in Al and Mn concentration with lower soil solution ratios is remarkable and probably related to the formation of soluble Al- and Mn-phosphate complexes.
An additional 1:100 $PO_4$-extraction was done, to closer estimate the amount of o,o-CuEDDHA adsorbed. When averaging the results of the racemic and meso o,o-EDDHA treatment, the extracted o,o-CuEDDHA accounts for 5.2 μM of the 27 μM o,o-EDDHA added; pore water content of the extracted soil (16.8%) and the CuEDDHA concentration therein (0.27 μM) were corrected for. No mass balance for the o,o-EDDHA ligands could be established and it is unclear which fraction of the adsorbed o,o-CuEDDHA was extracted. The results from the

extractions do however clearly indicate that o,o-CuEDDHA accounts for a substantial fraction of the o,o-EDDHA added, and that at least a factor 20 more o,o-CuEDDHA is adsorbed, than that is present in soil solution.

**Table 9.3: a)** Metal concentrations extracted from Santomera soil with 20 mM $PO_4$-extractant in various soil-solution ratios. Standard deviations are indicated between parentheses; **b)** P-values of the ANOVA; and **c)** P-values of the post-hoc test on treatment.

| (a) | | Al | Co | Cu | Mn | Ni |
|---|---|---|---|---|---|---|
| Extraction | Treatment | (µmol $l^{-1}$) | (nmol $l^{-1}$) | (nmol $l^{-1}$) | (nmol $l^{-1}$) | (nmol $l^{-1}$) |
| 1:1 | blank | 4.8 (0.54) | 5.8 (1.4) | 393 (9) | 23.7 (3.1) | 60.8 (0.7) |
| | racemic o,o-EDDHA | 5.2 (0.12) | 4.9 (0.2) | 520 (52) | 22.6 (1.5) | 50.9 (9.9) |
| | meso o,o-EDDHA | 5.9 (1.2) | 5.6 (0.7) | 520 (46) | 23.5 (2.3) | 46.3 (0.5) |
| 1:5 | blank | 16.5 (0.8) | 3.4 (0) | 345 (4) | 59.9 (5.9) | 40.9 (3.4) |
| | racemic o,o-EDDHA | 23.7 (4.0) | 3.4 (0.5) | 402 (19) | 87.0 (22.7) | 57.7 (11.3) |
| | meso o,o-EDDHA | 17.8 (-) | 3.4 (-) | 392 (-) | 76.1 (-) | 51.4 (-) |
| 1:25 | blank | 16.9 (1.6) | 1.4 (0.5) | 161 (6) | 91.0 (7.7) | 29.6 (2.4) |
| | racemic o,o-EDDHA | 19.7 (3.6) | 1.4 (0) | 186 (18) | 92.1 (4.1) | 29.5 (2.6) |
| | meso o,o-EDDHA | 16.4 (2.2) | 1.2 (0.2) | 205 (12) | 97.6 (6.2) | 23.5 (1.0) |
| 1:100 | blank | | | 85 (4) | | |
| | racemic o,o-EDDHA | | | 122 (3) | | |
| | meso o,o-EDDHA | | | 136 (17) | | |

| (b) | Effect of treatment | |
|---|---|---|
| Al | 0.059 | |
| Co | 0.761 | |
| Cu | 0.003 | (0.000)* |
| Mn | 0.222 | |
| Ni | 0.345 | |

| (c) | Cu | |
|---|---|---|
| blank - racemic | 0.005 | (0.001)* |
| blank – meso | 0.007 | (0.002) |
| racemic - meso | 1.000 | (0.909) |

* in between parentheses: p-values if 1:100 extraction is included.

**Plant experiment 1**

Although quantitatively of little importance, Co mobilization by EDDHA is a suitable indicator for the shuttle mechanism in soil-plant systems. Unlike Cu and Mn, Co largely remains in solution[10], the variance in concentration among replicates and over time tends to be small, and background Co concentrations in soil solution are generally very low compared to

[10] This is illustrated by the Co mobilization data from batch experiment 1, presented in the Appendix (Figure A9.7).

other metals. Therefore Co concentrations in the pore water of Santomera soil were examined as a function of time (Figure 9.5).

Both plant and FeEDDHA treatment were required to obtain elevated Co concentrations. Elevation in Co concentration became substantial in the 3rd week, when SPAD-indices of the youngest leaves in the blank treatment became significantly lower than those in the FeEDDHA treatments (Schenkeveld, et al., 2010a). This implies that, from the 3rd week onwards, plants receiving FeEDDHA treatments were evidently utilizing the Fe offered as FeEDDHA. So, utilization of Fe from FeEDDHA and elevation in Co concentration coincided. The extent to which Co concentrations were elevated, was determined by the o,o-FeEDDHA content of the treatment. Fe uptake positively correlates to the amount of o,o-FeEDDHA applied (Schenkeveld, et al., 2008), so larger Fe uptake was related to more Co mobilization. These findings supports the hypothesis that upon FeEDDHA facilitated Fe uptake, EDDHA ligands are released and mobilize metals, i.c. Co.

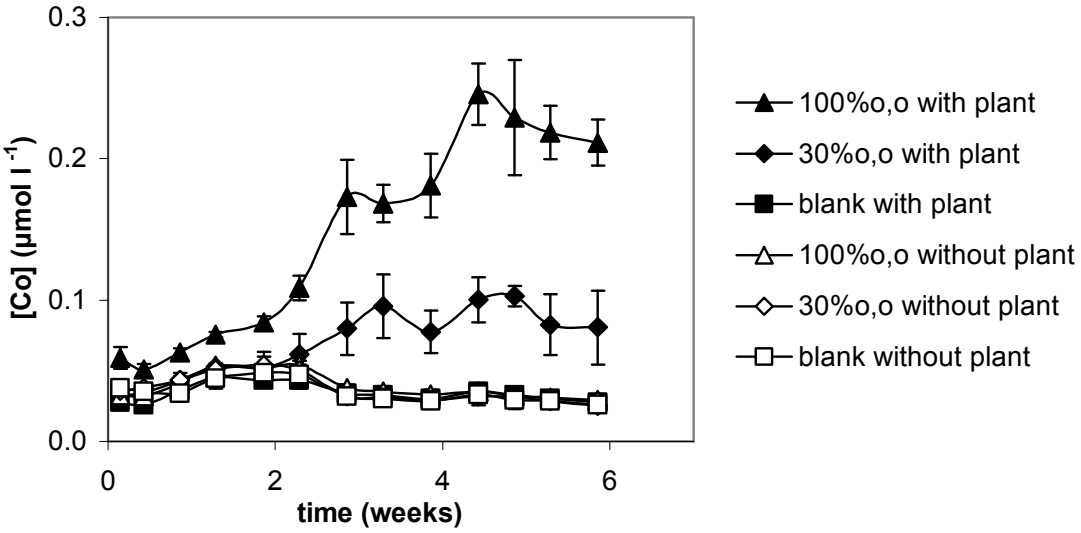



**Figure 9.5:** Co concentrations in the pore water of Santomera soil as a function of time for treatments with and without FeEDDHA application, and with and without plants.

**Plant experiment 2**

Because the kinetics involved with chelating and mobilizing metals are fast (see *batch experiment 1*), EDDHA ligands should be expected to mobilize metals from the direct vicinity of Fe uptake sites on the plasma membrane. Cell walls of root cells, both at the root surface and within the cortex, constitute a reactive surface onto which metals adsorb and metal hydroxides precipitate (Bienfait, et al., 1985; Kalis, et al., 2007). These metals are in close proximity to Fe uptake sites, and may comprise an important pool of metals for chelation by EDDHA ligands after Fe transfer. The effect of FeEDDHA application on metal availability at the root surface of soybean plants grown on Santomera soil was examined in plant

experiment 2 (Schenkeveld, et al., 2008) by means of an EDTA extraction of the roots. The amounts of Al, Co and Ni extracted from the root surface per gram dry weight root decreased linearly with increasing o,o-FeEDDHA content of the treatment (Figure 9.6). Blanks (without FeEDDHA treatment) have been excluded from the regression, because stress response mechanisms to Fe deficiency (rhizosphere acidification, excretion of chelating agents, etc (Marschner, et al., 1986)), cause metal mobilization from the root surface, unrelated to FeEDDHA application.

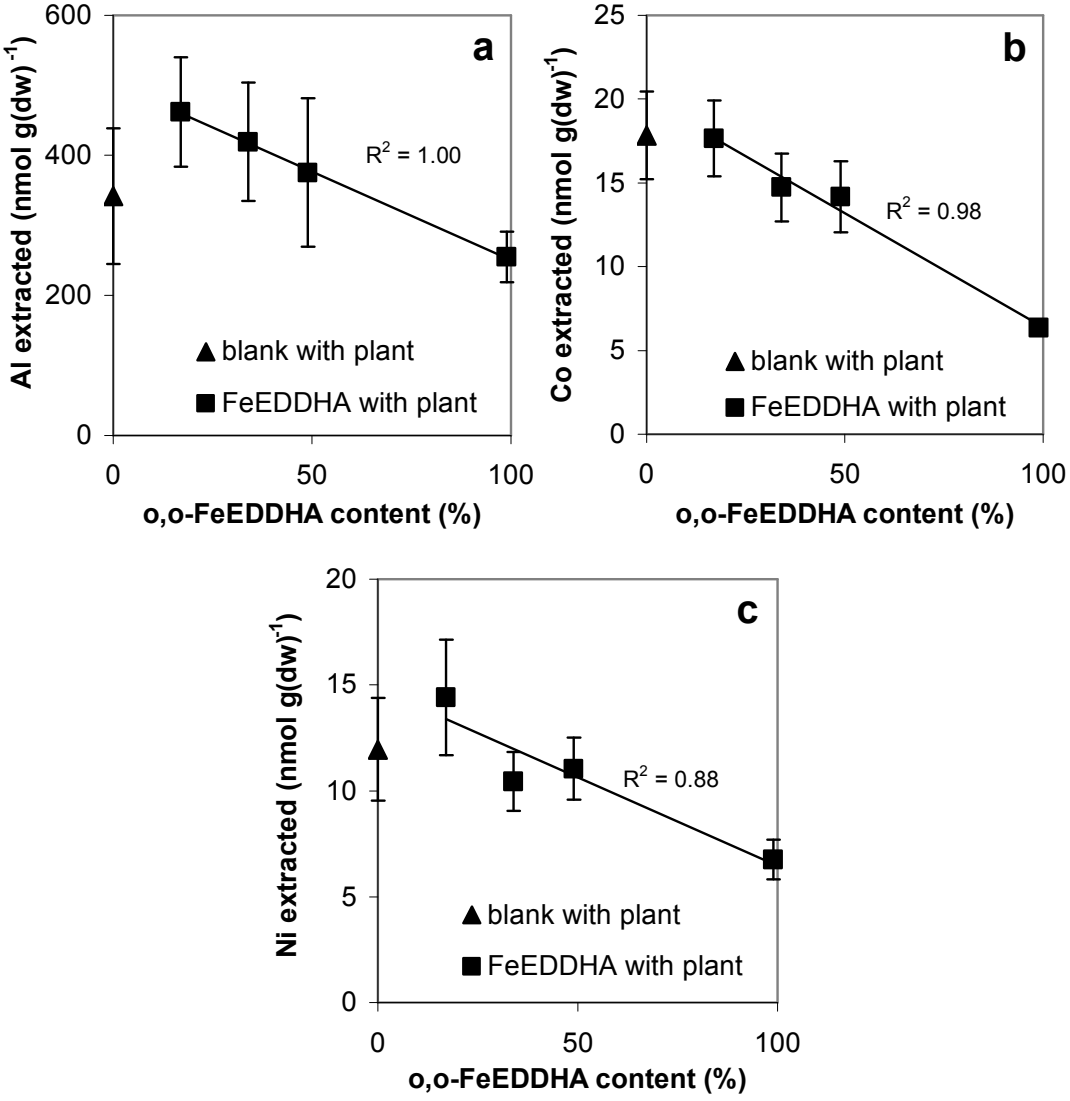



**Figure 9.6:** EDTA-extractable **a)** Al; **b)** Co; and **c)** Ni from roots of soybean plants grown on Santomera soil as a function of the o,o-FeEDDHA content of the treatment. The slopes of the regression lines for Al (p = 0.004), Co (p = 0.000) and Ni (p = 0.001) are significantly different from 0. Error bars indicate standard deviations.

Consistently, the concentrations of Al, Co and Ni in soil solution increased linearly with increasing o,o-FeEDDHA content of the treatment (Figure 9.7). In agreement with plant experiment 1, the data indicate that elevated metal concentrations are a combined effect of plant and FeEDDHA application: both the treatments with plant without FeEDDHA, and with FeEDDHA without plant result in lower metal concentrations than the treatments with both plant and FeEDDHA.

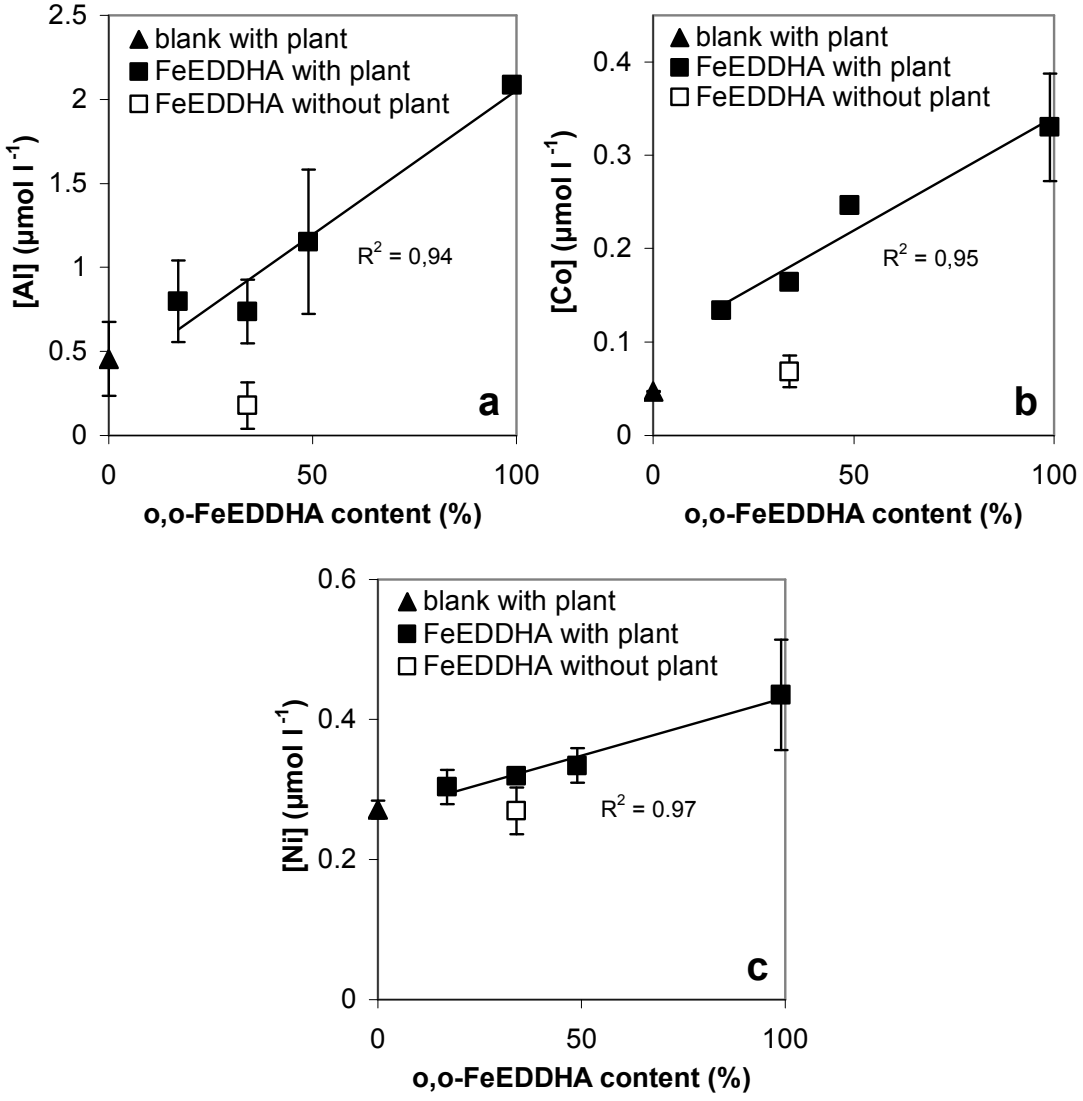



**Figure 9.7:** Al **(a)**, Co **(b)** and Ni **(c)** concentration in the pore water of Santomera soil as a function of the o,o-FeEDDHA content of the treatment. Slopes of the regression lines for Al ($p = 0.000$), Co ($p = 0.000$) and Ni ($p = 0.001$) deviate significantly from 0. Error bars indicate standard deviations.

For Fe and Cu, no significant decrease in EDTA-extractable amounts were observed as a function of the o,o-FeEDDHA content of the treatments. Possibly, the method applied does not deliver an adequate indication for Fe availability at the root surface due to the short extraction time and the relatively slow kinetics involved with EDTA-enhanced dissolution of Fe(hydr)oxides (Nowack and Sigg, 1997) from the root surface. Upon chelation of "root surface-Cu" by o,o-EDDHA, its high affinity for reactive surfaces may have caused o,o-CuEDDHA to adsorb to the root surface. If so, the amount of root surface-Cu remains unaffected by FeEDDHA treatment; possibly, part of the Cu in the EDTA extract was or had been chelated to o,o-EDDHA.

With the experimental setup of the plant experiments, it could not be determined whether the Fe chelated to o,o-EDDHA in soil solution was applied with the treatment or if it originated from the soil. Although, for this reason, the evidence of the shuttle mechanism remains circumstantial, the combined results of batch and plant experiments are convincing. Upon interaction of EDDHA ligands with soil in batch experiments, both Fe and competing metals were mobilized. In plant experiments, the soil solution concentration of competing metals, forming EDDHA chelates with limited affinity for the solid phase, were only elevated in treatments with plants receiving FeEDDHA; and elevation only started when plants had evidently started to utilize Fe from FeEDDHA. This suggests that, after Fe transfer to the plant, EDDHA ligands had mobilized competing metals; given the results from the batch experiments, it is only likely that EDDHA ligands will also have mobilized Fe.
If the efficiency of the shuttle is largely controlled by metal availability in the bulk soil, it is heavily compromised by competition from other cations. The Fe mobilizing efficiency amounted 21 - 52% for direct o,o-EDDHA application to calcareous soils; equilibration with electrolyte solution prior to o,o-EDDHA application to Santomera soil decreased the efficiency from 25 - 27% to 10 - 12%. However, root extraction data from plant experiments suggest that at least part of the metals mobilized by EDDHA were not adsorbed to soil, as suggested in Lindsay and Schwab's conceptual model, but to the root surface. Metal availability in the rhizosphere and the potential role of the apoplastic Fe pool in the roots (Bienfait, et al., 1985; Strasser, et al., 1999) need to be further examined in relation to metal chelation by EDDHA ligands. Tracer studies with isotope labeled FeEDDHA could aid this and help to provide a better understanding of the actual effectiveness of the shuttle mechanism in soil-plant systems.

# Appendix

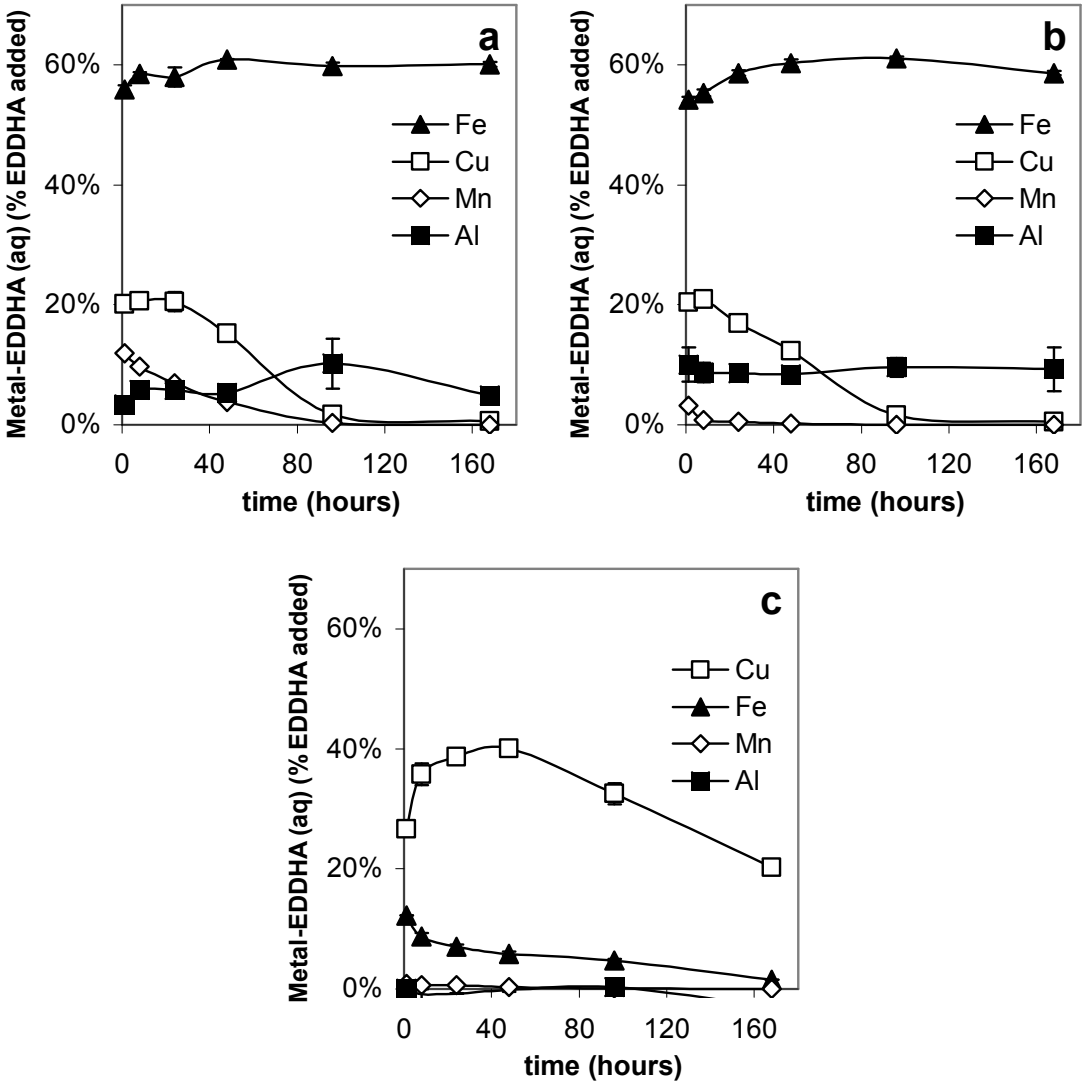



**Figure A9.1:** Percentage of **a)** racemic-o,o EDDHA; **b)** meso o,o-EDDHA; and **c)** o,p-EDDHA added, chelated to Fe, Cu, Mn and Al in solution upon interaction with Xeraco L soil as a function of time. Error bars indicate standard deviations.

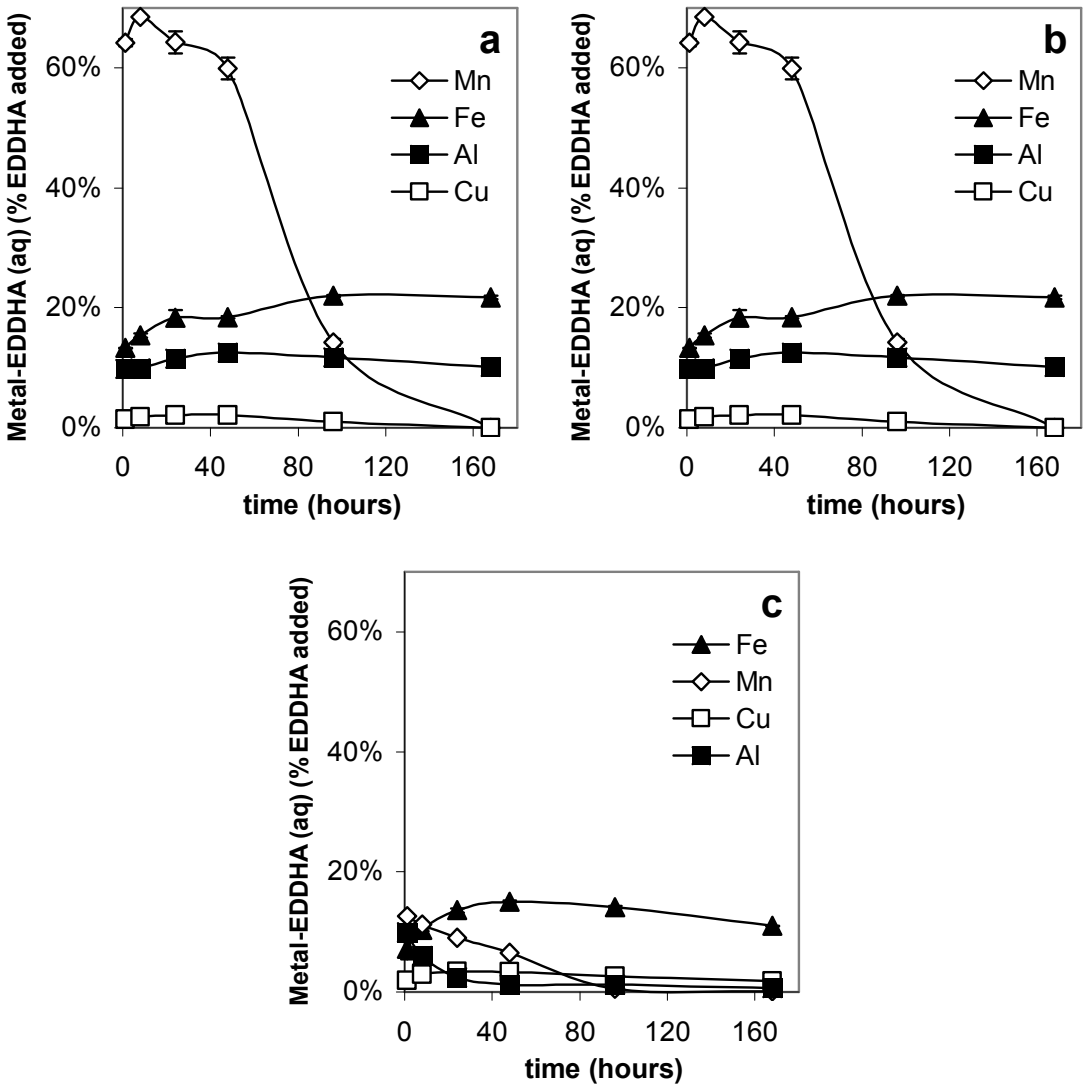



**Figure A9.2:** Percentage of **a)** racemic-o,o EDDHA; **b)** meso o,o-EDDHA; and **c)** o,p-EDDHA added, chelated to Fe, Cu, Mn and Al in solution upon interaction with Nadec soil as a function of time. Error bars indicate standard deviations.

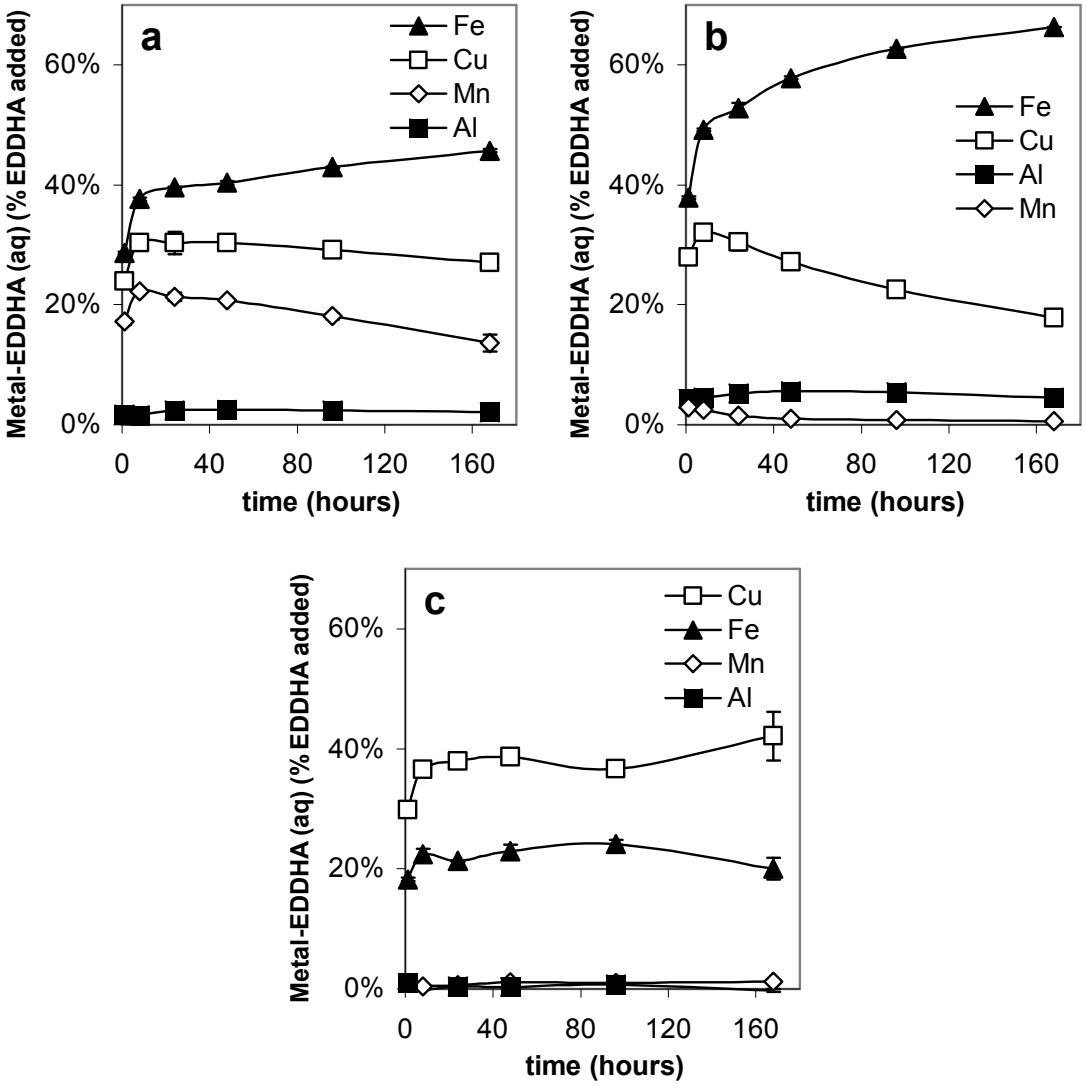



**Figure A9.3:** Percentage of **a)** racemic-o,o EDDHA; **b)** meso o,o-EDDHA; and **c)** o,p-EDDHA added, chelated to Fe, Cu, Mn and Al in solution upon interaction with Hofuf soil as a function of time. Error bars indicate standard deviations.

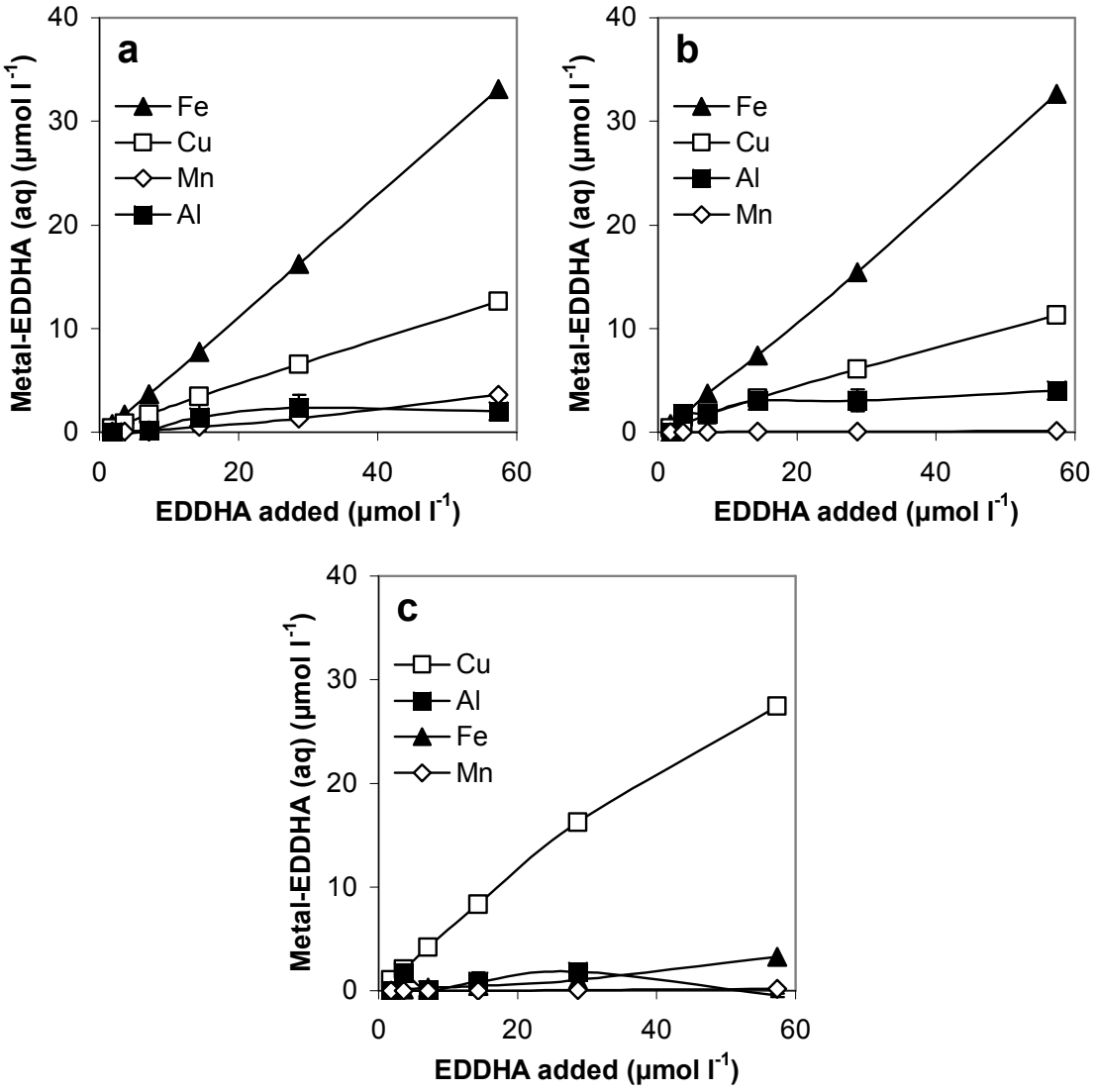



**Figure A9.4:** Metal-EDDHA concentrations in solution after 24 hours of interaction between EDDHA isomers and Xeraco L soil as a function of the concentration of **a)** racemic o,o-EDDHA; **b)** meso o,o-EDDHA; and **c)** o,p-EDDHA added. Error bars indicate standard deviations.

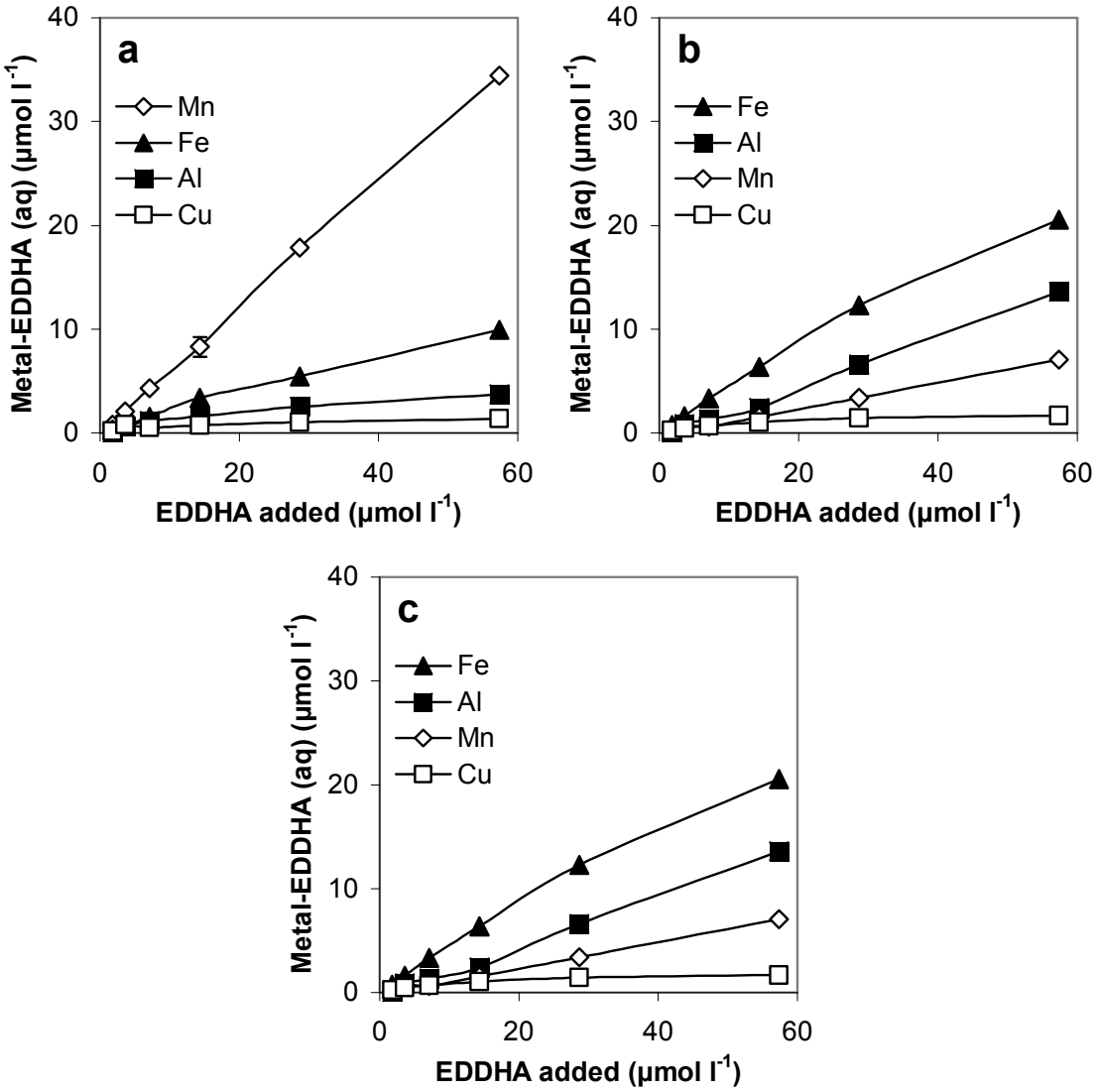



**Figure A9.5:** Metal-EDDHA concentrations in solution after 24 hours of interaction between EDDHA isomers and Nadec soil as a function of the concentration of **a)** racemic o,o-EDDHA; **b)** meso o,o-EDDHA; and **c)** o,p-EDDHA added. Error bars indicate standard deviations.

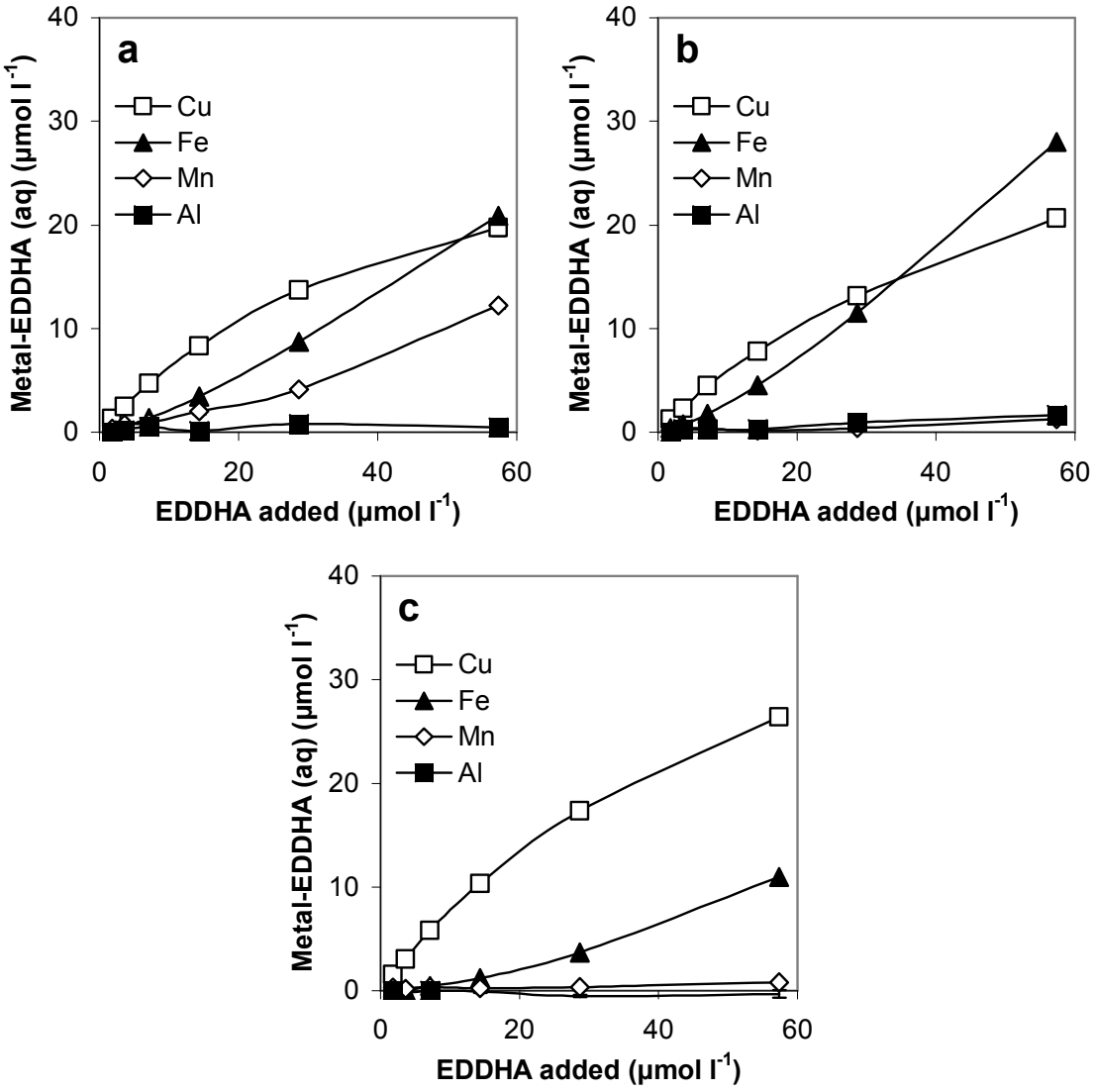



**Figure A9.6:** Metal-EDDHA concentrations in solution after 24 hours of interaction between EDDHA isomers and Hofuf soil as a function of the concentration of **a)** racemic o,o-EDDHA; **b)** meso o,o-EDDHA; and **c)** o,p-EDDHA added. Error bars indicate standard deviations.

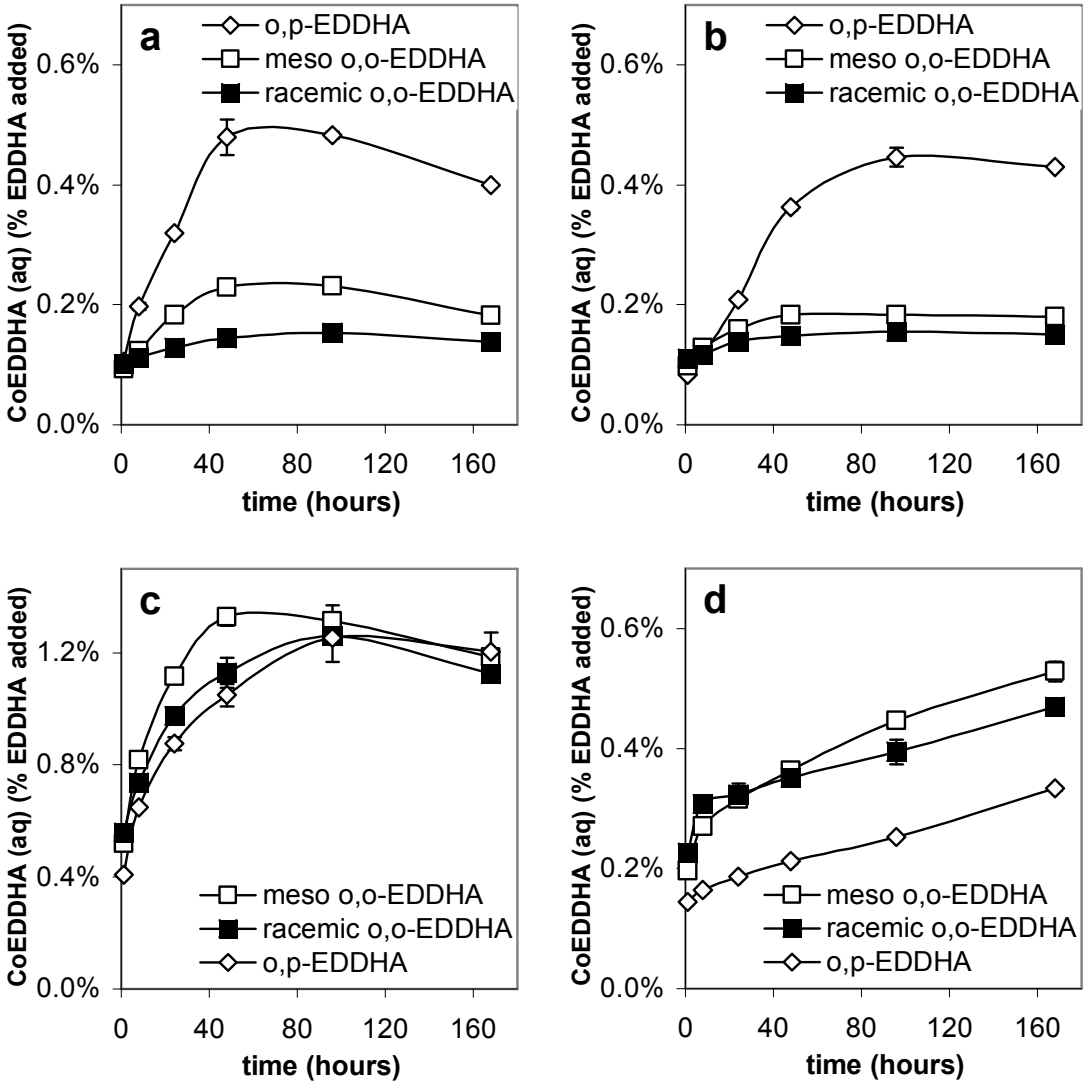



**Figure A9.7:** Percentage of EDDHA isomers added, chelated to Co in solution upon interaction with **a)** Santomera soil; **b)** Xeraco L soil; **c)** Nadec soil; and **d)** Hofuf soil as a function of time.

“Mann und Weib, und Weib und Mann,
  reichen an die Gottheit an.”

*trans*.
“Man and wife, and wife and man,
  reach to the height of godliness.”

Excerpt from the libretto of:
*“Die Zauberflöte”*
by Emanuel Schikaneder (1751 - 1812)

# Chapter 10

## Synthesis – A conceptual model for the behaviour of FeEDDHA components in soil-plant systems

# Introduction

In the general introduction (Chapter 1) it has been outlined, that, although FeEDDHA (iron (3+) ethylene diamine-N,N'-bis(hydroxy phenyl acetic acid)) products have been commonly applied to prevent and remedy Fe chlorosis in plants grown on calcareous soil since the 1950s, the fate and effectiveness of FeEDDHA components in soil-plant systems are still poorly understood. For an efficient use of FeEDDHA fertilizer, however, an advanced understanding of both the behaviour of FeEDDHA components in soil-plant systems, and of the efficiency of the individual FeEDDHA components in supplying plants with Fe is essential. Only with this understanding, moment and frequency of application, dosage and composition of the FeEDDHA treatment can be suitably matched with the Fe requirements of the crop and the characteristics of the soil.

In the preceding chapters several specific aspects regarding the behaviour of FeEDDHA components in soil-plant systems and the efficiency of FeEDDHA components as Fe fertilizer have been examined. The purpose of this concluding chapter is to present the key findings from this research study in a more coherent context, on the basis of a conceptual model. The model aims to identify and qualitatively describe the processes determining the fate of the individual FeEDDHA components, and is used as a starting point for discussing their effectiveness. It is based on the soil-plant system examined in the pot trial studies, presented in chapter 3 to 5, and was constructed from data collected in batch experiments, incubation experiments, pot trials and model calculations, described in the preceding chapters, supplemented with additional experimental data. The additional experiments are described in a materials and methods section, which is included in the Appendix.

First, the conceptual model is presented and elucidated on the basis of the observed concentration behaviour of individual EDDHA components. Then, the processes affecting EDDHA component concentrations are examined separately and more in-depth. Subsequently, the effectiveness of FeEDDHA components is discussed. In conclusion, the implications and limitations of the conceptual model and of the research presented in this thesis in general are considered, and suggestions for further research are made.

# Conceptual model

A conceptual model for the behaviour of FeEDDHA components in soil-plant systems is presented schematically in Figure 10.1. The backbone of the model consists of three processes, indicated with bold arrows:

1) FeEDDHA adsorption
2) Fe displacement from adsorbed FeEDDHA by Cu, followed by CuEDDHA release into solution
3) Slow re-adsorption of CuEDDHA

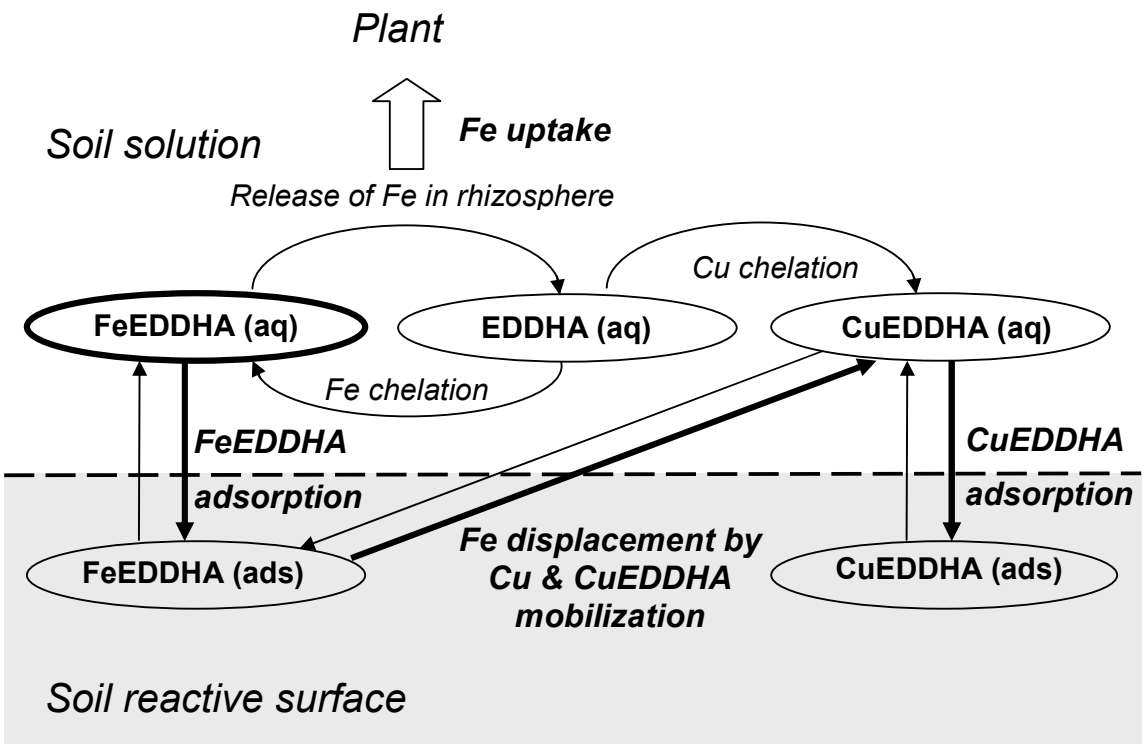



**Figure 10.1:** Conceptual model for the behaviour of FeEDDHA components in soil-plant systems. Bold arrows indicate the dominant direction of reversible processes.

Through mechanistic multi-surface modeling it has been affirmed there is a thermodynamic basis for assuming that cation competition from Cu may impact the soil solution concentration of all major FeEDDHA components in calcareous soils (Schenkeveld, et al., 2010d). The extent to which EDDHA is chelated to Cu under equilibrium conditions depends strongly on the EDDHA component as well as on soil characteristics like pH, Cu and reactive surface contents, and the solubility of the Fe(hydr)oxide phase. Complexation and mobilization of Cu upon interaction of EDDHA ligands with various calcareous soils confirmed the high relative affinity of the EDDHA ligands for Cu (Schenkeveld, et al., 2010f). In goethite suspensions it was demonstrated that the displacement of Fe from FeEDDHA by Cu is not kinetically inhibited (Schenkeveld, et al., 2010d) and predominantly occurs at a (soil) reactive surface, rather than in (soil) solution (Schenkeveld, et al., 2010e). This implies that FeEDDHA adsorption precedes Fe displacement (Figure 10.1). FeEDDHA adsorption is a relatively fast process; in goethite suspensions approximate adsorption equilibrium was attained within half an hour (Schenkeveld, et al., 2010e), in a soil incubation study the concentration drop as result of adsorption was completed within 24 hours (Schenkeveld, et al., 2010c).

Depending on the FeEDDHA component, Fe displacement by Cu leads to a strong release of CuEDDHA into solution (Schenkeveld, et al., 2010c; Schenkeveld, et al., 2007) (Figure 10.1). In goethite suspensions it was demonstrated that for a given FeEDDHA complex, the rate of

the displacement reaction in terms of decrease in FeEDDHA solution concentration depends on the available reactive surface area and on the FeEDDHA and Cu loading on this reactive surface area (Schenkeveld, et al., 2010e). For soil conditions, the rate equation of the displacement reaction can be simplified assuming: 1) adsorption is linear, 2) the Cu loading on the reactive surface remains constant, and 3) soil solution concentrations remain remote from equilibrium. The simplified rate equation can be solved to an exponential decay function in FeEDDHA solution concentration (Schenkeveld, et al., 2010e).
CuEDDHA released into soil solution gradually re-adsorbs (Figure 10.1). CuEDDHA has a high affinity for the soil solid phase (Schenkeveld, et al., 2010d). The strong tendency of CuEDDHA to adsorb greatly increases the impact of Cu competition on the FeEDDHA concentration in soil solution: as a result of the high degree of CuEDDHA adsorption, much more Fe needs to be displaced by Cu before the equilibrium ratio between FeEDDHA and CuEDDHA in soil solution is reached. The kinetics of CuEDDHA re-adsorption are much slower than the kinetics of FeEDDHA adsorption; after Cu had been rapidly mobilized from soil by EDDHA ligands, the CuEDDHA concentration remained approximately constant for approximately two days after which it gradually decreased in the course of days rather than hours (Schenkeveld, et al., 2010f).

In addition to the aforementioned processes, also Fe uptake is included into the conceptual model. In a pot trial it was shown that when FeEDDHA components are affected relatively little by cation competition, Fe uptake can lead to a pronounced decrease in FeEDDHA component concentration (Schenkeveld, et al., 2010a). It is generally assumed that the principal mechanism of Fe uptake from Fe chelates involves Fe reduction on the complex, chelate splitting and release of the ligand into soil solution (Chaney, et al., 1972; Lindsay and Schwab, 1982). A limited specificity of EDDHA ligands for chelating Fe from soil or root surfaces is the most probable cause for the contribution of Fe uptake to the decrease in FeEDDHA component concentration. In particular chelation of Cu was found to compromise efficiency of chelating Fe (Figure 10.1) (Schenkeveld, et al., 2010f).
Biodegradation was found not to affect FeEDDHA or CuEDDHA component concentration and has therefore not been included into the conceptual model (Schenkeveld, et al., 2010c)

## EDDHA isomer speciation in soil solution

Upon introduction into soil, the individual FeEDDHA components are exposed to the same conditions and processes. Still, the extent to which, and the rate at which the soil solution concentration of the FeEDDHA components is affected, differs distinctively as a result of differences in physico-chemical properties. This is illustrated for the quantitatively most important FeEDDHA components in FeEDDHA products: o,p-FeEDDHA (iron (3+) ethylene diamine-N-(2-hydroxy phenyl acetic acid)-N'-(4-hydroxy phenyl acetic acid) complexes) (Figure 10.2a), and racemic and meso o,o-FeEDDHA (iron (3+) ethylene diamine-N,N'-bis (2-hydroxy phenyl acetic acid) complexes) (Figure 10.2b). The o,p-FeEDDHA data were collected in a batch interaction experiment with FeEDDHA formulation "X" and Santomera

soil, described in the materials and methods section (SSR=1); the o,o-FeEDDHA data were collected in a pot trial study with soybean grown on Santomera soil, receiving FeEDDHA treatment ((Schenkeveld, et al., 2010a); treatment 100%o,oL; SSR≈6). The o,o-FeEDDHA isomer concentrations in the interaction experiment with formulation "X" (data not shown) declined much more slowly. Due to the lower soil solution ratio, less reactive surface area per unit solution was available for the displacement reaction, causing a much slower decline.
The three processes comprising the backbone of the conceptual model are well-illustrated from o,p-EDDHA speciation (Figure 10.2a). O,p-FeEDDHA was largely (for 86%) removed from soil solution within the first half hour, as a result of adsorption. Subsequently, o,p-CuEDDHA concentration increased, mainly as a result of Fe displacement from o,p-FeEDDHA by Cu, and release of o,p-CuEDDHA into solution. Formulation "X" contained a small fraction of o,p-EDDHA ligand, which also chelates and mobilizes Cu upon interaction with soil (Schenkeveld, et al., 2007; Schenkeveld, et al., 2010c). As a result, o,p-CuEDDHA concentration somewhat exceeded the initial o,p-FeEDDHA concentration after 1 day. After 3 days, o,p-CuEDDHA adsorption became the dominant process, which resulted in a net gradual decline in o,p-CuEDDHA concentration until the end of the experiment. At the same time the remaining o,p-FeEDDHA was removed from soil solution, probably as a result of a shift in solution equilibria due to o,p-CuEDDHA adsorption.

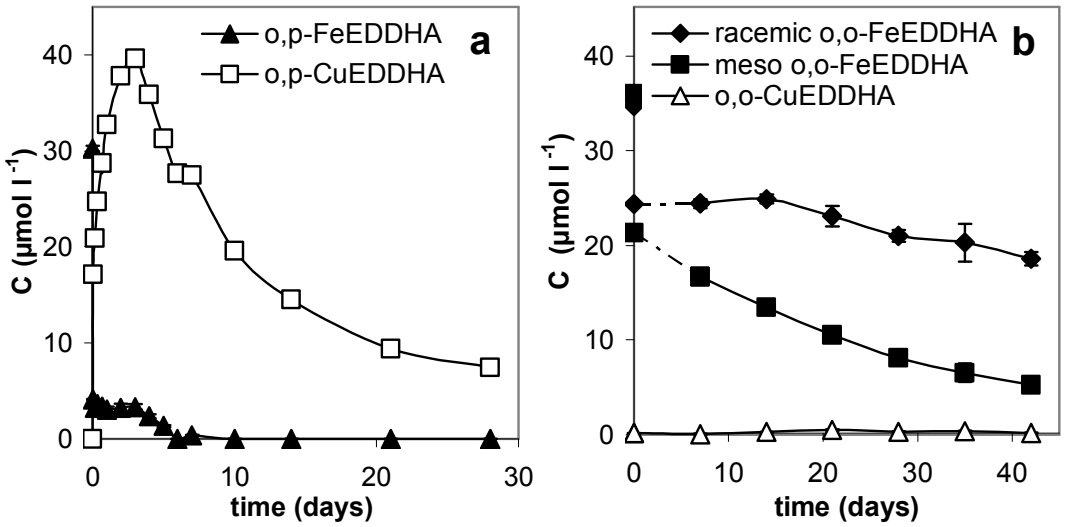



**Figure 10.2: a)** o,p-EDDHA speciation as a function of time in a batch experiment with FeEDDHA formulation "X" and Santomera soil (SSR = 1); **b)** o,o-EDDHA speciation in soil solution in a pot trial study with soybean plants grown on Santomera soil, receiving FeEDDHA treatment (Schenkeveld, et al., 2010a) (SSR = 6). Error bars indicate standard deviations.

In comparison to o,p-FeEDDHA, the initial concentration drop as a result of adsorption is much smaller for the o,o-FeEDDHA isomers, despite the much higher soil solution ratio (Figure 10.2a and 10.2b). After the initial drop, the meso o,o-FeEDDHA concentration

decreased exponentially as a function of time as a result of Fe displacement by Cu, in accordance with the simplified rate equation for soil conditions (Schenkeveld, et al., 2010a; Schenkeveld, et al., 2010e). For racemic o,o-FeEDDHA the exponential decline as a result of Fe displacement is less pronounced, because the concentration is also substantially affected by other processes, in particular racemic o,o-FeEDDHA facilitated Fe uptake by plants (Figure 10.1) (Schenkeveld, et al., 2010a). A significant effect of cation competition on the racemic o,o-FeEDDHA concentration, is however confirmed in batch and incubation experiments (Schenkeveld, et al., 2010c; Schenkeveld, et al., 2010f).

The sequence in rate of decline in FeEDDHA component concentration equals: o,p-FeEDDHA >> meso o,o-FeEDDHA > racemic o,o-FeEDDHA (Figure 10.2a and 10.2b). This sequence corresponds with both the affinity of the FeEDDHA components for the soil solid phase and the relative affinity of the corresponding EDDHA ligand for Cu in comparison to Fe (Schenkeveld, et al., 2010d; Schenkeveld, et al., 2010a).

Contrary to o,p-FeEDDHA, Fe displacement from o,o-FeEDDHA by Cu did not substantially increase the Cu concentration in soil solution (Figure 10.2b). This can be explained in view of the relation between the rate of Fe displacement and CuEDDHA release into solution ($r_{Cu\text{-}rel}$), and the rate of CuEDDHA adsorption ($r_{Cu\text{-}ads}$). If $r_{Cu\text{-}rel} > r_{Cu\text{-}ads}$, this leads to an increase in CuEDDHA concentration in soil solution (Figure 10.2a up until 3 days). If $r_{Cu\text{-}rel} < r_{Cu\text{-}ads}$, CuEDDHA concentration will decrease or no CuEDDHA mobilization will occur (Figure 10.2b and 10.2a after 3 days). Net o,p-CuEDDHA mobilization is kinetically favoured in comparison to o,o-CuEDDHA mobilization because: 1) o,p-FeEDDHA has a relatively high affinity for the soil solid phase where the displacement reaction takes place (Schenkeveld, et al., 2007), 2) Fe is bonded less strongly in the pentadentate o,p-FeEDDHA complex than in the hexandentate o,o-FeEDDHA complex; therefore Fe is probably displaced faster from o,p-FeEDDHA, and 3) o,o-CuEDDHA adsorption is considerably faster than o,p-CuEDDHA adsorption (Schenkeveld, et al., 2010f).

## Processes affecting FeEDDHA concentration

**FeEDDHA adsorption**

Adsorption of FeEDDHA components to soil is the net result of interaction with several soil reactive compounds, the most important ones being soil organic matter, clay and Fe(hydr)oxides (Hernandez-Apaolaza 2001). The extent to which FeEDDHA components adsorb is determined by both the affinity of the FeEDDHA components for the soil reactive compounds, and the occurrence of these compounds in the soil.

Pending questions regarding FeEDDHA adsorption in view of the conceptual model are: 1) which soil reactive compound dominates the adsorption process causing the initial concentration drop, as observed upon interaction of FeEDDHA components with Santomera soil (Figure 10.2); and 2) on which soil reactive compound does the displacement reaction take place?

The first question has been addressed by examining the adsorption behaviour of racemic and meso o,o-FeEDDHA to individual soil reactive compounds and interpreting this behaviour in relation to the adsorption isotherms of racemic and meso o,o-FeEDDHA to Santomera soil (Schenkeveld, et al., 2010a). Adsorption isotherms for racemic and meso o,o-FeEDDHA to Ca-montmorillonite and goethite were determined at pH 7, with $CaCl_2$ as background electrolyte (Figure 10.3). For meso o,o-FeEDDHA adsorption to goethite, the influence of ionic strength and adsorbed organic matter were also examined (Figure 10.3a). For racemic o,o-FeEDDHA only the isotherm for I = 0.01 M could be determined; at I = 0.1 M, with and without adsorbed HA, adsorption was too small to reliably measure (Figure 10.3b).
The adsorption isotherm with goethite and adsorbed HA was determined, because in soils, anions like phosphates and organic substances adsorb onto Fe(hydr)oxide surfaces thereby decreasing the electrostatic attraction between FeEDDHA and surface. For clay, an effect of anion adsorption has not been examined but is presumably smaller, because clay surfaces are already negatively charged. Adsorption to organic matter has not been considered, because the organic matter content of Santomera soil is low (≈ 1%) in comparison to the clay content (≈ 26%), and at neutral to alkaline pH, organic matter is strongly negatively charged, disfavouring FeEDDHA adsorption.

The adsorption isotherms (Figure 10.3) strongly suggest that the initial concentration drop observed for FeEDDHA components interacting with Santomera soil was predominantly caused by adsorption to clay, for three reasons:
First, the adsorption isotherms for racemic and meso o,o-FeEDDHA to Ca-montmorillonite are linear over the concentration range examined (Figure 10.3c), which corresponds with the shape of the adsorption isotherms to Santomera soil (Schenkeveld, et al., 2010a). Adsorption isotherms to goethite, however, bend off towards a maximum (Figure 10.3a and 10.3b). The negative effect of HA adsorbed onto the goethite surface on meso o,o-FeEDDHA adsorption shows clearly (Figure 10.3a).
Secondly, the ratio of the tangents of the isotherms for meso and racemic o,o-FeEDDHA is also comparable for adsorption to Ca-montmorillonte and Santomera soil: approximately 2.5. The ratio between racemic and meso o,o-FeEDDHA adsorption to goethite on the other hand, differs by approximately a factor 10.
And thirdly, by means of the presented adsorption isotherms, a first indication was obtained of how FeEDDHA adsorption to clay and Fe(hydr)oxides relate quantitatively in Santomera soil. For a given meso o,o-FeEDDHA concentration (0.2 mM), adsorption to clay and Fe(hydr)oxides were calculated, based on the reactive compound contents of Santomera soil (260 g $kg^{-1}$ clay ; 0.57 g $kg^{-1}$ reactive Fe(hydr)oxide and 10.6 g $kg^{-1}$ crystalline Fe(hydr)oxide). Adsorption to crystalline Fe(hydr)oxides was assumed to correspond with adsorption to goethite; the reactive Fe(hydr)oxides content was multiplied by six (based on the ratio in specific surface area between hydrous ferric oxide (HFO) and goethite) and added to the crystalline Fe(hydr)oxide content. The isotherm involving goethite with adsorbed HA was used for determining meso o,o-FeEDDHA adsorption. The calculated amount of adsorbed meso o,o-FeEDDHA was four times higher for clay than for Fe(hydr)oxides.

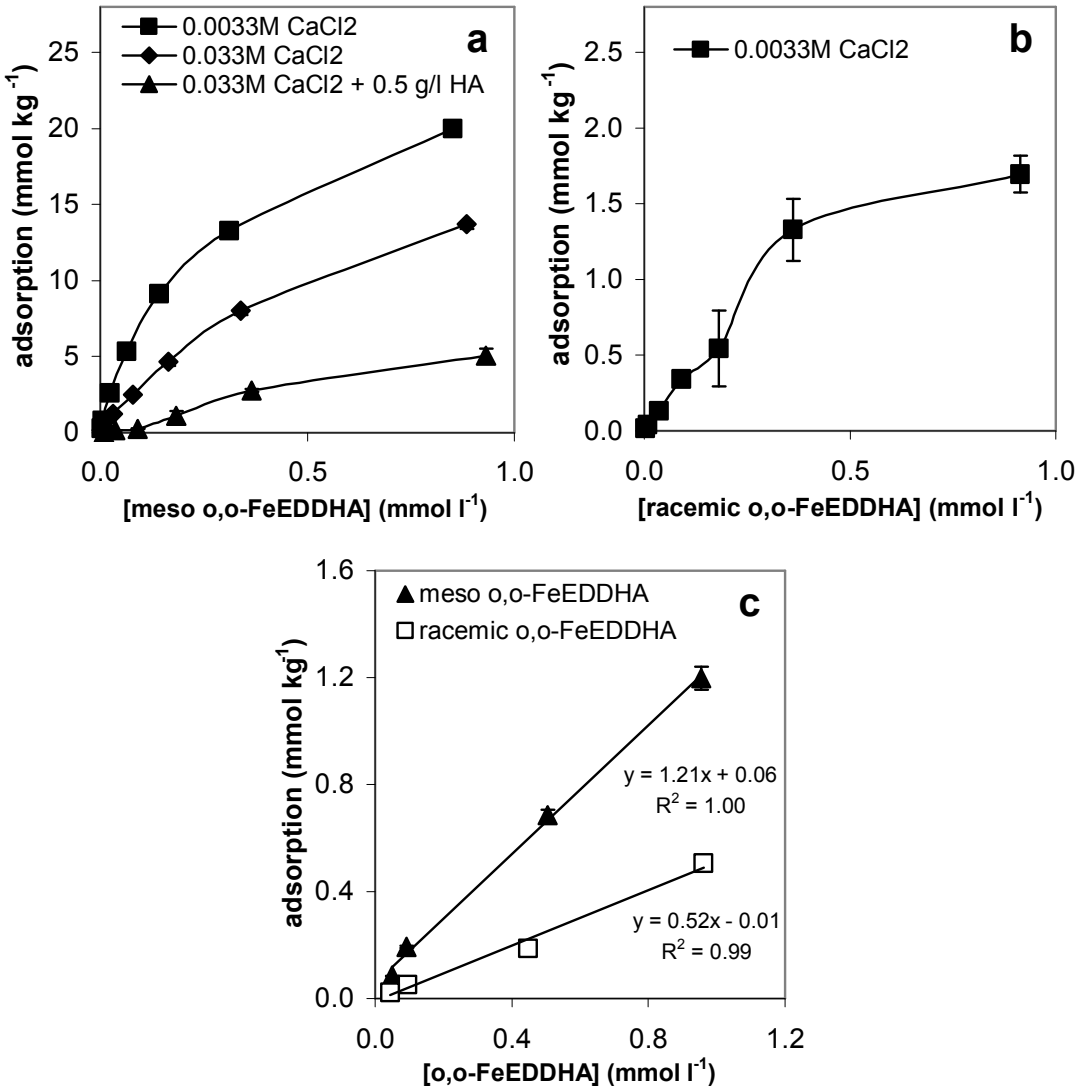



**Figure 10.3: a)** Adsorption isotherms for meso o,o-FeEDDHA to goethite at pH 7 with $CaCl_2$ as background electrolyte, I = 0.01 M and 0.1 M, with and without adsorbed HA; **b)** adsorption isotherm for racemic o,o-FeEDDHA to goethite at pH 7, with $CaCl_2$ as background electrolyte, I = 0.1 M; and **c)** adsorption isotherms for racemic and meso o,o-FeEDDHA to Ca-montmorillonite at pH 7.1 with $CaCl_2$ as background electrolyte; I = 0.3 M. Error bars indicate standard deviations.

Although adsorption to clay apparently dominates overall o,o-FeEDDHA adsorption, experimental data suggest that adsorption to other reactive surfaces also occurs, and that it plays an important role in the concentration behaviour of the o,o-FeEDDHA isomers.

Data from a batch experiment, in which FeEDDHA treatments interacted with eight soils (Schenkeveld, et al., 2007) show that the meso o,o-FeEDDHA concentration correlates much better with the reactive Fe(hydr)oxide content than with the clay content of the soils

(Figure 10.4). Linear regressions with $R^2 > 0.8$ were obtained for all sampling moments. Meso o,o-FeEDDHA concentrations gradually declined and the slope of the regression line steepened throughout the experiment. The fact that the linear relation was preserved over time strongly suggests that the reactive surface and the cause for the gradual decline in meso o,o-FeEDDHA concentration are related. This cause had been established in the conceptual model as Fe displacement by Cu. Ergo, the displacement reaction probably takes place at reactive Fe(hydr)oxide surfaces, answering the second pending question.

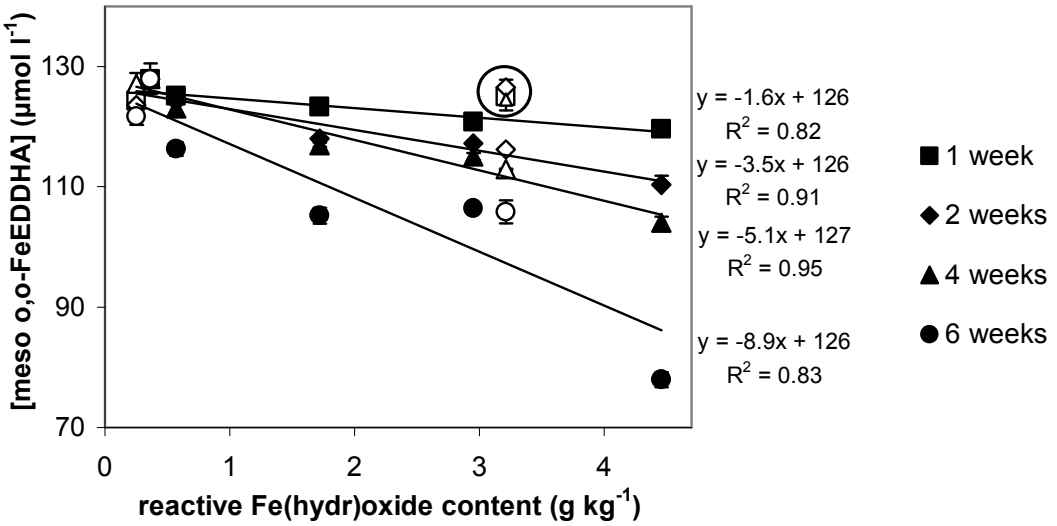



**Figure 10.4:** Meso o,o-FeEDDHA concentration as a function of the reactive Fe(hydr)oxide content of 8 soils. Data were collected in a 1:1 (w/v) batch interaction experiment, and are presented for 4 interaction times (Schenkeveld, et al., 2007). Open symbols represent sandy soils, closed symbols represent clay soils. The encircled data points concern a reference soil (Droevendaal) with a considerably lower pH. Error bars indicate standard deviations.

Correlation of meso o,o-FeEDDHA concentration to crystalline Fe(hydr)oxide content, and to a weighed sum (1:6) of crystalline and reactive Fe(hydr)oxide content were also checked but gave less good results. Meso o,o-FeEDDHA concentrations upon interaction with the reference soil Droevendaal (encircled in Figure 10.4) have not been included in the regressions. Despite its relatively high reactive Fe(hydr)oxide content, meso o,o-FeEDDHA concentrations remained constant throughout the experiment. Presumably this should be attributed to the considerably lower pH-$CaCl_2$ (5.3), in comparison to the other soils ($\geq$ 7.0). At lower pH, Fe displacement by Cu is thermodynamically less favourable.

It is important to note here that, although several experiments from which conclusions with respect to cation competition were drawn, were done specifically with Santomera soil, the relation between the rate of decline in meso o,o-FeEDDHA concentration and the reactive Fe(hydr)oxide content supports that the concept of Cu competition is more generally applicable for calcareous soils.

**Fe displacement by Cu**

With the displacement reaction taking place on soil Fe(hydr)oxide surfaces, and FeEDDHA adsorption to Fe(hydr)oxides being non-linear, in principal the linear adsorption assumption for simplifying and solving the rate equation of the displacement reaction to an exponential decay function in FeEDDHA solution concentration is violated (Schenkeveld, et al., 2010e). Still, given the accurate exponential fits of meso o,o-FeEDDHA concentration data (Schenkeveld, et al., 2010a; Schenkeveld, et al., 2010c), apparently, within the concentration ranges observed in individual FeEDDHA treatments, FeEDDHA adsorption could still be considered approximately linear. However, in a pot trial study with Santomera soil and FeEDDHA treatments containing meso o,o-FeEDDHA concentrations over a relatively large concentration range: 0.012 – 0.36 mmol (i.e. 0.68 – 20 mg $l^{-1}$ Fe) (Schenkeveld, et al., 2010a), it was observed that the decay constant declined with increasing initial meso o,o-FeEDDHA concentration. This can be explained by assuming that at higher meso o,o-FeEDDHA concentrations, a smaller fraction adsorbed onto Fe(hydr)oxide surfaces where the displacement reaction takes place, resulting in a lower relative displacement rate. This explanation is in line with the observed non-linear adsorption to Fe(hydr)oxides (Figure 10.3a). The effect of the decay constant decreasing with increasing initial meso o,o-FeEDDHA concentration may have been somewhat enhanced by plants, utilizing a higher meso o,o-FeEDDHA fraction for Fe uptake at lower initial meso o,o-FeEDDHA concentration, but was also observed in treatments with different initial meso o,o-FeEDDHA concentrations in which plants took up an equal amount of Fe (Schenkeveld, et al., 2010a).

In retrospective it is deduced that the displacement rate increased with increasing temperature and that soil sterilization decreased the displacement rate (Schenkeveld, et al., 2010c). Gamma-irradiation generated a lot of dissolved organic carbon (DOC), which in part adsorbed onto soil Fe(hydr)oxides surfaces. DOC adsorption decreased FeEDDHA adsorption to these surfaces (Schenkeveld, et al., 2010e) and reduced the rate at which meso o,o-FeEDDHA was removed from soil solution. The lack of a significant difference in initial concentration drop between sterilized and non-sterilized treatments confirms that the contribution of adsorption to Fe(hydr)oxide surfaces to overall adsorption is marginal.

**Re-adsorption of CuEDDHA**

The part of the conceptual model covering CuEDDHA adsorption is still unsatisfying; since the displacement reaction takes place on a soil reactive surface, a release of CuEDDHA into solution before adsorption, as observed for o,p-CuEDDHA (Figure 10.2a), seems counter-intuitive. Rapid mobilization and gradual adsorption of o,o-CuEDDHA upon interaction of o,o-EDDHA ligands with soil raises a comparable question regarding the underlying mechanism.

To explain these observations, a distinction between two mechanisms involved in CuEDDHA adsorption to soil is proposed: 1) a fast adsorption mechanism with limited adsorption capacity and 2) a slow adsorption mechanism with much larger adsorption capacity. Upon formation at the reactive surface, CuEDDHA is partly “instantaneously” adsorbed through the first

mechanism and partly released into solution, where it becomes subject to the second, slow adsorption mechanism, which is responsible for the observed gradual decline in CuEDDHA concentrations. The rate of CuEDDHA release ($r_{Cu\text{-}rel}$), mentioned before, equals the rate of CuEDDHA formation corrected for the extent of adsorption by the first adsorption mechanism, while $r_{Cu\text{-}ads}$ equals the adsorption rate by the second mechanism. The first mechanism is treated as an equilibrium process on the time scale of the slow adsorption reaction.

Thus far, little research on CuEDDHA adsorption to soil has been done. Therefore many questions regarding the soil reactive compounds involved and the mechanisms of action remain. Evidence for the fast adsorption mechanism was observed upon introduction of o,o-CuEDDHA into goethite suspensions (Schenkeveld, et al., 2010d); the concentration drop as a result of adsorption was completed within half an hour. Presumably this mechanism resembles the mechanism of FeEDDHA adsorption, which is comparably fast. Slow CuEDDHA adsorption was not observed in goethite suspensions, so other or additional soil compounds are involved.
In batch experiments, it was observed that o,o-EDDHA mobilized substantially less metals, in particular Cu, if the soil was first equilibrated with background electrolyte before o,o-EDDHA ligand was added (Schenkeveld, et al., 2010f). Hence, it was concluded that equilibration led to conditions more favourable for o,o-CuEDDHA adsorption. The CuEDDHA coordination complex has elongated bonds along the z-axis, or even a square planar structure as a result of a Jahn-Teller distortion due to the odd number of electrons in the $e_g$-orbitals (d-shell). This geometry makes the chelated Cu more susceptible to ligand exchange reactions than for instance Fe, which forms a relatively regular octahedral coordination complex with o,o-EDDHA. Functional groups of organic substances in the soil can participate in ligand exchange reactions. Therefore it is hypothesized that organic substances, which had coagulated while drying the soil, gradually unfolded upon rewetting and, in this unfolded form, were better able to participate in ligand exchange reactions, thereby enhancing the adsorption process.

To test this hypothesis, the influence of equilibration time and addition of organic substance on the adsorption rate of racemic o,o-CuEDDHA was examined in a batch experiment with Santomera soil, described in the Appendix.
Mobilization of Fe or any other metal, as a result of displacement, was negligible, indicating the Cu remained chelated by EDDHA. In the treatment which had not been equilibrated prior to CuEDDHA addition, CuEDDHA concentration displayed a small initial drop which is attributed to the fast adsorption mechanism. Subsequently, the concentration remained approximately constant for 2 days (Figure 10.5a), after which it started to gradually decrease due to the slow adsorption mechanism. In the three treatments which had been equilibrated before CuEDDHA addition, the decline in concentration set in straight away. Addition of organic substances had a considerable positive effect on the adsorption rate, and the effect was larger for FA than for HA; in the treatment without addition of organic substance, racemic

o,o-CuEDDHA was practically removed from solution after 96 hours, with HA addition after approximately 48 hours, and with FA addition after approximately 24 hours. In all treatments, the racemic o,o-CuEDDHA concentration declined linearly over time, indicating the rate of decline was independent of the CuEDDHA concentration, which corresponds with zero-order kinetics.

The slow adsorption mechanism presumably involves the formation of a ternary surface complex between CuEDDHA, organic substance and a clay or Fe(hydr)oxide surface. This is derived from the observations that: 1) organic substance is clearly involved in the mechanism, and 2) the extent of o,o-CuEDDHA adsorption proved related to the clay and crystalline Fe(hydr)oxide content of calcareous soils, and not to the organic matter content or DOC concentration (Schenkeveld, et al., 2010d). The formation of a ternary complex of this sort may also help to explain why only a fraction of CuEDDHA could be desorbed from soil, even with a large excess of phosphate (20 mM $PO_4$; SSR = 0.01) (Schenkeveld, et al., 2010f); large organic molecules like FA and HA participating in the ternary complex presumable bind more strongly to soil reactive compounds like Fe(hydr)oxides than individual chelates.

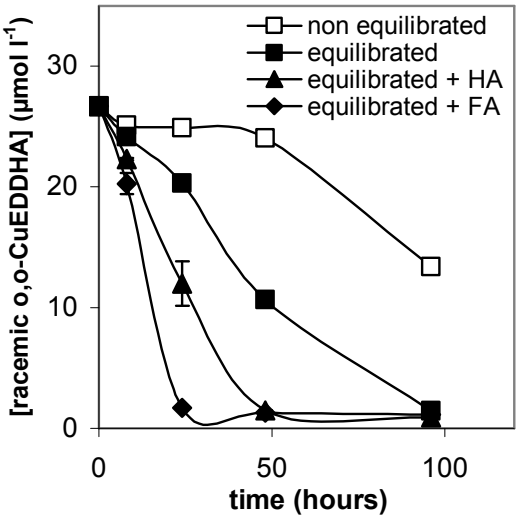



**Figure 10.5:** Racemic o,o-CuEDDHA concentration as a function of time upon interaction with Santomera soil in a 1:2 (w/v) batch experiment. Background electrolyte was 0.01 M $CaCl_2$. Treatments involved equilibration prior to CuEDDHA addition and addition of humic acid (HA) and fulvic acid (FA). Error bars indicate standard deviations.

The gradual decline in o,o-CuEDDHA concentration due to the slow adsorption mechanism, leads to gradual desorption of o,o-CuEDDHA adsorbed through the fast mechanism. The eventual near complete removal of CuEDDHA from soil solution, implies also the extent of adsorption by the fast mechanism becomes negligible. This has an important implication for the displacement reaction of Fe from FeEDDHA by Cu. It was concluded that the displacement reaction predominantly takes place on soil Fe(hydr)oxide surfaces (see

paragraph on FeEDDHA adsorption), and it was observed in goethite suspensions that (direct) CuEDDHA adsorption to Fe(hydr)oxides follows the fast mechanism. So despite the displacement reaction, the CuEDDHA loading on soil Fe(hydr)oxide surfaces remains low as a result of CuEDDHA desorption and re-adsorption through the slow mechanism (Figure 10.6). Therefore, CuEDDHA loading remains remote from the displacement equilibrium. This was one of the criteria for solving the displacement rate equation to an exponential function in FeEDDHA (Schenkeveld, et al., 2010e).
The Fe displacement related increase in o,p-CuEDDHA concentration to the extent presented in Figure 10.2a may be considered an experimental artifact. Because the soil had been dried, the slow adsorption mechanism had a lag time (Figure 10.5a) and hardly affected the o,p-CuEDDHA concentration for the first 48 hours, during which Fe displacement from o,p-FeEDDHA by Cu was almost complete. Still, also addition of o,p-FeEDDHA to equilibrated soil systems leads to substantial mobilization of o,p-CuEDDHA (Schenkeveld, et al., 2010c)

The presented data are inconclusive to whether CuEDDHA first binds to organic substances in solution and the two adsorb together as a complex, or CuEDDHA first adsorbs to a clay or Fe(hydr)oxide surface and organic substances bind to the adsorbed CuEDDHA complex. The zero-order kinetics, observed for the slow o,o-CuEDDHA adsorption, are typically found when a compound that is required for the reaction to proceed, is saturated by the reactant. Based on this notion, the following mechanism is hypothesized: organic substances capable of binding CuEDDHA through ligand exchange desorb from the soil solid phase (with a lag time if they first need to unfold); CuEDDHA binds in solution to a maximum extent (saturation) to dissolved organic substances with a limited number of suitable binding sites; the organic substances adsorb to the soil solid phase again, thereby decreasing the total CuEDDHA concentration in solution[11]. The overall DOC concentration remains approximately constant (Figure A10.1) as a result of simultaneous desorption of organic substances providing new binding sites for CuEDDHA. As long as the adsorbed organic substances binding CuEDDHA constitute a small fraction of the pool of desorbable organic substances, potentially binding CuEDDHA, the proposed mechanism continues to decline the CuEDDHA concentration at a constant rate. The continuous ad- and desorption of organic substances is comparable to the mechanism of a conveyor belt removing CuEDDHA from solution.
In this hypothesized mechanism, the rate of ad- and desorption of organic substances and the number of suitable binding sites per unit organic substance determine the rate of CuEDDHA removal from solution. The difference in rate of decline in CuEDDHA concentration between the treatments with HA and FA addition should be explained from a difference in these factors.

[11] Total CuEDDHA concentration comprises “free” CuEDDHA and CuEDDHA bonded to dissolved organic substances. Total CuEDDHA concentration was calculated as described in the Appendix; no distinction can be made between CuEDDHA species.

Based on the new insights in the processes affecting the concentration of FeEDDHA components, the conceptual model is revised and presented in Figure 10.6. The principal changes in comparison to Figure 10.1 are:

- A distinction in FeEDDHA adsorption to different reactive soil compounds, with adsorption to clay causing the initial concentration drop, and adsorption to Fe(hydr)oxides determining the rate of the reaction at which Cu displaces Fe from FeEDDHA.
- A distinction in two types of CuEDDHA adsorption: fast adsorption (and partial release into solution) upon CuEDDHA formation at the reactive surface, and slow CuEDDHA adsorption as ternary complex.

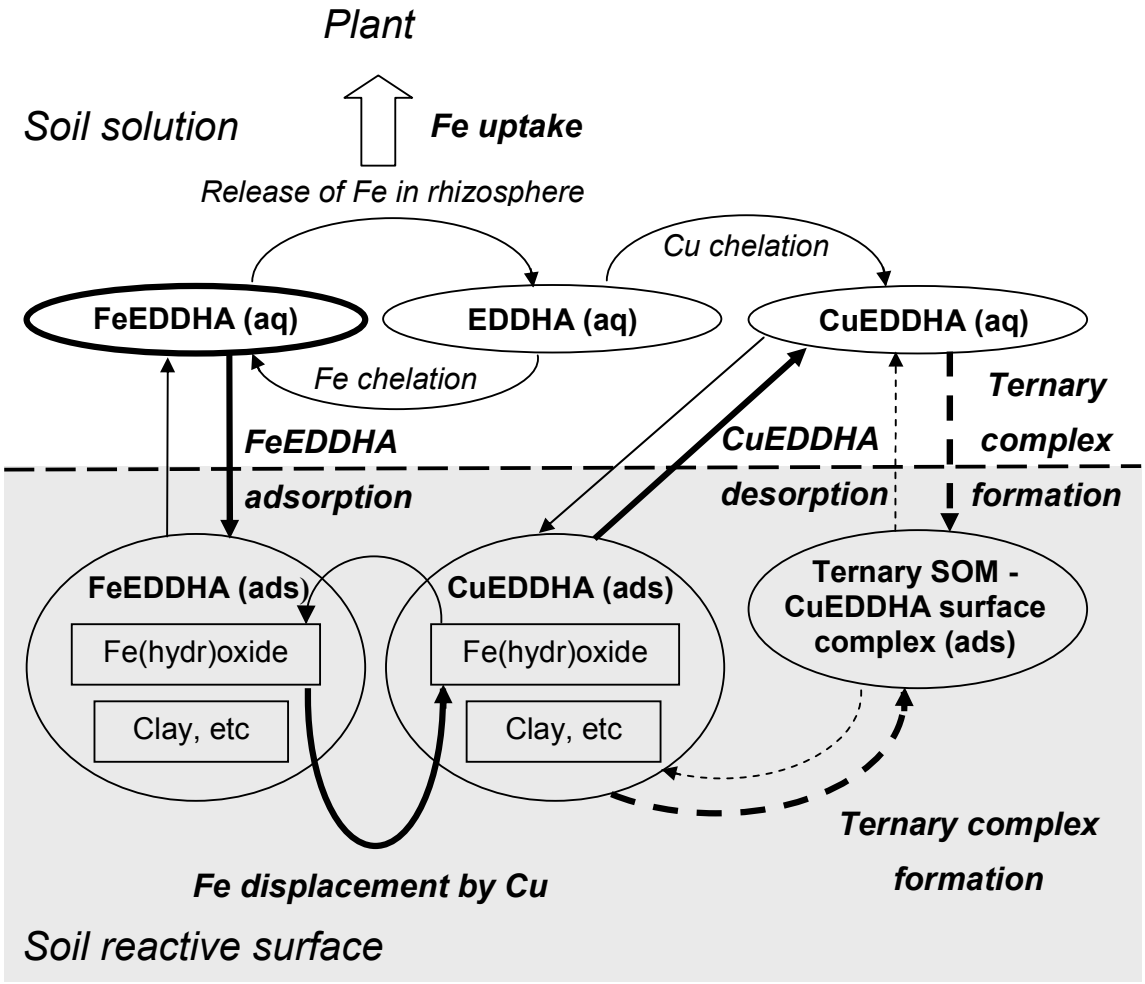



**Figure 10.6:** Revised conceptual model for the behaviour of FeEDDHA components in soil-plant systems. Bold arrows indicate the dominant direction of reversible processes. Dashed arrows indicate uncertainty on the mechanism by which an EDDHA species arrives in a certain state.

## Effectiveness

The effectiveness of Fe chelates as fertilizer is based on their ability to maintain Fe in soil solution and deliver it to the plant. From the findings presented in this thesis, as well as from literature, a list of requirements for an effective chelate-based Fe fertilizer has been assembled. Most requirements are not straight forward in the sense that "more is better"; features of the chelate should remain within a specific bandwidth.

Fe chelates used as fertilizer in soil application should:

- Bind Fe strongly in order to prevent complex dissociation and subsequent precipitation of Fe, — but not too strongly, that the chelated Fe becomes unavailable to plants.
- Be well-soluble and have limited affinity for soil reactive compounds in order to sufficiently increase Fe availability to meet the plants Fe demands, — but not readily leach out of the root-zone.
- Have a high relative affinity for Fe, so that cation competition does not substantially limit the effectiveness of the Fe chelate, and the ligand will bind Fe from the soil, once Fe has been delivered to the plant.
- Not be subject to biological or physico-chemical degradation throughout the growing season, — but neither be persistent in the environment.
- Facilitate Fe uptake, — but not be taken up by the plant as a whole.

**FeEDDHA component**

In the pot trial experiments conducted, it was found that the effectiveness of FeEDDHA components in delivering Fe to soil-grown plants is largely determined by the ability of FeEDDHA components to remain in solution. Characteristics that proved essential for this are: a limited affinity for the soil solid phase - to avoid a high degree of adsorption, and a high (relative) affinity for Fe - to avoid Fe displacement from the FeEDDHA component by competing cations, in particular Cu. Since the displacement reaction of Fe by Cu mainly takes place on a soil reactive surface, the processes of adsorption and Fe displacement should not be considered as independent threats to the effectiveness of FeEDDHA components.

The residence time of o,p-FeEDDHA in soil solution proved too short, to significantly contribute to facilitating Fe uptake (Schenkeveld, et al., 2010b). As discussed in view of the conceptual model, o,p-FeEDDHA displays a high degree of adsorption and a swift Fe displacement by Cu.

The residence time of both racemic and meso o,o-FeEDDHA was much longer and both isomers did contribute to Fe uptake (Schenkeveld, et al., 2010b). Although both o,o-FeEDDHA isomers are also subject to cation competition from Cu (Schenkeveld, et al., 2010c; Schenkeveld, et al., 2010f), the process goes much slower (Figure 10.2), at least in part

due to a much lower tendency to adsorb in comparison to o,p-FeEDDHA (Schenkeveld, et al., 2007). The extent to which racemic and meso o,o-FeEDDHA facilitated Fe uptake was approximately equal on the timescale considered (Schenkeveld, et al., 2010b). Lucena and Chaney (2006) reported that meso o,o-FeEDDHA was more effective in delivering Fe to hydroponically grown cucumber plants than racemic o,o-FeEDDHA, as a result of a lower stability favouring Fe reduction at the root surface. Possibly in soil, a preferential Fe uptake from meso o,o-FeEDDHA was balanced by a higher affinity for the solid phase and a faster decline in soil solution concentration (Figure 10.2b). Because the racemic o,o-FeEDDHA concentration in soil solution declines slower than the meso o,o-FeEDDHA concentration, the former isomer will remain effective for a longer time-span than the latter (Figure 10.2b). The extent to which racemic and meso o,o-EDDHA ligands mobilize Fe from soil via a shuttle mechanism is comparable (Schenkeveld, et al., 2010f).
The effectiveness of rest-FeEDDHA has not been separately assessed in soil plant-systems. In a pot trial study with FeEDDHA treatments differing in composition (Schenkeveld, et al., 2008) it was concluded that o,o-FeEDDHA governed Fe uptake and the contribution of rest-FeEDDHA was marginal, at most. The tendency of rest-FeEDDHA to (quickly) adsorb to soil compound (Hernandez-Apaolaza, et al., 2006; Schenkeveld, et al., 2007), is the primary cause for its poor effectiveness.

**Soil factors**
Soil factors affect the residence time of FeEDDHA components in soil solution and hence the time-span they remain effective as Fe fertilizer. For this reason, soil factors should be included in soil-specific Fe fertilization recommendations, affecting the recommended dosage and/or frequency of FeEDDHA application. In this thesis, the following soil factors have been identified to substantially affect FeEDDHA component concentrations in calcareous soils:

- Clay content – The initial drop in racemic and meso o,o-FeEDDHA concentration upon interaction with Santomera soil was mainly caused by adsorption to clay (Figure 10.2b; see section on FeEDDHA adsorption). In a batch interaction experiment with eight soils, the remaining o,p-FeEDDHA concentration also decreased with increasing clay content of the soils (Schenkeveld, et al., 2007).
- Fe(hydr)oxide content – Soil Fe(hydr)oxides provide the reactive surface on which the displacement of Fe from o,o-FeEDDHA by Cu takes place (see section on FeEDDHA adsorption). For meso o,o-FeEDDHA it has been demonstrated that the rate of decline in soil solution concentration increased with increasing reactive Fe(hydr)oxide content.
- Cu content – Cu constitutes an essential reagent in the aforementioned displacement reaction. In experiments with goethite suspensions, it has been demonstrated, that the decline in FeEDDHA solution concentration increased with increasing Cu loading on the Fe(hydr)oxide surface (Schenkeveld, et al., 2010e).

## Practical implications and limitations

The findings on the fate and effectiveness of FeEDDHA components in soil-plant systems presented in this thesis may:

- Serve appliers of FeEDDHA fertilizer to make a better selection out of the available products and help them to optimize the dosage and frequency of application. Clay-, reactive Fe(hydr)oxide- and Cu content of the farmland constitute important input for composing a tailor-made Fe fertilization plan.
- Serve producers of FeEDDHA fertilizers by providing leads for optimizing the compositions of their formulations and for effectively marketing their products.
- Provide points of attention for developing new, more efficient Fe fertilizers for soil application, based on alternative Fe chelates. For instance, besides a sufficiently high complexation constant to prevent Fe precipitation, the great importance of a high relative affinity for Fe in comparison to competing cations, and a limited tendency to adsorb have been demonstrated.

The presented conceptual model combines important insights in processes affecting the concentration of FeEDDHA components in a controlled, well-defined soil-plant system. These insights are of value to agricultural practice, because these same processes also take place in the field. A translation of the experimental results presented in this thesis to a field situation should however be treated with caution, because plant care and growth conditions differ strongly between the field and an academic setting, not all processes affecting FeEDDHA concentration in the field have been considered, and the relative impact of the individual process may well be different in the field than in a controlled environment.
In this thesis study, insight in processes was pursued on a level, transcending individual soils and crops. Still, for practical reasons, only one plant species (soybean) has been used, and one specific soil (Santomera) has received particular focus. This inevitably holds a risk of over-representation of soil-, species- or even cultivar specific peculiarities. The risk was minimized by seeking to explain experimental observations by the underlying mechanisms.

## Recommendations for further research

The following suggestions for complementary research are made, to further improve and expand the conceptual framework for understanding the fate and effectiveness of FeEDDHA components in soil-plant systems

- Fine-tuning the understanding of aspects already included into the conceptual model. The mechanisms underlying FeEDDHA and CuEDDHA adsorption to soil reactive compounds deserve particular attention in this respect, because of the important role of adsorption in cation competion, which has been demonstrated to have a large impact on FeEDDHA component concentrations in soil solution. Also the contribution of

FeEDDHA-facilitated Fe uptake to the total Fe uptake and to the decrease in FeEDDHA concentration in soil solution should be further examined.

- Examining factors not yet included, for potentially expanding the conceptual model to relate more closely to the field situation. Processes to consider include FeEDDHA leaching from the root zone, FeEDDHA precipitation on the soil surface resulting from evapo-transpiration induced transport, FeEDDHA uptake by plants, the behaviour of FeEDDHA under rhizosphere conditions and degradation of EDDHA ligands. FeEDDHA leaching has been briefly addressed by Lucena et al. (2005) and FeEDDHA uptake has been reported for plants grown on substrate (Bienfait, et al., 2004).
- Carrying out comparative studies with different soils, crops and possibly chelates to broaden the basis for the model, to examine if the fate of FeEDDHA components can be explained from the same leading processes, and to verify on which aspects the model requires refining.
- Conducting field trials to examine how the model relates to agricultural practice, and if relevant processes have been overlooked or should be weighed differently.

# Appendix

# Materials and methods

### Materials

*Soil* - Calcareous soil was collected from the top soil layer (0 – 20 cm) at a site located in Santomera (Spain). Plants grown on this soil became chlorotic, both under field conditions and in pot trials (Schenkeveld, et al., 2008; Schenkeveld, et al., 2010a). Pre-treatment consisted of air drying and sieving (2 mm). Relevant soil characteristics are presented in Table A10.1 and described in more detail in (Schenkeveld, et al., 2010a).

*Goethite* - A stock goethite suspension was prepared from $Fe(NO_3)_3$ according to Atkinson et al. (1967) as described in more detail by Hiemstra et al. (1989). The BET($N_2$) specific surface area amounted 98.6 $m^2$ $g^{-1}$ and electro conductivity (EC) was below 10 μS.

*Ca-montmorillonite* - Protonated montmorillonite (Montmorillonite K10 powder, Sigma Aldrich - product code: 69866; pH 3 - 4, specific surface area 250 $m^2$ $g^{-1}$) was successively washed with 1 M NaOH, 1 M and 0.1 M $CaCl_2$ solutions and ultra pure water until the electroconductivity of the suspension was below 5 μS $cm^{-1}$. The suspension was centrifuged at 2300 rpm, decanted and dried at 105 °C.

*Reagent solutions* - Depending on the experiment, metal-EDDHA solutions were prepared from a solid o,o-$H_4$EDDHA mixture (purity: 99%; 49% racemic o,o- $H_4$EDDHA, 51% meso o,o-$H_4$EDDHA), as described in Schenkeveld et al. (2008); from racemic o,o-$H_4$EDDHA (purity: 100%) and meso o,o-$H_4$EDDHA (purity: 99.5%) as described in (Schenkeveld, et al., 2010); or by dissolving an FeEDDHA formulation (Formulation "X"). Racemic and meso o,o-$H_4$EDDHA were obtained by separation of the o,o-$H_4$EDDHA mixture, as described in Bannochie and Martell (1989) and Bailey et al. (1981). The composition of metal-EDDHA solutions was examined at t=0 and at the end of the experiment by ICP and HPLC analysis.

$CO_2$-free 0.1 M NaOH solution, MOPS-buffer (3-(N-Morpholino)-propanesulfonic acid) and humic acid (HA) solutions were prepared as described in (Schenkeveld, et al., 2010e). Fulvic acid (FA) solution was prepared by dissolving FA extracted from the Bs horizon of a peaty podzol (Strichen Soil Association, Scotland), purified and freeze dried as described by Filius et al. (2000), with 0.1 M NaOH solution. Stock and experimental solution were prepared from analytical grade chemicals and ultra pure water.

### Experiments

Unless mentioned otherwise, extractions, soil interaction experiments and experiments for determining adoption isotherms were performed with 50 ml polypropylene test tubes (Greiner bio-one, Cat No 210296). Tubes were placed in an end-over-end shaker, rotating at 18 rpm in absence of light. Room temperature was kept at 20 (± 1) °C. After interaction, the samples were centrifuged for 10 minutes at 3,000 rpm. The pH and EC of the supernatant were measured. The supernatant was filtered over a 0.45 μm cellulose acetate micro pore filter (Schleicher & Schuell, ref no: 10462650) and the filtrate was further analyzed.

*Interaction of formulation "X" with Santomera soil*

The interaction of an FeEDDHA formulation "X" with Santomera soil was examined as a function of time in a of 1:1 (w/v) interaction experiment. The experimental solution comprised 50 μM racemic o,o-FeEDDHA, 47 μM meso o,o-FeEDDHA, 30 μM o,p-FeEDDHA and 65 μM rest-FeEDDHA[12]. The total Fe concentration equaled 10.8 mg $l^{-1}$ Fe. Sampling was done destructively after respectively 1, 4, 8 and 16 hours, as well as after 1, 2, 3, 4, 5, 6, 7, 10, 14, 21 and 28 days. 0.01M $CaCl_2$ was used as background electrolyte. Blank treatments were included for 7, 14, 21 and 28 days. To avoid drastic changes in redox conditions throughout the experiment, the tubes were taken out of the shaker, opened and left standing for 30 minutes every three to four days. The experiment was executed in triplicates.

*Adsorption isotherms for o,o-FeEDDHA isomers to Ca-montmorillonite*

Adsorption isotherms for racemic and meso o,o-FeEDDHA were determined together (o,o-FeEDDHA mixture) in 50 g $l^{-1}$ Ca-montmorillonite suspensions at pH 7.1 ± 0.1 and 20 °C. pH was set with 1 M HCl, no buffer was added. Interaction time amounted 7 days and 0.1 M $CaCl_2$ was used as background electrolyte. Total o,o-FeEDDHA concentration at t=0 was approximately 0.09; 0.18; 0.9 and 1.8 mM. The experiment was executed in duplicates.

*Adsorption isotherms for FeEDDHA isomers to goethite*

Adsorption isotherms for racemic and meso o,o-FeEDDHA were determined separately in 5 g $l^{-1}$ goethite suspensions at pH 7 and 20 °C. Isotherms were determined at ionic strengths 0.01 M and 0.1 M with $CaCl_2$ as background electrolyte, with and without HA adsorbed onto the goethite (0.5 g $l^{-1}$). The experiment was executed in duplicates.

Background electrolyte was added to a portion of the goethite stock solution in polyethylene bottles. pH was lowered to 5.5 and the suspension was purged overnight with washed (1 M $H_2SO_4$/1 M NaOH), moist $N_2$ gas to remove $CO_2$. The following day, pH was raised to around 7 with 0.1 M NaOH and MOPS-buffer (0.005 M; pH 7 ± 0.05) was added. Suspensions were made to volume with pre-boiled water. For the adsorption isotherm involving HA, MOPS buffer was omitted; HA served as pH buffer and was added to suspension after overnight purging. pH was raised to and maintained at 7 with 0.1 M NaOH, while the suspension equilibrated for 72 hours (Weng, et al., 2006) under continuous purging with $N_2$. DOC concentration in solution was determined after the suspension had been made to volume; at least 98% of the HA was adsorbed to goethite.

FeEDDHA solutions were added to goethite suspensions in gas-tight, 23.6 ml low-density polyethylene bottles (Rietra, et al., 2001) under $N_2$-atmosphere. FeEDDHA solutions had been purged with $N_2$ for 30 minutes prior to addition. The FeEDDHA concentrations at t=0 were approximately 3.6; 9.0; 36; 90 μM and 0.18; 0.36 and 0.90 mM. The bottles were placed in an end-over-end shaker, rotating at 24 rpm in absence of light. Sampling was done destructively after 72 hours. Samples were centrifuged for 10 minutes at 10,000 rpm. The supernatant was filtered over a 0.45 μm cellulose acetate micro pore filter (Schleicher & Schuell, ref no: 10462650). The filtrate was further analyzed.

[12] For rest-FeEDDHA, the concentration refers to Fe concentration

*Interaction of racemic o,o-CuEDDHA with soil*

The effect of equilibration time and addition of organic substances on the interaction between racemic o,o-CuEDDHA and Santomera soil was examined as a function of time in a 1:2 (w/v) interaction experiment in duplicates. The variables included were: equilibration time (0 or 3 days), racemic o,o-CuEDDHA concentration (0 or 27 μM) and DOC addition (none, 0.55 g $l^{-1}$ HA and 0.55 g $l^{-1}$ FA). HA and FA were included both to also examine the effect of the nature of the added organic substance. The amount of organic substance added, corresponded with 10% of the OM initially present in Santomera soil. Treatments without racemic o,o-CuEDDHA were included to determine the contribution of Cu bond to DOC to the total Cu concentration in corresponding treatments with racemic o,o-CuEDDHA. Not all possible combinations of the variables were included; the experiment involved 7 treatments (see Table A10.2)

Ionic strength was imposed with 0.01 M $CaCl_2$. In treatments involving equilibration, background electrolyte solution and, when required HA or FA solution, were added to the soil and equilibrated for 3 days in an end-over-end shaker at 18 rpm. After equilibration, racemic o,o-CuEDDHA solution or an equal quantity of ultra pure water was added, corresponding to 3% of the final solution volume. In treatments without equilibration, all reagent solutions were administered to the soil directly after each other. The moment all reagent solutions had been added, was recorded as t=0. Sampling was done destructively after 8, 24, 48 and 96 hours after, except in the treatment without equilibration, organic substance or CuEDDHA addition. In the latter treatment, sampling was done after 8, 24, 72 and 168 hours.

**Analysis**

Al, Co, Cu, Mn, Ni and Zn concentrations were measured by ICP-MS (Perkin Elmer, ELAN 6000), Ca, Mg and Fe concentrations by ICP-AES (Varian, Vista Pro). Samples were acidified with nitric acid before ICP-measurement. DOC concentrations were measured with a segmented flow analyzer (Skalar, SK12) by oxidation with persulphate and tetraborate and UV and IR-detection. FeEDDHA component concentrations were determined after separation through high-performance liquid chromatography (HPLC) as described in Schenkeveld et al. (2007). The Fe concentration chelated by rest-EDDHA was calculated by subtracting the Fe concentrations chelated by the other three FeEDDHA components and the Fe concentration in the blank treatment from the total Fe concentration as measured by ICP-AES. The concentration of metals (other than Fe) chelated to EDDHA components was calculated as the difference in metal concentration between corresponding treatments with and without addition of EDDHA chelates.

**Table 4.1:** Characteristics of Santomera soil.

| | | Extraction | | |
|---|---|---|---|---|
| **Origin/Name** | Santomera | **$CaCl_2$ (0.01 M)[g]** | **DOC (mg $l^{-1}$)** | 30 |
| **Region** | Murcia | **Oxalate[h]** | **Fe (g $kg^{-1}$)** | 0.30 |
| **Country** | Spain | | **Al (g $kg^{-1}$)** | 0.44 |
| **Soil classification** | entisol | **DTPA[i]** | **Fe (mg $kg^{-1}$)** | 3.5 |
| **Water holding capacity (g $kg^{-1}$)** | 320 | | **Mn (mg $kg^{-1}$)** | 4.6 |
| **pH-$CaCl_2$[a]** | 8.0 | | **Cu (mg $kg^{-1}$)** | 4.1 |
| **Electro conductivity (mS $m^{-1}$)[b]** | 23 | | **Zn (mg $kg^{-1}$)** | 0.9 |
| **SOC (g $kg^{-1}$)[c]** | 5.4 | **$HNO_3$ (0.43 M)[j]** | **Fe (mg $kg^{-1}$)** | 494 |
| **Clay (g $kg^{-1}$)[d]** | 260 | | **Mn (mg $kg^{-1}$)** | 179 |
| **$CaCO_3$ (g $kg^{-1}$)[e]** | 520 | | **Cu (mg $kg^{-1}$)** | 10 |
| **CEC (cmol $kg^{-1}$)[f]** | 10.3 | | **Zn (mg $kg^{-1}$)** | 5 |

[a] ISO/DIS 10390 Soil Quality – Determination of pH
[b] ISO/DIS 11265 Soil Quality – Determination of the specific electric conductivity
[c] Walinga et al. (1992)
[d] Houba et al. (1997)
[e] ISO 10693, Soil Quality – Determination of carbonate content, volumetric method
[f] ISO/DIS 11260 Soil Quality – Determination of cation exchange capacity and base saturation – method using barium chloride solution
[g] Houba et al. (2000)
[h] Schwertmann (1964)
[i] Lindsay and Norvell (1978) and Quevauvillier et al. (1996)
[j] Tipping et al. (2003). Fest el al. (2005)

**Table A10.2:** Treatment overview for the interaction of racemic o,o-CuEDDHA with Santemera soil.

| Treatment | racemic o,o-CuEDDHA | 3-day equilibration | DOC addition |
|---|---|---|---|
| 1 | - | - | - |
| 2 | - | x | HA |
| 3 | - | x | FA |
| 4 | x | - | - |
| 5 | x | x | |
| 6 | x | x | HA |
| 7 | x | x | FA |

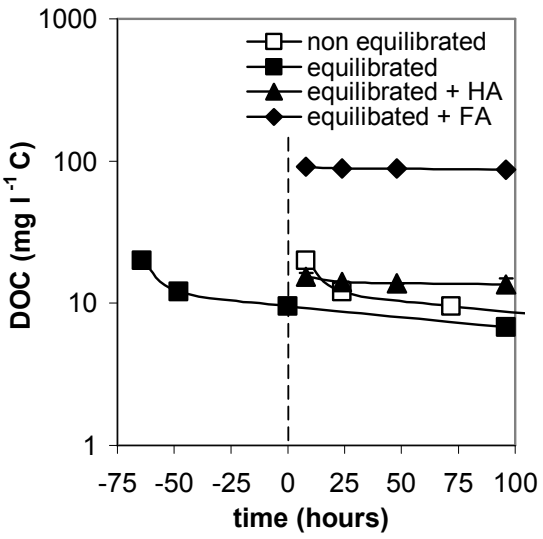



**Figure A10.1:** DOC concentration in the treatments without racemic o,o-CuEDDHA addition as a function of time in a 1:2 (w/v) batch experiment with Santomera soil. Background electrolyte was 0.01 M $CaCl_2$. Treatments involved equilibration prior to CuEDDHA addition and addition of humic acid (HA) and fulvic acid (FA). Error bars indicate standard deviations.

# References

Adams M L, Norvell W A, Philpot W D and Peverly J H 2000 Spectral detection of micronutrient deficiency in 'Bragg' soybean. Agron. J. 92, 261-268.

Ahrland S, Dahlgren A and Persson I 1990 Stabilities and hydrolysis of some iron(III) and manganese(III) complexes with chelating ligands. Acta Agr. Scand. 40, 101-111.

Ainscough E W, Brodie A M, Plowman J E, Brown K L, Addison A W and Gainsford A R 1980 Small molecule analogs for the specific iron-binding site of lactoferrin: A single-crystal X-ray structure of bis(methanol)bis[2-(5-methylpyrazol-3-yl)phenolato]iron(III) nitrate-methanol and spectroscopic studies on iron(III) phenolate complexes. Inorg. Chem. 19, 3655-3663.

Alvarez-Fernandez A, Abadia J and Abadia A 2006 Iron deficiency, fruit yield and fruit quality. In Iron nutrition in plants and rhizospheric microorganisms. Eds. L L Barton and J Abadia. pp 85-101. Springer.

Alvarez-Fernandez A, Garate A and Lucena J J 1997 Interaction of iron chelates with several soil materials and with a soil standard. J. Plant Nutr. 20, 559-572.

Alvarez-Fernandez A, Garcia-Lavina P, Fidalgo C, Abadia J and Abadia A 2004 Foliar fertilization to control iron chlorosis in pear (Pyrus communis L.) trees. Plant Soil 263, 5-15.

Alvarez-Fernandez A, Garcia-Marco S and Lucena J J 2005 Evaluation of synthetic iron(III)-chelates (EDDHA/$Fe^{3+}$, EDDHMA/$Fe^{3+}$ and the novel EDDHSA/$Fe^{3+}$) to correct iron chlorosis. Eur. J. Agron. 22, 119-130.

Alvarez-Fernandez A, Orera I, Abadia J and Abadia A 2007 Determination of synthetic ferric chelates used as fertilizers by liquid chromatography-electrospray/mass spectrometry in agricultural matrices. J. Am. Soc. Mass Spectr. 18, 37-47.

Alvarez-Fernandez A, Sierra M A and Lucena J J 2002 Reactivity of synthetic Fe chelates with soils and soil components. Plant Soil 241, 129-137.

Anderegg G and L'Eplattenier F 1964 Metallindikatoren 7. Aciditätskonstanten + eisenkomplex des [N,N]-äthylen-bis-(omikron-hydroxyphenyl-glycins) Helv. Chim. Acta 47, 1067-&.

Atkinson R J, Posner A M and Quirk J P 1967 Adsorption of potential-determining ions at ferric oxide-aqueous electrolyte interface. J. Phys. Chem. 71, 550-558.

Bailey N A, Cummins D, McKenzie E D and Worthington J M 1976 Crystal and molecular-structure of iron(III) compounds of sexadentate ligand N,N'-ethylene-bis-(o-hydroxyphenylglycine). Inorg. Chim. Acta 18, L13-L14.

Bailey N A, Cummins D, McKenzie E D and Worthington J M 1981 Iron(III) compounds of phenolic ligands. The chrystal and molecular structure of the sexadentate ligand N,N'-ethylene-bis-(o-hydroxyphenylglycine). Inorg. Chim. Acta, 111-120.

Bannochie C J and Martell A E 1989 Affinities of racemic and meso forms of N,N'-ethylenebis[2-(o-hydroxyphenyl)glycine] for divalent and trivalent metal ions. J. Am. Chem. Soc. 111, 4735-4742.

Banuls J, Quinones A, Martin B, Primo Millo E and Legaz F 2003 Effects of the frequency of iron chelate supply by fertigation on iron chlorosis in citrus. J. Plant Nutr. 26, 1985-1996.

Barak P and Chen Y 1987 Determination of FeEDDHA in soils and fertilizers by anion exchange chromatography. Soil Sci. Soc. Am. J. 51, 893-896.

Bates S S, Tessier A, Campbell P G C and Buffle J 1982 Zinc Adsorption and Transport by Chlamydomonas-Variabilis and Scenedesmus-Subspicatus (Chlorophyceae) Grown in Semi-Continuous Culture. Journal of Phycology 18, 521-529.

Bauer P and Hell R 2006 Translocation of iron in plant tissues. In Iron nutrition in plants and rhizospheric microorganisms. Eds. L L Barton and J Abadia. pp 279-288. Springer.

Bienfait H F, Garcia-Mina J and Zamareno A M 2004 Distribution and secondary effects of EDDHA in some vegetable species. J. Plant Nutr. Soil Sci. 50, 1103-1110.

Bienfait H F, Vandenbriel W and Meslandmul N T 1985 Free space iron pools in roots - Generation and mobilization. Plant Physiol. 78, 596-600.

Bihari S, Smith P A, Parsons S and Sadler P J 2002 Stereoisomers of Mn(III) complexes of ethylenebis[(o-hydroxyphenyl)glycine]. Inorg. Chim. Acta 331, 310-317.

Boxma R 1972 Bicarbonate as the most important soil factor in lime-induced chlorosis in the Netherlands. Plant Soil 37, 233-243.

Briat J F, Cellier F and Gaymard F 2006 Ferritins and iron accumulation in plants. In Iron nutrition in plants and rhizospheric microorganisms. Eds. L L Barton and J Abadia. pp 341-357. Springer.

Bruinsma J 1963 The quantitative analysis of chlorophylls a and b in plant extracts. Photochem. Photobio. Sci 2, 241-249.

Bucheli-Witschel M and Egli T 2001 Environmental fate and microbial degradation of aminopolycarboxylic acids. Fems Microbiol. Rev. 25, 69-106.

Caccavo F, Lonergan D J, Lovley D R, Davis M, Stolz J F and McInerney M J 1994 *Geobacter sulfurreducens* sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism. Appl. Environ. Microb. 60, 3752-3759.

Canellas L P, Olivares F L, Okorokova Facanha A L and Facanha A R 2002 Humic acids isolated from earthworm compost enhance root elongation, lateral root emergence, and plasma membrane $H^+$-ATPase activity in maize roots. Plant Physiol. 130, 1951-1957.

Cantera R G, Zamarreno A M and Garcia-Mina J M 2002 Characterization of commercial iron chelates and their behavior in an alkaline and calcareous soil. J. Agric. Food Chem. 50, 7609-7615.

Cerdan M, Juarez S A M, Jorda J D and Bermudez D 2006 Fe uptake from meso and d,l-racemic Fe(o,o-EDDHA) isomers by strategy I and II plants. J. Agric. Food Chem. 54, 1387-1391.

Cesco S, Nikolic M, Römheld V, Varanini Z and Pinton R 2002 Uptake of $^{59}$Fe from soluble $^{59}$Fe-humate complexes by cucumber and barley plants. Plant Soil 241, 121-128.

Cesco S, Römheld V, Varanini Z and Pinton R 2000 Solubilization of iron by water-extractable humic substances. J. Plant Nutr. Soil Sci. 163, 285-290.

Chaney R L 1984 Diagnostic practices to identify iron deficiency in higher plants. J. Plant Nutr. 7, 47-67.

Chaney R L, Brown J C and Tiffin L O 1972 Obligatory reduction of ferric chelates in iron uptake by soybeans. Plant Physiol. 50, 208-213.

Chen Y and Barak P 1982 Iron nutrition of plants in calcareous soils. Adv. Agron. 35, 217-240.

Cremonini M A, Alvarez-Fernandez A, Lucena J J, Rombola A, Marangoni B and Placucci G 2001 NMR analysis of the iron ligand ethylenediaminedi(o-hydroxyphenyl)acetic acid (EDDHA) employed in fertilizers. J. Agric. Food Chem. 49, 3527-3532.

De Kreij C 1998 Exchange of iron from chelate in the fertilizer against copper, manganese, and zinc in peaty substrates. Commun. Soil Sci. Plan. 29, 1897-1902.

Dijkstra J J, Meeussen J C L and Comans R N J 2004 Leaching of heavy metals from contaminated soils: an experimental and modeling study. Environ. Sci. Technol. 38, 4390-4395.

Dzombak D A and Morel F M M 1990 Surface complexation modeling - Hydrous ferric oxide. John Wiley & Sons. pp. 393.

FAO/AGL 2000 ProSoil - Problem soil database. FAO/OAL.

Fest E P M J, Temminghoff E J M, Griffioen J and Van Riemsdijk W H 2005 Proton buffering and metal leaching in sandy soils. Environ. Sci. Technol. 39, 7901-7908.

Filius J D, Hiemstra T and Van Riemsdijk W H 1997 Adsorption of small weak organic acids on goethite: Modeling of mechanisms. J. Colloid Interf. Sci., 368-380.

Filius J D, Lumsdon D G, Meeussen J C L, Hiemstra T and Van Riemsdijk W H 2000 Adsorption of fulvic acid on goethite. Geochim. Cosmochim. Ac. 64, 51-60.

Frost A E, Freedman H H, Westerback S J and Martell A E 1958 Chelating tendencies of N,N'-ethylenebis-[2-(o-hydroxyphenyl)]-glycine. J. Am. Chem. Soc 80, 530-536.

Gaber B P, Miskowsk V and Spiro T G 1974 Resonance Raman-scattering from iron(III)-transferrin and copper(II)-transferrin and an iron(III) model compound - Spectroscopic interpretation of transferrin binding-site. J. Am. Chem. Soc 96, 6868-6873.

Garcia-Marco S, Martinez N, Yunta F, Hernandez-Apaolaza L and Lucena J J 2006a Effectiveness of ethylenediamine-N(o-hydroxyphenylacetic)-N '(p-hydroxy-phenylacetic) acid (o,p-EDDHA) to supply iron to plants. Plant Soil 279, 31-40.

Garcia-Marco S, Torreblanca A and Lucena J J 2006b Chromatographic determination of Fe chelated by ethylenediamine-N-(o-hydroxyphenylacetic)-N '(p-hydroxyphenylacetic) acid in commercial EDDHA/$Fe^{3+}$ fertilizers. J. Agric. Food Chem. 54, 1380-1386.

Garcia-Marco S, Yunta F, De la Hinojosa M I M, Marti G and Lucena J J 2003 Evaluation of commercial Fe(III)-chelates using different methods. J. Plant Nutr. 26, 2009-2021.

Garcia-Mina J, Cantera R G and Zamarreno A 2003 Interaction of different iron chelates with an alkaline and calcareous soil: A complementary methodology to evaluate the performance of iron compounds in the correction of iron chlorosis. J. Plant Nutr. 26, 1943-1954.

Ghasemi Fasaei R, Ronaghi A, Maftoun M, Karimian N and Soltanpour P N 2003 Influence of FeEDDHA on iron-manganese interaction in soybean genotypes in a calcareous soil. J. Plant Nutr. 26, 1815-1823.

Gil-Ortiz R and Bautista-Carrascosa I 2004 Effects of Fe-EDDHA chelate application on evolution of soil extractable iron, copper, manganese, and zinc. Commun. Soil Sci. Plan. 35, 559-570.

Gomez-Gallego M, Fernandez I, Pellico D, Gutierrez A, Sierra M A and Lucena J J 2006 On the structure and spin states of Fe(III)-EDDHA complexes. Inorg. Chem. 45, 5321-5327.

Gomez-Gallego M, Pellico D, Ramirez-Lopez P, Mancheno M J, Romano S, De la Torre M C and Sierra M A 2005 Understanding of the mode of action of Fe(III)-EDDHA as iron chlorosis corrector based on its photochemical and redox behavior. Chem. Eur. J. 11, 5997-6005.

Gomez-Gallego M, Sierra M A, Alcazar R, Ramirez P, Pinar C, Mancheno M J, Garcia-Marco S, Yunta F and Lucena J J 2002 Synthesis of o,p-EDDHA and its detection as the main impurity in o,o-EDDHA commercial iron chelates. J. Agric. Food Chem. 50, 6395-6399.

Goos R J, Johnson B, Jackson G and Hargrove G 2004 Greenhouse evaluation of controlled-release iron fertilizers for soybean. J. Plant Nutr. 27, 43-55.

Goos R J and Johnson B E 2000 A comparison of three methods for reducing iron-deficiency chlorosis in soybean. Agron. J. 92, 1135-1139.

Gorby Y A, Caccavo F and Bolton H 1998 Microbial reduction of $cobalt^{III}EDTA^{-}$ in the presence and absence of manganese(IV) oxide. Environ. Sci. Technol. 32, 244-250.

Gruber B and Kosegarten H 2002 Depressed growth of non-chlorotic vine grown in calcareous soil is an iron deficiency symptom prior to leaf chlorosis. J. Plant Nutr. Soil Sci. 165, 111-117.

Grusak M A 1994 Iron transport to developing ovules of *Pisum Sativum;* 1. Seed import characteristics and phloem iron-loading capacity of source regions. Plant Physiol. 104, 649-655.

Grusak M A 1995 Whole-root iron(III)-reductase activity throughout the life-cycle of iron-grown *Pisum Sativum* L. (Fabaceae): Relevance to the iron nutrition of developing seeds. Planta 197, 111-117.

Grusak M A, Pearson J N and Marentes E 1999 The physiology of micronutrient homeostasis in field crops. Field Crops Res. 60, 41-56.

Halliwell B and Gutteridge J M C 1986 Oxygen free radicals and iron in relation to biology and medicine - some problems and concepts. Arch. Biochem. Biophys. 246, 501-514.

Hansen N C, Hopkins B G, Elsworth J W and Jolley V D 2006 Iron nutrition in field crops. In Iron nutrition in plants and rhizospheric microorganisms. Eds. L L Barton and J Abadia. pp 23-59. Springer.

Harrison P M and Arosio P 1996 Ferritins: Molecular properties, iron storage function and cellular regulation. BBA-Bioenergetics 1275, 161-203.

Hassler C S, Slaveykova V I and Wilkinson K J 2004 Discriminating between intra- and extracellular metals using chemical extractions. Limnol. Oceanogr.-Meth. 2, 237-247.

Heenan D P and Campbell L C 1983 Manganese and iron interactions on their uptake and distribution in soybean (Glycine max (L.) Merr.). Plant Soil 70, 317-326.

Heitholt J J, Sloan J J, MacKown C T and Cabrera R I 2003 Soybean growth on calcareous soil as affected by three iron sources. J. Plant Nutr. 26, 935-948.

Hell R and Stephan U W 2003 Iron uptake, trafficking and homeostasis in plants. Planta 216, 541-551.

Hering J G and Morel F M M 1988 Kinetics of trace-metal complexation: Role of alkaline-earth metals. Environ. Sci. Technol. 22, 1469-1478.

Hering J G and Morel F M M 1989 Slow coordination reactions in seawater. Geochim. Cosmochim. Ac. 53, 611-618.

Hernandez-Apaolaza L, Barak P and Lucena J J 1997 Chromatographic determination of commercial Fe(III) chelates of ethylenediaminetetraacetic acid, ethylenediaminedi(o-hydroxyphenylacetic) acid and ethylenediaminedi(o-hydroxyp-methylphenylacetic) acid. J. Chromatogr. 789, 453-460.

Hernandez-Apaolaza L, Garate A and Lucena J J 1995 Efficacy of commercial Fe(III)-EDDHA and Fe(III)-EDDHMA chelates to supply iron to sunflower and corn seedlings. J. Plant Nutr. 18, 1209-1223.

Hernandez-Apaolaza L, Garcia-Marco S, Nadal P, Lucena J J, Sierra M A, Gomez-Gallego M, Ramirez-Lopez P and Escudero R 2006 Structure and fertilizer properties of byproducts formed in the synthesis of EDDHA. J. Agric. Food Chem. 54, 4355-4363.

Hernandez-Apaolaza L and Lucena J J 2001 Fe(III)-EDDHA and -EDDHMA sorption on Ca-montmorillonite, ferrihydrite, and peat. J. Agric. Food Chem. 49, 5258-5264.

Hiemstra T, Dewit J C M and Van Riemsdijk W H 1989 Multisite proton adsorption modeling at the solid-solution interface of (hydr)oxides: A new approach. 2. Application to various important (hydr)oxides. J. Colloid Interf. Sci. 133, 105-117.

Hiemstra T and Van Riemsdijk W H 1996 A surface structural approach to ion adsorption: The charge distribution (CD) model. J. Colloid Interf. Sci. 179, 488-508.

Hiemstra T and Van Riemsdijk W H 1999 Surface structural ion adsorption modeling of competitive binding of oxyanions by metal (hydr)oxides. J. Colloid Interf. Sci. 210, 182-193.

Hill-Cottingham D G 1962 The paper chromatography of some complexones and their iron chelates. J. Chromatogr., 261-264.

Hill-Cottingham D G and Lloyd-Jones C P 1958 Behaviour of iron chelates in calcareous soils. II. Laboratory experiments with some further chelating agents. Plant Soil 9, 189-201.

Hill-Cottingham D G and Lloyd-Jones C P 1965 The behaviour of iron chelating agents with plants. J. Exp. Bot 16, 233-242.

Holmes R S and Brown J C 1955 Chelates as correctives for chlorosis. Soil Sci. 80, 167-179.

Holmgren G G 1967 A rapid citrate-dithionite extractable iron procedure. Soil Sci. Soc. Am. Pro. 31, 210-211.

Houba V J G, Temminghoff E J M, Gaikhorst G A and Van Vark W 2000 Soil analysis procedures using 0.01 M calcium chloride as extraction reagent. Commun. Soil Sci. Plan. 31, 1299-1396.

Houba V J G, Van der Lee J J G and Novozamsky I 1997 Soil and plant analysis. Wageningen University.

Jandl R and Sollins P 1997 Water extractable soil carbon in relation to the belowground carbon cycle. Biol. Fert. Soils 25, 196-201.

Jardine P M, Dunnivant F M, Selim H M and McCarthy J F 1992 Comparison of models for describing the transport of dissolved organic carbon in aquifer columns. Soil Sci. Soc. Am. J. 56, 393-401.

Jardine P M and Taylor D L 1995 Fate and transport of ethylenediaminetetraacetate chelated contaminants in subsurface environments. Geoderma 67, 125-140.

Jorda J, Sanchez-Andreu J and Juarez M 1992 Effect of temperature on the kinetics of FeEDDHA sorption on a calcareous soil. Soil Sci. 153, 48-52.

Julien J A L and Aymard A 1989 Nouveau procédé de preparation de l'acide ethylenediamine N,N'-bis(o-hydroxyphénylacétique) et de derives de celui-ci. In European Patent 0,331,556 A2.

Kaiser K, Kaupenjohann M and Zech W 2001 Sorption of dissolved organic carbon in soils: Effects of soil sample storage, soil-to-solution ratio, and temperature. Geoderma 99, 317-328.

Kalis E J J, Temminghoff E J M, Visser A and Van Riemsdijk W H 2007 Metal uptake by Lolium perenne in contaminated soils using a four-step approach. Environ. Toxicol. Chem. 26, 335-345.

Kalis E J J, Temminghoff E J M, Weng L P and Van Riemsdijk W H 2006 Effects of humic acid and competing cations on metal uptake by Lolium perenne. Environmental Toxicology and Chemistry 25, 702-711.

Keizer M G and Van Riemsdijk W H 1994 ECOSAT: Equilibrium Calculation Of Speciation And Transport. Wageningen University, Wageningen.

King E O, Ward M K and Raney D E 1954 Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44, 301-307.

Kinniburgh D G, Van Riemsdijk W H, Koopal L K, Borkovec M, Benedetti M F and Avena M J 1999 Ion binding to natural organic matter: Competition, heterogeneity, stoichiometry and thermodynamic consistency. Colloids Surface A 151, 147-166.

Kroll H 1957 The ferric chelate of ethylene-diamine di(O-hydroxyphenylacetic acid) for treatment of lime-induced chlorosis. Soil Sci. 84, 51-53.

Kroll H, Knell M, Powers J and Simonian J 1957 A phenolic analog of ethylenediamine-tetraacetic acid. J. Am. Chem. Soc. 79, 2024-2025.

Lindsay W L 1979 Chemical equilibria in soils. John Wiley and Sons: N.Y. pp. 449.

Lindsay W L 1988 Solubility and redox equilibria of iron compounds in soils. In Iron in soils and clay minerals. Eds. J W Stucki, B A Goodman and U Schwertmann. pp 37-62. D. Reidel Publishing Company, Dordrecht.

Lindsay W L and Norvell W A 1978 Development of a DTPA soil test for zinc, iron, manganese, and copper. Soil Sci. Soc. Am. J. 42, 421-428.

Lindsay W L and Schwab A P 1982 The chemistry of iron in soils and its availability to plants. J. Plant Nutr. 5, 821-840.

Lucena J J 2000 Effects of bicarbonate, nitrate and other environmental factors on iron deficiency chlorisis. A review. J. Plant Nutr. 23, 1591-1606.

Lucena J J 2003 Fe chelates for remediation of Fe chlorosis in strategy I plants. J. Plant Nutr. 26, 1969-1984.

Lucena J J 2006 Synthetic iron chelates to correct iron deficiency in plants. In Iron nutrition in plants and rhizospheric microorganisms. Eds. L L Barton and J Abadia. pp 103-128. Springer.

Lucena J J, Barak P and Hernandez-Apaolaza L 1996 Isocratic ion-pair high-performance liquid chromatographic method for the determination of various iron(III)chelates. J. Chromatogr. A 727, 253-264.

Lucena J J and Chaney R L 2006 Synthetic iron chelates as substrates of root ferric chelate reductase in green stressed cucumber plants. J. Plant Nutr. 29, 423-439.

Lucena J J and Chaney R L 2007 Response of cucumber plants to low doses of different synthetic iron chelates in hydroponics. J. Plant Nutr. 30, 795-809.

Lucena J J, Garcia-Marco S, Yunta F and Hernandez-Apaolaza L 2005 Theoretical modeling and reactivity of the iron chelates in agronomic conditions. In Biochemistry of chelating agents. Eds. B Nowack and J van Briesen. pp 348-363. American Chemical Society, Washington, DC.

Lucena J J, Manzanares M and Garate A 1992a Comparative study of the efficacy of commercial Fe-chelates using a new test. J. Plant Nutr. 15, 1995-2006.

Lucena J J, Manzanares M and Garate A 1992b A test to evaluate the efficacy of commercial Fe-chelates. J. Plant Nutr. 15, 1553-1566.

Machold O and Stephan U W 1969 Function of iron in porphyrin and chlorophyll biosynthesis. Phytochemistry 8, 2189-&.

Marschner H 1995 Mineral nutrition of higher plants. Academic Press, London. pp. 889.

Marschner H and Römheld V 1994 Strategies of plants for acquisition of iron. Plant Soil 165, 261-274.

Marschner H, Römheld V and Kissel M 1986 Different strategies in higher-plants in mobilization and uptake of iron. J. Plant Nutr. 9, 695-713.

Marschner H, Treeby M and Römheld V 1989 Role of root-induced changes in the rhizosphere for iron acquisition in higher plants. J. Plant Nutr. Soil Sci. 152, 197-204.

Mengel K 1994 Iron availability in plant tissues-iron chlorosis on calcareous soils. pp 275-283; .

Mengel K, Breininger M T and Bubl W 1984 Bicarbonate, the most important factor inducing iron chlorosis in vine grapes on calcareous soil. Plant Soil 81, 333-344.

Mengel K and Kirkby E A 2001 Principles of plant nutrition. International Potash Institute, Bern, Switzerland.

Milne C J, Kinniburgh D G and Tipping E 2001 Generic NICA-Donnan model parameters far proton binding by humic substances. Environ. Sci. Technol. 35, 2049-2059.

Milne C J, Kinniburgh D G, Van Riemsdijk W H and Tipping E 2003 Generic NICA-Donnan model parameters for metal-ion binding by humic substances. Environ. Sci. Technol. 37, 958-971.

Moraghan J T 1979 Manganese toxicity in flax growing on certain calcareous soils low in available iron. Soil Sci. Soc. Am. J. 43, 1177-1180.

Morel F M M and Hering J G 1993 Principles and applications of aquatic chemistry. John Wiley & Sons. pp. 588.

Morgan G T and Drew H D K 1920 CLXII.—Researches on residual affinity and co-ordination. Part II. Acetylacetones of selenium and tellurium. J. Chem. Soc. Trans 117.

Morris D R, Loeppert R H and Moore T J 1990 Indigenous soil factors influencing iron chlorosis of soybean in calcareous soils. Soil Sci. Soc. Am. J. 54, 1329-1336.

Mortvedt J J 1991 Correcting iron deficiencies in annual and perennial plants: Present technologies and future prospects. Plant Soil 130, 273-279.

Muneer M and Oades J M 1989 The role of Ca-organic interactions in soil aggregate stability. II. Field studies with $^{14}C$-labelled straw, $CaCO_3$ and $CaSO4.2H_2O$. Aust. J. Soil Res. 27, 401-409.

Neff J C and Asner G P 2001 Dissolved organic carbon in terrestrial ecosystems: Synthesis and a model. Ecosystems 4, 29-48.

Nikolic M and Römheld V 2002 Does high bicarbonate supply to roots change availability of iron in the leaf apoplast? Plant Soil 241, 67-74.

Novozamsky I, Van Eck R, Houba V J G and Van der Lee J J 1996 Solubilization of plant tissue with nitric acid-hydrofluoric acid-hydrogen peroxide in a closed-system microwave digestor. Commun. Soil Sci. Plan. 27, 867-875.

Nowack B 2002 Environmental chemistry of aminopolycarboxylate chelating agents. Environ. Sci. Technol. 36, 4009-4016.

Nowack B, Kari F G and Kruger H G 2001 The remobilization of metals from iron oxides and sediments by metal-EDTA complexes. Water Air Soil Poll. 125, 243-257.

Nowack B, Schulin R and Robinson B H 2006 Critical assessment of chelant-enhanced metal phytoextraction. Environ. Sci. Technol. 40, 5225-5232.

Nowack B and Sigg L 1997 Dissolution of Fe(III)(hydr)oxides by metal-EDTA complexes. Geochim. Cosmochim. Ac. 61, 951-963.

Nowack B, Lutzenkirchen J, Behra P and Sigg L 1996 Modeling the adsorption of metal-EDTA complexes onto oxides. Environ. Sci. Technol. 30, 2397-2405.

Nowack B and Sigg L 1996 Adsorption of EDTA and metal-EDTA complexes onto goethite. J. Colloid Interf. Sci. 177, 106-121.

O'Conner G.A., Lindsay W.L. and S.R. O 1971 Diffusion of Iron and iron chelates. Soil Sci. Soc. Am. Proc. 35, 407-410.

Orera I, Abadia A, Abadia J and Alvarez-Fernandez A 2009 Determination of o,oEDDHA - a xenobiotic chelating agent used in Fe fertilizers - in plant tissues by liquid chromatography/electrospray mass spectrometry: overcoming matrix effects. Rapid Commun. Mass Sp. 23, 1694-1702.

Pandeya S B, Singh A K and Dhar P 1998 Influence of fulvic acid on transport of iron in soils and uptake by paddy seedlings. Plant Soil 198, 117-125.

Papastylianou I 1990 Effectiveness of iron chelates and $FeSO_4$ for correcting iron chlorosis of peanut on calcareous soils. J. Plant Nutr. 13, 555-566.

Patch M G, Simolo K P and Carrano C J 1982 The cobalt(III), chromium(III), copper(II), and manganese(III) complexes of ethylenebis((ortho-hydroxyphenyl)glycine): Models for metallotransferrins. Inorg. Chem. 21, 2972-2977.

Patch M G, Simolo K P and Carrano C J 1983 Evaluation of iron(III) N,N'-ethylenebis((ortho-hydroxyphenyl)glycinate) as a model for the iron-binding site in the transferrins. Inorg. Chem. 22, 2630-2634.

Perez-Sanz A and Lucena J J 1995 Synthetic iron oxides as sources of Fe in a hydroponic culture of sunflower. In Iron nutrition in soils and plants. Ed. J Abadia. pp 241-246. Kluwer Academic Pulbishers.

Petree H E, Myatt H L and Jelenvsky A M 1978 Preparation of phenolic ethylenediaminepolycarboxylic acids. In U.S. Patent 4,-130,582.

Pietikainen J, Pettersson M and Baath E 2005 Comparison of temperature effects on soil respiration and bacterial and fungal growth rates. FEMS Microbiol. Ecol. 52, 49-58.

Pinton R, Cesco S, Santi S and Varanini Z 1997 Soil humic substances stimulate proton release by intact oat seedling roots. J. Plant Nutr. 20, 857-869.

Powlson D S and Jenkinson D S 1976 The effects of biocidal treatments on metabolism in soil. II. Gamma irradiation, autoclaving, air-drying and fumigation. Soil Biol. Biochem. 8, 179-188.

Pushnik J C and Miller G W 1989 Iron regulation of chlorophyll photosynthetic function: Mediation of PS I development. J. Plant Nutr. 12, 407-421.

Quevauviller P, Lachica M, Barahona E, Rauret G, Ure A, Gomez A and Muntau H 1996 Interlaboratory comparison of EDTA and DTPA procedures prior to certification of extractable trace elements in calcareous soil. Sci. Total Environ. 178, 127-132.

Rahnemaie R, Hiemstra T and Van Riemsdijk W H 2006 Inner- and outer-sphere complexation of ions at the goethite-solution interface. J. Colloid Interf. Sci. 297, 379-388.

Rajan K S, Mainer S, Rajan N L and Davis J M 1981 Studies on the chelation of aluminum for neurobiological application. J. Inorg. Biochem. 14, 339-350.

Reed D W, Lyons C G, Jr. and McEachern G R 1988 Field evaluation of inorganic and chelated iron fertilizers as foliar sprays and soil application. J. Plant Nutr. 11, 1369-1378.

Reuter D J, Robinson J B and Dutkiewicz C 1997 Plant analysis : An interpretation manual. CSIRO, Collingwood.

Rietra R P J J, Hiemstra T and Van Riemsdijk W H 2001 Interaction between calcium and phosphate adsorption on goethite. Environ. Sci. Technol. 35, 3369-3374.

Riley P E, Pecoraro V L, Carrano C J and Raymond K N 1983 Siderophilin metal coordination 3. Crystal-structures of the Cobalt(III), Gallium(III), and Copper(II) complexes of ethylenebis[(o-hydroxyphenyl)glycine]. Inorg. Chem. 22, 3096-3103.

Robinson N J, Procter C M, Connolly E L and Guerinot M L 1999 A ferric-chelate reductase for iron uptake from soils. Nature 397, 694-697.

Rojas C L, Romera F J, Alcantara E, Perez-Vicente R, Sariego C, Garcia-Alonso J I, Boned J and Marti G 2008 Efficacy of Fe(o,o-EDDHA) and Fe(o,p-EDDHA) isomers in supplying Fe to Strategy I plants differs in nutrient solution and calcareous soil. J. Agric. Food Chem. 56, 10774-10778.

Rombola A D and Tagliavini M 2006 Iron nutrition of fruit tree crops. In Iron nutrition in plants and rhizospheric microorganisms. Eds. L L Barton and A Abadia. pp 61-83. Springer.

Römheld V 2000 The chlorosis paradox: Fe inactivation as a secondary event in chlorotic leaves of grapevine. J. Plant Nutr. 23, 1629-1643.

Römheld V and Marschner H 1981 Rhythmic iron stress reactions in sunflower at suboptimal iron supply. Physiol. Plantarum 53, 347-353.

Römheld V and Marschner H 1983 Mechanism of iron uptake by peanut plants - I. Fe(III) reduction, chelate splitting, and release of phenolics. Plant Physiol. 71, 949-954.

Römheld V and Marschner H 1986 Evidence for a specific uptake system for iron phytosiderophores in roots of grasses. Plant Physiol. 80, 175-180.

Römkens P, Bouwman L, Japenga J and Draaisma C 2002 Potentials and drawbacks of chelate-enhanced phytoremediation of soils. Environ. Pollut. 116, 109-121.

Ryskievich D P and Boka G 1962 Separation and characterization of the stereoisomers of N,N'-ethylenebis-[2-(o-hydroxy-phenyl)]glycine. Nature, 472-473.

Saito T, Koopal L K, Van Riemsdijk W H, Nagasaki S and Tanaka S 2004 Adsorption of humic acid on goethite: Isotherms, charge adjustments, and potential profiles. Langmuir 20, 689-700.

Schenkeveld W D C, Dijcker R, Reichwein A M, Temminghoff E J M and Van Riemsdijk W H 2008 The effectiveness of soil-applied FeEDDHA treatments in preventing iron chlorosis in soybean as a function of the o,o-FeEDDHA content. Plant Soil 303, 161-176.

Schenkeveld W D C, Hoffland E, Reichwein A M, Temminghoff E J M and Van Riemsdijk W H 2010c The biodegradability of EDDHA-chelates under calcareous soil conditions This thesis Chapter 6.

Schenkeveld W D C, Reichwein A M, Bugter M, Temminghoff E J M and Van Riemsdijk W H 2010b The performance of soil-applied FeEDDHA isomers in delivering Fe to soybean plants in relation to the moment of application J. Agric. Food Chem. *accepted*.

Schenkeveld W D C, Reichwein A M, Temminghoff E J M and Van Riemsdijk W H 2007 The behaviour of EDDHA isomers in soils as influenced by soil properties. Plant Soil 290, 85-102.

Schenkeveld W D C, Reichwein A M, Temminghoff E J M and Van Riemsdijk W H 2010f Considerations on the shuttle mechanism of FeEDDHA chelates at the soil-root interface in case of Fe deficiency This thesis Chapter 9.

Schenkeveld W D C, Reichwein A M, Temminghoff E J M and Van Riemsdijk W H 2010e The effect of soil parameters on the kinetics of Fe displacement from FeEDDHA chelates by Cu This thesis Chapter 8.

Schenkeveld W D C, Temminghoff E J M, Reichwein A M and Van Riemsdijk W H 2010a FeEDDHA-facilitated Fe uptake in relation to the behaviour of FeEDDHA components in the soil-plant system as a function of time and dosage. Plant Soil 332, 69-85.

Schenkeveld W D C, Weng L P, Temminghoff E J M, Reichwein A M and Van Riemsdijk W H 2010d Evaluation of the potential impact from Cu competition on the performance of o,o-FeEDDHA in soil application. This thesis Chapter 7.

Schroder K H 1964 Study of polarographic behaviour of N,N]-ethylenebis-(2-)o-hydroxyphenyl)) glycine in presence of iron ions. Acta Chem. Scand. 18, 896-&.

Schwertmann U 1964 The differentiation of iron oxides in soil by extraction with ammonium-oxalate solution. J. Plant Nutr. Soil Sci. 105, 194-202.

Shi Y, Byrne D H, Reed D W and Loeppert R H 1993 Influence of bicarbonate level on iron-chlorosis development and nutrient uptake of the peach rootstock Montclar. J. Plant Nutr. 16, 1675-1689.

Siebner-Freibach H, Hadar Y and Chen Y 2003 Siderophores sorbed on Ca-montmorillonite as an iron source for plants. Plant Soil 251, 115-124.

Siebner-Freibach H, Hadar Y and Chen Y 2004 Interaction of iron chelating agents with clay minerals. Soil Sci. Soc. Am. J. 68, 470-480.

Spiller S C, Castelfranco A M and Castelfranco P A 1982 Effects of iron and oxygen on chlorophyll biosynthesis I. Invivo observations of iron and oxygen-deficient plants. Plant Physiol. 69, 107-111.

Stevenson F J 1994 Humus chemistry - Genesis, composition, reactions. John Wiley & Sons, Inc. pp. 496.

Strasser O, Kohl K and Römheld V 1999 Overestimation of apoplastic Fe in roots of soil grown plants. Plant Soil 210, 179-187.

Tandy S, Ammann A, Schulin R and Nowack B 2006 Biodegradation and speciation of residual SS-ethylenediaminedisuccinic acid (EDDS) in soil solution left after soil washing. Environ. Pollut. 142, 191-199.

Tandy S, Bossart K, Mueller R, Ritschel J, Hauser L, Schulin R and Nowack B 2004 Extraction of heavy metals from soils using biodegradable chelating agents. Environ. Sci. Technol. 38, 937-944.

Temminghoff E J M, Van der Zee S E A T M and De Haan F A M 1997 Copper mobility in a copper-contaminated sandy soil as affected pH and solid and dissolved organic matter. Environmental-science-and-technology 31, 1109-1115.

Terry N 1980 Limiting factors in photosynthesis I Use of iron stress to control photo-chemical capacity in vivo. Plant Physiol. 65, 114-120.

Tipping E, Rieuwerts J, Pan G, Ashmore M R, Lofts S, Hill M T R, Farago M E and Thornton I 2003 The solid-solution partitioning of heavy metals (Cu, Zn, Cd, Pb) in upland soils of England and Wales. Environ. Pollut. 125, 213-225.

Van Devivere P C, Saveyn H, Verstraete W, Feijtel T C J and Schowanek D R 2001 Biodegradation of metal-[S,S]-EDDS complexes. Environ. Sci. Technol. 35, 1765-1770.

Vasconcelos M and Grusak M A 2007 Status and future developments involving plant iron in animal and human nutrition. In Iron nutrition in plants and rhizospheric organisms. Eds. L L Barton and J Abadia. p. 477. Springer.

Venkatraju K and Marschner H 1981 Inhibition of iron-stress reactions in sunflower by bicarbonate. J. Plant Nutr. Soil Sci. 144, 339-355.

Villen M, Garcia-Arsuaga A and Lucena J J 2007 Potential use of biodegradable chelate N-(1,2-dicarboxyethyl)-D,L-aspartic acid/$Fe^{3+}$ as an Fe fertilizer. J. Agric. Food Chem. 55, 402-407.

Walinga I, Kithome M, Novozamsky I, Houba V J G and Van der Lee J J 1992 Spectrophotometric determination of organic carbon in soil. Commun. Soil Sci. Plan. 23, 1935-1944.

Wallace A 1962 A decade of synthetic chelating agents in inorganic plant nutrition, Los Angeles (California).

Wallace A 1966 Ten years of iron EDDHA use in correcting iron chlorosis in plants. In Current topics in plant nutrition. Ed. A Wallace. pp 1-2. Edwards Brothers, Inc., Ann Arbor, Michigan.

Wallace A and Cha J W 1986 Effects of bicarbonate, phosphorus, iron EDDHA, and nitrogen sources on soybeans grown in calcareous soil. J. Plant Nutr. 9, 251-256.

Wallace A and Lunt O R 1960 Iron chlorosis in horticultural plants, a review. Proc. Am. Soc. Hort. Sci. 75, 819-841.

Wallace A, Muerller R, Lunt O R, Ashcroft R T and Shannon L M 1955 Comparisons of five chelating agents in soils, in nutrient solutions, and in plant responses. Soil Sci. 80, 101-108.

Wallace A and Wallace G A 1983 Use of synthetic chelating agents in experimental and commercial nutrient solutions. J. Plant Nutr. 6, 513-525.

Wallace A and Wallace G A 1992 Some of the problems concerning iron nutrition of plants after four decades of synthetic chelating agents. J. Plant Nutr. 15, 1487-1508.

Weng L P, Koopal L K, Hiemstra T, Meeussen J C L and Van Riemsdijk W H 2005 Interactions of calcium and fulvic acid at the goethite-water interface. Geochim. Cosmochim. Ac. 69, 325-339.

Weng L P, Temminghoff E J M, Lofts S, Tipping E and Van Riemsdijk W H 2002 Complexation with dissolved organic matter and solubility control of heavy metals in a sandy soil. Environ. Sci. Technol. 36, 4804-4810.

Weng L P, Temminghoff E J M and Van Riemsdijk W H 2001 Contribution of individual sorbents to the control of heavy metal activity in sandy soil. Environ. Sci. Technol. 35, 4436-4443.

Weng L P, Van Riemsdijk W H, Koopal L K and Hiemstra T 2006 Adsorption of humic substances on goethite: Comparison between humic acids and fulvic acids. Environ. Sci. Technol. 40, 7494-7500.

Weng L P, Van Riemsdijk W H and Hiemstra T 2008a Humic nanoparticles at the oxide-water interface: Interactions with phosphate ion adsorption Environ. Sci. Technol. 42, 8747-8752

Weng L P, Van Riemsdijk W H, and Hiemstra T 2008b $Cu^{2+}$ and $Ca^{2+}$ adsorption to goethite in the presence of fulvic acids Geochim. Cosmochim. Ac. 72, 5857-5870

Wenzel W W, Unterbrunner R, Sommer P and Sacco P 2003 Chelate-assisted phytoextraction using canola (Brassica napus L.) in outdoors pot and lysimeter experiments. Plant Soil 249, 83-96.

Wu L H, Luo Y M, Xing X R and Christie P 2004 EDTA-enhanced phytoremediation of heavy metal contaminated soil with Indian mustard and associated potential leaching risk. Agr. Ecosyst. Environ. 102, 307-318.

Xue H B, Sigg L and Kari F G 1995 Speciation of EDTA in natural-waters: Exchange kinetics of Fe-EDTA in river water. Environ. Sci. Technol. 29, 59-68.

Yu Q Y, Kandegedara A, Xu Y and Rorabacher D B 1997 Avoiding interferences from Good's buffers: A contiguous series of noncomplexing tertiary amine buffers covering the entire range of pH 3-11. Anal. Biochem. 253, 50-56.

Yunta F, Garcia-Marco S and Lucena J J 2003a Theoretical speciation of ethylenediamine-N-(o-hydroxyphenylacetic)-N '-(p-hydroxyphenylacetic) acid (o,p-EDDHA) in agronomic conditions. J. Agric. Food Chem. 51, 5391-5399.

Yunta F, Garcia-Marco S, Lucena J J, Gomez-Gallego M, Alcazar R and Sierra M A 2003b Chelating agents related to ethylenediamine bis(2-hydroxyphenyl)acetic acid (EDDHA): Synthesis, characterization, and equilibrium studies of the free ligands and their $Mg^{2+}$, $Ca^{2+}$, $Cu^{2+}$, and $Fe^{3+}$ chelates. Inorg. Chem. 42, 5412-5421.

Yunta F, Sierra M A, Gomez-Gallego M, Alcazar R, Garcia-Marco S and Lucena J J 2003c Methodology to screen new iron chelates: Prediction of their behavior in nutrient solution and soil conditions. J. Plant Nutr. 26, 1955-1968.

Zhang C D, Römheld V and Marschner H 1995 Retranslocation of iron from primary leaves of bean-plants grown under iron deficiency. J. Plant Physiol. 146, 268-272.

# Summary

Iron deficiency chlorosis is a worldwide occurring nutritional disorder in plants, reducing crop yields both quantitatively and qualitatively, and leading to economic losses. It predominantly occurs in plants grown on calcareous soils and is caused by a low bioavailability rather than an absence of iron in the soil. This low bioavailability of iron is primarily related to the limited solubility of iron minerals at calcareous soil pH (7 - 8.5) and the presence of a bicarbonate buffer in soil solution. Chelate based Fe fertilizers are commonly applied to increase the solubility, and thereby the bioavailability of Fe. The synthetic chelate FeEDDHA (iron (3+) ethylene diamine-N,N'-bis(hydroxy phenyl acetic acid)) is among the most effective in preventing and remedying iron deficiency chlorosis in plants grown on calcareous soils. FeEDDHA fertilizer products consist of mixtures of chemical compounds that can be operationally divided into four groups of FeEDDHA components: racemic o,o-FeEDDHA, meso o,o-FeEDDHA, o,p-FeEDDHA and rest-FeEDDHA. The composition of commercially available FeEDDHA formulation varies, and so does the effectiveness of individual FeEDDHA components in delivering Fe to soil-grown plants.
An efficient use of FeEDDHA fertilizer is desirable, both in view of cost efficiency and from an environmental perspective. Maximizing the benefits from FeEDDHA application in terms of crop yield and Fe uptake by plants, while minimizing the applied dosage, requires application of the right fertilizer (right composition) at the right moment in the right quantity. In order to do so, an advanced understanding of the behaviour of FeEDDHA components in the soil-plant system is needed. The objective of this study was to establish the processes determining the fate of FeEDDHA components in soil-plant systems, and to relate these processes to the characteristics of FeEDDHA components, determining their effectiveness as Fe fertilizer.

First (**Chapter 2**), the interaction of FeEDDHA components and EDDHA ligands with soil was examined as a function of time in a batch experiment involving eight soils. The treatments consisted of mixtures of FeEDDHA components and EDDHA ligands. The o,o-FeEDDHA isomers remained largely in solution, while rest-FeEDDHA and o,p-FeEDDHA were largely removed within the first week. Removal of racemic o,o-FeEDDHA was related to the organic matter content of the soils, removal of meso o,o-FeEDDHA to the reactive Fe(hydr)oxide content and removal of o,p-FeEDDHA to the clay content. In treatments involving o,p-EDDHA, whether or not chelated to Fe, elevated Cu concentrations were found as a result of the formation of o,p-CuEDDHA. From one week onward, o,p-CuEDDHA concentrations declined.

**Chapters 3 to 5** describe three pot trial studies in which FeEDDHA-facilitated Fe uptake by soybean plants was examined. In **Chapter 3**, the influence of the composition of FeEDDHA treatments on Fe uptake was considered in an 8-week pot trial study involving eight soils. The o,o-FeEDDHA components largely determined the Fe concentration in soil solution. In plants grown on soils in which the blank treatment became chlorotic, Fe uptake was in turn a function of the Fe concentration in solution. Initially, Fe uptake increased strongly with increasing o,o-FeEDDHA content of the FeEDDHA treatment, but the slope flattened and an

uptake plateau was reached. Increased Fe uptake led to both an increase in Fe content of the shoot and an increase in dry weight yield.
In a 6-week pot trial study described in **Chapter 4**, the relation between FeEDDHA component concentrations in soil solution and FeEDDHA-facilitated Fe uptake was considered as a function of time. Rest-FeEDDHA and o,p-FeEDDHA were largely removed from soil solution within the first week, before plants in the blank treatment displayed symptoms of Fe deficiency. Racemic and meso o,o-FeEDDHA underwent an initial concentration drop as a result of adsorption, which could be described with linear adsorption isotherms. The concentration of both o,o-FeEDDHA isomers gradually declined further. For meso o,o-FeEDDHA, the decline could be described with an exponential decay function, and was largely independent of Fe uptake by plants. For racemic o,o-FeEDDHA, the decline in concentration was slower and to a much higher extent related to Fe uptake by plants, which was highest in the progressed vegetative stage and in the reproductive stage.
**Chapter 5** assesses the effectiveness of racemic o,o-FeEDDHA, meso o,o-FeEDDHA and o,p-FeEDDHA in soil application in relation to the moment of application in an 8-week pot trial. FeEDDHA components were applied both separately and in mixtures, at t=0 and in the aforementioned growth stages in which the plant's Fe demand was highest. Both meso and racemic o,o-FeEDDHA contributed to Fe uptake, approximately to the same extent. O,p-FeEDDHA did not significantly contribute to Fe uptake in any treatment. The moment of application significantly affected dry weight yield and remaining FeEDDHA component concentration in soil solution, but not Fe uptake by plants. The efficiency of plants in taking up Fe from FeEDDHA greatly increased, when plants were Fe deficient before FeEDDHA application.

**Chapters 6 to 8** study processes possibly responsible for the plant-independent decline in soil solution concentration of EDDHA chelates. In **Chapter 6** the role of biodegradation in the gradual decline in concentration of meso o,o-FeEDDHA and o,p-CuEDDHA (described in **Chapter 2 and 4**) was examined in a soil incubation experiment involving sterilized treatments. Biodegradation did not significantly affect the rate of decline in concentration of any EDDHA chelate, except for CoEDDHA, which is quantitatively of marginal importance. The rate of the process causing the decline in meso o,o-FeEDDHA and o,p-CuEDDHA concentration was higher at higher temperature and in soil which had not been sterilized.
The next process considered to explain the plant-independent decline in soil solution concentration of meso o,o-FeEDDHA (**Chapter 4**) was cation competion from Cu (**Chapter 7**). Predictions with mechanistic multi-surface models show that there is a thermodynamic basis for assuming that under equilibrium conditions a certain fraction of o,o-EDDHA ligands in soil solution can be chelated to Cu, in particular of meso o,o-EDDHA. Furthermore, in a batch interaction experiment with calcareous soils, o,o-CuEDDHA was demonstrated to have a high affinity for the soil solid phase. This suggests that the potential impact of Cu competition might be much larger than predicted by modeling, because for a given o,o-CuEDDHA concentration in soil solution, a much larger amount of o,o-CuEDDHA is adsorbed to the solid phase. Finally, the displacement of Fe from o,o-FeEDDHA by Cu was

reproduced in a single-surface goethite suspension, demonstrating the displacement reaction is not kinetically inhibited.
The influence of a number of soil parameters on the rate of the displacement reaction was examined in a series of batch experiments with goethite suspensions (**Chapter 8**). The displacement rate was co-determined by the FeEDDHA component. For equal conditions, the sequence in displacement rate was: o,p-FeEDDHA >> meso o,o-FeEDDHA > racemic o,o-FeEDDHA, which corresponds with the sequence in residence time in soils solution in soil-plant systems (**Chapter 4**). It was found that the displacement reaction predominantly takes place on a reactive surface rather than in solution. The rate of decline in FeEDDHA solution concentration proved dependent on the available reactive surface area, and the FeEDDHA and Cu loading on the reactive surface. Soil parameters decreasing FeEDDHA adsorption (high ionic strength, humic acid adsorbed onto the reactive surface, monovalent instead of divalent cations in the electrolyte), decreased the rate of displacement. For meso o,o-FeEDDHA, the rate equation of the displacement reaction was derived, which proved first order in FeEDDHA loading and half order in Cu loading. For soil conditions the rate equation could be simplified and solved to an exponential decay function in meso o,o-FeEDDHA solution concentration, which corresponds with findings in soil solution of soil-plant systems (**Chapter 4 and 6**).

**Chapter 9** is dedicated to the shuttle mechanism, a hypothetical cyclic mode of action in which the chelating agent is "recycled" by chelating and mobilizing Fe from the soil after delivering Fe at the root surface. The efficiency of the shuttle mechanism with EDDHA ligands was examined in batch interaction experiments with four calcareous soils. If metal availability in the bulk soil determines the shuttle's efficiency, it is heavily compromised as a result of chelation of competing cations: Al, Mn and in particular Cu. Additionally, data collected in the pot trial studies presented in **Chapter 3 and 4** were examined for experimental support for the shuttle mechanism. Specific metal mobilization was observed upon FeEDDHA-facilitated Fe uptake, indicating that EDDHA ligands do in fact mobilize metals after delivering Fe at the root surface. However, at least to a certain extent these metals originated from the root surface and not from the bulk soil.

In conclusion, a conceptual model for the behaviour of FeEDDHA chelates in soil-plant systems is presented in **Chapter 10**. The model integrates the findings from the previous chapters and describes the processes affecting the soil solution concentration of EDDHA chelates after administration of an FeEDDHA treatment. The backbone of the model comprises three processes: 1) FeEDDHA adsorption, 2) Fe displacement from FeEDDHA by Cu on a reactive surface, and subsequent release of CuEDDHA into soil solution, and 3) re-adsorption of CuEDDHA. The concentration behaviour of racemic o,o-FeEDDHA, meso o,o-FeEDDHA and o,p-FeEDDHA, observed in experiments, is interpreted within the framework of the conceptual model, and the three aforementioned processes are further explored, amongst others by means of additional experimental data.

The effectiveness of FeEDDHA components in soil application is largely determined by their ability to remain in solution. A limited affinity for the soil solid phase and a high (relative) affinity for Fe proved essential in this respect. Clay content, Fe(hydr)oxide content and Cu content are important soil parameters affecting the effectiveness of FeEDDHA components. The presented conceptual model provides a tool for both appliers of FeEDDHA products, for selecting the most suitable product and using it efficiently, and for producers of chelate based Fe fertilizers in view of product development and marketing. The chapter closes off with a discussion on the limitations of the research, and with recommendations for further research to verify and further expand the conceptual model.

# Samenvatting

IJzerchlorose is een voedingsstoornis in planten, die wereldwijd voorkomt. Het tast gewasopbrengsten zowel kwalitatief als kwantitatief aan, en leidt daardoor tot economische verliezen. IJzerchlorose komt voornamelijk voor in planten, die op kalkrijke gronden groeien, en wordt veroorzaakt door een lage biobeschikbaarheid van ijzer in de bodem; niet door de afwezigheid van ijzer. Deze lage biobeschikbaarheid hangt met name samen met de beperkte oplosbaarheid van ijzermineralen binnen de bodem-pH range van kalkrijke gronden (7 - 8.5), en met de aanwezigheid van een bicarbonaat pH-buffer in de bodemoplossing. Als remedie tegen ijzergebrek worden doorgaans ijzermeststoffen toegepast, die gebaseerd zijn op synthetische ijzerchelaten. Deze vergroten de oplosbaarheid en daarmee tevens de biobeschikbaarheid van ijzer. Het synthetische chelaat FeEDDHA (ijzer ethyleen diamine-N,N'-bis(hydroxifenyl azijnzuur)) behoort tot de meest effectieve meststoffen om ijzergebrek in planten op kalkrijke grond te voorkomen en te bestrijden. FeEDDHA producten bestaan uit mengsels van chemische verbindingen, die kunnen worden onderverdeeld in: racemisch o,o-FeEDDHA, meso o,o-FeEDDHA o,p-FeEDDHA en rest-FeEDDHA. De samenstelling van commercieel beschikbare FeEDDHA formuleringen varieert, zo ook de effectiviteit van de afzonderlijke FeEDDHA componenten als ijzermeststof in grondtoepassing. Een efficiënt gebruik van FeEDDHA is wenselijk, zowel vanuit het oogpunt van kostenefficiëntie, als vanuit milieuperspectief. Het maximaliseren van de baten van FeEDDHA toepassing voor wat betreft gewasopbrengst en ijzeropname door planten, bij een zo laag mogelijke FeEDDHA dosering, vereist toepassing van het FeEDDHA product met de juiste samenstelling, op het juiste moment en in de juiste hoeveelheid. Een goed begrip van het gedrag van FeEDDHA componenten in bodem-plant systemen is hiervoor essentieel. Het doel van het onderzoek, gepresenteerd in dit proefschrift, was het vaststellen van de processen, die het lot van FeEDDHA componenten in bodem-plant systemen bepalen, en het relateren van deze processen aan de eigenschappen van FeEDDHA componenten, die hun effectiviteit als ijzermeststof bepalen.

Eerst **(Hoofdstuk 2)** is de interactie van FeEDDHA componenten en EDDHA liganden met grond onderzocht in een batch experiment met acht gronden. De behandelingen bestonden uit mengsels van FeEDDHA componenten en EDDHA liganden. De o,o-FeEDDHA isomeren bleven grotendeels in oplossing, terwijl rest-FeEDDHA en o,p-FeEDDHA binnen een week grotendeels uit oplossing verdwenen. Verwijdering van racemisch o,o-FeEDDHA hing samen met het organisch stof gehalte van de gronden, verwijdering van meso o,o-FeEDDHA met het reactief ijzer(hydr)oxide gehalte en verwijdering van o,p-FeEDDHA met het kleigehalte. In behandelingen met o,p-EDDHA, al dan niet gecheleerd aan Fe, werden verhoogde Cu concentraties gemeten als gevolg van de vorming van o,p-CuEDDHA. Na de eerste week namen de o,p-CuEDDHA concentraties geleidelijk af.

De **Hoofdstukken 3 t/m 5** beschrijven drie potproeven, waarin FeEDDHA-gefaciliteerde ijzeropname door sojaboon planten is onderzocht. In **Hoofdstuk 3** is de invloed van de samenstelling van FeEDDHA behandelingen op de ijzeropname bekeken in een acht weken durend potexperiment met acht gronden. De o,o-FeEDDHA componenten bepaalden in hoge

mate de ijzerconcentratie in de bodemoplossing. Bij gronden, waarop de planten van de controlebehandeling chlorotisch werden, bleek de ijzeropname een functie van de ijzerconcentratie in de bodemoplossing. Aanvankelijk nam de ijzeropname sterk toe met het o,o-FeEDDHA gehalte van de behandeling, maar naarmate het o,o-FeEDDHA gehalte hoger werd nam het additioneel effect van meer o,o-FeEDDHA geleidelijk af, totdat een opnameplateau werd bereikt. Een toename in ijzeropname leidde zowel tot een toename in ijzergehalte in het plantenmateriaal als tot een toename in gewasopbrengst.
In een zes weken durend potexperiment, beschreven in **Hoofdstuk 4,** is de relatie tussen de concentraties van FeEDDHA componenten in de bodemoplossing en FeEDDHA-gefaciliteerde ijzeropname onderzocht als functie van de tijd. Rest-FeEDDHA en o,p-FeEDDHA waren binnen de eerste week grotendeels uit de bodemoplossing verdwenen, voordat de planten in de controle behandeling symptomen van ijzergebrek vertoonden. De concentratie van racemisch en meso o,o-FeEDDHA in de bodemoplossing onderging een sterke daling in de eerste week als gevolg van adsorptie, welke kon worden beschreven met lineaire adsorptie-isothermen. De concentratie van beide o,o-FeEDDHA isomeren daalde vervolgens geleidelijk verder. De afname in meso o,o-FeEDDHA concentratie kon worden beschreven met een exponentiële functie en was grotendeels onafhankelijk van ijzeropname door planten. De afname in racemisch o,o-FeEDDHA concentratie verliep langzamer en was in hogere mate gerelateerd aan ijzeropname door planten. De ijzeropname was het hoogst in het gevorderd vegetatieve en het reproductieve groeistadium van de sojaboon planten.
**Hoofdstuk 5** evalueert de effectiviteit van racemisch o,o-FeEDDHA, meso o,o-FeEDDHA en o,p-FeEDDHA in grond toepassing in relatie tot het moment van toediening in een acht weken durend potexperiment. De FeEDDHA componenten werden afzonderlijk en in mengsels toegediend, bij aanvang van het experiment en in de voorgenoemde groeistadia, wanneer de ijzerbehoefte het hoogst was. Zowel meso als racemisch o,o-FeEDDHA droeg bij aan de ijzeropname, ongeveer in gelijke mate. O,p-FeEDDHA leverde geen significante bijdrage aan de ijzeropname, in geen enkele behandeling. Het moment van toediening had een significant effect op het drooggewicht van de gewasopbrengst en op de resterende FeEDDHA component concentratie in de bodemoplossing, maar niet op de ijzeropname. De efficiëntie waarmee planten ijzer van FeEDDHA opnamen, nam sterk toe wanneer planten ijzergebrek hadden voor toediening van FeEDDHA.

De **Hoofdstukken 6 t/m 8** bestuderen processen, die mogelijk verantwoordelijk zijn voor de plant-onafhankelijke afname in EDDHA-chelaat concentraties in de bodemoplossing. In **Hoofdstuk 6** is de rol van biodegradatie in de geleidelijke afname in concentratie van meso o,o-FeEDDHA en o,p-CuEDDHA (beschreven in de **Hoofdstukken 2 en 4**) onderzocht in een grondincubatie experiment met gesteriliseerde behandelingen. Biodegradatie had voor geen enkel EDDHA chelaat een significant effect op de snelheid waarmee de concentratie in bodemoplossing afnam, met uitzondering van CoEDDHA, welk kwantitatief van marginaal belang is. De snelheid van het proces, dat de afname in meso o,o-FeEDDHA en o,p-CuEDDHA concentratie veroorzaakt, is hoger bij hogere temperatuur en in grond, die niet is gesteriliseerd.

Het volgend proces, dat is onderzocht als mogelijke verklaring voor de plant-onafhankelijke afname in meso o,o-FeEDDHA concentratie in de bodemoplossing **(Hoofdstuk 4)**, is kationcompetitie door koper **(Hoofdstuk 7)**. Voorspellingen met behulp van mechanistische multi-surface modellen laten zien, dat er een thermodynamische basis is om aan te nemen, dat onder evenwichtscondities een zekere fractie van o,o-EDDHA liganden in de bodemoplossing gecheleerd kan zijn aan koper, met name van meso o,o-EDDHA. Verder is in een schudproef met kalkrijke gronden aangetoond, dat o,o-CuEDDHA een hoge affiniteit heeft voor de vaste fase van de bodem. Dit suggereert, dat de potentiële impact van kopercompetitie veel groter zou kunnen zijn dan voorspeld aan de hand van de modellen, omdat voor een gegeven o,o-CuEDDHA concentratie in de bodemoplossing een veel grotere hoeveelheid o,o-CuEDDHA geadsorbeerd zit aan de vaste fase. Tot slot is de reactie waarin ijzer van o,o-FeEDDHA wordt verdrongen door koper gereproduceerd in goetietsuspensies, waarmee is aangetoond, dat de verdringingsreactie niet kinetisch verhinderd wordt.
De invloed van een aantal bodemparameters op de snelheid van de verdringingsreactie is onderzocht in een serie batchexperimenten met goetietsuspensies **(Hoofdstuk 8)**. De verdringingssnelheid wordt mede bepaald door de FeEDDHA component. Voor gelijke condities is de volgorde in verdringingsnelheid als volgt: o,p-FeEDDHA >> meso o,o-FeEDDHA > racemisch o,o-FeEDDHA; dit komt overeen met de volgorde in verblijftijd in bodemoplossing in bodem-plant systemen **(Hoofdstuk 4)**. Er is vastgesteld, dat de verdringingsreactie vooral op het reactief oppervlak plaatsvindt. De afnamesnelheid van de FeEDDHA concentratie in oplossing bleek afhankelijk van de hoeveelheid beschikbaar reactief oppervlak, en van de FeEDDHA- en koper-loading op het reactief oppervlak. Bodemparameters, die FeEDDHA adsorptie verminderden (hoge ionsterkte, geadsorbeerd humuszuur op het reactief oppervlak, en monovalente in plaats van divalante kationen in het electroliet), verminderden de verdringingssnelheid. Voor meso o,o-FeEDDHA is de reactiesnelheidsvergelijking van de verdringingsreactie afgeleid. Deze is eerste orde in FeEDDHA-loading en halfde orde in koper-loading. Voor bodemcondities kan de reactiesnelheidsvergelijking worden vereenvoudigd en geïntegreerd tot een exponentiële afnamefunctie in meso o,o-FeEDDHA concentratie in oplossing, hetgeen overeenkomt met bevindingen in de bodemoplossing van bodem-plant systemen **(Hoofdstukken 4 en 6)**.

**Hoofdstuk 9** is gewijd aan het shuttle mechanisme; een hypothetisch, cyclisch werkingsmechanisme, waarbij de chelant wordt "gerecycled" door ijzer uit de grond te cheleren en mobiliseren, nadat ijzer aan het worteloppervlak is afgeleverd. De efficiëntie van het shuttle mechanisme is onderzocht voor EDDHA liganden middels batch interactie-experimenten met vier kalkrijke gronden. Indien de metaalbeschikbaarheid in de bulkgrond maatgevend is voor de efficiëntie van de shuttle, dan wordt deze sterk beperkt door chelering van andere kationen: aluminium, mangaan en met name koper. Aanvullend is de data, verzameld in de potexperimenten gepresenteerd in de **Hoofdstukken 3 en 4**, onderzocht op experimentele ondersteuning voor het shuttle mechanisme. Er is specifieke metaalmobilisatie als gevolg van FeEDDHA-gefaciliteerde ijzeropname geconstateerd. Dit duidt erop, dat EDDHA liganden inderdaad metalen mobiliseren, nadat ijzer aan het worteloppervlak is

afgestaan. Echter, tenminste in zekere mate waren deze metalen afkomstig van het worteloppervlak en niet uit de bulkgrond.

Tot slot wordt in **Hoofdstuk 10** een conceptueel model voor het gedrag van FeEDDHA chelaten in bodem-plant systemen gepresenteerd. Het model integreert de bevindingen uit de voorafgaande hoofdstukken en beschrijft de processen, die de concentratie van EDDHA chelaten in de bodemoplossing beïnvloeden na toediening van een FeEDDHA behandeling. De basis van het model bestaat uit 3 processen: 1) FeEDDHA adsorptie, 2) de reactie op het reactief oppervlak, waarbij ijzer van FeEDDHA wordt verdrongen door koper, en het vervolgens in bodemoplossing gaan van CuEDDHA, en 3) her-adsorptie van CuEDDHA. Het concentratieverloop van racemisch o,o-FeEDDHA, meso o,o-FeEDDHA and o,p-FeEDDHA, zoals waargenomen in experimenten, wordt binnen het kader van het conceptueel model geïnterpreteerd, en de drie voorgenoemde processen worden nader verkend, onder andere aan de hand van additionele experimentele data.
De effectiviteit van de FeEDDHA componenten in bodemtoepassing wordt grotendeels bepaald door het vermogen van de FeEDDHA component om in oplossing te blijven. Een geringe affiniteit voor de vaste fase van de bodem en een hoge (relatieve) affiniteit voor ijzer zijn hiervoor essentieel. Kleigehalte, ijzer(hydr)oxidegehalte en kopergehalte zijn belangrijke bodemparameters, die de effectiviteit van FeEDDHA componenten beïnvloeden. Het gepresenteerde conceptueel model vormt een hulpmiddel voor zowel gebruikers van FeEDDHA producten, om het meest geschikte product te selecteren en het zo efficiënt mogelijk toe te passen, als voor producenten van ijzermeststoffen die gebaseerd zijn op chelaten, met het oog op productontwikkeling en marketing.
Het hoofdstuk sluit af met een korte bediscussiëring van de beperkingen van het onderzoek en met aanbevelingen voor verder onderzoek om het conceptueel model te verifiëren en uit te bouwen.

"What binds me has been slain,
what surrounds me has been overcome;
my desire has been ended,
and ignorance has died."

Excerpt from:
*The Gospel of Mary Magdalene*

# Dankwoord

"Acta est fabula" – met het schrijven van dit dankwoord zit mijn promotieonderzoek er gevoelsmatig dan echt definitief op. In retrospectief staat één ding voor mij als een paal boven water: ik heb met volle teugen genoten van m'n periode als AiO! Ik heb me afgevraagd, geëxperimenteerd, beschouwd, gepuzzeld en me verwonderd over de schoonheid der dingen, alvorens de volgende vragen weer opborrelden – een verslavende cyclus. Als ik het promotie-traject opnieuw in zou gaan, dan zou ik veel zaken beslist heel anders aanpakken - en waarschijnlijk is dat een goed teken.

Op deze plek wil ik graag de mensen met wie ik heb samengewerkt of die anderszins een belangrijke rol hebben gespeeld tijdens mijn promotie bedanken; mocht ik onverhoopt iemand vergeten zijn – mijn welgemeende excuses!
In de eerste plaats mijn promotor Willem en co-promotor Erwin. Willem, ik heb genoten van je begeestering voor, en je geweldige expertise op het gebied van bodemchemie. Onze gesprekken waren inspirerend voor mij, en ik heb me er meerdere malen – soms maanden later – over verbaasd, dat je hypotheses, tegen mijn verwachting in, toch juist bleken te zijn. Ook je snelle becommentariëring van mijn manuscripten en het intensievere contact richting de afronding van dit proefschrift heb ik erg gewaardeerd.
Erwin, dank dat je ervoor hebt gezorgd, dat ik in alle vrijheid, zorgeloos onderzoek kon doen – dat is enorm waardevol geweest voor mij. Ook heb ik veel van je geleerd ten aanzien van het structureren en helder presenteren van mijn onderzoeksresultaten.

Ook de mensen, die vanuit AkzoNobel bij het project betrokken zijn geweest ben ik veel dank verschuldigd.
Arjen, ik wil je hartelijk danken voor de erg fijne samenwerking! Jouw kennis op het gebied van chemie in het algemeen, en van complexvormers in het bijzonder is voor mijn onderzoek van onschatbare waarde geweest. Onze gesprekken over mijn onderzoeksresultaten, marktontwikkelingen en al dan niet recente publicaties waren zowel erg leuk als erg nuttig. Dank ook voor je belangstelling, je kritische blik, het meedenken en de duizenden HPLC en UV/VIS metingen, die ik in Arnhem heb mogen doen.
Marcel, je geloof in het onderzoek en je inspanningen om de resultaten onder de aandacht te brengen, ook buiten de wetenschappelijke gemeenschap, waren enthousiasmerend en voor mij mede aanleiding om, middels een derde potproef, een stapje extra te zetten.
Pascal Weijters wil ik graag bedanken voor zijn inspanningen bij het opstarten en op gang brengen van het project. De uitstekende voorselectie van de door jou geïmporteerde gronden ligt mede ten grondslag aan de geboekte onderzoeksresultaten.
Verder dank ik ook Staffan Asplund, Anders Mollenstahl en Teun Scheperman voor hun bijdragen.

Bij mijn onderzoekswerkzaamheden heb ik veel ondersteuning gehad, zowel in de kas als op het lab.
Jaap, dank voor al je praktische adviezen, vooral op het gebied van planten en bodemvruchtbaarheid; gevraagd of ongevraagd, maar altijd bijzonder welkom. Het succes van

de potproeven heb ik daar mede aan te danken. Ook je organisatorische hulp om mijn tweede potproef tot een goed einde te brengen, toen op het laatste moment de student, die me hierbij zou helpen, afhaakte, vond ik grandioos!
De heren van de VEP (Willem, Arie en Peter) wil ik hartelijk danken voor alle hulp bij het inzetten en verzorgen van mijn plantproeven, het malen en zeven van duizenden kilo's grond, en eindeloos wortelspoelen (super, Willem!).

Op het lab kon ik vooral aankloppen bij Peter en Gerlinde. Peter, bedankt voor het uitvoeren van de vele ICP-analyses; ik vond het leuk om gaandeweg steeds meer te leren over de kunst van het meten en daar ook over mee te denken. Gerlinde, dankjewel voor je adviezen en je fantastische ondersteuning bij mijn experimenten.
Ook de andere medewerkers van het Centraal Laboratorium Bodem en Gewasanalyses,
Andre, Arie, Carry, Johan, Karin, Kees, Monique, Simon, Tamas, Willeke, Wim en Wobbe, dank voor jullie hulp.

During my PhD-research, I was privileged to supervise two MSc students, which was a great learning experience for me. Rob and Marina, thank you very much for your dedication and hard work – the fruits of your labour are presented in Chapter 3 and Chapter 9 of this thesis.

Ook wil ik graag mijn ex-collega's bij de vakgroep bodemkwaliteit bedanken - de AiOs en post-docs Bert, Debby, Dorien, Ellen, Erwin, Franciska, Gerard, Gerwin, Gijs, Ingrid, Jacintha, Julia, Laura, Maria, Mabel, Monika, Nicole, Odair, Petra, Ram, Rasoul, Thomas, Xiaopeng; de vaste staf Ellis, Jan-Willem, Lijbert, Liping, Meindert, Paul, Rob, Ron, Thom en Tjisse en de dames van het secretariaat Caroline, Esther, Gerda, Riette en Winnie - dank voor de gezelligheid, de samenwerking en de fijne werksfeer!
Gerwin, dank ook dat je mij betrokken hebt bij jouw EDDS-project; voor mij een leuke zijstap en ik vond de samenwerking, die heeft geresulteerd in een mooie publicatie, erg plezierig! Ellis, Liping en Tjisse – ook erg bedankt voor jullie inhoudelijke bijdragen aan mijn onderzoek.

Een bijzonder woord van dank gaat uit naar mijn beide paranimfen Debby en Roald; ik vind het heel fijn dat jullie me bij de verdediging van dit proefschrift terzijde willen staan. Debby, het was een waar genoegen om ruim vier jaar een kamer met je te mogen delen – ik had me geen betere kamergenoot kunnen wensen. Goedlachs, altijd in voor een praatje en op z'n tijd een goed gesprek. Dank voor alle gezelligheid, een luisterend oor en een hart onder de riem als het even tegen zat. Roald, na twee gezamenlijke studies en een gelijktijdig promotie-onderzoek was een verdediging zonder jou als paranimf natuurlijk eigenlijk uitgesloten. Dank voor onze geweldige vriendschap, die weinig woorden nodig heeft, maar steeds weer bijzondere gesprekken voortbrengt.

Special thanks also go out to Claudio Jardim for designing the cover of this thesis, and to Dr. Goos for providing the soybean seeds I used in my pot experiments.

Verder wil ik mijn “nieuwe” collega’s bij ARCADIS bedanken; ik heb jullie interesse in mijn promotieonderzoek erg op prijs gesteld. In het bijzonder wil ik hier mijn teamleider Arjan Barbier noemen – ik ben je erg dankbaar dat je hebt meegedacht en meegewerkt aan een mogelijkheid voor mij om dit proefschrift af te ronden.

Het noodzakelijke tegenwicht aan al het onderzoeksgeweld werd steevast gewaarborgd door mijn familie en vrienden, waarvoor veel dank!

Joris, Marco en Siong – of het nu in Ierland, Estland, Duitsland of Nederland is, het is altijd weer goed om jullie te zien; ik ben dankbaar dat jullie mijn vrienden zijn.
Berry, Bob en Dennis – de mannen van de muziek; een taal waarin we elkaar bijzonder goed verstaan. De opnames van Camera Obscura en onze minitour in Portugal waren absolute hoogtepunten tijdens m’n promotieonderzoek!
Arno, Martijn, Remco en Roald, vanaf vrijwel de eerste dag NWBB klikte het – goed dat we elkaar weer met regelmaat zien!

Mijn ouders, en Ruben en Anna – dank voor jullie liefde, interesse en steun.
Fatima – obrigado pelo seu carinho e apoio.
En tot slot mijn lieve vriendin Carla – dank voor al je liefde, geduld en begrip; samen met jou is de wereld zo veel mooier.

# List of publications
# &
# Curriculum vitae

# List of publications

Schenkeveld, W.D.C., Reichwein, A.M., Bugter, M., Temminghoff, E.J.M. and Van Riemsdijk, W.H., (2010). 'The performance of soil-applied FeEDDHA isomers in delivering Fe to soybean plants in relation to the moment of application '. *Journal of Agricultural and Food Chemistry*, *(accepted).*

Schenkeveld, W.D.C., Temminghoff, E.J.M., Reichwein, A.M. and Van Riemsdijk, W.H., (2010). 'FeEDDHA-facilitated Fe uptake in relation to the behaviour of FeEDDHA components in the soil-plant system as a function of time and dosage'. *Plant and Soil*, 332 (1-2):69-85.

Van Rotterdam, A.M.D., Temminghoff, E.J.M., Schenkeveld, W.D.C., Hiemstra, T. and Van Riemsdijk, W.H., (2009). 'Phosphorus removal from soil using Fe oxide-impregnated paper: Processes and applications'. *Geoderma*, 151 (3-4):282-289.

Koopmans, G.F., Schenkeveld, W.D.C., Song, J., Luo, Y.M., Japenga, J. and Temminghoff, E.J.M., (2008). 'Influence of EDDS on metal speciation in soil extracts: Measurement and mechanistic multicomponent modeling'. *Environmental Science & Technology*, 42 (4):1123-1130.

Schenkeveld, W.D.C., Dijcker, R., Reichwein, A.M., Temminghoff, E.J.M. and Van Riemsdijk, W.H., (2008). 'The effectiveness of soil-applied FeEDDHA treatments in preventing iron chlorosis in soybean as a function of the o,o-FeEDDHA content'. *Plant and Soil*, 303 (1-2):161-176.

Schenkeveld, W.D.C., Reichwein, A.M., Temminghoff, E.J.M. and Van Riemsdijk, W.H., (2007). 'The behaviour of EDDHA isomers in soils as influenced by soil properties'. *Plant and Soil*, 290 (1/2):85-102.

# Curriculum vitae

Walter David Cornelis Schenkeveld was born on the 9th of November 1976 in Gouda, The Netherlands. He completed his secondary education at the Coornhert grammar school in Gouda in 1995. From 1995 to 2002, he studied Science & Policy, and Environmental Science at Utrecht University and completed both with a Master of Science degree. During his Master's curricula, he conducted a research study on the fractionation and charge density of dissolved organic matter in relation to soil profile layers at Alterra DLO, and performed an assessment of Fischer-Tropsch synthesis of biodiesel with pyrolysis oil as intermediate at Shell Global Solutions.

As a student, Walter was active in quality control of academic education as chairman of the Education Evaluation Program for Science and Policy, and as member of the Education Committee of Environmental Science. Furthermore, he was a tutor for secondary school pupils.

In March 2003 he started a PhD research project at the department of Soil Quality of Wageningen University, in cooperation with AkzoNobel. The main results of this project, entitled "Iron fertilization with FeEDDHA – the fate and effectiveness of FeEDDHA chelates in soil-plant-systems" are presented in this thesis. Since March 2008, Walter holds his present position as soil specialist for ARCADIS.

# Education certificate

Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment

# C E R T I F I C A T E

The Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment
(SENSE), declares that

## *Walter Schenkeveld*

born on 9 November 1976 in Gouda, The Netherlands

has successfully fulfilled all requirements of the
Educational Programme of SENSE.

Wageningen, 19 November 2010

the Chairman of the SENSE board

Prof. dr. Rik Leemans

the SENSE Director of Education

Dr. Ad van Dommelen

The SENSE Research School has been accredited by the Royal Netherlands Academy of Arts and Sciences (KNAW)

KONINKLIJKE NEDERLANDSE
AKADEMIE VAN WETENSCHAPPEN

The SENSE Research School declares that Mr. Walter Schenkeveld has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 40 ECTS, including the following activities:

SENSE PhD courses

- Environmental Research in Context
- Research Context Activity: Communication and knowledge transfer presentations on PhD research project for relevant stakeholder parties
- Speciation and Bioavailability
- Basic and Advanced Statistics

Other PhD and MSc courses

- Techniques for Writing and Presenting Scientific Papers
- Chemical Interactions Soil-Water-Sediment
- Speciation and Transport
- Soil Pollution and Soil Protection

Oral Presentations

- The behaviour of iron chelates as influenced by soil properties related to plant uptake, SENSE summer symposium, 23 June 2005, Ede, The Netherlands
- Fe fertilization with synthetic chelates. The fate and effectiveness of FeEDDHA isomers, Soil and Water symposium, 7 June 2007, Lunteren, The Netherlands

SENSE Coordinator PhD Education and Research

Mr. Johan Feenstra

The research described in this thesis was financially supported by AkzoNobel