Cacao (*Theobroma cacao* L.) in Peru:
Its varieties, quality potential, and risk for cacao farmer and final consumer

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Summary

Cacao (*Theobroma cacao* L.) is one of the most consumed luxury foods worldwide. Sweet industries are producing chocolate products especially for children but also for adults (then normally with higher cocoa concentration). Cocoa products originate basically from the cacao tree variations: the conventional bulk cacao trees (Forasteros) and fine and flavor cacao trees (Criollos) which are characterized by higher quality and special flavor compounds. Bulk cacaos are mostly cultivated in Africa with about 80% of the worldwide market. Only 5% of the cacao production is of fine and flavor cacaos and can be found especially in South America. Since the 1980th cacao is grown on a big scale in Peru supported the United Nations (UNODC) to replace illegal coca production by legal cacao cultivation as a stable income source for Peruvian farmers. During the first project phase, high-yield cacao clones with pronounced disease resistance such as the bulk cacao clone CCN-51 were spread to ensure reliable yields. In a second phase, also fine and flavor cacaos with higher organoleptic quality were promoted because of a higher stock price on the world-market and therefore higher income for farmers. Nowadays, cacaos native to Peru are increasingly discovered as a unique treasure for South America due to their outstanding flavor compositions. These cacaos with a high genetic variation were found in different regions of Peruvian Amazonia but until now genetic and quality analyses based on the identification of secondary metabolites and the description of yield characteristics (fruit-sizes, annual yields, disease susceptibilities etc.) are completely missing. Studies on the genetics of *Theobroma cacao* provide evidence for the origin of this species in the Amazonian lowlands and even for a complete revision of the traditional cacao classification in Forasteros, Criollos and Trinitarios (Forastero x Criollo hybrids). However, most studies used exclusively cacao material from different clone gardens in South America, whereas the analysis of native cacao samples from field collections in different Peruvian regions never have been done so far and may promise the discovery of further native varieties with unknown characteristics. Such novel cacaos may profoundly change the entire fine and flavor cacao market and provide farmers with safe income. However, the cacao tree also has its dark side known to be an accumulator of toxic metals. Several heavy metals (cadmium, copper, lead) were reported to exceed the legal limits in cocoa products permitted in the European Union regarding to the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO). Heavy metals such as cadmium (Cd), copper (Cu), and lead (Pb) can already be hazardous at small amounts. In particular children who consume chocolate as sweets are at risk to suffer heavy metal intoxication. Cocoa from South America has been reported to

exceed allowed Cd limits, whereas cocoa from Africa is more often polluted with Pb. Literature about other toxic metal pollution in cacao is scarce despite of the high health risk for humans. Several toxic metals have never (such as Cobalt and Manganese) or hardly (Aluminum, Nickel, Zinc) been studied in cocoa products. This work aimed to analyze a wide range of cacao types collected in Peruvian Amazonia in terms of their quality and yield characteristics and their accumulation of toxic metals in seeds. Overall, I sampled 328 cacao trees in three mayor cacao growing regions of the Peruvian lowlands over the years 2014 and 2016-2018. Cacao samples included the high-producing clone CCN-51, fine and flavor cacao clones, and native cacaos from the northern Cusco region, the central Huánuco region, and the middle-southern San Martin region in Peru. The sample were compared in terms of secondary metabolites in cocoa powder, yield parameters, disease susceptibility, and in toxic metal uptake and contents. Moreover, the influence of the farm management (e.g., pruning frequency, fertilizer use, age of trees, fruit set), and the environment (e.g., soil pH, electrical conductivity, soil toxic metal content, altitude a.s.l.) on secondary metabolites, yield parameters, disease susceptibility, and toxic metal uptake and contents in cacao seeds were analyzed. In this thesis, unfermented cocoa beans that have been processed into cocoa powder have been referred to as "cocoa powder", while other cocoa products are referred to as "cocoa" only after fermentation, and unfermented cacao seeds as "cacao seeds". In general, the native cacaos from Cusco showed the highest polyphenol contents (catechin hydrate: 0.11%; cyanidin arabinoside: 0.23%; cyanidin galactoside: 0.11%) except of epicatechin which was highest in the native cacaos from San Martin with 2.6%. Native cacaos from Huánuco, in contrast, showed overall the lowest polyphenol contents. Statistics revealed that polyphenol contents raised with the age of cacao trees and oldest trees were especially found in the Cusco Region. Also, caffeine as an important quality indicator was highest in native Cusco cacaos (1.15%), but lowest in native Huánuco cacaos (0.37%), and did not differ significantly between CCN-51, fine and flavor clones, and natives from San Martin. Theobromine with 2.92% in contrast was highest in CCN-51 and lowest in fine and flavor clones with 2.34%. Comparing the characteristics of the cacao types, the high-yield clone CCN-51 showed the longest fruits (23.8 cm) and biggest fruit-perimeters (31.2 cm), whereas native cacaos from Cusco were characterized by the shortest fruits (17.8 cm). The fine and flavor clones with the smallest fruit-perimeters (26.9 cm) also had the lowest annual yields (340 kg ha^{-1}), in contrast to the native cacaos from San Martin with the highest yields (1,516 kg ha^{-1}) even exceeding CCN-51 (1,176 kg ha^{-1}). In general, cacao clones (CCN-51, fine and flavor clones) were less infested with the fungi *Moniliophthora perniciosa*, *Moniliophthora roreri*, and *Phytophthora* sp., but more frequently attacked by insects compared with the native cacaos. Additionally, farm management and the environment were influencing secondary metabolites in cacao and yield parameters. Polyphenols decreased significantly at higher soil pH, whereby epicatechin and catechin hydrate also decreased with higher soil electrical conductivity. Epicatechin and the anthocyanins were higher when insect attacks were more frequent. In contrast, theobromine contents were positively correlated with the pruning frequency and caffeine content negatively. Comparing the toxic metal contents between the three regions, less toxic cobalt (Co: 1.13 mg kg^{-1}), copper (Cu 49.65 mg kg^{-1}), manganese (Mn: 78.54 mg kg^{-1}), and nickel (Ni: 27.17 mg kg^{-1}) were highest in cocoa powder samples from the Cusco Region. In the Huánuco Region, the highly hazardous Cd (2.13 mg kg^{-1}) and Pb (0.09 mg kg^{-1}), along with zinc (Zn: 84.76 mg kg^{-1}) showed highest concentrations, whereas San Martin was characterized by the highest Aluminum (Al) contents (16.56 mg kg^{-1}). Regarding to the legal limits for Cd, Cu, and Pb in the European Union, about 49% of all samples exceeded the Cd limit of 0.6 mg kg^{-1} in cocoa powder with a maximum value measured in Huánuco of 12.3 mg kg^{-1}. The limit of 50 mg kg^{-1} for Cu was exceeded in 39% of the cocoa powder samples with a maximum measured in Cusco of 86.5 mg kg^{-1}. Pb has a maximum allowed value of 1.0 mg kg^{-1} and 16% of the samples showed values higher than this limit. For formulating efficient mitigation measures of toxic metals in cacao seeds, I also analyzed the effects of farm management, and environmental factors and modelled toxic metal uptake from soil to cacao seeds and content in seeds using Boosted Regression Trees (BRT). BRTs revealed that Al, Cd, and Zn contents in cacao seeds decreased with cacao tree age, whereas Co and Cu were increased. Cd was the only toxic metal that raised in its content with the more frequent use of nitrogen (N), and phosphate (P) fertilizers. Annual pruning activities increased the transfer factors of Al, Cd, Cu, and Zn from the soil metal content to cacao seeds. Overall, the fine and flavor clones had higher toxic metal uptake rates than the other cacao types (Cd: 13.17; Co: 0.21; Cu: 3.52; Mn: 0.20; Ni: 1.35; Pb: 0.0073; Zn: 2.18). The CCN-51 had an even higher uptake of Cd with 13.26 which shows in general a trend of the clones to take up more toxic metals than native cacaos. Regarding environmental conditions, soil toxic metal content was among the most important predictors of toxic metal content in seeds. In addition, electrical conductivity and pH of soils were important predictors of toxic metal uptake with lower values leading to higher uptake of Al, Cd, Cu, Co, Mn, Ni, and Zn. The altitude a.s.l. was negatively correlated with Al, Cd, Cu, and Zn, but positively with Co and Ni. This dissertation is therefore the first study giving quality information, and tree characteristics about native cacaos from Peru showing their potential as high-quality cacao trees being cultivated on a bigger scale in future. Moreover, it demonstrates the actual risk of cacao consumption due to toxic metal pollution. To overcome this problem, I found various promising possibilities to mitigate toxic metal accumulation in cacao seeds. Mitigation strategies include soil pH and electric conductivity increase by liming and Mg amendment, the reduction of fertilization and pruning, and the use of cacao types with less pronounced toxic metal uptake such as the native cacaos.

Zusammenfassung

Kakao (*Theobroma cacao* L.) ist das weltweit am meisten konsumierte Genussmittel. Die Süßwarenindustrie stellt Schokoladenprodukte vor allem für Kinder her, aber auch für Erwachsene – und dann meist mit höherem Kakaoanteil. Kakaoprodukte stammen von zwei unterschiedlichen Kakaovarietäten: Den konventionellen Kakaos (sogenannte Forasteros) und den Edelkakaos (sog. Criollos), die besondere Geschmackqualitäten haben und höhere Preise am Weltmarkt erzielen. Konventioneller Kakao wird dabei zumeist in Afrika angebaut, wo 80% des gesamten Kakaos der Welt angebaut wird. Nur 5% des Kakaos weltweit gehören zu den Edelkakaos, die vor allem in Südamerika zu finden sind. Erst seit den 1980ern wird Kakao auch in Peru großflächig angebaut, was mit Unterstützung der vereinten Nationen (UNODC) vorangetrieben wurde, um den illegalen Kokaanbau in der Region zu verdrängen. In der ersten Projektphase wurden Hochleistungsklone wie der CCN-51 verteilt, der stabile Ernten versprach, jedoch zu den konventionellen Kakaos zählt. Später wurden auch Edelkakaoklone angebaut, die zwar krankheitsanfälliger sind, jedoch durch ihre höhere Qualität einen besseren Preis erzielen. Erst vor kurzem wurden heimische Kakaosorten aufgrund ihrer herausragenden geschmacklichen Eigenschaften als ein Schatz Südamerikas entdeckt. Diese ursprünglichen Kakaos besitzen eine hohe genetische Variabilität und wurden in den verschiedenen Regionen Perus gefunden. Bisher wurden sie jedoch kaum hinsichtlich ihrer Genetik, ihrer geschmacklichen Qualität (Kakaoinhaltsstoffe) sowie Erntecharakteristika (Fruchtgrößen, und -ernten, Krankheitsanfälligkeit, usw.) untersucht. Neuere genetische Studien sehen den Ursprung des Kakaobaums im amazonischen Tiefland und legen eine vollständige Revision der bisherigen Einteilung der Kakaovarietäten in die drei Gruppen Forasteros, Criollos und Trinitarios (Forastero x Criollo Hybride) nahe. Diese Studien basieren jedoch auf Kakaomaterial aus unterschiedlichen Klongärten Südamerikas. Wirkliche Feldstudien in verschiedenen Anbaugebieten Perus werden bisher jedoch nicht durchgeführt und versprechen deshalb die Entdeckung völlig neuer genetischer Variationen und vielversprechenden Edelkakaos. Solche neuen Kakaos könnten den weltweiten Edelkakaomarkt revolutionieren und für Bauern eine sichere Einnahmequelle bieten. Auf der anderen Seite hat der Kakaobaum jedoch auch seine Schattenseite, da er bekannt für Schwermetallbelastungen ist. Laut der Organisationen WHO (World Health Organization) und FAO (Food and Agriculture Organization of the United Nations), überschreiten Kakaoprodukte teilweise die europäischen Grenzwerte für Cadmium (Cd), Kupfer (Cu) und Blei (Pb). Diese Schwermetalle können bereits in kleinen Mengen vor allem für Kinder schwerwiegende gesundheitliche

Folgen haben. Kakaos aus Südamerika scheinen häufiger mit Cd belastet zu sein, während Kakao aus Afrika mehr Pb enthält. Im Allgemeinen gibt es jedoch trotz der gesundheitlichen Relevanz sehr wenig Literatur über Metallbelastungen im Kakao. Während über Kobalt (Co) und Mangan (Mn) in Kakao keine Literatur existiert, wurde über Aluminium (Al), Nickel (Ni) und Zink (Zn) nur mangelhaft berichtet. Diese Studie hat zum Ziel unterschiedliche peruanische Kakaos hinsichtlich ihrer Qualitäts- und Ernteparameter zu charakterisieren und ihre Eigenschaften in Bezug auf die Aufnahme und Akkumulation von toxischen Metallionen zu untersuchen. Insgesamt wurden im Zuge dieser Dissertation 328 Kakaobäume in drei Kakao-Hauptanbaugebieten Perus in den Jahren 2014, und 2016 bis 2018 beprobt. Das Inhaltsstoffprofil, Ernteparameter, Krankheitsanfälligkeit und Metallgehalte der verschiedenen Kakaotypen (native Kakaos aus Cusco, Huánuco und San Martin, CCN-51, Edelkakaoklone) wurden dabei miteinander verglichen und der Einfluss von Faktoren wie das Plantagenmanagement (z.B. Baumschnitt, Düngung, Alter der Bäume, Fruchtansatz) und Umweltbedingungen (z.B. Boden-pH, Leitfähigkeit, Bodenmetallgehalt, Höhe üNN) auf diese Eigenschaften untersucht. In dieser Arbeit wurden dabei die unfermentierten Kakaobohnen, die zu Kakaopulver verarbeitet worden sind als "cocoa powder" bezeichnet, während andere Kakaoprodukte erst nach einer Fermentation als „cocoa" bezeichnet werden und unfermentierte Kakaosamen als „cacao seeds". Die Ergebnisse zeigten, dass der heimische Kakao aus Cusco die höchsten Polyphenolgehalte (Catechin: 0,11%; Cyanidin-3-arabinosid: 0,23%; Cynidin-3-galaktosid: 0,11%) aufwies, mit Ausnahme von Epicatechin, welches am höchsten in nativen Kakaos aus San Martin war (2,6%). Im Gegensatz dazu zeigten heimische Kakaos aus Huánuco die geringsten Polyphenolgehalte. Zudem konnte ein Polyphenolgehaltanstieg in Kakaosamen mit dem Alter des Kakaobaums statistisch nachgewiesen werden, wobei die ältesten Bäume in Cusco gefunden wurden. Der Koffeingehalt, ein wichtiger Qualitätsparameter, war in heimischen Kakaos aus Cusco am höchsten (1,15%), aber am niedrigsten in heimischen Kakaos aus Huánuco (0,37%), während er zwischen den anderen Kakaotypen nicht signifikant variierte. CCN-51 zeigte hingegen den höchsten Theobromingehalt (2,92%) und die Edelkakaoklone den niedrigsten (2,34%). Bei den Erntecharakteristika zeigte der Hochleistungsklon CCN-51 die längsten Früchte (23,8 cm) und den größten Fruchtumfang (31,2 cm). Heimische Kakaos aus Cusco wiesen mit 17,8 cm die kleinsten Fruchtlängen auf. Die Edelkakaoklone hatten neben den kleinsten Fruchtumfängen auch die geringsten jährlichen Ernten (340 kg ha^{-1}) im Vergleich zum heimischen Kakao aus San Martin mit 1516 kg ha^{-1}, der sogar die Ernteparameter von CCN-51 (1176 kg ha^{-1}) übertraf. Die kommerziellen Kakaoklone (CCN-51, Edelkakaoklone) waren am wenigsten mit Pilzen befallen (*Moniliophthora perniciosa*, *Moniliophthora roreri*, and *Phytophthora* sp.), jedoch, verglichen mit den heimischen Kakaos, häufiger mit Insekten. Zusätzlich beeinflusste das Plantagenmanagement und Umweltbedingungen die Inhaltsstoffe in Kakaosamen sowie die Ernteparameter. Polyphenole in Kakaosamen sanken in ihrem Gehalt mit höherem Boden pH, wobei Epicatechin und Catechin auch bei steigender Bodenleitfähigkeit abnahmen. Epicatechin und die Anthocyane stiegen bei einem erhöhten Insektenbefall der Früchte an. Während Theobromin mit der Häufigkeit des Baumbeschnitts zunahm, sanken die Koffeinwerte in Kakaosamen. Der Gehalt and toxischen Metallionen in Kakaosamen unterschied sich deutlich zwischen den Anbauregionen. Cusco zeigte die höchsten Co (1,13 mg kg^{-1}), Cu (49,65 mg kg^{-1}), Mn (78,54 mg kg^{-1}) und Ni (27,17 mg kg^{-1}) Gehalte in Kakaosamen, während Cd (2,13 mg kg^{-1}), Pb (0,09 mg kg^{-1}) und Zn (84,76 mg kg^{-1}) am höchsten in Huánuco und Al (16,56 mg kg^{-1}) am höchsten in San Martin war. Bezogen auf die existierenden europäischen Grenzwerte, überschritten 49% der Kakaosamenproben den erlaubten Cd Wert von 0,6 mg kg^{-1} und der höchste gemessene Wert in Huánuco betrug 12,3 mg kg^{-1}. 39% der Kakaosamenproben überschritten den Grenzwert von Cu (50 mg kg^{-1}), während der Maximalwert 86,5 mg kg^{-1} (Cusco) betrug und 16% den Pb-Grenzwert von 1,0 mg kg^{-1}. Für die Formulierung von effizienten Minderungsmaßnahmen für toxische Metalle in Kakaofrüchten, wurden mithilfe von Boosted Regression Trees (BRT) Faktoren des Plantagenmanagement und der Umweltbedingungen auf ihren Einfluss auf die Metallaufnahme und -konzentration in Kakaosamen analysiert. Die Ergebnisse zeigten, dass Al, Cd und Zn Gehalte in Kakaosamen mit dem Alter der Kakaobäume abnahmen, während Co und Cu zunahmen. Cd nahm zudem in den Kakaosamen zu, wenn Stickstoff-(N-) oder Phosphat-(P-)Dünger verwendet wurden. Der jährliche Baumbeschnitt erhöhte die Aufnahmerate (den Transferfaktor) von Al, Cd, Cu und Zn aus dem Boden in Kakaosamen. Die toxischen Metalle variierten signifikant zwischen den Kakaotypen. Im Allgemeinen zeigten Edelkakaoklone die höchsten Transferfaktoren (Cd: 13,17; Co: 0,21; Cu: 3,52; Mn: 0,20; Ni: 1,35; Pb: 0,0073; Zn: 2,18). Der CCN-51 hatte sogar einen höheren Cd Transferfaktor mit 13,26, das den generellen Trend der Klone zeigt mehr toxische Metalle aufzunehmen als die nativen Kakaos. Der wichtigste Faktor für den Metallgehalt im Kakaosamen war der Metallgehalt des Bodens. Zusätzlich beeinflusste ein niedriger pH und eine niedrige Bodenleitfähigkeit die Aufnahme der meisten toxischen Metalle in Kakaosamen positiv. Kakaobäume in steigenden Höhenlagen üNN enthielten mehr Co und Ni in Kakaosamen, jedoch weniger Al, Cd, Cu und Zn. Diese Dissertation ist damit die erste Studie, die Qualitätsinformationen und Baumcharakteristika über native Kakaos aus Peru vorstellt und somit ihr Potenzial für einen flächenmäßigen Anbau in Zukunft aufzeigt. Außerdem demonstriert diese Studie das aktuelle Problem von Metallbelastungen in Kakao. Um dieses Problem zu bekämpfen, wurden einige vorbeugende Maßnahmen gefunden, die eine Erhöhung des Boden pHs und der elektrischen Leitfähigkeit durch Kalk- und Mg-Düngung einschließen sowie ein Herabsetzen der Düngung und des Baumbeschnitts im Allgemeinen. Zusätzlich können Kakaobäume kultiviert werden, die natürlicherweise weniger toxische Metalle aufnehmen, wie z.B. die vorgestellten nativen Kakaos.

Resumen

El cacao (*Theobroma cacao* L.) es uno de los alimentos de lujo más consumidos en todo el mundo. Las industrias de dulces están produciendo productos de chocolate especialmente para los niños, pero también para adultos – normalmente con una concentración mayor de cacao. Los productos de cacao tienen su origen en dos principales typos de cacao. Los cacaos convencionales (Forasteros) y los cacao finos y aromáticos (Criollos) que se caracterizan por una mayor calidad y compuestos de sabor especiales. Los cacaos convencionales se cultivan sobre todo en África, que produce app. 80% del monto total del mercado mundial. Sólo el 5% de la producción de cacao cuenta para los cacaos finos y aromáticos, que se producen especialmente en Sudamérica. Desde los años 1980, el cacao se cultiva a gran escala en el Perú con el apoyo las Naciones Unidas con fin de remplazar la producción ilegal de coca con un cultivo legal de cacao como fuente de ingresos estable para los agricultores peruanos. En primer lugar, se extendieron clones de cacao de alta producción con mayor resistencia a las enfermedades, tal como el CCN-51, para asegurar rendimientos altos. Posteriormente, se promovieron cacaos finos y aromáticos de mejor calidad que, debido al mayor precio de las acciones en el mercado mundial, por lo tanto rendieron un mayor ingreso para los agricultores. Hoy en día, los cacaos nativos del país fueron descubiertos como un tesoro de Sudamérica debido a su sabor extraordinario. Estos cacaos con una alta variación genética estan presentes en diferentes regiones de la Amazonía peruana, pero hasta ahora falta un análisis profundo de la genética, la calidad en cuanto a la identificación de metabolitos secundarios y las características de rendimiento (tamaño de los frutos, rendimiento anual, susceptibilidad a enfermedades, etc.). Hasta ahora, la literatura sobre la genética del cacao da evidencia que el origen del árbol de cacao está en las tierras bajas de la Amazonía que, más encima, implica una revisión completa de la clasificación tradicional del cacao en Forasteros, Criollos y Trinitarios (híbridos de Forastero x Criollo). No obstante, los estudios se basan exclusivamente en material de planta de diferentes jardines clónicos de Sudamérica. Por lo tanto, la investigación de muestras de cacaos nativos colectados en estudios de campo en diferentes regiones del Perú nunca se ha realizado y promete el descubrimiento de nuevos cacaos desconocidos. Nuevos cacaos con características extraordinarias podrían renovar el mercado de cacao fino y aromático y asegurar los ingresos de los agricultores. No obstante, el árbol de cacao también tiene su lado oscuro como es conocido por ser un acumulador de metales tóxicos. Varios metales pesados (Cadmio, Cobre, Plomo) excedían los límites en los productos de cacao permitidos en la Unión Europea con respecto a la Organización Mundial de la Salud (OMS) y

la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO). Los metales pesados como el cadmio (Cd), el cobre (Cu) y el plomo (Pb) pueden ya ser peligrosos en pequeñas cantidades. Especialmente los niños, que consumen chocolate como dulces, corren el riesgo de intoxicarse con metales pesados. Se informó de que el cacao procedente de Sudamérica supera los límites de Cd permitidos, mientras que el cacao procedente de África está más contaminado con Pb. Se carece de literatura sobre la contaminación por otros metales tóxicos en el cacao ya que hay un alto riesgo para la salud de los seres humanos. Hasta ahora, nunca se analisaron metales tóxicos como el cobalto (Co) y el manganeso (Mn) en los productos de cacao, mientras que para otros como aluminio (Al), níquel (Ni) und Zinc (Zn) existen sólo pocos estudios. El objetivo del presente estudio es el análisis de differentes cacao peruanos en cuanto a sus características de calidad y de rendimiento y la evaluación de la incorporación y accumulación de metales toxicos en la planta del cacao. Para esto, se tomaron muestras de 328 árboles de cacao en tres de las principales regiones productoras de cacao en Perú en los años 2014, y 2016 hasta 2018. Se compararon muestras de cacaos nativos, del clon de alta producción CCN-51, y de clones de cacaos finos y aromáticos de la región norte del Cusco, la región media de Huánuco y la región media-sur de San Martín en el Perú en cuanto a los metabolitos secundarios en el cacao en polvo, los parámetros de rendimiento, la susceptibilidad a enfermedades y el contenido de metales tóxicos. Además, se analizó el impacto del manejo de la finca (p.ej. frecuencia de poda, uso de fertilizantes, edad de los árboles, cuaje de frutos) y del medio ambiente (p.ej. pH del suelo, conductividad eléctrica del suelo, contenido de metales tóxicos en el suelo, altura sobre el nivel del mar) en los metabolitos secundarios, en los parámetros de rendimiento, en la susceptibilidad a enfermedades y en el contenido y la incorporación de metales tóxicos en las semillas de cacao. En este tesis, los granos de cacao sin fermentar que se han transformado en cacao en polvo se denominan "cocoa powder", mientras que otros productos de cacao se denominan "cocoa" sólo después de la fermentación y las semillas de cacao sin fermentar como "cacao seeds". En general, los cacaos nativos de Cusco tenían los mayores contenidos de polifenoles (catequina: 0,11%; cianidina arabinosida: 0,23%; cianidina galactosida: 0,11%), con excepción de la epicatequina que era más alta en los cacaos nativos de San Martín (2,6%). Por el contrario, los cacaos nativos de Huánuco mostraron los contenidos más bajos de polifenoles. Los contenidos de polifenoles aumentadoron con la edad de los árboles de cacao y los árboles más viejos se encontradoron especialmente en la región de Cusco. La cafeína mostró valores más alta en los cacaos nativos de Cusco (1,15%), pero más baja en los cacaos nativos de Huánuco (0,37%), y no se encontraron diferencias significativas entre los clones de CCN-51, finos y aromáticos y los nativos de San Martín. La teobromina, en cambio, fue más alta en el CCN-51 (2,92%) y más baja en los clones finos y aromáticos (2,34%). En cuanto a las características de rendimiento, el clon CCN-51 de alta producción tuvo los frutos más largos (23,8 cm) y de más grande perímetro de frutos (31,2 cm), mientras que los cacaos nativos del Cusco mostraron los frutos los más cortos (17,8 cm). Los clones finos y aromáticos mostaron los perímetros de fruta más pequeños (26,9 cm) y más encima tuvieron los rendimientos anuales los más bajos (340 kg ha^{-1}) comparado con los cacaos nativos de San Martín que rendieron todavía más (1516 kg ha^{-1}) que el CCN-51 (1176 kg ha^{-1}). Los clones de cacao (CCN-51, clones finos y aromáticos) se infestaron menos con los hongos *Moniliophthora perniciosa*, *Moniliophthora roreri* y *Phytophthora* sp., pero se atacaron más con insectos en comparación con los cacaos nativos. Adicionalmente, el manejo de la finca y factores del medio ambiente influenciaron los contenidos de metabolitos secundarios en el cacao y los parámetros de rendimiento. Los polifenoles se disminuyeron significativamente con mayor pH del suelo. Además, la epicatequina y la catequina hidratada también se redujieron con mayor conductividad eléctrica del suelo. La epicatequina y los antocianos se elevaron si los ataques de insectos fueron mayores. En cambio, el contenido de teobromina se aumentó con la frecuencia de la poda, mientras que la cafeína se disminuyó. Comparando los contenidos de metales tóxicos entre las tres regiones, Co (1,13 mg kg^{-1}), Cu (49,65 mg kg^{-1}), Mn (78,54 mg kg^{-1}), y níquel (Ni: 27,17 mg kg^{-1}) fueron los más altos en las semillas de cacao de la región del Cusco. En la región de Huánuco, el Cd (2,13 mg kg^{-1}), el Pb (0,09 mg kg^{-1}) y el zinc (Zn: 84,76 mg kg^{-1}) mostraron las concentraciones las más altas, y San Martín tuvo los contenidos los más altos de Aluminio (Al: 16,56 mg kg^{-1}). En cuanto a los límites legales de Cd, Cu, y Pb en la Unión Europea, cerca del 49% excedió el límite de Cd de 0,6 mg kg^{-1} en polvo de cacao con un valor máximo medido en Huánuco de 12,3 mg kg^{-1}. El límite de 50 mg kg^{-1} para el Cu fue excedido por el 39% de las muestras de cacao en polvo con un valor máximo medido en Cusco de 86,5 mg kg^{-1}. El Pb está limitado con 1,0 mg kg^{-1}, por lo que el 16% de las muestras mostraron valores más altos. Para poder formular medidas de mitigación eficientes contra la contaminación con metales tóxicos en los frutos de cacao, se analisaron la concentración y la absorción de los metales en cuanto a un effecto del manejo de la finca y los factores ambientales fueron utilizando *Boosted Regression Trees* (*BRT*). El modelaje reveló que los contenidos de Al, Cd y Zn en las semillas de cacao disminuyeron con la edad del árbol de cacao, mientras que los contenidos de Co y Cu fueron mayores. El contenido de Cd elevó en las semillas de cacao con el uso más frecuente con fertilizantes nitrogenados (N) y fosfatados (P). Las actividades de poda anual incrementaron las tasas de incorporación (factores de transferencia) de Al, Cu, Cd y Zn del contenido de metal del suelo a las semillas. Los contenidos de metales tóxicos en general difirieron entre los tipos de cacao. En general los cacaos finos y aromáticos tuvieron las absorciónes mas altas (Cd: 13,17; Co: 0,21; Cu: 3,52; Mn: 0,20; Ni: 1,35; Pb: 0,0073; Zn: 2,18). Solo el CCN-51 tuvo la absorción mas alta en Cd con 13.26 mostrando que la absorción de los metales toxicos fue mas alta en los clones que en los cacaos nativos. En general, el contenido de metales tóxicos en los suelos fue el factor lo más influyente para el contenido de metales en las semillas de cacao. También, una conductividad eléctrica y un pH más bajos influenciaron la incorporación de la mayoría de los metales tóxicos en las semillas de cacao positivamente. Con la altura a.s.l. se amuntó el contenido de Co y el Ni en las semillas de cacao, mientras que los contenidos de Al, Cd, Cu, y Zn se disminuyeron. Dentro de este proyecto de tesis, se caracterizaron genotipos desconocidos de cacao en cuanto a su potencial de qualidad y sus caracteristicas para descubrir su potencial de cultivarlos en futuro. Este estudio también demuestra el problema actual de la contaminación por metales toxicos en el cacao. Para combatir este problema, se han encontrado algunas medidas preventivas, entre ellas el aumento del pH del suelo y la conductividad eléctrica mediante la fertilización con calcio y Mg, y la reducción de la fertilización y la poda de árboles en general. Además, se pueden cultivar árboles de cacao que absorben naturalmente menos metales tóxicos, como el cacao nativo que se presenta.

1 General Introduction

1.1 The cacao tree (*Theobroma cacao* L.)

The cacao tree *Theobroma cacao* (Linné), which belongs to the Malvaceae family, is a crop plant cultivated in Central- and South America, South Eastern Asia and Africa, with the latter producing more than 70% of the global amount (Lieberei and Reisdorff 2012; Rohsius 2007). Cacao is an understorey tree that grows in tropical regions. Most commonly, it is cultivated in agroforestry systems which are characterized by high biodiversity of shadow trees and herb layer plants but also monocultures are frequent in all parts of the world (**Fig 1**; Kieck et al. 2016; Lieberei and Reisdorff 2007). Annual yields vary between 200 and 4,000 kg ha^{-1} and depend on a wide array of different factors (e.g. cacao type, farm management, environmental conditions; Motamayor et al. 2008; Kieck et al. 2016). Within the genus *Theobroma*, about 22 species have been described, but *Theobroma cacao* is the only species that is cultivated at large scale (Rohsius 2007). Wild-growing cacao trees reach heights of 15 to 20 m. On farms, it is normally pruned to a height of 4 to 8 m. The sympodially growing tree has oval evergreen leaves up to 30 cm in length. Cacao flowers are white or pink, about 1 cm small, and pollinated by small insects such as mosquitoes (**Fig. 2**). Self-pollination is excluded by the flower's morphology. Flowers are produced all year round in cushions located on the stem and large branches (cauli- and ramiflory), and fruits can be harvested continuously, albeit there are one or two main harvesting seasons per year. Depending on the genetics of cacao trees, fruits differ in color when ripe and reach a length up to 30 cm. Ripening requires a period of about 5 to 6 months (Lieberei and Reisdorff 2012).

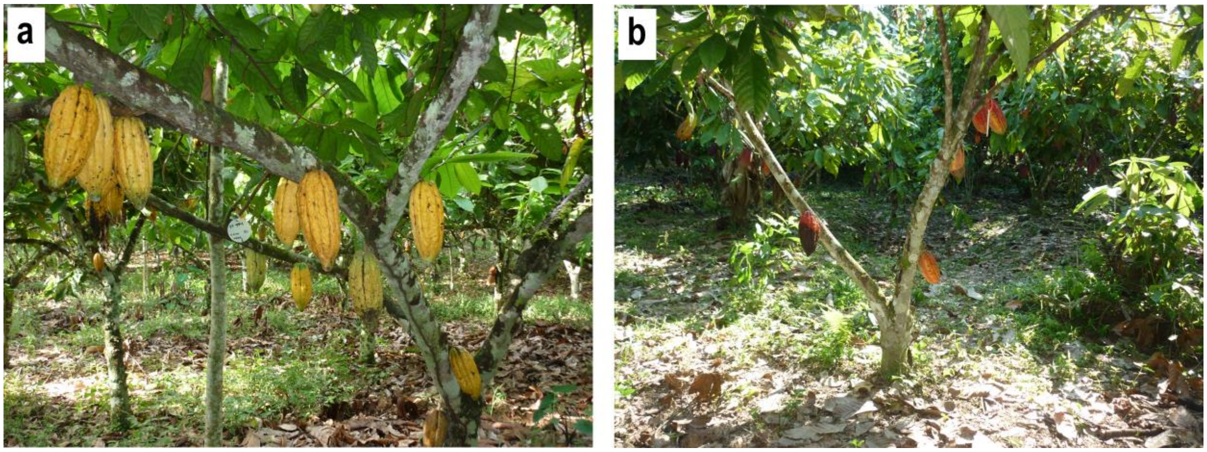

Figure 1: Cacao trees of different genotypes (**a**: native cacao; **b**: CCN-51) in an agroforestry system. Pictures taken by the author.

Cacao is divided into different cultivars: the so-called Forasteros produce high yields and are more resistant against diseases but show lower aromatic quality (Castro-Alayo et al. 2019). The bulk cacao and high-yield clone CCN-51 with origin in Ecuador is an example of a Forastero cacao (Boza etal. 2014) widely cultivated in the Peruvian lowlands. The Forasteros originate from the Amazonian basin (Rehm and Espig 1991). *Criollos* are a cultivar with outstanding aromatic quality. Its fruits are smaller in comparison with Forasteros and the trees are more susceptible to typical cacao diseases (Kieck et al. 2016; Motamayor et al. 2000). The Criollos were cultivated in Central America with southern extremes up to Columbia (Motamayor et al. 2008). The hybrid of both cacao cultivars is called Trinitario and has its origin in Trinidad. Hence, the Trinitarios have morphological and physiological characteristics of both Forasteros and Criollos (Wood and Lass 1989). Motamayor et al. (2008) and Zhang et al. (2006) provided genetic evidence that the species *Theobroma cacao* is indigenous to the Amazonian lowlands of South America. However, the differentiation into two main cultivars (Forasteros, Criollos) could not be supported based on genetic data. A recent study revealed a more accurate classification of cacao genotypes into 10 genetic clusters of cacaos from Central to South America, called Marańon, Curaray, Criollo, Iquitos, Nanay, Contamana, Amelonado, Purús, Nacional, and Guiana (Motamayor et al. 2006).

When the Spaniards reached South America in the 1500th century, they reported that Aztecs and Mayas prepared cacao as a drink. Cocoa beans were mixed with spices such as chili and vanilla in hot water. It was so valuable to the indigenous cultures that cocoa beans were even used as currency (Hoffmann

2008). When cacao was brought to Spain it was not consumed as a luxury product at high amounts because of its bitterness but sold as medicine by pharmacies. In the 1800th century, cacao was for the first time sold on the European luxury market together with coffee and tea and consumed by the rich (Alden 1976). The first chocolate was produced in England in 1848, and since then became one of the most successful sweets all over the world (Hoffmann 2008).

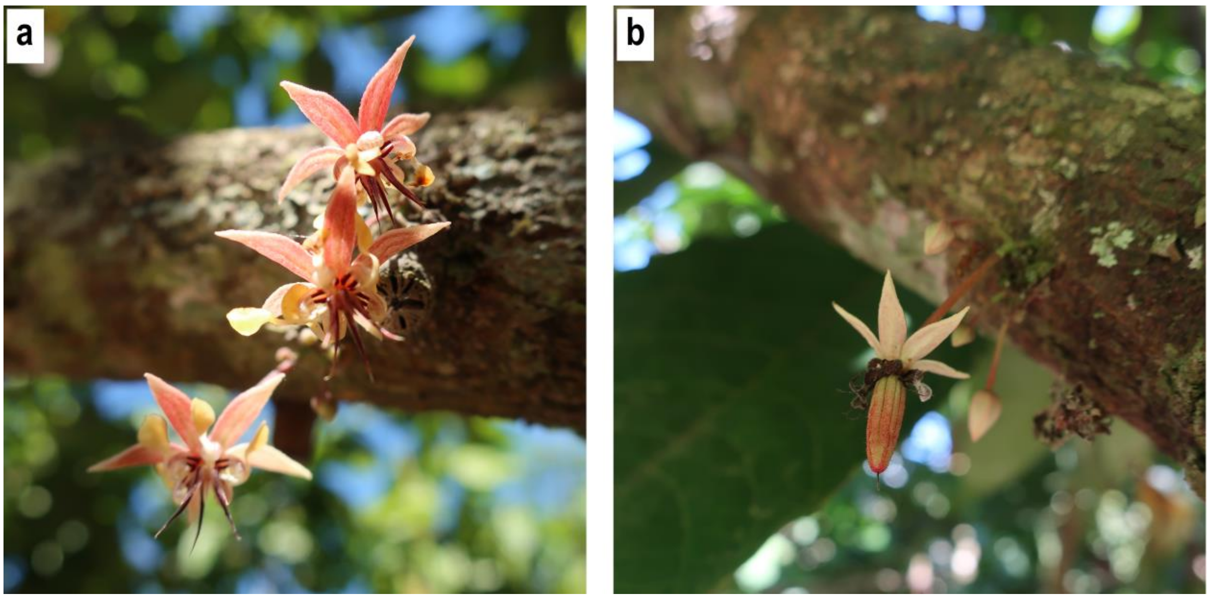

Figure 2: Cacao flowers growing on the stem (**a**). Flower with developing fruit (**b**). Pictures taken by the author.

1.2 Cacao in the study areas of Peru

In the Amazonian lowlands of Peru, cacao has been cultivated on a big scale since the 1980ies. Most cocoa is sold to the western multi-billion-dollar confectionary industries (Kieck et al. 2016; Motamayor et al. 2008), but an increasing amount is also processed by Peruvian companies. Before, agriculture in the Peruvian lowlands relied on illicit coca production (Peru was with Columbia the biggest coca-producing country). Several projects by UNODC - United Nations Office on Drugs and Crime in the regions of Huánuco and San Martin, Peru, started providing the farmers with the high-yield cacao clone CCN-51 as an alternative to coca to show legal ways to earn money (Boza et al. 2014; García Carrión 2012; Kieck et al. 2016; Lieberei and Reisdorff 2012; Zug et al. 2019). Later, the project also offered cacao clones characterized by higher aromatic quality such as the fine and flavor cacao clones ICS-1, ICS-6, ICS-39, ICS-95, and TSH-565 spread in the Huánuco, San Martin and Ucayali regions from the organization Alianza Cacao *Perú* in Tingo Maria (Alianza Cacao Perú 2020). Despite lower yield and resistance against diseases, fine and flavor cacaos may be promising to cacao farmers due to revenues higher than stock exchange prices for bulk cacao (Boza et al. 2014; Clough et al. 2011; García Carrión, 2012; Kieck et al. 2016; Zug et al. 2019). Besides well described cacao clones, also wild-growing cacaos native to different regions of Peru with presumably high genetic diversity can be found in forests or as relicts on cacao farms (Farmers interviews 2014, 2016, 2017, 2018; Kieck et al. 2016). In the Huánuco and San Martin regions of Peru, such native cacaos were exclusively found as relict trees growing on cacao farms nearby the high-producing clone CCN-51 or other cacao clones (**Fig 3**). In the Cusco region in contrast, native cacaos were cultivated on a commercial scale (**Fig 4**; Farmers interviews 2014, 2016, 2017, 2018; Kieck et al. 2016; Zug et al. 2019).

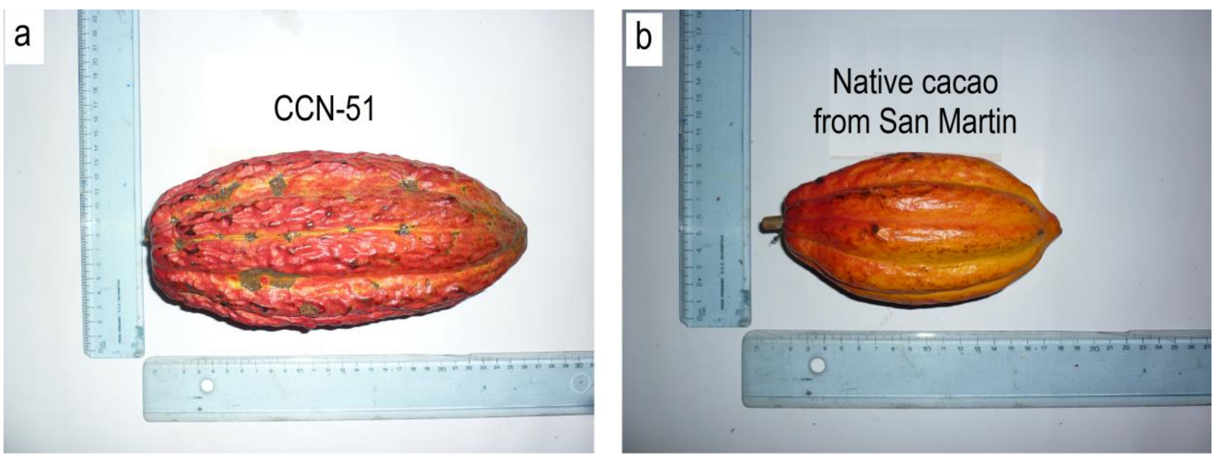

Figure 3: CCN-51 pod (**a**) and pod from a native cacao type from San Martin (**b**). Pictures taken by the author.

Figure 4: Ripe pods of different native cacaos from Cusco. Pictures taken by the author.

In the study regions of the present dissertation, cacao trees are frequently suffering from different fungal infestations. The fungus *Moniliophthora perniciosa* causes the growth of so-called “witches' brooms” on shoots (**Fig 5a**). Infested flowers are nearly not producing any fruits and infested fruits become moldy on the tree. Together with *Moniliophthora roreri*, the so-called “frosty rot pod” this fungus is one of the most important cacao pests in the study regions and results in pronounced yield losses when trees are infested (Mainhardt et al., 2008). *Moniliophthora roreri* affects the inside of fruits and provokes a whitish rotting of fruits (Aime and Phillips-Mora 2005). Infested plant parts must be cut off and burned to prevent transmission of spores (Evans 1981). While these two species of fungi are mainly distributed in tropical regions, the genus *Phytophthora* sp. is a worldwide problem (Nyasse 1995) and some species also affect cacao, causing yield losses up to 30%. Affected fruits show dark colored rotting and infestations are therefore called “black pod rot” (**Fig 5c**; Acebo-Guerrero et al. 2012). Moreover, cacao fruits are often attacked by insects which was also observed in this study (**Fig 5b**). The damaged plant tissue is often particularly prone to infestation with fungi.

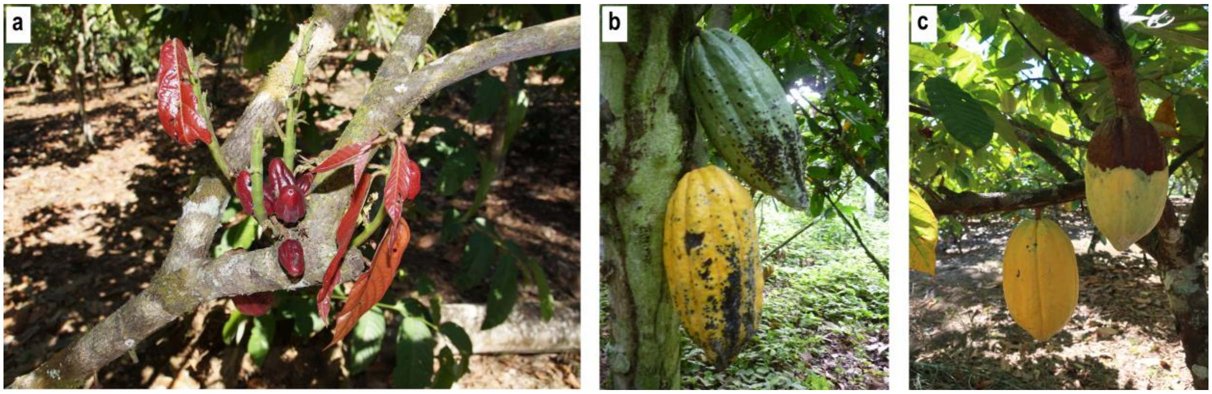

Figure 5: Cacao tree infested with "Witches broom" (**a**), cacao pod attacked by insects (**b**), and cacao pod infested by fungi (**c**). Pictures taken by the author.

1.3 The process of chocolate making

Cropping systems:

The cacao tree is commonly cultivated in agroforestry systems that comprise different shadow trees such as other fruit crops or species planted for wood use. Also, cacao plantations combined with coffee or banana plants are found. The herb and shrub species present on cacao fields depend on the use of herbicides and shadow management. Especially in the first year, young cacao trees need more shadow (Tscharntke et al. 2011). Cacao trees start to produce fruits at an age of 2 to 3 years and may then be used for more than 50 years (Rohsius 2007; Stenchly et al. 2011). After the first years, farmers remove some of the surrounding shadow trees for better growth of the cacao plants. In general, a dense shadow tree layer can lead to increased humidity on cacao farms which in turn enhances fungal pest incidence. However, cacao planted in monocultures may suffer from increased solar radiation which leads to physical and water stress (Schwendenmann et al. 2010; Tscharntke et al. 2011). Such stress conditions promote attack by fungi or insects which leads to yield losses or even the death of cacao trees. Apart from the benefit of a higher shadow tree diversity for the cacao trees, several ecosystem services are provided by agroforestry system such as carbon sequestration, increased soil fertility, improved drought resistance, and natural pest control (Tscharntke et al. 2011). Furthermore, the biodiversity of animals, as shown for insects, amphibians, and birds increases at higher tree diversity which contributes to biodiversity conservation (Bisseleua et al. 2008; Clough et al. 2009; Kieck et al. 2016; Stenchly et al. 2011; Tscharntke et al. 2011; Wielgoss et al. 2014). In turn, reduced animal diversity is known to lead to decreased cacao yields as a lack of pollinators impede fruit set (Groeneveld et al. 2010). In conclusion, cacao trees frequently benefit from surrounding shadow trees at moderate density and when they are pruned after the first years. A tight shadow tree management hence is pivotal for optimizing cacao yields (Tscharntke et al. 2011).

Fermentation:

After harvesting the cacao pods (**Fig 6**), the cacao pulp with the embedded seeds are fermented for a period of 2 to 9 days depending on the fermentation method, the genotype of the cacao, and the climate conditions (Afoakwa et al. 2008). Criollos are often fermented for shorter periods of time (up to 3 days), while Forasteros are fermented longer (up to 7 days; Afoakwa et al. 2008; Farmer interviews 2014, 2016, 2017, 2018; Stoll 2010). During the fermentation process, mainly the bitterness due to secondary substances is reduced and cocoa's characteristic aroma precursors are formed, which are important for the final chocolate aroma (Biehl and Adomako, 1983; Bytof et al. 2004). In the first step, the cacao fruit is opened to take out the cacao seeds including cacao pulp. Then, the pulp-seed-mass is collected in wooden boxes, in bags, or simply on a heap to start the fermentation. Sometimes, wooden boxes are lined with banana leaves to promote fermentation as they naturally contain yeasts and bacteria on their surface. During box fermentation, which is the most common method in Peru, the seeds are stirred daily (**Fig 7b**; depending on the method used) and transferred to a following box. Boxes located at different heights are particularly suitable for this (**Fig 7a**; Farmers interviews 2014, 2016, 2017, 2018; Rohsius 2007; Schwan et al. 1995; Stoll 2010). The fermentation process itself, is divided into two phases: During the first phase, the anaerobic fermentation phase, ethanol is produced through the decomposition of sugars by yeasts and lactic acid is produced by homofermentative lactic acid bacteria whereas heterofermentative lactic acid bacteria produce lactic acid, CO_2, and ethanol or lactic acid, CO_2, and acetic acid, and a sugar alcohol (manithol). Moreover, enzymes such as hemicellulases and cellulases decompose polymeric pulp components, which allows that pulp residues flow off and oxygen enters into the fermentation mass. The second phase is an aerobic oxidation phase, in which acetic acid bacteria use ethanol to produce acetic acid. During this phase, embryos in the seeds are killed by acetic acid entering through the picropyle and the increase in temperature of up to 55 °C. In this process, the cell interior of the seed tissues is de-compartmented and pH reaches values of 4.0 to 4.5 (Biehl and Adomako, 1983; Biehl 1973; Cocoa Atlas, 2002). PH decrease activates the seeds' own proteases, the aspartyl endoprotease and the carboxypeptidase. The former is responsible for splitting the storage proteins of the cacao seed in the area of the hydrophobic amino acids, resulting in oligopeptides with hydrophobic ends. In a second step, the carboxypeptidase splits the amino acids from the oligopeptides (Stoll 2010). In the last phase of the fermentation process, de-compartmentation causes the oxidation of polyphenols by its oxidases and a tanning reaction which changes the color of the seeds from purple to brown (Kim and Keeny 1984). The optimal pH of the fermented beans is about 5.0 to 5.3 (Biehl and Adomako, 1983).

Figure 6: Cacao pod harvesting on a cacao farm (**a**). Fresh cacao seed-pulp-mass ready for fermentation (**b**). Pictures taken by the author.

Figure 7: Fermentation boxes covered with jute bags (**a**). Stirring fermentation mass (**b**). Pictures taken by the author.

Drying of fermented beans:

The drying process of the fermented beans is important for the production of chocolate flavor and to facilitate further transportation and storage (Heinzler and Eichner 1991). Beans are normally sun-dried for about 7 days depending on climate conditions. Depending on availability, the cocoa is dried on special concrete or wooden frames, on bast mats fixed at about one meter above the ground, on plastic tarpaulins, grids (**Fig 8**; above the ground) or simply directly on the street. Some farmers and cooperatives use mobile rain tarps that can be pushed over the drying cocoa beans, otherwise the cocoa is gathered and stored at a dry place when the weather is changing. Rarely, cacao is dried in ovens. If such oven is fired with wood, an undesired smoky change of the raw cocoa taste may be the consequence (off-flavor; Afoakwa et al. 2008; Fowler 1995; Rohsius 2007). The fermented cocoa beans must be dried to a water content of less than 9% to prevent mold growth during further storage. In most cases, the dried beans are shipped directly in jute bags to avoid prolonged storage under moist conditions (Ney 1992; Stantschew 1976).

Figure 8: Drying of cacao beans on grids under roofs (**a**). Drying of cocoa beans on black plastic tarpaulins in the sun (**b**). Pictures taken by the author.

Roasting:

In consumer countries, cocoa is first roasted at 70 to 140°C for 10 to 45 minutes so that the cocoa's own aromas can be formed. Thereby, the cocoa's own aromas are created by the so-called Maillard reaction through the free amino acids, oligopeptides and reduced sugars produced during fermentation (Oracz and Nebesny 2019). After roasting, the cocoa is further processed. Cocoa beans shell can be removed, and the cocoa can be ground to a homogenic mass. To produce chocolate products, the cocoa is usually separated into consumer cocoa and fine flavored cocoa (Afoakwa et al. 2008; Lieberei and Reisdorff 2012; Stoll 2010).

The processing of cocoa:

The peeled raw cocoa beans are processed into cocoa powder and chocolate products. To produce cocoa powder, the fat content must be reduced to 10 to 24% by homogenizing and pressing of the cocoa beans. The cocoa powder can then be dried (Stoll 2010). For the production of chocolate, depending on the final product, sugar, milk powder, cocoa butter and spices are added to the chocolate mass. The chocolate mixture must then be homogenized by the so-called conching, a continuous stirring and rolling process. The chocolate mass can then be packed (Lieberei and Reisdorff 2012 Stoll 2010).

1.4 Cocoa and its quality

The quality of cocoa as a raw product is examined at many points before it is processed to chocolate. Usually after fermentation and drying, a first visual inspection is carried out using the cut test (**Fig 9a**). The cocoa is evaluated according to the percentage of beans with brown color, which indicates the homogeneity of the fermentation process (**Fig 9b**; Costa et al. 2015). In addition, attention is paid to contaminations with fungi, insects, foreign particles, etc. During and after drying and storage, the water content is regularly determined. When beans are sold, they are usually checked again by the purchaser. Here, the origin of the cocoa, the homogeneity of seed size and color, water content, contaminations and taste are considered. The taste is checked for aroma intensity, acidity, bitterness, astringency, off-flavor, and generally for aromas that are not typical for cocoa. In the last step, which is usually implemented by the chocolate manufacturer, the quality is checked again, and fat content and fat solubility are additionally determined (Afoakwa et al. 2008; Costa et al. 2015; Rohsius et al. 2008).

Figure 9: Cut-test of fermented cocoa beans (**a**). Bean color during the fermentation (**b**). Pictures taken by the author.

Analyses of organic acids such as acetic and lactic acid, along with amino acids, polyphenols, and methylxanthines can additionally give an overview about cacao flavor compounds of fermented and dried beans (amino acids), of its acidity (acetic acids), the bitterness of fermented and dried cocoa beans or fresh cocoa beans (methylxanthines, polyphenols), and the astringency (polyphenols) of fresh or processed material (Afoakwa et al. 2008). In the case of this dissertation, polyphenols (epicatechin, catechin hydrate, cyanidin-arabinoside, cyanidin-galactoside) and methylxanthines (theobromine, caffeine) were analyzed, as exclusively fresh cacao beans were used that were harvested from the tree and dried directly without fermentation.

Organic acids in cacao:

Acids in cocoa are classified into volatile acids (acetic, butyric, iso-butyric, isovaleric, propionic) with about 0.47 to 1.02% and nonvolatile (citric, lactic, malic, oxalic, succinic, tartaric) with about 1.01 to 1.88% (Jinap 1994). The main acids, determining the acidity of the final cocoa bean (after fermenting and drying) are lactic and acidic acids which are entering into the cacao bean during the de-compartmentation during the fermentation process. Fermentation has a great influence on final acid contents in cocoa beans which change with the duration, heat, microorganisms that are present (Jinap 1994; Jinap and Dimick 1990; Jinap and Thien 1994). Also drying has an impact on organic acid contents as volatile acids evaporate during drying (Jinap and Thien 1994). The content of acids also differs depending the country of origin (Jinap and Dimick 1990).

Free amino acids in cocoa:

During the fermentation-process a great amount of free amino acids are produced due to the enzymatic decomposition of storage proteins in the seeds. The total amount of free amino acids found in samples from Rohsius (2007) varied between 5.0 to 25.2 mg g^{-1} in the fat free dry matter (ffdm) of cocoa beans with differences according to the origin of cocoa samples. In general, free amino acids such as the hydrophobic ones (alanine, leucin, phenylalanine, tyrosine, valine, isoleucine), the acidic amino acids (asparagine, asparagine acid, glutamine, glutamine acid, histidine), and other amino acids such as tryptophan, lysine, serine, glycine, arginine, threonine, and GABA (γ-aminobutyric acid) can be measured in cocoa beans (Kirchhof et al. 1989; Puziah et al. 1998). Flavor compounds in cocoa beans besides bitterness, astringency or acidity derived from amino acids (e.g. 3-methylbutanol, phenylacetaldehyde, 2-methyl-3-(methyldithio)furan, 2-ethyl-3,5-dimethyl, 2,3-diethyl-5-methylpyrazine) are presented in **Table 1** compiled by Afoakwa et al. (2008) from two publications (Belitz and Grosch 1999; Schnermann and Schieberle 1997).

Table 1: Dominant odor-active volatiles in cocoa mass (Afoakwa et al. 2008).

Compound	Odour quality	Flavour dilution factor
2- and 3-methylbutanoic acid[b]	sweaty	2048
3-Methylbutanal[a,b]	Malty	1024
Ethyl 2-methylbutanoate[a,b]	Fruity	1024
Hexanal[a,b]	Green	512
Unknown[a]	Fruity, waxy	512
2-Methoxy-3-isopropyrazine[a,b]	Peasy, earthy	512
(E)-2-Octanal[a,b]	Fatty, waxy	512
Unknown[a]	Tallowy	512
2-Methyl-3-(methyldithio)furan[a,b]	Cooked meat-like	512
2-Ethyl-3,5-dimethylpyrazine[a,b]	Earthy roasty	256
2,3-Diethyl-5-methylpyrazine[a]	Earthy roasty	256
(E)-2-Nonenal[a,b]	Tallowy green	256
Unknown[a,b]	Pungent, grassy	128
Unknown[a,b]	Sweet, waxy	128
Phenylacetaldehyde[a,b]	Honey-like	64
(Z)-4-Heptanal[a,b]	Biscuit-like	64
δ-Octenolactone[a,b]	Sweet, coconut-like	64
γ-Decalactone[b]	Sweet, peach-like	64

Sources: [a]Belitz and Grosch (1999); [b]Schnermann and Schieberle (1997).

Polyphenols:

Polyphenols cause bitterness and astringency in cocoa mass. On the other hand, polyphenols are antioxidants and therefore recommended in a healthy diet for humans (Aprotosoaie et al. 2016). During the de-compartmentation, these substances are oxidized and polymerized by polyphenol-oxidases. In an intact plant cell, polyphenols are stored in idioplasts and polyphenol-oxidases separately from them in thylakoid membranes of chloroplasts (Sullivan et al. 2004). Only when the cell is destroyed, polyphenols and oxidases get together and a browning reaction takes place, whereby the oxidation of mono- and diphenols to quinones with reduction of oxygen occurs. The quinones react with each other or with amino and sulfhydryl groups of proteins and enzymes, so that polymerization and condensation reactions between proteins and phenols take place (González et al. 1999; Stoll 2010).

Cacao beans contain about 14 to 20% polyphenolic cells (Osman et al. 2004). The total polyphenol content in cacao beans is about 34 to 60 mg g^{-1} ffdm (fat free dry matter), in cocoa powder about 20 to 62 mg g^{-1} (Nazaruddin etal. 2001). There are three major polyphenol groups with catechins accounting to about 37% (e.g. epicatechin, catechin hydrate), anthocyanins to about 4% (e.g. cyanidin-3-α-L-arabinoside, cyanidin-3-β-D-galactoside), and proanthocyanidins (e.g. flavan-3,4-diols) to about 58% of the total polyphenol contents (Kim and Keeney 1984; Romanczyk et al., 1997). Forastero cacaos are in general characterized by higher polyphenol amounts than Criollos. In the ffdm of Forasteros between 15 and 20% polyphenols can be found before fermentation, which should fall to 5% during the fermentation-process. Criollos normally reach polyphenol contents of two-thirds of the content of Forasteros. The color-giving anthocyanins of the cacao seeds are higher concentrated in Forasteros than in Criollos (Lange and Fincke 1970; Hansen et al. 2000).

Methylxanthines:

Methylxanthine contents also differ among cacao genotypes. Criollo cacaos have higher caffeine contents (up to 0.6%) than Forasteros (about 0.19%), whereas the theobromine contents are higher in Forasteros (about 3.95%) in comparison to those of Criollos (2.85 to 3.43%; Afoakwa et al. 2008). As polyphenols, methylxanthines are provoking a bitter taste of cocoa (Afoakwa et al. 2008; Taylor et al. 2002). The total methylxanthine content is about 4% in raw cacao beans. Methylxanthines are stored together with polyphenols in polyphenolic cells (Aprotosoaie et al. 2016; Franco et al. 2013; Kadow et al. 2013). Ho et al. (2014) reported that theobromine contents decrease down to 40% during fermentation, whereas caffeine decreases down to 54% which may be due to the dissolving of alkaloids in the run-off of the first fermentation phase (Nigam and Singh 2014). Methylxanthines (e.g., caffeine) are stimulating the nervous system and have cardiovascular and metabolic effects that can also lead to improved intellectual performance (Aprotosaie and Stănescu 2010; Franco et al. 2013).

Cacao quality and native cacaos:

Cacao varieties native to different regions of the Peruvian Amazonia have been collected in various germplasms in Peru since 1937 (Pound 1938). Breeding of cacao trees focused mainly on higher resistance against diseases. Nowadays, these native cacaos from germplasms are used to analyze the genetic diversity and the geographic origin of the cacao tree (Bartra 1993; Gonzáles 1996; Motamayor et al. 2008; Pound 1938, 1943; Zhang et al. 2006). Studies about the quality and yield characteristics of such native cacaos have so far not been realized. The knowledge about disease susceptibility, annual yield potential, fruit set, and the beans' secondary compound profile (polyphenols, methylxanthines) is important to assess the trees' potential on the market. It may help cacao farmers to select cacao trees with special flavor and yield potential, providing them with increased income.

1.5 The risk of chocolate consummation

In contrast to the health-promoting compounds such as anti-oxidative polyphenols or a high proportion of trace elements and amino acids that are essential for humans, cocoa also has a dark side (Afoakwa et al. 2008; Aprotosoaie et al. 2016; Zug et al. 2019). It is widely acknowledged that the cacao plant accumulates particularly large quantities of heavy metals (Bansah and Addo 2016). To reduce the risk to human health, maximum allowed values have been set for heavy metals, that are to be found in cocoa at large quantities, in the European Union (*EFSA* 2012, 2018; *European commission* 2014). These legal limits were recently adjusted when new studies revealed the harmful effects of these heavy metals on human and especially childrens health (e.g. Cd; WHO 2011). This is the case for cadmium (Cd) that provokes for example bone fractures, osteoporosis, and kidney insufficiency—a syndrome, first reported in Japan as Itai-Itai disease caused by Cd polluted crops (WHO 2010). The heavy metal lead (Pb) is known for neurological intoxication (Hodge 1981), and high amounts of copper (Cu) leads to arthritis, fatigue, osteoporosis, heart disease, memory loss. In addition, these heavy metals are carcinogenic (Izah et al. 2016a, b; Lanre-Iyanda and Adekunle 2012). The maximum allowed values for Cd, Cu, and Pb in cocoa are a content of 0.6 mg Cd per kg cocoa powder, 1.0 mg Pb kg^{-1}, and 50 mg Cu per kg cocoa bean (EFSA 2012, 2018; European commission 2014). Cd and Pb are more or less well studied in cocoa and chocolate products. Cd pollution (0.3 to 3.0 mg kg^{-1}; Argüello et al. 2019; Chavez et al. 2015; Mounicou et al. 2002a, b) is often higher in cacaos from South America, whereas samples from Africa are more frequently contaminated with Pb (0.01 to 3.45 mg kg^{-1}; Aikpokpodion et al. 2013; Mounicou et al. 2003; Prugarová and Kováč 1987). In contrast, there exists nearly no literature about Cu in cacao, albeit Lee and Low (1985) found an average of 93.9 mg kg^{-1} in cocoa beans, whereas Aikpokpodion et al. (2013) found 26.1 mg kg^{-1}. In accordance, other heavy metals such as Co or Mn have not been studied yet regarding their pollution level in cocoa products, although they are known to produce hazardous effects on human health when consumed at higher amounts with Co causing goiter and Mn neurological toxicity (Berk et al 1949; Izah and Angaye 2016; WHO 2011). At the same time, they are needed in small concentration, for enzyme activation (Mn) for example in the vitamin B_{12} synthesis (Co; Berk et al. 1949). The same applies to zinc (Zn) which is sometimes supplemented to food stuff but causes for example vascular shock, dyspeptic nausea, vomiting, diarrhea, and pancreatitis at higher amounts (Aikpokpodion et al. 2013). Nickel (Ni) damages the gastrointestinal, hematological, neurological, and immune systems and is therefore limited by European law in drinking water (20 µg L^{-1}; EFSA 2015 and 2011; He et al.

2005): However, Ni has also not been analyzed so far in cocoa products. Finally, aluminum (Al) may impose a great health risk for humans as it is assumed to be related to the Alzheimer disease (Perl and Moalem 2006; Stahl et al. 2011).

The pronounced lack of scientific literature on hazardous metals in cocoa products produced by the confectionary industry especially for consumption by children, highlights that further studies on this issue are urgently needed. As shown for Cd, Pb, and Cu, cacao has a high potential to incorporate and accumulate these metals in beans that are further processed for human consumption (Aikpokpodion et al. 2013; Argüello et al. 2019; Bansah and Addo 2016; Chavez et al. 2015; Lee and Low 1985; Mounicou et al. 2002a, b, 2003; Prugarová and Kováč 1987). Apart from description of toxic metal pollution in different cacao products, it is also important to identify the underlying mechanisms for such contamination to develop mitigation strategies concerning farm management, environmental or geographic factors, and the cacao genotype. The main factors influencing the Cd pollution in cocoa were the Cd content of soils and soil pH (Aikpokpodion et al. 2013; Chavez et al. 2015; Fauziah et al.2001; Gramlich et al. 2017; He and Singh 1994a, b; He et al. 2005; Mounicou et al. 2002a, b and 2003). However, other factors such as the farm management (pruning frequency, fertilization, age of cacao trees, cacao genotype) and environmental conditions (electrical conductivity, altitude a.s.l., biodiversity of surrounding vegetation), which are partly easier to be influenced by farmers, may also be identified as important drivers of metal accumulation and should therefore be included into mitigation options to counteract the pollution of cacao with toxic substances.

1.6 Objectives of the thesis

The high genetic diversity of cacao trees in the Peruvian Amazonia implies a treasure of unknown and so far never examined native cacaos (Motamayor et al. 2008; Zhang et al. 2011). Some of these cacaos such as the native cacaos from the Cusco Region are already on the market and sold as a fine and flavor cacao with special quality characteristics (e.g. from Perú Puro and Cacaosuyo), although they have not yet been analyzed systematically. Therefore, the following dissertation project studied a wide range of Peruvian cacaos from different regions (San Martin, Huánuco, Cusco) concerning their biochemical profile (polyphenol and methylxanthine contents), yield parameters (fruit-set, fruit-size, annual yield), and disease susceptibility (infestations with *Moniliophthora perniciosa*, *Moniliophthora roreri*, *Phytophthora* sp., attacks by insects). The aim of this dissertation was to compare these native cacaos with well-described commercial cacao clones (CCN-51, ICS-1, ICS-6, ICS-39, ICS-95, and TSH-565), and to generate a broad overview on yield and quality characteristics (Chapter 2). In addition, detailed field studies and analyses of the environmental conditions were conducted to find potential reasons for the pollution of cacao with toxic metals (Cd: Chapter 3; Studies combining different metals: Cd, Co, Cu, Mn, Ni, Pb, Zn, Al: Chapters 4 and 5). Cd and Pb accumulation in cacao is known to be a particular problem, but other toxic metals have never been studied before, albeit their potential risk to human health. Furthermore, this study aims at comparing heavy metal pollution in cacaos from the different regions of Peru (Chapter 4).

In particular, I worked on the following hypotheses handled in the different chapters (2, 3, 4, 5) of this dissertation:

Chapter 2: Yield and quality characteristics of native cacaos from three different regions of Peru - genetic differences and environmental drivers.

- Hypothesis 1: The native cacaos show significant differences among each other and to other varieties concerning the content in secondary metabolites (polyphenols, methylxanthines)
- Hypothesis 2: The high-yield clone CCN-51 is characterized by bigger fruits and higher yields and is less infested by diseases compared to the other cacaos.
- Hypothesis 3: Secondary metabolites in unfermented cocoa seeds and yield parameters are in general influenced by farm management (pruning, fertilization, planting distance) and environmental conditions (soil pH, electric conductivity, fungi infestation).

Chapter 3: Cadmium accumulation in Peruvian cacao (*Theobroma cacao* L.) and opportunities for mitigation.

- Hypothesis 1: Cd contents from Peruvian cacao seed samples are at least sometimes higher than the allowed limits.
- Hypothesis 2: Cd contents in CCN-51 are higher than in fine and flavor cacao clones due to its faster growth and metabolism.
- Hypothesis 3: Soil conditions, field management, and plant diversity influence Cd absorption in cacao seeds.

- Hypothesis 4: Quality (plant secondary compounds) and yield (annual yield per hectare, pods per tree, pod size, pest incidence) parameters are related to Cd accumulation in cacao seeds.

Chapter 4: Toxic metal contents of cocoa seed samples (*Theobroma cacao* L.) from Peruvian Amazonia are related to soil, region, and cacao type.

- Hypothesis 1: Heavy metal contents in cocoa powder samples depend on the heavy metal concentration in soil samples.
- Hypothesis 2: The content of heavy metals (Cd, Co, Cu, Mn, Ni, Pb, Zn) and Al in cacao powder from unfermented seeds depend on the samples` origin (San Martin, Huánuco, Cusco).
- Hypothesis 3: The contents of heavy metals and Al from the collected cacao clones (fine and flavor cacao clones, CCN-51) are in general higher than those of native cacaos from San Martin, Huánuco, and Cusco.

Chapter 5: Farm management and environmental factors drive toxic metal accumulation in Peruvian cacao (*Theobroma cacao* L.).

- Hypothesis 1: The content and transfer of toxic metals in cacao seeds is significantly influenced by farm management (e.g. fertilizer application, pruning frequency, age of study trees, fruits per study tree along with cacao type).
- Hypothesis 2: Environmental conditions (e.g. soil pH, electric conductivity, soil metal contents, altitude a.s.l. along with sampling region) have an influence on toxic metal contents and transfer in cacao seeds.
- Hypothesis 3: Toxic metals content in cacao is affecting yield parameters such as annual yield in kg ha^{-1}, fruit-size, or disease infections of fruits.

2 Yield and quality characteristics of native cacaos *from three* different regions of Peru – Genetic differences and environmental drivers.

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Manuscript

2.1 Abstract

The market for high quality cacao has been raising worldwide during the past decades. However, only 5% of the total production are fine and flavor cacaos which yield above-market stock price, whereas 80% of the worldwide bulk cacao is produced in Africa. Peru is well known for its fine and flavor cacaos. In addition to frequently cultivated cacao clones, Peru holds a treasure of native cacaos in its different regions, both as a relict growing between cultivated clones and on a big scale on plantations, e.g., in the Cusco region. These native cacaos with a wide genetic variation promise a high potential as fine and flavor cacaos with potentially unique characteristics. However, the Peruvian native cacaos have not been analyzed in terms of yield and quality traits so far. In the present study, 328 cacao samples collected in 3 different regions of the Peruvian Amazonia were compared regarding secondary metabolites (polyphenols, methylxanthines) of cacao seeds, disease susceptibility, fruit-size, and yield. We analyzed native cacaos from the regions San Martin, Huánuco, and Cusco, and compared them to the high-production clone CCN-51 and several fine and flavor cacao clones cultivated in Peru. Native cacaos from Cusco significantly showed the highest polyphenol contents with exception of epicatechin which was highest in native San Martin cacaos. The native cacao trees from Cusco were the oldest and polyphenol contents clearly increased with tree age. Native cacaos from Huánuco exhibited significantly the lowest polyphenol values. CCN-51 showed the highest theobromine contents, but caffeine contents were highest in native Cusco cacaos. The CCN-51 clone differed in terms of anthocyanins between the sampling regions, but we found neither any regional differences in other secondary compounds nor in yield parameters or disease susceptibility. High soil pH was associated with lower catechin hydrate in cacao seeds, whereas higher electric conductivity was related to reduce theobromine content. In general, native cacaos were more frequently infested by fungi (*Moniliophthora perniciosa*, *Moniliophthora roreri*, *Phytophthora* spp.), but less attacked by insects compared to the clones. Highest annual yields were recorded from native San Martin cacaos followed by significantly lower yields from native Huánuco cacaos and CCN-51. Overall, native Peruvian cacaos showed promising characteristics in terms of yield and contents of plant secondary compounds and have a high potential for use in chocolate and confectionary industry.

Main findings:

Flavor-, and fruit characteristics, disease susceptibility, and yield of native cacao trees from different

Peruvian regions compared with common cacao clones. Flavor characteristics, and disease susceptibility depended on soil conditions, and the age of study trees.

Key words: cacao biodiversity, cacao flavor characteristics, agroforestry, South America, genetically native cacaos, food chemistry.

2.2 Introduction

Cocoa and coffee are the most consumed luxury products on earth (Motamayor et al. 2008). The main cocoa producing areas are West Africa, South America and South East Asia (ICCO, 12.05.2019), with 80% of the production deriving from Africa. This cacao is traded as bulk cacao, whereas the market for higher quality, so called fine and flavor cacao is constantly raising (Motamayor et al. 2008). South America is the main cultivating area of these cacaos but produces only 5% of the worldwide cacao.

The cacao tree *Theobroma cacao* L. has its origin in Peruvian Amazonia. Consequently, Peru holds the highest genetic diversity of wild native cacaos in its regions and has therefore a pronounced potential to produce cacaos with an extraordinary quality (Motamayor et al. 2008; Zhang et al. 2011). In Peru, *T. cacao* is most commonly planted in agroforestry systems, leading to higher species richness of plants and animals in fields compared to full-sun monocultures (Clough et al. 2009; Clough et al. 2011; Lieberei and Reisdorff 2012; Tscharntke et al. 2011). Native cacaos are growing predominantly uncultivated and unmanaged within cacao plantations between planted clones (Kieck et al. 2016) or as pure native cacao cultivations (e.g., in the Cusco Region). Cacao cultivation in the different Peruvian regions was intensified in the 1980s with the help of several projects (e.g. UNODC – United Nations Office on Drugs and Crime, Kieck et al. 2016) to minimize the illicit coca production. Especially the high producing bulk clone CCN-51 was distributed to ensure income generation (García Carrión 2012; Kieck et al. 2016). Later, also fine and flavor cacaos with better quality were distributed in the country in order to guarantee a more reliable income source for farmers (Alianza Cacao, 05.10.2018; Zug et al. 2019).

Due to the high diversity of wild native cacaos in Peru, the genetics of different clones were investigated in several studies (Motamayor et al. 2008; Zhang et al. 2006; Zhang et al. 2011). In different germplasms, wild native cacaos have been collected all over Central and South America to conserve the genetic diversity. Motamayor et al. (2008) analyzed the genetics of 952 individuals of these native cacaos and presented 10 genetic clusters, which is in clear contrast to the known system of three cacao groups

Criollos (fine and flavor cacaos), Forasteros (bulk cacaos), and Trinitarios (hybrids of Criollo × Forastero). Additionally, samples from the Huallaga and Ucayali lowland showed a strict genetic separation of germplasm groups from these Peruvian Regions (Zhang et al 2006). Genetic analyses and native cacao collections in germplasms may help to protect the diversity of cacao (Zhang et al. 2011). The quality, disease susceptibility, and yield are essential to assess the economic potential of collected material but have not been studied yet. Such data exclusively can exclusively be gathered in field studies which are at present not available.

Cacao quality assessment is based on the content of secondary compounds as has been done in all bigger cacao cultivation areas of the world (e.g. Afoakwa et al. 2008; Aprotosaie et al. 2016; Niemenak et al. 2006). Theobromine contents of unfermented bulk cacao beans from West Africa is in average 3.95% (ffdm – fat free dry matter) and caffeine 0.19% (ffdm), whereas fine and flavor cacaos from South America show clearly higher caffeine contents up to 0.6% (ffdm) and less theobromine with 2.85 – 3.43% (ffdm) (Afoakwa et al. 2008). Consequently, the theobromine/caffeine ratio has been proposed as an indicator of cocoa quality with a lower ratio describing higher quality (Rohsius et al. 2008). Theobromine and caffeine are known as stimulants (Franco et al. 2013), whereas polyphenols in cacao seeds lead to astringent taste along with a health benefit effect (Krähmer et al. 2015). Seeds contain about 2.5 to 4.6 mg/g of epicatechin, the polyphenol with the highest concentration present in bulk cacaos and in fine and flavor cacaos (Afoakwa et al. 2008). In contrast, anthocyanin glucosides which are responsible for the violet color of bulk cacao seeds show much lower content in fine and flavor cacaos (Aprotosoaie et al. 2016). In addition to genetics and regional differences in cacao quality, also environmental and management conditions are known to influence the content of secondary metabolites involved in pathogen defense mechanisms of plants and plant-plant interactions (Kieck et al. 2016, Zug et al. 2019).

The aim of this study was to assess the quality, disease susceptibility, and yield parameters of native cacaos from three different regions in Peru based on the comparison with other commonly grown cacao varieties such as the high-yield clone CCN-51. This clone originates from Ecuador and has now been cultivated in entire America. It is appreciated for its fast growth, huge fruits, and its lower disease susceptibility (Boza et al. 2014; ICGD 12.05.2019). Furthermore, the native clones were compared with some well-described fine and flavor clones. Apart from genetic and regional differences, we also included management (annual frequency of pruning) and environmental (soil conditions, diseases) parameters into the analysis on cacao quality and yield potential.

In particular, we assessed the following hypotheses: 1) The native cacaos show significant differences among each other and to other varieties concerning the content in secondary metabolites (polyphenols, methylxanthines); 2) The high-yield clone CCN-51 is characterized by bigger fruits and higher yields and is less infested by diseases compared to the other cacaos; 3) Secondary metabolites in unfermented cocoa seeds and yield parameters are in general influenced by farm management (pruning, fertilization, planting distance) and environmental conditions (soil pH, electric conductivity, fungi infestation).

2.3 Material and Methods

2.3.1 Study area

The study area was conducted in three different regions of Peruvian Amazonia. Sampling took place in the regions San Martin, Huánuco, and Cusco during the dry seasons between April and June in the years 2014 – 2018. The mean annual temperature in these regions is 24 °C with a maximum of 29 °C and a minimum of 19.8 °C (Soberanis et al. 1991). The natural vegetation in Amazonian lowlands in Peru is evergreen rainforest with a mean annual precipitation of 3000 mm and a mean relative humidity of 81%, but the landscape on the eastern slope of the Andes is nowadays widely transformed to a mosaic of agrarian areas and forest remnants. Soils are mainly Inceptisols with low content in organic matter (Zimmermann et al. 2009).

In Peru, the high-yield clone CCN-51 is by far the most common one, whereas fine and flavor cacao clones are cultivated clearly less frequently (Clough et al. 2011). Due to the fact that CCN-51 promised high yields at reduced management intensity and that the fine cacao clones obtain a potentially higher stock price (García Carrión 2012; *International Germ Plasm Database* (*IGPD*) 12.05.2019), native cacao varieties can be scarcely found as relict trees growing on fields between the CCN-51 or fine and flavor cacao trees in the San Martin and Huánuco regions. The Cusco Region is the only studied region where a native variety called Chuncho is cultivated on a larger scale by many farmers.

2.3.2 Study design

In 2014, data collection was conducted in the San Martin region in the Amazonian lowland. Each 24 samples of the CCN-51 clone and 24 native cacao samples were randomly collected on 14 different plantations. In the Huánuco region, 30 CCN-51 and 30 fine and flavor cacao clones were randomly sampled on 40 different farms in 2016. We sampled the following fine cacao clones: ICS-1, ICS-6, ICS-39, ICS-95, TSH-565. In 2017, 105 native cacao samples were randomly collected from 7 different plantations in the proximity of Ivochote in the Cusco region. Moreover, 27 samples of the native cacaos from Huánuco and 24 samples of the CCN-51 were randomly collected on 12 different cacao farms in 2018. In the San Martin region, 36 native cacaos and 28 CCN-51 were randomly sampled on 14 farms in 2018 (**Tab 2**). Overall, 328 cacao samples on 87 randomly selected farms were taken in three different regions in the Peruvian Amazonia, of which samples from 106 CCN-51, 30 fine and flavor cacao clones, 105 samples of native Cusco cacaos, 27 samples of native Huánuco cacaos, and 60 native cacaos from San Martin were analyzed as shown in **Table 2** (Kieck et al. 2016; Zug et al. 2019).

Table 2: Sampling in the three regions (San Martin, Huánuco, Cusco) with the sample year, the cacao sample type, and numbers of sampled cacao, and farms.

	Sample year	**Cacao sample type**	**Number of native cacaos**	**Number of CCN-51**	**Number of fine cacaos**	**Total sample number**	**Number of farms**
San Martin	2014, 2018	Native cacaos, CCN-51	60	52	0	112	28
Huánuco	2016, 2018	Native cacaos, CCN-51, fine and flavor cacaos	27	54	30	111	52
Cusco	2017	Native cacaos	105	0	0	105	7

On every selected farm within the study areas, data and samples were collected following a standardized sampling design. We randomly selected study trees with at least two ripe fruits and with a minimum distance of 10 m from the field's edge. At each study tree, we recorded GPS data and the height above sea level. Furthermore, we counted the total number of fruits, along with the numbers of ripe, unripe, and diseased fruits longer than 8 cm to calculate fruit set and the proportion of infested fruits. We recorded diseases caused by the fungi *Phytophthora* sp., *Moniliophthora roreri*, and attacks by insects as well as the number of branches infested with the fungus *Moniliophthora perniciosa* (**Tab 3**). Two to three ripe fruits were collected for biochemical analyses of cacao seeds and to determine fruit-length and fruit-perimeter. Cacao seeds were removed, and sun dried without fermentation. As a mixed sample, 30 cacao seeds per study tree were randomly chosen for analyses.

Table 3: Mean values (± standard error) of secondary compounds (polyphenols, methylxanthines), fruit number, and infestation with diseases, fruit sizes, as well as farm management information.

	CCN-51	Fine cacao clones	Native Cusco	Native Huánuco	Native San Martin
Mean polyphenol contents in cocoa powder					
Epicatechin (%)	2.15 (± 0.01)	1.93 (± 0.21)	2.15 (± 0.10)	0.40 (± 0.08)	2.62 (± 0.21)
Catechin hydrate (%)	0.10 (± 0.01)	0.09 (± 0.01)	0.11 (± 0.01)	0.02 (± 0.01)	0.09 (± 0.00)
Cyanidin arabinoside (%)	0.09 (± 0.01)	0.14 (± 0.02)	0.23 (± 0.01)	0.005 (± 0.002)	0.16 (± 0.01)
Cyanidin galactoside (%)	0.06 (± 0.01)	0.07 (± 0.01)	0.11 (± 0.01)	0.001 (± 0.001)	0.08 (± 0.01)
Mean methylxanthine contents in cocoa powder					
Theobromine (%)	2.92 (± 0.04)	2.34 (± 0.08)	2.66 (± 0.05)	2.49 (± 0.07)	2.80 (± 0.05)
Caffeine (%)	0.52 (± 0.02)	0.55 (± 0.03)	1.15 (± 0.04)	0.37 (± 0.03)	0.57 (± 0.03)
Mean fruit number and infested fruits per tree					
Total fruit number	14.26 (± 0.79)	14.10 (± 1.87)	29.67 (± 2.00)	19.07 (± 2.44)	25.33 (± 3.05)
Ripe fruits (%)	30.48 (± 1.92)	36.82 (± 2.22)	32.53 (± 3.23)	24.84 (± 4.39)	20.82 (± 2.18)
Unripe fruits (%)	66.96 (± 2.07)	63.18 (± 2.22)	67.59 (± 3.20)	75.16 (± 4.39)	73.84 (± 2.64)
Infested fruit (%)	19.17 (± 2.02)	26.27 (± 44.47)	18.72 (± 1.72)	12.35 (± 2.84)	14.18 (± 1.73)
Fruits attacked by insects (%)	10.50 (± 1.80)	20.34 (± 4.29)	2.15 (± 0.57)	3.41 (± 1.25)	1.14 (± 0.63)
Moniliophthora roreri (%)	4.42 (± 0.76)	4.72 (± 1.24)	3.89 (± 0.93)	6.71 (± 1.85)	6.10 (± 1.20)
Phythophthora spp. (%)	4.24 (± 0.70)	0.80 (0.43)	4.27 (0.91)	1.57 (0.65)	6.48 (1.03)
Number of branches infested with *Moniliophthora perniciosa*	1.89 (± 0.28)	1.60 (± 0.58)	8.01 (± 1.06)	4.78 (± 1.16)	4.87 (± 0.77)
Fruit-size					
Fruit-length (cm)	23.80 (± 0.26)	20.97 (± 0.29)	17.84 (± 0.28)	20.50 (± 0.72)	22.22 (± 0.45)
Fruit-perimeter (cm)	31.20 (± 0.28)	26.90 (± 0.37)	27.88 (± 0.29)	28.46 (± 0.79)	30.22 (± 0.41)
Farm management & information					
Annual frequency of pruning	5.94 (± 0.93)	1.97 (± 0.10)	1.14 (± 0.15)	2.85 (± 0.14)	10.82 (± 1.82)
Age of cacao trees (years)	10.20 (± 0.64)	3.60 (± 0.19)	40.71 (± 1.68)	17.00 (± 2.34)	18.76 (± 1.41)
Annual yield (kg ha^{-1})	1175 (± 68)	340 (± 59)	362 (± 10)	1179 (± 86)	1516 (± 80)

Additionally, four soil samples were taken in a depth of 0-30 cm around the study tree and with a distance of 50-100 cm to the stem. The soil samples were mixed sundried and subsequently dried for 48 hours at 105 °C.

To obtain information about farm management practices, we conducted farmer interviews. Specifically, we asked the farmers about the annual yield in kg per hectare, the use of fertilizers and pesticides, pruning activities, planting distance, and the age of cacao trees (**Tab 3**).

2.3.3 Lab analyses

Extraction of secondary metabolites

Seed shells from 30 seeds of each tree sample were removed and seeds were homogenized in a mortar. Subsequently, 2 g of the homogenized seed sample were milled with 10 mL n-hexane in a ball mill (MM200 Retsch, Haan, Germany) for 10 minutes and with a frequency of 20 s^{-1}. In a Büchner funnel with a 45-µm filter, the grist was washed with 25 mL petroleum ether and the resulting filter cake was vacuum dried at room temperature for one hour (Araujo et al. 2014; Kieck et al. 2016; Niemenak et al. 2006; Zug et al. 2019). The degreased cocoa powder was used for biochemical analyses of secondary metabolites.

For the extraction of the polyphenols epicatechin, catechin hydrate, cyanidin arabinoside, and cyanidin galactoside, 100 mg cocoa powder was homogenized in 5 mL methanol with an ULTRA TURRAX T25 agiator (Janke and Kunkel, Staufen, Germany) for 30 to 40 s. Extraction vials were sonicated in an ultrasonic bath for 3 min and cooled down on ice for 15 min. After a centrifugation for 10 min and 4100 U min^{-1}, the extraction process was repeated twice with a cooling step on ice for 3 min only. The resulting methanol-supernatant was reduced in a rotary evaporator at 40 °C and 100 mbar. The reduced extract was resolved in 3 mL methanol in the ultrasonic bath and filtered with a 45 µm syringe filter. Polyphenols were determined using HPLC (high-performance liquid chromatography) at wavelengths of 225 to 540 nm, (Zug et al. 2019) against a calibration series with the respective polyphenols (Kieck et al. 2016).

The methylxanthines theobromine and caffeine were extracted in boiling water with 100 mg cocoa powder boiled in 40 mL distilled water for 30 minutes and subsequently cooled down in a water bath to 20 °C. To clarify the extract, 0.2 mL Carrez I solution, and 0.2 mL Carrez II solution were added. Extraction was filled-up with distilled water to a volume of 50 mL and filtered with a 45 µm syringe filter.

The methylxanthines were detected with a HPLC at a wavelength of 274 nm against calibration series with caffeine and theobromine (Arauje et al. 2014; Elwers et al. 2009; Kieck et al. 2016; Zug et al. 2019).

Analyses of soil conditions

Soil samples were dried for 48 hours at a temperature of 105 °C and sieved with a 2 mm soil sieve. For soil pH and electric conductivity analyses, 10 g of the soil samples were stirred with 25 mL distilled water for 1.5 hours and measured with a VWR symphony SP90M5 (Radnor, PA, USA; Zug et al. 2019).

2.3.4 Statistical analyses

To compare contents of secondary metabolites, fruit-size, yield parameters, and pathogen incidence within the different cacao types (native cacaos from Cusco, Huánuco, San Martin, the CCN-51 as well as fine and flavor cacao clones), a Two-way Analysis of Variance (ANOVA) and a Tukey-Test as a Post-hoc-Test was conducted with the Software R, version 3.5.1. (R Development Core Team 2014). To test differences in secondary compounds, yield parameters, and disease susceptibility within the clone CCN-51 of the two different regions, we used an ANOVA that includes the independent variables of the sample regions (San Martin, Huánuco).

To determine factors that are predicting secondary metabolites and yield parameters, generalized linear mixed models (GLMM) were calculated with the Software R, version 3.5.1. (R Development Core Team 2014). Farm management parameters, soil conditions, contents of secondary metabolites, and pathogen incidence were included in the models as fixed effects. The different cacao types on each farm and the farms in the different locations and regions were considered as random effects of the nested study design (Zuur et al. 2009). The variables were modelled with the lme function within the nlme and MASS package (Pinheiro and Bates 1995; Ripley 2015).

2.4 Results

2.4.1 Differences in secondary metabolites between cacao types

Polyphenol and methylxanthine contents in cocoa powder from native cacaos and cacao clones from the Cusco, Huánuco and San Martin regions differed significantly. The most abundant polyphenol epicatechin was significantly higher in the native cacao from the San Martin region in comparison to the other native cacaos and cacao clones (**Fig 10a**). In contrast, the native cacao from the Huánuco region showed the significantly lowest epicatechin contents (**Fig 10a**). The cocoa powder from the Cusco region, the fine cacao clones and CCN-51 clone were not differing significantly among each other in their epicatechin contents (**Fig 10a**). Catechin contents in native cacaos from the San Martin and Cusco regions as well as in fine and flavor cacao clones and the CCN-51 clone did not differ significantly, but in accordance with epicatechin contents also the catechin content was significantly lower in the Huánuco region (**Fig 10b**). Moreover, cyanidin arabinoside and cyanidin galactoside contents were significantly lower in native cacao from the Huánuco region compared to the other cacao types (**Fig 10c, d**). Both anthocyanins (cyanidin arabinoside, cyanidin galactoside) showed the significantly highest contents in native cacaos from Cusco with the other types showing intermediate contents (**Fig 10c, d**). Considering the methylxanthines, the clone CCN-51 had the highest theobromine contents but did not differ significantly from the native cacaos from the San Martin region (**Fig 10e, f**). The fine cacao clones had the lowest theobromine contents but did not differ significantly from the Huánuco region (**Fig 10e**). Cocoa powder from Cusco exhibited significantly the highest caffeine contents, whereas cocoa from Huánuco showed the lowest, not differing significantly from fine cacao clones and the CCN-51 clone but from San Martin (**Fig 10f**).

Also the origin of the cacao tree can influence polyphenol contents, as seen in significantly different epicatechin, catechin, and methylxanthine contents within the CCN-51 clone from the different regions San Martin and Huánuco (epicatechin: $p < 0.01$; catechin: $p < 0.01$; caffeine: $p < 0.01$; theobromine: $p = 0.01$). Both, the polyphenols, and methylxanthines were higher in San Martin than in Huánuco. However, we found no regional differences concerning the anthocyanins.

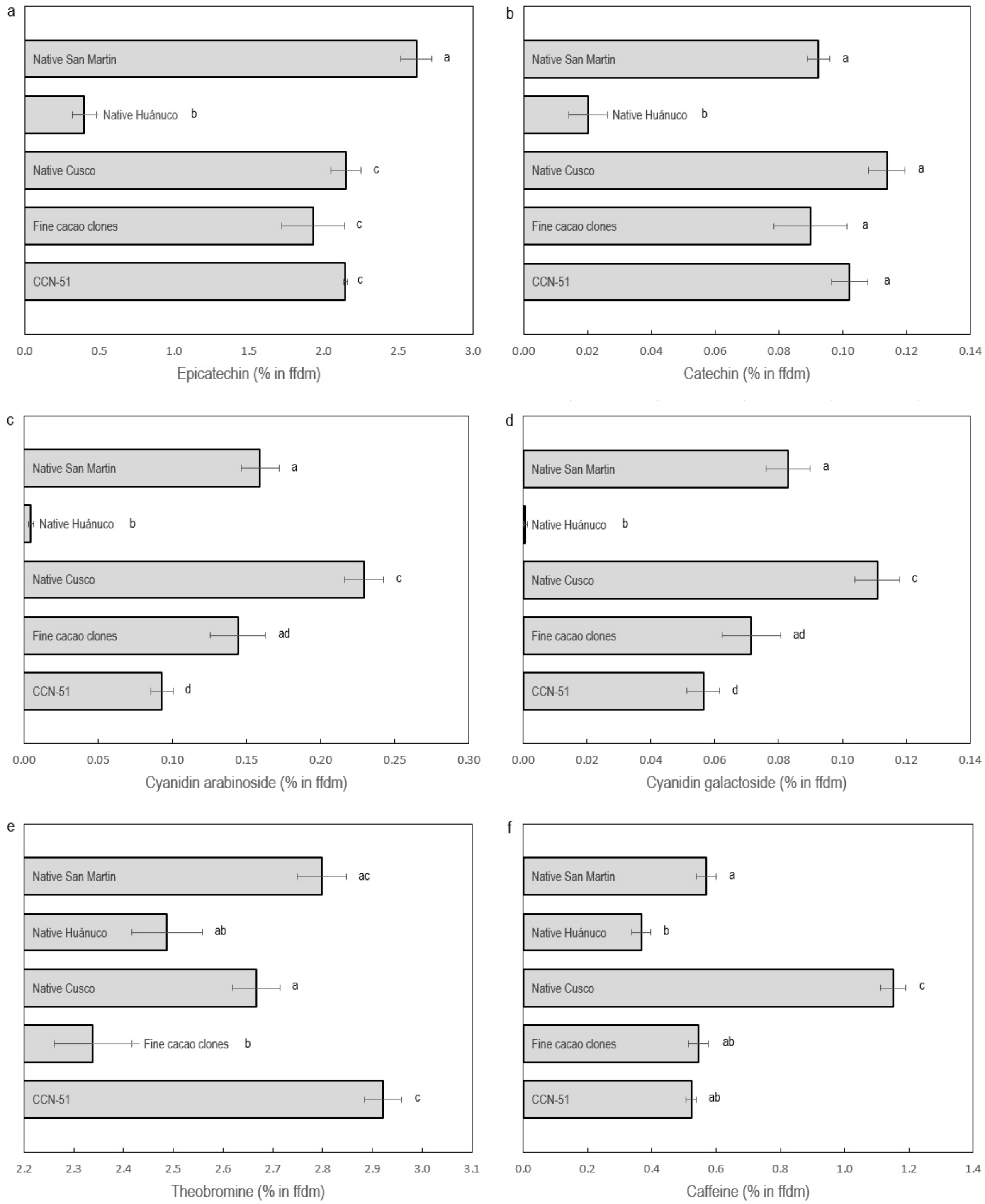

Figure 10: Differences in the polyphenol (epicatechin, catechin, cyanidin arabinoside, cyanidin galactoside) and methylxanthine (theobromine, caffeine) contents (%) in the fat free dry matter (ffdm) of cocoa seeds of the different cacao types (native cacaos from the San Martin, Huánuco, Cusco regions,

fine cacao clones, CCN-51). Different lower-case letters show significant differences between the cacao types. Averages with standard errors. Software R 3.2.3. ANOVA, Tukey-Test.

2.4.2 Comparison of yield parameters and disease susceptibility

Yield parameters such as fruit-size, number of fruits per tree and the annual yield showed clear differences between the native cacaos from the San Martin, Huánuco and Cusco regions as well as the fine cacao clones and the CCN-51 clone (**Fig 11; Tab 3**). The high producing clone CCN-51 produced significantly longer fruits than the others, whereas the native cacao from the Cusco region significantly had the shortest (**Fig 11a**). Moreover, the CCN-51 and the native cacao from San Martin had significantly bigger perimeters compared to other cacao types except native cacao from San Martin, whereas the fine cacao clones had the smallest perimeter, albeit not significantly different from the native cacaos from Cusco and Huánuco (**Fig 11b**). The cacao clones (CCN-51 and fine and flavor cacao clones) exhibited the lowest fruit numbers per tree and the native cacao from the Cusco region significantly the highest but without a significant difference to native San Martin cacao (**Fig 11c**). The native cacaos from the San Martin region significantly had the highest annual yields, whereas the yields of fine and flavor cacao clones and the cacao from the Cusco region significantly were the lowest (**Fig 11d**).

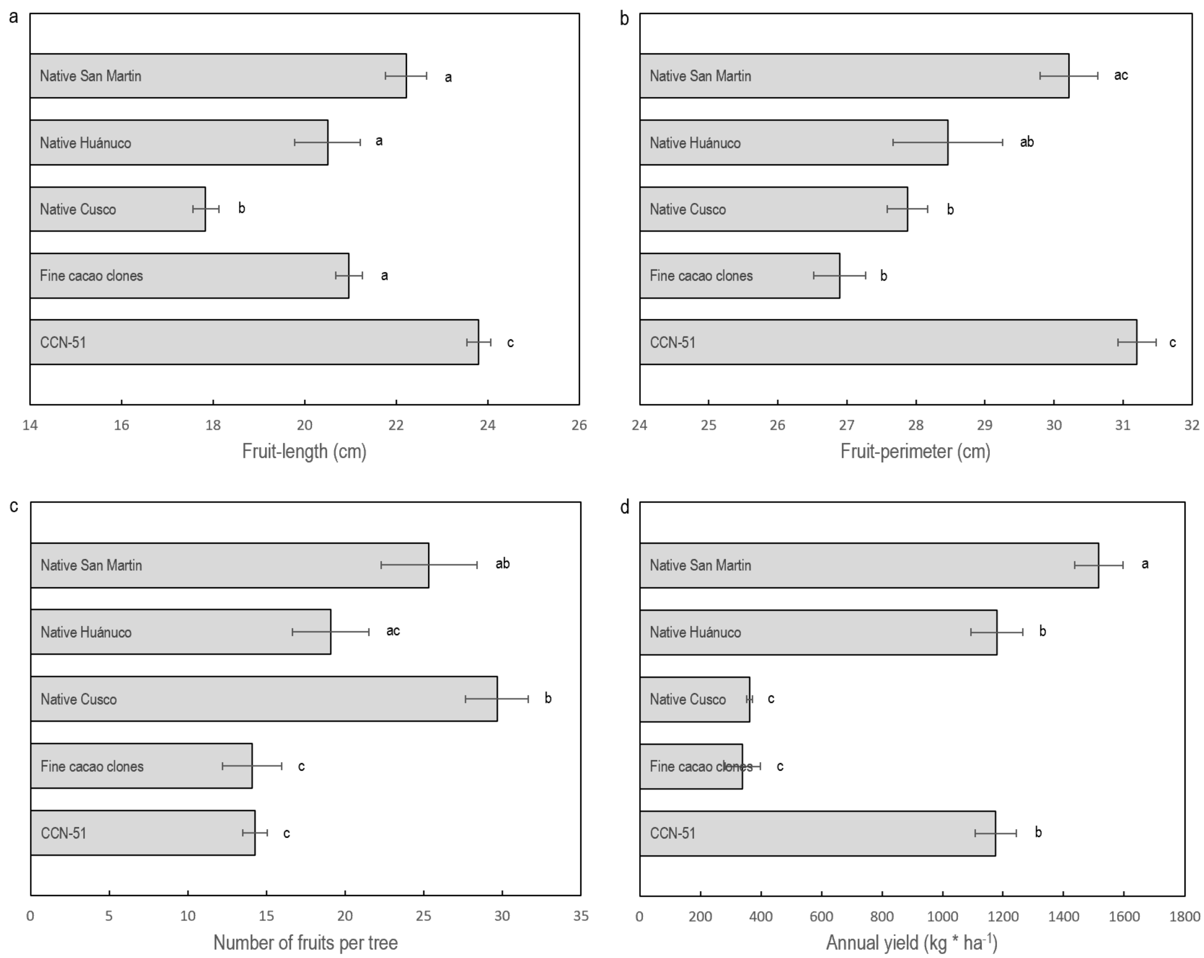

Figure 11: Differences between the fruit-sizes (length, perimeter) in cm, the number of fruits per tree and the annual yield in kg per hectare of the different cacao types (native cacaos from the San Martin, Huánuco, Cusco Regions, fine cacao clones, CCN-51). Different lower-case letters show significant differences between the cacao types. Averages with standard errors. Software R 3.2.3. ANOVA, Tukey-Test.

The cacao types were also characterized by different infection rates depending on the disease (**Fig 12; Tab 3**). The fruits of fine cacao clones were significantly more frequently attacked by insects than the other cacao types, whereas the native cacaos from San Martin and Cusco showed significantly lower insect attack rates than CCN-51 and fine and flavor cacaos (**Fig 12a**). Branches of cacao trees from Cusco had the highest infection rates with the fungus *Moniliophthora perniciosa* but did not differ from native cacao from San Martin and Huánuco. Fine cacao clones and the CCN-1 clone had the significantly lowest infection rates with *Moniliophthora perniciosa* (**Fig 12b**). Fruits of the native cacaos from San Martin were more affected with the fungus *Phytophthora* sp. than the other cacao types but the infestation rates of this cacao did only differ significantly from the fruits of native Huánuco cacaos and the fine and flavor cacao clones which had the lowest infections (**Fig 12c**). Fruits of the native cacaos from Huánuco had the highest infestation rates with the fungus *Moniliophthora roreri*, closely followed by fruits from San Martin. But we did not find significant differences between cacao types (**Fig 12d**).

Within the clone CCN-51, we found significantly more attacked fruits by insects in the Huánuco region than in San Martin ($p < 0.01$) and vice versa a significantly higher infestation rate with *Phytophthora* sp. in San Martin compared to Huánuco ($p < 0.01$).

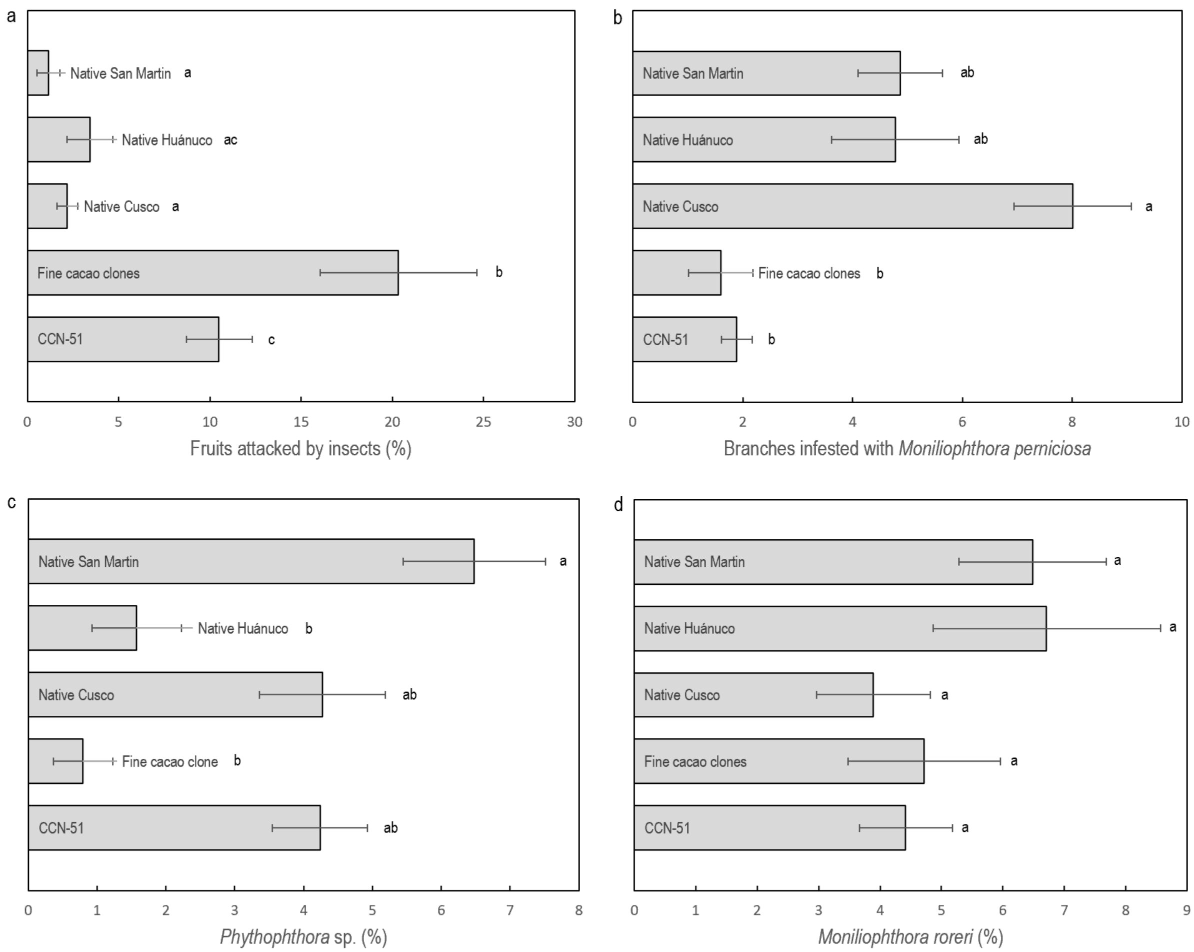

Figure 12: Differences of infestation rates with the fungi *Moniliophthora roreri*, *Phytophthora* sp. and *Moniliophthora perniciosa* as well as the attack by insects of the different cacao types (native cacaos from the San Martin, Huánuco, Cusco regions, fine cacao clones, CCN-51). Different lower-case letters

show significant differences between the cacao types. Averages with standard errors. Software R 3.2.3. ANOVA, Tukey-Test.

2.4.3 Environmental and management effects on cacao

In general, the environment such as soil conditions and farm management can have an effect on the cacao tree. Catechin in cocoa powder decreased significantly with higher soil pH (**Fig 13a**), whereas theobromine contents decreased significantly with higher soil electric conductivity (**Fig 13b**). Epicatechin ($p < 0.002$), cyanidin arabinoside ($p < 0.03$), and cyanidin galactoside ($p < 0.004$) contents were significantly lower with increasing soil pH. In addition, epicatechin ($p < 0.002$), and catechin ($p < 0.01$) decreased significantly with higher electric conductivity. Farm management factors such as the number of pruning activities per year influenced the methylxanthines (**Fig 13c, d**). Theobromine increased significantly with the number of pruning activities per year (**Fig 13c**), whereas caffeine decreased (**Fig 13d**). Moreover, diseases with fungi and insects influenced secondary metabolites. The epicatechin ($p < 0.027$), cyanidin arabinoside ($p < 0.035$), and cyanidin galactoside ($p < 0.001$) contents in cocoa powder were significantly higher when insects attack of the fruits increased, which by trend could also be seen in catechin ($p = 0.085$), albeit the significance is just marginal. Cacao trees with more branches infested with *Moniliophthora perniciosa* had significantly higher cyanidin arabinoside contents ($p < 0.015$) in cacao seeds. In contrast, the cyanidin arabinoside contents were significantly lower with increased infection with *Phytophthora* sp. ($p < 0.016$). Older cacao trees showed significantly higher epicatechin contents ($p < 0.027$) and marginally significantly higher catechin contents ($p < 0.094$) in seeds. Theobromine content increased significantly with the fruit-size as indicated by length ($p < 0.001$) and perimeter ($p < 0.049$). In contrast, the caffeine ($p < 0.046$), and cyanidin arabinoside ($p < 0.049$) contents decreased significantly with the fruit-perimeter.

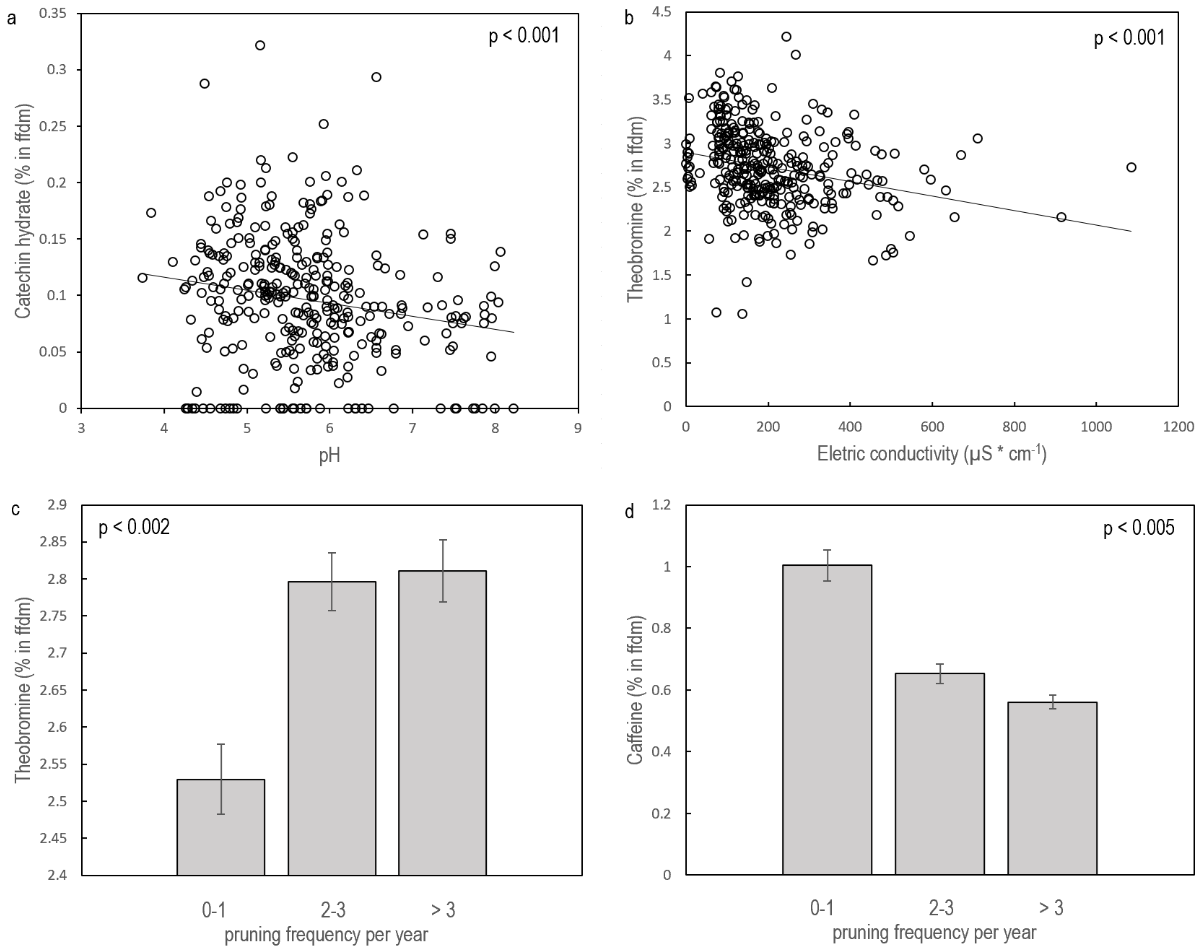

Figure 13: Influence of soil and management on polyphenol contents: **a)** The content of catechin (%) in the fat free dry matter (ffdm) of cocoa powder from different cacao types (native cacaos from the San Martin, Huánuco, Cusco regions, fine cacao clones, CCN-51) with the soil pH; **b)** The theobromine (%; ffdm) content with the electric conductivity; **c)** The theobromine (%; ffdm) content with the number of pruning activities per year; **d)** The caffeine (%; ffdm) content with the number of pruning activities per year. Generalized Linear Mixed Models (GLMM), R 3.2.3. ± Standard errors.

Environmental conditions and farm management influenced also annual yield and yield parameters, such as ripe fruits per tree, diseased fruits, and fruit size. Cacao trees with less ripe fruits (%) had significantly higher fruit-length ($p < 0.004$), and fruit-perimeter ($p < 0.001$). The number of total fruits per tree decreased significantly with the fruit perimeter ($p < 0.035$). However, fruit-length ($p < 0.04$) and fruit-perimeter ($p < 0.001$) increased significantly with lower electric conductivity of the soil. Moreover, the percentage of ripe fruits ($p < 0.029$) and diseased fruits in general ($p < 0.025$) per cacao tree decreased significantly with the number of diseased branches with *Moniliophthora perniciosa*. The annual yield (kg ha^{-1}) was significantly higher with more frequent pruning activities per year ($p < 0.001$), whereas the annual yield decreased with the age of cacao trees ($p < 0.001$).

2.5 Discussion

2.5.1 Influence of the genetics of cacao on secondary metabolites

The quality of raw cacao depends on the composition of secondary metabolites which is known to show pronounced differences among cacao genotypes (Afoakwa et al. 2008; Counet et al. 2004; Luna et al. 2002; Niemenak et al. 2006; Kadow et al. 2013). Many of the available studies have concentrated on so called fine and flavor cacao clones which are characterized by their particularly high flavor and quality properties in comparison to the bulk cacao CCN-51, which is predominantly cultivated to gain high and stable yields (Boza et al. 2014). In contrast, native cacaos from different regions of Peru have so far poorly been studied (Motamayor et al. 2008). Some native cacaos are appreciated for their unique flavor, which is the reason why the so called *Chuncho* cacao is cultivated in the Cusco Region. Chuncho has a high genetic variation and is supposed to originate from this Peruvian region. For other native cacaos the scientific background is completely missing, and the potential of these cacaos remained unexplored. This study provides for the first time data on quality and yield traits of these unknown native cacaos.

Concerning polyphenols, being responsible for bitter flavor, such as epicatechin, the mean values were the highest with 2.6% in cocoa powder from native cacao of the San Martin region and lowest with 0.4% from native cacao of Huánuco, whereas the CCN-51 clone showed 2.1%, native cacao from Cusco 2.1%, and the fine cacao clones 1.9%. Catechin reached the highest value (0.1%) in native cacaos from the Cusco region, followed by the bulk clone CCN-51 (0.1%). In general, there was no significant difference between CCN-51 and the fine and flavor cacao clones regarding to the epicatechin and catechin. This contrasts with the general perception that bulk cacao such as CCN-51 show more bitter flavor characters due to high polyphenol contents, whereas fine cacaos are characterized by lower polyphenol content (Clapperton et al. 1994; Kattenberg and Kemming 1993; Luna et al. 2002).

Dry cocoa beans can reach polyphenol contents up to 15% (Krähmer et al. 2015) and the most abundant polyphenol epicatechin amounts to about 35% of the total phenolic content (Kim and Keeney 1984). Cocoa powder of defatted and unfermented cacao beans from Malaysia contained 3.8% of the most abundant polyphenol epicatechin (Nazaruddin et al 2001), whereas the study from Kim and Keeney (1984) showed epicatechin values up to 4.3%. With 0.4 to 2.6% and a mean value of 1.8% the epicatechin content of all three regions in Peru is lower than those of other commercially used cocoa worldwide.

Anthocyanin contents are about 4% of the total phenolic compounds and determine the color of fresh cacao beans from white to deep purple (Afoakwa et al. 2008; Jahurul et al. 2013; Kim and Keeney 1984). Criollo cacaos contain less polyphenols in general and nearly no anthocyanins, which is the reason why fresh beans are white (Lange and Finke 1970; Hansen et al. 2000). Regarding this assumption, the native cacaos analyzed in the present study from the Cusco region may not be grouped into the Criollo group with the highest anthocyanin contents (cyanidin arabinoside: 0.23%, cyanidin galactoside: 0.11%). Also, the native cacaos from the San Martin region and the fine and flavor cacao clones showed higher anthocyanin contents than the CCN-51 clone, whereas the native cacaos from Huánuco had the lowest values with 0.005% cyanidin arabinoside and 0.0007% cyanidin galactoside.

Raw cacao beans contain about 4% methylxanthines, of which 2 to 3% is theobromine (Afoakwa et al. 2008). In the present study, theobromine contents were highest in the bulk clone CCN-51 with 2.9% and lowest in fine and flavor cacao clones with 2.3%, whereas the native cacaos showed diverging results. Theobromine content of unfermented bulk cocoa powder from West Africa reaches up to 3.95%, while the caffeine content is 0.19% (Aprotosoaie et al. 2016). In many studies, caffeine was only found in small amounts (0.2%; Franco et al. 2013; Kadow et al. 2013). Fine and flavor cacaos from South America are richer in caffeine (0.3% to 0.6% ffdm) and poorer in theobromine (2.85% to 3.43% ffdm; Afoakwa et al., 2008). In general, our study showed methylxanthine values within the range of literature values for fine and flavor cacaos from South America with lower theobromine contents (2.3-2.9%) and higher caffeine contents. Caffeine values of native cacaos ranged from 0.4% in Huánuco to 1.2% in Cusco.

2.5.2 Fruit size, disease susceptibility and yield are depending on the cacao varieties

Cacao clones which are cultivated on a big scale in different countries such as the CCN-51 in South America are appreciated for their higher disease resistance, big fruits and higher yields. In contrast, fine and flavor cacao clones are less resistant, need higher management intensity, and have smaller fruits (Afoakwa et al. 2008; Boza et al. 2014; Fowler 2009; IGPD 2018; Rusconi and Conti 2010; Wood and Lass 1988; Ziegleder et al. 1990). In contrast to the study of Kieck et al. (2016), the CCN-51 clone in our study area had significantly longer fruits (23.8 cm) and fruit-perimeter was significantly bigger (31.2 cm) than most other cacao types. However, highest annual yields were reported for native cacaos from San Martin with 1516 kg ha^{-1}, whereas CCN-51 had 1176 kg ha^{-1} on average. This is in contrast to Boza et al. (2014) who reported a 89% higher productivity of CCN-51 compared with Nacional cacaos from Ecuador. The native cacaos showed significantly higher total fruit numbers in comparison with CCN-51 and fine cacao clones. This may be an effect of less frequent pruning resulting in higher growth of these native cacao trees and therefore higher fruit numbers (according to farmer interviews).

The resistance especially against the fungus *Moniliophthora perniciosa* of CCN-51 has been reported to be higher than of Nacional cacaos, and disease resistance of CCN-51 is expected to be generally higher (Boza et al. 2014; IGPD, 2019). However, the study from Kieck et al. (2016) revealed no significant difference in disease susceptibility (*Moniliophthora roreri*, *Moniliophthora perniciosa*, *Phytophthora* spp., attacks by insects) between the CCN-51 clone and native cacaos from the San Martin region. In our study, the clone CCN-51 (10.5%) was significantly more frequently attacked by insects compared to the native cacaos from San Martin (1.1%) and Cusco (2.2%), albeit fine and flavor cacao clones had the highest proportion of attacked fruits by insects (20.3%), which is in accordance with the assumption of higher disease susceptibility in the literature (Boza et al. 2014; Rusconi and Conti 2010; Ziegleder 1990 and 1991). For other pathogens, our study demonstrates significantly lower infestation rates with *Moniliophthora perniciosa* of CCN-51 (1.9) compared to the other cacao types, but there were no significant differences in infestation rates of *Moniliophthora roreri* and *Phytophthora* sp. between CCN-51 and the other cacao types.

The comparison of CCN-51 between the different sampling regions showed no significant differences in yield parameters and anthocyanins, but disease susceptibility, some polyphenols and methylxanthines which may be attributed to effects of the environment in the two different regions or the management on farms. Several studies indicated that apart from cacao genotype, polyphenol contents also depend on the origin, climate conditions, the ripeness of fruits, as well as the post-harvest processing of cacao seeds (Kieck et al. 2016; Kothe et al. 2013; Niemenak et al. 2006; Rusconi and Conti 2010).

Mislabeling of cacao clones on cacao plantations or the distribution of a mislabeled clone on plantations can also be reasons for different flavor- and fruit characteristics (Herrmann et al. 2015). Herrmann et al. (2015) found a difference in the CCN-51 clones' genome which indicates a variability within the CCN-51. However, there is a lack of studies on the drivers of such differences in flavor-, and fruit characteristics.

2.5.3 Influence of environmental and management factors on secondary metabolites and yield

Environmental conditions such as soil, climate, disease incidence, and management factors may influence composition of secondary compounds in cacao (Aprotosoaie et al. 2016; Afoakwa et al. 2008; Kieck et al. 2016; Zug et al. 2019). In particular, soil conditions have been reported to influence secondary compound contents, as for instance higher soil pH leads to lower polyphenol contents in cacao seeds, presumably due to indirect stress reduction (Elwers et al. 2009; Kieck et al. 2016). Accordingly, also in the present study we found that higher pH led to lower catechin contents as well as higher electric conductivity of soils which may be accompanied by higher pH led to lower theobromine contents.

Moreover, theobromine content responded to management factors and raised with the number of pruning activities per year, again as possible stress reaction to plant injury while pruning. In general, plant injuries cause higher synthesis of secondary metabolites as a plant defense mechanism (Stoll 2010; Vaughn and Duke 1984). Consequently, Ndoumou et al. (1995) found higher flavonoid contents in cacao seeds with higher resistance against *Phytophthora* sp. Also, the increase of procyanidins in cacao leads to lower infection rates with the fungus *Moniliophthora perniciosa* (Andebrhan et al. 1995; Brownlee et al. 1990 and 1992; Kieck et al. 2016). In turn, an increase in *Phytophthora* sp. infection causes higher phenolic content in fruit shells (Blaha and Letode 1987), while caffeine contents showed the opposite with pruning. Also, in this study, we found a positive correlation of polyphenol contents and incidence of insects and *Moniliophthora perniciosa* infestation.

Cacao yield is highly affected by diseases, which can cause yield losses of up to 30% and even kill up to 10% of cacao trees per year (Acebo-Guerrero et al. 2011; Clough et al. 2009). In contrast, frequent pruning can increase cacao yields and reduce disease incidence (Clough et al. 2009; Tscharntke et al. 2011). These findings could be confirmed by our study as ripe fruit number was lower at higher *Moniliophthora perniciosa* infestation rate, and yield was higher with more frequent pruning. In addition, we found that fruits increased in length and perimeter when less ripe fruits per tree were present and fruit-perimeter also increased with lower total number of fruits per tree. Such negative fruit number-size relationship in cacao has also been reported by other authors (Beer 1988; Groeneveld et al. 2010; Muñoz and Beer 2001).

We found that yield decreased with the age of cacao trees, whereas Zug et al. (2019) reported an increase of annual yield with the age of trees. This may be attributed to the fact that maximum tree age reached 10 years in the study of Zug et al. (2019), while in the present study maximum age was 60 years. Also, other studies reported losses in yield due the age of crop trees along with diseases, pollination-reduction, as well as water and nutrient limitation (Acebo-Guerrero et al. 2011; Bisseleua et al. 2008; Cierjacks et al. 2016; Groeneveld et al. 2010; Moser et al. 2010; Schwendenmann et al. 2010; Tscharntke et al. 2011). As in our study the polyphenol contents of epicatechin and catechin increased with the age of cacao trees, maybe indicating higher infestation rate at higher age, which is probably the reason for the found yield reduction (see also Tscharntke et al. 2011).

2.6 Conclusion

This study is the first that investigates native cacaos from Amazonian lowlands regarding to their secondary metabolites, fruit sizes, disease susceptibility, and yield potential. Native cacaos from different regions of the Peruvian Amazonia differed significantly from each other and the known high-yield clone CCN-51 or fine and flavor cacao clones. The native cacaos from Huánuco had lowest polyphenol contents which are responsible for bitterness and astringent mouth-feeling, whereas the native cacaos from San Martin showed highest epicatechin contents. Native cacaos from Cusco had the highest caffeine contents which is typical for fine and flavor cacaos (Afoakwa et al. 2008; Aprotosoaie et al. 2016). Moreover, older trees had higher epicatechin and catechin contents, with the native Cusco trees showing highest mean age.

Overall, native cacaos showed less insects incidence, but high infestation rates with fungi, in particular *Moniliophthora perniciosa* which points to the need of more frequent health care of native trees. Fruits were biggest from the high-yield clone CCN-51 but yields of native cacaos in San Martin higher. We therefore assume a good potential for high revenues from native cacaos in the San Martin region. Native cacaos from Cusco were the only cacaos grown wild without shape pruning and with lowest yields. Overall, results showed an increase in yield and fruit size with higher pruning frequencies and lower fruit number per tree which makes shape pruning a promising measure.

Cacao in general is affected by many factors such as climate, soil, and management conditions, as well as the surrounding flora and fauna (Clough et al. 2009; Clough et al. 2011; Groeneveld et al. 2010; Moser et al. 2010; Kieck et al. 2016; Schwendenmann et al. 2010; Tscharntke et al. 2011; Zug et al. 2019), which can also lead to differences in flavor composition in the same cacao variety. Accordingly, we found significant differences in epicatechin, catechin, and methylxanthine contents within the same clone CCN-51 of different regions San Martin and Huánuco (compare Afoakwa et al. 2008; Aprotosoaie et al. 2016). The analysis of different environmental factors revealed that higher electric conductivity and pH of soils decreased secondary metabolites, possibly due to stress reaction in the course higher nutrient supply (Elwers et al. 2009; Kieck et al. 2016). Consequently, liming to increase soil pH may be a possibility to decrease stress reactions in cacao.

2.7 Acknowledgements

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3 Cadmium accumulation in Peruvian cacao (*Theobroma cacao* L.) and opportunities for mitigation.

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3.1 Abstract

Crops are the main source of toxic cadmium for humans due to uptake from naturally or anthropogenically polluted soils. Chronic Cd ingestion causes kidney, liver, and skeletal damage along with an increased risk of cancer. Cacao is known to accumulate Cd and may therefore be potentially harmful to human health. Consequently, cocoa production on intensely polluted soils should be avoided. Cocoa products from South America in particular often exceed the limits for Cd, but the factors that drive Cd uptake are as yet poorly studied. In this study, we measured Cd concentrations in defatted cocoa powder from unfermented seeds of 40 different trees on 20 farms in the Huánuco Region, Peru, and associated the Cd levels with the farms' soil, field management, and nearby vegetation diversity. The mean Cd concentration found in cocoa of the study region was 2.46 mg kg^{-1} with a range of 0.2–12.56 mg kg^{-1}. The maximum content measured was an order of magnitude higher than the allowed limit of 1.5 mg kg^{-1} and was the highest reported so far in the literature. Soil Cd content was the most relevant driver of Cd concentration in cacao. In addition, fertilizer use caused significantly higher Cd concentration in cocoa. Higher biodiversity of herbs was positively correlated with Cd contents in cocoa. The study shows that, apart from the known correlation of soil conditions with Cd accumulation in cacao seeds, changes in fertilization and plant composition may be promising measures to counteract Cd contamination in regions with high soil Cd content.

Keywords: Biodiversity, Heavy metal pollution, Agroforestry, South America, Food security, Food chain

3.2 Introduction

For both plants and animals, Cd is one of the most hazardous heavy metals (Hoseini and Zargari 2013). In humans, Cd toxicity was reported for the first time as Itai-Itai disease in Japan, which was later attributed to the consumption of Cd contaminated crops. It is characterized by osteoporosis, painful bone fractures, kidney stones, and kidney damage. The most common cause is industrially Cd polluted water, which contaminates soils and subsequently crops and is thus integrated into the food chain of the human population (Chavez et al. 2015; Greger et al. 2016; Wagner 1993; WHO 2010). While soils often naturally contain dangerous levels of heavy metals, industrialization has increasingly led to more manmade pollution as a consequence of mining activities or smoke contamination (Jung and Thornton 1996). Heavy metals differ in their frequency (Pb > Zn > Cd > Cu) and in their availability for living cells. Some, such as Co, Cr, Cu, Ni, and Zn, are essential for plants and therefore nontoxic at low concentrations, whereas others, such as Ag, As, Cd, Hg, Pb, and Sb, are toxic at any level (Aikpokpodion et al. 2012; Holm et al. 1995; Hoseini and Zargari 2013; Ogunlade and Agbeniyi 2011). For the nonsmoking population, contaminated crops are the main source of Cd (EFSA 2012) as it is relatively mobile in the soil–plant system (Chavez et al. 2015; McLaughlin and Singh 1995). Consequently, tolerable Cd limits are now regulated for crop species depending on the expected consumption (EFSA 2012; Hoseini and Zargari 2013).

In plants, Cd is accumulated in different tissues depending on the species (He and Singh 1994a, b; Ji et al. 2011; Li et al. 2017), and different plant species accumulate pronouncedly different amounts of Cd (Chaney et al. 1997; Ji et al. 2011; Salt et al. 1995). In particular, banana, cocoyam, and cacao are known to accumulate Cd at high levels and are therefore recommended to be planted exclusively on noncontaminated soils. However, many tropical soils in South America, especially andesite soils of volcanic origin, show naturally high concentrations of Cd (Bansah and Addo 2016; Chavez et al. 2015; Fauziah et al. 2001; Prugarová and Kovác 1987). The aforementioned crops are among the main cultivated crops in the tropical regions of South America (Lieberei and Reisdorff 2012). As in many cases, soil quality is not determined (Chen et al. 2015) before planting, and farmers are at risk of losing their entire harvest if levels of heavy metals are found to exceed allowed concentrations.

Contamination of different tissues of the cacao plant translates to different levels in the related cocoa products (e.g., fermented seeds, liquor, butter, cocoa powder). Cacao seeds from South America in particular often exceed maximum allowed values (Chavez et al. 2015; Mounicou et al. 2003; Prugarová and Kovác 1987). Seeds are fermented and dried mostly on farms and subsequently processed into cocoa-based products such as chocolate (Mounicou et al. 2003; Villa et al. 2014). Villa et al. (2014) showed that Cd content in chocolate bars from Brazil increased with the cocoa content in the bars (0.002–0.1 mg kg^{-1}).

Considering the dangers to humans of excess consumption and that the levels of Cd differ in different cocoa products, maximum allowed Cd limits have been defined by the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) for the different products, ranging from 0.2 to 2.0 mg kg^{-1} (**Tab 4**). Based on new studies that demonstrated even higher sensitivity of humans to Cd, the European Food Safety Authority (EFSA) has set even lower limits for cocoa products that will go into effect in 2019 (EFSA 2012; European commission 2014).

In many tropical countries, including Peru, cocoa production is the main source of income and therefore of pivotal importance for millions of farmers. In Peru, cacao cultivation has been re-intensified since the 1980s in several regions to overcome illegal coca production and to provide farmers with a reliable source of income (García Carrión 2012). Both the highly productive bulk cacao clone CCN-51 and promising fine and flavor cacao clones were distributed (García Carrión 2012). Unfortunately, many cacao farmers are located in the reach of the Andean mountains, which makes the existence of Cd in soils probable (Fauziah et al. 2001). However, little is known about Cd accumulation and the underlying mechanisms in these cacao varieties.

The high socioeconomic importance of cacao together with the pronounced potential for Cd accumulation in South American cocoa products requires an improved understanding of factors determining Cd accumulation in cacao. With the new Cd limits for cocoa products set for 2019, this study aims to untangle the main drivers of Cd accumulation in cacao, which may help to sustain cacao cultivation in regions at risk of heavy metal contamination. In particular, we hypothesized that (1) Cd contents from Peruvian cacao seed samples are at least sometimes higher than the allowed limits; (2) Cd contents in CCN-51 are higher than in fine and flavor cacao clones due to its faster growth and metabolism; (3) soil conditions, field management, and plant diversity influence Cd absorption in cacao seeds; and (4) quality (plant secondary compounds) and yield (annual yield per hectare, pods per tree, pod size, pest incidence) parameters are related to Cd accumulation in cacao seeds.

Table 4: Maximum Cd concentrations in cocoa products according to the World Health Organization (WHO), Food and Agriculture Organization (FAO), 2016 and the European commission, 2014.

Products	Cadmium maximum limit (mg kg^{-1})	Cadmium maximum limit in 2019 (mg kg^{-1})
1. Milk chocolate with <30% total dry cocoa solids	0.20	0.10
2. Chocolate with <50% total dry cocoa solid; milk chocolate with ≥30% total dry cocoa solids	0.60	0.30
3. Chocolate with ≥50% total dry cocoa solids	2.00	0.80
4. Cocoa powder sold to the final consumer	1.50	0.60

3.3 Materials and Methods

3.3.1 Study area and land use

The study was conducted in the Huánuco region, central Peru (9° 17' 43" S, 75° 59' 51" W) during the dry season from March to July 2016. The climate of the area is tropical.

Mean annual precipitation of the study region is approximately 3000 mm, and precipitation distribution is characterized by a pronounced rainy season from November to March. Mean annual temperature is 24 °C (maximum 29 °C, minimum 19.8 °C) and mean relative humidity is 81% (Soberanis et al. 1991). Due to the proximity to the Andes, soils are inceptisols characterized by a weak horizon formation and nearly no accumulation of organic matter.

The study area was formerly covered by evergreen rainforest (Walter and Breckle 2004) but has been widely transformed for agricultural activities. In Peru, cacao was first cultivated in the eighteenth century (Zhang et al. 2011) but was abandoned in many areas in the twentieth century in favor of increased cultivation of coca mainly for the illicit manufacture of cocaine (Strug 1985). Starting in the 1980s, several projects of the UNODC (United Nations Office on Drugs and Crime) and the Alianza Cacao Perú were implemented in the regions of Huánuco, San Martín, and Ucayali that aimed at replacing coca cultivation with cacao to offer farmers a legal income alternative. In the first phase of the UNODC project, the high-yield clone CCN-51 was distributed to farmers for quick income generation. This clone is considered more resistant to infestations of fungi, especially *Moniliophthora perniciosa*, and relatively easy to cultivate (Clough et al. 2011). Additionally, fine and flavor cacaos of high quality have been involved in many projects, e.g., Alianza Cacao Perú. These clones may yield a price above the official stock price, but they are considered less resistant to fungi and, therefore, need more intense management, such as pruning activities (International Cacao Germplasm Database (ICGD), 2018).

3.3.2 Study design and data collection

In the proximity of the city of Tingo Maria (9° 17' 43" S, 75° 59' 51" W), 20 cacao farms were randomly selected in 13 different locations (**Tab 5**). Only farms that cultivated both CCN-51 and fine and flavor cacao clones were included in the study. The fine and flavor cacao clones included ICS-1, ICS-6, ICS-

39, ICS-95, and TSH-565. On each farm, one tree of the clone CCN-51 and one of a fine and flavor cacao clone with at least two ripe pods were selected. The study trees were at least 10 m from the edge of the plantation. Accordingly, we analyzed 40 study trees on 20 cacao farms located at 13 locations. Each tree represented the center of a study plot for soil and vegetation analyses.

Cacao pests were assessed by counting pods infested with *Phytophthora* spp., *Moniliophthora roreri*, or insects and deformed twigs affected by *Moniliophthora perniciosa*. In addition, we counted ripe and unripe pods on the study tree that were at least 8 cm long to calculate fruit set and to determine the proportion of infested pods. Two to three ripe pods per tree were collected for seed analyses. To calculate pod size, perimeter and length of the collected pods were measured. For biochemical analyses, cacao seeds were removed from the pods and sun-dried without fermentation. For each tree, we used a separate seed sample (30 seeds) mixed from the collected pods per tree.

For vegetation and plant diversity assessments of the herb and shrub layer, we delimited an area of 5 × 5 m^2 around each cacao tree. The abundance of each occurring species was determined based on the methodology of Braun-Blanquet (1964). Identification of herbaceous plants and shrubs was conducted at the *Universidad Nacional Agraria de la Selva* (*UNAS*) in Tingo Maria, Peru. In addition, we counted and identified the shade trees (all individuals > 4 m in height) in a 20 × 20 m^2 area around each cacao tree and also estimated their relative cover based on the methodology of Braun-Blanquet (1964). The trunk circumference at breast height of each shade tree was used to calculate the basal area of shade trees.

For soil analyses, four 175 cm^3 soil samples (0–20 cm in depth) were taken within a radius of 1 m around each study tree and mixed. To gather information about field management such as pruning activities, planting density, field size for cacao cultivation, use of fertilizers (Roca fosforica, Guano de Isla, Sulfomag, urea, Cal-Mag, NPK, Compomoster cacao) and pesticides (Sulpocal, unspecified herbicides, Folistar), yield per hectare, and age of cacao trees, we interviewed the farmers (**Tab 5**).

Table 5: Yield, tree age, field management, and mean soil parameters (± standard errors) of all farms analyzed. Numbers in "Fertilizer use" refer to different fertilizers (1 = Roca fosforica, 2 = Guano de Isla, 3 = Sulfomag, 4 = Urea, 5 = Cal-Mag, 6 = NPK, 7 = Compomoster cacao). Letters in "Pesticide use" refer to different pesticides (A = Sulpocal, B = Herbicidos, C = Folistar).

Farm	Yield (kg ha^{-1}* year)		Tree age (years)		Fertilizer use	Pesticide use	Pruning per year	Soil			
	CCN-51	Fine and flavor cacao	CCN-51	Fine and flavor cacao				pH (H_2O)	E. conductivity (µS cm^{-1})	Water content (%)	Cadmium content (mg kg^{-1})
1	60	300	1.5	2	1, 2, 6	-	4	5.10 (0.31)	355.0 (28.5)	30.4 (0.0)	1.42 (0.43)
2	100	50	5	3	4	-	4	5.09 (0.35)	243.0 (55.5)	38.7 (2.8)	0.67 (0.24)
3	800	600	5	4	-	B	1	7.47 (0.01)	229.0 (25.0)	31.8 (4.2)	0.32 (0.01)
4	900	1000	8	3	1, 2	-	3	4.87 (0.06)	263.5 (6.0)	25.5 (0.3)	0.08 (0.01)
5	130	200	5	3	1, 2	-	3	4.63 (0.05)	181.4 (24.6)	32.4 (2.0)	0.04 (0.00)
6	500	800	5	3	-	-	1	5.11 (0.01)	206.0 (47.6)	31.7 (1.3)	0.09 (0.00)
7	800	0	7	2	-	-	2	5.48 (1.09)	270.7 (121.8)	27.2 (6.1)	0.08 (0.05)
8	2000	0	8	2	-	-	2	4.33 (0.05)	182.3 (7.9)	28.3 (2.5)	0.08 (0.00)
9	0	100	5	7	-	-	4	4.92 (0.66)	247.0 (42.1)	33.6 (1.3)	0.18 (0.05)
10	700	700	7	5	1, 2, 3	A	1	5.07 (0.34)	211.6 (38.4)	26.9 (3.2)	0.19 (0.09)
11	900	0	8	2	-	-	1	7.40 (0.10)	306.0 (49.0)	25.8 (4.5)	0.20 (0.01)
12	800	0	5	1	-	-	2	5.32 (1.22)	363.9 (216.1)	26.2 (2.2)	0.01 (0.01)
13	900	700	6	8	-	-	1	5.56 (1.32)	256.3 (75.7)	30.3 (4.0)	0.17 (0.12)
14	0	0	2.5	2.5	1, 5	-	2	5.31 (0.04)	191.8 (11.7)	23.0 (4.5)	0.10 (0.01)
15	500	500	4	4	-	-	1	5.85 (0.00)	187.8 (37.2)	27.5 (0.9)	0.23 (0.01)
16	700	800	3.5	3	7	-	1	4.55 (0.11)	169.4 (6.6)	34.0 (1.8)	0.04 (0.01)
17	400	400	3	3	2	-	1	4.50 (0.05)	135.1 (1.8)	31.4 (0.5)	0.04 (0.00)
18	1000	900	10	10	-	C	1	4.26 (0.42)	129.7 (37.9)	30.5 (4.1)	0.05 (0.01)
19	500	300	3.5	3.5	-	-	1	5.18 (0.14)	156.0 (29.4)	35.5 (1.7)	0.14 (0.04)
20	350	70	3	3	-	-	2	5.23 (0.13)	194.6 (19.4)	37.1 (3.5)	0.38 (0.11)

3.3.3 Lab analyses

Cd analysis

The seed samples of each cacao tree were dried for 48 h at 40 °C. Subsequently, the seed shell was removed from 30 seeds. Seeds were ground in a mortar, and 2 g of the resulting seed samples were transferred to a ball mill (MM200, Retsch, Haan, Germany). The samples were milled with 10 mL n-hexane at a frequency of 20 s^{-1} for 10 min. The grist was washed three times with 25 mL petroleum ether using a 0.45 µm filter in a Büchner funnel. The degreased filter cake was vacuum-dried at room temperature for 1 h (Araujo et al. 2014; Kieck et al. 2016; Niemenak et al. 2006). For Cd analyses of seed shells, we also milled the removed material in a ball mill (MM200, Retsch, Haan, Germany).

Soil samples were dried at 105 °C for 48 h, homogenized through a 2 mm sieve, and accordingly milled in a ball mill (MM200, Retsch, Haan, Germany). The soil samples, the degreased cocoa powder, and seed shell samples were treated with a microwave-assisted acid digestion (CEM 2004; Hoenig and de Kersabiec 1996). First, 2 mL distilled water, 3 mL 30% H_2O_2, and 5 mL 65% HNO_3 were added to 0.5 g of sample. After 90 min of microwave digestion under pressure using CEM MARS 5 (microwave routine protocol BPlant Tissue 2^; CEM 2001; CEM 2004), the samples were cooled down to room temperature, supplemented with distilled water to a final volume of 25 mL, and centrifuged for 2 min at 1800 rpm (HERAEUS MEGAFUGE 8, Thermo Scientific). Each sample was prepared twice and analyzed for Cd in a graphite furnace atomic absorption spectrometer (GF- AAS, Analytic Jena, ContrAA 700). The *AAS* method was used to achieve comparability to maximum allowed values as *WHO* and *FAO* also utilize this method (*FAO* and *WHO* 2016). Measurement was conducted in double runs following a calibration curve. Calibration was performed in a concentration range between 0.4 and 2.0 μgL^{-1}. Finally, 20 µL of each sample was automatically pipetted in a graphite furnace where the sample vaporizes due to high temperature. The atomization temperature was 1550 °C. Cd content was measured in micrograms per liter (Villa et al. 2014; Welz and Sperling 2008), which was then transformed to milligrams per kilogram fat-free dry matter.

To ensure that no contamination with Cd occurred during the preparation of cacao samples, flour samples were treated similarly to dried seeds or dried seed shells. Neither the untreated nor the treated flour was found to be contaminated with Cd (0.0 μgL^{-1}).

Analyses of soil conditions

Soil samples were weighed before and after drying at 105 °C for 48 h to determine the water content. Further soil conditions are based on dried, sieved soil samples. Soil pH was measured in both distilled water and 10 mM $CaCl_2$ solution after stirring 10 g of soil in 25 mL liquid for 1.5 h. In addition, conductivity was measured in the soil solution with distilled water. Soil pH and electrical conductivity were measured using a VWR symphony SP90M5 (Radnor, PA, USA). For statistical analyses, we used the results from pH tests in water.

Analyses of secondary compounds

To extract methylxanthines (theobromine, caffeine), 40 mL boiling distilled water was added to 0.1 g of fat-free and dried cocoa powder. The samples were kept at 100 °C for 30 min and afterwards cooled down to 20 °C. Subsequently, 0.2 mL Carrez I solution (150 g L^{-1} $K_4[Fe(CN)_6]\cdot 3H_2O$) and 0.2 mL Carrez II solution (300 g L^{-1} $ZnSO_4\cdot 7H_2O$) were added to clarify the sample solution. Distilled water was added to reach a final volume of 50 mL. Finally, the solution was filtered through a 0.45 µm syringe filter. Caffeine and theobromine were quantified using high-performance liquid chromatography (HPLC) at a wavelength of 274 nm using the appropriate calibration series for quantification (for further details, see Araujo et al. 2014; Elwers et al. 2009; Kieck et al. 2016).

Polyphenolic compounds such as epicatechin, catechin, cyanidine-3-arabinoside, and cyanidine-3-galactoside were extracted with methanol. First, 5 mL methanol was mixed with 0.1 g fat-free and dried cocoa powder for 30–40 s using an ULTRA-TURRAX T25 agitator (Janke & Kunkel, Staufen, Germany). The extract was sonicated for 3 min, cooled on ice for 15 min, and centrifuged at 4100 U min^{-1}. The extraction process was repeated two times, except for the cooling time, which was reduced to 3 min. The collected supernatant was concentrated in a rotary evaporator (40 °C, 100 mbar), resolved in 3 mL methanol in an ultrasonic bath, and filtered through a syringe filter of 0.45 µm. The phenolic compounds were detected using HPLC at wavelengths ranging from 225 to 540 nm against calibration series of the respective polyphenols (Araujo et al. 2014; Elwers et al. 2009; Kieck et al. 2016; Niemenak et al. 2006).

3.3.4 Statistical analyses

Vegetation data of the herb, shrub, and shade tree layer were transformed to mean cover percentages following Frey and Lösch (2010) to calculate diversity indices. To assess diversity within each study plot, we used the following α-diversity indices: species richness (number of species), species evenness (distribution of species), the abundance of the vegetation in the different layers, and Shannon and Simpson indices with the latter two involving both the number of species and their distribution in an area (Magurran 1988). Whereas the Shannon index is sensitive to rare species, the Simpson index focuses on frequently occurring species and indicates the likelihood that two randomly chosen individuals in a defined area belong to the same species (Magurran 1988; Peet 1974). As a high Simpson value indicates a low diversity, we used the inverse (Magurran 1988). The diversity indices were calculated using *R*, version 3.1.1 (R Development Core Team 2014) with the package BiodiversityR (Kindt and Coe 2005). We calculated separate indices for (1) the herb and shrub layer, (2) the shade tree layer, and (3) all vegetation.

To detect variables that influence the content of Cd in seeds, seed shells, and soil, generalized linear mixed models (GLMM) were calculated using R, version 3.1.1 (R Development Core Team 2014). Field management parameters; Cd content in seeds, seed shells, and soil; diversity indices; soil parameters; and pathogen incidence were included in the models as fixed effects. The nested study design with paired trees on each farm and with more than one farm per location was considered in the random effects of the model (Zuur et al. 2009). The glmer function in the lme4 package was used for fruit counts and yield because these showed Poisson distribution (Bates 2010). Normally distributed response variables were modeled with the lme function within the nlme and MASS package (Pinheiro and Bates 1995; Ripley 2015).

To detect variables which potentially predict the total soil Cd contents, we initially considered (1) soil variables: pH, water content, and soil bulk density; (2) farm management variables: fertilizer and pesticide application, the age of cacao trees, the distance between planted cacao trees; and (3) environmental variables: richness, abundance, the Shannon and Simpson indices, and species evenness of the herb layer, the shade tree layer, and the total vegetation. The same variables were used to describe the total Cd content in the cocoa powder from seeds and in seed shells as well as polyphenol content in cocoa powder, but we included total soil Cd content, the location, the number of pruning activities per year, and the clone as additional variables. Variable reduction was performed by stepwise elimination of predictor variables with p value higher than 0.05.

3.4 Results

3.4.1 Cd content in different cacao varieties

Cd concentrations in defatted cocoa powder were 2.46 mg kg^{-1} on average and ranged from 0.02 to 12.56 mg kg^{-1} (**Tab 6**). Fine and flavor cacao showed slightly higher Cd concentrations in cocoa powder and seed shells than CCN-51, but the differences were not significant (**Tab 6**).

Table 6: Cd content, pod number and size, pathogen incidence, plant secondary compounds in cacao seeds, plant abundance, and diversity indices of 40 study trees and plots (means ± standard errors of 20 CCN-51 and 20 fine and flavor cacao trees). Values in bold refer to significant differences between CCN-51 and the fine and flavor cacao clones in generalized linear mixed models (GLMMs). ffdm = fat free dry matter.

	CCN-51	Fine and flavor cacao
Mean cadmium and lead content		
Cadmium in soil samples (mg kg^{-1})	0.18 ± 0.05	0.27 ± 0.09
Cadmium in seed shells (mg kg^{-1})	0.72 ± 0.27	0.71 ± 0.30
Cadmium in cocoa powder from unfermented seeds (mg kg^{-1} ffdm)	2.26 ± 0.56	2.66 ± 0.75
Mean number and size of cacao pods per tree		
Total pod number per tree	11.6 ± 1.6	15.9 ± 2.6
Percentage of ripe healthy pods (%)	41.8 ± 4.1	35.6 ± 5.1
Percentage of unripe pods (%)	58.2 ± 4.1	64.4 ± 5.1
Mean perimeter of ripe pods (cm)	**30.2 ± 0.5**	**27.2 ± 0.5**
Mean longitudinal length of ripe pods (cm)	**23.6 ± 0.5**	**21.2 ± 0.4**
Mean pathogen incidence per tree		
Percentage of infested pods (%)	**38.5 ± 5.3**	**21.8 ± 4.2**
Percentage of pods infested with insects (%)	27.8 ± 5.7	17.8 ± 4.2
Percentage of pods infested with *Phytophthora* spp. (%)	2.8 ± 1.7	0.7 ± 0.4
Percentage of pods infested with *Moniliophthora roreri* (%)	6.7 ± 2.0	2.7 ± 1.1
Number of branches deformed by *Moniliophthora perniciosa*	1.1 ± 0.6	1.5 ± 0.7
Plant secondary compounds (means of at least two ripe pods per tree)		
Theobromine (mg kg^{-1} ffdm)	2.59 ± 0.05	2.45 ± 0.10
Caffeine (mg kg^{-1} ffdm)	**0.36 ± 0.02**	**0.58 ± 0.04**
Ratio theobromine/caffeine	**7.69 ± 0.41**	**4.56 ± 0.38**

Catechin (mg kg^{-1} ffdm)	0.93 ± 0.15	0.96 ± 0.14
Epicatechin (mg kg^{-1} ffdm)	20.68 ± 2.21	20.79 ± 2.52
Cyanidine-3-galactoside (mg kg^{-1} ffdm)	0.93 ± 0.13	0.79 ± 0.11
Cyanidine-3-arabinoside (mg kg^{-1} ffdm)	1.49 ± 0.18	1.60 ± 0.22
Mean plant abundance and diversity per study plot		
Richness of the entire vegetation	9.55 ± 0.73	9.70 ± 0.80
Shannon index of the entire vegetation	1.75 ± 0.10	1.73 ± 0.12
Inverse Simpson index of the entire vegetation	0.76 ± 0.03	0.75 ± 0.04
Evenness of the entire vegetation	0.65 ± 0.02	0.66 ± 0.04
Basal area of shade trees in m^2 per 400 m^2	3.58 ± 1.14	2.17 ± 0.52
Richness of shade trees per 400 m^2	2.85 ± 0.37	2.45 ± 0.30
Shannon index of shade trees per 400 m^2	0.45 ± 0.09	0.37 ± 0.08
Inverse Simpson index of shade trees per 400 m^2	0.23 ± 0.05	0.20 ± 0.04
Evenness of shade trees per 400 m^2	0.66 ± 0.04	0.73 ± 0.05
Richness of herb layer per 25 m^2	6.85 ± 0.68	7.75 ± 0.78
Shannon index of herb layer per 25 m^2	1.48 ± 0.12	1.58 ± 0.13
Inverse Simpson index of herb layer per 25 m^2	0.68 ± 0.05	0.70 ± 0.05
Evenness of herb layer per 25 m^2	0.74 ± 0.03	0.72 ± 0.04

3.4.2 Impact of soil conditions, field management, and plant diversity on Cd absorption in cacao trees

The Cd content of both cocoa powder (**Fig 14a**) and seed shells (**Fig 14b**) was positively correlated with soil Cd concentration. The locations of the studied farms showed pronouncedly different Cd concentrations in soils ($p = 0.0179$), and the highest Cd concentrations were measured in cacao seeds from trees growing on farms 1, 2, 14, and 20 (**Tab 5**; **Supplementary Material**, **Tab A.1**). Farms 1, 2, and 20 were also the farms with the highest Cd contents in soil (farms 1 and 20 are adjacent to each other).

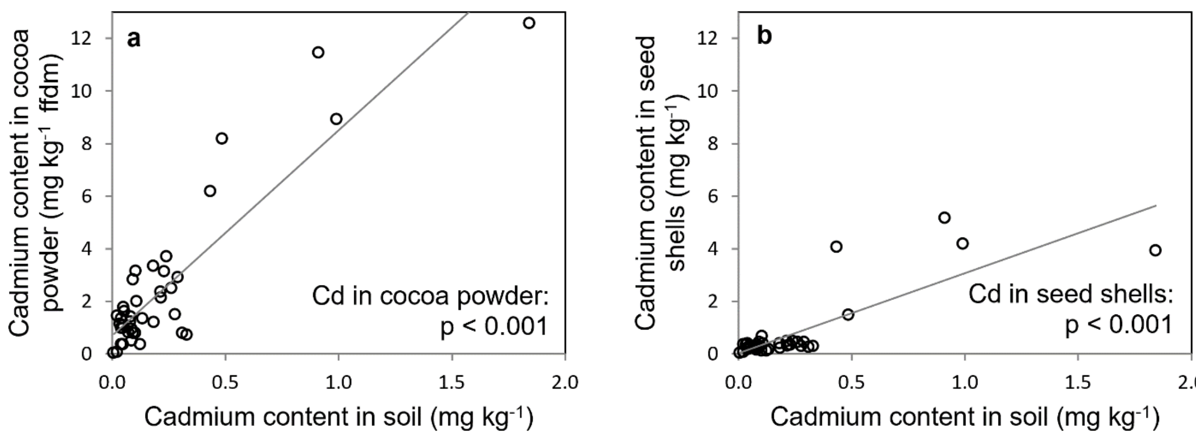

Figure 14: Correlation of **a** Cd content in cocoa powder of seeds [lme (Cd in cocoa powder of seeds ~ Cd in soil, random = ~ 1│Location/Farm/Clone)] and **b** Cd content in dried seed shells [lme (Cd in seed shells ~ Cd in soil, random = ~ 1│Location/Farm/Clone)], with the Cd content in soils. Model documentation according to GLMM. ffdm = fat-free dry matter. Lines show linear regressions of the respective variables to indicate the slope of significant correlations.

The use of fertilizers significantly increased the Cd content in cocoa powder from seeds (**Fig 15**). Eight farms were fertilized since at least 1 year, whereas 12 did not use fertilization (**Tab 7**). Cd contents in cocoa powder from plantations using N-fertilizers (Guano de Isla, urea, NPK; **Tab 7**) were significantly higher ($p = 0.0161$), and those using P-fertilizers (Roca fosfórica, Guano de Isla, NPK; **Tab 7)** were marginally higher ($p = 0.0511$). Moreover, Cd contents in soil were significantly positively related to the use of P-fertilizers ($p = 0.0139$).

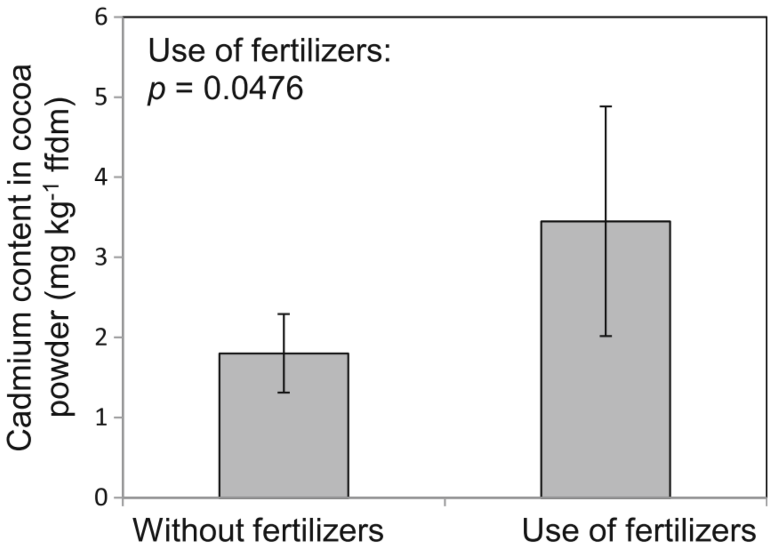

Figure 15: Differences of the Cd content in cocoa powder of seeds regarding the use of fertilizers [lme (Cd in cocoa powder of seeds ~ fertilizers in total, random = ~ 1 | Location/Farm/Clone)]. Mean values with standard errors. Model documentation according to GLMM. Lme = linear mixed effects. ffdm = fat-free dry matter.

Table 7: Fertilizer use, type of fertilizer, and nutrient presence in different fertilizer types (Roca fosforica, Guano de Isla, Sulfomag, urea, Cal-Mag, NPK, Compomoster cacao) used on the studied farms. "+" indicates whether the respective fertilizers contain nitrogen or phosphate; "−" refers to absence of fertilizer or nutrient.

Farm	Fertilizer use	With nitrogen	With phosphate
1	Roca fosforica, Guano de Isla, NPK	+	+
2	Urea	+	-
3	-	-	-
4	Roca fosforica, Guano de Isla	+	+
5	Roca fosforica, Guano de Isla	+	+
6	-	-	-
7	-	-	-
8	-	-	-
9	-	-	-
10	Roca fosforica, Guano de Isla, Sulfomag	+	+
11	-	-	-
12	-	-	-
13	-	-	-
14	Roca fosforica, Cal-Mag	-	+
15	-	-	-
16	Compomoster cacao	-	-
17	Guano de Isla	+	+
18	-	-	-
19	-	-	-
20	-	-	-

The vegetation assessment revealed a considerable plant diversity in the studied agroforestry systems. Overall, we identified 124 plant species, 69 of them to the species level, 32 to the genus level, 15 to family level, and 8 as morphospecies (**Supplementary Material**, **Tab A.2**). Per plot (combining 5 × 5 and 20 × 20 m^2 plots), species number ranged from 4 to 18 species. Cd content in cocoa powder was positively correlated with the inverse Simpson index of the total vegetation ($p = 0.0047$), indicating that Cd content in cacao increases with diversity. Soil Cd content was correlated with the farm's plant abundance and showed higher Cd values when the abundance of the herb layer was higher ($p < 0.0001$). In contrast, a higher abundance of shade trees was marginally correlated with lower Cd concentrations in soils ($p = 0.0955$). Banana tree abundance exhibited a marginally negative correlation with the Cd contents in cocoa powder (p = 0.0905) and soil (p = 0.0883). In addition, inverse Simpson indices of both the herb layer (p = 0.0082) and all vegetation (p = 0.0001) were positively correlated with Cd content in soils, whereas both Shannon index and evenness of the herb layer (Shannon index: p = 0.0486; evenness: p = 0.0011) and all vegetation (Shannon index: p = 0.0025; evenness: p < 0.0001) were negatively correlated with Cd in soils. Also, fertilizer use influenced biodiversity. The use of fertilizers significantly increased the inverse Simpson index of the total vegetation (p = 0.019) and the herb layer (p = 0.0144) along with the Shannon index of the total vegetation (p = 0.0218) and the herb layer alone (p = 0.0241).

3.4.3 Relation of Cd accumulation to quality and yield parameters

Statistical results provide evidence for a relationship between Cd content and polyphenol content in seeds (**Fig 16**). All polyphenols considered (epicatechin, catechin, cyanidin arabinoside, cyanidin galactoside) were significantly negatively correlated with the Cd content in cocoa powder samples (**Fig 16**). In contrast to polyphenols, methylxanthines (theobromine, caffeine) showed no relation to Cd accumulation in cacao seeds (**Tab 6**). Moreover, significant negative correlations of three polyphenols and the use of fertilizer were observed (epicatechin: p = 0.0207; cyanidin arabinoside: p = 0.0124; cyanidin galactoside: p = 0.0139). The use of N-fertilizers marginally decreased the content of catechin (p = 0.0563).

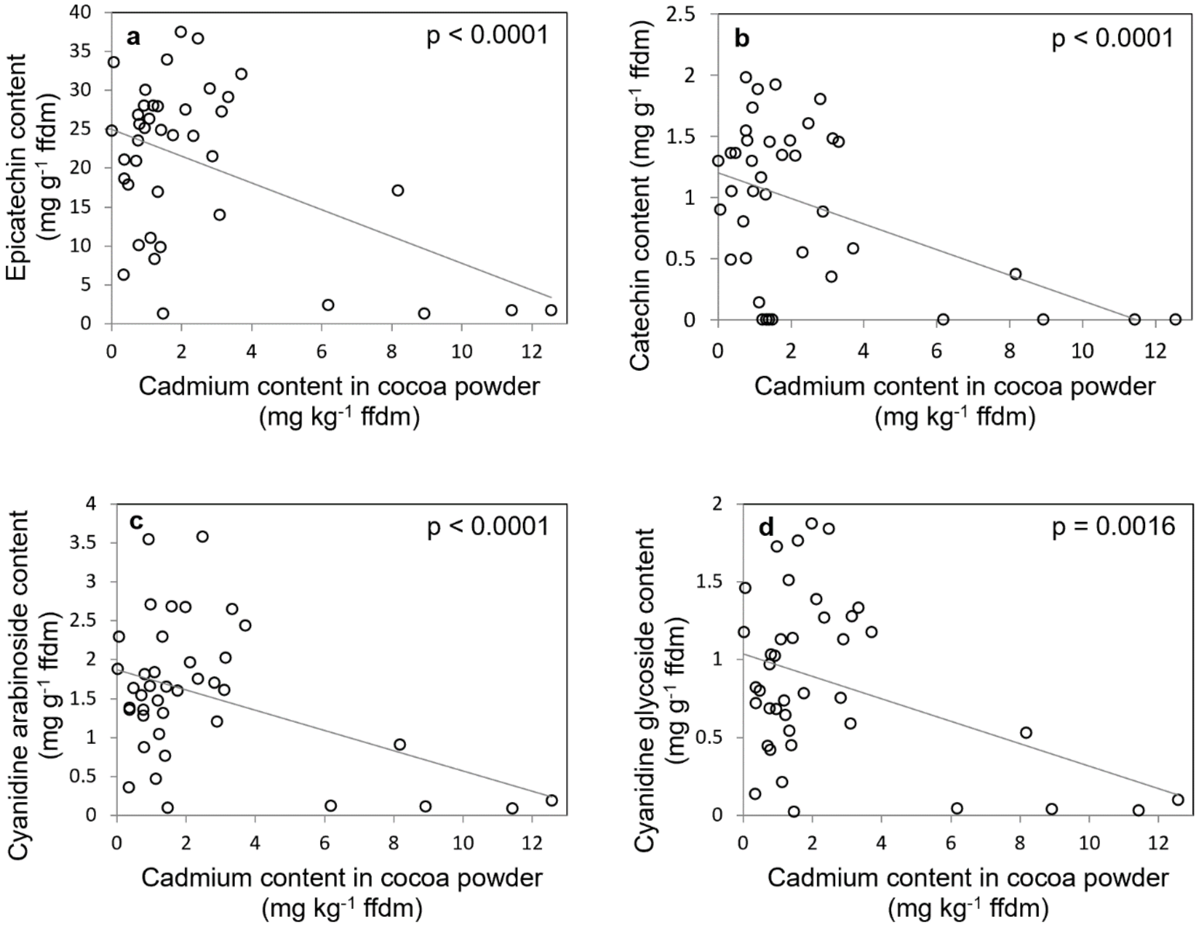

Figure 16: Correlation of Cd content in cocoa powder of seeds with the contents of **a** epicatechin, **b** catechin, **c** cyanidin arabinoside, and **d** cyanidin glycoside in cacao seeds [lme (polyphenol in cocoa powder of seeds ~ Cd in cocoa powder of seeds, random = ~ 1|Location/Farm/Clone)]. Model documentation according to *GLMM*. ffdm = fat-free dry matter. Lines show linear regressions of the respective variables to indicate the slope of significant correlations.

Polyphenol and theobromine contents did not differ between CCN-51 and fine cacao clones (**Tab 6**), but we found significant differences in caffeine concentration with fine and flavor cacao clones showing higher contents ($p < 0.0001$) and lower theobromine/caffeine ratio than CCN-51 ($p < 0.0001$).

For most yield parameters, such as the overall annual field yield in kilograms per hectare, the size of pods (perimeter, length), or pest incidence, we found no significant correlation with the content of Cd in soils, seed shells, or cocoa powder. However, the total number of pods per sample tree was significantly related to the Cd content in cocoa powder, the water content in soil, and the inverse Simpson index of the total vegetation (**Fig 17**). As Cd contents increased in pods, the pod number decreased. In contrast, higher water content and diversity around the study tree correlated positively with the number of pods on the cacao trees. The annual yield in kilogram per hectare proved to be influenced by the age of the cacao tree ($p < 0.0001$) along with the infestation rates of *Moniliophthora roreri* ($p = 0.0527$). Fields with older cacao trees showed higher yields, whereas higher pathogen infestation rates reduced yields. Further, we found that overall infestation rate was significantly related to clone type. Surprisingly, CCN-51 exhibited significantly more total infested pods than the fine and flavor cacao clones ($p = 0.0087$; **Tab 6**). Infestations of CCN-51 pods with insects ($p = 0.0818$) and *Moniliophthora roreri* ($p = 0.063$) individually were marginally significantly higher (**Tab 6**). In addition, the length ($p = 0.0011$) and perimeter ($p < 0.0001$), measurements of direct yield parameters, were significantly higher, and hence, the pods of the CCN-51 clone were bigger than the fine and flavor cacao clones (**Tab 6).**

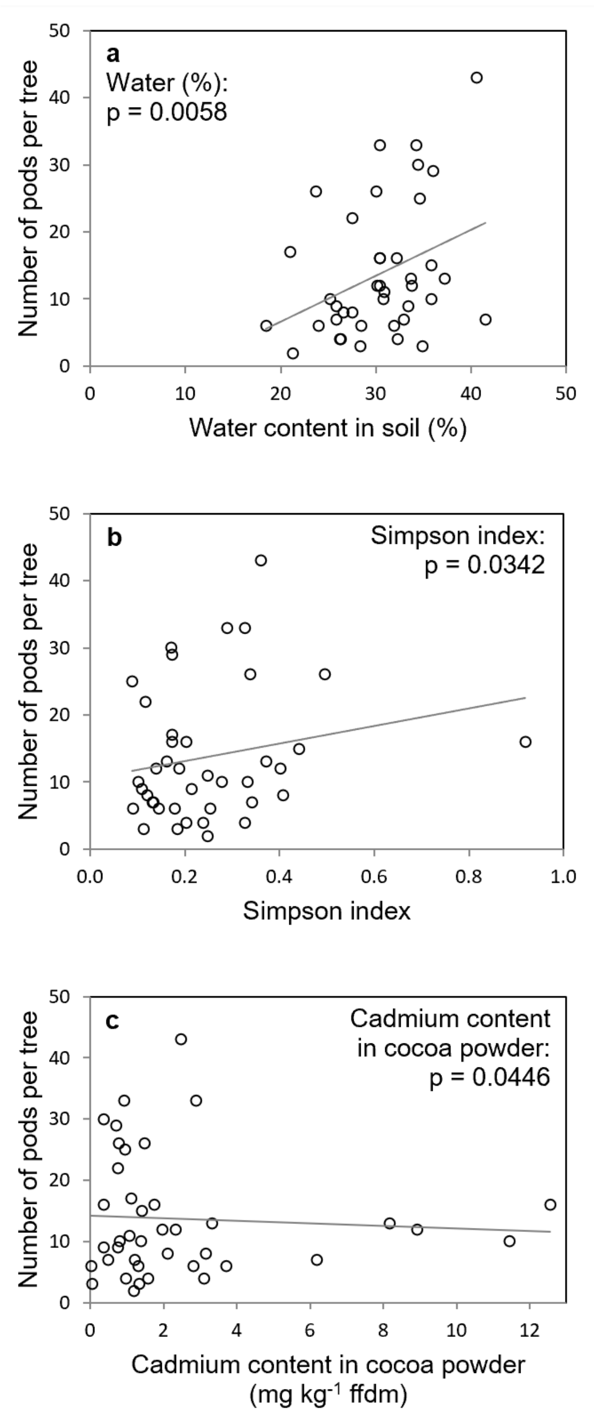

Figure 17: Correlation of **a** water content of the soil, **b** inverse Simpson index of the total vegetation, and **c** Cd content in cocoa powder of seeds with the number of pods per tree [lme (pods per tree ~ water content in soil + inverse Simpson index + Cd in cocoa powder of seeds, random = ~ 1│Location/Farm/Clone)]. Model documentation according to *GLMM*. ffdm = fat-free dry matter. Lines show linear regressions of the respective variables to indicate the slope of significant correlations.

3.5 Discussion

The present study demonstrates that there is evidence for several interactions of Cd contents in cacao seeds with soil Cd contents, field management, and plant diversity, along with plant secondary compounds and fruit set. Cd contamination of cocoa products has frequently been reported, and contents are often far too high for consumption (Chavez et al. 2015; Arévalo-Gardini et al. 2017; Fauziah et al. 2001; Mounicou et al. 2002a, b; Prugarová and Kovác 1987; Sager 2012; Villa et al. 2014). However, to our knowledge, there are no studies that have demonstrated Cd contents as high as in the present study. Recent research suggests that the human body is about three times more sensitive to the presence of Cd than assumed previously (EFSA 2012). The tolerable weekly intake for Cd was reduced from 7.5 to 2.5 µg kg^{-1} body weight after studies highlighted the pronounced sensitivity of the human body to Cd intake (ESFA 2012; *European Commission* 2014; Mounicou et al. 2002a, b, 2003; Schroeder et al. 1966; Villa et al. 2014). Thus, it is increasingly important to understand reasons for high Cd contents in crops. While numerous studies have shown various effects of plant or animal biodiversity on yield, pathogen incidence, or secondary metabolism in cacao agroforestry systems (Acebo-Guerrero et al. 2012; Cicuzza and Kessler 2011; Clough et al. 2011, 2009a, b; Kieck et al. 2016; Stenchly et al. 2011, 2012; Tscharntke et al. 2011), the relationships between field management, biodiversity, and the absorption of heavy metals such as Cd in cacao trees have not yet been investigated comprehensively (as claimed by Mounicou et al. 2003). Many studies highlighted that soil pH and soil Cd at the location where cacao trees are cultivated are important factors determining the availability of Cd for plants (Chavez et al. 2015). Our study additionally provides novel insights into the less understood effects of field management and plant diversity on Cd contents in cacao tissues.

3.5.1 Cd contents

Cd values measured in defatted cocoa powder from the sample seeds ranged from 0.02 to 12.56 mg kg^{-1}, the latter being far too high for human consumption according to EFSA (2012). In general, chocolate has relatively high contents of Cd compared with other sweets (Sager 2012). New allowed values set by the European Commission (2014) range from 0.1 to 0.8 mg kg^{-1} for chocolate products with a limit of 0.6 mg kg^{-1} for defatted cocoa powder. The limits must be complied with in European countries by 2019. In the present study, 40% of the analyzed cocoa powder samples showed higher Cd contents than currently allowed, and 85% of the samples were higher than the maximum tolerable value of 0.6 mg kg^{-1} set to take effect in 2019. As opposed to our hypothesis that Cd contents would be higher in CCN-51 than in fine and flavor cacao clones due to the former's faster growth and metabolism, we found no significant differences between the two varieties. In contrast, Arévalo-Gardini et al. (2017) reported differences in Cd accumulation rates in farms with different cacao clones. Leaves and beans from farms with CCN-51 alone had lower Cd concentrations than samples from farms with a combination of CCN-51 and ICS-95 together. Also, for other species such as rice, differences in Cd accumulation depending on the clone type have been reported (Liu et al. 2016).

Most studies analyzed the Cd content of the kernel of fermented seeds, whereas this study refers to the Cd content of defatted cocoa powder of dried seeds as Cd is almost entirely retained in the cocoa powder and not in the cocoa butter (Mounicou et al. 2003). Consequently, Cd concentration in peeled cocoa beans from Ecuador revealed lower mean Cd concentrations (0.90 mg kg^{-1}; Argüello et al. 2019) than in our study. Our results showed a strong correlation among Cd concentrations in seed shells and in the cocoa powder of seeds with the Cd concentration in the shells being lower than in cocoa powder. Accordingly, Chavez et al. (2015) reported a decrease in Cd concentrations in cacao plant parts in the order of seed (whole seed, without seed shell) > shell > leaf, whereas Gramlich et al. (2017) and Fauziah et al. (2001) demonstrated higher values in the leaves than in the seeds (whole seed). Prugarová and Kovác (1987) found higher Cd contents in seed shells than in seeds (whole seed, without seed shell) from the Ivory Coast, but samples from Ecuador had higher contents in seeds compared to shells. Future studies should therefore investigate which cacao varieties or cultivation conditions cause the observed differences in Cd accumulation in different plant parts. The recent work of Thyssen et al. (2018) who visualized Cd distribution within cocoa bean tissues may facilitate such studies. Possibly, also the production process from fermentation to the bean roasting has an influence on resulting Cd concentrations.

High Cd contents in cocoa products were found in earlier studies and differed with geographical origin. In western Africa and Brazil, where about 80% of cacao is cultivated, relatively low Cd contents have been measured (0.3 ± 0.1 mg kg^{-1} in cocoa powder, Mounicou et al. 2002a, b), whereas Cd contents in the rest of South America were higher, with concentrations up to 1.8 mg kg^{-1} in cocoa powder (Mounicou et al. 2002a, b). Gramlich et al. (2017) found an average of 1.1 mg kg^{-1} Cd in beans (whole seeds, without seed shell) from Honduras. Chavez et al. (2015) reported contents that ranged from 0.02 to 3.00

mg kg^{-1} in cacao seeds without shells from Ecuador with 12 of 19 samples exhibiting Cd over the tolerable limits.

3.5.2 Impact of soil conditions, field management, and plant diversity on Cd absorption in cacao trees

The present study provides further evidence for the pivotal importance of soil for Cd accumulation in plants. The total Cd content in soil was a significant driver of Cd content in cacao seeds (seed shells and cocoa powder). We further found differences in Cd concentration in cacao seeds from the different locations, presumably based on the natural abundance of Cd in soils. Fauziah et al. (2001) demonstrated that the content of Cd in cacao seeds is higher from trees grown on developed andesite (volcanic origin) compared to those from cacao trees grown on alluvium. Accordingly, the soils in our study area are mainly of mountainous origin with higher likelihood of Cd occurrence. In contrast, Gramlich et al. (2017) found the highest Cd values in cocoa bean (whole bean, without seed shell) growing on alluvial substrates. Even when soil Cd contents are low, concentrations in cacao tissues can be higher due to accumulation processes (Fauziah et al. 2001). In addition to natural Cd occurrence in soil, sewage sludge, aerial disposition (Grant et al. 1996; Mullins et al. 1986; Williams and David 1976), and contaminated fertilizers may increase Cd pollution in soils of different locations. Industrial pollution from metal mining in Huánuco may also be a reason (Purser and Purser 2008).

Moreover, soil pH is known to drive availability and subsequent uptake by plants of nutrients (He and Singh 1994a, b) as well as Cd from soil solution (Fauziah et al. 2001; Prasad 1995). In general, higher soil pH leads to lower Cd concentrations in crops (as shown for carrot, spinach; He and Singh 1994a). Accordingly, Gramlich et al. (2017) found that the available Cd in soil is an influencing factor of cocoa bean contamination. Negative charges with higher pH induce adsorption of Cd and, hence, a lower availability for plants (Hanafi and Maria 1998; He and Singh 1994b). Zhu et al. (2016) reported a decreased phytoavailability of Cd in rice fields with an increased lime application due to a higher soil pH. Liming was also reported as a potential measure for mitigating Cd uptake in cacao. Further studies should therefore measure both available Cd and total contents in soil for more detailed information about this issue. In addition to soil conditions, we identified some field management parameters that significantly influenced Cd contents in cacao. The use of fertilizers in general and the use of nitrogen-fertilizer dramatically increased Cd in cocoa powder of seeds. Many fertilizers, particularly phosphate fertilizers, are known to be Cd-polluted (Fauziah et al. 2001; Grant et al. 1996; He and Singh 1994a, b; Williams and David 1976). Moreover, Cd from phosphate fertilizers can accumulate in soils over time (Grant et al. 1996). He and Singh (1994a, b) tested the effect of NPK fertilizers on Cd contents in crops (oat, ryegrass, carrot, and spinach) and found a positive correlation of Cd contents and NPK fertilizer application. This may also be the case in our study where the positive correlation of phosphate-fertilizers and soil Cd content suggests additional soil contamination by these fertilizers. In the study of He and Singh (1994a, b), this effect could be counterbalanced by increasing soil pH.

However, in the present study, it was less the use of phosphate-fertilizers, but rather potentially Cd-free nitrogen-fertilizers that increased Cd in cocoa powder—presumably due to increased growth rate of cacao trees and related mineral uptake along with higher Cd availability. This is supported by the study of Grant et al. (1996) who demonstrated a Cd increase in the grain of durum (*Triticum turgidum*) after fertilizer application, irrespective of whether it was nonpolluted nitrogen, phosphate, or a combination of both. They suggested that the higher Cd content in soil solution could be attributed to the desorption of Cd from soil colloids after fertilizer treatment. In accordance, the use of mycorrhizal fungi as biofertilizers in cacao fields increased Cd contents in the leaves and stems (Ramtahal et al. 2012). Overall, the use of fertilizers, polluted or not, can cause higher Cd levels in crops due to a direct pollution with Cd, greater mobility of Cd in acidic soils, faster growth of the plant itself, or higher Cd absorption by soil mycelia (Hahne and Kroontje 1973; McLaughlin and Singh 1995; Ramtahal et al. 2012). Further studies preferably under controlled conditions in greenhouses are required to verify the effect of individual fertilizers because various fertilizers were used on the farms analyzed in our study (**Tab 5**).

Our study revealed several interactions of plant diversity and abundance with Cd content in soil and cacao seeds. The inverse Simpson index, a measure of alpha diversity that responds to more common species, was positively related to soil Cd content, whereas both the Shannon index and evenness decreased with higher soil Cd content. These findings point to a change in the relative abundance of species due to Cd contamination of soil with a greater dominance of potentially Cd tolerant species in more contaminated soils and reduced abundance of sensitive ones. As a consequence, evenness and the Shannon index decreased, and the more frequently appearing species were distributed more evenly with positive effects on inverse Simpson index. In fact, the supply of various elements, such as Cd and elements for plant nutrition, affects and shapes vegetation abundance and communities (Mengel and

Kirkby 1978; Kabata-Pendias 2010), and plant species are known to differ in their potential to accumulate heavy metals (Ji et al. 2011). In general, the accumulation of Cd of different crop species growing in the same soil decreases in the following order: leafy vegetables > root vegetables > grain crops (He and Singh 1994a, b). Some species are even used as hyperaccumulator plants in phytoremediation projects, thereby offering a cost-effective and environmentally friendly green technology to counteract heavy metal pollution of soils (Chaney et al. 1997; Ji et al. 2011; Tang et al. 2016).

In our study, Cd accumulation in cacao seeds was significantly higher when overall plant diversity and herb abundance were higher. The inverse Simpson index again showed a correlation with Cd content in seeds, whereas other indices were not significant in the statistical analyses. This correlation may imply an indirect effect of soil Cd leading to both higher content of Cd in seeds and higher inverse Simpson index, but a direct positive effect of plant diversity on both nutrient and contaminant availability due to complementary plant–plant interactions also seems possible (see Smith et al. 2010; Kieck et al. 2016). Moreover, this finding may be the result of fertilizer application, which may have increased Cd concentrations in plants (Ogunlade and Agbeniyi 2011; Williams and David 1976), while leading to a more abundant herb layer with a higher inverse Simpson index. Our results give evidence for such an interaction. The use of fertilizers was significantly positively correlated to the inverse Simpson index and Shannon index of the total vegetation and the herb layer alone. In contrast to the herb layer, increased shade tree abundance, especially banana tree abundance, led to marginally significantly lower Cd contents in seeds. Higher banana abundance also decreased Cd in soil marginally, which can be explained by the high Cd accumulation ability of banana plants (Bansah and Addo 2016) and points to a certain potential of this species for phytoremediation, when not used as food source. Gramlich et al. (2016) reported lower Cd contents in cacao leaves coming from agroforestry systems of the Alto Beni Region of Bolivia compared to leaves from monocultures, which supports this assumption.

Overall, our study suggests that the use of fertilizers should be reduced to counteract Cd accumulation in cacao seeds. In addition, shade trees and, in particular, banana may play a role in Cd accumulation of contaminated soils; further experimental evidence is needed to support large-scale application.

3.5.3 Relation of Cd accumulation to quality and yield parameters

We found significant negative correlations of different polyphenols and Cd contents in cacao seeds. Polyphenols are relevant substances in plant defense mechanisms and plant–plant interactions (e.g., Tugwell and Branch 1989). In addition, the content of polyphenols is relevant for flavor attributes and health effects of cocoa products and therefore determines final product quality (Niemenak et al. 2006). In general, polyphenols and their oxidases are separated in different cell compartments. In case of injury to the plant tissue by herbivores or pathogens, polyphenols are oxidized by their oxidases as a plant defense mechanism, and the synthesis of more polyphenols and oxidases is stimulated (Stoll 2010; Vaughn and Duke 1984). The resulting quinones are toxic for herbivores. Several studies reported stress reactions at higher Cd concentrations. Sunflower seedlings (*Helianthus annuus*) had increased levels of abscisic acid in roots and decreased level of total protein and chlorophyll, and seedling growth was negatively affected (Kirbag Zengin and Munzuroglu 2006). Dudjak et al. (2004) showed that barley seedlings (*Hordeum vulgare*) have higher polyphenol levels as Cd content increases in roots and shoots. Cadmium also causes an accumulation of strong oxidases including phenolic oxidases (Amal et al. 2009; Dudjak et al. 2004; Kováčik et al. 2009; Lavid et al. 2001). The negative correlation of Cd and polyphenol content in our study could again be explained by indirect effects of fertilization, which may have caused high biomass accumulation and reduced stress reaction of the plants. In addition, fertilizers may have increased soil pH, which leads to reduced polyphenol synthesis (Elwers et al. 2009; Kieck et al. 2016). The significant negative correlations with fertilizer use and the polyphenols in our results give evidence for a stress reduction. Although a final explanation for this clear finding is as yet lacking, the result points to clearly reduced constitutive defense of the cacao tree as a consequence of Cd intoxication.

Cd content in seeds in interaction with soil water content and plant diversity also reduced the total pod number per tree. In contrast to Hanafi and Maria (1998), who documented significant increases in plant heights and dry matter yields in a greenhouse experiment with cacao seedlings after application of Zn and Cd, our data found detrimental consequences of Cd uptake on the cacao fruit set. The positive effect of soil water content is supported by Schwendenmann et al. (2010) and Moser et al. (2010), who reported a strong yield reduction (50%) after a 13-month drought period, which indicates that sufficient water supply is required for pod production. High plant biodiversity is further known to be advantageous or at least not disadvantageous for high yields in cacao (Bisseleua et al. 2008; Clough et al. 2011; Schroth et al. 2016) as well as for other perennial crops such as oil palm (Samedani et al. 2014) or coconut and banana (Cierjacks et al. 2016). In accordance, Kieck et al. (2016) documented a positive impact of diversity in the herb and shrub layer on the number of ripe and healthy pods per tree.

Nevertheless, annual yield per hectare was not related to Cd or biodiversity in our study. We found that fields with older cacao trees had higher annual yields. In addition, higher infestation with fungi decreased yield, as also shown by Clough et al. (2009a). Concerning pest incidence, we found unexpected differences between the cacao varieties with higher infestation rates in CCN-51 than in fine and flavor cacao clones. In CCN-51, the infestation rates by *Moniliophthora roreri* and by insects were 40 and 64% higher, respectively, than in fine and flavor clones. *Moniliophthora perniciosa* alone was slightly lower in CCN-51. CCN-51 is known for its fast growth and increased resistance against pests in general, but particularly against *Moniliophthora perniciosa* (Boza et al. 2014; *ICGD* 2018), compared to other varieties. However, CCN-51 performance is best documented from Ecuador where CCN-51 has become one of the most planted cultivars (Boza et al. 2014) and climatic condition is pronouncedly different from our study region with a long cloudy dry season (May to November) and only 1 or 2 h of sunshine per day. In areas with high rainfall, as in Peruvian Amazonia, CCN-51 shows no comparable tolerance according to the International Cacao Germplasm Database (ICGD 2018), which may be an explanation for the observed infestation rates in this study. Still, CCN-51 in our study area showed bigger pods than fine and flavor clones, which may justify the cultivation of CCN-51 in Peru.

Interestingly, the expected positive effects of fertilizer application on yield (e.g., Ahenkorah et al. 1987) could not be confirmed in this study in accordance with other studies (Cierjacks et al. 2016; Groeneveld et al. 2010). The reduction or complete cessation of fertilizers in cacao agroforestry systems may therefore be an option for mitigating Cd accumulation in our study area.

3.6 Conclusion

The present study shows alarmingly high Cd contents in nonfermented seed samples of different cacao varieties cultivated in the Huánuco Region, Peru, with values up to 12.56 mg kg^{-1}. It therefore clearly highlights the need for stricter controls of Cd contents in cocoa products and for finding management solutions that allow farmers to grow cacao on contaminated soils without suffering yield and income loss. This study provides first indications of possible connections of Cd in cacao seeds and the Cd content in soil, the use of fertilizers, and the vegetation around the sample trees. The data further suggest that Cd content in plant tissues affects both fruit set and the secondary metabolism of cacao.

Although fertilizer application is often seen as necessary for adequate nutrient availability to ensure yield and pathogen resistance (Ahenkorah et al. 1987), such fertilizers also increase plant Cd absorption. In our study, we could not confirm a positive impact of the applied fertilizers on yield parameters, which makes reduction or even cessation of fertilization feasible. Phytoremediation with special Cd-accumulating plant species in agroforestry systems may be an option to further reduce Cd contents in crops. Our data imply that a higher abundance of shade trees and particularly banana may lead to lower Cd concentrations in soils, which makes cultivation of more shade trees in agroforestry systems with high soil Cd contents promising. However, many parameters (e.g., availability of macro- and micronutrients in the soil) which we did not measure in this study may have further influence on Cd accumulation, and therefore, further experimental evidence is needed to confirm the success of such management adaptations and to optimize yield while achieving minimum seed Cd content on polluted soils.

3.7 Acknowledgements

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4 Toxic metal contents of cocoa seed samples (*Theobroma cacao* L.) from Peruvian Amazonia are related to soil, region, and cacao type.

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4.1 Abstract

Cacao is, along with coffee, the most consumed luxury food worldwide, but may also be a relevant source of hazardous heavy metals and other toxic elements. In particular, cadmium (Cd), copper (Cu), and lead (Pb) have strict legal concentration limits in cocoa powder and other chocolate products due to their toxic effects for humans already at low contents. In this study, we analyzed heavy metal and aluminum (Al) contents in Peruvian cacao along with the most important drivers for accumulation in cocoa powder from unfermented seeds collected from 280 cacao trees and corresponding soil samples. We determined toxic metal contents, such as Cd, cobalt (Co), Cu, manganese (Mn), nickel (Ni), Pb, zinc (Zn), and Al, and subsequently modelled the impacts of soil metal concentration, sampling region (San Martin, Huánuco, Cusco), and cacao type (CCN-51, fine and flavor cacaos and native cacaos from San Martin, Huánuco, Cusco) using Boosted Regression Trees (BRTs). In the Cusco region, we detected the highest Co (1.13 mg kg^{-1}), Cu (49.65 mg kg^{-1}), Mn (78.54 mg kg^{-1}), and Ni (27.17 mg kg^{-1}) contents in cocoa powder samples, whereas Cd (2.13 mg kg^{-1}), Pb (0.09 mg kg^{-1}), and Zn (84.76 mg kg^{-1}) contents were highest in Huánuco and Al contents (16.56 mg kg^{-1}) reached maximum values in San Martin. However, BRT modeling revealed that heavy metal content in soil was the most important driver of increased contents in seeds in the case of Cd, Co, Cu, Mn, Ni, and Zn (42.0-78.8% of explained variance), in comparison to the regions which showed the lowest impact (3.4-19.1% of explained variance). Exclusively for Al, the regional differences explained 44.1% of the explained variance. For Pb, differences of the cacao types were most decisive for accumulation in seeds (73.7% of explained variance), with Pb contents in seeds from native Huánuco cacaos showing the highest value with 0.215 mg kg^{-1}, followed by CCN-51 (0.055 mg kg^{-1}), fine and flavor clones (0.050 mg kg^{-1}), native cacaos from Cusco (0.019 mg kg^{-1}), and native cacao from San Martin (0.010 mg kg^{-1}). Overall, 49.29% of cocoa powder samples exceeded their maximum allowed values in Cd, 38.57% in Cu, and 0.71% in Pb, which clearly highlights the need for efficient mitigation measures in cacao. For all other toxic metals, we give recommendations for a consumption-limit.

4.2 Introduction

Cocoa and coffee products are worldwide the most frequently consumed luxury foods (Aprotosoaie et al. 2016; Castro-Alayo et al. 2018). In particular, children consume cocoa products in sweets or chocolate drinks (EFSA 2012; Villa et al. 2014). Recent studies revealed alarming results about high levels of hazardous heavy metals in these products (Abt et al. 2018; Anyimah-Ackah et al. 2019; Arévalo-Gardini et al. 2017; Argüello et al. 2019; Chavez et al. 2015; EFSA 2015, 2018; Fechner et al. 2019; Zug et al. 2019), with higher Cd contents in South America than in the worldwide biggest cocoa producing area in West-Africa, whereas Pb contents are supposed to be higher in Africa. However, little is known about the abundance of other heavy metals and toxic elements in cocoa products, although the responsible interstate agencies such as FAO/WHO (Food and Agriculture Organization/World Health Organization) or the EFSA (European Food Safety Authority) are constantly reporting recent findings on potential health risks for humans (European Commission 2011, 2012, 2014, 2015, 2018; WHO 1982, 2010a, b, 2011). Consequently, further studies are needed to find potential regional hotspots of heavy metal and toxic elements accumulation in cocoa products and to better understand the underlying mechanisms.

In general, heavy metals and Al have toxic effects at high concentrations for plants and humans, but some are even harmful in small amounts, such as Cd and Pb (Hanafi and Maria 1998; He and Singh 1994b; WHO 2010b; Yang et al. 2005). Because of their toxicity for e.g., the neurological system (Pb) and bones or kidneys (Cd), maximum allowed limits for foods must be complied with in Europe (EFSA 2012; WHO 2010a, b). For cocoa powder, limits were set at 0.6 mg kg^{-1} for Cd and at 1.0 mg per kg^{-1} for Pb (COPAL 2004; European commission 2014). Co is one of the heavy metals which is harmful for plants at small concentrations, but necessary for animals and humans in small amounts (He et al. 2005). There is no legal limitation of Co concentration in foods, but higher exposure is known to cause goiter and reduced thyroid activity (Berk et al 1949). Cu consumption over 5 mg per kg body weight can damage liver and kidneys (Izah et al. 2016a, b; Lanre-Iyanda and Adekunle 2012) but is also essential in lower concentrations for cells (Yang et al. 2005). Therefore, the EFSA (2018) set a limit for cacao beans of 50 mg kg^{-1} Cu which must be complied with in Europe. Moreover, Mn and Zn are essential elements for the human body system and are playing a role in e.g., enzyme activation (Mn) and in immune system function (Zn; Fraker and King 2004; Izah and Angaye 2016; WHO 2011). A daily exposure of more than 0.06 mg kg^{-1} body weight Mn and 0.3 to 1.0 mg kg^{-1} Zn can be harmful for the human body (Izah and Angaye 2016; JECFA 2011; WHO 1982, 2011). Ni is considered a toxic element for living cells and a daily intake is therefore recommended not to exceed 2.8 µg kg^{-1} body weight (EFSA 2015; He et al. 2005). Albeit not a heavy metal, Al is known to be toxic at higher concentration. In recent studies, it was related to the Alzheimer disease in humans and therefore decreased in its tolerated weekly consumption from 7 to 1 mg kg^{-1} body weight (JECFA 2011; Perl and Moalem 2006; Stahl et al. 2011). In general, heavy metals and Al are naturally present in the earth crust and are transferred from soils into plant systems (Chavez et al. 2015; He and Singh 1994a, b; He et al. 2005). Anthropogenic pollution of soils or plants with these substances can occur due to fertilizer, pesticide, biosolid or compost use, mining activities, or leaded petrol aerosols (Aikpokpodion et al. 2013; Fauziah et al. 2001; Grant et al. 1996; He and Singh 1994a, b; Hebbar 2007; He et al. 2005; Mullins et al. 1986; Ogunlade and Agbeniyi 2010; Williams and David 1976; Zug et al. 2019).

Several studies analyzed heavy metal and toxic element contents in cacao and its products (Aikpokpodion et al. 2013; Bertoldi et al. 2016; Chavez et al. 2015; Fechner et al. 2019; Gramlich et al. 2017; Kruszewski et al. 2018; Lee and Low 1985; Lockard and Burridge 1965; Mounicou et al. 2003; Stahl et al. 2011; Steinberg et al. 2003; Vītola and Ciproviča 2016; Zug et al. 2019). Cd and Pb are the most intensely analyzed heavy metals in cacao owing to their pronounced health risk for humans. Cd contents in cocoa powder samples ranged from 0.3 mg kg^{-1} in Africa and Brazil to 1.8 mg kg^{-1} in South America (Mounicou et al. 2002a, b). In peeled cacao beans, Cd contents were between 0.9 mg kg^{-1} and 3.0 mg kg^{-1} in Ecuador (Argüello et al. 2019; Chavez et al. 2015), but highest contents were measured by Zug et al. (2019) with 12.56 mg kg^{-1} in cocoa powder samples from Peru. Aikpokpodion et al. (2013) found Cu contents of 25-261 mg kg^{-1} in cocoa beans from Africa with some contents far too high for human consumption regarding the recommendation of EFSA (2018) of 50 mg kg^{-1}. In accordance, also Lee and Low (1985) found an average content of 93.9 mg kg^{-1} Cu in cocoa powder from the local market of Malaysia. For Co and Mn, no literature concerning in cacao or cocoa products has been published so far. Also, literature about Ni levels in cocoa products remains relatively scarce. A recent study presented maximum values of Ni in cocoa beans of 12.1 mg kg^{-1} and 4.5 mg kg^{-1} in chocolate (Kruszewski et al. 2018), whereas the EFSA (2015) found a content of 9.8 mg kg^{-1} in cocoa. Pb contents in African cocoa beans measured between <0.0005 mg kg^{-1} (Rancin et al. 2005), 0.01-0.03 mg kg^{-1} (Prugarová and Kováč 1987), and 0.4-3.45 mg kg^{-1} (Aikpokpodion et al. 2013). Another study analyzed highest Pb values in Brazil with 0.769 mg kg^{-1}, and lowest in Ecuador with 0.011 mg kg^{-1} (Mounicou et al. 2003). Zn seems to be a heavy metal with a relative low toxicity and cocoa samples are containing about 43.4 up to 111 mg kg^{-1} (Aikpokpodion et al. 2013; Harland and Oberleas 1985; Steinberg et al. 2003). Soils are containing about 7% Al which can be potentially taken up by plants (Foy 1988; Kochian et al. 2004; Sposito 1996; Zhang et al. 2006). Several studies analyzed Al contents in cocoa powder and found average values up to 274 mg kg^{-1} in Africa, 41 mg kg^{-1} in Central America or 16 mg kg^{-1} in Ecuador (Bertoldi et al. 2016;

Vītola and Ciproviča 2016). Cocoa products from the German market contained about 118-171 mg kg^{-1} (Fechner et al. 2019).

Overall, some heavy metals such as Cd or Pb are relatively well represented in existing literature about cacao contamination, while others (Co, Cu, or Mn) have hardly been studied. Due to the general toxicity of heavy metals and Al, foods that tend to accumulate higher amounts of heavy metals than others, such as cacao (Bansah and Addo 2016), represent a potential risk for humans and should therefore be carefully monitored. In particular, products consumed by children require rigorous controls (EFSA 2012). Moreover, the factors that drive heavy metal and Al accumulation in cacao plants need more in-depth studies to prevent contamination in affected areas. In general, heavy metal and Al contents in cacao seem to be related to the geographic origin (Mounicou et al. 2003; Zug et al. 2019) and the concentration of the respective elements in soil (Zug et al. 2019). In addition, different accumulation behavior may be expected in different cacao types, but it remains unclear which of these factors is most important. To clarify this question, we selected three regions in the Peruvian Amazonia (San Martin in the north, Huánuco in the center, Cusco in the south) and collected 280 cacao seed and soil samples for heavy metal analyses. The study tests three hypotheses: 1) Heavy metal contents in cocoa powder samples depend on the heavy metal concentration in soil samples; 2) The content of heavy metals (Cd, Co, Cu, Mn, Ni, Pb, Zn) and Al in cacao powder from unfermented seeds depend on the samples` origin (San Martin, Huánuco, Cusco); 3) The contents of heavy metals and Al from the collected cacao clones (fine and flavor cacao clones, CCN-51) are in general higher than those of native cacaos from San Martin, Huánuco, and Cusco.

4.3 Material and Methods

4.3.1 Study area

The study was carried out in the Amazonian lowland of Peru, collecting unfermented seed and soil samples in three different regions: The Cusco region (southern Peru), the Huánuco region (central Peru), and the San Martin region (northern Peru). The natural vegetation is represented by evergreen rainforests with Peruvian Amazonian lowlands being characterized by a marked rainy and dry season. Sampling took place in the dry season between March to June in the years 2016, 2017, 2018. The annual average temperature in the lowland rainforests is 24 °C but may reach up to 29 °C and drop to approx.

20 °C (Soberanis et al. 1991). The annual precipitation is in average 3000 mm with a humidity of 80%. Soils in the area are mainly inceptisols with no or a small content of organic matter in the upper horizon. Nowadays, the majority of the eastern foothills of the Andes are used for agriculture (Zimmermann et al. 2009), with cacao and oil palms representing the most frequent crops after cessation of illicit coca production.

In particular, the high-yield cacao clone CCN-51, which is originally from Ecuador (International Germ Plasm Database (IGPD), 2019), has been widely planted in Peru. It produces big fruits and promises high yields within a short time with a lower disease susceptibility, whereas fine and flavor cacao clones have been cultivated for their special flavor qualities and higher stock price on the international market (Boza et al. 2014). In addition, also native cacao types with genetic origin in different regions of Peru can be found, but most trees are relicts in cacao fields between the cultivated cacao clones in San Martin and Huánuco (Kieck et al. 2016). Exclusively in the Cusco region, native cacaos known as Chuncho are cultivated on a big scale due to their specific flavor (Zug et al. submitted).

4.3.2 Study design

Due to the large extent of the study region, sampling was carried out in different study years. In June 2016, 30 fine and flavor cacao clones (ICS-1, ICS-6, ICS-39, ICS-95, TSH-565), and 30 samples of CCN-51 were collected on 40 different cacao plantations in the Huánuco region. The Cusco region was sampled in May and June 2017 collecting 105 samples of native cacaos from 7 cacao farms. In March 2018, 27 samples of native cacaos and 24 samples of CCN-51 were collected on 12 different cacao farms in the Huánuco region, and in April 2018, 36 native cacao and 28 CCN-51 samples were gathered from 14 cacao farms in the San Martin region. Overall, we analyzed 280 samples from 73 randomly selected cacao farms in three different regions (Kieck et al. 2016; Zug et al. 2019, see an overview on sampling sites and dates in Zug et al. submitted).

Field work was conducted using the same approach in each region and on each farm. Cacao farms were randomly selected in each region based on interviews with farmer cooperations, agro-technicians or private farmers. On each farm, the study trees were randomly selected with a minimum distance to field edge of 20 m and had at least two ripe fruits for enabling later toxic metal analysis in seeds (Kieck et al. 2016; Zug et al. 2019). Once a study tree was chosen, GPS coordinates were taken, and two to three ripe cacao pods were harvested. The seeds were removed and sundried without fermentation process. Dried seed were mixed for each sample tree and later defatted and processed into cocoa powder. Around every sample tree, four soil samples were taken at a depth of 0-50 cm and distance of one meter to tree stem. At a first measure to counteract further biological and chemical soil processes, soil samples were sundried. In the lab, soil samples were dried again for 48 h in an oven at 105 °C (Kieck et al. 2016; Zug et al. 2019).

4.3.3 Toxic metal analyses

For heavy metal (Cd, Co, Cu, Mn, Ni, Pb, Zn) and Al analyses, the seed shells of the dried cacao seeds were removed, and seeds were ground in a mortar for homogenization. Four g of homogenized seeds were mixed with approx. 20 mL of n-hexane and ground for 10 minutes at a frequency of 20 s^{-1} in a ball-mill (MM2000 Retsch, Haan, Germany). The cacao grist was washed three times with 25 mL of petrol ether through a 45 µm filter in a Büchner funnel, and later dried for one hour (Araujo et al. 2014; Kieck et al. 2016; Niemenak et al. 2006; Zug et al. 2019). The decreased cocoa powder was then used for toxic metal analyses.

The sun- and oven-dried soil samples were sieved through a 2 mm sieve and then used for metal analyses. Heavy metal and Al contents in cocoa powder, and soil samples from the three different Peruvian regions are presented in **Table 8**.

The toxic metal contents of the soil samples were analyzed after an aqua regia digestion according to A2.4.3.1 (VDLUFA, 1991). Two g of sample (cocoa powder or soil) were mixed with $HCl:HNO_3$ (3:1), boiled for two h at 130 °C, made up to 50 mL with distilled water and then filtered. The determination of the metal concentrations of the filtrates was carried by ICP-OES (inductively coupled plasma optical emission spectrometry) against a respective calibration curve of the metal measured.

4.3.4 Statistical analyses

Boosted Regression Trees (BRT) were used to calculate the importance of predictor variables for the toxic metal contents in seeds and transfer-factors of these metals (Buston and Elith 2011; Elith et al.

2008; Elith et al. 2006). BRTs were conducted in R, Version 3.6.1, using the packages dismo, Version 1.1-4, and gbm, Version 2.1.5 (Buston and Elith 2011; R Development Core Team 2007; Ridgeway 2007). In general, the BRT method is a combination of a single regression method and boosting, which fits multiple simple models combining them for prediction and generating a more robust model (Buston and Elith 2011; Elith et al. 2008).

As predictor variables for the toxic metal contents in cocoa powder samples, the region (Cusco, Huánuco, San Martin) in which cacao samples were taken, the cacao type (genetically native cacao, fine and flavor cacao clones, CCN-51 clone) which was sampled, and the respective heavy metal or Al content in soil samples were selected. During BRT modelling, some parameters (*learning rate*; *tree complexity*) must be adjusted; with the *learning rate* being important for reducing the contribution of each tree added to the model and the *tree complexity* determining the number of nods in a tree. Both, *learning rate* and *tree complexity* affect the optimal number of trees in a model. To minimize model error, we choose a rather high *tree complexity* of 5 for modelling. A smaller *learning rate* leads to a higher number of trees which is preferable in modelling BRTs (Buston and Elith 2011; Elith et al. 2008). In this study, the *learning rate* was selected to reach a minimum of 1000 trees and consequently differed between the modelling of the different toxic metal contents in seeds (Al: 0.003; Cd: 0.004, Co: 0.0006; Cu: 0.001; Mn: 0.1; Ni: 0.005; Pb: 0.0007; Zn: 0.001).

BRT modelling calculates the *relative influence* of each predicting variable based on the number of times the variable is selected during modelling and its improvement for the model. The sum of the *relative influence* of each predictor adds up to 100% (Buston and Elith 2011). Moreover, BRT modelling is not producing p values, instead the *explained variance* of a model was calculated using the total mean deviance and the estimated *cv*- (*cross-validation*) *deviance*. The resulting *explained variance* informs about the power/significance of the model. In the present study, only the metal contents in seeds of Al (46.2%), Cd (48.6%), and Ni (64.4%) showed *explained variances* high enough for interpretation, all other models showed an *explained variance* under 8%. Although this implies that the most important drivers of heavy metal and toxic element accumulation in cacao have not been analyzed in this study, the models still help to rank the predictor variables. In general, BRT modelling was used to visualize the most important factor influencing the toxic metal content in seeds. For the graphic illustration of the models, *partial dependence plots* were presented, which show the effect of one single predictor variable on response controlling for the average effect of all other predictors (Buston and Elith 2011).

To provide test statistics with p-values, differences in toxic metal contents of soil and cacao seed samples between the three Peruvian regions and between the different cacao types were determined using Analysis of Variance (ANOVA) and a Post-hoc test (Tukey-test) Calculations were carried out with the Software R, version 3.6.1. (R Development Core Team 2014). To calculate if toxic metal contents in cacao seeds depend on the corresponding metal contents in soil, generalized linear mixed models (GLMM) were used in the Software R, version 3.6.1. (R Development Core Team 2014). The toxic metal contents in the soil samples were set as the fixed effects in the model. The nested study design includes the cacao type in its regions as random effects (Zuur et al. 2009). The models were run with the lme function within the package nlme and MASS (Pinheiro and Bates 1995; Ripley 2015).

Table 8: Means (± standard errors), minimum, and maximum values of aluminum, cadmium, cobalt, copper, manganese, nickel, lead, zinc in cocoa seed powder-, and soil samples of different Peruvian regions (San Martin, Huánuco, Cusco). Different lower-case letters show significant differences of cocoa powder and soil samples between the three regions. The maximum allowed limits for Cd, Cu, and Pb in cocoa powder are given, as well as the recommended daily consumption limits per kg body weight for Al, Cd, Cu, Mn, Ni, Pb, and Zn.

	Max. allowed limit	Recommended consumption limit	San Martin		Huánuco		Cusco	
			cocoa	soil	cocoa	soil	cocoa	soil
Aluminum (Al) in mg kg^{-1}	-	0.14	16.56 (± 1.09)^{a}	23,491 (± 1,080)^{a}	7.57 (± 0.49)^{b}	20,727 (± 772)^{b}	4.42 (± 0.31)^{c}	12,024 (± 425)^{c}
Min. – max. values			6.23 – 59.71	6,170 – 47353	2.38 – 33.59	5,371 – 52,317	1.39 – 26.96	4,086 - 20,562
Cadmium (Cd) in mg kg^{-1}	0.6	0.36	0.96 (± 0.08)^{a}	0.09 (± 0.02)^{a}	2.13 (± 0.21)^{b}	0.29 (± 0.03)^{b}	0.21 (± 0.02)^{c}	0.12 (± 0.01)^{a}
Min. – max. values			0.31 – 3.25	0 – 0.47	0.08 – 12.31	0 – 1.99	0.05 – 1.12	0 – 0.46
Cobalt (Co) in mg kg^{-1}	-	-	0.95 (± 0.07)^{ab}	10.32 (± 0.77)^{a}	0.69 (± 0.07)^{a}	11.52 (± 0.78)^{a}	1.13 (± 0.11)^{b}	22.67 (± 0.09)^{b}
Min. – max. values			0.10 – 2.13	3.33 – 27.58	0.07 – 5.00	0.48 – 51.71	0.14 – 8.43	1.31 – 55.01
Copper (Cu) in mg kg^{-1}	50	5.0	46.93 (± 1.58)^{ab}	16.42 (± 0.91)^{a}	44.06 (± 1.12)^{a}	18.60 (± 0.94)^{a}	49.65 (± 1.34)^{b}	25.74 (± 0.76)^{b}
Min. – max. values			20.41 – 79.42	6.47 – 36.46	18.93 – 76.94	3.19 – 41.54	13.87 – 86.51	10.38 – 45.13
Manganese (Mn) in mg kg^{-1}	-	0.06	55.18 (± 3.63)^{a}	723 (± 53)^{a}	57.88 (± 3.69)^{a}	866 (± 55)^{a}	78.54 (± 3.92)^{b}	1,713 (± 82)^{b}
Min. – max. values			14.09 – 137.18	111 – 2846	12.89 – 211	34.91 – 3300	29.59 – 232	192 – 4773
Nickel (Ni) in mg kg^{-1}	-	0.028	8.69 (± 0.67)^{a}	11.23 (± 1.00)^{a}	10.38 (± 0.71)^{a}	17.35 (±1.50)^{b}	27.17 (± 0.85)^{b}	23.69 (± 1.04)^{c}
Min. – max. values			1.79 – 22.00	3.11 – 32.59	0.74 – 33.89	0.64 – 124.96	11.54 – 59.91	1.93 – 47.31
Lead (Pb) in mg kg^{-1}	1.0	0.0036	0.03 (± 0.01)^{a}	14.30 (± 0.66)^{a}	0.09 (± 0.03)^{a}	27.77 (± 2.79)^{b}	0.02 (± 0.01)^{a}	21.91 (±0.96)^{b}
Min. – max. values			0 – 0.43	4.55 – 31.55	0 – 2.79	4.76 – 236	0 – 0.25	3.39 – 44.35
Zink (Zn) in mg kg^{-1}	-	0.3-1.0	79.47 (± 1.39)^{a}	51.01 (± 3.75)^{a}	84.76 (± 1.36)^{b}	65.43 (± 4.37)^{a}	78.03 (± 1.42)^{a}	85.12 (± 3.79)^{b}
Min. – max. values			48.85 – 115	8.40 - 128	45.37 – 122	12.39 – 271	52.63 – 136	10.39 – 207

4.4 Results

4.4.1 The importance of soil metal contents for its accumulation in cacao seeds

BRT modelling gave the most robust models with *explained variances* of 46.2% for Al, 48.6% for Cd, and 64.4% for Ni. All other heavy metals (Cu, Co, Pb, Zn) had explained variances lower than 8% and were used to visualize the ranking of important variables predicting the toxic metal contents in cocoa powder samples. The models clearly revealed that the metal content in soil is the most important factor influencing the resulting metal content in cacao seeds in most of the elements analyzed (**Fig 18**). For Cd in cocoa powder samples with a relative influence of 78.8% (**Fig 18b**), Co with 72.7% (**Fig 18c**), Cu with 73.6% **(Fig 18d**), Mn with 69.5% (**Fig 18e**), Ni with 42.0% (**Fig 18f**), and Zn with 65.9% (**Fig 18h**), the respective heavy metal contents in soil were the most important factor. In Al and Pb, soil content was the second most important factor.

However, some heavy metals such as Cd, and Cu showed even higher contents in cocoa powder samples than in the corresponding soils (**Tab 8**), which further provides evidence for the classification of the cacao tree as heavy-metal-accumulating plant. The BRT results were supported by the GLMM statistics which resulted in significant correlations of Al, Cd, Cu, Mn, Ni, and Zn contents in cocoa powder with those of the soil samples of the respective trees. Exclusively correlations of Co and Pb contents in cocoa and in soil were not significant.

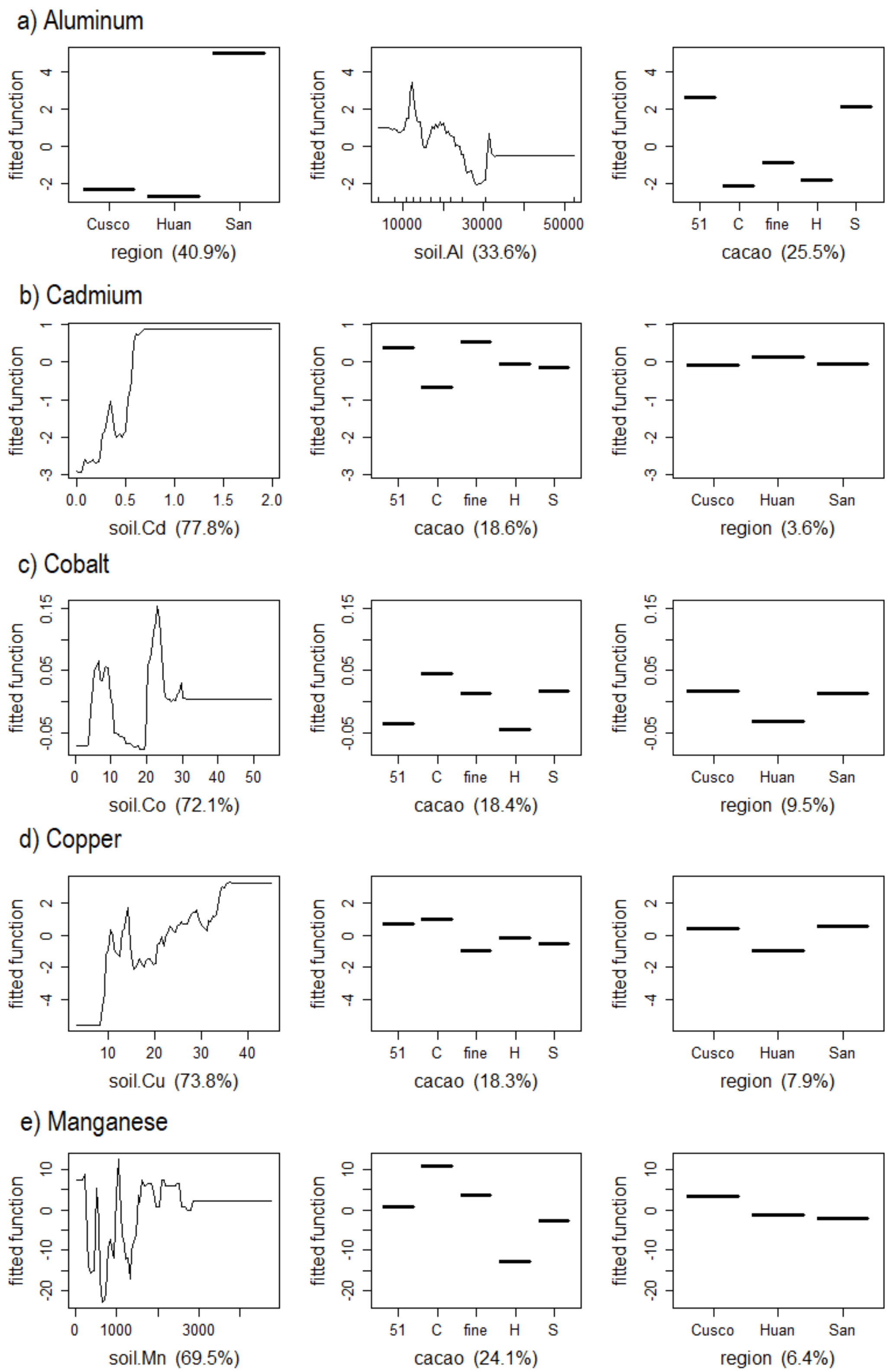

Figure 18: Figure continued on page 94.

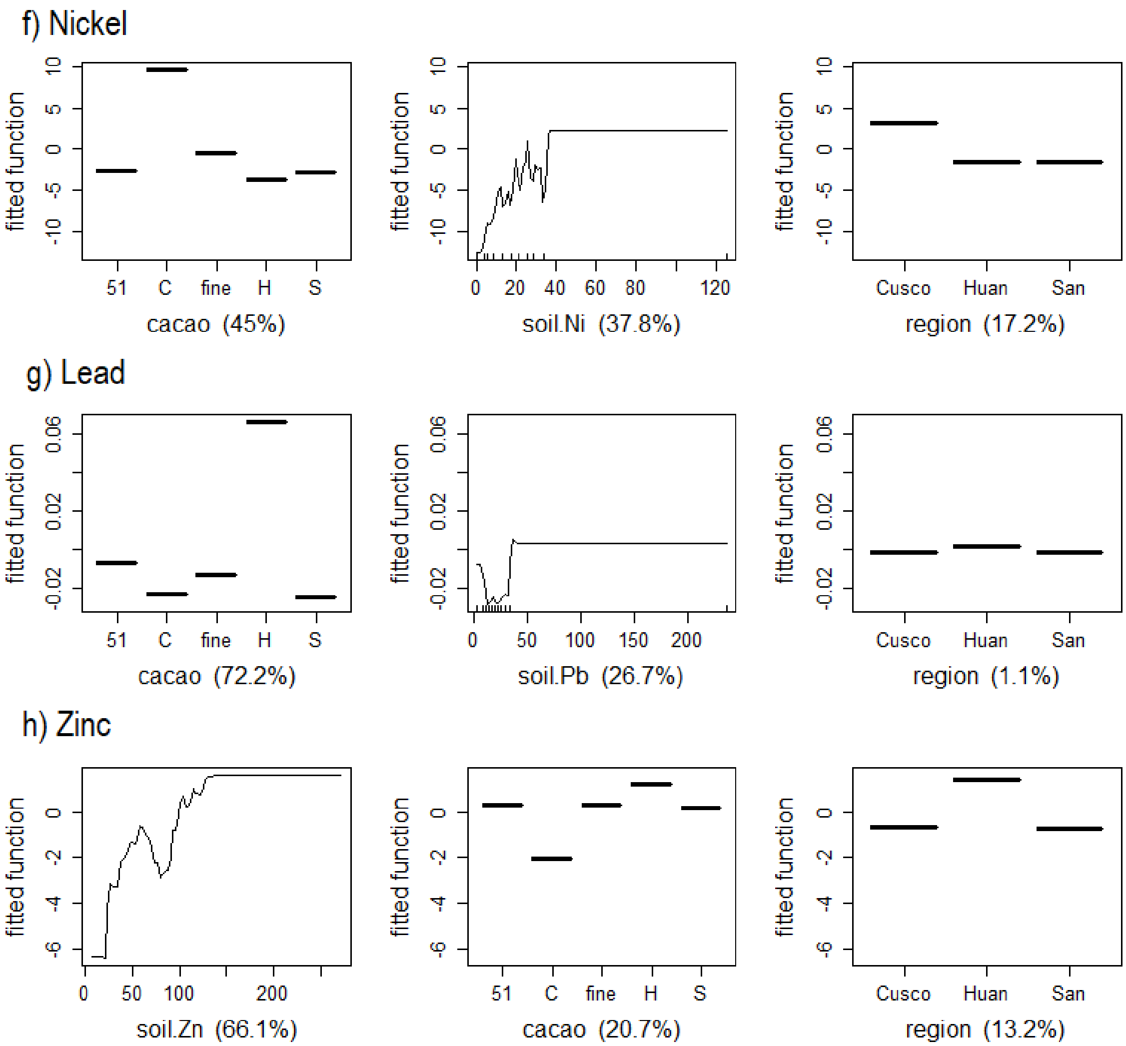

Figure 18: Partial dependence plots of the predicting variables (soil metal contents, cacao type, region) on the toxic metal contents (Al, Cd, Co, Cu, Mn, Ni, Pb, Zn) in cocoa powder samples. The respective models had explained variances of 46.2% for Al, 48.6% for Cd, 1.8% for Co, 5.4% for Cu, 7.3% for Mn, 64.4% for Ni, 0.0% for Pb, and 5.1% for Zn. Fitted lines represent the mean estimate and relative influences of predicting variables are given in%. Software R 3.6.1. Boosted Regression Trees.

4.4.2 Toxic metal contents and its dependence on regions

BRT results for the analyzed metals showed that exclusively for Al, the most important factor for its accumulation in seeds was the region in which samples were taken with a relative influence of 44.1% followed by the soil Al content (31.0%) and the cacao type (24.9%; **Fig 18a**). In general, toxic metal contents differed significantly between the sample regions (Cusco, Huánuco, San Martin). In the case of Al, soil and cocoa powder contents differed significantly between each region with the lowest contents in Cusco, intermediate values the middle region Huánuco and highest values in San Martin (**Tab 8**). In contrast, the content of Cd in cocoa powder samples showed highest values in Huánuco, intermediate values in San Martin, and lowest values in Cusco. Interestingly, the Cd contents were higher in cocoa powder than in soils which again points to pronounced Cd accumulation in cacao (**Tab 8**). Accordingly, Cd contents in soil did not correspond to cocoa powder samples and were lowest in soils from San Martin but highest in cocoa powder than samples from Huánuco (**Tab 8**). Co concentrations were higher in soils than in cocoa powder and overall higher in the Cusco region, intermediate in Huánuco, and lowest in San Martin but only differed between Cusco with Huánuco and San Martin (**Tab 8**). Co in cocoa powder was highest in Cusco, and lowest in Huánuco, and significantly differed between those two regions. Cu in soil samples decreased from Cusco and Huánuco to San Martin, and Cusco differed significantly from Huánuco and San Martin. (**Tab 8**). In contrast, cocoa powder samples differed significantly between Cusco with highest Cu values and Huánuco with lowest values (**Tab 8**). Mn contents in soils were higher than in cocoa powder samples and both, soil and cocoa powder contents differed significantly between the high mean from Cusco and the lower values in Huánuco and San Martin (**Tab 8**). Ni showed the same trend as Mn but, however, had only in Cusco higher contents in cocoa powder samples than in the corresponding soil samples (**Tab 8**). San Martin soils were lowest in Pb content whereby Huánuco were highest, with San Martin differing significantly from Huánuco and Cusco (**Tab 8**). In comparison with soil Pb contents, the cocoa powder contents were extremely low and showed no significant differences (**Tab 8**). Highest Zn soil contents were detected in Cusco and decreased from Huánuco to San Martin with significant differences between Cusco and the other two regions (**Tab 8**). Contrary to cocoa powder contents of Huánuco and San Martin, the Cusco samples had lower Zn contents in cocoa powder than in soil samples (**Tab 8**). Highest Zn contents were detected in cocoa powder from Huánuco which differed significantly from Cusco and San Martin (**Tab 8**).

Differences in soil and cocoa powder metal contents indicate diverging transfer of elements from soil to the cacao seed. We therefore calculated transfer factors for all elements and analyzed possible differences among regions and clones (**Tab 9**). The transfer factor of Al was, compared to the heavy metals, the lowest. Al showed the highest transfer-factor in San Martin, differing significantly from the cacaos from Cusco and from Huánuco (**Tab 9**). The transfer-factor of Cd was clearly higher, and San Martin and Huánuco differed significantly from Cusco (**Tab 9**). Co showed the same trend as Cd and had its lowest transfer-factor in Cusco which differed significantly from San Martin and Huánuco (**Tab 9**). Moreover, the Cu transfer factor was the lowest in Cusco and differed significantly from San Martin and from Huánuco (**Tab 9**). The Mn transfer-factor was highest in Huánuco and differed only significantly from the lowest transfer-factor in Cusco (**Tab 9**). Ni showed its highest transfer-factor in Cusco contrary to the other heavy metals and differed significantly from Huánuco and from San Martin (**Tab 9**). The transfer-factors of Pb showed no significant differences among regions but had the lowest value in Cusco and the highest in Huánuco (**Tab 9**). The lowest transfer-factor of Zn in Cusco differed significantly from the highest in San Martin and from Huánuco (**Tab 9**).

Table 9: Transfer factors and standard errors of the different elements absorbed by the cacao tree and accumulated in its seeds with minimum and maximum values. Different lower-case letters show significant differences of the transfer-factors between the three regions.

	San Martin	**Huánuco**	**Cusco**
Aluminum (Al)	0.00090 (± 0.00011)^{a}	0.00046 (± 0.00004)^{b}	0.00039 (± 0.00002)^{b}
Min. – max. values	0.00021 – 0.00587	0.00005 – 0.00275	0.00011 – 0.00134
Cadmium (Cd)	12.41 (± 1.45)^{a}	10.62 (± 0.10)^{a}	2.37 (± 0.21)^{b}
Min. – max. values	2.50 – 46.46	1.17 – 55.43	0.33 – 11.56
Cobalt (Co)	0.13 (± 0.01)^{a}	0.12 (± 0.02)^{a}	0.06 (± 0.01)^{b}
Min. – max. values	0.008 – 0.503	0.008 – 1.066	0.005 – 0.418
Copper (Cu)	3.28 (± 0.17)^{a}	3.18 (± 0.20)^{a}	2.19 (± 0.12)^{b}
Min. – max. values	1.17 – 7.12	0.87 – 11.79	0.72 – 6.89
Manganese (Mn)	0.11 (± 0.02)^{ab}	0.16 (± 0.03)^{a}	0.07 (± 0.01)^{b}
Min. – max. values	0.01 – 0.69	0.02 – 2.39	0.01 – 1.21
Nickel (Ni)	1.07 (± 0.12)^{a}	0.93 (± 0.07)^{a}	1.58 (± 0.13)^{b}
Min. – max. values	0.21 – 3.92	0.08 – 4.23	0.43 – 9.49
Lead (Pb)	0.0025 (± 0.0009)^{a}	0.0050 (± 0.0017)^{a}	0.0014 (± 0.0005)^{a}
Min. – max. values	0 – 0.04	0 – 0.13	0 – 0.04
Zink (Zn)	2.25 (± 0.19)^{a}	1.85 (± 0.11)^{a}	1.27 (± 0.10)^{b}
Min. – max. values	0.46 – 7.58	0.31 – 6.27	0.40 – 6.85

4.4.3 Importance of the cacao type for toxic metal accumulation in cacao

Looking at BRT modelling with the toxic metal contents in cocoa powder samples, the cacao type (CCN-51, fine and flavor cacao clones, native cacaos from Cusco, Huánuco, San Martin) was the most important predicting variable for Pb (**Fig 18g**), but only the second most important for all other heavy metals and toxic elements (**Fig 18b-h**).

To compare the cacao types, we again used transfer factors which are independent from soil content. The cacao types like the native cacaos from Cusco, Huánuco, and San Martin, as well as the fine and flavor cacao clones and the high-yield clone CCN-51 showed significant differences in its transfer factors (**Fig 19**). In the case of Al, transfer factors were relatively low which indicates a low absorption rate from soil to the fruit. Native cacaos from San Martin had the highest Al transfer factor with 0.00077, followed by CCN-51 with 0.00075 (**Fig 19a**). Significantly lower were with 0.00022 the Al transfer factors of native cacaos from Huánuco compared to the natives of San Martin and CCN-51 (**Fig 19a**). Moreover, the native cacaos from Cusco had a significantly lower Al transfer factor (0.00039) than native types from San Martin, and CCN-51 (**Fig 19a).** The native cacaos from Cusco showed overall a significantly lower transfer factor of Cd (2.37) compared with native cacaos from San Martin (8.98), the fine and flavor cacao clones (13.16), and CCN-51 (13.26; **Fig 19b**). Also the native cacaos from Huánuco with a transfer factor of 6.44 accumulated significantly less Cd than fine and flavor cacao clones and CCN-51 (**Fig 19b**). The highest Co transfer factors were calculated for fine and flavor cacao clones at 0.21 and differed significantly from native Huánuco cacaos (0.06), from the native cacaos from Cusco (0.06), and from CCN-51 (0.10; **Fig 19c**). Moreover, the Co transfer factor from native cacao from Cusco was significantly lower than that of native cacaos from San Martin (0.14; **Fig 19c**). Native cacaos from Cusco (2.19) had a significantly lower transfer factor of Cu compared to CCN-51 (3.28), the native cacao from San Martin (3.32), and fine and flavor cacao clones (3.52; **Fig 19d**). Fine and flavor cacaos had the highest absorption rate of Mn with a transfer factor of 0.20 and differed significantly from native cacaos from Cusco (0.07; **Fig 19e**). In addition, the mean Mn transfer factor was significantly lower in native Cusco cacaos than in CCN-51 (0.17; **Fig 19e**). The transfer factor of Ni showed the highest value with 1.58 in native cacaos from Cusco and differed significantly from the native cacaos from Huánuco (0.54), as well as from CCN-51 (0.96; **Fig 19f**). Moreover, the native cacaos from Huánuco had significantly lower transfer factors of Ni than the fine and flavor cacao clones (1.35 **Fig 19f**). The absorption rate of Pb was lowest with 0.0009 in native cacaos from San Martin and the highest in native cacaos of Huánuco but there are no significant differences (**Fig 19g**). The native cacaos from Cusco showed the lowest transfer factor of Zn with 1.27 and differed significantly from native cacaos from San Martin with the highest Zn transfer-factor (2.20), from fine and flavor cacao clones (2.18), and CCN-51 (2.04; **Fig 19h**).

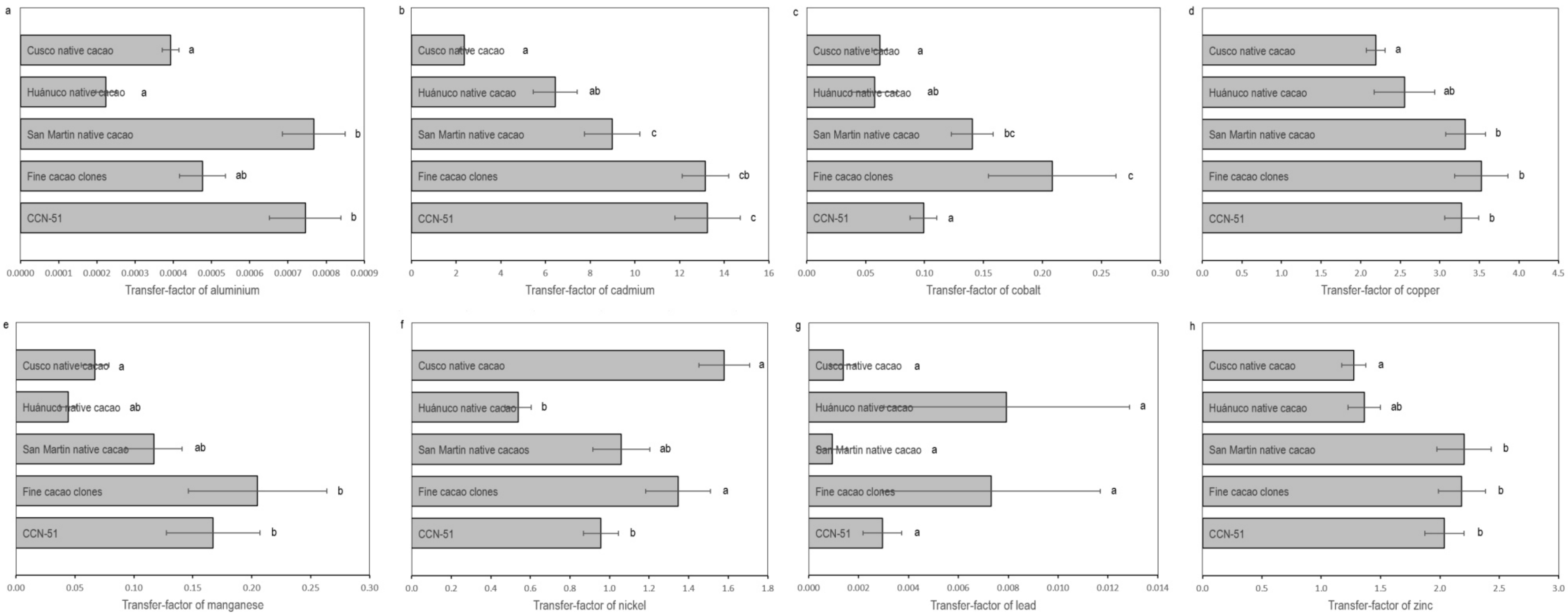

Figure 19: Transfer-factors of the toxic metals (Al, Cd, Co, Cu, Mn, Ni, Pb, Zn) from different cacao types (native cacaos from Cusco, Huánuco, San Martin, fine and flavor cacao clones, and CCN-51). Different lower-case letters show significant differences between the cacao types. Averages with standard errors shown. Software R 3.2.3. ANOVA, Tukey-Test.

4.5 Discussion

To the best of our knowledge, this study is the first that screened a huge array of toxic metal contents in 280 cacao and soil samples from different regions (Cusco – Huánuco – San Martin) of Peru. Based on the data set, we were able to rank the impact of soil, region and cacao type using BRTs. Moreover, the transfer factors of toxic metals were compared between different regions and cacao types (genetically native cacaos from each region, fine and flavor cacao clones, CCN-51). The transfer factors also showed which cacao types were naturally absorbing less toxic metals than others. 40 samples from the Huánuco region from our former study (Zug et al. 2019), which showed highest Cd samples ever reported in literature, were included in this study and samples were remeasured with the same results. Some of the metals are hazardous for plants and animals (Al, silver (Ag), arsenic (As), Cd, mercury (Hg), Pb, antimony (Sb)), whereas other metals such as Co, Cr, Cu, Ni, and Zn are essential in low concentrations (Aikpokpodion et al. 2012; He et al. 2005; Holm et al. 1995; Hoseini and Zargari 2013). In general, crops are the main source for a toxic metal consumption for humans, and therefore the major risk for intoxication of the human body (Yukimasa et al. 1975).

4.5.1 Soil as the source for toxic metal accumulation in plants

Regarding to the BRT results, soil toxic metal contents proved to be the most important driver of Cd, Co, Cu, Mn, Ni, and Zn accumulation in cacao plants in the present study. Soil toxic metals are differing in its abundance due to soil type and geographic origin as well as anthropogenic contamination, as described by several studies (Chavez et al. 2015; Fauziah et al. 2001; Grant et al. 1996; Mullins et al. 1986; Zug et al. 2019). The present study confirms that almost every measured metal (Al, Cd, Cu, Mn, Ni, Zn) and its content in cacao correlated significantly with soil contents.

4.5.2 Toxic metals, their differences between regions, and their risk for humans

The toxic metals in the present study showed individual trends in their occurrence in different regions. Overall, Co, Cu, Mn, Ni, and Zn contents were higher in the Cusco regions, whereas Cd and Pb prevailed in the central region of Huánuco. San Martin was characterized by the highest Al contents found in this study. However, the region was only the most important predicting factor for toxic metal contents in cacao seeds for Al. Reasons for the differences among regions can be various, such as the natural variety in abundance of metals in soils in different regions, the origin of soils, e.g. volcanic origin of andesites, contamination through sewage sludge or areal disposition (Fauziah et al. 2001; Grant et al. 1996; He et al. 2005; Zug et al. 2019). Further anthropogenic reasons can be the use of contaminated fertilizers (normally with Cd, Cu, Pb, Zn), biosolids or compost (Ni, Zn), pesticides, fungicides, herbicides (Al, Cd, Co, Cu, Mn, Ni, Pb, Zn) or mining processes (He and Singh 1994a, b; He et al. 2005; Mullins et al. 1986; Williams and David 1976; Zug et al. 2019). Pb is reported to get into the food chain over leaded gasoline which contaminates crop plants or due to the use of lead-acid batteries that are used against termite outbreaks in cacao plantations (Aikpokpodion et al. 2013). The soil pH is also playing an important role in soil processes (precipitation-dissolution, adsorption-desorption, complexation-dissociation, oxidation-reduction) which in turn regulates the abundance of metals in the soil solution and hence its potential uptake by plants (Chavez et al. 2015; Hanafi and Maria 1998; He and Singh 1994b; He et al. 2005; WHO 2010b Zug et al. 2019). However, each toxic element shows clear characteristics concerning transfer to plant tissues (He et al. 2005).

Aluminum is one of the toxic metals which occurs at high concentrations in soil but in rather low concentrations in cocoa powder. This can be attributed to the low solubility of Al in the soil solution as it is strongly bound to soil particles, with strong impact of the soil's chemical conditions (Clemens et al. 2002; Zhang et al. 2006). Al forms stable complexes with soil organic matter but can build soluble complexes (with sulfates, nitrates, and silicates) which are stable at different soil pH (May et al. 1979; Sposito 1996). In general, average soils contain about 7% Al, which enters into the soil solution at low pH and results in Al toxicity in plants growing on acidic soils (Foy 1988; Kochian et al. 2004; Sposito 1996; Zhang et al. 2006). In particular in the rainforests of Peru, acidic soils are frequently observed which implies a high risk of Al toxicity (Hanafi et al. 1998; Kieck et al. 2016; Zug et al. 2019). At high concentrations, Al can even limit the growth of plants (Foy 1988). In the case of cacao, Al is known to cause yield reductions (Baligar and Fageria 2004), nutrient reduction in seedlings (Moreira de Almeida et al. 2015; Quinteiro Ribeiro et al. 2013), and a root and shoot growth reduction (Baligar and Fageria 2005). Highest average content was reported in San Martin (soil: 23491 mg kg^{-1}; cocoa powder: 16.56 mg kg^{-1}) and lowest in Cusco (soil: 12024 mg kg^{-1}; cocoa powder: 4.42 mg kg^{-1}; **Tab 8**). The relatively low transfer factors provide evidence for low mobility of Al in the analyzed soils (He et al. 2005). As Al was recently related to the Alzheimer disease (Perl and Moalem 2006; Stahl et al. 2011), the tolerable weekly consumption content of Al was decreased from 7 mg kg^{-1} body weight to 1 mg kg^{-1} body weight (JECFA 2011). Stahl et al. (2011) found a minimum Al value of 80 mg kg^{-1} and a maximum value of 312 mg kg^{-1} in cocoa powder samples with an average of 165 mg kg^{-1} which is clearly higher than the maximum value found in cocoa powder in this study (59.71 mg kg^{-1}). Average contents in cocoa powder from the German market ranged between 118 mg kg^{-1} and 171 mg kg^{-1} (Fechner et al. 2019). In general, higher Al values were found in Africa with averages of 54, 155, 274 mg kg^{-1} than in Ecuador with 16 mg kg^{-1}, Central America with 41.1 mg kg^{-1}, and in South America, and Asia between 90.2 and 89.1 mg kg^{-1} (Bertoldi et al. 2016; Vītola and Ciproviča 2016). Hence, Peruvian cacao seems to show a below-average risk for Al intoxication in humans.

Cadmium showed the opposite trend of Al in this study: Cd contents in soils were relatively low (max. 1.99 mg kg^{-1}), whereas Cd content in cocoa powder samples reached a maximum value of 12.31 mg kg^{-1} in Huánuco (as already shown in Zug et al. 2019). The accumulation of Cd in cacao even at low soil Cd contents was also reported from Fauziah et al. (2001). The authors showed that Cd accumulation in plants and also in cacao depends on soil pH and the related Cd sorption at soil particles. Cd concentrations in soil solution are increasing with lower pH and can than more easily be taken up by plants. Cd has a high toxicity for plants in even low concentrations and cause a decrease in plant growth, as well at it is hazardous for humans causing several health problems (EFSA 2012; Hanafi and Maria 1998; He and Singh 1994b; WHO 2010b), and a yield reduction in cacao trees (Zug et al. 2019). Several factors, such as the natural occurrence of high Cd contents in soil (e.g. soils with a volcanic origin), aerial disposition, or man-made contamination due to sewage sludge or fertilization seem to increase Cd contents (Fauziah et al. 2001; Grant et al. 1996; He and Singh 1994a, b; Mullins et al. 1986; Williams and David 1976; Zug et al. 2019). With the stricter Cd limits in chocolate products set by the European Commission (2014), only 0.6 mg Cd per kg cocoa powder are allowed in the European market. It is recommended not to consume more than 2.5 µg Cd per kg body weight and week (FAO and WHO 2016). In the present study, about 49.29% of cocoa powder samples were higher than the actually allowed Cd limit with 83.78% in Huánuco, 64.06% in San Martin, and 3.81% in Cusco. Until now, highest Cd values in cocoa powder were reported by Zug et al. (2019) in Huánuco which were included and remeasured in this study (max. 12.31 mg kg^{-1}). Former studies reported relatively low Cd concentrations in cocoa products with 0.3 to 3.0 mg kg^{-1} in South America, Arica, and Honduras (Argüello et al. 2019; Chavez et al. 2015; Gramlich et al. 2017; Mounicou et al. 2002a, b).The transfer factors for Cd in the present study showed a high absorption rate especially of cacaos from San Martin (12.41) compared to the cacaos in

Cusco (2.37), which may be the result of differences between regions or cacao types (as shown in this study), environmental conditions or management (Arévalo-Gardini et al. 2017; Zug et al. 2019).

Cobalt is a toxic element for plants, but essential for animals and humans at low concentrations (He et al. 2005), as it is needed in vitamin B_{12} synthesis (Barceloux and Barceloux 1999). However, an excessive consumption is clearly harmful to humans (Berk et al 1949). Co concentration is relatively low in soils (10 – 40 mg kg^{-1} or lower; He et al. 2005), which can be compared to the contents in the present study ranging from 10.32 mg kg^{-1} in San Martin to 22.67 mg kg^{-1} in the Cusco Region. Co is introduced in soils through weathering of minerals in igneous rocks, or anthropogenically as part of pesticides and fungicides. It is represented in soils mostly as arsenides, oxides, and sulfides (Barceloux and Barceloux 1999; He et al. 2005). Its absorption by plants again depends on soil pH and the related sorption with soil colloids but Co has a lower mobility as e.g. Cd in soils (Barceloux and Barceloux 1999; He et al. 2005). The transfer factors measured in this study were relatively low (< 1), which supports the view of its low mobility (He et al. 2005). There was no literature found about cobalt concentration in cacao or its products. Co in coffee samples, ranged from 4.6 – 9.6 mg kg^{-1} (Saulea et al. 2004). Moreover, there are no limits in foodstuff for Co.

Copper is one of the essential heavy metals such as Co for humans but can cause diseases in higher concentrations than 5 mg kg^{-1} body weight. It is toxic especially for the gastrointestinal system and can cause a huge array of diseases such as arthritis, fatigue, insomnia, scoliosis, osteoporosis, heart disease, cancer, migraine, heart seizures, gum diseases, and memory loss (Izah et al. 2016a, b; Lanre-Iyanda and Adekunle 2012). In plants, Cu can reduce growth as shown for rice (Xu et al. 2005). Cu is present in soil minerals such as malachite, azurite, and tenorite, which are soluble particularly in acid soils. Furthermore, Cu may also occur as the mineral cupric ferrite and the less soluble Cu sulfides. In average, soils are containing about 20 mg kg^{-1} of Cu (He et al. 2005), which is comparable with our results of 16.42 mg kg^{-1} to 25.74 mg kg^{-1}. Reasons for a high Cu pollution in cacao can be natural high Cu concentrations in soil but also Cu contaminated pesticides and fungicides (Aikpokpodion et al. 2013; Hebbar 2007). The EFSA (2018) set a limit for Cu in cocoa beans of 50 mg kg^{-1}. Average contents did not exceed the limit (highest average of 49.65 mg kg^{-1} in Cusco) in the present study. However, in total 38.57% of the cacao samples exceeded the limits, with 50.48% in Cusco, 29.73% in Huánuco, and 34.38% in San Martin samples. Lee and Low (1985) found Cu contents of 93.9 mg kg^{-1} in cocoa powder and 95.9 mg kg^{-1} in raw cacao beans which also exceeded *EFSA* limits. Cocoa beans from Nigeria contained between 25 mg kg^{-1} and 26.1 mg kg^{-1} in average (Aikpokpodion et al. 2013), which shows that Peruvian cocoa has a higher risk of Cu toxicity than African one.

Manganese is one of the most abundant on earth crust. Mn occurs naturally in soil, water and hence also in foods (*WHO* 2011) or anthropogenically in fertilizers (He et al. 2005). Mn is most stable as the mineral $MnCO_3$ in soils and clearly more soluble in acidic soils, but it also occurs as MnOOH and MnO_2 (He et al. 2005; Sanders 1983). Mn is essential for plants and animals and plays a role in oxidative phosphorylation, fatty acids or the activation of specific enzymes. Humans should not exceed a daily consumption limit of 2.3 mg Mn for men and 1.8 mg for women or an average of 0.06 mg kg^{-1} body weight to prevent neurological toxicity (Izah and Angaye 2016; WHO 2011). Shuman (1979) found Mn concentrations in different soil types ranging from 0.2 to 1673 mg kg^{-1}. Soil Mn content in our study ranged between 723 mg kg^{-1} (San Martin) to 1713 mg kg^{-1} (Cusco). Regarding to the WHO (2011), foods such as grains and grain products contain up to 40.7 mg kg^{-1} of Mn and fruits up to 10.38 mg kg^{-1}, but there is nearly no literature about Mn in cacao. Lockard and Burridge (1965) analyzed Mn in cacao leaves and found an average of 279 mg kg^{-1}. Mn contents were in general lower in our cocoa powder (with 55.18 in San Martin to 78.54 mg kg^{-1} in Cusco) than in soils which may be attributed to low mobility of Mn in soils (He et al. 2005).

Nickel is present all over the earth crust and therefore also in drinking water and food. Ni occurs in natural or anthropogenic (e.g. use of biosolids / compost) processes and is considered a toxic element for living systems (EFSA 2015; He et al. 2005). Worldwide, soils contain about 40 mg Ni kg^{-1} with metal rich soils ranging between 800 and 8000 mg Ni kg^{-1} (He et al. 2005). Soils in the present study contained about 10.32 mg kg^{-1} in San Martin and up to 23.69 mg kg^{-1} in Cusco which is clearly below the worldwide average. There is no legal limitation for Ni in foods, but studies are raising concerns about the toxicological consequences of Ni consumption. Ni exposure can have negative effects for the gastrointestinal, hematological, neurological, and the immune systems. Allergic reactions are also reported (EFSA 2015). However, recommendation for a daily intake level of 2.8 µg kg^{-1} body weight are given by the EFSA (06.08.2019), as well as drinking water should not exceed a level of 20 µg L^{-1}. Regarding to the EFSA (2015), highest Ni values in foods were reported in cacao and cocoa based products with 9.8 mg kg^{-1} in cocoa beans. Even the average contents of Ni in Huánuco with 10.38 mg kg^{-1}, and Cusco with 27.17 mg kg^{-1} in the present study clearly exceed the EFSA (2015) findings, which indicates the need for a detailed examination of health risks and limitation of Ni in foods. Kruszewski et al. (2018) reported a maximum Ni value of 12.1 mg kg^{-1} in raw cocoa and 4.5 mg kg^{-1} in chocolate. Transfer factors were in average higher in Cusco (1.58) than in Huánuco (0.93) which could be due to the different cacao types (as shown in our study) or the management of plantations (Arévalo-Gardini et al. 2013; Zug et al. 2019).

Lead is present in relatively low concentrations in earth crust and mainly occurs as lead sulfide (WHO 2010a). In average, soils are containing about 10 to 150 mg kg^{-1} of Pb (He et al. 2005) which is within the range of what we found in Peruvian soils with 14.3 mg kg^{-1} in San Martin, 21.91 mg kg^{-1} in Cusco, and 27.77 mg kg^{-1} in Huánuco. In nature, Pb is found more likely in soils of volcanic origin, but Pb is one of the heavy metals which are widely spread due to anthropogenic influence (Fauziah et al. 2001; WHO 2010a) such as mining activities, use of leaded petrol (more often in developing countries), or the use of Pb contaminated fertilizer or pesticides often used in agroforestry systems (He et al. 2005; Ogunlade and Agbeniyi 2010; WHO 2010a). Pb is known for its toxic effect on the neurological, haematological, gastrointestinal, cardiovascular, and renal systems in the human body (WHO 2010a). Therefore, legal limits were set from the European Union for cocoa butter with 0.1 mg kg^{-1}, and 1.0 mg kg^{-1} for cocoa mass and cocoa powder must be complied (COPAL 2004). In addition, a tolerable weekly consumption of 25 µg Pb kg^{-1} body weight (WHO 2010a) is applied for children. Pb contents in cocoa powder (highest average in Huánuco with 0.09 mg kg^{-1}) of our study were relatively low compared to the soil Pb concentrations (highest average in Huánuco with 27.77 mg kg^{-1}), which points to a relatively low mobility of Pb in soils (He et al. 2005). However, Pb contents exceeded in 0.71% of all samples the maximum limit of 1.0 mg kg^{-1} for cocoa powder, with 1.8% in Huánuco. Pb concentrations ranging from <0.0005 to 3.45 mg kg^{-1} were found in cocoa products in several studies (Abt et al. 2018; Aikpokpodion et al. 2013; Anyimah-Ackah et al. 2019; Guérin et al. 2017; Duran et al. 2009; Lee and Low 1985; Lo Dico et al. 2018; Mounicou et al. 2002a and 2003; Prugarová and Kováč 1987; Rancin et al. 2005; Villa et al. 2014; Yanus et al. 2014). Reasons, such as the use of leaded gasoline (which is still used in African countries), an application of lead-acid batteries against termite attacks or simply the soil Pb content were discussed (Aikpokpodion et al. 2013). According to Mounicou et al. (2003), Pb concentrations in cocoa powder differ between its origin. Our study clearly shows the risk of Pb toxicity from Peruvian cacaos. Still, transfer factors were relatively low owing to low Pb mobility in soil and low absorption rate of cacao.

Zinc, which is essential in low concentrations for plants and animals, mostly occurs as sulfite in soils (He et al. 2005). In the geosphere and biosphere, Zn is related with Cd with Cd/Zn ratio ranging from 1:500

to 1:5000 (Schroeder et al. 1966). Fertilizers, biosolids, or pesticides frequently contain Zn. Soils show in average a Zn content of 10-300 mg kg^{-1} (He et al. 2005). In our study, soils of cacao plantations contained about 51.01 mg kg^{-1} in San Martin to 85.12 mg kg^{-1} in Cusco (**Tab 8**). Zn in the analyzed cocoa powder was in general present at higher concentrations than in soils (78.03 mg kg^{-1} in Cusco to 84.76 mg kg^{-1} in Huánuco), which implies high mobility of Zn in soils (He et al. 2005). There are no limits for Zn consumption, but a high exposure may cause tachycardia, vascular shock, dyspeptic nausea, vomiting, diarrhea, pancreatitis ad damage of parenchyma (Aikpokpodion et al. 2013). The Joint FAO/WHO Expert Committee on Food Additives (JECFA 2011; WHO 1982) recommended an intake of 15 mg Zn per day for adults and a tolerable intake of 0.3-1.0 mg kg^{-1} body weight per day. In the present study, Zn contents in cocoa powder ranged from 78.03 mg kg^{-1} in Cusco to 84.76 mg kg^{-1} in Huánuco. Lockard and Burridge (1965) found Zn levels in cacao leaves of 38 mg kg^{-1} which increased after fertilization. Zn contents in cocoa bean samples from Africa ranged from 77 to 111 mg kg^{-1} (Aikpokpodion et al. 2013). Steinberg et al. (2003) found 79.55 mg kg^{-1} of Zn in cocoa powder. Other studies found Zn concentrations of 43.4 mg kg^{-1} in cocoa (Harland and Oberleas 1985), which is within the range of our results.

4.5.3 Cacao types and its absorption of heavy metals

In addition to the differences between the Peruvian regions, also cacao types proved to be divergent in terms of seed metal contents and absorption rates regarding to BRT results. To detect differences between cacao types, we used transfer factors of toxic metals absorbed from soil by cacao plants and accumulated in their seeds. As the transfer factors include soil and cocoa powder contents, values are comparable irrespective of natural variations in toxic metal soil. Transfer factors with a value higher than one indicate accumulation of toxic metal contents in cocoa powder, which could be observed in Cd, Cu, Ni (only in samples from native cacao in San Martin, Cusco, and the fine and flavor cacao clones), and Zn. Both high mobility of these elements in soils and higher absorption by cacao may be assumed as possible reasons (Bansah and Addo 2016; He et al. 2005). In general, all transfer factors (including those of Al, Co, Cu, Mn, and Zn) showed the same trend and were the lowest in native cacaos from Cusco and Huánuco (which provides evidence for our third hypothesis), whereas native cacaos from San Martin, fine and flavor cacao clones, and the CCN-51 clone had relatively high values. Consequently, our study supports findings of other studies which showed genetical differences in terms of metal uptake between these cacao types (Arévalo-Gardini et al. 2017). Contrary to the study from Zug et al. (2019), in this study, clear differences seem to exist within the cacao types. Arévalo-Gardini et al. (2017) found higher Cd contents in beans and leaves of cacao plantations with combinations of CCN-51 and ICS-95 than in plantations planted exclusively with CCN-51. Another study reported strong variation in Cd contents of different rice clones (Liu et al. 2016). Despite these genetic differences, soil condition proved to be the main driver of metal uptake in cacao which is modified by the solubility of each metal in soil solution in relation to soil pH (Gramlich et al. 2017; Hanafi and Maria 1998). Moreover, it is known that environmental and management conditions have an influence on cacao quality (Afoakwa et al. 2008; Aprotosaie et al. 2016; Counet et al. 2004; Kieck et al. 2016; Niemenak et al. 2006; Zug et al. 2019) and subsequently also on toxic metal accumulation in cacao. In accordance, plant diversity and use of fertilizers have been shown to influence Cd uptake in cacao (Zug et al. 2019). Consequently, further investigation studies are needed to better understand the mechanisms of toxic metal accumulation in cacao.

4.6 Conclusion

The present study is the first that systematically analyzed a huge sample of cocoa powder and soils from three different Peruvian regions (San Martin, Huánuco, Cusco) concerning its content in Al and heavy metals (Cd, Co, Cu, Mn, Ni, Pb, Zn). Based on BRT analysis, we were able to disentangle the roles of soil metal content, geographic origin (Cusco, Huánuco, San Martin), and cacao type (native cacaos from San Martin, Huánuco, Cusco, fine and flavor cacao clones, and CCN-51) with soil and cacao type explaining the highest share of variance in the models. In particular, most toxic metal contents (Al, Cd, Cu, Mn, Ni, Zn) in cocoa powder significantly correlated with the corresponding contents in soils. Consequently, mitigation strategies to counteract toxic metal contamination in cocoa should focus on soil measures (e.g., liming, phytoremediation).

The toxic metals clearly differed in terms of toxicity to the human body with Cd, Cu, Pb being hazardous even at small amount and therefore limited in their concentrations in cocoa products. Many cocoa powder samples in this study exceeded the legal limits in the European Union for Cd (64.06% in San Martin, 83.78% in Huánuco, 3.81% in Cusco), for Cu (34.38% in San Martin, 29.73% in Huánuco, 50.48% in Cusco), and for Pb (0% in San Martin, 1.8% in Huánuco, 0% in Cusco) (COPAL 2004; EFSA 2018; European Commission 2014). Other heavy metals, such as Co, Cu, Mn, Zn are essential at low concentrations for the human body and only cause toxic reactions with increased exposure (He et al.

2005; Izah and Angaye 2016; Lanre-Iyanda and Adekunle 2012; WHO 2011). Interestingly, Al and Ni are not limited in their concentrations in food although these metals can cause several health effects (EFSA 2015; Perl and Moalem 2006; Stahl et al. 2011). Our results clearly indicate a stricter control for potentially harmful metals in cocoa products along with the need of further studies on the mechanisms of metal uptake in plants and possibilities for mitigation.

The heavy metals Co, Cu, Mn, and Ni showed maximum values in cocoa powder samples from Cusco, with a pronounced high transfer factor for Ni. In contrast, cocoa samples from Huánuco were highest in Cd, Pb, and Zn content, but transfer factors reached the highest values in this region for Mn and Pb. San Martin was characterized by high Al contents in cocoa powder and showed maximum transfer factors in Al, Cd, Co, Cu, and Zn. Absorption rates also differed significantly between the cacao types and give evidence for a clone-dependent absorption of toxic metals. The clone CCN-51 and fine and flavor cacao clones were the cacao types that showed relatively often the highest or second highest transfer factors (CCN-51 in its Al, Cd, Cu, and Mn contents; fine and flavor cacao clones in Cd, Co, Cu, Ni, Pb, and Zn contents). In contrast, native cacaos showed lower metal contents with the native cacaos from San Martin being characterized by among the highest contents of less hazardous metal such as Al, Co, Cu, Mn, and Zn. Further studies are needed to clarify the reasons for these differences.

4.7 Acknowledgements

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5 Farm management and environmental factors drive toxic metal accumulation in Peruvian cacao (*Theobroma cacao* L.)

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5.1 Abstract

Toxic metal pollution in food stuff can be hazardous for humans as it may cause several diseases, depending on substance and concentration. In particular, children's health is threatened and must strongly be protected. Consequently, legal limits for foodstuff such as chocolate which is predominantly consumed by children and known to be contaminated by toxic metals have to be complied with in the European Union. This applies for the heavy metals Cadmium (Cd), Copper (Cu), and Lead (Pb) in chocolate products. Other heavy metals such as Cobalt (Co), Manganese (Mn), Nickel (Ni), and Zinc (Zn), as well as the toxic metal Aluminum (Al) are known to be harmful for humans at certain concentrations. Despite the high relevance for human health, the impact of environmental and management conditions on metal uptake in cacao seeds have hardly been studied comprehensively. In the present study, these toxic metals were measured in cacao seeds and soil from 280 different cacao trees in three mayor cacao producing regions of the Peruvian Amazonia (San Martin, Huánuco, Cusco) in relation to farm management and the environment. Our analyses provide evidence that farm management practices such as a more frequent pruning raised the transfer of Al, Cu, and Zn from soil to the cacao seeds. In contrast, pruning along with the fertilization with nitrogen-(N-)fertilizers increased the transfer factors of Cd. Older cacao trees showed in general lower Al, Cd, and Zn contents in cacao seeds, whereas Co, Cu, and Mn contents increased with age. Environmental factors such as soil pH, electric conductivity, and soil metal content, as well as the altitude a.s.l. proved to be important predictors of metal content and transfer factors. With increasing altitude a.s.l., lower Al, Cd, Cu, Mn, and Zn values in cacao were found, whereas Co and Ni contents increased. However, the content of metals in corresponding soil samples was among the most important drivers of metal content in cacao seeds, except for Al and Pb. Moreover, the transfer factors of all analyzed metals increased with lower soil electric conductivity, and the transfer factors of Al, Cd, Cu, Co, Mn, Ni, and Zn were higher with lower soil pH. We furthermore found clear differences among the planted cacao types (CCN-51, fine and flavor cacao clones, native cacaos from San Martin, Huánuco, Cusco) in their toxic metal contents and transfer factors. Our results clearly contribute to formulate new mitigation strategies for toxic metal accumulation in cacao seeds and chocolate products, which may be implemented already on farm level.

5.2 Introduction

Theobroma cacao L. was once discovered in central America and brought by the Spaniards to Europe in the 15^{th} century (Hoffmann 2008; Lieberei and Reisdorff 2012). During the first decades, cacao was used for medicinal purposes but nowadays it is cultivated on a huge scale in the tropics and consumed all over the world as luxury food (Hoffmann 2008; Motamayor et al. 2011). About 80% of the standard bulk cacao is produced in western Africa (mainly Ivory Coast), whereas cacaos with special aroma qualities (so called fine and flavor cacaos that receive a higher stock market price) are predominantly grown in South America (ICCO, 2019; Motamayor et al. 2008). With more pronounced cultivation and consumption of cacao, cocoa products revealed a high potential for toxic metal accumulation (Bansah and Addo 2016). Some heavy metals such as lead (Pb) occurred mostly in cacaos from Africa, whereas cadmium (Cd) was more frequently detected in cacaos from South America (Aikpokpodion et al. 2013; Chavez et al. 2015; Mounicou 2002a, b, 2003; Rankin et al. 2005; Zug et al. 2019). The health risk for humans may indeed be high, in particular for children who are consuming a high amount of chocolate in sweetened products (EFSA 2012; European commission 2014). Despite this problem, little is known about the mechanisms which influence the accumulation of toxic metals in the cacao plant and subsequently in chocolate products.

Different heavy metals and other toxic elements lead to varying symptoms of intoxication. The Itai-Itai disease, first occurred in Japan, is caused by Cd intoxication and is accompanied with osteoporosis, bone fractures, and kidney insufficiency (Greger et al. 2016; Wagner 1993; WHO 2010). Pb is known to cause neurological symptoms, which were frequently observed due to old lead-pipes for water supply (Hodge 1981). Both heavy metals are also known to occur at high amounts in chocolate products. Several studies revealed that Cd and Pb are absorbed and accumulated by the cacao plant when cultivated on polluted soils (Aikpokpodion et al. 2013; Chavez et al. 2015; Fauziah et al.2001; Gramlich et al. 2017; Mounicou et al. 2002a, b and 2003; Zug et al. 2019). In addition, other harmful metals exist at small amounts in the environment that could potentially be accumulated by the cacao plant, which received clearly less attention in scientific literature, such as cobalt (Co), copper (Cu), manganese (Mn), nickel (Ni), and zinc (Zn) or the toxic metal aluminum (Al). These metals are clearly less harmful than Cd or Pb or are even needed in small amounts in human metabolism, but studies indicate a high health risk when consumed in excess (Aikpokpodion et al. 2013; Berk et al 1949; EFSA 2011, 2015, 2018; Izah et al. 2016a, b; Lanre-Iyanda and Adekunle 2012; Perl and Moalem 2006; Stahl et al. 2011; WHO 2011). Al was recently found to be related to the Alzheimer disease (Perl and Moalem 2006; Stahl et al. 2011). Co, albeit necessary in the vitamin B_{12} synthesis, cause an intoxication leading to goiter and a reduced thyroid activity (Berk et al 1949). Cu is one of the heavy metals that is even limited by law and should not exceed 50 mg kg^{-1} in cacao beans (EFSA 2018), as this heavy metal at high concentrations causes several diseases such as arthritis, fatigue, insomnia, scoliosis, osteoporosis, heart disease, cancer, migraine, heart seizures, gum diseases, and memory loss (Izah et al. 2016a, b; Lanre-Iyanda and Adekunle 2012). Also, Mn is needed at small concentrations by the human body but is known for its neurological toxicity at high amounts (Izah and Angaye 2016; WHO 2011). Because of potentially high Ni concentrations in drinking water, it is limited by law in Europe to a concentration of 20 µg L^{-1} (EFSA 2015). It provokes damages to the gastrointestinal, hematological, neurological, and the immune system (EFSA 2015 and 2011; He et al. 2005). Zn is less toxic and sometimes even supplemented in foodstuff but may, at increased concentration, cause tachycardia, vascular shock, dyspeptic nausea, vomiting, diarrhea, pancreatitis and damage of parenchyma (Aikpokpodion et al. 2013). Consequently, there is increasing awareness about the health risk of toxic metal in foodstuffs, but little is known about contamination of chocolate products and possible mechanisms of uptake into the cacao plant of these substances, except for Cd and Pb (Aikpokpodion et al. 2013; Chavez et al. 2015; Mounicou 2002a, b, 2003; Rankin et al. 2005; Zug et al. 2019).

Cd seems to occur most frequently in cacaos from South America with contents of 1.8 mg kg^{-1} (Mounicou et al. 2002a) or between 0.9 mg kg^{-1} and 3.0 mg kg^{-1} in Ecuador (Argüello et al. 2019; Chavez et al. 2015), whereas in Africa or Brazil lower contents with an average of 0.3 mg kg^{-1} have been reported (Mounicou et al. 2002a, b). Cacao beans from Honduras showed an average of 1.1 mg kg^{-1} Cd. In contrast, Pb has more frequently been detected in Africa with contents ranging from 0.40 to 3.45 mg kg^{-1} (Aikpokpodion et al. 2013), but also lower contents between 0.01 and 0.03 mg kg^{-1} have been found (Prugarová and Kováč 1987). Mounicou et al. (2003) found higher Pb contents in Brazil with 0.769 mg kg^{-1} and the lowest content in Ecuador with 0.011 mg kg^{-1}. There is so far no literature about toxic metals such as Co and Mn in cocoa products and only a few studies dealt with Cu contents, reporting 93.9 mg kg^{-1} in cocoa powder (Lee and Low, 1985) which exceeded the legal limit of 50 mg kg^{-1} in cocoa beans (EFSA 2018). In Africa, 26.1 mg kg^{-1} Cu were found in cocoa beans (Aikpokpodion et al. 2013). Ni was measured at relatively high amounts in cocoa beans with 9.8 mg kg^{-1} by EFSA (2015) but Kruszewski et al. (2018) even found higher values with up to 12.1 mg kg^{-1}. Zn contents up to 111 mg kg^{-1} were analyzed in cocoa beans from Africa (Aikpokpodion et al. 2013) and about 79.5 mg kg^{-1} in cocoa powder (Steinberg et al. 2003). Finally, Al contents of 16 to 274 mg kg-1 were found in cocoa products in several studies (Bertoldi et al. 2016; Fechner et al. 2019; Vītola and Ciprovīča 2016).

Possible mitigation strategies include measures along the entire chocolate production chain from cacao growing at farm level using different cacao types, fermentation, and drying to grinding and conching. Unfortunately, there are so far nearly no studies available that give recommendations for the mitigation of metal contamination into cocoa products (Zug et al. 2019). There is broad evidence that metal contents in soils are the main predictor of metal content in cocoa beans, which implies that the origin of cocoa beans plays an important role as for instance soils with a volcanic origin contain more heavy metals than deeply weathered oxisols (Aikpokpodion et al. 2013; Chavez et al. 2015; Fauziah et al.2001; Mounicou et al. 2002a, b and 2003; Zug et al. 2019). Moreover, the soil pH clearly influences the uptake of metal ions from soil into plant parts (Chavez et al. 2015; Fauziah et al. 2001; He and Singh 1994a, b; He et al. 2005; Zug et al. 2019). In Cd, a lower soil pH leads to higher solubility in soil solution and uptake in plants (Fauziah et al. 2001; Gramlich et al. 2017; Hanafi and Maria 1998; He and Singh 1994a, b; Prasad 1995). In addition, also farm management may be relevant for heavy metal pollution as many fertilizers contain heavy metals which leads to increased contents in plant tissues (Fauziah et al. 2001; Grant et al. 1996; He and Singh 1994a, b; Williams and David 1976). Recently, Zug et al. (2019) additionally reported higher Cd contents when plant biodiversity was higher around sample trees and plant growth was promoted by nitrogen-(N-)fertilizers, which is not expected to be contaminated by heavy metals. Moreover, there is evidence that heavy metals such as Cd also negatively influence cocoa yield (Zug et al. 2019). However, these insights are not sufficient to change the existing cropping system fundamentally. In particular, studies should include also other toxic metals and focus on detecting and ranking factors that drive their uptake in plants.

In the present study, we aimed at analyzing drivers for toxic metal accumulation in cacao in terms of environment and farm management. This was realized for a wide range of cacao and soil samples from three mayor growing regions (San Martin, Huánuco, Cusco) in the Peruvian Amazonia, which is particularly prone to toxic metal contamination (e.g., Zug et al. 2019). In addition to farm management and environment, we included the region and cacao type into the analysis—both of which being known to play a role in metal uptake (Zug et al. submitted). In particular, we tested three mayor hypotheses: 1) the content and transfer of toxic metals in cacao seeds is significantly influenced by farm management (e.g. fertilizer application, pruning frequency, age of study trees, fruits per study tree along with cacao type); 2) environmental conditions (e.g. soil pH, electric conductivity, soil metal contents, altitude a.s.l. along with sampling region) have an influence on toxic metal contents and transfer into cacao seeds; and 3) toxic metals contents in cacao is affecting yield parameters such as annual yield in kg ha^{-1}, fruit-size, or disease infections of fruits. Based on the results, we provide possible mitigation pathways to counteract toxic contamination of cacao seeds.

5.3 Material and Methods

5.3.1 Study area

The study was carried out in the Amazonian lowland of Peru including three mayor cacao growing regions. The Cusco Region is situated in the central south of Peru, the Huánuco Region in the center and the San Martin Region in the north. More information related to the study areas can be found in Kieck et al. (2016), Zug et al. (2019), and Zug et al. (submitted). In total, we analyzed toxic metal contents (Al, Cd, Co, Cu, Mn, Ni, Pb, Zn) of 280 cacao seed samples and 280 soil samples and calculated transfer factors for toxic metal uptake in the seeds of the sampled cacao trees.

5.3.2 Study design

In Huánuco, 60 cacao and corresponding soil samples were taken on 40 different cacao farms in 2016, 30 of which were taken from the high-yield clone CCN-51, and 30 samples from fine and flavor cacao clones (ICS-1, ICS-6, ICS-39, ICS-95, TSH-565). In 2018, additionally 51 cacao and soil samples were collected from 12 farms in the Huánuco Region, which included 24 CCN-51 trees and 27 cacao trees native to the Huánuco region. In total, both campaigns led to 111 cacao and soil samples taken in Huánuco. In 2017, 105 cacao and soil samples were collected from cacao trees native to the Cusco region from 7 farms. Furthermore, 64 cacao and soil samples in the San Martin region from 14 different farms in 2018, 28 of which were CCN-51 and 36 were cacao types native to the San Martin region. Overall, we randomly selected 280 cacao trees for cacao seed and soil sampling on 73 different farms. Sampling is described more in detail in Zug et al. (2019).

Sampling procedure was identical during all field campaigns and followed Kieck et al. (2016), Zug et al. (2019), and Zug et al. (submitted). On each farm, study trees were randomly selected but had to have at least 2 to 3 ripe pods for seed harvesting. GPS data, as well as its altitude above sea level (a.s.l) were recorded. For each study tree, the length and perimeter of the ripe sample pods were measured. Seed samples from the ripe sample pods were mixed and sun dried without subsequent fermentation and used for later laboratory analyses. On each study tree, we counted the number of ripe, unripe, and infested pods. A distinction was made between pods or branches invested with different fungi (*Moniliophthora roreri*, *Moniliophthora perniciosa*, *Phytophthora* sp.) or pods attacked by insects. Around the stem foot of each study tree, 4 soil samples in a depth of 20 to 50 cm and with a distance of 100 cm to the tree were taken, mixed, sun-dried, and oven-dried for later laboratory analyses. Moreover, farmers were interviewed to gain information about the use of fertilizers, pruning frequency per year, age of the study trees, and the annual cacao yield per hectare. For later statistical analyses, the fertilizers were separated in phosphate (P) and nitrogen (N) fertilizers as described by Zug et al. (2019) to test, whether different fertilizer types influence toxic metal accumulation in cacao seeds.

5.3.3 Lab analyses

The laboratory procedures are described more in detail in Kieck et al. (2016) and Zug et al. (2019). In general, the sun-, and oven-dried soil samples were sieved and prepared in distilled H_2O for pH and electric conductivity analyses, which were conducted using a VWR symphony SP90M5 (Radnor, PA, USA). Seed shells were removed from cacao seed samples for homogenization in a mortar. 2 g of the mixed sample was ground in a ball mill (MM200, Retsch, Haan, Germany) with n-hexane. The grist was washed with petroleum ether using a Büchner funnel, and subsequently, the degreased cocoa cake was vacuum-dried at room temperature.

The degreased cocoa powder samples and the dried and sieved soil samples were used for toxic metal analyses using inductively coupled plasma optical emission spectrometry (ICP-OES). Toxic metal contents were determined after an aqua regia digestion according to A2.4.3.1 (VDLUFA, 1991). Samples were prepared weighing 2 g of each sample and mixed with $HCl:HNO_3$ (3:1), boiled for two hours at 130 °C, made up to 50 mL with distilled water, and then filtered. The analyzation of the metal concentrations of the filtrates was carried out against a respective calibration curve of the metal measured.

5.3.4 Statistical analyses

We analyzed data using Boosted Regression Trees (BRTs) to select and rank each predictor variable. BRTs are used to determine the *relative influence* of different factors on a selected variable. In this case, BRTs were performed to calculate the *relative influence* of environmental conditions (soil pH, soil electric conductivity, altitude a.s.l., soil metal content, sample region), and farm management factors (annual pruning activity, use of fertilizers, use of N or P fertilizers, age of study tree, number of fruits per study tree, cacao type) on the content of seven heavy metals (Cd, Co, Cu, Mn, Ni, Pb, Zn) and the toxic metal Al in cacao seed samples. In addition, we run the same analysis for transfer factors from soil to seed of these toxic metals. BRT modelling represents a robust method which combines the regression method with boosting (Buston and Elith 2011; Elith et al. 2008).

To run a BRT model, some parameters that affect the optimal number of trees in a model must be adjusted in the R instructions. The *tree complexity* sets the number of trees in a model with more complex trees leading to a minimum error. In our case, we set a *tree complexity* of 5 for all model calculations. The second parameter adjusted is the *learning rate* which reduces the contribution of each tree added to a model. The smaller the *learning rate*, the higher is the tree number and the better the model (Buston and Elith 2011; Elith et al. 2008). The *learning rate* was adjusted to obtain a minimum of 1000 trees during *BRT* modelling. The *learning rate* differed regarding to the modelled toxic metal content: We calculated Al with 0.003, Cd with 0.006, Co with 0.002, Cu with 0.002, Mn with 0.1, Ni with 0.005, Pb with 0.001, and Zn with 0.001; Learning rate of the transfer factors of toxic metals were set as follows: Al with 0.009, Cd with 0.004, Co with 0.001, Cu with 0.001, Mn with 0.0007, Ni with 0.007, Pb with 0.001, and Zn with 0.01.

The BRT model calculates the relative influence of each predicting variable on a selected variable (metal content in seeds, transfer factors), based on the frequency the predicting variable is chosen during the modelling for model improvement. The *relative influences* of all predicting variables included into a model always sums up to 100% (Buston and Elith 2011). The power of the resulting BRT model is expressed by the *explained variance* which is calculated by dividing the *estimated cross-validation (cv) deviance by* the *total mean deviance* of each model (Buston and Elith 2011; Elith et al. 2008). A model with an *explained variance* over 10% was considered robust. Irrespective of the *explained variances*, all models were used to rank the predictor variables among each other.

BRTs were calculated using the Software R, Version 3.6.1, with the packages dismo, Version 1.1-4, and gbm, Version 2.1.5. For the graphical illustration of the results, partial dependence plots of each variable within the calculated models are shown. Partial dependence plots show the effect of a single variable on the response variable and controlling at the same time the average effect of all other predictors (Buston and Elith 2011).

To test potential correlations between yield parameters and the toxic metal contents in cacao seeds, generalized linear mixed models (GLMM) were conducted using the lme function within the packages nlme, Version 3.1-140, and MASS, Version 7.3-51.4 (Pinheiro and Bates 1995; R Development Core Team 2014; Ripley 2015) in the Software R, Version 3.6.1. In each GLMM, the toxic metal contents in cacao seeds were set as fixed effects on the response variables annual yield in kg ha^{-1}, the fruit length and perimeter of ripe fruits, and the proportion of infested fruits (infections with *Moniliophthora roreri*, *Moniliophthora perniciosa*, *Phytophthora* sp., attacks by insects). GLMMs permit a nesting of data which is given in our data structure. Therefore, our cacao types (CCN-51 clone, fine and flavor cacao clones, native cacaos from San Martin, Huánuco, Cusco) were nested in the different cacao farms and sampling regions of Peru (San Martin, Huánuco, Cusco; Zuur et al. 2009).

5.4 Results

5.4.1 Farm management as driver of toxic metal pollution in cacao

The farm management factors showed a clear influence on the toxic metal contents in cacao seeds (**Fig 20**). The models of the Al (49.2%), Cd (54.1%), Co (12.8%), Cu (17.6%), Mn (24.7%), and Ni (70.3%) contents had *explained variances* over 10% and were therefore considered as robust models, whereas model performance of Pb (0%), and Zn (5.4%) was insufficient. Irrespective of the explained variances, all models were used to rank the predictor variables among each other. In the case of the toxic metal Al, the cacao type with 15.2%, and the age of the study trees with 10.2% were the most predicting management factors within the BRT model explaining overall 49.9% for the variance in Al content (**Fig 20a**). Highest Al contents were found in native cacaos from San Martin (15.0 mg kg^{-1}), followed by the CCN-51 clone (12.5 mg kg^{-1}), fine and flavor cacao clones (6.7 mg kg^{-1}), native cacaos from Huánuco (4.8 mg kg^{-1}), and native cacaos from Cusco (4.4 mg kg^{-1}). Older study trees had in general lower Al contents than younger. The age of the sampled tree (15.7%) and the cacao type (8.4%) also had a great influence on Cd accumulation in cacao seeds, with older trees containing less Cd than younger (overall model power: 54.1% of *explained variance*; **Fig 20b**). Fine and flavor clones with 2.1 mg kg^{-1}, native clones from Huánuco with 1.9 mg kg^{-1}, and CCN-51 with 1.9 mg kg^{-1} showed highest Cd content values, whereas native cacaos from San Martin (0.8 mg kg^{-1}) and Cusco (0.2 mg kg^{-1}) showed clearly lower values (**Fig 20b**). Also, Co accumulation in cacao seeds responded to the age of study trees (16.3%) but showed the opposite trend as Al and Cd with older trees having higher Co contents. The *explained variance* of the overall model was low with 12.8%. In Cu, a lower fruit number led to below average Cu contents (with a *relative influence* of 11.3%; **Fig 20d**). The cacao type (7.5%) also predicted the Cu contents with highest contents in native cacaos from Cusco (49.7 mg kg^{-1}), Huánuco (46.1 mg kg^{-1}), and San Martin (45.2 mg kg^{-1}), followed by the CCN-51 clone (44.5 mg kg^{-1}), and the fine and flavor cacao clones (41.7 mg kg^{-1}; **Fig 20d**). Younger cacao trees had below average Cu contents with a *relative influence* of 6.7% and a less frequent pruning led to above average Cu contents in seeds (6.7%; **Fig 20d**). However, overall *explained variance* of the model was with 17.6% rather low. Mn contents in cocoa powder samples raised with the age of cacao trees (*relative influence*: 17.2%; **Fig 20e**). The cacao type had a *relative influence* of 13% in the Mn model and showed the highest Mn contents in native cacaos from Cusco (78.5 mg kg^{-1}), followed by the fine and flavor cacao clones (66.3 mg kg^{-1}), the CCN-51 (59.7 mg kg^{-1}), the native cacaos from San Martin (54.4 mg kg^{-1}), and the lowest contents in native cacaos from Huánuco with 41.2 mg kg^{-1} (**Fig 20e**). The Mn contents in cocoa powder increased with the fruit number per sample tree (*relative influence*: 11.2%; **Fig 20e**). The BRT model on Mn content had an overall low *explained variance* of 24.7% in contrast to the Ni model exhibited a high overall *explained variance* of 70.3%. Ni contents in cocoa powder clearly varied between the cacao types (with an *relative influence* of 40%) and decreased from the native cacaos of Cusco (27.2 mg kg^{-1}), over the fine and flavor cacao clones (11.7 mg kg^{-1}), the native cacaos from Huánuco (10.3 mg kg^{-1}), the CCN-51 (9.4 mg kg^{-1}) to the native cacaos from San Martin (8.6 mg kg^{-1}; **Fig 20f**). Ni content in cacao seeds was below average at lower pruning frequency (7.5% *relative influence*), and above average with more fruits per tree (6.1% *relative influence*; **Fig 20f**). The same applied to the Pb content in cacao seeds that decreased a higher number of fruits per tree (15.2% *relative influence*; **Fig 20g**). The cacao type showed a *relative influence* of 16.3% on Pb content with higher values in native cacaos from Huánuco (0.22 mg kg^{-1}) and lower values in CCN-51 (0.06 mg kg^{-1}), fine and flavor cacao clones (0.05 mg kg^{-1}), native cacaos from Cusco (0.02 mg kg^{-1}), and native cacaos from San Martin (0.01 mg kg^{-1}; **Fig 20g**). Nevertheless, model power for Pb was only at 0%. The most influencing management factor for the content of Zn in cacao seeds was the age of the cacao tree (16.6%) with the Zn content decreasing with the age of trees (**Fig 20h**). Furthermore, Zn contents in seeds were higher with more fruits per tree (11.5% *relative influence*; **Fig 20h**) and the cacao type influenced Zn content with 7.2% *relative influence*. Here, native cacaos from Huánuco showed the highest Zn contents with 87.7 mg kg^{-1}, followed by the fine and flavor cacao clones with 83.1 mg kg^{-1}, the CCN-51 clone with 82.3 mg kg^{-1}, the native cacaos from San Martin with 80.1 mg kg^{-1}, and the native cacaos from Cusco with the lowest value of 78.0 mg kg^{-1}. Again, model power of the BRT for Zn was low with 5.4%.

a) Aluminum

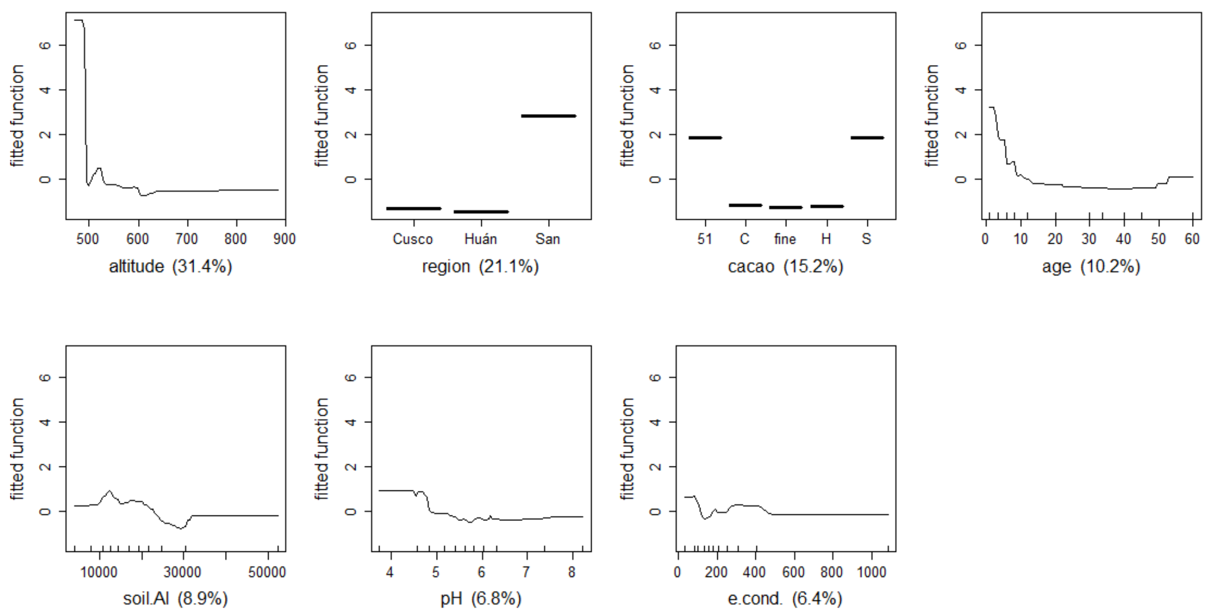
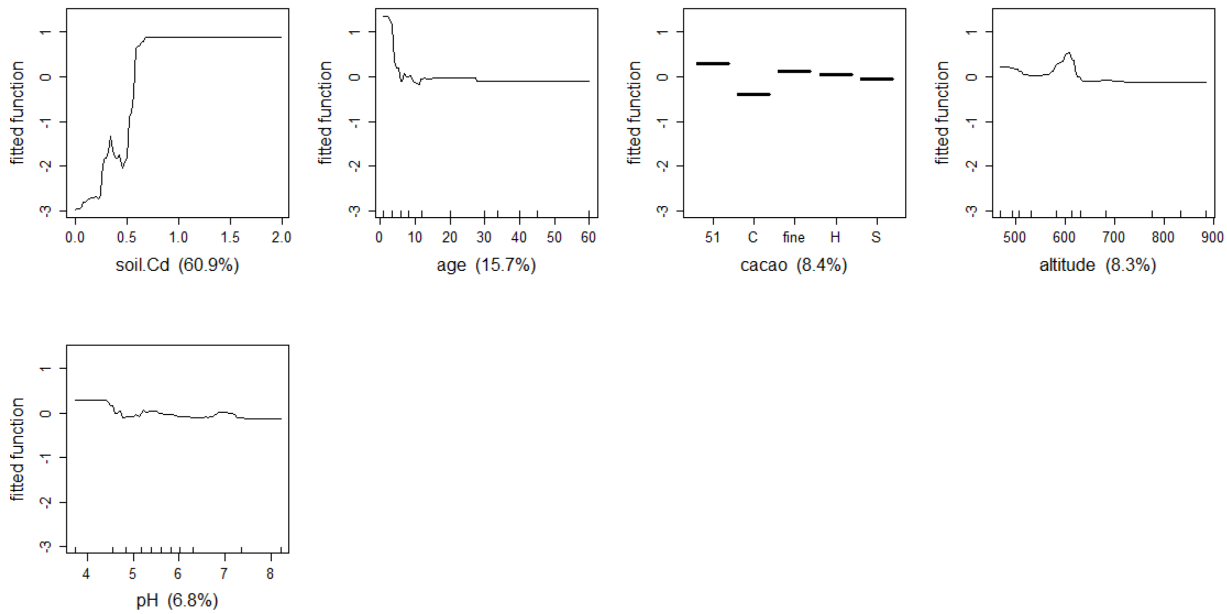


Figure 20: Continued on page 120.

b) Cadmium



c) Cobalt

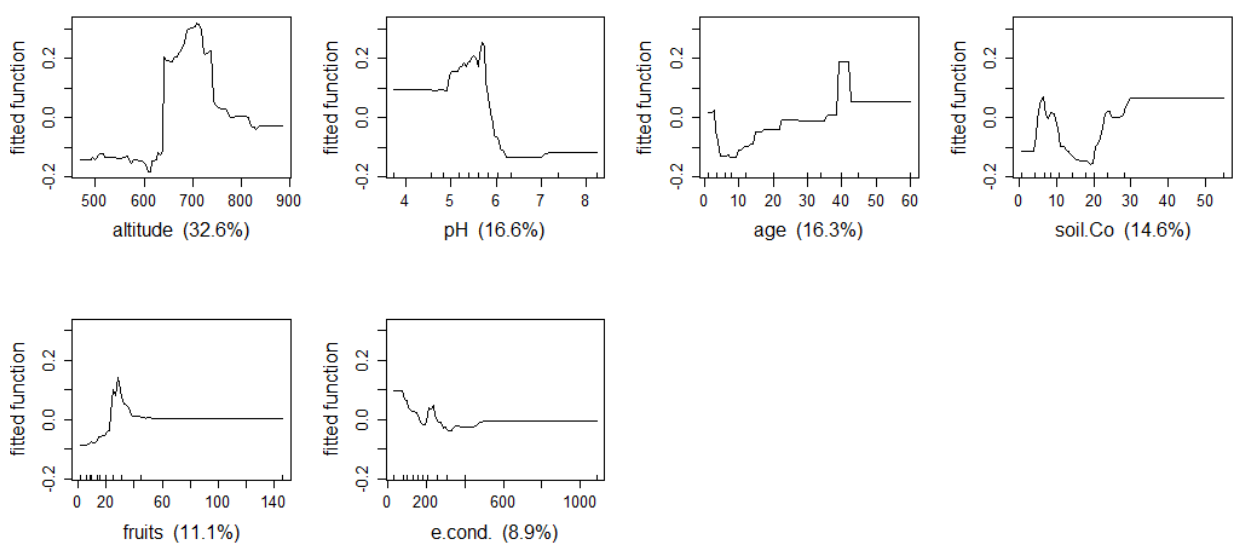
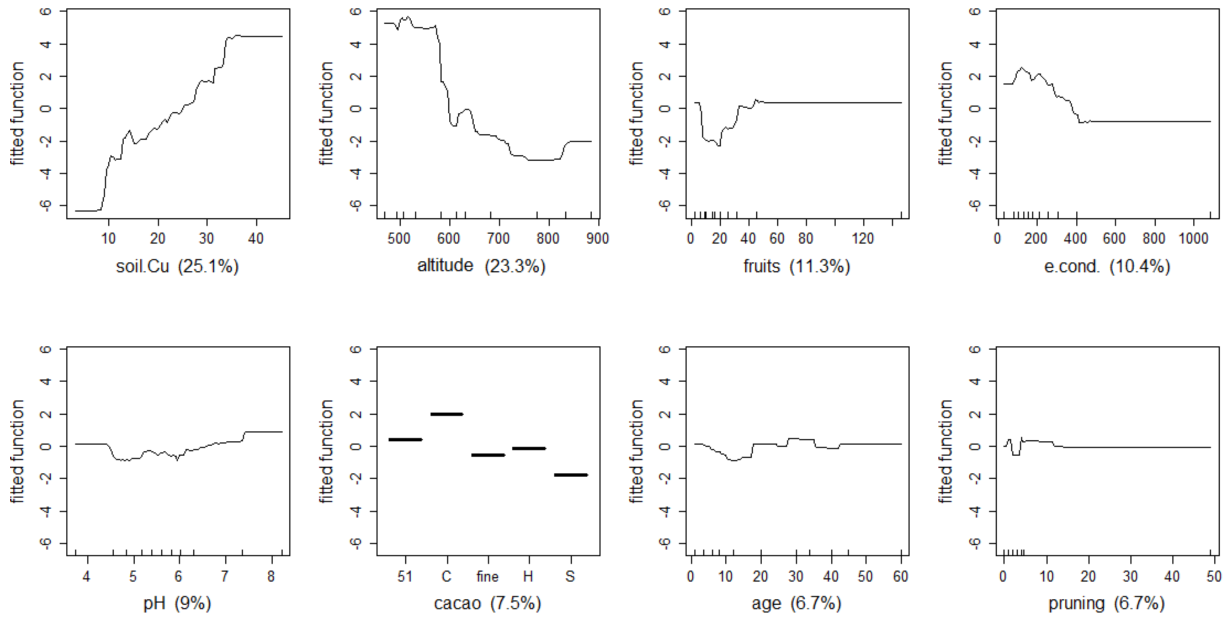


Figure 20: Continued on page 121.

d) Copper



e) Manganese

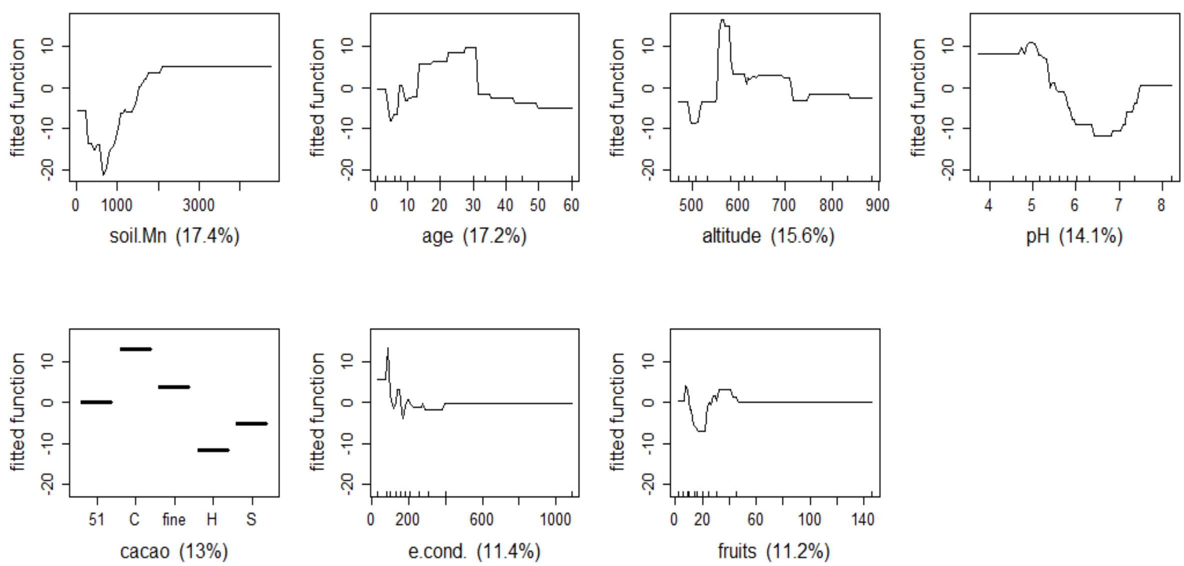


Figure 20: Continued on page 122.

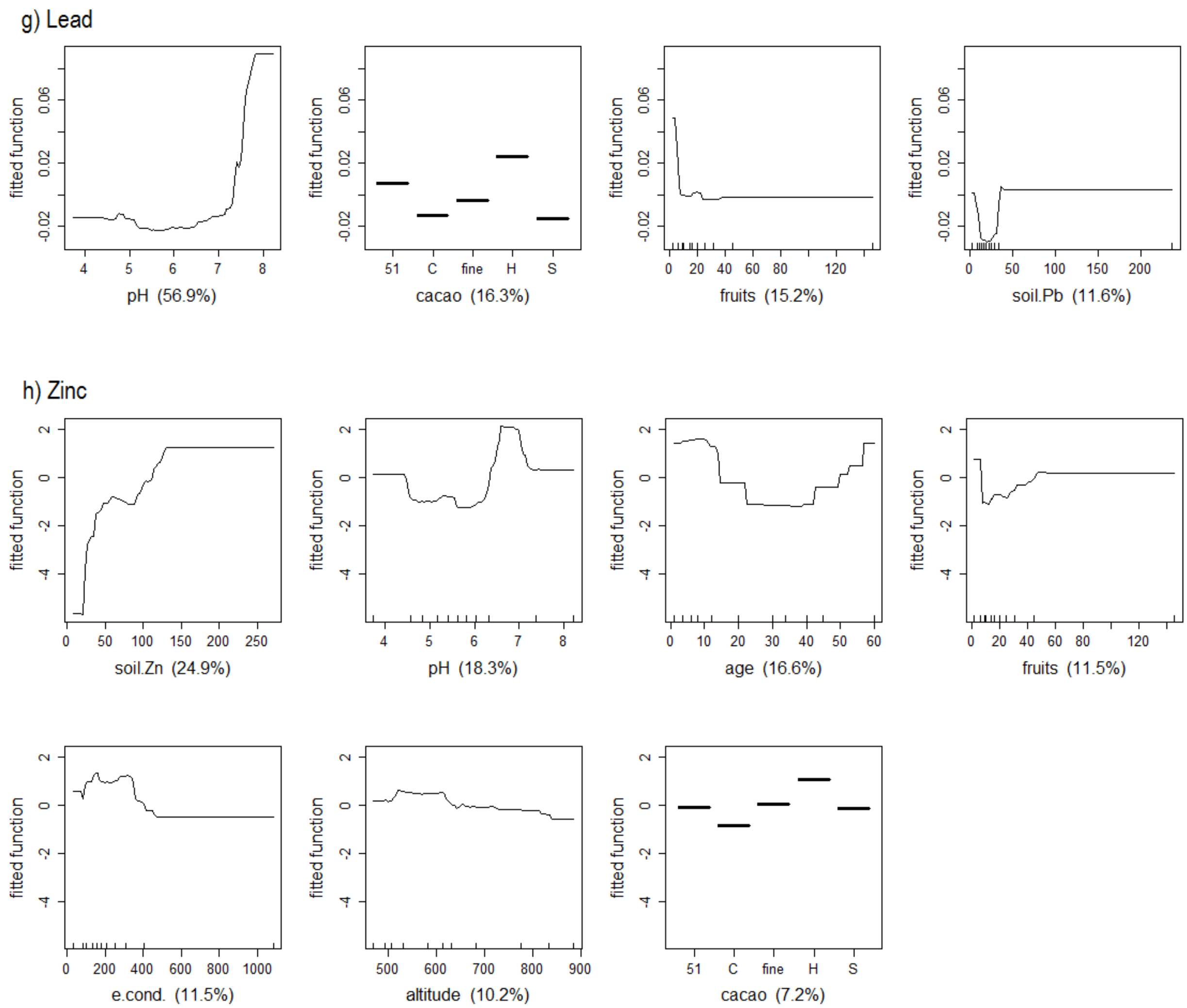


Figure 20: Partial dependence plots of the predicting variables: soil toxic metal contents in mg kg^{-1} (soil Al, Cd, Cu, Co, Mn, Ni, Pb, or Zn), cacao type (cacao), region (region), soil pH (pH), soil electrical conductivity in S m^{-1} (e.cond.), altitude a.s.l. (altitude), age of study trees in years (age), fruit-set in numbers of fruits per tree (fruits), annual pruning frequency (pruning), number of used fertilizers in total (fert.no.), plantations with or without fertilization (fert.), total number of used N-fertilizers (N.no.), number of used P-fertilizers (P.no.), plantations with or without N-fertilization (N.fert.), and plantations with or without P-fertilization on the toxic metal contents (**a**) Aluminum, **b**) Cadmium, **c**) Cobalt, **d**) Copper, **e**) Manganese, **f**) Nickel, **g**) Lead, **h**) Zinc) in cocoa powder samples. Fitted lines represent the mean estimate and relative influences of predicting variables are given in%. Regions: Cusco = Cusco; Huán = Huánuco; San = San Martin. Cacao types: 51 = CCN-51; fine = fine and flavor cacao clones; C = native

cacaos from Cusco; H = native cacaos from Huánuco; S = native cacaos from San Martin. Software R 3.6.1. Boosted Regression Trees.

The transfer factors of the toxic metals from soil to cacao seeds also clearly responded to farm management. For the transfer factors, we obtained the following *explained variances*: Cd (27.3%), Cu (29.3%), Ni (40.1%), and Zn (26.2%) had relatively high *explained variances* compared to Al (0%), Co (5.6%), Mn (1.7%), and Pb (0%). The transfer factor of Al was influenced with 12.1% by the annual pruning activities showing below-average metal content at low pruning frequency (**Fig 21a**). Al uptake furthermore depended on cacao type with similar findings as for Al content (highest values in native cacaos from San Martin and CCN-51). Also, Cd proved to have below-average transfer factors at lower pruning frequency: (6.1% *relative influence*; **Fig 21b**), but Cu (7.4% *relative influence*; **Fig 21d**), Ni (6.5% *relative influence*; **Fig 21f**), and Zn (6.8% *relative influence*; **Fig 21h**) exhibited above-average transfer factors at low pruning frequency. The transfer factors of all studied toxic metals were clearly influenced by the age of the study trees with younger cacao trees showing higher transfer factors (Al: 9.6% *relative influence*; Cd: 21.5% *relative influence;* Co: 23.6% *relative influence*; Cu: 21.9% *relative influence*; Mn: 29% *relative influence*; Ni: 20% *relative influence*; Pb: 9.6% *relative influence*; Zn: 20.9% *relative influence*; **Fig 21**). Moreover, also the cacao type was an important factor for the transfer factors of most studied heavy metals (Cd: 16.6% *relative influence*; Co: 22.8% *relative influence*; Cu: 8.1% *relative influence*; Ni: 12.4% *relative influence*; Pb: 19.2% *relative influence*; Zn: 8.8% *relative influence*; **Fig 21**), except Mn. Transfer factors of all toxic metals were consistently among the highest in fine and flavor cacaos (Cd: 13.17; Co: 0.21; Cu: 3.52; Mn: 0.20; Ni: 1.35; Pb: 0.007; Zn: 2.18. Uptake of Cd was exclusively exceeded by CCN-51 (13.26). Co (0.14), Cu (3.32), and Zn (2.20) transfer factors were also high in native cacaos from San Martin, whereas Ni showed highest values in Cusco (1.58) and Pb in Huánuco (0.008). The transfer factor of Cd was also influenced by the number of fruits per sample tree (10.2% *relative influence*; **Fig 21b**), with above-average transfer factors at low fruit numbers. This effect was equally observed in Cu (11.7% *relative influence*; **Fig 21d**), Pb (11.9% *relative influence;* **Fig 21g**), and Zn (10.5% *relative influence*; **Fig 21h**). Only the transfer-factors of Co (13.3% *relative influence*; **Fig 21c**) and Ni (10.7% *relative influence*; **Fig 21f**) showed the opposite effects with increasing transfer factors with more fruits per tree. The transfer factor of Cd was the only one influenced by the frequency of N-fertilizer use (6.7% *relative influence*; **Fig 21b**). The transfer-factor rose the more N-fertilizers were used. In general, transfer factors higher than 1 indicate that the metal content is higher in cacao seed samples than in the corresponding soil samples which implies toxic metal accumulation in plants tissues. For the heavy metals Cd, Cu, Ni, and Zn, we found transfer factors higher than 1. In contrast, uptake of Al (average: 0.0005) and Pb (average: 0.0031) by the cacao plants was nearly not existent.

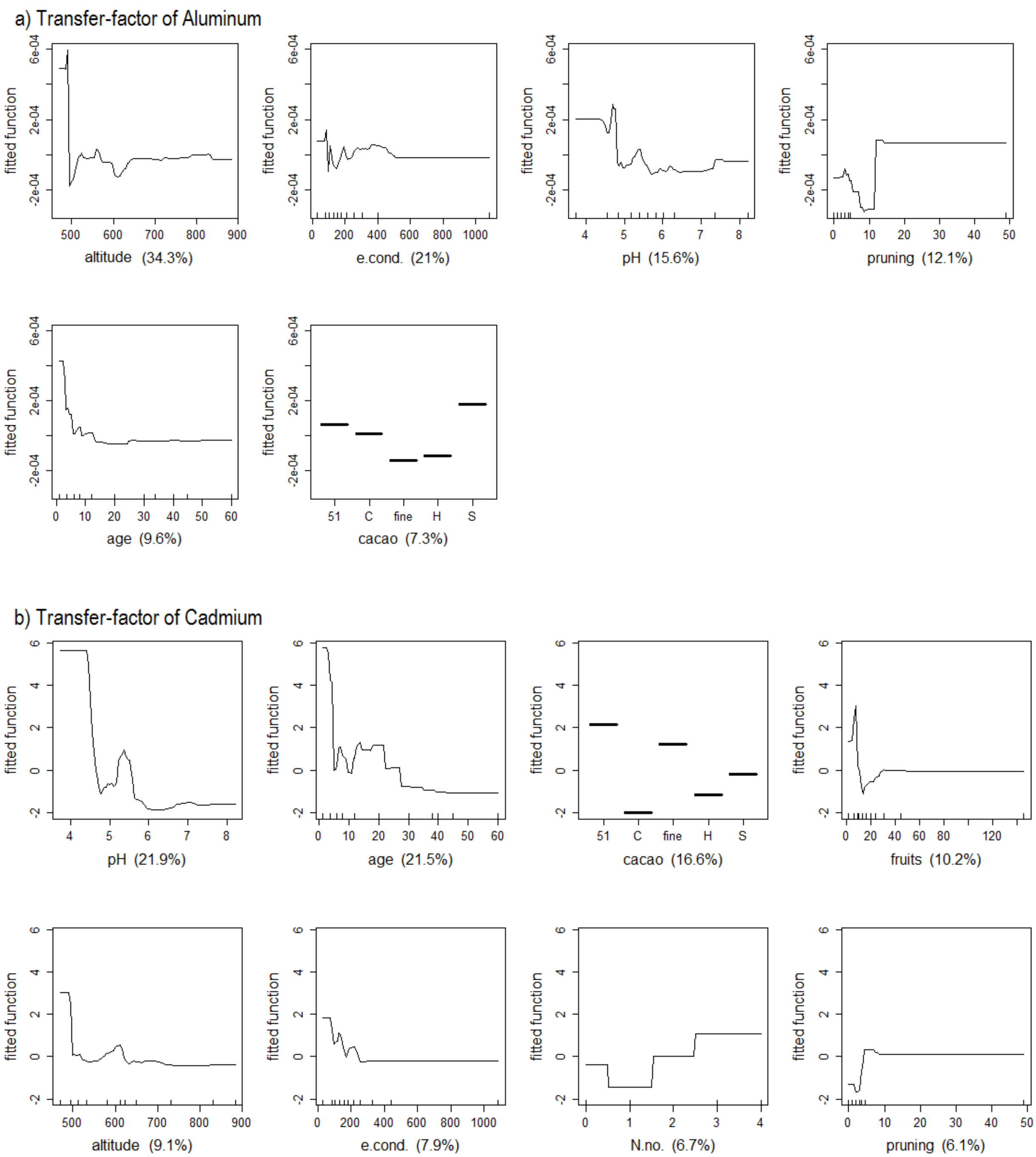
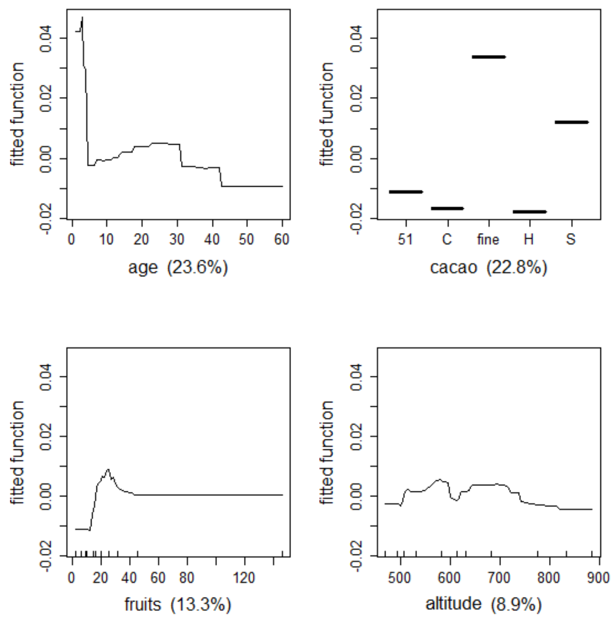


Figure 21: Continued on page 125.

c) Transfer-factor of Cobalt



d) Transfer-factor of Copper

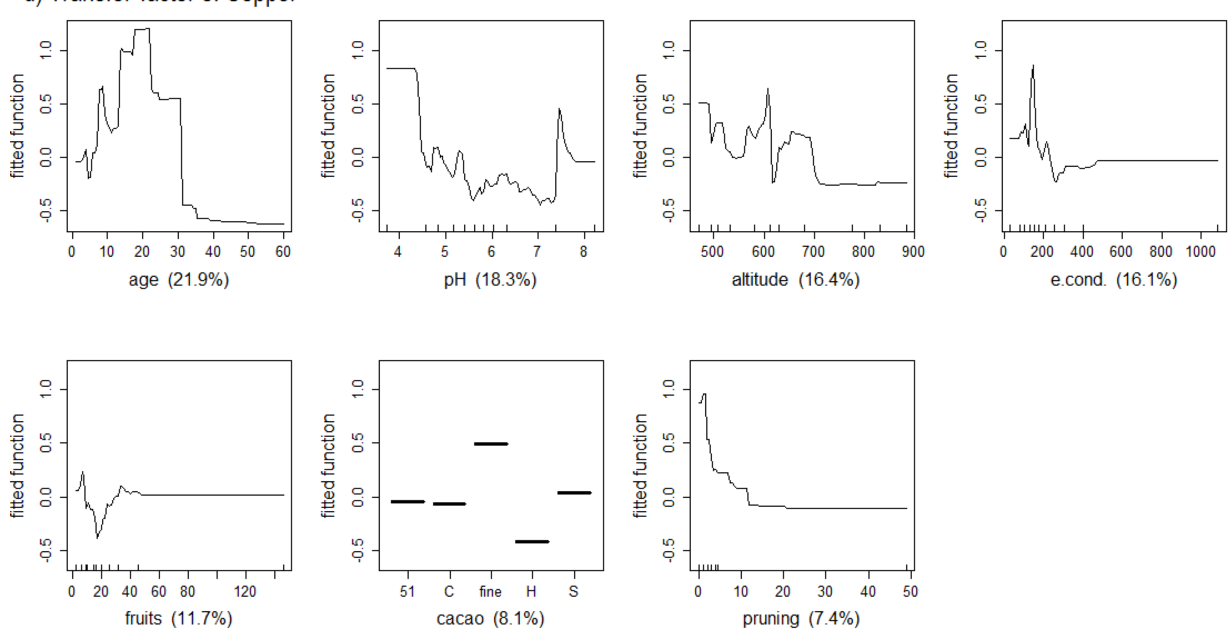


Figure 21: Continued on page 126.

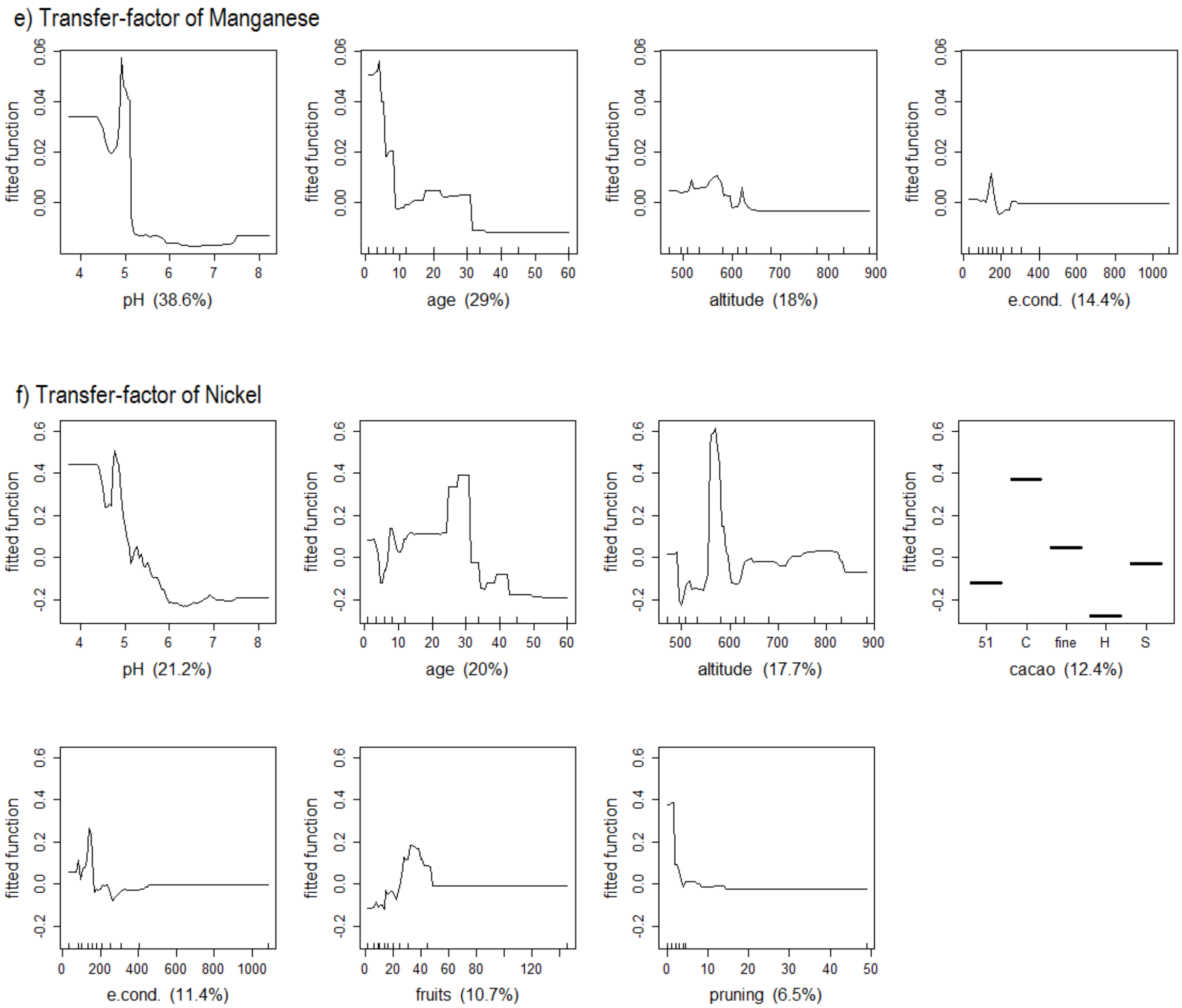
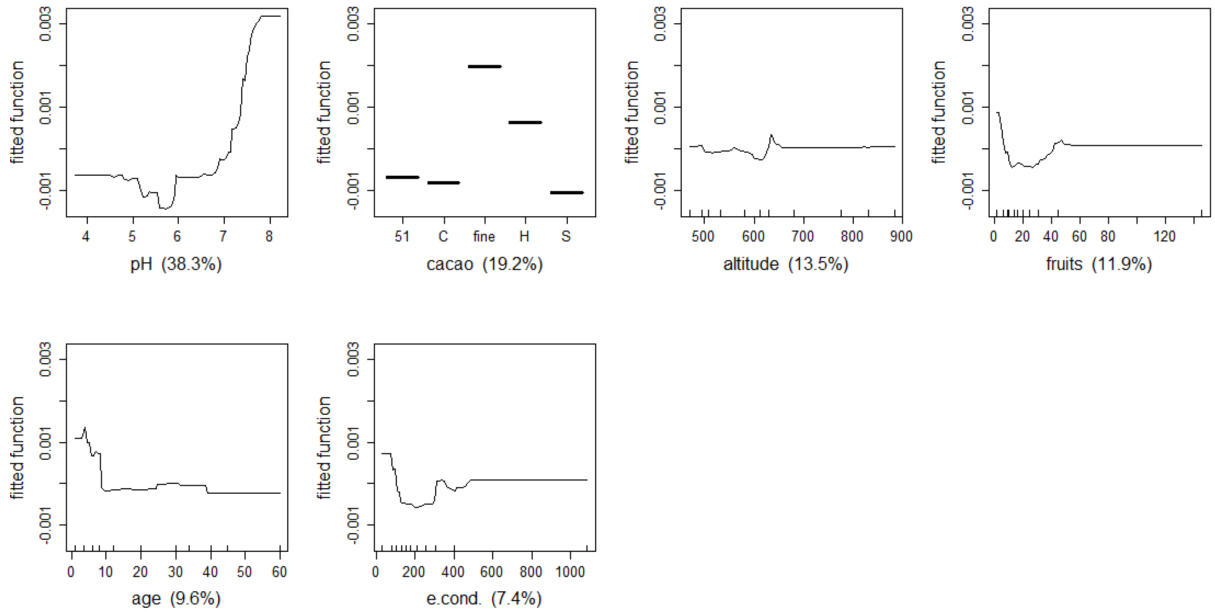


Figure 21: Continued on page 127.

g) Transfer-factor of Lead



h) Transfer-factor of Zinc

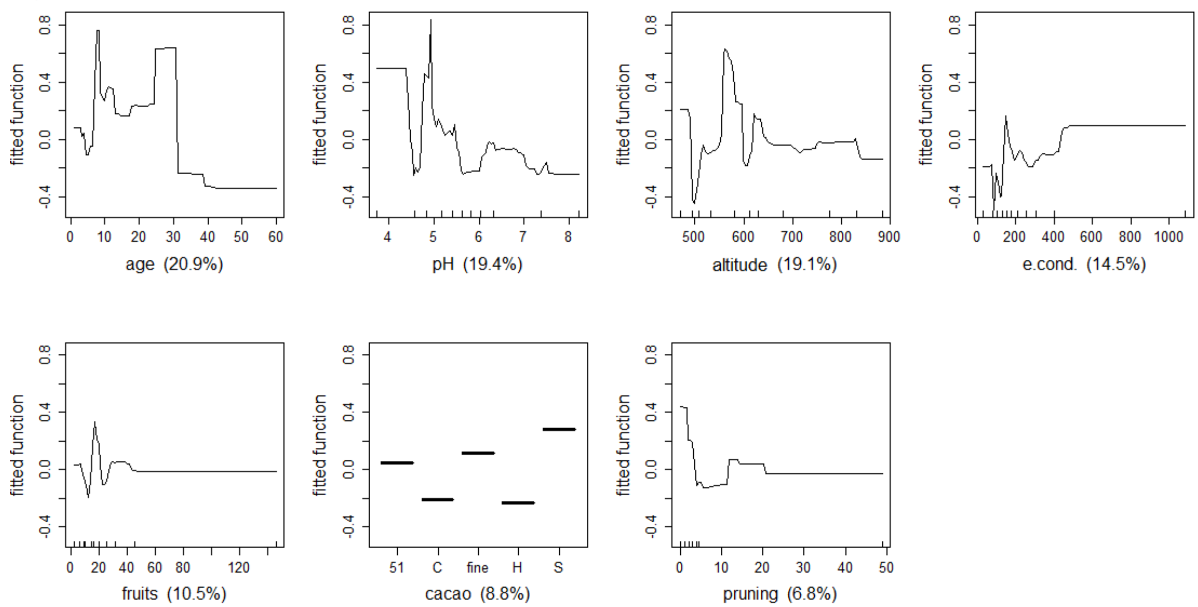


Figure 21: Partial dependence plots of the predicting variables: cacao type (cacao), region (region), soil pH (pH), soil electrical conductivity in S m^{-1} (e.cond.), altitude a.s.l. (altitude), age of study trees in years (age), fruit-set in numbers of fruits per tree (fruits), annual pruning frequency (pruning), number of used fertilizers in total (fert.no.), plantations with or without fertilization (fert.), total number of used N-fertilizers (N.no.), number of used P-fertilizers (P.no.), plantations with or without N-fertilization (N.fert.), and plantations with or without P-fertilization on the transfer-factors of toxic metals (**a**) Transfer-factor of

Aluminum, **b**) Transfer-factor of Cadmium, **c**) Transfer-factor of Cobalt, **d**) Transfer-factor of Copper, **e**) Transfer-factor of Manganese, **f**) Transfer-factor of Nickel, **g**) Transfer-factor of Lead, **h**) Transfer-factor of Zinc). Fitted lines represent the mean estimate and relative influences of predicting variables are given in%. Regions: Cusco = Cusco; Huán = Huánuco; San = San Martin. Cacao types: 51 = CCN-51; fine = fine and flavor cacao clones; C = native cacaos from Cusco; H = native cacaos from Huánuco; S = native cacaos from San Martin. Software R 3.6.1. Boosted Regression Trees.

5.4.2 Environmental factors as driver of toxic metal pollution in cacao

Apart from the farm management factors, our study provides evidence that environment profoundly influences toxic metal content and uptake in cacao fruits. The Al content in cocoa powder samples was most relevantly influenced by the altitude a.s.l. with a *relative influence* of 31.4%: Al content decreased with a decreasing altitude (**Fig 20a**) which also holds true for other toxic metals, such as Cd (8.3% *relative influence*), Cu (23.3% *relative influence*), Mn (15.6% *relative influence*), and Zn (10.2% *relative influence;* **Fig. 20b,d,e,h**). In contrast, Co (31.4% *relative influence*) and Ni (8.8% *relative influence*) contents clearly increased with altitude (**Fig 20c,f**). The region (San Martin, Huánuco, Cusco) in which samples were taken seemed to be less important for toxic metal content. Exclusively Al (21.1% *relative influence*) and Ni (14.7% *relative influence*) contents in cacao seeds were influenced by the region (**Fig 20a,f**). with Al contents being highest in San Martin (16.56 mg kg^{-1}) and Ni showing highest values in Cusco (27.17 mg kg^{-1}). One of the most important drivers of toxic metal content in cocoa power was the metal content in corresponding soil samples. The heavy metals showed a clear positive correlation of soil and seed metal content (Co with 13.8% *relative influence*; Ni with 16.4%; Pb with 11.6%; Cd with 60.9%; Cu with 25.1%; Mn with 17.4%; Zn with 24.9%; **Fig 20**). In Cd, Cu, Mn, and Zn, soil content was the most important predictor. In contrast, Al content in seeds was higher at lower soil Al content and correlation was weak (8.9%). In addition to soil metal content, the soil pH was an important variable with lower pH leading to high metal contents in seeds for Al (6.8% *relative influence*), Cd (6.8% *relative influence*), Co (16.1% *relative influence*), Mn (15.6% *relative influence*), and Ni (6.4% *relative influence*; **Fig 20a,b,c,e,f**), whereas Cu (9.0% *relative influence*), Zn (18.3% *relative influence*), and Pb (56.9% *relative influence*; **Fig 20d,g,h**) contents raised with increasing soil pH. Moreover, low electric conductivity led to higher seed contents of Al (6.4% *relative influence*), Co (8.5% *relative influence*), Cu (10.4% *relative influence*), Mn (11.4% *relative influence*), and Zn (11.5% *relative influence*; **Fig 20a,c,d,e,h**).

The transfer factors of these toxic metals were most strongly influenced by soil pH, soil electric conductivity, and altitude a.s.l. The altitude was the most important predictor of the Al transfer factor with a *relative influence* of 34.3% (**Fig 21a**) with lower transfer factor with decreasing altitude a.s.l. The same trend was also found for all heavy metals: Cd with 9.1% *relative influence*, Co with 8.9%, Cu with 16.4%, Mn with 18.0%, Ni with 17.7%, Pb with 13.5%, and Zn with 19.1% (**Fig 21**). However, soil pH was the most relevant predictor variable for the transfer factors of the heavy metals Cd with 21.9% *relative influence*, Mn with 38.6%, Ni with 21.2%, and Pb with 38.3%, among the four most important variables for Al with 15.6%, Co with 14.3%, Cu with 18.3%, and Zn with 19.4% (**Fig 21**). The transfer factors of most metals increased with decreasing pH, except from Pb which showed the opposite trend. In accordance to pH, the transfer factors of all studied toxic metals except Zn correlated negatively with electric conductivity in soil samples. BRT modelling revealed *relative influences* of electric conductivity on the transfer factors of Al of 21.0%, Cd of 7.9%, Co of 17.1% Cu of 16.1%, Mn of 14.4%, Ni of 11.4%, Pb of 7.4%, and Zn of 14.5% (**Fig 21**).

5.4.3 Influence of toxic metals on yield parameters

The contents of toxic metals showed only weak influence on yield parameters of cacao. GLMM modelling revealed that Al in cacao seeds correlated positively with mean fruit size measured as fruit length ($p = 0.009$) and fruit perimeter ($p = 0.047$). Accordingly, higher Cu contents in seeds were related to significantly increased fruit perimeter ($p = 0.003$). The annual yield in kg per ha was marginally significantly and negatively correlated by the Ni contents in cacao seeds ($p = 0.073$). Moreover, fruits were marginally significantly less infected with *Phytophthora* sp. when the Mn content in cacao seeds was higher ($p = 0.072$).

5.5 Discussion

The present study gives detailed insights into the complex interplay of toxic metal uptake and content in cacao plant tissues (*Theobroma cacao* L.). Most studies until now have focused on factors such as the metal content in soil solution, soil pH, and contaminated fertilizers or pesticides as main drivers of toxic metal uptake in plants (Apikpokpodion et al. 2013; Chavez et al. 2015; Fauziah et al. 2001; Gramlich et al. 2017; Grant et al. 1996; He and Singh 1994a, b; He et al. 2005; Mounicou et al 2002a, b, 2003; Williams and David 1974; Zug et al. 2019). In addition, the cultivation of a cacao clone combination in contrast to a single cacao clone cultivation was identified to influence the Cd accumulation in cacao fruits (Arévalo-Gardini et al. 2017). Our study supports the strong impact of soil and cacao type but broadens the view to further environmental factors such as the soils' electrical conductivity and the altitude a.s.l. or farm management such as the pruning frequency, fruit-set, and the age of the sampled cacao, which have so far never been reported as potentially important drivers. Moreover, our statistic approach using *BRT* allows for the ranking of predictors which facilitates to focus on the most important drivers when developing mitigation measures. The correlation of toxic metals with yield parameters showed that, despite the pronounced health relevance for humans, current metal concentrations are rather beneficial to the cacao tree, which may explain the pronounced accumulation of heavy metals in plant tissues in this species prevailing on highly weathered soils. Still our finding on Ni and a former study on Cd show a potential toxicity of heavy metals for plant metabolisms, as well.

5.5.1 Farm management as driver of toxic metal pollution in cacao

Farm management was hardly in the focus for explaining toxic metal pollution in crops, albeit it may be particularly promising for mitigating metal uptake. Our study provides clear evidence that farm management has pronounced impact on metal accumulation in cacao plants. Most studies revealed that metal contents in soil and soil pH are the main factors for contents of toxic metals in plants (Apikpokpodion et al. 2013; Chavez et al. 2015; Gramlich et al. 2017; Mounicou et al 2002a, b, 2003; Zug et al. 2019) factors that can hardly be influenced except by liming.

It is also widely acknowledged that fertilizers or compost used in agriculture are frequently polluted with Cd, Mn, Ni, Pb, and Zn, and Co, Cu, Pb, and Zn also occur in pesticides or fungicides (He and Singh 1994a, b; He et al. 2005; Fauziah et al. 2001; Zug et al. 2019). In particular, Cd was reported to occur in phosphate fertilizers (Fauziah et al. 2001; Grant et al. 1996; He and Singh 1994a, b; He et al. 2005; Williams and David 1974). But also non-polluted N-fertilizers are known to increase Cd content in cacao seeds (He and Singh 1994a, b; Zug et al. 2019). Cd from P-fertilizers may accumulate in soils over a longer time (Grant et al., 1998). Grant et al. (1996) suggested that higher Cd contents in the soil solution are related to the desorption of Cd from soil colloids as a consequence of fertilizer treatment. Pb pollution of cacao seeds has been reported as a result of the use of lead-acid batteries as herbicide or of leaded fuel still used in Africa (Apikpokpodion et al. 2013). Our study confirmed the influence of N-fertilizers on increased Cd transfer in cacao plants as formerly reported by Zug et al. (2019).

Different cacao types clearly show diverging toxic metal contents and transfer factors as previously reported by Zug et al. (submitted). Our study may hence be relevant for finding varieties with less pronounced toxic metal accumulation, as recently claimed by Maddela et al. (2020). We showed that the cacao type was among the most important drivers for transfer factors of many toxic metals (Al, Cd, Co, Cu, Ni, Pb, Zn) as well as of actual metal contents in cacao seeds (Al, Cd, Cu, Ni, Pb, Zn) (Zug et al., submitted). Interestingly, fine and flavor cacaos we characterized by higher transfer factors than many native types. In addition, the frequently grown CCN-51 shows particularly high Cd accumulation: a fact that has not been reported in former studies (e.g., Zug et al. 2019). Differences in toxic metal transfer and content have scarcely been studied so far but Arévalo-Gardini et al. (2017) reported lower Cd contents in cacao plants of CCN-51 monocultures compared with CCN-51 and ICS-95 combinations, both of which showed a high transfer factor in comparison to native types in our study.

A novel aspect of this study is that the age of the study cacao tree was a clear indicator for toxic metal pollution with transfer factors of older trees being lower (Al, Cd, Co, Cu, Mn, Pb, Zn), except for the toxic metal Ni. Zug et al (2019) showed that Cd contents in cacao seeds increased with the age of trees, but the study trees only reached a maximum age of 10 years in their study, whereby ages in this study varied between 1 and 60 years. Another reason could be that oldest trees were sampled from the native Cusco cacaos which also had higher transfer factors for Ni than the other trees (transfer factor for Ni in Cusco: 1.58, compared with 1.07 in San Martin and 0.93 in Huánuco). This may be attributed to the fact that soils in the Cusco region naturally contain more Ni (23.69 mg kg^{-1}) than the soils in other regions (Huánuco: 17.35 mg kg^{-1}; San Martin: 11.23 mg kg^{-1}). Still, the general decrease of toxic metal transfer in older trees indicates that a generally reduced tree growth at higher age also diminishes uptake of minerals. A similar explanation was given by Zug et al. (2019) for the impact of N fertilization on Cd content in seeds. Unfortunately, it remains difficult to use this fact for mitigation of toxic metal accumulation, as increasing age of cacao trees has also been connected with yield reduction due to aging processes and decreasing disease defenses (Clough et al. 2009).

Moreover, fruit set and transfer factors of Cd, Cu, Pb, and Zn were negatively correlated. This may again be related to tree age, as older trees produce a higher number of fruits per tree. This view is supported by the fact that the transfer factors of Ni (and also Co) are higher with more fruits per tree as stated above. In accordance to these findings, Zug et al. (2019) reported lower fruit set with higher Cd contents in cacao seeds. However, many different factors are connected with fruit set. A positive effect of the diversity of the herb and shrub layer around trees on ripe and healthy cacao fruits per study trees was found by Kieck et al. (2016). In general, a high plant diversity within the plantation also proved to be beneficial or at least not disadvantageous for cacao yields (Bisseleua et al. 2008; Clough et al. 2011; Herion et al. under review; Kieck et al. 2016; Schroth et al. 2016; Zug et al. 2019). In contrast, yield losses in cacao have been attributed to drought (Moser et al. 2010; Schwendenmann et al. 2010), loss of pollinators (Groeneveld et al. 2010), nutrient limitation (Ahenkorah et al. 1987), diseases with fungi (Tscharntke et al. 2011; Clough et al. 2009). Also heavy metals such as Cd (Hanafi and Maria 1998; He and Singh 1994b; WHO 2010b), Co (He et al. 2005), Cu (shown for rice from Xu et al. 2005), Ni (Yusuf et al. 2010) were reported to be highly toxic for plants in even low concentrations and to reduce plant growth. Equally, Pb has several effects on plants such as photosynthesis inhibition, chlorosis, and blackening of roots, and may even lead to stunted growth (Sharma and Dubey 2005). The same applies to Al which is toxic for plants and can reduce nutrient content in seedlings, plant growth and subsequently yield of, for instance, cacao (Baligar and Fageria 2004; Foy 1988; Moreira de Almeida et al. 2015; Quinteiro Ribeiro et al. 2013). Mn inhibits plant growth, but many plant species are well adapted to Mn toxicity by different biochemical pathways in which Mn is absorbed and metabolized (El-Jaoual and Cox 1998). However, Hanafi and Maria (1998) even showed increased growth in cacao seedlings after an application of Cd in combination with Zn. In view of the few rather positive effects of toxic metal content and yield parameters (see below), the observed negative correlation of fruit set and transfer factors seems to be rather a matter of tree age and canopy surface than of metal toxicity.

Transfer factors of Al, Cd, Cu, and Zn also increased with annual pruning frequency of cacao trees, but Ni transfer decreased. Many fruit trees are pruned to increase yields and limit the diffusion of diseases in plantations which also applies to the cacao tree (shape and sanitary pruning). Already two to three pruning activities of shade trees per year in coffee or cacao plantations can be sufficient to replace the use of fertilizers to obtain higher productivity (Beer 1988; Muñoz and Beer 2001; Imbach et al. 1989; Sepkoski 1988). Pruned trees tend to show increased growth and nutrient uptake which results in better fruit growth and yields (Beer 1988) but may also stimulate uptake of toxic metals in cacao fruits.

Overall, less pronounced growth of trees using plantations for longer periods with older trees, reducing fertilizer input and pruning frequency seem to be promising mitigation strategies for toxic metal pollution in cacao seeds and chocolate products. Also, the use of novel cacao types such as the native varieties analyzed in this study may be an efficient mitigation measure. However, all those possibilities are accompanied by reduced yield. Consequently, the confectionary industry should compensate farmers for these income losses to provide consumers with healthy products.

5.5.2 Environmental factors as driver of toxic metal pollution in cacao

Several studies confirmed that the metal content in soil plays a pivotal role for the metal content in plants (Apikpokpodion et al. 2013; Chavez et al. 2015; Fauziah et al. 2001; Gramlich et al. 2017; Grant et al. 1996; Mounicou et al 2002a, b, 2003; Mullins et al. 1986; Williams and David 1974; Zug et al. 2019). Consequently, soils are the most commonly used predictors of cacao contamination (Chavez et al. 2015; Fauziah et al. 2001). However, the presence of toxic metals in cacao seeds may have several reasons: naturally high heavy metal content due to the soil's element composition (Gramlich et al. 2017; Zug et al. 2019) or the volcanic origin of soils (Fauziah et al. 2005), and natural acidity of soil (soil pH; Hanafi and Maria 1998); also anthropogenic reasons, such as mining activities (Purser and Purser 2008), pollution due to contaminated water (WHO 2010), or the use of contaminated fertilizers (Fauziah et al. 2001; Grant et al. 1996; He and Singh 1994a, b; He et al. 2005; Williams and David 1974; Zug et al. 2019), and pesticides (both counting to farm management factors in our study; Apikpokpodion et al. 2013) may have caused high toxic metal content in soil. This may explain the observed differences between the sampling regions of our study. Our study also confirmed the known impacts of soil pH on toxic metal uptake in plants. Plants mostly absorb higher metal contents with lower soil pH (Fauziah et al. 2001; He et al. 2005; He and Singh 1994a, b; Prasad 1995). Metals are less intensely bound to colloids at lower soil pH and therefore more soluble in the soil solution (Fauziah et al. 2001; Gramlich et al. 2017; He and Singh 1994b). Moreover, metal ions are normally better available in acidic soils typically found in tropical soils (Hanafi and Maria 1995). However, each element has its own optimal soil pH at which it is soluble and therefore available to plants (He et al. 2005). Also plants show species- and site-specific uptake mechanisms (El-Jaoual and Cox 1998; He and Singh 1994a, b; He et l. 2005), which is the reason why we also analyzed transfer factors in this study that are comparable independent of soil metal content.

This is clearly observable in Pb uptake, which is neglectable even at high soil concentrations, whereas Cd was accumulated at high amounts in cacao seed even at minimal Cd contents in soil.

Moreover, we found that the altitude a.s.l. influenced the metal contents in cacao seeds with some metals increasing with altitude (Co, Ni), and others decreasing (Al, Cd, Cu, Mn, and Zn). A possible reason may be the observed differences in the origin of samples with diverging natural occurrence of the respective metals (Chavez et al. 2015; Fauziah et al. 2001; Gramlich et al. 2017; Mounicou et al 2002a, b, 2003; Zug et al. 2019). In addition, also reduced plant growth at higher altitudes may have played a role.

Another novel environmental factor that influenced the transfer factors of metals in our study was soil electrical conductivity. The values ranged between 32 and 1085 S m^{-1} and lower conductivity increased metal uptake in cacao. In general, higher salinity with higher electric conductivity of soils can be crucial for plants and for nutrient absorption, growth, and yield under humid conditions (Flowers and Yeo 1989; Tank and Saraf 2010). However, nutritive salts and toxic metal ions are not differentiated in soil electrical conductivity (Rhoades et al. 1989). In the case of our study, low pH and a low concentration of ions in the soil solution are positively related to each other and toxic metals are presumably more soluble in soil solution at low electrical conductivity. In addition, the lack of nutrients in the soil solution indicated by low electric connectivity may have forced the cacao tree to take up other metal ions. This is supported by a lower transfer factor of Zn at lower electric conductivity in contrast to the other toxic metals. A lack of Zn may promote uptake of other divalent ions such as Cd. Fertilization with less toxic Zn or even non-toxic calcium or magnesium may therefore be a possible mitigation measure to counteract Cd uptake (Saison et al. 2004).

5.5.3 Influence of toxic metals on yield parameters

Fruit size proved to be positively influenced by higher Al and Cu contents in seeds. A pronounced growth and fruit set may have promoted uptake of these elements. The cacao tree seems to be relatively tolerant to Al toxicity as it is highly adapted to deeply weathered acidic soils with high Al solubility. The accumulation of metal ions such as Cd even at low soil concentrations in addition points to a highly efficient mineral uptake. In general, yield increases with the supply of elements (Mengel and Kirkby 1978; Kabata-Pendias 2010) and cacao seems to poorly differentiate between necessary nutrients and toxic metals. The marginally significant negative effect of Ni on yield may be related to limited plant growth caused by several heavy metals (see section 4.1 and Baligar and Fageria 2004; Foy 1988; Hanafi and Maria 1998; He and Singh 1994b; He et al. 2005; Moreira de Almeida et al. 2015; Quinteiro Ribeiro et al. 2013; WHO 2010b; Xu et al. 2005). Also Zug et al. (2019) stated reduced yield with higher Cd seed content. However, this study revealed rather positive effects of toxic metals in cacao seeds such as reduced fruit attacks by *Phytophthora* sp. at higher Mn content which may be explained by a growth limiting effect of Mn on fungi in general (Babich and Stotzky 1981; Thompson and Medve 1984). Concentrations between 100 and 1500 ppm inhibited growth of different fungi (Babich and Stotzky 1981), which is within the range of our study with a mean of 65 mg kg^{-1} and a maximum value of 232 mg Mn kg^{-1} in cocoa seeds.

5.6 Conclusion

Toxic metal pollution seems to be a serious problem in foodstuffs that tend to accumulate toxic metals such as the cacao, coco, or banana plant (Bansah & Addo 2016). For these foodstuffs, several legal limits for metal pollution have been set in the European Union. For cocoa products, limits have exclusively been formulated for the heavy metals Cd (0.6 mg kg^{-1} in cocoa powder), Cu (50 mg kg^{-1} in cocoa beans), and Pb (1.0 mg kg^{-1} in cocoa powder) (COPAL 2004; EFSA 2018; European commission 2014). However, several other toxic metals are accumulated at hazardous concentrations in cocoa products, as shown in this study for Al, Co, Mn, Ni, and Zn in three mayor cacao growing regions in Peru based on a total of 280 samples. The aim of the present study was to disentangle and rank the main factors concerning farm management and environmental conditions that drive the toxic metal uptake and accumulation in cacao seeds to find possibilities for efficient mitigation.

The farm management clearly influenced uptake and content of toxic metals in cacao seed samples. Our study found that the sampled cacao type affected the content of the metals Al, Cd, Cu, Mn, Ni, Pb, and Zn, as well as their transfer-factors Al, Cd, Co, Cu, Mn, Ni, Pb, and Zn. In particular, fine and flavor cacao showed high transfer factors. CCN-51 exhibited the highest Pb and the second highest Cd transfer factor. Hence, novel native cacao types may increasingly be used to obtain lower contents of particularly harmful Cd and Pb, albeit contents in less harmful Al, Co, Cu, Mn Ni, and Zn may be higher in these varieties. As Al, Cd, Mn, and Zn contents in cocoa powder samples decreased in older cacao trees, the longer use of cacao field even with lower productivity may be a mitigation option. The finding on fruit-set, which reduced transfer factors of Cd, Co, Cu, Pb, and Zn, also implies that older trees may be an option to limit metal uptake. Finally, measures promoting excessive plant growth such as frequent pruning and the use of nitrogen-fertilizers (see Gramlich et al. 2017; Grant et al. 1996; Zug et al. 2019) proved to enhance toxic metal uptake and should therefore be avoided or reduced. In particular, fertilization may be ceased as several studies failed to find a significant positive effect of common N- and P-fertilizers on cacao yield (Kieck et al. 2016; Zug et al. 2019).

The environmental factor with the highest influence on the content of all analyzed toxic metals in cacao seed samples was the metal content in soil as confirmed by several former studies on Cd (Chavez et al. 2015; Fauziah et al. 2001; Gramlich et al. 2017). However, soil toxic metal contamination from varying sources is difficult to influence. It may be more applicable to influence soil conditions such as pH or electric conductivity (by e.g. liming, Fauziah et al. 2001; He and Singh 1994a, b), which both proved to be relevant for toxic metal uptake. Furthermore, the amendment of less toxic (Zn) or non-toxic divalid cations (Ca or Mg) may be a valuable mitigation option that may be tested in future studies. Another important factor influencing metal uptake was altitude a.s.l., which implies regional differences in soil metal content. Decreasing uptake with higher altitude may in addition be related to lower growth rate of the cacao tree. Shifting cacao cultivation towards higher altitudes may therefore be a mitigation option when agricultural area is available.

Overall, our study provides various promising possibilities to mitigate toxic metal accumulation in cacao seeds originating from Peruvian Amazonia. Mitigation measures include liming and Mg amendment to increase soil pH, electric conductivity, and sufficient supply with nutrients, the reduction or cessation of fertilizers and pruning, the prolonged use of existing fields (thereby also reducing utilization of further area for agriculture), and use of cacao types with less pronounced toxic metal uptake. Our results clearly highlight the accumulation capacity of toxic metals in the cacao plant, which may result in contaminated cacao products and potential intoxication of humans and, even more severe, children. Further studies that test the proposed mitigation measures are therefore urgently needed in different cacao producing countries.

5.7 Acknowledgement

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6 Synthesis

In this chapter, the main findings of this dissertation project will be presented according to the analyzed hypotheses and discussed with regard to the current state of research. In addition, the implications of this thesis for the future cocoa cultivation are highlighted. In particular, I address the relevance of the findings for cacao farmers in Peru and I will propose possible mitigation measures to prevent toxic metal contamination of cocoa products.

6.1 Key findings

The following key findings are ordered according to the different chapters and the underlying hypotheses:

Chapter 2: Yield and quality characteristics of native cacaos from three different regions of Peru – genetic differences and environmental drivers.

- Native cacaos from Cusco (in catechin hydrate, and anthocyanins) and native cacaos from San Martin (in epicatechin) showed the highest polyphenol contents, whereas the native cacaos from Huánuco had overall the lowest.
- The caffeine content of the native cacaos from Cusco were the highest, whereas those from native varieties from Huánuco were the lowest. Theobromine was significantly higher in CCN-51 than in all other cacao types and lowest in fine and flavor cacao clones.
- In accordance to the hypotheses, CCN-51 had the longest fruits and biggest fruit-perimeters. However, native cacaos from San Martin and Huánuco had higher annual yields than CCN-51.
- CCN-51 and the fine and flavor cacao clones were less infested with *Moniliophthora perniciosa* in comparison with the native cacaos, but native cacaos had significantly less attacks by insects.
- Polyphenol contents in seeds increased significantly with the age of cacao trees.
- The polyphenol catechin hydrate significantly decreased with soil pH, whereas theobromine increased with the soil's electrical conductivity.

- Attacks by insects significantly correlated with the theobromine contents in seeds, the infestation of the tree with *Moniliophthora perniciosa* correlated significantly positively with the cyanidin arabinoside contents, and *Phytophthora* spp. with the epicatechin contents.

Chapter 3: Cadmium accumulation in Peruvian cacao (*Theobroma cacao* L.) and opportunities for mitigation.

- With an average of 2.46 mg of Cd per kg cocoa powder, 85% of the cocoa powder samples exceeded the allowed limit of 0.6 mg kg^{-1} of the European Union.
- The maximum measured value of 12.56 mg kg^{-1} was the highest reported so far in literature.
- Cd contents were not differing significantly between CCN-51 and the fine and flavor cacao clones.
- Soil Cd content was the most relevant diver of Cd concentration in cocoa.
- The use of N- and P-fertilizer significantly increased Cd in cocoa powder samples.
- Cd contents in cocoa samples correlated positively with the biodiversity of the herb layer.
- The polyphenol contents were negatively correlated with the Cd contents in cocoa samples.
- Fruit set of cacao trees correlated significantly negative with the Cd content in cacao seeds.

Chapter 4: Toxic metal contents of cocoa seed samples (*Theobroma cacao* L.) from Peruvian Amazonia are related to soil, region, and cacao type.

- For Cd, Co, Cu, Ni, and Zn in cocoa seeds, the soil metal content was the most influencing factor.
- Highest Co (1.13 mg kg^{-1}), Cu (49.65 mg kg^{-1}), Mn (78.54 mg kg^{-1}), and Ni (27.17 mg kg^{-1}) contents in cocoa powder samples were measured in Cusco, whereas Cd (2.13 mg kg^{-1}), Pb (0.09 mg kg^{-1}), and Zn (84.76 mg kg^{-1}) contents had their maximum values in Huánuco, and Al (16.56 mg kg^{-1}) in San Martin.
- Only for Al contents in cocoa seeds, the region was the most important driver.
- 49.3% of the cocoa samples exceeded the allowed Cd limit of 0.6 mg kg^{-1}, 38.6% the Cu limit of 50 mg kg^{-1}, and 0.71% the Pb limit of 1.0 mg kg^{-1}.
- In contrast to the hypothesis, the commercial cacao clones showed only the highest values in Al (CCN-51 with 12.5 mg kg^{-1}), and in Cd contents (fine and flavor cacao clones with 2.1 mg kg^{-1}).

- The fine and flavor clones had higher transfer-factors than the other cacao types (Cd: 13.17; Co: 0.21; Cu: 3.52; Mn: 0.20; Ni: 1.35; Pb: 0.0073; Zn: 2.18), whereby CCN-51 had an even higher transfer-factor of Cd with 13.26.

Chapter 5: Farm management and environmental factors drive toxic metal accumulation in Peruvian cacao (*Theobroma cacao* L.).

- Frequent pruning correlated positively with the transfer factors of Al, Cu, and Zn.
- N-fertilization increased the transfer factor of Cd.
- Co, Cu, and Mn contents in cocoa powder were higher in older trees, whereas Al, Cd, and Zn decreased with the age.
- Al, Cd, Cu, Mn, and Zn values in cocoa seeds decreased with the altitude a.s.l. but Co, and Ni contents raised.
- Overall, the transfer-factors increased with lower soil electrical conductivity.
- Lower soil pH increased transfer of Al, Cd, Cu, Co, Mn, Ni, and Zn from soil to cacao seeds.
- Yield parameters were not affected by toxic metal content in cacao fruits. On the contrary, cacao fruits were significantly bigger with increasing Al and Cu content in cacao seeds. However, the annual yield was marginally reduced by Ni.

6.2 Quality and yield characteristics of native cacao types

The origin of the cacao tree was first considered to be in Central America and now assumed to be in South America with its focus on Peru based on genetic analyses done in the last years with genetic material of cacao all over the Amazonian regions. Peru is known to be a geographic focus of cacao genetic diversity (Boza et al. 2014; Motamayor et al. 2008; Zhang et al. 2011; Zhang et al. 2006). First genetic collections of cacaos for a germplasm in Iquitos, Peru started in 1937. Cacao trees were collected with the aim to find cacaos that are more resistant against pests such as the fungus *Moniliophthora perniciosa:* one of the most severe diseases of cacao. In the following breeding programs, the clones, now known as "International clones," were created (Bartra 1993; Gonzáles 1996; Pound 1938, 1943; Zhang et al. 2006). In accordance, the major aim of more recent cacao germplasms (e.g. in my study region Tingo Maria, San Martin) was also to breed more resistant and productive cacao trees (Evans et al. 1998; Zhang et al. 2006). However, despite these breeding programs with cacao trees native to the different regions of Peru, the clone CCN-51 from Ecuador has become the most frequently cultivated variety in all Peruvian regions (Herrmann et al. 2015; Zhang et al. 2006). This is presumably related to the social unrests during the end of 1980 and the resulting loss of information about the bred cacao clones (Zhang et al. 2006). During this time, the United Nations Office for Drug and Crime (UNODC) started to spread the Ecuadorian clone CCN-51 (Colección Castro Naranjal 51), which is characterized by high yields, easy cultivation, and higher resistance against diseases, as an alternative to the illicit coca cultivation (García Carrión 2012; Kieck et al. 2016). In addition, these events also led to the loss of knowledge on the diversity of the native cacaos from the different Peruvian regions. Consequently, the potential of native cacaos has never been fully exploited. In the last years, especially genetic methods analyzing genetic material from different cacao germplasms were used to find mislabeling, to assess cacao diversity, and to study the origin and dispersal of cacao (Boza et al. 2014; Cervantes-Martinez et al. 2006; Herrmann et al. 2015; Motamayor et al. 2002, 2003, 2008; Zhang et al. 2006, 2011). Latest studies found simple sequence repeats (SSR) located in the nuclear genome, so called microsatellite markers, suitable for genetic analyses of cacao. Motamayor et al. (2008) used 106 microsatellites to analyze 1241 cacaos from different cacao germplasms in South America. Based on their statistical analyses, they proposed a completely new classification of cacaos in 10 genetic clusters in contrast to the former separation in three groups (Forastero, Criollo, Trinitario). The different clusters and their origin can be seen in **Figure 22** from the study of Motamayor et al. (2008).

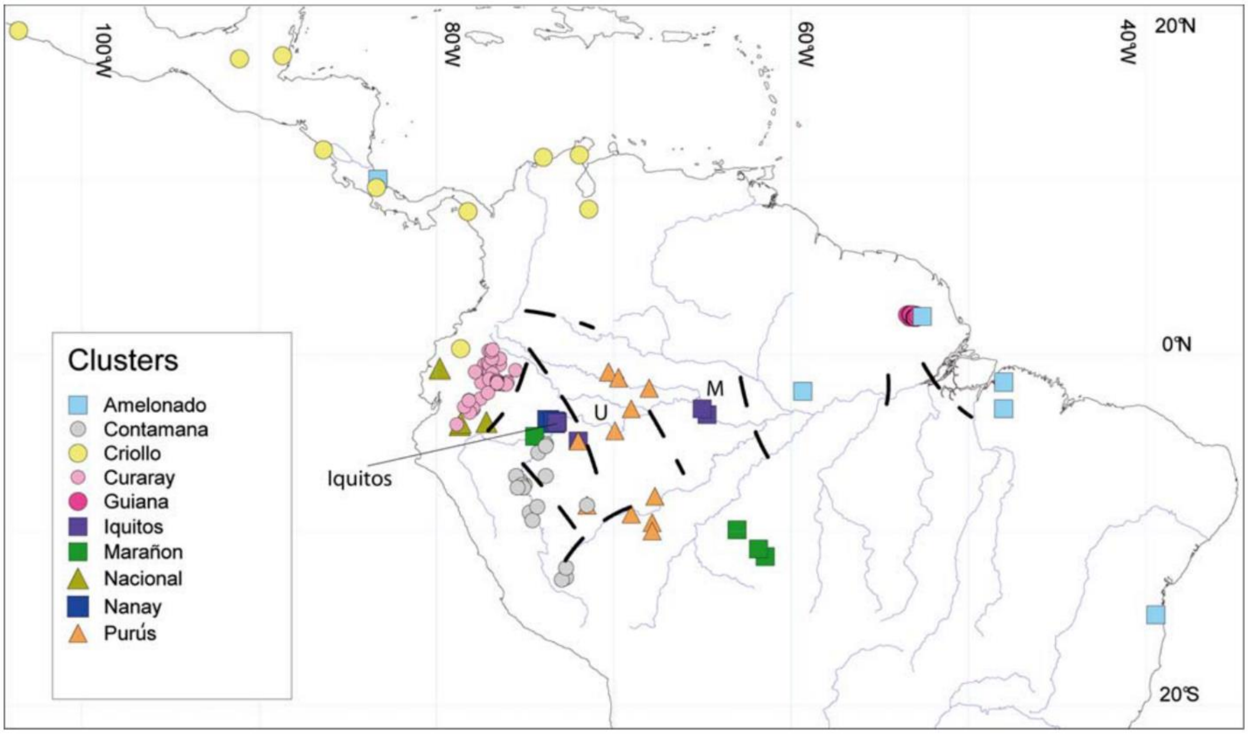

Figure 22: Localization of the origin of individuals analyzed; colors indicate the inferred genetic cluster to which they belong (Figure from Motamayor et al. 2008).

A comparison of cacaos genetically from germplasms from the Ucayali and the Huallaga valley in Peru revealed that these populations can be genetically separated, with a clearly higher genetic diversity found in the Ucayali valley. The analyses showed the cacaos origin in Upper Amazon Forasteros (Zhang et al. 2006, 2011). In general, the cacao diversity was extremely high in these Peruvian regions (Zhang et al. 2006). Until now, these highly interesting cacaos from the Peruvian regions were exclusively examined genetically to asses diversity, population dynamics, and conservation. In contrast, these cacao types have never been studied regarding their flavor quality and yield potential. Moreover, most studies rely on cacao samples from germplasms, whereas field sampling campaigns have so far not been reported. The present dissertation is completing this knowledge gap in literature by providing comprehensive analyses on the profile of secondary compounds (polyphenols, methylxanthines) and tree characteristics (disease susceptibility, fruit-sizes, fruit-set, annual yields) from native cacao trees in three different Peruvian regions. The comparison of these native cacaos from the different regions and a comparison with known cacao clones gave evidence about their potential as fine and flavor cacaos and its possibilities for more intense cultivation. The native cacaos differed significantly from each other and showed some fine and flavor characteristics such as a high caffeine contents (Cusco), or lower polyphenol contents (Huánuco). Native cacaos from San Martin had significantly higher yields and the native cacaos in general were less attacked by insects in comparison with the cultivated clones. The findings are clearly separating the native cacaos from the cacao clones and show their benefits in secondary metabolites, disease susceptibility, and yield parameters. Due to the fact that native cacaos are definitely differing in secondary compounds and therefore in their aroma profile in comparison to the known cacao clones, it holds the potential of cultivating these cacaos on a bigger scale and selling them for a better price on the local markets. Native cacaos from Cusco are already in the focus of the sweets industry (Perú Puro, Cacaosuyo) because of its special cacao aromas and therefore cultivated on a bigger scale (farmers interviews). This could serve as a model for the other regions San Martin and Huánuco and their native cacaos.

6.3 Drivers of cacao contamination with toxic metals and possible mitigation measures

The EFSA, WHO and FAO recommended consumption limits for several toxic metals such as Al, Cd, Cu, Mn, Ni, Pb, and Zn (presented in chapter 4; EFSA 2012, 2018; European Commission 2011, 2012, 2014, 2015, 2018; WHO 1982, 2010a, b, 2011). For some of these metals with particularly high toxicity for the human body (Cd, Cu, and Pb) limits have been set in Europe especially for cocoa products (Assa et al. 2018; EFSA 2012, 2018; WHO 2010a, b) also because cacao accumulates these toxic metals (Chavez et al. 2015; Engbersen et al. 2019). Cd is the most studied toxic metal in the last decade in cocoa because the maximum allowed values for Cd in cocoa powder were reduced from 1.5 mg kg^{-1} to 0.6 mg kg^{-1}. This new limit must be complied with since January 2019. Several studies reported Cd values above this limit from Peru such as Arévalo-Gardini et al. (2017) with 40% of the cacao samples exceeding the legal limit and from Trinidad and Tobago with up to 4.5 mg kg^{-1} in cocoa beans (Ramtahal et al. 2016). To counteract these alarming findings, mitigation strategies have been developed in different countries of the world with focus on highly polluted regions as found in the northern regions of Peru (Arévalo-Gardini et al. 2017; Zug et al. 2019). Some strategies proved to be effective such as liming (Ramtahal et al. 2018), use of genotypes accumulating less Cd (Arévalo-Gardini et al. 2017; Lewis et al.

2018), use of vermicompost to reduce available Cd in soils (Chavez et al. 2016). Others explored the effect of mycorrhizal fungi and showed higher Cd levels in mycorrhized cacaos (Ramtahal et al. 2012). Similarly, a higher density of the soil macrofauna increased available Cd and Pb in cacao trees (Huauya and Huamaní 2014). Moreover, other plant species such as *Carludovica palmata* may be used for phytoremediation of soil on contaminated farms (Llatance et al. 2018).

Literature about Pb is also available and shows that Pb occurs more frequently in African countries due to the use of leaded gasoline or pesticides containing Pb (Aikpokpodion et al. 2013; Mounicou et al. 2003; Rankin et al. 2005). However, mitigation strategies for Pb have so far not been proposed, which highlights the importance of studies such as this dissertation on toxic metal accumulation in cacao. In addition, there are further toxic metals in soils potentially incorporated into the cacao plant, which impose a risk for cocoa product consumers. Assa et al. (2018) measured As, Cd, Hg, and Pb contents in cocoa beans from Indonesia, but values were below detection limits. Only the contents of Cu were measurable and about 10.4 mg kg^{-1} to 19.3 mg kg^{-1} which is still lower than the average Cu content with 46.9 mg kg^{-1} in cocoa powder of this study. A study of Arévalo-Gardini et al. (2016) analyzed Cd, Cu, Fe, Mn, Ni, Pb, and Zn concentrations in soils of different Peruvian regions. The concentrations in soils were consistently below the critical phytotoxic limits. The values of Fe (4.3 mg kg^{-1}), Mn (1275 mg kg^{-1}), Ni (24.7 mg kg^{-1}), Pb (21.8 mg kg^{-1}), and Zn (96.8 mg kg^{-1}) were higher in the southern Cusco region, as I confirmed by this dissertation for soil Mn (1713 mg kg^{-1}), Ni (23.7 mg kg^{-1}), and Zn (85.1 mg kg^{-1}). In contrast, the measured Pb value (27.8 mg kg^{-1}) in soils was highest in the Huánuco region, whereas the San Martin region was characterized by higher levels of Cd (0.2 mg kg^{-1}) and Cu (15.4 mg kg^{-1}) than in Huánuco, which was vice versa in this dissertation with higher Cd (0.3 mg kg^{-1}), and Cu (25.7 mg kg^{-1}) values in the southern Cusco region. Still, the range of values from this dissertation is comparable to the one reported by Arévalo-Gardini et al. (2016). In general, literature about toxic metal contents in soils and cacao of Peruvian regions is scarce (e.g. Cd, Cu, Mn, Ni, Pb, Zn) or completely missing (e.g. Al, Co), which demonstrates the need for a better knowledge about polluted regions worldwide and a better understanding of toxic metal accumulation in crops such as cacao and possibilities for mitigation. Studies such as this one offer first insights about the pollution grade of cacao and its soils and can therefore give an overview about toxic metal pollution in different Peruvian regions. This study also offers new findings about mitigation strategies. It is the first to show that native cacaos from three different Peruvian regions generally transferred less toxic metals from soil to cacao seed (in the case of Cd, Co, Cu, Mn, Ni, Pb, and Zn) in comparison with known cacao clones (fine and flavor cacao clones, CCN-51). Therefore, presenting these cacaos as high-potential cacaos to prevent high levels of cacao contamination. Mitigation strategies were also found in the farms' management with less pruning and less fertilization promising lower toxic metal rates in cacao seeds in general. Especially N- and P-fertilizers seem to drastically increase cacao seeds Cd contents in particular which leads to the recommendation of less fertilization with these fertilizers. The modification of environmental conditions such as soil pH and electrical conductivity are further promising measures. Liming or Ca- and Mg amendment can increase the soils' pH and electrical conductivity which were shown to decrease the transfer of toxic metals from soil to cacao seeds.

6.4 Cacao characteristics needed for future cacao cultivation in the aspect of changing climate conditions

This dissertation thesis provides new findings on the pollution of cacao with toxic metals in Peruvian regions with naturally high metal concentrations in soils. These findings imply an urgent need for mitigation strategies to save the future of cacao cultivation in Peru. Another aspect is, that the pressure to cultivate cacao on a bigger scale in South American countries may be expected to raise drastically in the next few decades because cultivation in West African countries, which currently produce about 80% of the entire global cacao amount will be increasingly challenging due to climatic and political reasons. Climate change is already limiting yields in Africa due to raising temperatures and missing precipitation. Cacao trees suffer from longer droughts and higher infestation with diseases because of more intense solar radiation (Georgen 2020). Droughts, together with the loss of pollinators, and resource limitation (Groeneveld et al. 2010) are the main reason for yield losses and can lead to losses up to 50% such as pointed out by Schwendenmann et al. (2010) and Moser et al. (2010). Physiological stress from solar radiation and dry periods lead to a higher disease susceptibility, which altogether reduces yields. These factors can even lead to the breakdown of entire cacao farms and many fields must be burned due to fungus outbreaks (Tscharntke et al. 2011). Due to these problems, Läderach et al. (2013) forecasted that by 2050 up to 90% of the nowadays cultivated areas in West Africa will be unsuitable for cacao cultivation. This means that about 600,000 cacao farmers only in Ivory Coast will lose their income source (Georgen 2020). Therefore, a shift in cultivation areas may be expected and cacao farmers in South America may profit from increasing cacao prices and higher demand on the world market. Moreover, the raising problem of severe cacao pests requires further investigation in cacao varieties with higher resistance, which may also be introduced to West African countries. Another, in many countries well-established, possibility to deal with droughts and higher pest incidence are agroforestry systems with higher plant and cacao diversity. Higher diversity is known to increase resilience of ecosystems and to prevent mass outbreak of pests (Clough et al. 2006; Tscharntke et al. 2011; Wanger et al. 2010; Wielgoss et al. 2014). Furthermore, shadow trees keep higher humidity on fields, which may buffer drought effects (Clough et al 2009a). Climate change may already limit yields in African countries but will also reach the Amazonian lowlands in future which shows the need for a more intense investigation of cacao types with the aim of finding those more resistant against diseases and dry periods. On the one hand, this dissertation supports the view as the native cacaos with high genetic diversity suffer less from insect attacks. On the other hand, the analyzed cacao clones (CCN-51, fine and flavor cacao clones) suffer less from fungi infestations (especially from *Moniliophthora perniciosa*) which is a promising factor not to lose yields on a bigger scale with more intense droughts. Results in this study are confirming the researched facts about the high-producing clone CCN-51 resisting fungi and achieving high yields at the same time. Another result presented in chapter 3 shows a yield decrease with less water contents measured in soil samples around cacao trees, whereby CCN-51 and fine and flavor cacao clones were investigated. This result argues against the studied clones considering future problems to be overcome. Regarding to the pollution problem with toxic metals in some Peruvian regions, the search for more resistant cacaos taking up less toxic metals, such as the native cacaos, and breeding programs should be intensified to deal with future challenges.

6.5 Importance of the scientific findings for cacao farmers

The present dissertation provides several novel findings that are clearly relevant to practitioners and in particular farmers and may be used to optimize cacao cultivation. The native cacaos showed some characteristics that may be used in systematic breeding programs. The cacaos native to the San Martin Region showed the highest yields, the second biggest fruits, and the lowest rate of attack by insects. These native cacaos may be used by farmers who aim at high yields and high genetic diversity to decrease the incidence of cacao diseases. The native cacaos from Huánuco, in contrast, were characterized by lower transfer factors for the toxic metals Al, Cd, Co, Cu, Mn, Ni, and Zn (except Pb), high yield, and had the lowest polyphenol contents indicating less bitterness and astringency. These cacaos may therefore yield higher prices on the local and also international market. The native cacaos from Cusco did not show very high yields per ha but were characterized by the highest number of fruits per tree and the highest caffeine contents in cocoa powder as a characteristic of fine and flavor cacaos. Native cacaos from Cusco are the only studied type that is already cultivated on a big scale and sold as fine and flavor cacao to the industry (e.g. Perú Puro, Cacaosuyo) with a price clearly higher than the world market price for bulk cacao. In addition, the Cusco trees exhibited in general low transfer factors for the toxic metals Al, Cd, Co, Cu, Mn, Pb, and Zn (except for Ni). This may be a promising example for farmers in other regions of Peru. Concerning the clone CCN-51, this thesis confirmed that it has relatively big fruit-sizes and provides high yields. Moreover, the CCN-51 together with the fine and flavor cacao clones were less infested by fungi in comparison to the native cacao, as aimed by the breeding programs since 1937 (Zhang et al. 2006). The present study also revealed pronounced differences in environmental conditions (soil pH, soils electric conductivity, soils toxic metal contents) among the three study regions. The toxic metal contents in soils of Co (22.7 mg kg^{-1}), Cu (25.7 mg kg^{-1}), Mn (1,713 mg kg^{-1}), Ni (23.7 mg kg^{-1}), and Zn (85.1 mg kg^{-1}) were highest in the Cusco region. Al (23,491 mg kg^{-1}) contents were the highest in San Martin, and Cd (0.3 mg kg^{-1}) and Pb (27.8 mg kg^{-1}) highest in Huánuco. As toxic metal contents in soils are directly positively correlated with the contents in cacao seeds, it is necessary to increase the knowledge about natural occurrence of toxic metals in soils. This dissertation is the first that presents soil data for Al, Cd, Co, Cu, Mn, Ni, Pb, and Zn in three different Peruvian regions. The soil pH was lower in Huánuco and San Martin in comparison with Cusco, which increased the transfer factors for toxic metals in these two regions. A possible mitigation measure to counteract uptake of toxic metals is increasing soil pH and electrical conductivity by liming and Mg amendment. Furthermore, farm management proved to influence Cd contents in seeds with higher Cd contents after N or P fertilizer application. Less intense fertilization may therefore reduce transfer of Cd into the plant. Also, the annual pruning frequency was positively correlated with the transfer factors of some toxic metals. The reduction of pruning frequency 1 or 2 campaigns per year may result in lower toxic metal contents in cacao seeds. These suggestions for possible mitigation measures, particularly in polluted regions, seem important to farmers in Peruvian Amazonia for improving cacao production in terms of low toxic metal contents and high prices on the cacao market. To disseminate this information to cacao farmers, workshops will be carried out at different farmer cooperatives in the studies regions of Peru in 2022.

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Supplementary material

Table A.1: Average, minimum, and maximum distance (in m) between farms.

Average (m)	Maximum (m)	Minimum (m)
14180	48846	296

Table A.2: List of plant species found on the study plots.

Family	Genus	Species	Author
Asteraceae	*Ageratum*	*Ageratum conyzoides*	L.
Amaranthaceae	*Amaranthus*	*Amaranthus hybridus*	L.
Amaranthaceae	*Amaranthus*	*Amaranthus spinosus*	L.
Annonaceae	*Annona*	*Annona muricata*	L.
Asclepiadaceae	*Asclepias*	*Asclepias curassiva*	L.
Poaceae	*Axonopus*	*Axonopus compressus*	Beauv.
Asteraceae	*Bidens*	*Bidens pilosa*	L.
Bixaceae	*Bixa*	*Bixa urucurana*	Willd.
Brassicaceae	*Brassica*	*Brassica campestris*	L.
Caricaceae	*Carica*	*Carica papaya*	L.
Urticaceae	*Cecropia*	*Cecropia engleriana*	Loefl.
Meliaceae	*Cedrela*	*Cedrela odorata*	L.
Fabaceae	*Cedrelinga*	*Cedrelinga cateniformis*	Ducke
Poaceae	*Chloris*	*Chloris ciliata*	Sw.
Rutaceae	*Citrus*	*Citrus medica*	L.
Rutaceae	*Citrus*	*Citrus reticulata*	Blanco.
Rutaceae	*Citrus*	*Citrus sinensis*	L.
Arecaceae	*Cocos*	*Cocos nucifera*	L.
Araceae	*Colocasia*	*Colocasia esculenta*	L.
Commelinaceae	*Commelina*	*Commelina diffusa*	Burm.
Poaceae	*Cortaderia*	*Cortaderia selloana*	Asch. Graebn.
Euphorbiaceae	*Croton*	*Croton lechleri*	Müll. Arg.
Cupressaceae	*Cupressus*	*Cupressus lusitanica*	Mill.
Cyperaceae	*Cyperus*	*Cyperus esculentus*	L.
Cyperaceae	*Cyperus*	*Cyperus ferax*	L.
Cyperaceae	*Cyperus*	*Cyperus rotundus*	L.
Cyperaceae	*Cyperus*	*Cyperus rotundus*	L.
Poaceae	*Digitaria*	*Digitaria sanguinalis*	Scop.
Fabaceae	*Diplotropis*	*Diplotropis martiusii*	Benth.

Poaceae	*Echinochloa*	*Echinochloa colona*	L.
Poaceae	*Eleusine*	*Eleusine indica*	Gaertn.
Erythroxylaceae	*Erythroxylum*	*Erythroxylum coca*	Lam.
Euphorbiaceae	*Euphorbia*	*Euphorbia heterophylla*	L.
Euphorbiaceae	*Euphorbia*	*Euphorbia hirta*	L.
Euphorbiaceae	*Euphorbia*	*Euphorbia hyssopifolia*	L.
Arecaceae	*Euterpe*	*Euterpe oleracea*	Mart.
Moraceae	*Ficus*	*Ficus urbaniana*	Warb.
Cyperaceae	*Fimbristylis*	*Fimbristylis miliaceae*	Vahl
Malvaceae	*Guazuma*	*Guazuma crinita*	Lam.
Fabaceae	*Inga*	*Inga edulis*	Mart.
Convolvulaceae	*Ipomoea*	*Ipomoea nil*	L.
Anacardiaceae	*Mangifera*	*Mangifera indica*	L.
Euphorbiaceae	*Manihot*	*Manihot esculenta*	Crantz
Arecaceae	*Mauritia*	*Mauritia flexuosa*	L.
Sapindaceae	*Nephelium*	*Nephelium lappaceum*	L.
Poaceae	*Paspalum*	*Paspalum conjugatum*	Bergius
Lauraceae	*Persea*	*Persea americana*	Mill.
Phytolaccaceae	*Petiveria*	*Petiveria alliaceae*	L.
Phyllanthaceae	*Phyllanthus*	*Phyllanthus niruri*	L.
Solanaceae	*Physalis*	*Physalis angulata*	L.
Myrtaceae	*Pisidum*	*Pisidum guajava*	L.
Pteridaceae	*Pityrogramma*	*Pityrogramma calomelanos*	L.
Portulacaceae	*Portulaca*	*Portulaca oleracea*	L.
Urticaceae	*Pourouma*	*Pourouma cacropiifolia*	L.
Sapotaceae	*Pouteria*	*Pouteria sapota*	More Stearn
Dennstaedtiaceae	*Pteridium*	*Pteridium aquilinum*	L.
Fabaceae	*Pueraria*	*Pueraria phaseoloides*	Benth.
Euphorbiaceae	*Ricinus*	*Ricinus communis*	L.
Poaceae	*Rottboellia*	*Rottboellia exaltata*	L.
Polygonaceae	*Rumex*	*Rumex crispus*	L.
Fabaceae	*Schizolobium*	*Schizolobium amazonicum*	Huber
Poaceae	*Setaria*	*Setaria geniculata*	Kerguelen
Malvaceae	*Sida*	*Sida acuta*	Burm.
Solanaceae	*Solanum*	*Solanum sessiliflorum*	Dunal
Asteraceae	*Sonchus*	*Sonchus oleraceus*	L.
Anacardiaceae	*Spondias*	*Spondias mombin*	L.
Malvaceae	*Theobroma*	*Theobroma cacao*	L.
Urticaceae	*Urera*	*Urera baccifera*	Gaudich.
Euphorbiaceae	*Acalypha*		
Amaranthaceae	*Amaranthus*		

Marantaceae	*Calathea*
Rubiaceae	*Capirona*
Urticaceae	*Cecropia*
Rubiaceae	*Coffea*
Commelinaceae	*Commelina*
Cucurbitaceae	*Cucumis*
Cyperaceae	*Cyperus*
Fabaceae	*Desmodium*
Fabaceae	*Erythrina*
Euphorbiaceae	*Euphorbia*
Polypodiaceae	*Microgramma*
Musaceae	*Musa spec.*
Lauraceae	*Ocotea 1*
Lauraceae	*Ocotea 2*
Rubiaceae	*Palicourea*
Poaceae	*Paspalum*
Passifloraceae	*Passiflora*
Piperaceae	*Peperomia*
Arecaceae	*Phytelephas*
Pinaceae	*Pinus*
Piperaceae	*Piper*
Polypodiaceae	*Polypodium*
Solanaceae	*Solanum*
Asteraceae	*Taraxacum*
Urticaceae	*Urtica*
Asteraceae	*Vernonia 1*
Asteraceae	*Vernonia 2*
Asteraceae	*Vernonia 3*
Asteraceae	*Vernonia 4*
Lamiaceae	*Vitex*
Asteraceae	
Bambusoideae	
Cyperaceae	
Fabaceae 1	
Fabaceae 2	
Fabaceae 3	
Fabaceae 4	
Fabaceae 5	
Fabaceae 6	
Orchideacea	
Poaceae 1	

Poaceae 2
Poaceae 3
Poaceae 4
Poaceae 5
Unknown 1
Unknown 2
Unknown 3
Unknown 4
Unknown 5
Unknown 6
Unknown 7
Unknown 8

Author contributions

Chapter 1 Katharina L.M. Zug wrote this chapter. Figures 1 to 9 are photos done by Katharina L.M. Zug during the field studies in Peru. Table 1 presents findings from the publication of Afoakwa et at. (2008).

Chapter 2 Katharina L.M. Zug designed and set up the field studies, conducted the fieldwork, analyzed most of the data in the laboratory as well as she did all statistical analyses, and wrote the initial manuscript. Yva H. Heroin and Bela C. Bruhn conducted a part of the secondary compound analyses from the field study in 2018.

Chapter 3 Katharina L.M. Zug designed and set up the field studies, conducted the fieldwork, analyzed all data in the laboratory as well as she did all statistical analyses, and wrote the initial manuscript.

Chapter 4 Katharina L.M. Zug designed and set up the field studies, conducted the fieldwork, analyzed most of the data in the laboratory as well as she did all statistical analyses, and wrote the initial manuscript. The team of Nadine Herwig from the Julius Kühn-Institut, Berlin conducted the metal analyses of cacao samples.

Chapter 5 Katharina L.M. Zug designed and set up the field studies, conducted the fieldwork, analyzed most of the data in the laboratory as well as she did all statistical analyses, and wrote the initial manuscript. The team of Nadine Herwig from the Julius Kühn-Institut, Berlin conducted the metal analyses of cacao samples.

Chapter 6 Katharina L.M. Zug wrote this chapter. Figure 22 presents findings of Motamayor et al. (2008). All findings in 6.1 are based on the research and data analysis by Katharina L.M. Zug.

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