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FACULTEIT BIO-INGENIEURSWETENSCHAPPEN

The uptake of cadmium by cacao seedlings as affected by the root distribution and bioavailable cadmium

Cadmium opname door cacao zaailingen: de rol van het wortelstelsel en het biobeschikbaar cadmium

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Preface

The Maya and Aztecs believed that the cacao tree was a gift from the gods. I would exaggerate if I'd claim that this thesis was a gift from the gods for me. However, I feel honored that I was a part of this project and could hopefully contribute to the future of chocolate and particularly the cacao farmers in Latin America.

First of all, I need to thank David Argüello. David was a great supervisor, on many sides. The practically challenging pot experiment succeeded thanks to his hard work, experience and patience. In the greenhouse, we have spent days of just grinding (the never ending amount of) soil. In the field, we got stung by killer bees. However, David always kept me motivated to see the beauty of this research. Not only did David teach me about scientific research, but also about Ecuador, making this experience an enrichment in my cultural development as well. Next, I want to thank dr. Daniela Montalvo for all her support. Although not officially my supervisor, it certainly felt like that. Her work mentality, efficiency and scientific knowledge are truly inspirational. Her revisions were always incredibly accurate, and very crucial in the fulfillment of this thesis. Daniela was as well always ready to answer my (load of) questions, although she was swimming in the workload. Of course, I cannot otherwise than to thank the driving force behind this research. Prof. Erik Smolders, thank you for being such an inspirational and motivating professor. Throughout this thesis and his courses he unlocked an enthusiasm about the environmental sciences in me, which I will hopefully carry with me through the rest of my life since it makes working/studying much more of a pleasure.

I hope all readers now realize that I had professionally the perfect team behind me to fulfill this thesis. However, I also need to thank the people in my private life. Without the care of my family, I would probably never have been in the position to write this thesis. Mom and dad, thank you for always believing in me and enlightening my path with your experience and advice (except for the neus spoelen part). Eline and Marijn, I also need to thank you guys, since without your help and intelligence, getting a degree of bioscience engineer would have been quite a challenge. Special credit goes to Marijn, the LaTeX-master, since he taught me how to make this thesis more of a beauty for the eye. Lastly, I also need to remind the world of my beautiful friends, who made studying truly, so far, the best part of my life. It's with a bit of grief that I'm closing this chapter of my life, however, I feel that time has come to explore new areas of life.

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Contents

Abstract

Cadmium (Cd) is a highly-toxic trace metal that can be taken up by plant roots and may accumulate in edible parts of the plant. Recent studies have reported high levels of Cd in cacao (*Theobroma cacao* L.) beans from Ecuador (average 0.9 mg Cd/kg bean), which has raised concerns on its future trade to international markets due to difficulties to comply with food safety regulations. As a consequence, there is an urgent call to find mitigation strategies to reduce the concentration of Cd in cacao beans. Managing soil pH through the application of lime is an obvious and promising method to reduce the bioavailability of Cd in soils. However, lime can only be applied to the top layer of the soil whilst cacao is a perennial crop with deep-penetrating roots. Therefore, it is crucial to understand the functionality of cacao root structures on Cd uptake. Hence, the purpose of this research was to determine the role of the different rooted soil compartments (surface soil versus subsoil) and bioavailable Cd on the uptake of Cd by cacao seedlings. This better understanding would allow the development of more effective mitigation practices.

A pot experiment was conducted in a glasshouse in Guayaquil (University of ESPOL) with cacao seedlings using the stable isotope ^{108}Cd as a tracer. Pots contained superimposed soils (top and bottom) that differed in Cd availability either by applying liming amendments or by using soils with contrasting properties (surface soil (0-15 cm, pH 5.2, 1.38 mg Cd/kg) or subsoil (15-30 cm, pH 5.5, 0.77 mg Cd/kg)). Three soil amendments were tested: lime (CaCO3), gypsum (CaSO4) and magnesium oxide (MgO). The experiment consisted of 24 treatments replicated three times with pots

arranged in a completely randomized design. After four months of growth, plants were harvested to determine ^{108}Cd and ^{111}Cd in new leaves by means of ICP-MS. Additionally, on selected treatments the roots of the top and bottom layer were separated and collected.

It was found that the vertical heterogeneity of the soils and liming indeed affected the concentration of Cd in cacao leaves. In the homogeneous pots (surface soil, subsoil only) the total soil Cd concentration could explain the 50% reduction in leaf Cd when grown on subsoil instead of surface soil since in the subsoil half the amount of Cd was present. In the contrasting soil treatments (surface on subsoil), the reduction in leaf Cd concentration (10%) was less than expected based on total soil Cd concentrations. The stable isotope study indicated an increase of 18% in Cd derived from the top layer. Thus, cacao roots tended to be more active in the uptake of Cd in the nutrient-rich surface layer than in the subsoil. In pots with surface soil only the application of lime in both compartments reduced Cd in the leaves with 42%, whereas when only top compartment was limed the reduction was of 16%. Here, most of Cd derived from the bottom compartment (64%). This result reveals the dynamics of cacao roots and the possibility to counteract the Cd immobilization since uptake of Cd from the bottom layer increased. In contrasting soils, leaf Cd significantly reduced by 40% when the top compartment was limed and overall there were no differences between the tested amendments (lime, gypsum and magnesium oxide).

In conclusion, liming of the soil may be advocated as an effective mitigation method for cacao trees when subsoil layers are not rich in Cd. Otherwise, the use of amendments capable to reach deeper soil layers is recommended.

Nederlandse samenvatting

Cadmium is een toxisch spoormetaal dat kan accumuleren in eetbare delen van een plant. Recente studies tonen aan dat in cacaobonen uit Ecuador hoge Cd concentraties (gemiddeld 0.9 mg Cd/kg boon) aanwezig zijn. Er is dus een hoge nood om een aanpak te ontwikkelen waarbij de opname van Cd door cacaobomen ingeperkt wordt. Een veelbelovende werkwijze, die traditioneel al veel gebruikt wordt, is het aanbrengen van kalk op de bodem. Het is echter problematisch dat kalk alleen de bovenste oppervlakte laag beïnvloedt, terwijl cacaowortels diepe structuren ontwikkelen. Daarom is het van belang om de rol van de verschillende wortelstructuren (oppervlakte t.o.v. diepere) in de opname van Cd te begrijpen, opdat een geschikte aanpak uitgewerkt kan worden om de opname van Cd te verlagen.

Daarom werd een pot experiment opgesteld te Guayaquil (Universiteit van ESPOL) met cacao zaailingen. Het stabiel isotoop ^{108}Cd diende als merker om de bijdrage van de twee bodem compartimenten aan de opname van Cd te meten. In de potten werden twee bodem horizonten aangebracht. Deze verschilden in biobeschikbaar Cd door bekalking of een verschillend bodemtype (oppervlakte bodem: 0-15 cm, pH 5.2, 1.38 mg Cd/kg, onderliggende bodem: 15-30 cm, pH 5.5, 0.77 mg Cd/kg). Drie bodemverbeteraars werden getest: kalk ($CaCO_3$), magnesium oxide (MgO) en gips ($CaSO_4$). Het experiment bestond in totaal uit 24 behandelingen met drie herhalingen. Na vier maanden van groei, werden de zaailingen geoogst en de concentraties aan ^{108}Cd en ^{111}Cd bepaald met de ICP-MS.

Het onderzoek wees uit dat de verticale heterogeniteit van de bodem in biobeschikbaar Cd en het bekalken van de bodems inderdaad een effect had op de opname van Cd. De homogeen verdeelde potten toonden aan dat de concentraties aan Cd in de bladeren verklaard konden worden door de bodem Cd concentraties. De pot met alleen onderliggende bodem bedroeg ongeveer de helft aan Cd t.o.v. de oppervlakte bodem, wat leidde tot een halvering van de Cd concentratie in het cacao blad. Wanneer de potten de heterogene bodems bevatten (oppervlakte op onderliggende bodem), was de reductie aan Cd concentraties minder dan verwacht werd op basis van de bodemconcentraties. In dit geval, kon de studie met het stabiele isotoop verklaren dat er 18% meer Cd van de bodemlaag werd opgenomen t.o.v. de homogene behandelingen. Dit kan aantonen dat de cacaowortels liever in de nutriëntrijke laag vertoeven, waardoor ze hier actiever Cd opnemen. Het bekalken van beide bodemlagen zorgde voor een reductie in Cd concentratie van 42%, terwijl het bekalken van de toplaag slechts voor een reductie van 16% zorgde. In dit geval, kwam het grootste aandeel in de opname van Cd uit de onderste laag (64%). Dit legt de dynamica van het wortelsysteem bloot. Hierbij is het mogelijk dat de immobilisatie van Cd tegengewerkt wordt doordat er meer Cd uit de onderliggende lagen opgenomen wordt wanneer de toplaag bekalkt wordt. In de potten met de verschillende bodemlagen verminderde Cd in het blad van de zaailing met 40% wanneer de toplaag bekalkt werd. Voor de andere bodemverbeteraars (gips en magnesium oxide) werd een soortgelijk resultaat vastgesteld.

Kortom, het pleiten voor het bekalken van de bodem om Cd in cacao planten te verminderen is gepast wanneer de onderliggende lagen arm zijn aan Cd. Anders wordt het gebruik van bodembehandelingen aangeraden die meer mobiel zijn en diepere lagen kunnen bereiken.

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Abbreviations

ANOVA	One-way Analysis of Variance
Cd	Cadmium
CEC	Cation Exchange Capacity
d.w.	Dry Weight
DOM	Dissolved Organic Matter
ECEC	Effective Cation Exchange Capacity
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization
IARC	International Agency for Research on Cancer
ICP-MS	Inductively Coupled Plasma Mass Spectroscopy
IA	Isotopic Abundance
IR	Isotopic Ratio
K	Equilibrium Constant
LOQ	Limit of Quantification
OC	Organic Carbon
TDI	Tolerable Daily Intake
TF	Transfer Factor
TWI	Tolerable Weekly Intake

Symbols

L	Surface soil with lime ($CaCO_3$)
L-$CaSO_4$	Surface soil with gypsum ($CaSO_4$)
L-MgO	Surface soil with MgO
NL	Surface soil without amendment
NLB	Subsoil without amendment
*	Spiked soil with ^{108}Cd

Chapter 1

Context and objectives

1.1 Context

Europeans love chocolate. Covering only 6% of the world's populations, Europeans manage to lead the competition of biggest chocolate consumers (about half of the world's chocolate consumption is on their count). Recently, concerns have arisen regarding this consumption of chocolate. Evidence exists of the presence of cadmium (classified as a human carcinogen) in chocolate. To address this health issue, the European Union and the Codex Alimentarius imposed new maximum levels for cadmium in chocolate.

The domestic cacao production in Europe is nonexistent, thus the European Union relies on Latin America, Asia and Africa for the import of cacao. The young volcanic soils from Latin America are naturally enriched in cadmium (Cd), therefore a big part of their beans may not be exported to Europe due to the regulation of Cd in chocolate. Hence, great anxiety exists about the socio-economic impacts of these regulations on the resource-poor small-scale cacao farmers. As a consequence, the research for mitigation methods to reduce Cd concentrations in cacao is on the fast track.

The application of lime is generally advocated as the most promising mitigation method to reduce the bioavailability of Cd in soils (total soil Cd, pH and organic carbon content are the most important factors controlling Cd bioavailability). However, from previous research in field experiments on crops with deep-penetrating roots like cacao, the application of lime does not consistently affect Cd uptake. This is in contrast with pot experiments where liming undoubtedly reduces the uptake of Cd. The discrepancy between the field and pot experiments might be related to the vertical heterogeneity of the soils in the field where the surface soil contains higher bioavailable Cd than the subsoil. Liming may affect the root activity of cacao trees which possibly counteracts the targeted Cd immobilization by increment of Cd uptake from the subsoil layers. Since cacao is a perennial crop with deep-rooted soil horizons it is important to understand the functionality of root structures on Cd uptake. Hence, the purpose of this research was to determine the role of the different rooted soil compartments (surface versus deep) and bioavailable Cd on the uptake of Cd by cacao seedlings. This better understanding would allow the development of more effective mitigation practices. Therefore, the following research question was formulated.

> What is the role of the root distribution and bioavailable Cd on the uptake of Cd in cacao seedlings?

1.2 Objectives

To answer the research question, following objectives were appointed:

- To quantify the contribution of rooted soil compartments (top- or bottom soil) on the concentration of Cd in cacao leaves as affected by liming and superimposed soils with different bioavailable Cd
- To determine the effect of different amendments (lime ($CaCO_3$), magnesium oxide (MgO) and gypsum ($CaSO_4$)) on the uptake of Cd in cacao

A novel and original approach was used to quantify the contribution of the rooted soil compartments in a pot experiment with cacao seedlings. The stable isotope ^{108}Cd functioned as a tracer to determine from which 'compartment' the Cd in the cacao plant was originating. Different liming amendments ($CaCO_3$, MgO and $CaSO_4$) were tested in the search of the most appropriate mitigation method.

1.3 Content of the thesis

First, an extensive literature review will take the reader throughout the knowledge regarding Cd, with a focus on the environmental chemistry of Cd. Also, the agronomic properties of cacao are described, together with a brief summary of the literature involving the interaction between Cd and cacao. The literature review ends with some examples of previous studies that used isotopes of Cd to research the uptake of Cd by crops. The following materials and methods section introduces the practicalities of the pot experiment and the calculations performed with stable isotopes. The corresponding chemical and statistical analyses are as well described, which are indispensable in the understanding of this research. The probably most interesting chapter, results and discussion, will present and discuss our own research with the most important results. The focus lies on the concentrations of Cd in the leaves for the different treatments. The stable isotope study indicates the contribution of the two soil compartments to these leaf Cd concentrations, allowing us to analyze the Cd uptake patterns for cacao seedlings. Finally, we end with the conclusion where the key findings, the relevance of this research and the outlook to the future are described. In appendix A, a side-experiment is described that was performed out of curiosity, attributing to a better chemical understanding of the gypsum amendment used in the pot experiment.

Chapter 2

Literature review

Cadmium has been extremely studied over the last decades, hence the literature about Cd is numerous. Therefore, in this literature review, the emphasis is on the environmental chemistry of Cd and its relation with agricultural crops, like cacao. The focus in this literature review is put on recent publications. The gaps in the knowledge in this particular field, as well as the cutting edge results from the research, are as well covered to ensure a good understanding of Cd and its relation to cacao.

2.1 Cadmium (Cd)

2.1.1 Chemical properties of Cd

Cadmium (atomic number 48) is a heavy, non-essential, toxic metal, located in group 12 in the Periodic System of the Chemical Elements between zinc (Zn) and mercury (Hg) [82]. The abundant speciation of Cd in the environment is Cd^{2+} with a completely filled d-shell (like Pd^0) as electronic configuration, resulting in a flexible coordination and an absence of redox chemistry. Following the HSAB (Hard-soft-Lewis acid-base) principle, Cd is a soft Lewis acid [63] (Cd has an electron cloud that is polarizable and an intermediate ionic radius (0.95 Å) [81]). Hence, Cd can participate in covalent binding with surfaces and form inner-sphere complexes with high selectivity. The element has a higher affinity for sulfur ligands than Ca

since Cd^{2+} is more thiophilic than Ca^{2+} (a hard cation). As a consequence, Cd will form less stable complexes with oxygen and nitrogen ligands. Therefore, the biochemistry of Cd involves strong complexation with proteins and molecules like glutathione, which have sulfur ligands [81]. Since Cd is a non-essential metal, this creates the toxic nature of Cd.

2.1.2 Chemistry of Cd in soils

The origin of Cd in soils

Cadmium is naturally present in soils. The ambient Cd concentration in soils (away from point sources) is typically 0.10-1 mg/kg dry weight (dw) and is a function of the parent material, the localization and the land use of the soil [82, 27, 43]. Cadmium originates mainly from Zn minerals (e.g. sphalerite (ZnS)) [43]. Concentrations of Cd in agricultural soils have generally risen due to anthropogenic activities like the use of phosphate fertilizers, application of sewage sludge on soils and atmospheric pollution [28, 82]. The retention times for Cd in the upper soil layers are roughly around hundreds of years. Hence, the fluxes of Cd out of the topsoil are small compared to the Cd content in the soil [63]. A recent (2018) stable isotope study in agricultural fields reveals that the Cd mass balances depend on the crop type grown and fertilizer Cd concentrations. The study concludes that the mass balances are stabilized, indicating that Cd will not further accumulate in soils if correct limits of Cd in fertilizers are applied [50].

The fate of Cd in soils

Trace elements, like Cd, are distributed over different soil compartments [43]:

- the soil solution (aqueous species, complexes, suspended colloids)
- the soil solid phase (weakly adsorbed to clays, strongly adsorbed to Fe, Mn, and Al oxides, sorption with organic matter, sparingly soluble precipitates).

Cadmium in the soil solution phase

In soil solution, the significant part of Cd occurs as the free-hydrated cation. The remainder forms complexes (with inorganic and organic ligands) or binds to suspended colloids, enhancing the mobility of Cd in soils. For instance, in saline soils, the Cd solubility is increased because of the presence of chlorides (Cl^-), forming complexes with Cd ($CdCl^+$ and $CdCl_2{}^0$). These complexes can increase transport to plant roots and may be readily taken up by plants [82, 43, 62]. In soils with high organic matter content, dissolved organic matter (DOM) complexes can play a major role in the solubility and mobility of Cd [63].

The fraction of Cd in soil solution represents only a small fraction of the total Cd. Generally speaking, over 99% of the Cd content is present in the soil solid phase whilst less than 1% is found in the soil solution. Usually, the concentrations of total dissolved Cd in the soil solution of natural soils is less than 50 nM [63]. Although the fraction in solution is minimal, the importance of the soil solution must be stressed, since the soil solution is the medium of contact between the soil reservoir and the plant root [57]. Soil extractions with dilute neutral salts (e.g.$CaCl_2$, NH_4OAc and NH_4NO_3) can be used as surrogates to estimate concentrations of Cd in pore water. They can mimic soil solution and are unlikely to extract non-labile or fixed Cd [25, 98, 64].

Cadmium in the soil solid phase

In the soil solid phase, the speciation of Cd is determined by sorption, precipitation and occlusion reactions in minerals. There is little evidence that Cd minerals are important solid phases in agricultural soils or in most contaminated soils [63]. Precipitation only controls Cd solubility at high Cd concentrations (in contaminated areas) in combination with alkaline soils (pH $>$ 7) [18].

Sorption is the most important factor controlling solution Cd. Sorption strength is larger at low Cd concentrations than at high concentrations (as expected according

to the concept of site-heterogeneity). A survey on agricultural soils (n=3045) in the United States in 1993 by Holgrem et al. reported a positive correlation between Cd concentrations, soil pH and organic carbon (OC) content when pH < 7. At pH > 7, there was a less significant correlation between Cd and OC [42]. Speciation modeling predicts that in acid soils (pH < 6.5) most Cd is adsorbed to organic matter whilst in soils with pH > 6.5 the Cd is associated with Fe-oxides [13]. Naidu et al. concluded after reviewing different studies about Cd sorption that key factors controlling adsorption are soil organic matter, nature of constituent minerals and composition of the ambient soil solution which is all related to the particle surface charge density [67]. The most important factors and their mechanisms are shortly discussed in next paragraphs.

1) Effect of soil pH on sorption

The acidity of the soil has the most pronounced effect on Cd sorption in most soils (per unit pH increase, the sorption enhances 3-5 times) [18, 26]. Protons are the main competitors for Cd^{2+}sorption on carboxylic and phenolic groups in organic polymers, O and OH groups in oxide surfaces or edge faces of the kaolinite crystal (reaction 2.1) [82, 93]. Increasing soil pH leads to an increase in net negative surface charge which contributes to an enhanced affinity for Cd [67]. It is important to keep in mind that the actual effect of pH depends on the specific soil and the pH range [63].

$$\mathrm{S{-}OH} + \mathrm{Cd^{2+}} = \mathrm{S{-}OCd^{+}} + \mathrm{H^{+}} \tag{2.1}$$

2) Effect of organic matter on sorption

In organic matter, the ion binding sites are the surface oxygen atoms of carboxylic or phenolic groups of humic and fulvic acids. Organic constituents in the soil play a dual role in Cd speciation [63]. The low-molecular-weight components can serve as carriers of Cd in soil solution whilst the high-molecular-weight components serve as a sink for Cd sorption.

3) Effect of Fe, Al and Mn hydroxides on sorption

The Fe, Al and Mn oxyhydroxides have a surface hydroxyl group to bind cations. The Cd-O bound is substantially covalent, resulting in the specific adsorption of Cd when liberating H^+ from surface metal OH groups [63].

$$\mathrm{Al{-}OH} + \mathrm{Cd^{2+}} = \mathrm{Al{-}OCd^{+}} + \mathrm{H^{+}} \tag{2.2a}$$

$$2\,\mathrm{Al{-}O} + \mathrm{Cd^{2+}} = \mathrm{Al{-}OCdO{-}Al} + 2\,\mathrm{H^{+}} \tag{2.2b}$$

4) Effect of layer silicates on sorption

The crystal structure of clay minerals (or layer silicates) arises due to the sharing of oxygen atoms between contiguous silica and alumina sheets. For smectites and vermiculites, isomorphic substitution in the crystal results in a permanent negative charge of these minerals [93]. For kaolinite, the charge occurs due to lattice imperfection, exposed structural hydroxyl groups, and broken bonds at edges of particles [63]. The binding of Cd with kaolinite is mainly specific whilst for smectite and vermiculite it is non-specific. Clay minerals typically do not play a big role in sorption of Cd^{2+}. At relevant conditions the selectivity for Cd:Ca selectivity is less than five hence the competition of Ca^{2+} ions is too large [35, 82]. Nevertheless, in soils with low to moderate pH and little organic matter, clay is an important adsorbent for Cd [13].

Cd sorption on variable charge soils

The relation between Cd sorption and soil characteristics has been researched mainly for soils in temperate regions. However, the findings for one geographical region may not apply to other regions. The soils in the tropics are mainly dominated by Oxisols. They typically consist out of low-activity sesquioxide minerals and clays with variable charge surfaces, being highly weathered due to high-intensity rainfall and high temperatures [67]. As a consequence, they adsorb less Cd than temperate soils because of the high surface positive charge and lower net surface negative charge density.

In temperate soils, an increase in ionic strength results in a decrease of Cd sorption. However, in variable charge soils, there is a characteristic pH (point of net zero charge (PZC)) above which Cd sorption decreases with an increase in ionic strength, but below which the reverse occurs [66]. In permanent charge soils, the competition effect of the index cations is the main driver behind the decrease in Cd sorption when increasing ionic strength. For variable charge soils, there is an effect of ionic charge on the double-layer thickness which adjusts the surface-charge characteristics of the soil [67]. At pH values below the PZC, a rise of ionic strength results in a less positive potential in the plane of adsorption which consequently increases Cd sorption. This hypothesis suggests that Cd sorption occurs by specific and non-specific interactions [67].

2.1.3 Toxicology of Cd

Cadmium interferes with numerous transporters, signaling molecules and metalloproteins. One of the hypotheses for the toxicity is that Cd interferes with the cellular Zn homeostasis. The log K (equilibrium constant) values of cysteine (9.2 for Zn^{2+} and 11.0 for Cd^{2+}) indicate that elevated Cd concentrations in organisms can replace Zn in enzymes with S ligands [81]. Especially when Zn deficiency occurs, this mechanism will come to expression. The mechanism blocks critical biological pathways, leading to inhibition of DNA repair, effects on gene expression and endocrine dysfunction according to the European Food Safety Authority (EFSA) [28].

Environmental toxicology

Although Cd is mostly studied in soils, Cd also occurs in aquatic systems. It infiltrates in rivers and lakes by weathering and erosion of Cd containing minerals as well as by the discharge of industrial and municipal effluent [28]. The ambient air Cd concentrations are fairly low. Only around areas of industrial point sources, the influence of atmospheric deposition is proven [82].

The literature about the environmental toxicology of Cd is rather moderate, since the research in this field is often challenging. Moreover, the concentrations of Cd which cause toxicity on soil dwelling biota are commonly higher than the concentrations affecting humans [82]. Species like grasses, food crops, earthworms, poultry, cattle, horses, and wildlife seem capable to accumulate Cd [28]. Soil biota and plants can thus have Cd concentrations that are already a risk for mammals whilst showing no physical disorders. Hence, the greatest risk of Cd exists in the accumulation in the food chain. The study of the effects of soil Cd to mammals and birds via the food chain is challenging, however, some tissue Cd concentrations suggest risk to moles, shrews and beavers in contaminated sites [27].

Human toxicology

Cadmium is classified as *carcinogenic to humans* (Group 1) (International Agency for Research on Cancer (IARC)) [48], being associated with increased risk of cancer in bladder, lung, pancreas, endometrium, and breast. The biological half-life of Cd is 20 to 30 years, accumulating in the organs (particularly in the kidneys). The toxicity of Cd is thus more of a chronic than an acute nature since the doses for acute Cd intoxication rarely occur. Hence, the lifetime exposure is the best indicator to estimate the effects of Cd [82]. The kidney in humans is the main target organ where Cd causes cell dysfunction [81]. However, also the liver, respiratory system, and bones can be affected. An example of a fatal Cd intoxication is the *itai-itai* disease, with symptons as osteomalacia, osteoporosis, an increase of bone fractures, and renal tubular dysfunction [3]. Around 1950 the *itai-itai* disease manifested in Japan due to the consumption of rice that was irrigated with Cd contaminated water from a Zn mining area [70].

For non-smoking humans, the main source of Cd is originating from foodstuffs [28]. Food items with relatively higher concentrations of Cd are shellfish, offal, grains, and seed. EFSA suggested in 2011 a tolerable weekly intake (TWI) of 2.5 µg/kg

body weight (resulting in a tolerable daily intake (TDI) of 0.36 µg/kg b.w.). For a person weighing 60 kg, this is a tolerable intake of 21.6 µg/day which is close to the average dietary intake for an adult (8-20 µg/day). As a consequence, there is a fine line between the average Cd consumption and the tolerable intake of the human body. Four subgroups (vegetarians, children, people living in Cd-contaminated areas and smokers) are even found to be two times above the TWI of 2.5 µg/kg b.w. [74].

EU legislation

Based on the health impacts on humans the European Union reviewed existing maximum levels of Cd in foodstuff. The review focused especially at the protection of infants and young children. Chocolate was targeted by these new maximum levels. Three maximum levels in chocolate were set (Table 2.1) and entered into force on 1 January 2019. The strictest levels are imposed on chocolates that are most consumed by children (like milk chocolate) [29]. The corresponding threshold for the concentrations of Cd in cacao beans to meet the regulations in the final product is estimated at 0.6 mg Cd/kg dry weight [16].

Table 2.1: Maximum Cd concentrations (mg/kg wet weight) on specific cocoa and chocolate products (1 January 2019) [29]

Description	mg Cd/kg
Milk chocolate with <30 % total dry cocoa solids	0.10
Chocolate with <50 % total dry cocoa solids; milk chocolate with ≥ 30 % total dry cocoa solids	0.30
Chocolate with ≥ 50 % total dry cocoa solids	0.80
Cocoa powder sold to the final consumer or as an ingredient in sweetened cocoa powder sold to the final consumer (drinking chocolate)	0.60

In July 2018 the commission of the *Codex Alimentarius* of the Food and Agriculture Organization of the United Nations (FAO) has set as well limits for Cd in different types of chocolate (0.8 or 0.9 mg/kg of chocolate, depending on the cocoa content) [21].

Need for mitigation strategies to reduce Cd levels in cacao

Bertoldi (2016) did a fingerprint analysis to report the content of 56 macro-, micro- and trace- elements of 61 cacao beans in 12 countries of East and West Africa, Asia and Central and South America. In South America, the concentrations of Cd in cacao beans were about three to four times larger than those from East and West Africa. The analysis suggests that these elevated Cd concentrations are unrelated to point pollution, but are from geogenic origin [9]. To manage to satisfy the international trade restrictions on Cd levels in cacao, it is of striking importance to investigate mitigation practices to reduce Cd concentrations in cacao beans, especially for Latin America.

2.2 The crop cacao: the food of the Gods

Theobroma cacao L. delivers its scientific name from the Greek words for Gods (theos) and food (broma). Cacao is a perennial tropical tree of the family *Sterculiaceae (Malvacear sensu lato*), order *Malvales* [11]. The 'cacao belt' lies between latitudes 10°S and 20°N. The climate in this belt satisfies the conditions to grow the cacao tree: i) rainfall of 1250-3000 mm per annum with a dry season of no more than three months with less than 100 mm rain per month ii) maximum and minimum temperatures varying between 30-32°C and 18-21°C iii) no persistent strong winds [95]. Full-sunlight monocultures and agroforestry systems are the most relevant farming methods for cacao trees. In agroforestry systems palm, timber and fruit trees are grown together with the cacao trees [38, 80].

The seeds of *Theobroma cacao* L., normally called beans, are the basis for chocolate production. They contain carbohydrates, fats, proteins, natural minerals, flavonoids,

and vitamins. The main varieties of *T. cacao.* are: Criollo, Forastero, Trinitario and Nacional [1]. Forastero is the most produced and is used for basic cacao products. Nacional is known for its fine flavor. Criollo is mostly affected by diseases and therefore not cultivated on a big scale. Trinitario is a hybrid of Forastero and Criollo.

Economic relevance of cacao in Ecuador

Most cacao is produced by Ivory Coast, Ghana, Indonesia, Brazil, Nigeria, Cameroon and Ecuador (95% of total production). According to the Quarterly Bulletin of Cocoa Statistics of the International Cocoa Organization (ICCO), 4.7 million tonnes of cacao beans were globally produced in the year 2016/2017. In Ecuador, cacao is the most planted crop (area of 434418 ha) of the country [75]. Ecuador exported in 2017 cacao for a value of 688.98 million US dollars. Together with the cultivation of bananas and coffee the share of cacao for the gross domestic product (GDP) of Ecuador was 1.86% in 2016 [22]. Ecuador is especially known for the cultivation of the Nacional variety with its special flavor and aroma, giving the chocolate a high value in the chocolate industry [15]. Because of the susceptibility of Nacional to diseases (especially the witches' broom disease, frosty pod or moniliasis disease and mal de machete or ceratocystis wilt disease) the cultivar is substituted more and more by higher yielding cultivars [11]. The CCN-51 (*Coleccion Castro Naranjal*) is such a highly productive cultivar. However, it does not meet the qualification of fine-flavored beans. In 2014, CCN-51 was planted in Ecuador for an area of 80 000 ha. Most production of cacao is in the hands of small-scale farmers [33]. As a consequence, a big social impact on these Ecuadorian cacao farmers is expected due to the new regulations concerning Cd in cacao.

2.2.1 Cacao: uptake and translocation of Cd

Cadmium is a non-essential element for plants and has no beneficial effects, but when Cd is available in soils, plants tend to take it up [63]. Recent studies showed that cacao can be termed as a Cd accumulator, suggesting that the potential uptake

of Cd by cacao is large [39, 5, 16]. Therefore, in next section, the different factors controlling the concentrations of Cd in general in crops are mentioned. Afterwards, these generalities are reflected upon the studies that involve the uptake of Cd by the cacao crop.

Soil factors controlling uptake of Cd in crops

In section 2.1.2 the different parameters controlling the bioavailability of Cd in soils are mentioned (total Cd, pH, % organic matter, chloride content,...). These factors are as well important measures for crop Cd concentrations since the Cd bioavailability determines among other things Cd uptake by crops. However, a study of plant metal concentrations and speciation of metals in soils showed that pore water concentrations did not explain the crop Cd concentrations [17]. This could be due to the fact that other species than Cd^{2+} (like chloro-Cd complexes) can possibly be taken up by plants and most models are based on the free ion activities, biasing the uptake by plants [45]. Other processes which are possibly clarifying this surprising result are rhizosphere processes and ion competition effects. Rhizosphere processes alter the pore water competition compared to the sampled solution, whilst competition effects change the uptake rate of the free ion [82]. Soil solution characteristics evolve during rhizosphere development and affect the bioavailability of Cd, complicating an assessment of the bioavailability of Cd for crops [57].

Generally, it is found that crop Cd concentrations increase with increasing total Cd and with decreasing pH and with decreasing % OM. Therefore, Freundlich type functions are often used to relate the Cd concentration in the crop (mg/kg) with the Cd content in the soil (mg/kg), pH and % OM. [31, 82].

$$\log(Cd_{plant}) = a + b * \log(CdT_{soil}) + c * pH + d * \log(\%OM) \tag{2.3}$$

Additionally, increasing salinity is found to be strongly and positively associated with the accumulation of Cd in potatoes and durum wheat in saline soils [62, 71]. There is clear evidence that the uptake of Cd^{2+} occurs through Ca^{2+}, Fe^{2+}, and Zn^{2+} trans-

porters/channels of low specificity [19]. Therefore, some of these metals can inhibit Cd uptake from the rhizospheric solution by competing at the binding sites [36]. A study about the relationship between Zn and Cd in spinach and lettuce demonstrates that accumulation of Cd in young leaves decreases exponentially by increasing Zn in the soil solution. However, in sites with high contamination of Cd and Zn no effect was revealed since only an antagonistic effect was reported at low solution Cd [60].

Based on previous findings, mitigation strategies to reduce Cd content in crops were developed based on soil liming (increasing soil pH), organic matter amendment (increasing sorption) and Zn fertilization (increasing competition on the transmembrane carriers) [82]. These mitigation strategies have inconsistent results, varying with type of soil or crop, indicating that mitigation strategies should be advocated with care and need a case specific approach.

Biotic factors (plant species, crop cultivar, root activity, rooting patterns and rhizosphere root-associated microorganisms,...) are as well essential in the understanding of the uptake of Cd by crops [91, 20]. For instance, a study shows that mycorrhizal root colonization can decrease the uptake of Cd [51]. Additionally, the age of orchard may explain effects on Cd concentrations as shown in poplars [55]. Here, Cd concentration in leaves of the poplars reduced with augmenting age. However, this trend can again be linked to abiotic factors, like growth dilution, change in bioavailable soil Cd, and root distribution over time.

Uptake of Cd by roots

Cadmium absorption in the roots is driven by the electrochemical potential difference between the activity of Cd^{2+} in the cytoplasm and the activity in the root apoplasm [63]. This negative membrane potential is relatively large and produces sufficient energy to drive Cd^{2+} uptake even when the activity of Cd in the apoplasm is low. The most limiting factor in the uptake of the aqueous species of Cd is the

Table 2.2: Biotic and abiotic factors affecting Cd uptake by plants. After McLaughlin et al., 1999 [63]

Biotic factors	Abiotic factors
Plant species	Total soil Cd
Crop cultivar	Soil pH
Plant tissue	Clay content
Leaf age	Metal oxides (Fe, Mn and Al)
Root activity	Redox potential
Rooting pattern	Organic matter
Rhizosphere and root associated microorganisms	Complexing ligands
	Soluble salts
	Soil management practices

diffusion to the root surface absorptive sites [91] (Figure 2.1). As discussed before, the underlying hypotheses when assessing bioavailability of Cd is that the concentration of Cd in pore water is mostly affecting the Cd uptake. However, plant roots excrete substances that can utterly change the chemistry in the soil solution near the root, altering the interionic effects and the speciation of Cd [52, 91]. Complexation of Cd can change the mobility and uptake thoroughly since complexes are also absorbed by roots [63].

Transport of Cd in plants

The existence of Cd into edible portions of crops is related to transport and deposition. Cadmium transport into the organs varies among different crops. Rice and corn, for instance, accumulate little Cd whilst sunflower and flax accumulate more Cd [91]. Cadmium concentrations in grain and potatoes are somewhere in between these extremes but because of the large consumption of these crops, the importance is not negligible. To understand the Cd concentration in these edible plant organs, translocation is studied [82]. In some crops, internal distribution rather than root uptake is causing the variation in Cd accumulation in crops [91].

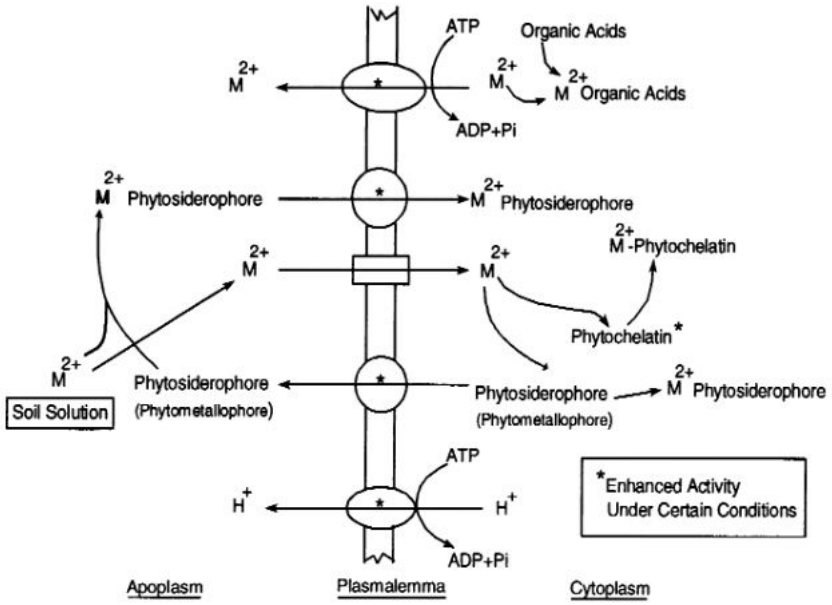


Figure 2.1: Root processes for a heavy metal divalent ion by Welch [63]. Circles indicate membrane transport protein systems; rectangles channel protein; ovals enzyme-activated membrane ion pumps or reductases.

In many plants, translocation of Cd into shoots by the xylem is shown to be a major factor for the accumulation of Cd in shoots [76, 86]. However, for potato, a crop with low rate of transpiration, the use of xylem is reduced and it is shown that Cd is as well rapidly distributed via the phloem to all tissues [78]. This suggests that Cd has high mobility in xylem and phloem and that the stem has an important function in the transfer between these pathways. In xylem sap the most common species of Cd are the inorganic cation (Cd^{2+}) and complexes with organic molecules, whilst in phloem sap almost all Cd^{2+} is complexated (with cysteine and other sulfhydril containing compounds and various organic acids and amino acids) [91]. The mobility of Cd in plants is thus high, Cd is not simply deposited in a tissue but stays recirculating in the plant tissues. This is contrasting with other heavy metals like Pb, Cr and Cu which are immobilized and deposited in the roots [76]. There is some evidence that Cd ions might compete with Zn ions when accumulating in the shoots (i.e. these ions can have the same translocation pathways). For the translocation, mobile binding partners in the cytosol are es-

sential, as well as efflux pumps to transport Cd from the root cells to the xylem. The uptake and sequestration to the cells follow similar pathways as for root cells [19].

Often there are different concentrations found in the different organs of the crop. For instance, in cacao leaves, Cd concentrations are often higher than in the beans [5, 38]. Metal ions are generally transported from roots to leaves through the xylem whilst transport to seeds requires xylem-to-phloem transfer, toughening the transport to the beans since more membranes need to be crossed [20].

Uptake, translocation and accumulation of Cd by cacao

The short-term kinetics of uptake of Cd are comparable to enzyme kinetics (Michaelis-Menten kinetics) where first the uptake increases linearly with higher Cd concentration, until the uptake of Cd by the roots saturates. Most Cd concentrations in soils range within the linear part of this kinetics. Here, transfer factors (also known as bioconcentration factors) between the crop and soil can be defined [82]. A transfer factor is the ratio of Cd in the plant to Cd in the soil. This concept is used in risk assessment to estimate the soil-plant transfer. However, these transfer factors are often fluctuating because of the different abiotic and biotic factors mentioned above (summarised in Table 2.2). Cacao might be classified as a Cd accumulator [5]. The transfer factors from different studies are ranging between 0.13-16.3 [5, 39, 6]. The transfer factors of most fruits/grains are inferior to the TF of cacao.

As with the other crops, uptake of Cd by cacao plants is associated with the mobility of Cd in soils [5]. However, Cd uptake by cacao is not only dependent on soil factors, but also on plant and management factors [38]. Between different cacao cultivars there could, for instance, exist a great variation in Cd uptake [23, 20, 56]. A very recent study (2018) shows strong variations (with a factor 10) between cultivars grown on conditions with compareable bioavailable Cd [56]. Nonetheless, there is a complete knowledge gap on how Cd is transported from the soil to the beans, making

the research on the genetics of Cd accumulation challenging. Moreover, cultivar effects can be related to growth effects, rather than the involvement of a physiological difference in uptake between cultivars [38]. Another recent study shows that cacao trees in agroforestry systems accumulate less leaf Cd than trees in monocultures (because of greater competition with other trees) [38]. A positive correlation between Zn concentrations in beans and Cd concentration in leaves was found in a study in cacao plantations in Peru, indicating a synergism between these elements in cacao plants sampled [4]. However, more research is needed about the synergism of Zn and Cd in cacao, since in other crops (wheat and corn) the interaction between the two metals can vary among different cultivars of the same species [68]. In the study of Argüello et al. [5], agronomic management practices were as well evaluated to explain the bean Cd concentrations in cacao, but none of them were significant except for age of orchard and organic vs. conventional farming.

2.2.2 Root distribution of cacao

The roots of *T. cacao* seedlings develop as a tap root which grows straight down into the ground. Early in the development of cacao plants, lateral roots arise in a collar in the top layer of the soil (0-15 cm from the soil surface). In later growth stages, the structure of the roots is affected by soil, water, and air relations. When soils are poorly drained (high water table) the tap root does not infiltrate the soil further than 45 cm and terminates in a club. The opposite occurs with well-drained soils, where the tap root infiltrates the soil much deeper [95].

In the humid and sub-humid tropics, perennial crops and shade trees are likely to concentrate the fine roots in the topsoil [72]. This is confirmed by a study in a humid tropical agroforestry system where roots of cacao were superficial (since most nutrients were taken from the thick litter layer). In this case, water deficit was extremely rare, hence no deep rooting of the tree was necessary [72]. In Ghana most of the root activity (75%) was located in the top 10 cm, however in India the

root activity was more abundant in the first 57 cm of the soil [54], showing the large variety in rooting systems of cacao.

The literature about the role of these different root structures in *T. cacao* is modest. High rooting density in the topsoil is explained for other crops by the more favorable conditions in the topsoil. The small surface roots are supposed to be more important for nutrient uptake whilst the deeper roots are more involved in the uptake of water and the mechanical stability of the tree. However, nutrient uptake from subsoil can be relevant. Especially when there is low water content in the topsoil, root elongation and nutrient uptake from the subsoil layer is stimulated [59]. When subsoil root activity is preferred for optimal use of nutrients and water, cacao roots have the ability to explore subsoil resources [54].

Much existent root research assumes that all roots have similar functional characteristics. However, there is clear evidence that differences in size, shape, and development of roots result in distinct functional characteristics. For instance, fine roots (diameter <1 mm) embody 90% or more of the total root length. Consequently, they play an important role in the nutrient uptake of the plant and often there are interactions found between these two factors. However, due to their challenging measurement, they are often neglected in studies. For cacao, it was shown that the fine roots became longer and thinner with rising concentrations of nitrate [99].

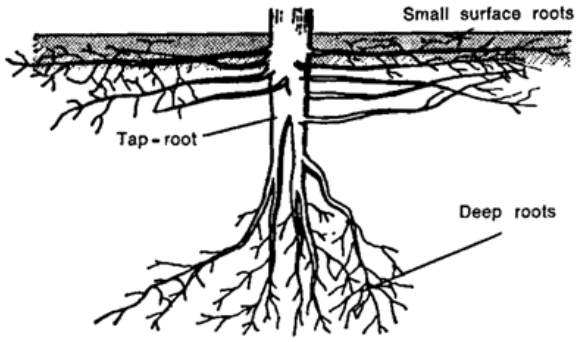


Figure 2.2: Root structure of a cacao tree. Taken from FAO [34].

2.2.3 Nutrient concentrations in cacao leaves

Wessel (1971) studied the nutrient concentrations in cacao leaves, concluding that leaf analysis is advantageous for detecting nutrient deficiencies. The nutrient composition of cacao leaves is highly correlated with light intensity and age of the leaf. When light intensity increases, N and K content decrease, whilst Ca content rises. The age is highly associated with P, K and Ca content of the leaf. A way to avoid the problem of age correlation is to express the concentrations as fresh weight since there was also a correlation with the dry matter content. A summary of nutrient concentrations and the ranges is found in the criteria of nutrient concentrations in Table 2.3. The criteria proposed by Murray correspond probably to leaves of 5-10 weeks old, the criteria of Loué to leaves somewhat older [92, 89].

Table 2.3: Nutrient concentrations in normal and deficient cacao leaves (in % of leaf mass(dw))

Nutrient	**Criteria***			**Criteria****		
	normal	**low**	**deficient**	**normal**	**low**	**deficient**
N	2.35-2.50	1.80-2.00	<1.80	>2.00	1.80-2.00	<1.80
P	>0.18	0.10-0.13	0.08-0.10	>0.30	0.13-0.20	<0.13
K	>1.20	1.00-1.20	<1.00	>2.00	1.20-2.00	<1.20
Ca				>0.40	0.30-0.40	<0.30

Criteria* according to Loué (1961) and criteria** according to Murray (1967) [92].

2.3 Soils in cacao plantations of Ecuador

The mountains of the Andes segregate Ecuador in the Coastal Plane, Highlands, and Amazonia. These natural regions each have a complex system of climate, landscapes, soils, and biodiversity. The soils in Ecuador are visualized in Figure B.2 according to parental material. Most cacao plantations are located in the coastal plane, where 43% of the land is used for the cultivation of tropical crops [32]. Soils in the plain have typically high fertility (typical soils are Andisols in the humid zone and Mollisols in the dryer zone) [32]. Precipitation is the most important factor in the soil formation

of these soils. Three different groups of soils from the coastal plain are i) soils from old rocks (sedimentary, metamorphic or igneous) ii) soils with volcanic ash depositions and iii) alluvial soils [32].

The type of soils in cacao plantations is diverse (clayey highly eroded soils, volcanic sands and silty soils with pH varying between 4 and 7). For optimal growth, cacao needs well drained clay loamy soils with high organic matter content [6]. Soils in cacao plantations of Ecuador commonly have a top layer which accumulates soil organic matter (SOM) [95]. Cacao plantations are often on Alfisols, typically having a subsoil with an argillic horizon where there is a significant accumulation of translocated layer silicate clay [84].

2.3.1 Occurence of Cd in Ecuadorian cacao plantations

Until 2015, not much research took place about the provenance of Cd in the soils of Ecuador. A head start was a study of Chavez et al. (2015) that concluded that Cd is accumulating in the top layer of cacao plantations in South Ecuador. From the surface layer (0-15 cm) to the subsurface layers (15-50 cm) the total recoverable Cd decreased about 42%, while M3-extractable Cd reduced by 78%. The number of sampling sites (n=19) was however rather limited [16].

Barraza et al. conducted a study in 2017 to investigate if oil activities are a source of Cd in cacao and to understand mechanisms of Cd transfer to edible parts of cacao (n=31). The study concluded that the Cd distribution in soils from the Amazon and Pacific coastal regions is associated with natural (soil properties, geochemical background conditions) and anthropogenic (agricultural uses) factors. The agricultural crop management practices include the use of fertilizers and pesticides, and the decomposition of leaves and pods on the soils in cacao plantations. In the topsoil layer the Cd concentrations were again higher than in the bottom soil layers. Half of the beans sampled in this survey had a higher Cd content than 0.8 mg/kg [6].

A nationwide survey (n=560) of soil-bean samples was conducted by Argüello et al. in 2019. The highest concentrations of Cd in soil were located in alluvial soils originating from sedimentary material (Mollisols, Entisols, Inceptisols; 0.41-0.67 mg/kg) [5]. These soils also corresponded with the plantations where the beans with highest Cd concentrations were grown. Although the average concentrations measured in the soils were within ranges for non-contaminated soils, up to 45% of the beans sampled had a higher concentration than the selected threshold of 0.6 mg/kg (see section 2.1.3). This high concentration is associated with the large uptake potential of cacao combined with soil factors [5].

All these studies suggest that concentrations of Cd are higher in the top layer than in the bottom layer of the soils. Hereafter, it seems necessary to have a closer look at the properties of surface and subsurface soils and determine their relations with the uptake of Cd by cacao.

2.3.2 Properties of surface soils and subsurface soils and the relation with Cd

Agricultural practices (e.g. fertilizing, liming, applying of organic matter, ...) modify the upper 12-18 cm of the soil. This altered soil part is often referred to as the surface soil or topsoil, being the primary zone for root development of crop plants. Generally, it contains most nutrients and water moisture [12]. Concentrations of heavy metals in soils are usually measured in this top layer [88].

However, plant roots also penetrate into the subsoil, the layer under the topsoil. Commonly, this layer is not affected by physical agricultural practices like soil tillage. Nonetheless, this layer is as well relevant as a reservoir of moisture and nutrients (and heavy metals) [12].

Gramlich et al. (2018) reported higher Cd concentrations in the topsoils (0.25 ± 0.02 mg/kg) than the subsoils (0.16 ± 0.01 mg/kg) of cacao plantations in Honduras [39]. The hypothesis from Chavez (2015) that Cd is accumulating in the top layer of cacao plantations is hence confirmed by other studies [16]. One hypothesis, confirmed by a study with stable isotopes, is that plants cycle Cd from the subsoil layers to the upper soil layers [50]. The accumulation may be a result of leaf litter accumulating Cd since litter decomposition is contributing to the mineral compositions of topsoils in cacao plantations [77].

Van Lune (1997) found that for most crops studied Cd showed a linear increase with increasing depth of Cd addition, illustrating that most crops are indeed taking up Cd from subsoil layers [88]. For cacao it is unclear what the source of Cd in the tree is. However, to find suitable mitigation methods to reduce Cd in cacao beans, this needs to be investigated. If cacao is taking up more Cd from the subsoil layers, then liming of only topsoil layers might not be an adequate strategy to reduce Cd concentrations.

2.3.3 Mitigation strategies to reduce crop Cd concentrations

It is necessary to develop mitigation strategies to reduce the concentration of Cd in cacao beans under the threshold of 0.6 mg/kg. As mentioned before, decreasing the bioavailability of Cd is a possibility to achieve this goal. The main factors controlling soil solution Cd in cacao plantations are total soil Cd, soil pH, soil organic matter [5].

Change of soil acidity by agricultural limes

Adding carbonates, oxides or hydroxides of calcium and magnesium decreases the soil acidity [12]. These compounds are referred to as agricultural limes. In soils these compounds react with water to form OH^- and $HCO_3{}^-$ ions . The quantity of lime needed is determined by i) the required pH, ii) the buffer capacity of the soil iii) the chemical composition of liming materials, and iv) the coarseness of the lime [12, 93].

When lime is applied to a moist soil, it slowly dissolves because of hydrolysis by the following reaction [93]:

$$CaCO_3 + 2\ H_2O \longrightarrow Ca(OH)_2 + H_2CO_3$$

The strong base $Ca(OH)_2$ reacts with CO_2 and forms $Ca(HCO_3)_2$, resulting in a net reaction of:

$$CaCO_3 + H_2O + CO_2 \longrightarrow Ca(HCO_3)_2 + H_2CO_3$$

For the MgO-application, the same reactions occur resulting in following net reaction:

$$MgO + H_2O + CO_2 \longrightarrow Mg(HCO_3)_2$$

Soil pH is generally the primary statistical factor explaining Cd in soil solution [10, 26, 82]. Sorption studies show a factor 3-5 stronger sorption of Cd in soils per unit pH increase [18, 26]. The research indicating reductions in Cd phyto-availability by increment of pH mainly result from pot experiments [40]. However, in the field the results are less consistent, i.e. increasing pH does not always decrease uptake of Cd by crops [31]. A hypothesis suggested is the mechanism of deep-root absorption of Cd from soil layers below the limed top layer, where the bioavailability of Cd is not affected [40, 7]. This is particularly of interest for cacao trees since these perennial crops have deep rooting systems. Other speculated reasons are i) that concentrations of metals in plants grown in pots in a greenhouse are higher than in plants grown on the field [24], ii) that the use of other quality of irrigation water under experimental conditions can have a different effect on the mobility of Cd in the soils [62], iii) that the uptake of trace elements like Zn is also lowered when the pH of the soil increases, which can enhance the uptake of Cd [82], or iv) that because of ineffective mixing in the field the lime is not reacting well with the soil as is shown in the studies of next paragraph.

Following studies even indicated an increase in the uptake of Cd with rising soil pH. A study by Maier et al. (1997) showed that under controlled greenhouse conditions, liming was decreasing Cd concentrations in tubers of potatoes. However, in the field there was either no effect or even increased Cd concentrations. The reason suggested were the ineffective mixing of lime, or the competitive desorption of Cd by Ca (as also confirmed by Christensen in another study) [58, 18]. Sparrow et al. (1997) also studied the effects of lime on Cd uptake in potatoes and carrots, but on Tasmanian ferrosols, and concluded as well that deep mixing of the lime is needed to reduce Cd concentrations in the crops [83]. A general explanation therefore might be that these soils have low affinity for Cd and the pH is causing minimal change in the Cd retention by the soil surfaces [40].

For optimal growth of cacao, soil pH ranges preferably between 6-7.5 [95]. Nonetheless, there are also cacao plantations on more acid soils [89]. A recent field experiment in Trinidad and Tobago by Ramtahal et al. (2018) researched the effect of liming on the availability of Cd in soils and its uptake in cacao. Hydrated lime ($Ca(OH)_2$ was mixed with sieved soil and this was applied on a selected experimental site. The soil pH in the experimental field was altered in the lime treatments from 4.8 to 5.1 after eighteen months. Not only the Cd concentrations in the leaves decreased, but in the control (untreated) treatment as well. The rate of decline was however three times stronger for the limed treatments. The liming caused a decrease from around 5.5 total nitric extractable leaf-Cd µg/g to 3 µg/g after eighteen months. The study is however a small-scale preliminary study (n=12 trees) to develop a full-scale research experiment [77].

Effect of liming on the root architecture

Dose and source of liming can have an effect on root anatomy. A study on willows showed that liming the soil can ameliorate the anatomy of roots in weakly acidic contaminated soils. There was, however, only a positive effect found at a low dose

(7.3 g lime/kg soil). A suggestion for the deterioration of the roots at higher dose is that of beginning of phytotoxic effects on roots connected with formation of hydroxides [90]. Acidity can restrict root growth in the subsoil. In pecan seedlings, liming of the subsoil (from pH 5.1 to 6.5) increased the growth of the taproot with 64% in the subsoil [54].

Application of gypsum

As discussed before, the liming of the soil often implies mixing with the soil to get an effective reduction of Cd in the crops. Nevertheless, the mixing is labor-intensive and difficult on plantations with perennial trees like cacao. Therefore, no-till systems, like the application of gypsum on soils, are an interesting pathway in the search of a sustainable strategy to reduce Cd in cacao.

Gypsum ($CaSO_4$) is a traditional soil amendment (often being reused industrial waste). It is applied on the soil surface and can leach to acidic subsoils, where it can improve the root growth and the absorption of water and nutrients by the roots [84, 14]. Gypsum does not affect soil pH since it is unable to consume protons (H^+), therefore it cannot be classified as a liming material. It is more soluble than $CaCO_3$ ($pK_{sp} = 8.48$) and can consequently reach deeper soil layers [49, 93].

$$CaSO_4 \cdot 2\,H_2O \longrightarrow Ca^{2+} + {SO_4}^{2-} + 2\ H_2O \ (pK_{sp} = 4.6)$$

Nevertheless, gypsum can enhance pH in subsoil layers by replacing OH^- on Fe and Al hydrated oxides with ${SO_4}^{2-}$ [49]. Gypsum is as well beneficial to reduce Al^{3+} toxicity in soils, by forming ${AlSO_4}^+$ species [37]. Exchangeable Ca also increases all over the soil profile when applying gypsum [49].

Amending the soil with gypsum can increase the retention of Cd in the soil, by increasing the negative charge on soil surfaces, especially in variable charge soils where Fe-and Al hydrated oxides dominate as surface charges. Also, Al-hydroxy polymers

can form in gypsum-amended soils which are promoting retention of Cd [44, 49]. However, noting that the availability to crops of $CdSO_4{}^0$ is similar to the availability of Cd^{2+}, the application of gypsum can also increase Cd uptake by plants since SO_4 treatments increase the activity of $CdSO_4{}^0$ in soil solution and Cd may be displaced from adsorption sites by Ca [63, 61].

A study by Salardini in 1993 reported that gypsum addition in pots of poppies increased seed Cd concentrations [79]. The effect can be ascribed to the increase in Ca and ionic strength in soils solution, enhancing Cd in soil solution. In contrast, a study from 2018 shows gypsum application is effective to remediate soil Cd contamination since the labile Cd pool and the concentration of Cd in rice decreased [97].

As gypsum reaches deeper soil layers because of its solubility, this might be an interesting amendment for the reduction of Cd in the perennial cacao trees that have deep roots. However, the results with other crops were highly variable and dependent on a load of environmental factors. The speciation of the sulfate ultimately defines whether the uptake of Cd will increase or decrease. Optimal for a decrease in Cd content in the crop when applying gypsum to the soil, is i) the abundance of Fe- and Al- oxyhydroxides in the subsoil layers so they can react with the sulfate and ultimately sorb Cd, ii) low Ca-Cd competition at the surface binding sites, and iii) high Ca-Cd competition at the root membrane.

2.4 Stable isotopes

Isotopes of an element have the same atomic number, but a varying number of neutrons (and consequently, different atomic weight). Stable isotopes are the non-radioactive isotopes of atoms, i.e. they are persistent throughout time since they do not decay. Assuming that the isotopes of one element function identically, they allow informative and safe tracing of sources and processes. As a consequence, there is a great exploitation of stable isotopes in multiple applications, like water and soil management, environmental studies, nutrition assessment studies, and forensics [46, 47].

Two pool mixing models are used in stable isotope chemistry to quantify pool sizes or determine proportions from different pools. The method relies on the fact that the isotopic composition of a mixed sample ('sink') is controlled by the isotopic signature and contribution of the original sources [46]. This method is only reliable if i) the sources have different isotopic ratios, ii) the isotope fractionation in the system is known or negligible, and iii) additional sources are eliminated from the system [94]. Fractionation is the state of unequal stable isotope composition in different materials, associated by a reaction or process [30]. Noteworthy is the fact that if highly enriched substances are used, there is no need to take fractionation effects into account [46].

Soil-plant Cd transfer studies with isotopes of Cd

In several studies, isotopes of Cd are used to study the behavior of Cd in soil-plant systems. The isotopes of Cd and their natural abundances are shown in Table 2.4. A recent study compared radio and stable isotope techniques that were used to trace the pathway of Cd in P fertilizer through the soil-wheat system [94]. The stable isotope technique used the naturally occurring differences in the Cd stable isotopes ^{110}Cd and ^{114}Cd, assuming that there is a change in the isotopic composition ($\delta^{114/110}$Cd) of the plant-available Cd pool to lighter isotopes through the addition of soluble mineral P fertilizer. The radio isotope technique exploited an enrichment in the mineral P fertilizer with ^{109}Cd. Wiggenhauser et al. (2019) concluded that the radio

isotope technique was more robust than the one with the stable isotopes at natural abundance since the isotopic composition of the soil and the fertilizer was too similar. With the help of the isotopes, they concluded that fertilizer applications can lead to a build-up of Cd pool in soils in the long term since only 3% of the Cd from the fertilizer reached the plant shoot [94]. Qin et al. (2013) studied the distribution of ^{108}Cd in split-root marigold seedlings. They exposed a part of the roots to an enriched isotope solution of ^{108}Cd and the other part to a similar solution without ^{108}Cd. In this way, the study indicated that the translocation of ^{108}Cd from roots to shoots via xylem and phloem is an important mechanism in the loading of Cd in the seedling [76]. A last example is the use of ^{109}Cd to study the mobility of Cd in potato plants [78]. Here, they added ^{109}Cd to soil where potato plants were grown in a split pot system. In this way, they suggested that Cd has high mobility in plants in both xylem and phloem, and that stem has an important role in controlling the transfer of these pathways [78].

Table 2.4: The isotopic composition of Cd with the natural abundances (fractions) and atom masses. Taken from the IUPAC technical report of 2013 [8].

	^{106}Cd	^{108}Cd	^{110}Cd	^{111}Cd	^{112}Cd	^{113}Cd	^{114}Cd	^{116}Cd
Natural abundance	0.0125	0.0089	0.1249	0.128	0.2412	0.1222	0.2873	0.0750

Isotope analysis: ICP-MS

Traditionally, isotope analysis is performed with isotope ratio mass spectrometry (IRMS). However, for heavier stable isotopes the inductively coupled plasma mass spectrometry is more used [46]. The ICP-MS has excellent sensitivity and is user-friendly. The precision is limited by signal stability, polyatomic mass spectral interferences, and mass discrimination [69].

Chapter 3

Materials and methods

A pot experiment was conducted in Ecuador to determine the uptake of Cd by cacao seedlings as affected by the root distribution and bioavailable Cd. Two superimposed soil layers of different chemical composition (different bioavailable Cd) were combined in all possible combinations in which one compartment was labeled with the stable isotope ^{108}Cd as a tracer to determine Cd provenance. Furthermore, the effect of various soil amendments ($CaCO_3$, $CaSO_4$ and MgO) on the uptake of Cd was investigated.

3.1 Pot experiment

The experiment was carried out in a greenhouse at ESPOL university facilities in Guayaquil, Ecuador for approximately five months (August-December). The popular cacao cultivar CCN-51 was used, propagated vegetatively by the technique of rooting cuttings, a clonal propagation technique. This method allowed to obtain an exact copy of the genome of a mother plant, reducing the genetic variance in the experiment [65, 85]. Roots were induced to form on a piece of stem from a donor plant. Meristematic, undifferentiated cells were exploited which differentiated into roots [85].

The technique was practiced in so called nurseries where shade, water and protection from the wind is provided. Cuttings were grouped together (after dipping in an hormone solution) and covered with a polythene sheet in a shaded nursery. In four weeks the cuttings were well rooted and removed from the polythene sheet for a gradually increasing period [95]. The seedlings were then placed in polythene bags with preferably a sandy loam soil and transported to the greenhouse in Guayaquil prior to the transplantation to pots.

Figure 3.1: Setup of pot experiment with cacao seedlings in the greenhouse

3.1.1 Soil

For this study, a surface soil (0-15 cm depth) and subsoil (15-30 cm depth) were collected from a cacao plantation in the coastal region of Ecuador (province of Azuay) (figure B.1). The soil is classified as an Alfisol by U.S. soil taxonomy [87]. In a previous survey [5] the total elemental composition of the surface soil, was determined by ICP-MS after aqua regia digestion which indicated an average Cd concentration of 0.85 mg Cd/kg in this Alfisol. The same survey determined the pH of the surface soil as 5.05 (in a 0.001 M $CaCl_2$ solution) [53]. This soil was thus chosen since it is a commonly occurring soil in cacao plantations and moreover contained a high Cd concentration. Furthermore, the soil pH allowed, theoretically, to benefit from the application of liming amendments to reduce Cd accumulation in the cacao seedlings.

Approximately 400 kg of surface soil and 140 kg of subsoil were collected. The soil was spread in the greenhouse to let it air-dry for one week. Afterwards, the soil particles were sieved using a 4-mm mesh. After the drying and sieving procedure, approximately 250 kg of surface soil and 100 kg of subsoil was left over. The soils were divided in plastics bags each containing 7.5 kg of dried and sieved soil, to facilitate the application and thorough hand-mixing of the stable isotope spiking solution and respective soil amendments.

Spiking of the soil with ^{108}Cd

Preparation of the spike solution

A cadmium metal with an enrichment of 71.1% in ^{108}Cd was dissolved in nitric acid to obtain following reaction.

$$Cd\ (s) + 2\ HNO_3 \longrightarrow Cd(NO_3)_2 + H_2$$

Therefore, Cd metal (20.0 mg), concentrated HNO_3 suprapur (70 µL) and Milli-Q water (2mL) were added in a vial. The solution was then pipetted in a 15 mL tube and diluted to 10 mL with Milli-Q water. The measured concentration was 1630 mg ^{108}Cd/L (with a measured ^{108}Cd abundance ratio of 71.2%).

The spiking solution was prepared by diluting 5 mL of the ^{108}Cd stock solution with deionized water to a final volume of 7.7 L. Next, 350 mL of this spike solution was sprayed in the bags with the soils where ^{108}Cd needed to be enriched. Afterwards, the soil in the bag was thoroughly mixed with the solution. The soil was then put to rest for three days to reach equilibrium. With this method, the concentration of the freshly added Cd was 0.049 mg Cd/kg soil. The theoretical enrichment of the spiked surface and subsoil can then be calculated with a mixing Equation 3.1. The values can be found in Table 3.1. In a later section, there will be more explanation about

the meaning of isotopic abundances (IA) and isotopic ratios (IR). Nevertheless, they are shown here to indicate the enrichment which was aimed for in the soil.

$$IA = \frac{Cd_{spike} * IA_{spike} + Cd_{natural} * IA_{natural}}{Cd_{spike} + Cd_{natural}} \tag{3.1}$$

Table 3.1: The theoretical isotopic abundance (IA) (in %) and isotopic ratio (IR)

	soil	spike solution	spiked surface soil	spiked subsoil
IA ^{111}Cd	12.8	4.74	12.5	12.3
IA ^{108}Cd	0.89	71.1	3.29	5.08
IR	0.070	15.0	0.26	0.41

The IA and IR which was aimed for in the pot experiment. The calculation is based on concentrations of Cd_{spike}=0.049 mg/kg soil, $Cd_{surface,na}$=1.38 mg/kg soil and Cd_{sub}=0.77 mg/kg soil (Eq. 3.1). Hence, surface soil was labeled with 3% enrichment in ^{108}Cd, subsoil with 5% enrichment in ^{108}Cd.

Amendments

Three commercially available amendments (lime ($CaCO_3$), gypsum ($CaSO_4$), magnesium oxide (MgO)) were considered for the experiment. The $CaCO_3$ and MgO amendments were chosen because in the field they are often applied and $CaSO_4$ since it is more mobile, being able to reach deeper soil layers as explained in the literature review (Section 2.3.3). The purpose of the amendments was to increase the pH with one unit. Therefore, concentrations of 1.54 g (0.015 mol) $CaCO_3$/kg soil, 1.54 g (0.011 mol) $CaSO_4$/kg soil and 0.77 g (0.019 mol) MgO/kg soil were considered for the experiment. This approaches four tons of $CaCO_3$ and $CaSO_4$ and two tons of MgO per hectare, quantities that are usually applied on the field.

Hence, in the selected bags 11.55 g $CaCO_3$ and $CaSO_4$ and 5.775 MgO was added. Next, the soil was moisturized until 50% water hold capacity and mixed to assure the lime would react with the pore water. The soil was then put to rest for three days to reach equilibrium. In this manner, the lime caused a pH change of the soil

from 5.6 to 6.5 for $CaCO_3$, 6.6 for MgO and 5.6 for $CaSO_4$ (pH measurement in deionized H_2O solution with a 1:5 soil/solution ratio, measured after the equilibrium time of three days).

3.1.2 Pot experiment

The soil was moisturized further until all pots had a water hold capacity of 70%. The six-week old cacao plants, coming from a cacao nursery, were removed from the substrate in the polyethylene bags. The roots of the plants were washed by immersing them in distilled water to remove adhere substrate. They were then planted in the pots containing 5 kg of soil (dry weight basis). Every day, they were weighted and watered with deionized water to maintain a homogeneous WHC (water hold capacity) of 70% in the pots. After the first week, 16 plants were replanted since they wilted. To ensure the growth of the seedlings, they were fertilized weekly in the first month with a solution of 3.925 mg/kg soil *Menorel Super*® [2] (containing following components: N, P, K, Fe, S, Zn, giberellic acid, Mn, B, Co, Cu, Mo). Every month, soil fertilizer (containing 80% Multi K 16 (16-16-16) [41] (N, P, K) and 20% Rafos (12-24-12) [96] (N, P, K, Mg, S, Zn) of 440 mg/kg soil was applied to the pots.

After six weeks of growth in the pots, newly grown leaves were harvested and transported to Belgium for chemical analysis. The experiment finished after sixteen weeks when all the biomass of the seedling (leaves, roots, stems) was harvested. Roots were collected on selected pots (treatments 4, 5, 7, 8, 10, 11, 13, 15, 16,20, 21, 23, 24) in a special manner to make sure the root biomass from each soil layer was identified and collected. The pots were cut through the center horizontally to separate the top and bottom compartment (2.5 kg soil each) and subsequently washed with deionized water to remove soil particles from the roots. All biomass was oven-dried at 60 °C prior to transport to Belgium for further chemical analysis.

3.1.3 Treatments

To answer the research questions in this experiment different combinations in pots were created (Table 3.2). The combinations depend on

- the position of the treatment in the pot (top or bottom compartment)
- the soil type (surface- or subsoil (B))
- the amendment (no amendment (NL), $CaCO_3$ (L), $CaSO_4$ (L-$CaSO_4$), or MgO (L-MgO))
- whether the soil was spiked (*) with ^{108}Cd or not.

In the following chapters, 'top'- or 'bottom' will always refer to the physical location of the treatment in the pot whilst 'surface' and 'subsoil' specify the soil type used. Figure 3.2 shows two examples of possible combinations. The first seedling is grown on the surface only that is non-limed (NL/NL) whilst the second seedling is grown on the contrasting soils without application of lime (NL/NLB). Treatments 1-12 were carried out on one soil, the surface soil. Treatments 14-18 and 20-24 were performed on soils with contrasting properties, on top the surface soil was used and in the bottom compartment the subsoil (B). The amendment applied was $CaCO_3$, except for treatment 23 (MgO) and 24 ($CaSO_4$) (Table 3.2). All treatments were replicated three times which gave a total of 72 pots.

Figure 3.2: Two examples of possible treatments.
(NL: surface soil, non limed, NLB: subsoil, non limed)

Table 3.2: Different treatments used in the pot experiment

Treatment	**1**	**2**	**3**	**4**	**5**	**6**
Top	L	NL	L	NL*	NL	NL*
Bottom	L	NL	NL	NL	NL*	NL*
Treatment	**7**	**8**	**9**	**10**	**11**	**12**
Top	L*	L	L*	L*	L	L*
Bottom	NL	NL*	NL*	L	L*	L*
Treatment	**13**	**14**	**15**	**16**	**17**	**18**
Top	NLB	NL	NL*	NL	NL*	L
Bottom	NLB	NLB	NLB	NLB*	NLB*	NLB
Treatment	**19**	**20**	**21**	**22**	**23**	**24**
Top	NLB*	L*	L	L*	L-$CaSO_4$	L-MgO
Bottom	NLB*	NLB	NLB*	NLB*	NLB	NLB

The structure of the different pots. When there is no specification of the soil, surface soil is used. The other labels symbolize the following; B: subsoil, L: $CaCO_3$, L-$CaSO_4$: $CaSO_4$, L-MgO: MgO, NL: no amendment, *: enriched in ^{108}Cd

3.2 Chemical analysis

3.2.1 Soil analysis

Soil samples were taken from the top layer in each pot (n=72) and oven dried (105°C). Afterwards, the samples were put in the digestion block for 3-4 hours in boiling aqua regia (1:3 Suprapur HCl:HNO_3). Next, the digested samples were 10x diluted with Milli-Q water. The total elemental composition and the ^{108}Cd and ^{111}Cd isotopes were measured by inductively coupled plasma mass spectroscopy (ICP-MS, Agilent 7700x, Agilent Technologies). Determination of ^{108}Cd and ^{111}Cd isotopes occurred in the He gas mode. The instrument was calibrated with certified synthetic water solutions for trace elements. For quality assurance certified reference material (BCR-142R light sandy soil) was included in duplicates in the digestion batch (measured concentration: 0.236 ± 0.026 mg Cd/kg). When calculating the final concentrations the blank was subtracted and a conversion to dry weight (mg/kg soil) was made. The % recovery with respect to the certified value from the references was 94.26% % which is in the range for a correct measurement of Cd.

Soil chemical properties

The measurement of soil pH was performed in Milli-Q water or in a 0.001 M $CaCl_2$ solution with a 1:5 soil/solution ratio. Prior to analysis of total % organic carbon (%OC), the soil was ground to a powder with a pestle and mortar and acidified with 20-50 µL of 10% hydrochloric acid (HCl). Then %OC was determined with elemental analyser (Carlo Erba EA1108). The cation exchange capacity (CEC) was determined using cobalt hexamine trichloride extractant solution as described in the standardized protocol ISO 23470 (2007). This method follows a onestep centrifuge extraction with 0.0166 M cobalt(III)hexamine chloride solution (Cohex) $[Co[NH_3]_6]Cl_3$. The decrease in Co concentration is a measure for the CEC whilst the supernatant solution contains all exchangeable cations. To quantify ammonium oxalate extractable aluminium (Al-ox), iron (Fe-ox) and manganese (Mn-ox) a 1:50 soil to ammonium oxalate extract was used (buffered at pH 3). The extractions were

put into an end over end shaker for two hours in dark tubes to avoid the incidence of light. The supernatant solution was then filtered through a 0.45 µm membrane. The extract was analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES, iCAP 7400 series, Thermo Scientific) for concentrations of Al, Fe and Mn.

3.2.2 Cacao leaves analysis

The analysis on the leaves was performed after six and sixteen weeks of growth. For the first measurement, the largest newly grown leaf was harvested. For the last measurements, all newly grown leaves were collected. The collected leaves were oven-dried at 60 °C and ground to a fine powder. Afterward, 50 mg of the dried plant material was digested with concentrated Suprapur nitric acid (HNO_3) in open block digestion (4-5 hours). The total elemental composition, and the ^{108}Cd and ^{111}Cd isotopes were measured by ICP-MS in the He gas mode for the 2x diluted samples. In each digested batch, the certified reference materials included were NIST 1573a tomato leaves (1.40 ± 0.045 mg Cd/kg (six weeks), 1.41 ± 0.005 mg Cd/kg (sixteen weeks)) and the BCR 679 white cabbage (1.61 ± 0.060 mg Cd/kg (six weeks), 1.70 ± 0.022 mg Cd/kg (sixteen weeks)). The respective % recoveries with respect to the certified value from the references were -96.98%, 102.63%, 92.03%, 107.14%. The limit of quantification (LOQ) was defined as the largest value from three times the standard deviation of the blanks, the maximum value of the blanks or the LOQ defined by the instrument and was for Cd 0.004 mg/kg.

3.3 Calculations and statistics

3.3.1 Determination of isotopic abundances

Working principle of ICP-MS

The ICP-MS allows a determination of the isotopic abundance of an element in a sample. First, the sample is prepared by a laser which focuses on a sample, vaporizing it. A plume of ablated material is formed which is then introduced in plasma of

10 000 °C where it decomposes into atoms which subsequently are ionized. Next, these ions are separated based on their mass-charge ratio in the mass spectrometer [46]. Here, the counts of every isotope is registered by the detector.

Mass bias correction

In isotope ratio measurements by ICP-MS mass discrimination occurs due to the space-charge effect and other phenomena. Lighter ions are deflected more than the heavier ones, resulting in a space-charge effect (because of the Coulomb repulsion of charged ions produced in the plasma and the resulting loss of transmission through the ion optical lens system) [69]. This mass bias was corrected by using a mass bias correction factor (MBC). The mass bias correction was done for the measured 108/111Cd isotope ratio. Therefore a linear relationship between mass bias and mass difference was assumed. The mass bias correction factor was calculated as the following.

$$f_{MBC} = \frac{IR_{theory}}{IR_{measured}} = \frac{0.0695}{0.0610} = 1.140 \tag{3.2}$$

With $IR_{measured}$ the isotopic ratio measured for a standard of 100 ppb Cd single element ($IR_{measured} = 0.0610$). By multiplying the counts of ^{108}Cd of all samples by the mass bias correction factor, a mass bias corrected value was obtained.

3.3.2 Isotopic abundances

The isotopic abundance (IA) is the relative number of atoms of a particular isotope in a mixture of the isotopes of an element, expressed as a fraction of all the atoms of the element [73]. The IA in the leaves of the cacao seedlings were calculated from ICP-MS analysis of ^{108}Cd and ^{111}Cd. For the first measurement after six weeks, only ^{108}Cd and ^{111}Cd were measured (in cps). Hence to calculate the IA's, the next procedure was used.

First the IR (isotopic ratio) was calculated for each sample with the counts measured by ICP-MS (cps).

$$\mathrm{IR}_{\mathrm{measured}} = \frac{^{108}Cd}{^{111}Cd} \tag{3.3}$$

This value was then used to determine the isotopic abundance of ^{111}Cd for the different samples.

$$\mathrm{IA}^{111}\mathrm{Cd} = \frac{^{111}Cd}{^{106}Cd + ^{108}Cd + ^{110}Cd + ^{111}Cd + ^{112}Cd + ^{113}Cd + ^{114}Cd + ^{116}Cd} \tag{3.4}$$

$$\mathrm{IA}^{108}\mathrm{Cd} = \frac{^{108}Cd}{^{106}Cd + ^{108}Cd + ^{110}Cd + ^{111}Cd + ^{112}Cd + ^{113}Cd + ^{114}Cd + ^{116}Cd} \tag{3.5}$$

$$= \frac{1}{\frac{^{106}Cd}{^{111}Cd} + ... + \mathrm{IR}_{\mathrm{measured}} + \frac{^{110}Cd}{^{111}Cd} + 1 + ... + \frac{^{116}Cd}{^{111}Cd}} \tag{3.6}$$

With this isotopic abundance the total counts were calculated.

$$\text{Total (cps)} = \frac{^{111}Cd(\text{cps})}{\mathrm{IA}^{111}\mathrm{Cd}} \tag{3.7}$$

And finally, the isotopic abundance of ^{108}Cd could be calculated. For the measurement after sixteen weeks, it was possible to immediately use following formula since all Cd isotopes were measured.

$$\mathrm{IA}^{108}\mathrm{Cd} = \frac{^{108}Cd(\text{cps})}{\text{Total (cps)}} \tag{3.8}$$

3.3.3 Fractions of Cd in the leaf (from the top or bottom soil)

In this experiment, the stable isotope ^{108}Cd served as a tracer. The cacao plant had two possible Cd sources, namely the top compartment or the bottom compartment. With the *two pool mixing model*, the fractions of Cd derived by the plant from these two sources were calculated. This model is based on the principles of i) mass balance and ii) conservation of matter. It states that the isotopic composition of a mixed sample is defined by the contribution and the isotopic signature of the two sources [47].

The seedling can take the Cd from either the compartment with the natural abundance of Cd or from the compartment with the spiked abundance (with f the fraction of Cd).

$$f_{na} + f_{sp*} = 1 \tag{3.9}$$

The mass balance with isotopic abundances looks then like the following.

$$\mathrm{IA}_{leaf} = f_{na}.\mathrm{IA}_{na} + f_{sp*}.\mathrm{IA}_{sp*} \tag{3.10}$$

From equation 3.9 and 3.10, the fraction from the spiked compartment can be derived.

$$f_{sp*} = \frac{\mathrm{IA}_{leaf} - \mathrm{IA}_{na}}{\mathrm{IA}_{sp*} - \mathrm{IA}_{na}} \tag{3.11}$$

Thus in order to quantify the fraction from each compartment, the $\mathrm{IA_{leaf}}$, $\mathrm{IA_{na}}$ and $\mathrm{IA_{sp}}$ need to be determined. In Figure 3.3a, this is shown for the pot where there is only surface soil and the top and bottom compartment are entirely limed. These pots correspond with treatments 1, 10, 11, 12 (Table 3.2). In treatment 1, where no spiking was applied, the natural IA of the leaf is determined. Treatment 12, the fully spiked pot, gives the spiked IA of the leaf. From pot 10, where only the top is spiked, the IA of the leaf is determined. The same corresponds to pot 11, where only the bottom part is spiked.

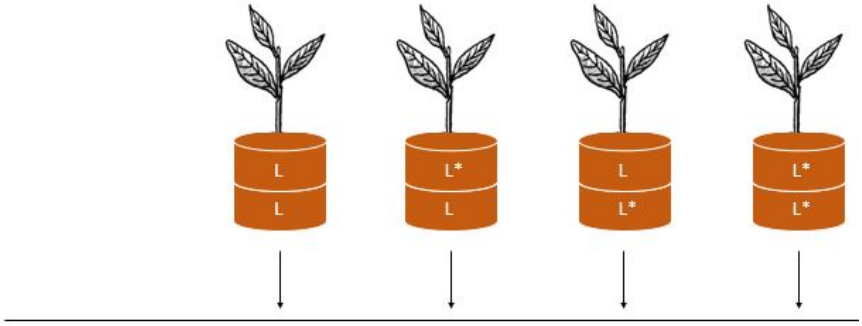
$$f_{top*} = \frac{\mathrm{IA}_{leaf,top*,10} - \mathrm{IA}_{na,1}}{\mathrm{IA}_{sp*,12} - \mathrm{IA}_{na,1}} \tag{3.12}$$

$$f_{bottom*} = \frac{\mathrm{IA}_{leaf,bottom*,11} - \mathrm{IA}_{na,1}}{\mathrm{IA}_{sp*,12} - \mathrm{IA}_{na,1}} \tag{3.13}$$

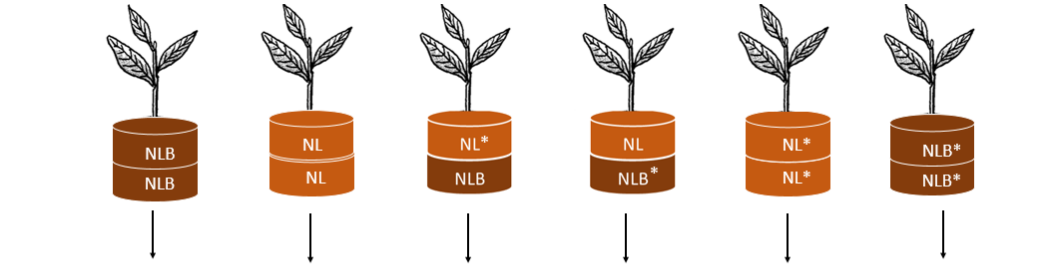
When there are two types of soils in the pots, the situation gets more complicated. Now, six treatments are involved in the calculation since the natural and spike abundance differs for the two soil types (Fig. 3.3b).

$$f_{top*} = \frac{\mathrm{IA}_{leaf,top*,15} - \mathrm{IA}_{na,13}}{\mathrm{IA}_{sp*,6} - \mathrm{IA}_{na,13}} \tag{3.14}$$

$$f_{bottom*} = \frac{\mathrm{IA}_{leaf,bottom*,16} - \mathrm{IA}_{na,2}}{\mathrm{IA}_{sp*,19} - \mathrm{IA}_{na,2}} \tag{3.15}$$



(a) Two compartments with only one soil (Treatment 1, 10, 11, 12)



(b) Two compartments with two contrasting soils (Treatment 13, 2, 15, 16, 6, 19)

Figure 3.3: Visualization of the different sources of isotopic abundances for the calculation of the fractions

Table 3.3 shows all the treatments used for the isotopic abundances of the different soil types and amendments. This table indicates why all the different treatments were necessary. There is no need to look at the soil to determine where the cadmium is originating from. With these different treatments, only the isotopic abundances of the seedlings need to be determined (that are grown in a non-spiked pot, a pot with the top spiked and bottom non-spiked, a pot with the top non-spiked and bottom spiked and a fully-spiked pot) to ascertain the source of Cd in the pot.

Table 3.3: The treatments used for the calculations of the fractions of Cd derived from top and bottom compartment.

			Treatments used for IA			
Soil type	Amendment	Fraction	IA natural	IA leaf, top	IA leaf, bottom	IA spiked
Surface	Limed	Top	1	10		12
		Bottom	1		11	12
	Top limed	Top	1	7		12
		Bottom	2		8	6
	No liming	Top	2	4		6
		Bottom	2		5	6
Surface/sub	Top limed	Top	13	20		12
		Bottom	1		21	19
	No liming	Top	13	15		6
		Bottom	2		16	19

3.3.4 Statistical data analysis

The statistical analysis of data was conducted with JMP statistical package version 14 (SAS Institute). One-way analysis of variance (ANOVA) was performed to determine if the effect of liming, lime position, soil type and different soil amendment had a significant effect on leaf Cd in the plant. Differences between treatments were analyzed with the post hoc Tukey HSD test (level of significance $p<0.05$ level). These tests were done by using one-way ANOVA followed by the post hoc Tukey HSD test (level of significance: $p<0.05$).

Prior to the one-way ANOVA analysis the following assumptions were checked:

- The dependent variable should be continuous. In this case the dependent variable was often a concentration, making the assumption fulfilled.
- The independent variable should be two or more categorical independent groups. Here the independent variables were the type of amendment, the type of soil,...
- The observations should be independent. When doing the analysis it was ensured that there was no relation between the observations in each group or between the groups themselves.
- The residuals of the dependent variable should be approximately normally distributed for each category of the independent variable. This was tested for each model with the Shapiro-Wilk W test in JMP.
- The homogeneity of variances is the last assumption which has to be fulfilled to conduct a one-way ANOVA analysis. This was as well tested with the JMP software. When this assumption was not accomplished, the Welch test was used.

Chapter 4

Results and discussion

This section presents the key results, followed by an immediate interpretation. First, the focus lies on the concentrations of Cd in the leaves for the different soil types. Second, the effect of the liming amendments on the concentrations of Cd in the leaves is presented. Next, the stable isotope study is depicted, revealing the contribution of the two rooted soil compartments on the uptake of Cd. Table 4.1 shows the concentrations of Cd in the leaves for all different treatment and the relative change compared to the reference treatment, which is a welcome guidance in the understanding of following sections.

Table 4.1: Leaf Cd concentrations (*aqua regia* soluble: mean (std. dev.))

Treatment	Cd (mg/kg leaf)	Reference	% Change (to ref.)
NL/NL	10.13 (1.05)		
L/NL	8.54 (1.06)	NL/NL	-16
L/L	5.84 (0.97)	NL/NL	-42
NL/NLB	9.16 (1.85)	NL/NL	-10
L/NLB	5.42 (0.44)	NL/NLB	-40
L-Gyp/NLB	5.97 (0.33)	NL/NLB	-35
L-MgO/NLB	5.05 (0.39)	NL/NLB	-45
NLB/NLB	5.17 (0.87)	NL/NL	-49

4.1 Effect of soil type on leaf Cd concentrations

4.1.1 Characteristics of surface and subsurface soil

The properties of the surface soil (0-15 cm) and the subsurface soil (B) (15-30 cm) are presented in Table 4.2. The surface soil contained about 1.8 times more total Cd than the subsurface soil. The surface soil was slightly more acidic than the subsoil. The nutrient concentrations were overall larger in the surface soil. The oxalate extractable Fe and Mn (representing the amorphous Fe- and Mn-oxides in the soil) and %OC (organic carbon) were as well higher in the surface soil, but this trend was not followed by the oxalate extractable Al (i.e. the amorphous Al -oxides). The ECEC was only slightly higher in the surface soil.

The soil profile roughly follows the properties of Alfisols and in particular of cacao plantations, only the values of the exchangeable bases are quite high compared to other cacao plantations [89]. Also the total P values are relatively low compared to cacao plantations in Costa Rica, especially in the subsoil (where in Costa Rica a value of 616 mg P/kg was reported). Total P is mostly higher in the surface soil due to the contribution of organic P in the surface layer [92]. All properties fall within the ranges of the survey conducted in cacao plantations of Ecuador (n=560) on surface soils, except for the oxalate-extractable Mn (3.73 g/kg), which is really high compared to the average value of 0.58 g/kg (with a range from 0.01 to 2.67 g/kg) in this survey [53].

The surface soil contains more total Cd than the subsoil, confirming the research of Chavez and Barraza, stating that in cacao plantations of Ecuador Cd concentrations decrease with depth [16, 6]. The concentrations of competing ions (H^+, Ca^{2+} and Mg^+) are as well higher in the solution of the surface soil, promoting the solubility of Cd. As a consequence, one could expect a larger uptake of Cd by the seedlings from the surface layer. However, this effect may be counteracted by the higher presence of adsorbents in the surface soil (organic matter and Fe, Al and Mn hydroxides).

Table 4.2: Properties of the two contrasting soils

	Surface soil	Subsoil
	Mean (std dev)	Mean (std dev)
pH (water)**	5.18	5.46
Organic Carbon (%)	2.62	1.45
Oxalate extractable Fe (g/kg)	10.3	6.54
Oxalate extractable Al (g/kg)	2.03	2.09
Oxalate extractable Mn (g/kg)	3.73	2.37
ECEC ($cmol_c$/kg)	16.62	14.61
Cd (mg/kg)*	1.38 (0.26)	0.77 (0.13)
Ca (g/kg)*	3.52 (0.27)	2.81 (0.17)
Mg (g/kg)*	3.41 (0.54)	3.60 (0.32)
Zn (mg/kg)*	124 (7.32)	115 (7.04)
Mn (g/kg)*	3.34 (0.32)	3.25 (0.40)
Fe (g/kg)*	60.8 (4.27)	72.6 (5.16)
P (mg/kg)*	465 (14.9)	293 (67.7)
Ca-exchangeable ($cmol_c$/kg)	11.4	9.48
Mg-exchangeable ($cmol_c$/kg)	4.78	3.63
K-exchangeable ($cmol_c$/kg)	0.36	0.24

**aqua regia* soluble, **1:5 soil: water ratio, ECEC: effective cation exchange capacity

To make an estimation which effect is determining the phytoavailability of Cd, the concentrations of Cd in the bean are predicted. According to the model and the accessory Freundlich type equation (Eq 4.1) proposed by Argüello et al. (2019) [5], the predicted Cd concentration in the cacao bean is 2.86 mg/kg d.w. of bean for a cacao tree grown on the surface soil. When grown on the subsurface soil the formula estimates a concentration of 2.0 mg/kg d.w. bean. Taking the transfer factors described in that paper (TF soil-bean = 1.60, TF soil-leaf = 4.46), for the surface soil treatments a concentration of 7.98 mg/kg leaf and for the subsoil treatments 5.58 mg/kg leaf is predicted. Hence, on the basis of this model it is expected that

the phytoavailable Cd is higher in the surface soil than in the subsoil.

$$\log(Cd_{bean}) = 1.66 + 0.94 * \log(CdT_{soil}) - 0.21 * pH - 0.63 * \log(\%OC) \quad (4.1)$$

4.1.2 Concentrations of Cd in the cacao leaves

The concentrations of Cd in the leaves for all treatments are indicated in Table 4.1. A one-way between subjects ANOVA was conducted to compare the effect of the contrasting soils on the total Cd concentration in the leaf. The seedlings were grown on surface soil only, surface on subsoil and subsoil only (NL/NL, NL/NLB, NLB/ NLB). There was a significant difference at the 0.05 alpha level for the Cd content in the leaf for the different soil types [F(2, 27)=25.2, p<0.0001]. Post hoc comparisons using the Tukey HSD test indicated that the mean score for the seedling grown on subsoil only (5.17 ±0.87) was significantly different from the conditions where the seedling grew on surface soil (10.13 ±1.05) and contrasting soils (9.16 ±1.86).

Interpretation on the effect of soil type on the Cd in the cacao leaf

When the cacao seedling grew on subsoil only there was a striking decrease in Cd content in the leaves. The concentration of Cd in the leaves was nearly two times lower in plants grown on the subsoil only compared to the surface soil only, which relates to the total Cd concentration in these soils (1.8 times higher in surface soil than in subsoil). Hence, for the seedlings grown on the surface on subsoil, a reduction of 25% on the concentrations of Cd in the leaves was expected (predicted concentration is then 7.5 mg Cd/kg leaf). Nonetheless, the measured concentration was 9.16 mg Cd/kg leaf, which is only a reduction of 10%. This result suggests that a larger fraction of Cd is taken from the top compartment where the soil has higher total Cd.

Hence, the key finding is that the total concentration of Cd in the soil may be a good predictor for the uptake of Cd by cacao when the soil is homogeneously distributed (i.e. the pots with surface soil only and subsoil only in this experiment).

However, cacao plantations are vertically heterogeneous, since Cd concentrations are decreasing with depth [16, 6]. In these pots with vertical heterogeneity, the decrease in Cd uptake is less than solely expected from Cd concentrations in the soil. To explain this peculiarity, the fractions of both compartments and the root distribution are studied in later sections.

4.2 Leaf Cd concentrations after amending the soil

4.2.1 Effect of amendments on the surface soil

The change in pH during the experiment is depicted in Fig. 4.1. The pH increased one unit at the start of the experiment for the soils where MgO and $CaCO_3$ were applied. The $CaSO_4$ treatment did not affect the soil pH (the curve follows approximately the same trend as the curve of the non-limed soil). This behaviour was expected since gypsum has no alkalizing effect (it does not consume protons). It is however used, as other liming amendments, to reduce Al toxicity by forming $AlSO_4{}^+$ species [37]. For all amendments, the pH decreased in time and for the MgO and $CaCO_3$ the decrease was more pronounced. This effect can be addressed to the buffer capacity of the soil. Solid-phase components can buffer soil pH against rapid change when natural or anthropogenic addition of acids and bases occurs in soils. The buffering reactions are proton desorption and adsorption reactions by organic and mineral materials. Nonetheless, ion exchange, dissolution and precipitation can also play a major role in the buffering of the soil [84]. Total Mg in the soil was higher for the MgO treatments, whereas the total Ca was higher for the $CaCO_3$ treatments (Table 4.3).

Table 4.3: Concentrations of total Mg and Ca (*aqua regia* soluble) in the soils for the different amendments

	No amend.	Gypsum	Lime	MgO
Mg (g/kg)	3.28 (0.57)	3.58 (0.37)	3.44 (0.23)	4.31 (0.73)
Ca (g/kg)	3.51 (0.61)	3.89 (0.38)	4.09 (0.21)	3.88 (0.72)

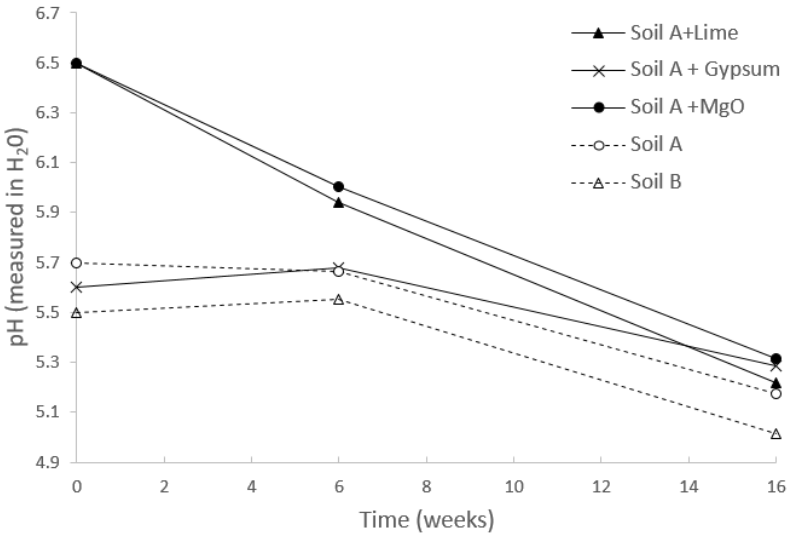


Figure 4.1: Soil pH as a function of time for different amendments in the experiment. The measurement was performed in a 1:5 soil: water ratio. Soil A is the surface soil, soil B the subsoil.

4.2.2 Effect on leaf Cd concentrations for different amendments

Effect of liming surface soil on Cd in the leaf

The concentrations of Cd for following comparison can be found in Table 4.1 in the beginning of this chapter. The effect of the different liming positions (NL/NL, L/NL, L/L) on the concentrations of Cd in the leaves were compared with one-way ANOVA. There was a significant effect of liming on the concentrations of Cd in the leaves at the $p<0.05$ level for the three conditions [$F(2, 33)=53.7$, $p<0.0001$]. A post hoc comparison with the Tukey HSD test pointed out that the mean concentration of Cd in the leaf was different for each group.

Effect of amendments in contrasting soils on leaf Cd concentrations

For a comparison on the leaf Cd concentrations between the different amendment types (Fig. 4.2), a Welch test was performed since the assumption of homogeneity of variance was not met. The test was significant at the 0.05 alpha level [$F(3, 7)=15.59$, $p=0.0019$]. All amendments decreased the Cd concentrations in the leaves by 35-45% (Table 4.1).

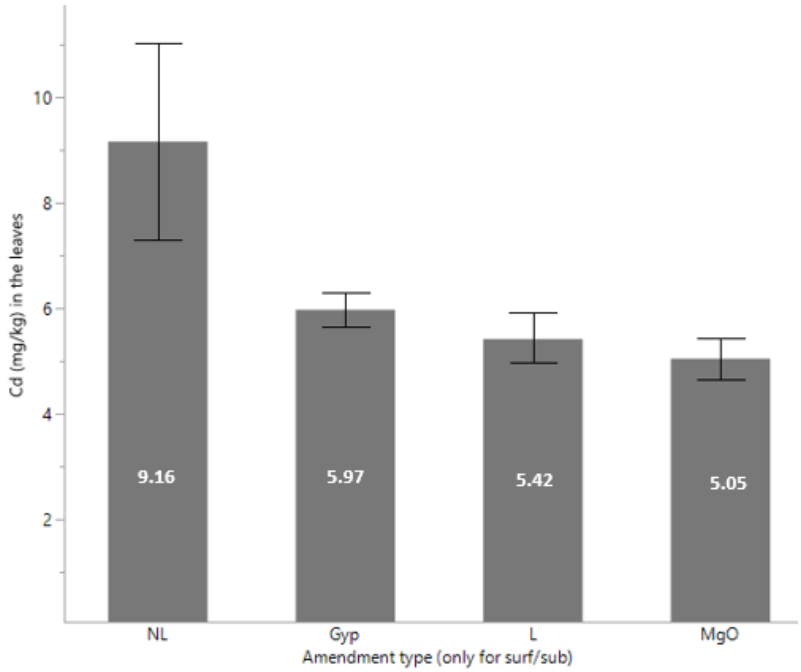


Figure 4.2: Cd concentrations in cacao leaves for the different amendments in contrasting soils.
(NL: no amendment, Gyp:$CaSO_4$, L:$CaCO_3$)

Interpretation of the effect of amendments on Cd in the leaf

For the treatments with the surface soil only, liming the whole pot reduced the concentrations of Cd in the leaf by 42%. The reduction when only the top layer is limed is only about 16%, hence the decrease is again not linear. This may indicate that more Cd is taken up from the bottom layer, where the bioavailability did not decrease, as will be discussed in following section where the sourcing of Cd is described.

All of the amendments on the contrasting soils are reducing the uptake of Cd by the cacao seedlings effectively. For $CaCO_3$ and MgO, the change of pH is assumed as the main driver behind the reduction. Sorption studies show a factor 3-5 stronger sorption of Cd in soils per unit pH increase [18, 26]. The plant concentrations in the leaves decreased with a factor 1.5-1.8. The decrease is not surprising, since in almost all pot experiments where lime was mixed well with the soil, Cd reduces the

uptake of Cd in crops [40, 82]. Now, this is also confirmed for cacao seedlings grown in pots. Our results may also suggest why on the field the decrease in the uptake of Cd is not as desired. When in the field the same pattern applies as in the pots of this experiment where more Cd is taken from lower layers when liming the top layer, the targeted Cd immobilization may possibly be counteracted. Especially for crops with deep-rooting structures like cacao, this mechanism may be noticeable.

For gypsum, literature is inconsistent about the effect on the uptake of Cd by crops. Some studies show increasing Cd concentrations in crops [79, 83], however McLaughlin et al. (1998) indicates that these studies are equivocal and open to conflicting interpretation [61]. In this particular study, the addition of sulfate to the soil did not affect the Cd uptake consistently while SO_4 increased Cd concentrations in soil solution. For the cacao seedlings grown on this particular Alfisol, the gypsum application is clearly reducing the uptake of Cd. Hence, it may be expected that Cd is retained more in the soil by new negative surface charges created by the adsorption of ${SO_4}^{2-}$ on Fe, Al and Mn hydroxides [14]. The reduction may have been this effective since the soil contains large amounts of Fe, Al and Mn hydroxides. Another hypothesis is that gypsum enhances the solubility of ${SO_4}^{2-}$, able to form ${CdSO_4}^0$ complexes which may not be available for plants [61]. Moreover, it is possible that the cation Ca^{2+} is increased in solution, augmenting the competition with Cd^{2+} on the binding sites of the cacao roots [18, 14]. To find out which mechanism was behind the decrease in the uptake of Cd in cacao, an additional solubility experiment was conducted on the surface and subsoil which can be found in appendix A. The conclusion of the experiment was that the main driver behind the reduction in the uptake of Cd was the increased activity of Ca^{2+} in soil solution, augmenting the competition with Cd^{2+} on the binding sites of the cacao roots. The effect may be so pronounced in the surface soil with high DOM content, increasing the activity of Cd-DOM complexes, which consequently reduces the phytoavailability of Cd in the soil solution.

4.3 Source of Cd in cacao seedlings

4.3.1 The isotopic ratio of the soils

In Figure 4.3 the isotopic ratio (^{108}Cd/^{111}Cd) of the spiked topsoils and non-spiked topsoils is depicted. Not surprisingly, the IR of the non-spiked soils was hovering around 0.070 (the natural IR, see Table 3.1). In a first measurement, the average IR of the non-spiked soils was remarkably around 0.090. When measuring the samples again, it was found that ZrO was interfering with ^{108}Cd. Therefore, the counts of the ^{92}Zr isotope were subtracted from the counts of ^{108}Cd which resulted in an average IR of 0.075 (Figure 4.3). For the spiked treatments, the IR was on average 0.33. Treatment 19 (subsurface soil) had a remarkably higher IR (0.50) than the other spiked treatments. The cause is the lower Cd content in the subsoil (see Table 4.2), resulting in a larger isotopic enrichment in ^{108}Cd compared to the surface soil. The theoretical calculation of the enrichment in the soil corresponded to an IR in the surface soil of 0.26 and for the subsurface soil of 0.41 (see Table 3.1). These values confirm that the spiking of the soil succeeded.

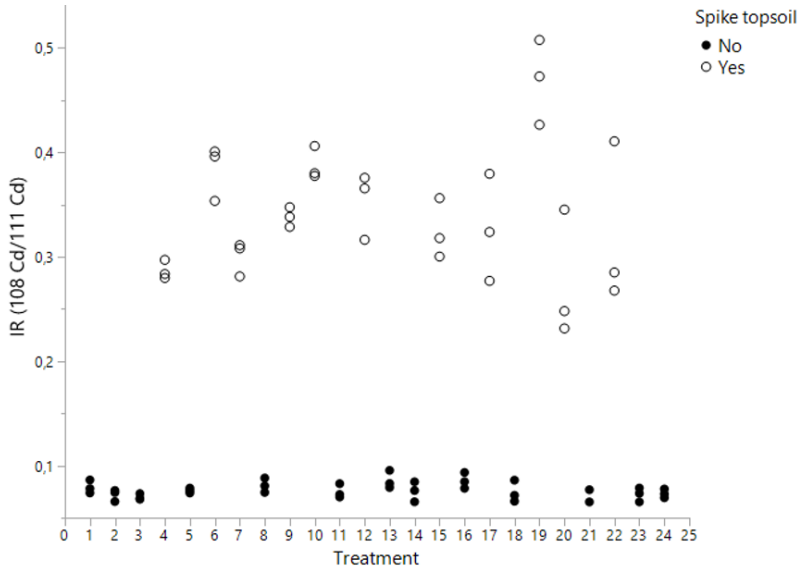
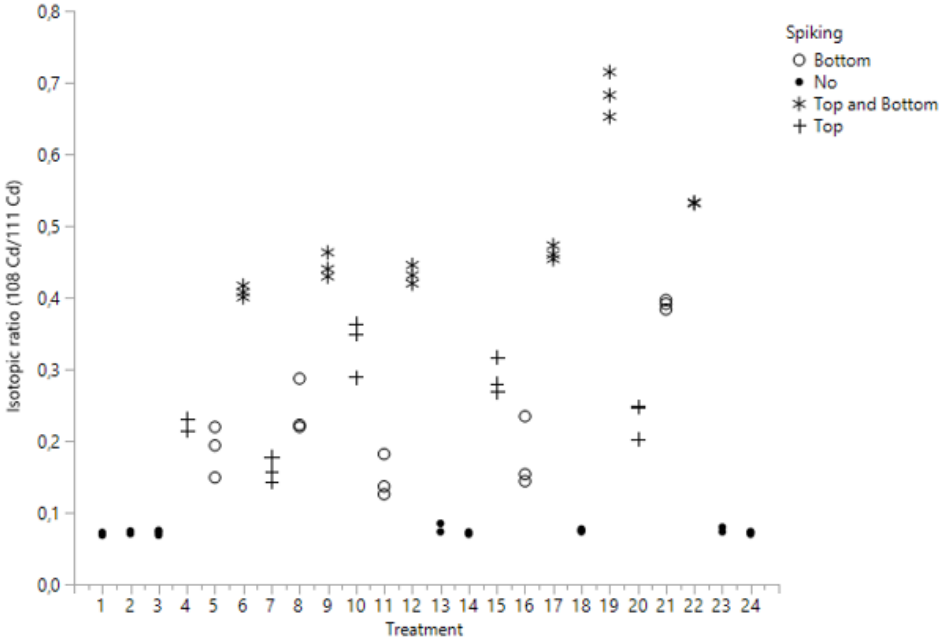


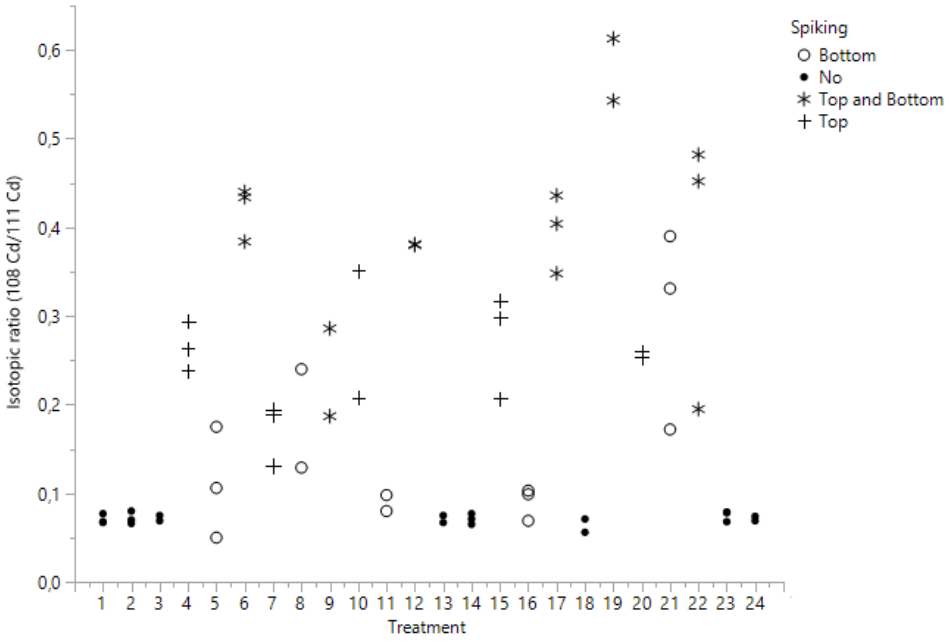
Figure 4.3: The effect of spiking the soil with ^{108}Cd on the soil IR (^{108}Cd/^{111}Cd). Only the topsoils were measured, hence all treatments are surface soils except for treatment 13, 19.

4.3.2 The isotopic ratio of the leaves

The isotopic ratio measured in the leaves of the cacao seedlings is shown in Figures 4.4 (two measurements were performed, after six and sixteen weeks). The natural IR (of the seedlings grown on non-spiked soils) was again hovering around 0.070. Treatment 19 with the seedling grown on subsurface soil only, had again the highest IR (0.68). The IR of the leaves at the end of the experiment (sixteen weeks) was largest for the seedlings grown on a fully spiked soil (IR = 0.46). For the seedlings grown on a spiked soil with one compartment, the IR was more or less equal (top: IR = 0.25, bottom: IR = 0.23) which was contrasting with the measurement after six weeks. Here, the IR was higher when the seedling was growing on soil with the topsoil spiked compared to the treatments where only the bottom soil was spiked. This might indicate that after six weeks, the roots of the seedling were not developed well in the bottom layer. However, after sixteen weeks the equal IR in both compartments indicates that the roots developed well in the bottom layer. Therefore, in the further interpretation of the data, only the measurements of the leaves after sixteen weeks of growth will be used.



(a) After six weeks of growth



(b) After sixteen weeks of growth

Figure 4.4: The effect of spiking the soil with ^{108}Cd on the IR (^{108}Cd/^{111}Cd) in the leaves of the cacao seedlings.
There are four different groups, depending on the position of the spiking in the pot.

4.3.3 Origin of Cd in cacao seedlings

With the two pool mixing equation described in the materials and methods section 4.2 the relative contribution of the top- and bottom compartment in the pot to the Cd in the leaf was calculated for the NL/NL, L/NL, L/L, NL/NLB, and L/NLB treatments (Figure 4.5). To avoid confusion, keep in mind whilst interpreting this graph that it depicts *relative* contributions. The bars do not relate to the total concentrations of Cd in the leaves (discussed in previous sections). The 'reference' group in Figure 4.5 is NL/NL, where no amendment was applied and only one soil type (surface soil) was present in the pot. The contribution of both layers to the Cd is marginally higher for the top compartment (56%) in this reference group.

$$f_{sp*} = \frac{\mathrm{IA}_{leaf} - \mathrm{IA}_{na}}{\mathrm{IA}_{sp*} - \mathrm{IA}_{na}} \tag{4.2}$$

Effect of the contrasting soils on the source of Cd in cacao leaves

When comparing NL/NL with NL/NLB (Fig. 4.5), the uptake of Cd from the bottom compartment clearly reduces (with 19%) which is confirmed by an ANOVA analysis, indicating a significant effect between these treatments [$F(1, 10) = 7.11$, $p = 0.0236$]. This decrease in the uptake of Cd from the bottom compartment was expected since the Cd in the subsoil is less bioavailable (see Table 4.2).

Effect of liming on the source of Cd in cacao leaves

A one-way ANOVA between subjects was conducted to compare the effect of liming with $CaCO_3$ on the source of Cd in cacao leaves grown on surface soil only (L/L, L/NL, NL/NL in Figure 4.5). There was a significant effect of liming on the fractions of Cd coming from the top layer at the $p<0.05$ level for the three conditions [$F(2, 15)=15.89$, $p=0.0002$]. A post hoc comparison with the Tukey HSD test also revealed that the mean fraction of Cd in the leaf was different for each group.

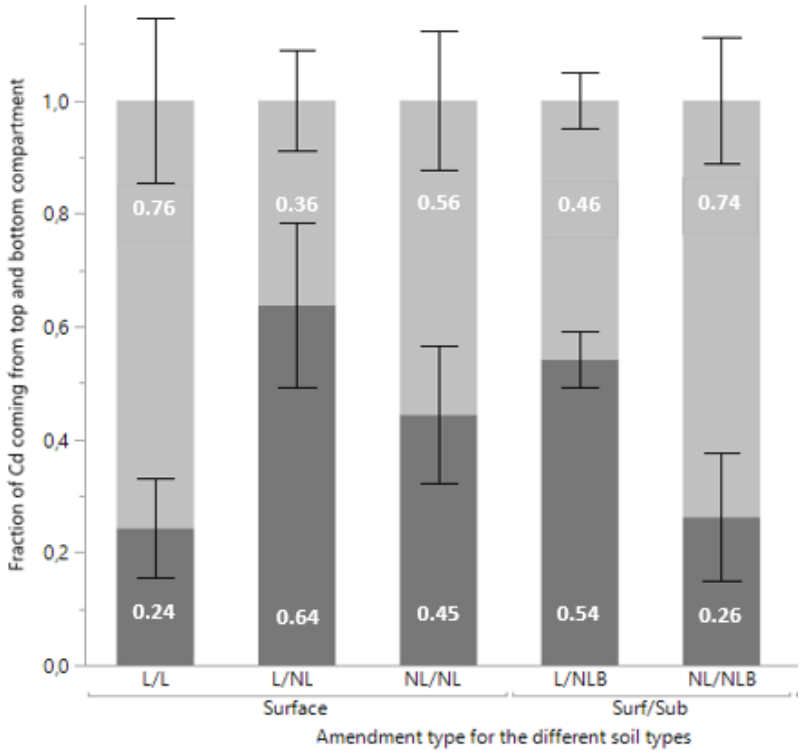


Figure 4.5: The relative contribution of the top and bottom compartment of the soil to the Cd in the leaf of the seedling (calculated with Eq. 3.11).
The dark grey indicates the bottom fraction, the light grey the top fraction. The error bars express the standard deviation.

This result implies that when the top layer is limed with $CaCO_3$, less Cd was taken up from the top layer than when no lime was applied (reduction of 20%). This agrees with the fact that Cd is more bioavailable in the bottom compartment that is not limed compared to the limed top compartment. Curiously, in the L/L treatments there is a spectacular rise in the uptake from the top compartment (from 56% to 76%). This is remarkable because the properties of the soil in the top and bottom compartment are presumably equal. Hence, the bioavailability of Cd is supposed to be similar in both layers (and consequently, the uptake is expected to be similar). This suggests that there may be other mechanisms involved in the uptake of Cd by cacao than only the bioavailability of Cd. Therefore, in later sections, the biomass of the roots in each compartment and the nutrient composition of the leaves will be investigated to try to explain this peculiarity.

A one-way ANOVA also indicated a significant difference between the non-limed (NL/NLB) and the top limed (L/NLB) treatments [$F(1, 10) = 30.96$, $p = 0.0002$]. The uptake from the top compartment reduced 28% when the top was limed (from 74% to 46%). Thus, there is a greater reduction in the contribution from the limed top layer for the seedlings on contrasting soils than on the homogeneous soils.

The root distribution in the pot

Consistently around 70% of the root biomass was located in the top compartment (Figure 4.6). No significant differences between the different groups were reported when comparing root masses with ANOVA. However, the standard deviations on the measurements are quite high as indicated with the error bars in Fig. 4.6. The fine roots were not distinguished, and these may have changed during the experiment since they do not contribute majorly to the weight of the roots. As a consequence, although the root weight stayed constant throughout all treatments, it is impossible to conclude that the root architecture was not affected by the different treatments.

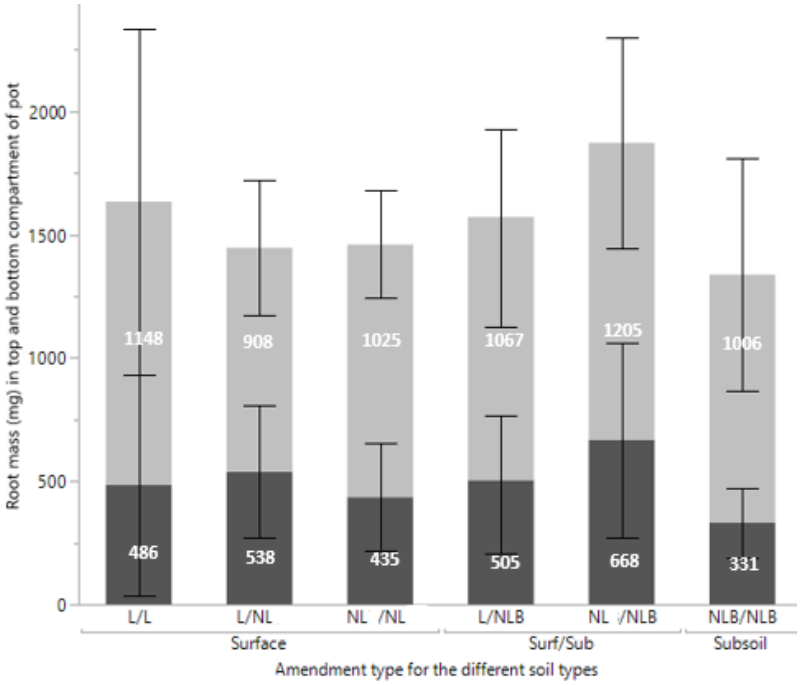


Figure 4.6: The mass of roots (mg) in the top and bottom compartment of the pot.

Interpretation of the origin of Cd in cacao seedlings

The weight of the roots did not differ throughout the different treatments. Contrasting are the fractions of Cd (Figure 4.5), where liming and soil type are both influencing the sourcing of Cd. For the 'reference' treatments without amendment and with only one soil type (NL/NL), the uptake is only marginally higher in the top compartment. There is more root biomass in the top layer present (70%), thus the roots in the top layer may be less 'active' in the uptake of Cd than the roots in the bottom layer when comparing them on weight basis. The data suggests that the roots in the top tend to get more active in the uptake of Cd when the seedling is growing on either i) contrasting soils or ii) soils with liming in the top and bottom compartment.

The first effect could be addressed to the change in bioavailable Cd in the bottom layer. However, it may also be related to the fact that the roots of cacao are a dynamic system in which the fine roots evolve according to water and nutrient accessibility [54, 99]. Since there are more nutrients available in the surface layer (see Table 4.2), the fine root architecture might be affected as was the case in the study of Zobel et al. (2007) where the fine roots of cacao became longer and thinner with increasing nitrate concentrations in the soil [99]. The second effect is conflicting since the availability of the nutrients (and Cd) in both layers should be similar, hence it is contradictory that the contribution from the top layer increases remarkably.

Nonetheless, the overall (logic) trends on the sourcing of Cd in cacao leaves which both relate to the bioavailability of Cd in the soil:

- liming in the top reduces the contribution of Cd in the leaves from the top layer,
- relatively less Cd is taken up from the bottom compartment consisting out of subsoil.

4.4 Concentrations of Cd with the contributions from top and bottom compartment

The interpretation of the fractions is confusing since it does not take into account the total concentrations of Cd in the leaves. To that end, the contribution of the two rooted soil compartment to the Cd concentrations in the leaf is visualized in Figure 4.7. Primarily, the effect of the soil type on the Cd leaf concentration is not only due to the change of bioavailability. The concentration in the top layer increases from 5.67 mg Cd/kg leaf to 6.78 mg Cd/kg, although the same soil type with the same bioavailable Cd is present here (surface soil). Therefore, it is suggested that the root activity in this top layer increases because of the better conditions in the surface soil compared to the subsoil. This may also explain the lower decrease in Cd concentrations in the treatments with contrasting soils than expected based on the available Cd (see section 4.1.2). Secondly, the concentration in the bottom compartments increases when the top layer is limed (from NL/NL (4.46 mg Cd/kg leaf) to L/NL (5.47 mg Cd/kg leaf) and from NL/NLB (2.38 mg Cd/kg leaf) to (2.87 mg Cd/kg leaf). Following previous reasoning, it may be that the activity of the roots increases in the bottom layer because liming is negatively influencing the environment for the roots. The dose applied may have been too high for the cacao seedlings, resulting in the direction of the roots to the bottom layer [90]. This may also attribute to the lower reduction than expected in this pot experiment and the possibility to counteract the Cd immobilization since these deeper soil layers are not targeted when applying lime on the top (see section 4.2.2). Another dose of lime may have lead to the improvement of the root structure in the top layer [90, 54]. Nonetheless, the dose used in this study corresponds to values applied in the field.

The bioavailability of Cd is the major factor controlling the Cd concentration patterns in cacao seedlings. However, previous sections show that the effects of the different treatments may not be entirely attributed to the bioavailable Cd. A first key message of this thesis may be that more Cd is taken from the surface layer. More Cd is

available here and roots are more likely to be active in the subsoil since the conditions for the roots are better. Secondly, liming only the top soil may not be effective when the underlying layer contains high Cd (like in the L/NL-treatment in Fig. 4.7). Application of lime in the top layer increases the root activity in the bottom layer which may counteract the desired reduction in Cd uptake if the availability of Cd is high in this soil layer.

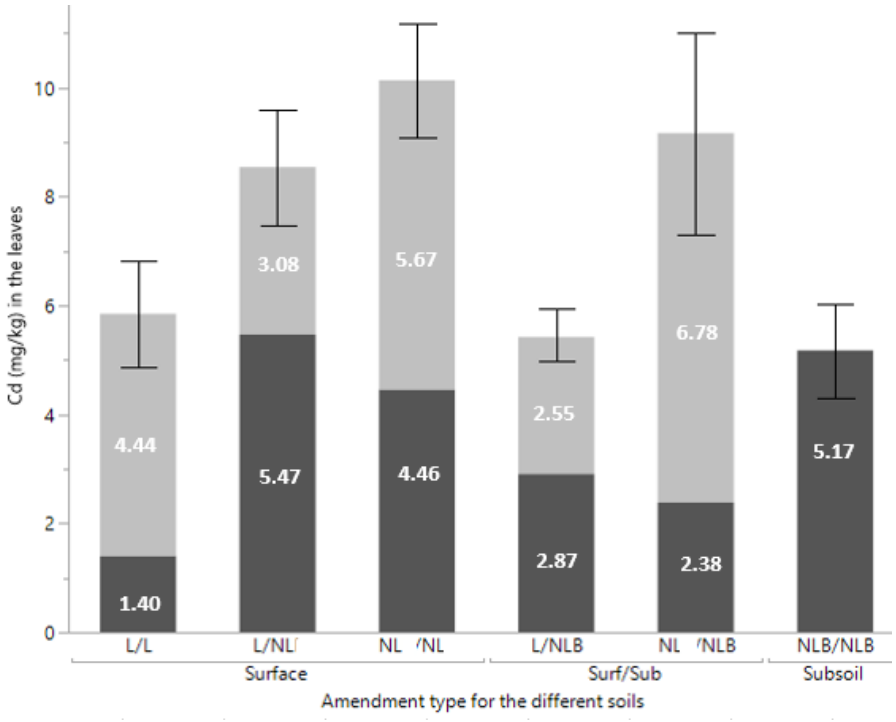


Figure 4.7: Cd concentrations (mg/kg leaf) (*aqua regia* soluble) with the contribution of the two soil compartments.
The dark grey bar indicates the contribution from the bottom compartment, the light grey bar from the top compartment according to the fractions of Fig. 4.5. For the seedlings grown on the subsoil, there is no calculation for the contribution of both compartments carried out. The error bar indicates the standard deviation of the total Cd in the leaf.

4.5 Elemental composition of the leaves

To see how the different nutrients are interacting with each other in the cacao seedlings, the leaf mineral composition is shown in Table 4.4. The values for P, K and Ca (N content was not measured) fall within the range of adequate nutrient concentrations for normal growth (according to Wessel [92], see Table 2.3). As a consequence, the seedling was probably not disturbed in its growth. This is confirmed by the leaf biomass (dry weight), where no significant differences were reported between the different treatments. The concentration of the nutrients was quite constant for all treatments.

Uptake patterns of divalent ions

When glancing at the trends of the concentrations of divalent ions in the cacao seedlings, a similar trend manifests in the concentrations of Cd and Zn (Fig. 4.8). Hence, liming and soil type are affecting Zn concentrations in tissues in the same way as Cd. Although it is suggested that Zn concentrations are somewhat constant while Cd concentrations are varying in tissues of crops [82], in this experiment *Cd is acting like the chemical analog of Zn.* Overall, it looks like there is a synergistic effect playing between Zn and Cd in the leaves of the cacao seedlings.

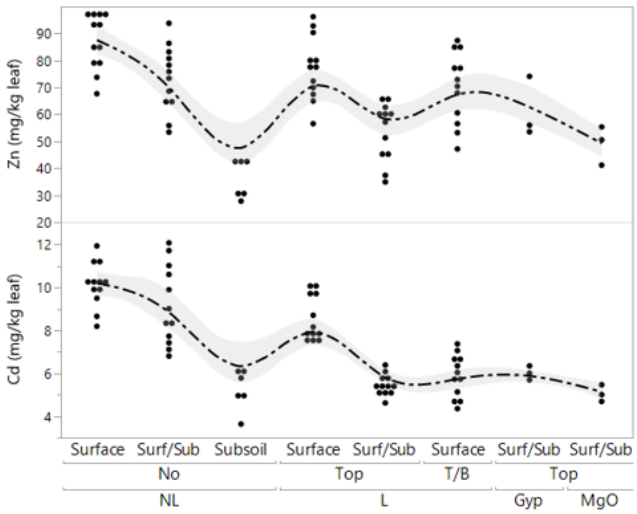


Figure 4.8: Concentrations of Cd and Zn in leaves for different treatments.

In cacao plantations of Peru a synergism between Cd and Zn is as well reported [4]. For wheat and corn tissues in field conditions there are synergistic effects between Cd and Zn, but under nursery conditions synergism and antagonism are both manifesting [68]. In lettuce and spinach leaves the interactions between Zn and Cd are complex and a function of the relative concentrations of Cd and Zn, plant species and plant tissue. However, a strong antagonistic Zn effect on Cd content in the leaf at low solution Cd was found in these crops [60]. In this experiment similar patterns manifest as in previous studies (see Fig. 4.9). In the subsoil seedlings, where the concentrations of Cd and Zn in the leaf are low, more Zn in the leaf is related with a lower Cd content in the leaf (antagonistic effect). For the conditions that are more realistic to the field (surface soil and surface on subsoil), an increase of Zn in the leaf is associated with a higher Cd content in the leaf (synergistic effect).

The concentrations of Mn and Ni in the leaves are peaking in the seedlings grown on the subsoil whereas the the concentrations of Cd and Zn are plunging in these seedlings (Table 4.4). The belief is that Cd^{2+} is introduced into plant cells by Fe^{2+}, Ca^{2+} and Zn^{2+} transporters/channels of low specificity [19]. In the leaves grown on the subsoil the concentration of Cd reduced with 49%, which can be addressed to the lower concentration of Cd in the subsoil. However, looking at the concentrations in the leaves it may also be attributed to a higher competition effect at the divalent ion-carrier sites of the cacao roots because the uptake of Zn, Mn and Ni is antagonistic. Questions arising with this hypothesis are i) why is there only an antagonistic effect between Cd and Zn when growing on the subsoil, ii) why is the seedling taking up more Mn and Ni in this soil type (in the data of the soil there is no significant difference in Mn and Ni content between subsoil and surface soil (Table 4.2)). Therefore, further research should investigate cationic interactions in the rhizosphere of cacao, to truly understand what is affecting the uptake of Cd by roots.

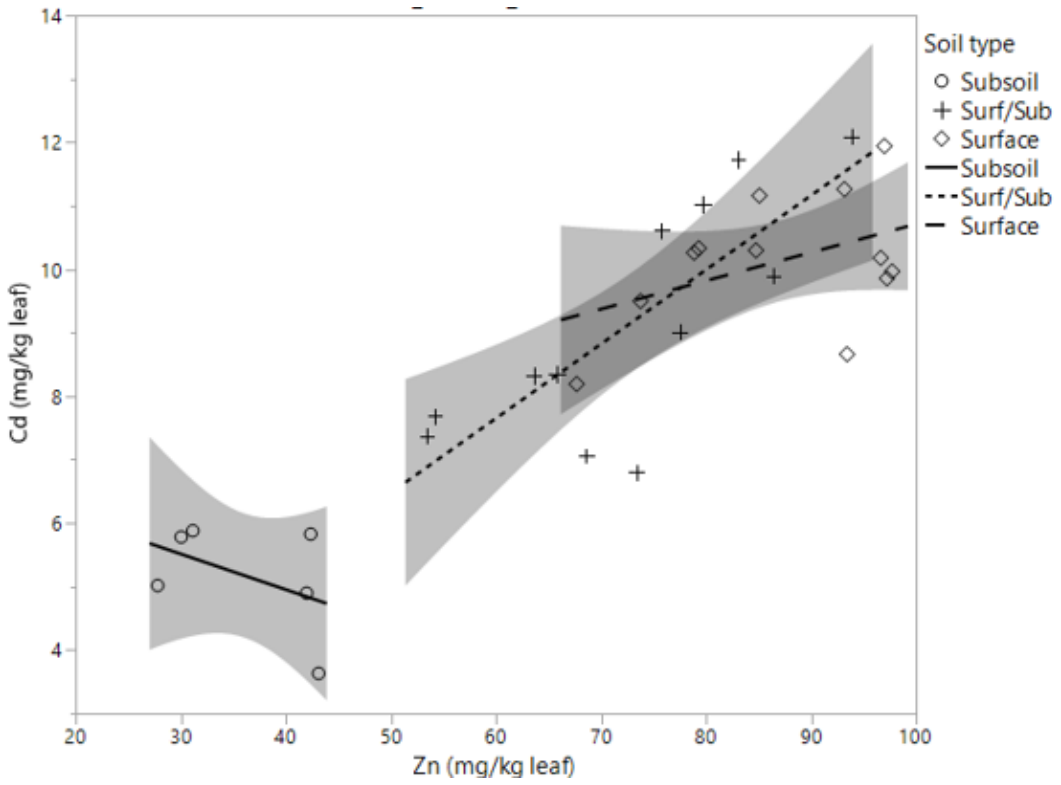


Figure 4.9: Relation between Cd and Zn in the leaf for the different soil types. The grey area is the confidence region for the fitted line ($\alpha = 0.05$).
Surface soil: Cd= $6.25 + 0.045{*}$Zn (R^2 =0.19)
Surface on subsoil: Cd= $-0.62 + 0.12{*}$Zn (R^2 =0.61)
Subsoil: Cd= $7.20 - 0.056{*}$Zn (R^2 =0.21)

Table 4.4: The leaf mineral composition for all treatments (mg/kg leaf) (*aqua regia* soluble)

Amend.	**Compartm.**	**Soil type**	P (g/kg)	K (g/kg)	Ca (g/kg)	Mn (g/kg)	Fe (mg/kg)	Zn (mg/kg)	Cd (mg/kg)
No		Subsoil	2.21	18.5	17.3	1.58	209	36.1	5.17
		Surf/Sub	2.01	17.8	19.2	1.43	137	72.9	9.16
		Surface	2.52	19.9	19.1	1.28	120	87.1	10.1
$CaCO_3$	Top + bottom	Surface	2.40	20.0	19.2	0.72	177	69.8	5.85
	Top	Surf/Sub	2.55	20.3	17.2	0.94	115	53.8	5.42
	Top	Surface	2.40	20.1	19.1	1.04	147	76.8	8.54
MgO	Top	Surf/Sub	2.57	20.6	16.4	1.11	132	49.0	5.05
$CaSO_4$	Top	Surf/Sub	2.43	21.5	15.87	1.2	201	60.8	5.98

Chapter 5

Conclusions

> "What is the role of the root distribution and bioavailable Cd on the uptake of Cd in cacao seedlings?"

This research aimed to unravel why the uptake of Cd was not always reduced when applying lime to the soil in field conditions, by focusing on the relation between the roots and the soil characteristics in the surface and subsoils of cacao plantations. It was hypothesized that the surface and subsoil roots were acting differently in the uptake of Cd, leading to different Cd uptake patterns when applying lime.

Key findings

The vertical heterogeneity in bioavailable Cd of the soils indeed affected the concentration of Cd in cacao leaves. In the homogeneous pots (surface soil, subsoil only) the total soil Cd concentration could explain the 50% reduction between these treatments since in the subsoil half the amount of Cd was present. In the contrasting soil treatments (surface on subsoil), the reduction in leaf Cd concentration (10%) was less than expected based on total soil Cd concentrations. The stable isotope study indicated that Cd derived from the top layer increased with 18% relative to the bottom layer. Hence, the cacao roots tended to be more active in the uptake of Cd in the nutrient-rich surface layer than in the subsoil, reducing the uptake of Cd less than would be expected based on bioavailable Cd.

In pots with surface soil the application of lime in both compartments reduced Cd in the leaves with 42%, whereas when only top compartment was limed the reduction was 16%. For this treatment, most of Cd in the leaf derived from the bottom compartment (64%). This result reveals the dynamics of cacao roots and the possibility to counteract the Cd immobilization since uptake of Cd from the bottom layer increased. All amendments applied to the contrasting soil treatments (lime, gypsum, MgO) were reducing the uptake of Cd in the cacao leaves by more or less the same rates (35-45%). Increasing pH by applying lime and MgO reduces the availability of Cd. For gypsum, the driver behind the decline in the uptake of Cd is the enhancement of Ca in the soil solution, leading to augmented competition at the cacao root binding sites.

In conclusion, bioavailable Cd has the most important role in the determination of the uptake pattern of Cd by cacao. The deviations from the expectations based on the Cd concentrations in the soil may be attributed to the changing root activity in the surface and subsoil, according to the environmental conditions.

Mitigation strategies to reduce Cd uptake by cacao trees

The amendments $CaCO_3$, MgO, $CaSO_4$ are clearly reducing the uptake of Cd which is confirming other experiments performed in greenhouse conditions. Our data suggest why on the field this desired result is not consistent for crops with deep penetrating roots. When in the field the same pattern applies as in the pots of this experiment where more Cd is taken from lower layers when liming the top layer, the targeted Cd immobilization may possibly be counteracted. Hence, caution is needed when applying only lime to the surface soil in case the subsoil contains also a high concentration in Cd. In this scenario, it is necessary to lime the surface and subsurface layers which is to be avoided because of the challenging practicalities and the correspondingly high costs. For the MgO a similar mitigation strategy

can be expected since it behaves analogously to $CaCO_3$. Furthermore, the gypsum amendment deserves as well some caution. The effects on the uptake of Cd are dependent upon the crop and the soil type used. Our study on gypsum was very limited (n=3) and may be rather seen as a preliminary study. The solubility experiment in appendix A showed that the solubility of Cd increased when applying gypsum, however, due to the high DOM-content and activity of Ca in the soil, the uptake in the cacao seedlings may have reduced. Nonetheless, it is unclear if this effect will be similar in other soil and crop types, hence some further research may be required before advocating gypsum as a mitigation strategy to reduce Cd in crops.

Recommendations for further research

Our data suggest that the uptake is changing by the different bioavailability of mineral elements in the soil, but the mechanism behind the uptake at the roots is still a mystery. Therefore, in further research, it is recommended to focus more on the fine root dynamics of the cacao tree. In this research, there was a lack of knowledge about the real root architecture of the seedlings, since only the biomass was measured and literature states that this may not be a good measure for root activity. Furthermore, more knowledge about the different rooting structures of cacao cultivars is necessary, since there is a wide variety in cacao rooting structures. With these insights, a more adequate extrapolation of our findings to the field may be performed.

For the cases where liming and application of gypsum are not appropriate, another interesting mitigation method may be the screening of different cacao cultivars with low Cd uptake potential. Therefore, the unraveling of the Cd transfer from soil to bean with attention to Cd uptake and transport pathways seems a next step to lower Cd concentrations in cacao beans.

Nonetheless, we believe that this thesis reveals some interesting findings of the relation between bioavailable Cd and cacao and can be beneficial for the small-scale farmers in Latin-America. The study shows that liming is effective in most cases because generally, the availability of Cd is lower in the subsoil, so liming the topsoil will decrease the uptake of Cd. Therefore, farmers can consider the application of lime in their search to a sustainable, cost-effective measure to reduce the Cd concentrations in cacao beans.

Appendix A

Effect of gypsum on the soil solubility of Cd

A.1 Introduction

Gypsum ($CaSO_4$) is a traditional soil amendment that is capable of reaching deeper soil layers because of its high mobility [14]. The application of gypsum in the surface soil of the previous pot experiment decreased the concentrations of Cd in the leaves of the cacao seedlings with a factor 1.5 compared to the control (see Fig. 4.2). Since gypsum was not affecting pH, curiosity was raised about the mechanism behind this reduction. To that end, the effect of gypsum on the solubility of Cd in soil solution was investigated. Possible hypotheses for the decrease in the uptake were that i) Cd was retained more in the soil solid phase by the increment of negative charges (SO_4^{2-}) on the exchange sites [14, 49], ii) dissolution of gypsum increases soluble SO_4^{2-} which can form $CdSO_4^0$ complexes which are not available for plants [61] or iii) the increased concentration of Ca^{2+} in solution, augmented the competition with Cd^{2+} on the binding sites of the cacao roots [18, 61, 49]. To explain the observed reduction of Cd in the cacao leaves, a short-term batch experiment was conducted with the surface soil and subsoil used in the pot trial to determine the effect of gypsum on the concentration and speciation of Cd in solution.

A.2 Materials and methods

Determination of solubility of Cd as affected by different amendments

The two soils of the previous pot experiment were air-dried and sieved (< 2mm) and weighted (10.0 g for each treatment). Besides the gypsum ($CaSO_4$) treatment, three other amendments were used for this solubility experiment. The Na_2SO_4 amendment was applied to determine the effect of the index cation Ca^{2+} compared to the gypsum treatment. Also, a treatment with $NaNO_3$ was added, to have treatments with equivalent Na salinity, so that the effect of sulfate could be researched. Lastly, a CaO treatment was included, to notify the difference in chemistry between the lime and the gypsum treatments. The amendments were applied to the soils with different concentrations to obtain equal change of ionic strength in soil solution (except for CaO treatments). For the $CaSO_4$, Na_2SO_4 and CaO treatments concentrations of 0, 5, 15, 25 mM were used, for the $NaNO_3$ treatments concentrations of 10, 30 and 50 mM. The soil solution concentrations of Cd and other elements were measured with a 0.001 M $CaCl_2$ extract [25]. Ten mL of 1 mM $CaCl_2$ was added to the soils, to obtain a 1:1 extraction ratio. The extraction solutions with the different amendments were placed on the end-over-end shaker for 24 hours. Afterward, the pH was measured in the suspension. Then, the samples were centrifuged for 15 minutes at 3000 RPM. Cadmium concentrations in the supernatant were measured in the ICP-MS (10x dilution). Concentrations of Na, Mg, K, S, Al, Fe, Mn, Zn, Ca and P were determined with ICP-OES (10x dilution). The DOC in each sample was as well measured with a TOC (total organic carbon) analyser (Shimadzu, TOC-L). Each treatment was duplicated to assure the quality of the measurements.

Speciation of Cd in soil solution

The speciation of Cd in the soil solution was predicted with Visual MINTEQ ver. 3.1, using the measured pH and total elemental concentrations in the solution extract as input data. Sulphur and phosphorus were assumed to be present as sulphate and phosphate. The concentrations of Cu were added based on the total

elemental concentrations from *aqua regia* digestion of the soil. Complexation of Cd by organic ligands was as well considered, by adding the DOC values in the input data. Precipitation of gypsum and lime (CaO) was allowed by adding them as possible solid phases.

A.3 Results

Total dissolved Cd

The measurements of pH and total dissolved Cd together with the modeled species are summarized in Table A.1 (surface soil) and Table A.2 (subsoil). The effect of the amendment on the soil pH was only significant ($p<0.05$) for the liming treatment (CaO) in the subsoil where soil pH increased with one unit. The application of CaO did only decrease solution Cd in the subsoil. For the surface soil, there was no reduction, however the pH change was also less pronounced. There was an increase in total dissolved Cd concentrations for the treatments with $NaNO_3$, $CaSO_4$, and Na_2SO_4. Remarkable are the concentrations of dissolved Cd in the surface soil at 25 mM. They are nearly double as high for the $CaSO_4$ treatments compared to the Na_2SO_4 treatments. All treatments were affecting the ionic strength by similar rates, except for the CaO treatment that did not alter the ionic strength. The DOM in solution increased as well for all treatments by similar rates, however, for the CaO treatments there was a sharp decrease in DOM.

Modeled species

The activities of Cd^{2+} increased for the $CaSO_4$, $NaNO_3$, and Na_2SO_4 treatments, but the increase was more pronounced for the first two treatments. The activities of this free ion were greater in the subsoil than in the surface soil (about three times higher). The activities of $CdSO_4{}^0$ were similar in surface and subsoil and were clearly enhanced in the sulfate treatments. In both soils, the levels of Cd bound to dissolved organic matter (Cd-DOM) significantly increased for $CaSO_4$, Na_2SO_4, and $NaNO_3$

treatments. The effect was especially marked for the $CaSO_4$ treatment in the surface soil where the Cd-DOM species increased with a factor 6.2 compared to the control treatment.

A.4 Discussion

In this experiment, gypsum is clearly enhancing Cd in solution (in both soils). At first sight this was surprising since there was such a marked reduction in the concentrations of Cd in the cacao leaves when gypsum was applied (see Fig. 4.2). Contrasting are the liming treatments (CaO) where Cd in solution decreased as expected according to the pot experiment and literature stating that soil pH is the primary statistical factor explaining Cd in soil solution [10, 26, 82] (although the effect is not really pronounced in the surface soil).

Effect of ionic strength and index cation Ca on the soil solubility of Cd

A first observation was that *increasing the ionic strength is enhancing Cd in solution.* The study of Naidu et al. (1994) suggests that for variable charge soils, increasing the ionic strength to a pH value above the PZC (point of net zero charge) of the soil, enhances Cd in solution by a decrease in negative potential in the plane of sorption [66]. For the Na_2SO_4 treatments, the effect of Cd desorption is less strong than for the other treatments. This may be addressed to the greater activity of Ca^{2+} in soil solution for the $CaSO_4$ and $NaNO_3$ treatments compared to the Na_2SO_4 treatments. Hence, a second observation was that *Ca^{2+}-ions may compete with Cd^{2+}-ions on the soil surface exchange sites, increasing the activity of Cd in soil solution.* This is also confirmed by the study of Naidu et al. (1994) where adsorption of Cd approximately doubled when Na instead of Ca was used as the index cation, since Na^+ is less competitive with Cd for adsorption sites than Ca^{2+} [66]. These results are supported by a study of Christensen (1984) [18], who relates the increase in soil solution Cd concentrations to the displacement of Cd by Ca from exchange sites on soil into the soil solution. Both $CaSO_4$ and $NaNO_3$ are increasing Ca^{2+}

in soil solution, hence the increase is not only related to the addition of Ca when applying gypsum on the soil, but also to the increase in ionic strength. The Na_2SO_4 treatment is also increasing ionic strength, but for this treatment, the activity of Ca^{2+} has not increased due to complexation of Ca with sulfate ($CaSO_4$).

Effect of the amendments on the speciation of Cd in solution

In the $CaSO_4$ and Na_2SO_4 treatments, Cd is also complexating with sulfate to form $CdSO_4{}^0$. Literature is ambiguous about the fact if this complex is available for crops or not [61]. However, whether it is available for plants or not, in soils with large amount of organic matter, the complexation of Cd with DOM (dissolved organic matter) is of way greater importance as can be seen in both tables. Most of the Cd is bound to the DOM in both soils. The main difference in the solution of the surface soil and subsoil is the concentration of DOM (two times higher for the surface soil). This results in a lower activity of Cd^{2+}-ion in the surface soil. Contrasting is the higher activity of Ca^{2+} in the surface soils. Thus, increasing DOM will markedly decrease the free ion activity of Cd^{2+}, whilst it even slightly increases the activity of Ca^{2+}. Therefore, it may be possible that gypsum is reducing the uptake of Cd by plants when DOM is sufficiently present in the soil since Cd is more bound to the DOM (which is not available for the plant) and the increased activity of Ca is competing with Cd at the transmembrane carriers of the roots.

A.5 Conclusion

Gypsum increased Cd in the soil solution of the surface and subsoil. The ionic strength and the increased activity of Ca^{2+}-ions were clearly the main drivers behind the desorption of Cd since pH was not affected. However, gypsum decreased the uptake of Cd in the cacao seedlings. By this solubility experiment, we can cross out the hypothesis that Cd retention was enhanced by an increment of negative charges on the exchange sites in the soils. The following hypothesis was that gypsum increased the possibility for Cd to form complexes that are not available for plants.

The increase in DOM complexes was most remarkable, especially in the surface soil with high DOM content and these complexes are definitely not available for plants. Also, $CdSO4^0$ was formed in the sulfate treatments (but to a minor extent), which may also decrease the phytoavailability for plants. However, there was still an increased activity of the free ion Cd^{2+} when applying gypsum, so the decrease in uptake cannot be solely addressed to the formation of non-available complexes. To that end, we suggest that gypsum is increasing the competition of Ca with Cd thoroughly at the cacao root binding sites and that this competition effect is most pronounced in soils with high DOM content since Cd has higher affinity for DOM than Ca.

Table A.1: Effect of amendment added on the activity of different species in surface soil solution

Amend.	Conc.	pH	IS	DOC	Tot. Diss. Cd	Cd-DOM	Cd^{+2}	$CdSO4^{0}$	Ca^{+2}	$CaSO_4$
	mmol/L		M	mg/L	nM	nM	nM	nM	mM	µM
$CaSO_4$	0	5.5	0.004	118	13.6	13.0	0.54	0.03	0.44	32.7
	5	5.3	0.014	118	32.9	28.7	1.30	0.79	1.90	529
	15	5.2	0.034	123	76.8	56.1	2.68	5.94	6.55	2371
	25	5.2	0.050	135	121	80.6	3.68	13.42	10.60	4553
Na_2SO_4	0	5.5	0.004	118	13.6	13.0	0.54	0.03	0.44	32.7
	5	5.4	0.015	150	28.1	26.3	0.79	0.40	0.76	406
	15	5.3	0.035	141	45.0	39.6	1.08	1.77	1.55	1209
	25	5.3	0.059	144	64.7	53.7	1.29	4.13	2.40	2170
CaO	0	5.5	0.004	118	13.6	13.0	0.54	0.03	0.44	32.7
	5	6.1	0.006	18.7	10.7	2.9	0.78	0.29	4.79	46.4
	25	6.2	0.007	17.5	14.9	3.7	0.97	0.49	6.68	69.0
$NaNO_3$	0	5.5	0.004	118	13.6	13.0	0.54	0.03	0.44	32.7
	10	5.3	0.012	118	31.9	27.6	1.41	0.09	2.39	50.0
	30	5.2	0.024	122	54.3	43.7	2.27	0.14	5.23	58.0
	50	5.2	0.037	119	75.4	57.4	2.94	0.31	8.03	110

Table A.2: Effect of amendment added on the activity of different species in subsoil solution

Amend.	Conc.	pH	IS	DOC	Tot. Diss. Cd	Cd-DOM	Cd^{+2}	$CdSO4^0$	Ca^{+2}	$CaSO_4$
	mmol/L		M	mg/L	nM	nM	nM	nM	mM	µM
$CaSO_4$	0	5.2	0.003	53.7	8.96	7.71	0.83	0.01	0.48	7.18
	5	5.1	0.014	62.0	21.3	15.5	2.67	0.94	1.51	516
	15	5.0	0.026	73.0	48.2	30.4	6.39	4.55	2.36	1643
	25	5.0	0.042	81.0	82.2	44.0	10.89	11.80	3.60	3810
Na_2SO_4	0	5.2	0.003	53.7	8.96	7.71	0.83	0.01	0.48	7.18
	5	5.2	0.010	60.6	38.3	30.3	3.89	1.32	0.59	195
	15	5.2	0.029	70.8	49.6	37.5	3.80	3.79	0.79	766
	25	5.1	0.048	80.0	60.5	43.6	4.11	6.17	0.98	1430
CaO	0	5.2	0.003	53.7	8.96	7.71	0.83	0.01	0.48	7.18
	5	5.7	0.004	9.53	10.7	7.6	2.02	0.06	0.59	16.1
	15	6.2	0.005	12.1	2.7	1.8	0.56	0.03	0.76	40.3
$NaNO_3$	0	5.2	0.003	53.7	8.96	7.71	0.83	0.01	0.48	7.18
	10	5.0	0.010	63.4	46.5	31.5	8.85	0.07	1.24	9.71
	30	4.9	0.022	66.9	59.5	35.3	12.25	0.08	2.16	13.7
	50	4.8	0.034	68.4	71.7	39.0	15.07	0.09	2.73	15.7

Appendix B

Additional tables and figures

B.1 ANOVA

Prior to the one-way ANOVA analysis, the assumptions described in the Materials and Methods section, need to be fulfilled. The residuals of the dependent variable should be approximately normally distributed for each category of the independent variable, which was tested with the Shapiro-Wilk W test in JMP. Another assumption tested in JMP with the Bartlett test was the homogeneity of variances. When this assumption was not met, the Welch test was performed instead of an ANOVA. The significant results of the statistical tests performed during the experiment are reported in Table B.1.

Table B.1: Shapiro-Wilk and Bartlett-test and ANOVA/Welch results, p- values with * are significant on a 95% confidence interval.

Dependent var.	**Indep. var.**	**Shapiro-Wilk**	**Bartlett**	**ANOVA/ Welch**	
		p-value	p-value	F ratio	p-value
Cd conc. in leaf	Soil type	0.75	0.080	F(2,27)=25.2	<0.0001*
	Liming pos.	0.38	0.95	F(2,33)=53.7	<0.0001*
	Amendment	0.14	<0.0001*	F(3,7)=15.6	0.0019*
Fractions of Cd	Soil type	0.37	0.85	F(1,10)=7.11	0.024*
	Liming pos.	0.26	0.58	F(2,15)=15.9	0.0002*

B.2 Maps

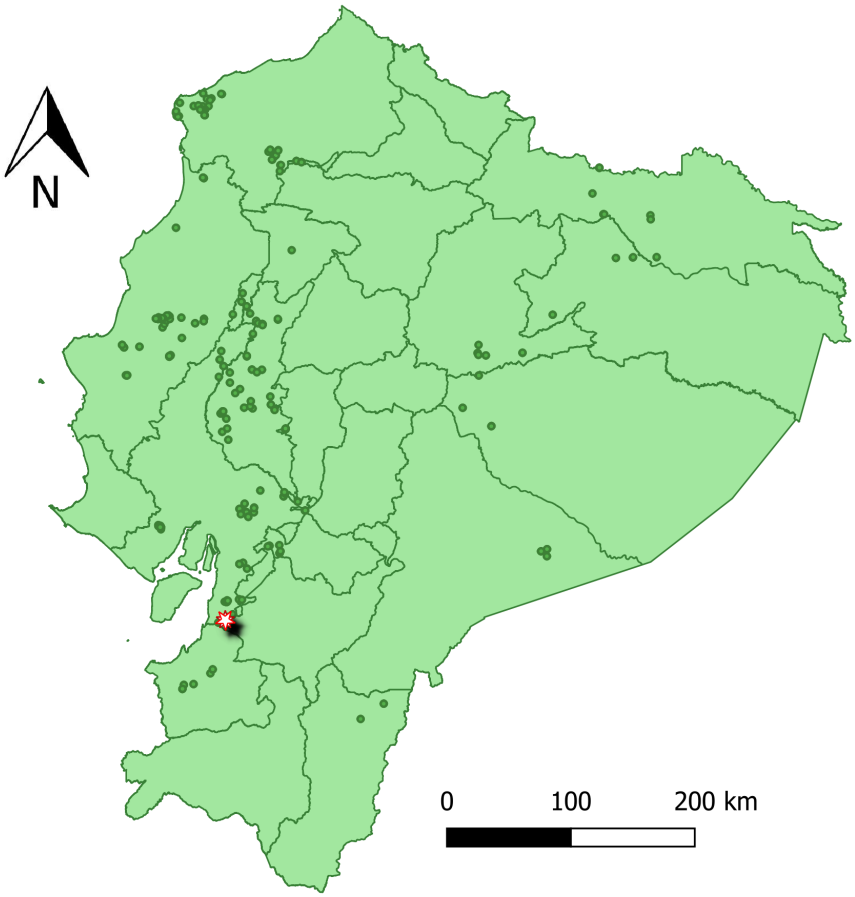


Figure B.1: Basemap of Ecuador with the sampled farms (green dots) in the survey of Arguello [5].
The star indicates the sampled farm for this experiment (in the province of Azuay).

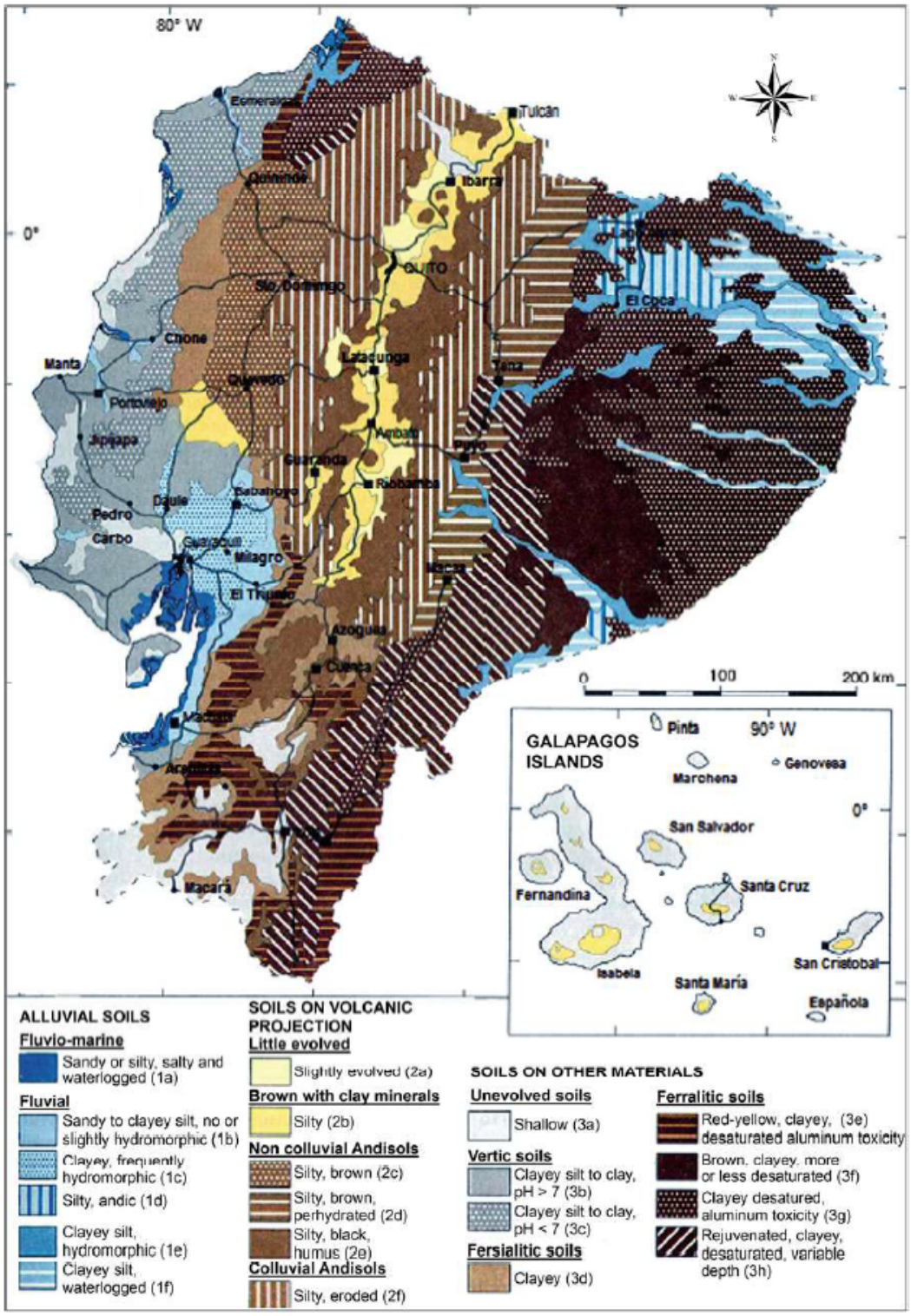


Figure B.2: Classification of Ecuadorian soils according to parent material [32]

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Popularized summary

Chocolate may contain cadmium, a toxic heavy metal. To protect chocolate consumers, the European Commission entered new threshold limits into force for cadmium. This limit largely affects the cacao producers in South America as the natural background concentrations of cadmium in the young soils of the Andes are high, resulting in the presence of cadmium in cacao beans. Hence, a major threat hangs over the cacao sector where 90% of the production is in the hand of resource-poor small-scale farmers. There is thus a great urgency to find strategies to reduce the concentration of cadmium in cacao.

A promising method is the application of lime on the soil, which is a traditional practice in agriculture to fight soil acidity. This soil amendment may also be appropriate to decrease the uptake of cadmium by cacao trees. However, in the field, the application of lime does not always lead to the expected decrease of cadmium. In this thesis we therefore investigated why this soil amendment was sometimes lacking the ability to reduce the uptake of cadmium by cacao trees. These perennial trees have deep rooting structures, thus it may be that liming only the upper layer of the soil is not sufficient for reducing the uptake of Cd. Hence, we researched which rooting structures (the surface and deep roots) are most responsible for the uptake of cadmium. The research was performed in pots with cacao seedlings that were grown for four months in Guayaquil, Ecuador.

We figured out that there is indeed a difference in the uptake of cadmium from the two rooting structures. The roots like to settle more in the nutrient-rich surface soil compared to the deeper soil layers and consequently, the uptake of cadmium is higher in this surface soil. However, when this surface layer is limed, the total Cd uptake by the plants decreases. But it decreases less than expected when the bottom layer is rich in Cd since the uptake from this compartment slightly increases. To that end, we only recommend the application of lime when the subsoil layers are not rich in cadmium. Otherwise, we suggest using amendments that are capable of reaching deeper soil layers.