LICENTIATE THESIS

Fractionation of Cu and Fe Isotopes in Metal-Rich Mine sites: Biotic and Abiotic Processes

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Cover photo: View of the Laver tailings.

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Abstract

After mineral exploitation the residual grinded and milled material, rich in sulphide minerals and heavy metals, is often left exposed to the atmospheric variables. This weathered mine waste material can lead to the formation of acid mine drainage (AMD) which has negative effects to the environment. The fractionation of stable isotope of metals such as Cu and Fe can be measured using innovative analytical techniques developed recently and could offer a detailed hindsight of the geochemical processes occurring in mine contaminated sites.

Tailings profiles from Northern Sweden with high content of Cu and Fe sulphides and in different stages of weathering and/or remediation, along with plant and soil samples from a phytoremediation test site in Ronneburg, Germany were analysed using MC-ICP-MS to measure the isotope ratios of $^{65}Cu/^{63}Cu$ and $^{56}Fe/^{54}Fe$. The analytical method used requires anion exchange chromatography to extract Cu and Fe from a complex matrix prior to the proper isotope ratio measurement.

The samples from the tailings profile were useful to interpret the geochemical processes that can lead to a fractionation of Cu and Fe in the field, since redox-driven reactions such as rock oxidation and mineral precipitation are present in such environment. This study shows that precipitation of covellite in a redox-boundary zone in a mine tailings can cause a clear fractionation of Cu ($\Delta^{65}Cu_{rock\text{-}covellite}$= -5.66±0.05‰) and a depletion of the lighter Cu isotope in the oxidised areas of the tailings due to dissolution of the remaining Cu-sulphides. Precipitation of Fe(oxy)hydroxides as a result of the oxidation process of sulphide-bearing rocks can also fractionate Fe, being the precipitated mineral slightly enriched in ^{56}Fe.

The influence of soil bacteria and plant uptake in the fractionation of Cu and Fe was investigated in pot and field experiments at the Ronneburg site, where organic amendments were used. The results showed that the plant material was enriched in the lighter Fe isotope compared to the substrate used in the pot and field experiments, in spite of the application of a bacterial consortium. Cu isotope fractionation is more susceptible to the changes in the amendments used, being those bacterial consortium, mychorriza or compost than Fe isotope fractionation. There are differences in the fractionation values in pot and field trials, regardless of the type of organic amendment applied. As an overall view, leaves are enriched in the heavier Cu isotope compared to the soils, regardless of the amendment used

The application of the results obtained in this work would help not only to offer a view in the cycle of Fe and Cu in the surface environment, and the understanding of the (bio)geochemical processes occurring in sulphide soil surfaces. But also in the way that current remediation techniques of metal contaminated sites could be evaluated, having in mind that simplified systems show a different Cu and Fe fractionation compared to natural systems where more variables are needed to take into account.

Keywords: Cu and Fe isotopes, fractionation, tailings, plants, redox processes, covellite, Cu and Fe cycle, soil bacteria, organic amendments, phytoremediation.

List of papers

This thesis is based on the following 2 papers, henceforth referred to by their roman numerals.

I. Nathalie Pérez Rodríguez, Emma Engström, Ilia Rodushkin, Peter Nason, Lena Alakangas, Björn Öhlander. 2012. Copper and iron isotope fractionation in mine tailings at the Laver and Kristineberg mines, northern Sweden. *Manuscript submitted to Applied Geochemistry*.

II. Nathalie Pérez Rodríguez, Francesca Langella, Ilia Rodushkin, Emma Engström, Erika Kothe, Lena Alakangas, Björn Öhlander. 2012. The role of bacteria and organic amendments in the Cu and Fe isotope fractionation in plants studied in phytoremediation of mine contaminated sites. *Manuscript.*

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1. Introduction

The mining and use of metals by humans is dated to thousands of years ago and already during the Roman times, metal (especially Cu) pollution was present (Hong et al., 1996). At the present time when mining and smelting rates are higher, excessive accumulation of wastes in environmental reservoirs can occur. During mine operation, residual rock material (tailings) is often left untreated. In sulphide-bearing ore mines, the tailings composition often contains minerals such as pyrite (FeS_2) and pyrrhotite ($Fe_{1-x}S$). Oxidation of these sulphide minerals could lead to the release of important amounts of acid and heavy metals, so called Acid Mine Drainage (AMD), into stream waters.

Pyrite oxidises in the presence of oxygen (O_2) or ferric iron (Fe(III)), releasing Fe and sulphuric acid. Both pathways are summarised in the reactions 1 and 2 (INAP, 2009).

$$FeS_2 + 7/2O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \qquad \text{React. 1}$$

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \qquad \text{React. 2}$$

Fe^{2+} could further oxidise to Fe-(oxy)hyroxides or precipitate as Fe-oxy(hydr)oxy-sulphates, for example (Valente et al., 2011). Cu concentration in AMD from abandoned sulphide mines can reach the order of tens of mg/l (Sarmiento et al., 2011). Even though metals such as Fe and Cu, are important nutrients for organisms, their presence above certain threshold concentration can be potentially toxic for living organisms (An, 2006), causing bioaccumulation, metabolic malfunction and the reduction of the primary production.

To reduce or stop the formation of AMD in the tailings, the contact of dissolved oxygen to the sulphide-rich tailings should be avoided. Several techniques have been developed, including the addition of alkaline materials, flooding or the application of a dry cover. The establishment of a vegetation cover is also part of the process of mine closure strategy (INAP, 2009).

1.1. Scope of the thesis

The general objective of this thesis is to understand the geochemical processes governing the isotopic fractionation of Cu and Fe, in metal-rich mine sites. Samples from non-remediated tailings, recently phyto-remediated sites and tailings remediated over 50 years ago were analysed. Organic material present in those sites also was also analysed to create a better perception of the Cu and Fe transport in polluted environments.

2. Isotope Geochemistry of Copper and Iron

In nature, the atoms of elements are present with slight differences in its weight, due to the variation of the number of neutrons that a given element has in its nucleus. Those atoms which have a different number of neutrons but the same number of protons and

electrons are called isotopes. Isotopes are the product of processes such as radioactive decay, cosmic ray interactions and anthropogenic activities, among others (Faure and Mensing, 2005). This change in atomic mass allows the isotopes to behave differently during chemical reactions. Isotopes are divided in two classes: radioactive and stable isotopes.

Two stable Cu isotopes are present in natural conditions, ^{63}Cu (mass 63, abundance 69.15%) and ^{65}Cu (mass 65, abundance 30.85%). Iron has four stable isotopes ^{54}Fe (mass 54, abundance 5.84%), ^{56}Fe (mass 56, abundance 91.75%), ^{57}Fe (mass 57, abundance 2.12%)and ^{58}Fe (mass 58, abundance 0.28%) (De Laeter et al., 2003).

2.1.Standards and Notation

In isotope geochemistry reference standards are used to provide an indication of the isotopic composition of an element in an unknown material. Nearly every published study in Cu isotope report their results using the NIST 976 commercial reference standard. However, this material is unavailable at the moment and instead the ERM-AE633 standard reference was used for the Cu isotope concentration during this study. The certified ^{65}Cu/^{63}Cu value of the ERM-AE633 standard is 0.445±0.002, identical to the reported value for the NIST 976 standard (IRMM and ERM, 2002; NIST, 1994). The later means that the results obtained from either reference material are traceable between each other.

The Cu isotope composition of a given sample is reported as δ^{65}Cu in parts per mil (‰), as described in equation 1:

$$\delta^{65}\mathrm{Cu}(‰) = \left[\frac{\left(^{65}\mathrm{Cu}/^{63}\mathrm{Cu}\right)_{\mathrm{sample}}}{\left(^{65}\mathrm{Cu}/^{63}\mathrm{Cu}\right)_{\mathrm{ERM-AE633}}} - 1 \right] \times 1000 \qquad \text{(eq. 1)}$$

Data comparison among laboratories can be a hard work when new isotope systems progress in the scientific area. The Fe isotope system is not an exception and several standards (external and internal) have been developed to compare results. Still, the IRMM-014 reference material has been a common reference standard that allows interlaboratory comparisons, and it has been used for this study. Iron isotope composition is reported as δ^{56}Fe in parts per mil (‰), as described in equation 2:

$$\delta^{56}Fe(‰) = \left[\frac{\left(^{56}Fe/^{54}Fe\right)_{sample}}{\left(^{56}Fe/^{54}Fe\right)_{IRMM-014}} - 1 \right] \times 1000 \qquad \text{(eq. 2)}$$

Uncertainty for δ^{65}Cu and δ^{56}Fe measurements is commonly reported with either the 2σ for the long term uncertainty of the replicate measurement of the samples, or 2σ for the instrumental uncertainty only. In this work the 2σ for the instrumental uncertainty was used.

2.2.Development of Cu isotope work

During the late 1950's the relative isotopic abundance of Cu was measured in a number of minerals and plant materials, using a mass spectrometer of the Nier type but the reported variations, although small, were larger than the experimental error (0.1% $^{63}Cu/^{65}Cu$) (Walker et al., 1958). Another work which carried out measurement of Cu isotope variations in natural materials was performed by Shields et al. (1965), but the experimental error was still large. With the use of Thermal Ionisation Mass Spectrometer (TIMS) Scientists were able to measure Cu isotope variations, compared to a reference material (later known as NIST 976) but the analytical precision of this work was $\pm$1.5%, which is relatively poor. It was indeed necessary to have instruments with better analytical resolution to achieve reliable results.

With the arrival in the market of the first Multi Collector Inductively Coupled Plasma Mass Spectrometer (MC-ICP-MS), plasma 54 (VG Elements), key advances occurred in the field of metal isotope chemistry. The main advance was done by Maréchal et al. (1999) who created and developed a method for purifying Cu from environmental samples. This method consists in passing acid-digested samples in 7N HCl thorough an anion exchange resin (AG MP-1; Bio Rad), which is extremely basic. The recovery is achieved up to 100%, and this method is available for chromatographic elution of not only Cu, but also for Fe and Zn.

Several modifications of the Marechal et al (1999) technique of separation have been performed, especially in sea and river water (Archer and Vance, 2004; Bermin et al., 2006), but the basic principle is still present and it is by far the standard method used in the research of Cu isotope measurement.

2.3.Development of Fe isotope work

The first efforts to analyse Fe isotopes are traced back to the late 1940's (Valley and Andersson, 1947). The biggest challenges to achieve precise Fe isotope measurements were the low ionization efficiency of Fe and the necessity to correct for instrumental mass fractionation while using TIMS (Beard and Johnson, 1999).

Just as for Cu, Fe isotope studies had a higher development with the introduction of high resolution MC-ICP-MS instruments. Methods have been developed that avoid the use of a double spike for Fe and other systems, simplifying analytical procedures (Anbar, 2004). However, isobaric interferences from ArN^+, ArO^+ and $ArOH^+$ at masses 54, 56 and 57 can still affect the accuracy of the measurements and even residual matrix effects can alter the results obtained, with shifts between samples and standards of 0.1-0.5‰ for Fe (Arnold et al., 2003). Despite the possible complications in their measurement, Fe isotope works have become a routine while studying biological and non-biological processes in the terrestrial environment.

2.4.Copper isotope variation in environmental samples

The concentration of Cu isotopes in nature varies according to the type of material in which it is contained. Several studies of Cu isotope fractionation in different sample materials and in different environments have been published. Figure 1 shows a summary of those findings (modified from Bigalke, 2010b).

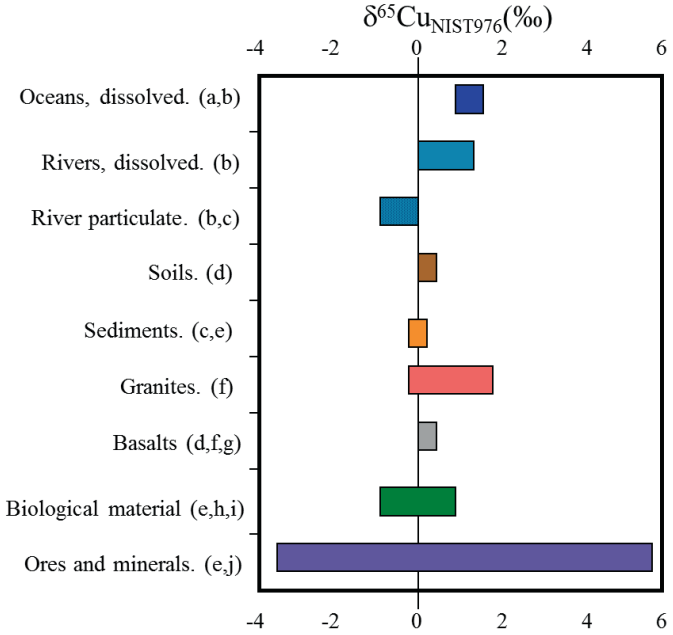

Fig 1. $\delta^{65}Cu$ values of several natural environmental samples.

a) (Bermin et al., 2006)
b) (Vance et al., 2008)
c) (Petit et al., 2008)
d) (Bigalke et al., 2010c)
e) (Marechal et al., 1999)
f) (Li et al., 2009)
g) (Archer and Vance, 2004)
h) (Zhu et al., 2002)
i) (Weinstein et al., 2011)
j) (Asael et al., 2007)

2.4.1. Ore minerals

Copper ore minerals can be found in both *hypogene* (result of cooling of hydrothermal solutions) and *supergene* (precipitation from low temperature aqueous fluids) environments. Chalcopyrite ($CuFeS_2$), chalcocite (Cu_2S) and bornite (Cu_5FeS_4) are examples of minerals that are found in the hypogene environment. In the supergene environment minerals such as covellite (CuS), cuprite (Cu_2O) and malachite ($Cu_2CO_3(OH)_2$) among others can be found. Among the primary/secondary Cu(I)-sulphides the range of $\delta^{65}Cu$ is approximately 9‰, in which approximately 85% of the primary chalcopyrite values are between the -0.5 to 0.5 range, similar to the Cu standard reference used (NIST 976). (Asael et al., 2007; Gale et al., 1999; Larson et al., 2003; Marechal et al., 1999; Markl et al., 2006; Mathur et al., 2005; Zhu et al., 2002)

The variability observed in secondary Cu minerals is larger than the one of the primary Cu minerals, with a broad window of 11‰ in their $\delta^{65}Cu$ values. More than 80% of the values are between -2 and 2‰.(Gale et al., 1999; Larson et al., 2003; Marechal et al., 1999; Mathur et al., 2005).

2.4.2. Soils and sediments

The δ^{65}Cu values for soils depend greatly on the type of soil and also where in the soil horizon the sample was taken. Bigalke et al. (2010c) studied the abundance of Cu isotope ratios in hydromorphic soils with results within the range of -0.34 to 0.16 ‰ for cambisols and -0.26 to 0.33 ‰ for gleysols. The main difference in the values is due to the vertical distribution of the δ^{65}Cu values within a profile, as a consequence of dissolution, accumulation and transport processes, and the chemical conditions. These processes are discussed in section 2.5. The variations in the Cu isotope ratios in soils from the surroundings of a smelter (-0.12 to 0.36 ‰) presents a pattern were geochemical processes such as sorption on oxy-(hydr)oxides and organic matter probably occurs. As a consequence, the isotopically lighter Cu isotope is preferentially leached out of the system or to deeper soil horizons (Bigalke et al., 2010a). Other studies shows that the difference in δ^{65}Cu values can reach 1‰ in podzols, with the organic horizon having a lighter Cu isotopic composition (Bigalke et al., 2011).

Sediment samples from the Atlantic Ocean were analysed by Marechal et al.(1999) and the main difference was observed in the first 2.5 cm, where the δ^{65}Cu value was lower than the samples taken at greater depths (2500m). The values of δ^{65}Cu of the samples range from 0.08 to 0.35 ‰. In the sample from the Mediterranean Sea the value obtained was -0.09‰. In an urban lake the variability of the δ^{65}Cu values was 0.29‰, ranging from 0.8 to 1.1‰. However, this difference was sufficient to track changes in the metal sources in the lake (Thapalia et al., 2010).

2.4.3. Water

The mean δ^{65}Cu value for major rivers worldwide is 0.66‰ and range between 0.02 and 1.45‰ (Vance et al., 2008). Rivers with high Cu concentrations tend to have δ^{65}Cu values closer to 0‰. AMD affected streams are generally enriched in the heavy Cu, with values ranging from -0.4 to 1.69. In spite of this, δ^{65}Cu values do not change during a diel cycle (Balistrieri et al., 2008; Borrok et al., 2008; Kimball et al., 2009).

Due to the low concentration of Cu in the oceans, a new procedure had to be developed to measure the Cu isotope concentration in seawater samples. The δ^{65}Cu values range from 0.75 to 1.44‰ for samples from the Pacific Ocean, Indian Ocean and English Channel (Bermin et al., 2006; Vance et al., 2008). The heavy isotopic composition of the oceans is inferred to be originated from intra-oceanic processes.

2.4.4. Organic samples

The measurements of the Cu isotope composition in organic material have shown a particular increase in the recent years, because of the importance that Cu has as a micronutrient in organisms. Maréchal et al (1999) measured the δ^{65}Cu values in mussel tissue (0.08 $\pm$ 0.04 ‰) and human blood (0.3 $\pm$ 0.04‰). In the same way, Pokrovsky et al. (2008) measured the Cu isotope fractionation in cells of *P. aureofaciens* with values in the range of -0.6 to -1.7‰. In plants the studies of Navarrete et al. (2011b) and Weinstein

at al. (2011) showed a wide window in the $\delta^{65}Cu$ values, 0.3 to -1.5‰ in desert shrubs (*P. pubecens*) and -0.32 to -0.75 ‰ in Virginia Wild Rye (*Elymus virginicus*). In organic compounds such as humic acids, $\delta^{65}Cu$ values go from 0.17 to 0.7 depending on the fraction of Cu complexed to the humic solution (Bigalke et al., 2010b).

2.5.Processes of Copper isotope fractionation

2.5.1. Abiotic and biotic mineral dissolution

During the dissolution of sulphide minerals, redox transformations play an important part in the isotopic fractionation of Cu, as pointed out in several research papers. Oxidative dissolution of sulphide minerals such as chalcopyrite and chalcocite, at pH 2.3 and 25ºC, showed an enrichment of the heavier Cu isotope in the aqueous Cu fraction (Mathur et al., 2005). In the same way, leach experiments of enargite showed that the leachate was enriched in the heavier Cu isotope (Kimball et al., 2009). This effect is attributed to the preferential oxidation of $^{65}Cu(I)$ at the interface of the isotopically homogeneous mineral and the oxidized layer. This redox effect was noticed by Fernandez and Borrok (2009), under acidic conditions when the oxidative weathering of sulphide-rich rocks resulted in fluids enriched in the heavier Cu isotopes.

The overall result during the oxidative dissolution of sulphide minerals is that the leached fluid is enriched in the heavier Cu isotope regardless of a Rayleigh-type kinetic effect (Wall et al., 2011) or a mixing of isotopic reservoirs (Fernandez and Borrok, 2009). Other factors such as pH and salinity can control the partitioning of Cu between the vapour and liquid phases and therefore its isotopic fractionation magnitude (Maher et al., 2011). In the latter case, the leached phase is enriched in the lighter Cu isotope under anoxic conditions, which is of importance in the hypogene ore forming systems.

When bacterial activity is involved in mineral dissolution, the results are different from the abiotic process under oxic conditions. Aqueous copper from leached chalcocite and chalcopyrite inoculated with *Thiobacillus ferrooxidans* was isotopically similar to the starting material, assuming that the bacteria acts as a sink for the heavy Cu isotope (Mathur et al., 2005). Similar result was obtained by Kimball et al. (2009) who propose the hypothesis of a preferential association of $^{65}Cu_{aq}$ with *A. ferroxidans* cells and related precipitates.

2.5.2. Mineral precipitation

Malachite precipitation from $Cu(NO_3)_2$ and $CuCl_2$ solutions at 30ºC resulted in a measured Cu fractionation for $\Delta^{65}Cu_{aq\text{-}min}$ of 0.20±0.06‰ and 0.38±0.06‰, respectively. The values were slightly lower at 50ºC (Marechal and Sheppard, 2002). Cu isotope zonation in Australian porphyry deposits demonstrate that variations in Cu isotope fractionation can be caused by fractionation of Cu between brine, sulphide and vapour during porphyry mineralisation, as a response to the decrease in the temperature and precipitation of minerals such as chalcopyrite and bornite (Li, 2010). However, variations in the Cu isotope fractionation are larger where redox processes are involved.

2.5.3. Redox processes

Copper is a redox sensitive element and the changes between oxidising and reducing conditions can cause a fractionation in the isotopes of the metal. Several studies point out the relevance of redox processes in the isotopic fractionation of Cu and examples in the dissolution of minerals are given by Asael et al. (2006). The authors carried out an experiment where dissolved Cu(II) reacted with pyrite and pyrrhotite ($Fe_{(1-x)}S$) in anoxic conditions and the remaining solution was enriched in ^{65}Cu relative to the sulfides by 3.02±0.14‰. Following the oxidative weathering processes in rocks, Fernandez and Borrok (2009) performed leaching experiments of chalcopyrite-bearing rocks. According to the authors, this process involves two main steps: the oxidation of the surface layer by the air, which causes an enrichment of the heavier isotope on that layer. The second step is the subsequent redox transformation in water which preferentially leaches the heavier isotope during the experimental stage, except at pH~5 where the precipitated Fe-(oxy)hydroxides minerals were the primary control for the isotope fractionation of Cu. The oxidative weathering of sulphate minerals was recently addressed by Wall et al. (2011) proposing that the mechanisms by which the Cu isotope fractionation of sulphide minerals are the result of Rayleigh-type processes or kinetic isotope effects instead of the mixing between the air-oxidised layer and the bulk mineral proposed by Fernandez and Borrok (2009). Nevertheless, there is no doubt that during the dissolution of sulphide minerals oxidation reactions are the dominant influence on the apparent isotope fractionation, resulting in a mineral reservoir depleted in the heavier Cu isotope.

An example of reduction-driven processes is the precipitation of covellite from Cu(II) and solid Cu(I) in an experiment performed at 20ºC. This results in a isotopically depleted covellite relative to the aqueous Cu(II) ($^{65}Cu_{aq\text{-}min}$ = 3.06±0.15‰), with increased fractionation at lower temperatures (Ehrlich et al., 2004). In the same way, the role of the reduction step of Cu(II) to Cu(I) in the Cu isotope fractionation was studied by Pekala et al. (2011), with other factors such as complexing ligands which might have a minor role in the overall fractionation. The transfer of the lighter Cu isotope, ^{63}Cu, is from the solution into the precipitating mineral phase. As long as the reaction continues the increasing depleted Cu signatures will be found in the mineral and the enriched (heavier) ones in the equivalent solution.

Redox transformations induced by biological organisms can isotopically fractionate Cu. In adsorption experiments, live bacteria cells preferentially incorporate the lighter Cu isotope as a result of the reduction of Cu (II) to Cu(I) by proteins across the intracellular membrane. While in dead cells the preference is for the heavier Cu isotope, due to Cu complexation processes (Navarrete et al., 2011a)

Examples of the impact of redox processes in Cu isotope fractionation in natural settings are described by Asael et al. (2007) and Bigalke et al. (2010c). In the Timna Valley, Israel, Asael et al. (2007) observed a significant fractionation of Cu isotopes between reduced Cu-sulphide minerals (covellite, chalcocite) and coexisting oxidized Cu minerals. Low $\delta^{65}Cu$ values of Cu-sulphides are related to the reduction of $Cu(II)_{aq}$ and *in*

situ oxidation of the Cu-sulphides to positive fractionation. In hydromorphic soils, Bigalke et al. (2010c) found out that changes in Cu speciation may fractionate Cu isotopes due to changes of the redox state. Both studies support the idea that Cu isotopes can be used as a proxy for the elucidation of biogeochemical processes and the cycling of Cu in different matrixes and pollution scales.

2.5.4. Copper sorption

Copper can be sorbed into both inorganic and organic compounds. In the case of inorganic materials, Cu can be sorbed to ferrihydrite, being the mineral enriched in the heavier isotope, compared to the aqueous Cu in a solution at pH=3 (Balistrieri et al., 2008). Goethite and gibbsite are also enriched in the heavier Cu isotope at pH between 4-6 after Cu adsorption. This study also proposes that strong, bidentate, inner-sphere complexes of tetrahedrally coordinated Cu on metal oxides surfaces have an impact in the enrichment of the heavier isotope in the metal oxides (Pokrovsky et al., 2008)

Organisms such as bacteria and algae can adsorb Cu onto their surfaces. Pokrovsky et al. (2008) found out that uptake of Cu by algae at pH 7.5-8 had no effect in the isotopic composition of the remaining dissolved Cu, having the same result in experiments with aquatic and soil bacteria and diatoms at the same pH. However, at lower pH (1.8 - 3.5) the soil bacterium *Pseudomonas aureofaciens* was depleted in ^{65}Cu compared to the solution. The authors suggest that acidic and neutral environments cause a difference in the protonation of functional groups on cell surfaces, affecting the fractionation of Cu in those surfaces.

Humic acids can also adsorb metals in their structure. In the case of Cu, (Bigalke et al., 2010b) performed Cu sorption experiments at pH 2-7 with insolubilized humic acid (IHA) and measured the Cu isotope fractionation. The results obtained show enrichment in ^{65}Cu for the IHA compared to the dissolved Cu in solution. This is explained due to differences in vibrational frequencies and zero point energies of the different isotopes, being the heavier isotope bound to the functional groups with higher bond strength. Metal coordination might be also a critical control of isotope fractionation in organic complexes (Schauble et al., 2009).

2.5.5. Biological processes

The δ^{65}Cu from the protein azurite expressed in *Escherichia coli* and *Pseudomona aeruginosa* was measured, being the protein depleted in ^{65}Cu compared to the dissolved Cu by 1.53±0.07‰ and 0.98±0.07‰, respectively (Zhu et al., 2002). This difference shows that mass fractionation of Cu isotopes occurs stepwise along the biological pathways within a single cell, assuring that transition metal isotopes can contribute to the understanding of the kinetic pathways within biological systems.

In the work of Mathur et al. (2005), chalcocite and chalcopyrite batch dissolution experiments with bacteria (*Thiobacillus ferroxidans*) resulted in a similar Cu isotopic content as the original value. This can be interpreted as that the cell walls of the bacteria

act as a sink for Cu, while analysing the content of the cell walls, the precipitated Cu nanoparticles were enriched in the heavier Cu isotope compared to the source medium by as much as 2.88±0.23‰. In the same way, Pokrovsky et al. (2008) corroborated a heavy Cu isotope enrichment onto amorphous Fe-(oxy)hydroxides and on metal hydroxide precipitates on the external membranes of several bacteria such as *Pseudomonas aureofaciens* and *Rhodobacter sp*. Both investigations show the potential effects of kinetic and equilibrium isotope effects during redox transformations in the presence of microorganisms.

In higher plants, the mechanisms of which Cu is isotopically fractionated were studied (Navarrete et al., 2011b; Weinstein et al., 2011). Both studies point out a systematic enrichment in the Cu lighter isotope in comparison to the soil where they grow. The incorporation of the lighter Cu isotope continues during the vertical translocation of the plants, from roots, to stems and finally leaves. Reduction reactions associated with biological incorporation by different enzymes as well as a combination of diffusion and transport through cell membranes can explain the enrichment of the lighter Cu as you go higher on the plant. A model for the Cu isotope fractionation in plants has been proposed by Jouvin et al. (2012). The dominant processes involve the speciation and diffusion of the metal in the solution, equilibrium adsorption on the root's cell walls, and reduction of Cu(II) at the surface of the plasma membrane by a Cu-reductase. Translocation of the metals from roots to shoots involves the complexation of the metal with organic acids, aminoacids or peptides produced by the plants during xylem and phloem transport.

2.6.Iron isotope variation in environmental samples

Since the development of precise Fe isotope measurement techniques, a high number of samples from different natural materials have been measured in order to elucidate processes such as Earth accretion or metal transport between reservoirs. A summary of those values are shown in figure 2.

2.6.1. Minerals and rocks

Iron ore minerals are mostly oxides or oxy(hydro)oxides of the metal, examples are hematite, which Fe isotope variability is minor, with reported δ^{57}Fe values of 0.740‰ (Saunier et al., 2011), δ^{57}Fe= 0.775‰ and δ^{56}Fe=0.521‰ (Poitrasson and Freydier, 2005). (Whitehouse and Fedo, 2007) measured the δ^{56}Fe values of pyrite (-0.399‰) and magnetite (-0.375‰). Special mention is given to the reported values of magnetite by (Marin-Carbonne et al., 2011), which show a variation of 4‰ in one single grain of magnetite and the rim of this magnetite showed an unprecedented δ^{56}Fe value of 5.2±0.2‰. Modifications of fluid isotopic composition during Fe-phases precipitation can explain this difference.

Earth rocks show little variation in their Fe isotope composition, δ^{57}Fe value for the mafic Earth (mean) is 0.102±0.032‰ (Poitrasson and Freydier, 2005). The Fe isotope homogeneity of igneous rocks is remarkable δ^{56}Fe = 0.00±0.05‰ (Beard et al., 2003) and

similar to lunar basalt samples. δ^{56}Fe values for meteorites range between 0.2 to 1.5‰, and for several chondrites the variation is larger between positive and negative values. However it is hypothesized that the early solar nebular had a homogeneous Fe isotope signature that was fractionated during planetesimal accretion (Zhu et al., 2001). Banded Iron Formation samples (BIF) have Fe isotope compositions that vary over a relatively wide range, from δ^{56}Fe +0.9 to -1.2 ‰ (Beard and Johnson, 1999)

2.6.2. Water

Given the homogeneity of Fe isotope variation in Earth rock samples, significant Fe isotopic variation input from the continents to the oceans seems unlikely. Values for δ^{56}Fe in the oceans range from 0.2 to 0.5‰ (Beard et al., 2003). In the same way, dissolved Fe and the suspended sediment from the Amazon River samples are isotopically light relative to igneous rocks. However, results from the Negro River were distinct. The dissolved fraction of the Fe was isotopically heavy (+0.28‰); whereas the suspended sediment Fe was isotopically light (−0.9‰), establishing the importance of organic ligands that can bound the dissolved Fe (Bergquist and Boyle, 2006). A negative δ^{56}Fe value was also observed in the Kalix River, Sweden, where Fe-C colloids play an important role as a metal carrier from the river to the sea (Ingri et al., 2006).

2.6.3. Soil and sediments

Zhu et al. (2000) measured the δ^{56}Fe values for a Chinese loess that were in the range from 0.6 to 4.8‰, as a way to establish a value for the upper continental crust. In the same article, Fe-Mn crusts samples from the North Atlantic showed a substantially change in the Fe isotope fractionation over the past 6 million, with positive values in the recent years from negative in the oldest.

Several studies have pinpointed the importance of measurement of Fe stable isotopes in soils to trace the biogeochemical cycle of this metal. In redoximorphic soils the mobility of the lighter Fe isotope compared to the heavier isotopes is greater during pedogenesis, with values of about 0.3‰ in δ^{57}Fe in total soil digests (Wiederhold, 2007a). In podzol profiles, systematic variations in the Fe isotope signature were found with negative peaks of δ^{57}Fe in the horizon were the Fe translocation was present (aprox. -0.5 ‰) (Wiederhold, 2007b). Biotite and chlorite weathering are key processes for soil formation. Kiczka et al. (2011) studied the grain size fractions in an alpine glacier finding that the clay sections with the newly formed Fe-oxy(hydro)oxides were enriched in the light Fe isotope, while the coarser fraction remained in the range of 0.4‰ of δ^{56}Fe.

Special attention should be given to the study of the Fe isotope fractionation in mine tailings at Kristineberg done by Herbert and Schippers (2008), where they report δ^{56}Fe values of roughly 0.9‰ for the bulk tailings above the oxidation front and slightly higher values below the oxidation front.

2.6.4. Biological material

One of the first reports of Fe fractionation by soil microbes was presented by Brantley et al. (2001), showing that the δ^{56}Fe value of dissolved Fe is by as much as 0.8‰ lighter than the bulk Fe in the starting mineral, suggesting that bacteria preferentially take up heavy, inorganically complexed Fe. To study the absorption of Fe in humans and animals, samples of blood, liver, muscle tissue and faeces were analysed by Walczyk and von Blackenburg (2002) reflecting differences in intestinal absorption between individuals. Human blood presented δ^{56}Fe values in the range from -2.0 to -3.1‰; liver from -0.9 to -1.6‰; muscle tissue from -2.1 to -3.4‰ and faeces with the heaviest Fe signature -0.7 to -1.1‰, which favours the theory of the discharge of heavier isotopes in organisms.

Isotope fractionation of Fe associated with the growth of higher plants was studied by Guelke and von Blackenburg (2007), reporting that according to different parts of strategy I (monocots) and strategy II (dicots) plants their δ^{56}Fe values varies from +0.7 to -1.64 ‰. Strategy I plants had a lower δ^{56}Fe value than the available Fe in the soil, while strategy II plants had a slightly higher δ^{56}Fe value than the plant available soil. In alpine plants the overall range of fractionation was 4.5‰ in δ^{56}Fe for the uptake and in-plant fractionation process (Kiczka et al., 2010b).

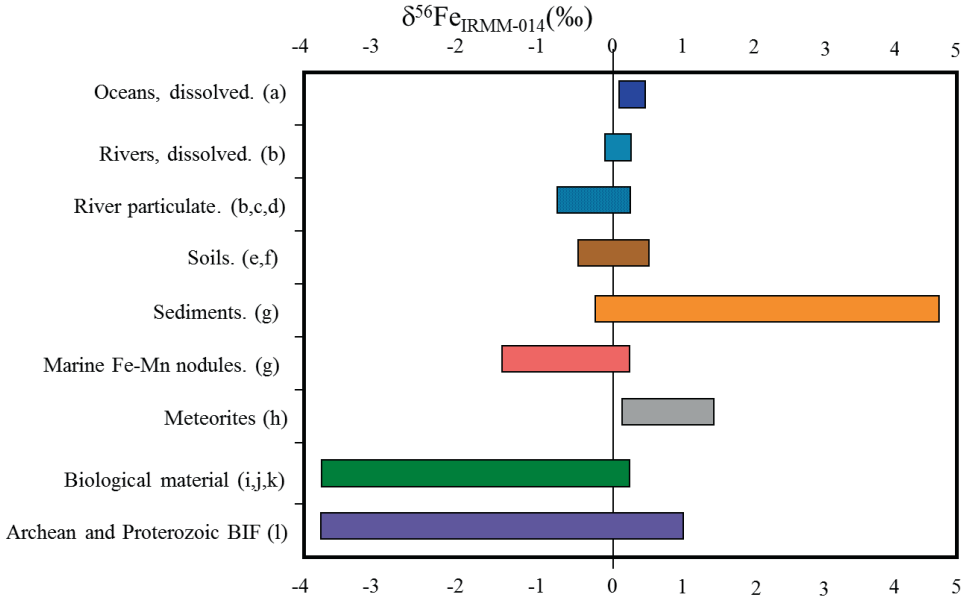

Fig 2. δ^{56}Fe values of several natural environmental samples. a)(Beard et al., 2003) b)(Bergquist and Boyle, 2006) c)(Song et al., 2001) d) (Ingri et al., 2006) e)(Kiczka et al., 2011) f)(Wiederhold, 2007b) g)(Zhu et al., 2000) h)(Zhu et al., 2001) i)(Guelke and von Blackenburg, 2007) j)(Walczyk and von Blackenburg, 2002) k)(Brantley et al., 2001) l)(Beard and Johnson, 1999).

2.7.Processes of Iron isotope fractionation

2.7.1. Non-redox processes

Fe isotope fractionation can occur as the result of chemical processes at room temperature in the absence of biotic factors, as reported by Anbar et al. (2000). This study showed that chromatographic experiments (ion exchange column in 7M HCl) can fractionate Fe from δ^{56}Fe values of+3.6‰ in the earliest eluted Fe to -3.4‰ in the later fractions. Ligands present in the chemical environment of any aqueous Fe reactions are just as important as the redox state to drive isotopic fractionation of iron-bearing species. A potential equilibrium fractionation of 1.5-2.5‰ between ferric chloride complexes is observed (Hill et al., 2009).

In nano and microrod experiments of Fe sorbtion with goethite at room temperature over 30 days, the isotopic fractionation at equilibrium between $Fe(II)_{aq}$ and $Fe(II)_{sorb}$, ($\Delta^{56}Fe_{Fe(II)aq\text{-}Fe(II)sorb}$) is -1.24‰ (Beard et al., 2010). During rapid precipitation of hematite from dissolved Fe(III), the fractionation of Fe favours the lighter in the precipitate. In this case kinetic isotope effects are the main reason for this fractionation (Skulan et al., 2002). Another example is given by the abiotic leaching of Fe from hornblende in the presence of chelating ligands, which favours the lighter Fe isotope dissolved in solution (Brantley et al., 2001). Despite some uncertainties, the non-redox reactions cause an isotopic fractionation of Fe, even when redox reactions can produce larger fractionation values, but non-redox reactions contribute to some extent to the Fe isotope signature seen in nature.

2.7.2. Mineral dissolution

Abiotic acid dissolution of Fe-bearing minerals such as hematite does not produce measureable Fe isotope fractionation, as demonstrated by Skulan et al. (2002). In this process the dissolution front advances into the mineral faster than solid state diffusion at low temperatures. In the same way, goethite dissolution during proton promoted mechanisms (in acid) will not cause isotope fractionation (Wiederhold et al., 2006). If dissolution is also accompanied by precipitation, however, an isotopic contrast might be expected between the solid and fluid phases (Skulan et al., 2002).

When mechanisms such as ligand controlled (oxalate-dark) and reductive (oxalate-light) dissolution of goethite, a kinetic isotope effect causes an enrichment of the lighter Fe isotope in the dissolved fractions during the early stages of the dissolution (Wiederhold et al., 2006). Conversely, the latter dissolved fractions show a depletion of the light Fe isotope which is caused by an equilibrium isotope effect between the goethite surfaces and complexed Fe(III) in solution. In the case of hematite dissolution via acid hydrolysis at 98ºC performed by Skulan et al. (2002), the initial Fe isotopic fractionation is also inferred to be originated from kinetic effects and in a long term the equilibrium isotope effect is the main cause for the isotope fractionation. Both studies show the heterogeneities regarding Fe isotope fractionation effects that might occur in nature.

Studies regarding the isotope fractionation during the oxidative dissolution of Fe-sulphide minerals such as pyrite have not being published due to the incongruent nature of its weathering and the development of complex surface layers. Oxidative dissolution experiments have been carried out mostly between pairs of pyrite/hematite and pyrite/siderite to assess the validity of theoretical calculations (Blanchard et al., 2009) and in sulphide-rich rocks (Fernandez and Borrok, 2009), which will be discussed in the section 2.7.4.

Dissolution of Fe-bearing phyllosilicates such as biotite and chlorite can isotopically fractionate Fe by different mechanisms. Kiczka et al. (2010a) demonstrated that under anoxic conditions the leached fraction was enriched in the light Fe isotope relative to the dissolving biotite and chlorite, based on a kinetic isotopic effect. Similar to Fe-(oxy)hydroxides, the later dissolution stages in a ligand control dissolution the heavy Fe isotopes were preferentially removed, being influenced by an equilibrium isotope effect.

2.7.3. Mineral precipitation

Precipitation of Fe minerals in the supergene environment can be driven by redox and non-redox reactions. Rapid precipitation of hematite from dissolved Fe(III) favours the light Fe isotope in the precipitate, where kinetic isotope effects cause this fractionation, along with a strong Fe isotope zonation (Skulan et al., 2002). In a similar way to non-redox reaction, redox transformations such as a rapid precipitation of ferrihydrite from Fe(II) is also controlled by kinetic effects, and the solid phase is enriched in the lighter Fe isotope (Bullen et al., 2001).

As it has been pointed out in section 2.7.2, following the precipitation of Fe-(oxy)hydroxides an isotope equilibrium between both phases can be reached after some time. In the case of synthesis of hematite, the equilibrium between the mineral and $[Fe(III)(H_2O)_6]_{aq}^{3+}$ reverses the fractionation caused by kinetic isotope effects, leading to identical Fe isotope composition in both phases (Welch et al., 2003). An important determinant in the isotope fractionation between solids and liquid phases is the coordination environment, where higher coordination favours the lighter isotopes. For example, Fe in hematite (octahedral coordination) is lighter than Fe in magnetite (octahedral-tetrahedral coordination) by ~3‰ (Johnson et al., 2002).

2.7.4. Redox transformations

Iron oxidation states have significant differences in solubility, being Fe(II) soluble under acidic conditions, near neutral pH and low Eh (anoxic) conditions. At redox boundaries, Fe changes in oxidation states are known for causing large Fe isotope fractionations and likely to provide records of modern and ancient oxidation conditions (Schauble, 2004). Redox reactions can be mediated in an inorganic or biotic way, resulting in significant metal stable isotope fractionation, being the reduced species of the metal generally lighter than the oxidised species (Bullen, 2011).

Examples of Fe isotope fractionation caused by redox reactions are well documented, as the case of non-biological oxidation and precipitation of ferrihydrite from dissolved Fe(II) at neutral pH, that favours the lighter Fe in the oxidised precipitate (Bullen et al., 2001). Even though abiotic oxidation of Fe can produce metal isotope fractionation, acidophilic Fe(II)-oxidising bacteria are more efficient at oxidation and can have a larger fractionation record.

Oxidative weathering of pyrite-rich rocks produces Fe isotope variations such as $\Delta^{56}Fe_{solution\text{-}py\ rock}$ = -1.75±1.0‰, releasing the lighter Fe isotope into solution, especially at low pH where the fluid phase is enriched in the heavier Fe isotope and this change is caused by electron-exchange reactions at the surfaces of the sulphide-bearing minerals (Fernandez and Borrok, 2009).

2.7.5. Fe Sorption

Isotope fractionation associated with sorption of transition and post-transition metals is highly element specific (Bullen, 2011). In the case of Fe, nanorod and microrod goethite was used to achieve complete isotope exchange among sorbed Fe(III), surface Fe (III) and bulk goethite in a series of experiments performed by Beard et al. (2010). These experiments presented that sorbed Fe(III) on goethite had a heavier isotope composition than $Fe(II)_{aq}$ and it is interpreted to reflect the differences in Fe(III) bonding between surface Fe and Fe in bulk goethite. There were also differences between both sizes of particles in their isotope composition, which is likely to be originated by significant differences in surface area for these iron oxides. Sorption of Fe onto goethite in the presence of bacterial activity has also been reported (Icopini et al., 2004). In this study the heavier Fe isotope preferentially sorbs into goethite.

2.7.6. Biological processes

Metabolic processes that involve multiple steps are able to produce significant Fe isotope fractionation (Beard et al., 1999). The same study reported that the Fe-reducing bacteria *Shewanella algae* grown on a ferrihydrite substrate yielded a dissolved Fe isotopically lighter than the ferrihydrite substrate. Similar results were found by Brantley et al. (2001) in which *Arthrobacter sp.* and *Streptomyces sp.* (both soil bacteria) grew in the presence of hornblende. In this case, it is suggested that the bacteria preferentially takes up the heavy, inorganically complexed Fe, which agrees with the production of catecholate siderophores that have a high Fe association. Biological Fe(II) oxidation is expected to occur in environments that promote redox cycling. Fe (II) oxidation pathways that involves the initial Fe(III) mineral precipitation outside the cell do not reflect equilibrium conditions because oxide/hydroxide redox cycling may be inhibited (Kappler et al., 2010).

Anbar (2004) suggests that processes such as microbial reduction of Fe could be enzymatically catalyzed stressing the importance of kinetic isotope effects. Since the dissociation of Fe-O and Fe-N are key bonds in Fe biochemistry, those bonds can coordinate the Fe in solution to be able to bind Fe to enzyme active sites.

Processes of Fe uptake and translocation by plants are able to isotopically fractionate Fe along the different steps in which the Fe fulfill a function in the plant metabolism. The first published study about this by Guelke and von Blackenburg (2007) concluded that according to the plant metabolism (strategy I and II plants) Fe can undergo different pathways to be incorporated into the biomass. For strategy I plants, Fe reduction is the main process that fractionate Fe, causing enrichment of the lighter isotope in the uptake from the soils and translocation from roots to stem and seeds. In the case of strategy II plants the isotopic fractionation of Fe is minimal for both uptake and translocation, suggesting Fe complexation as a way to incorporate the metal into the plant. In contrast, in the species *Agrostis* (strategy II plant) from an alpine glacier the Fe isotope fractionation from roots to stems, leaves and flowers showed a significant fractionation towards an enrichment in the lighter isotope (Kiczka et al., 2010b). The authors do not contradict the work of Guelke and von Blackenburg (2007), but add that the Fe isotope signature of plant biomass depends not only on the Fe uptake strategy but also on the nutrient availability in the substrate. In the same debate, oat plants (strategy II) grown on Fe(III)-EDTA are enriched in the lighter Fe isotope (Guelke-Stelling and von Blackenburg, 2012). It is supposed that a constitutive reductive uptake mechanism of Fe in the nutrient solution is the prevailing determinant of this fractionation.

A difference in the way that Fe is isotopically fractionated due to metabolic processes by humans and other animals is shown by Walczyk and von Blackenburg (2002). They present that dietary Fe absorption leaves a distinct Fe isotope signature in the blood of different individuals, especially between men and women.

3. Study areas

3.1. Laver Mine

Laver mine is located in northern Sweden, 120 km west of Luleå at an altitude of 350 m above sea level (figure 3). It is a former copper mine, being the main ore minerals chalcopyrite and pyrrhotite, with minor quantities of pyrite. Mine operations ended in 1946 after being in production for 10 years (Ljungberg and Öhlander, 2001).

Minor reclamation measures had been done in the tailings site, which includes the liming, fertilization and seeding with a grass mixture of the previously eroded parts of the tailings (Lindgren, 1975). Currently the site has a vegetation cover and layers of iron hydroxides precipitates can be seen in the discharge areas.

There is a chemical zonation within the tailings as reported by Holmström et al. (1999). This zonation consists in three well defined areas in the profile: A strongly oxidised zone in the top layer, depleted in sulphide bound metals. A secondary copper enrichment zone right below the oxidised zone where Cu content is at its peak (up to 4800 ppm) and secondary formed covellite is found. At the bottom of the profile is located a non-oxidised zone, clearly identifiable by its blue color and the presence of primary Cu ore minerals.

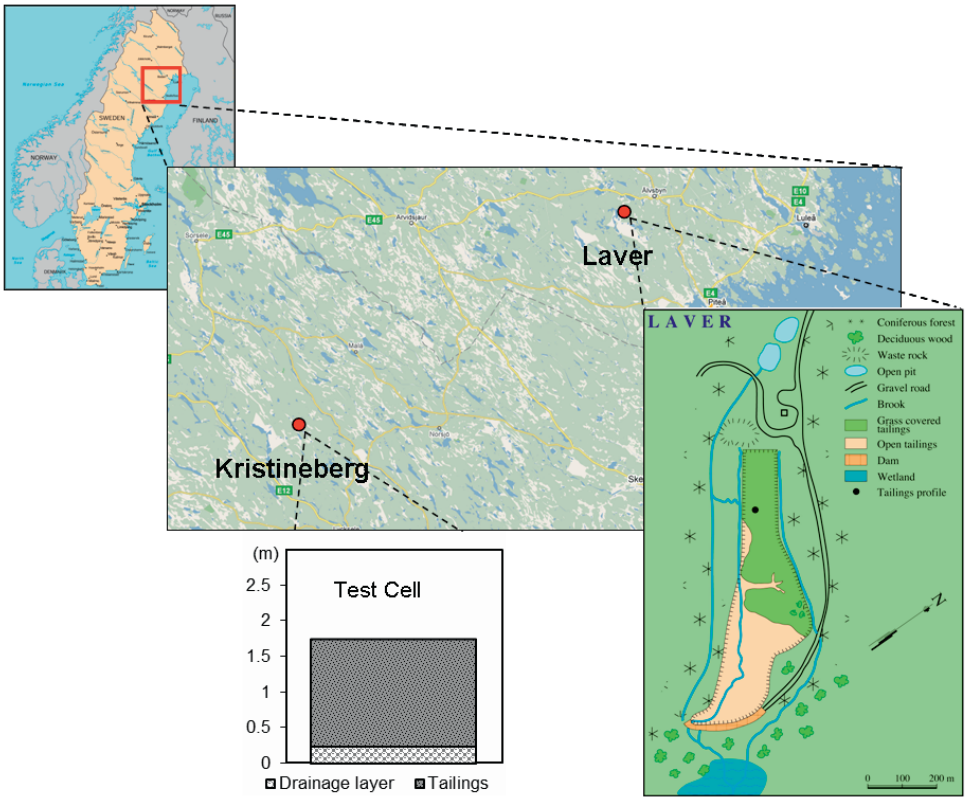

Fig. 3. Location of the study sites Laver and Kristineberg (paper I).

3.2. Kristineberg test cells

Six test cells were constructed at the Kristineberg mine site, in 2001. They contained fresh unoxidised tailings from the Kristineberg Pb-Zn-Cu mine, being their main sulphide minerals pyrite and pyrrhotite. The cells were covered with different materials for research purposes, but one cell was left without covering as a reference and is the one used in this study. Location and sketch of the test cell is shown in the figure 3. The uncovered cell showed evidence of oxidation after 5 years of being constructed (Alakangas and Öhlander, 2006).

3.3. Gessenwiese test site

The Gessenwiese test site is located in the former mining district of Ronneburg (Thuringia, Germany) where U was mined from 1952 to 1990 (Merten et al., 2004) (Fig. 4); after 1960 the low grade substrate was leached on the ancient heap Gessenhalde with acid mine drainage (AMD) and this heavy metal enriched leachate infiltrated the barrier soil and retained in the glacial sediments underneath (Neagoe et al., 2009). In the north-

west side of the former leaching area, the test site Gessenwiese was created in 2004 covering 2500m^2 (Grawunder et al., 2009).

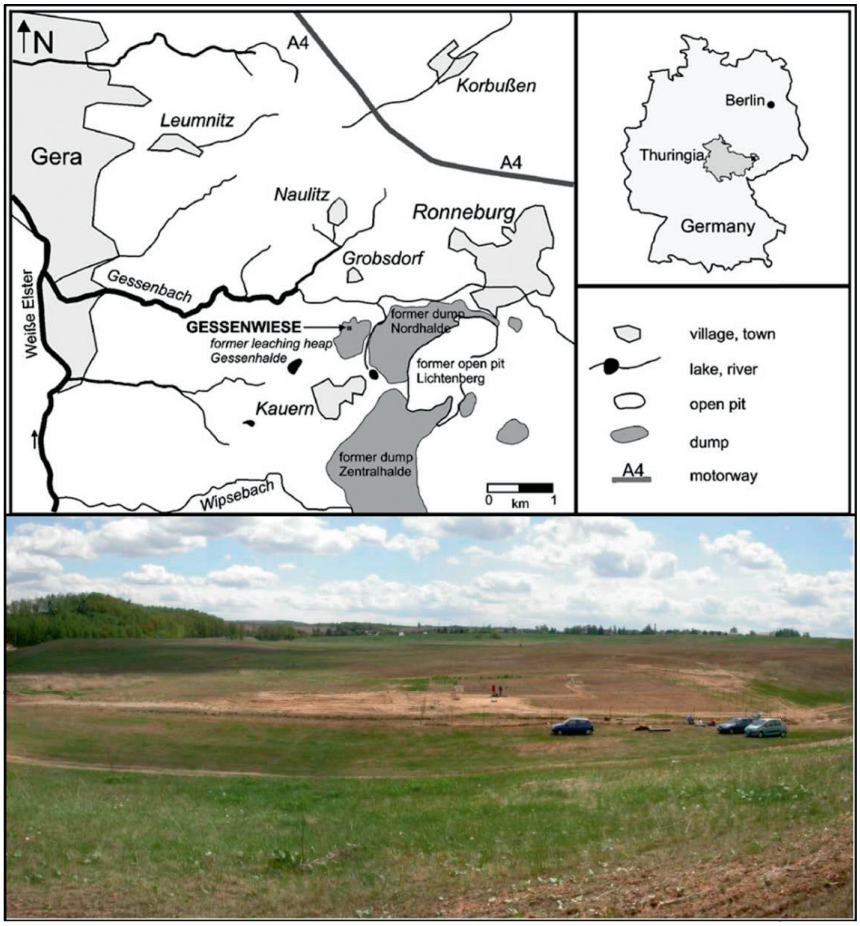

Fig. 4. Location of the Gessenwiese test site (paper II). Map taken from (Grawunder et al., 2009) and photography courtesy of A. Grawunder.

This test site is used for research and to try out new technologies for low-cost decontaminations strategies, such as bioremediation, for the extraction of heavy metals by plants (Lonschinski et al., 2011). Over time radionuclides (U, Th), heavy metals (Cd, Co, Cr, Cu, Fe, Mn, Ni, rare earth elements (REE) and Zn have leached towards the sediments underneath (Grawunder et al., 2009). One interesting observation of the past studies in this area is the finding of two autochthonous *Streptomyces mirabilis* strains that are resistant to high concentrations of Ni and Zn. However resistance to Cu could not be detected (Schmidt et al., 2009).

4. Materials and methods

4.1. Sampling and experimental planning

4.1.1. Paper I

In order to obtain a clean profile from the tailings at Laver, the samples were taken from a pit dug using a mechanical excavator and collected manually using metal-free tools. This sampling session was done in October 2009 and the coordinates of the dug pit are N 65° 46.183' E 20° 14.958' (Figure 3). The tailings samples were collected at 20 cm intervals until a depth of 150 cm and the humus (organic layer) sample was taken at a depth of 0-2 cm. All samples were stored in plastic non diffusive bags that were kept refrigerated until later preparation and analysis was performed.

Biological material, such as grass (*Festuca* sp.) samples were taken in the surrounding areas where the pit was dug, and the white cottongrass (*Eriophorum scheuchzeri*) samples were taken from a boggy area where the groundwater from the tailings reaches the surface.

Tailings samples in a profile from the cell at Kristineberg were taken during the summer of 2009 using a mechanical excavator and collected every 10 cm to a depth of 1.1 m carefully avoiding oxygen contamination. The samples were stored in closed plastic cups and kept refrigerated until further analysis (Nason, 2012).

4.1.2. Paper II

To study the effect of bacteria in the Fe and Cu isotope signature in plants, two experiments were designed. The first experiment was performed using 10 pots of 1 kg filled with 800g of the topsoil from the Gessenwiese test site and seeded with untreated sunflower seeds (*Helianthus annuus*). Of those 10 pots 5 were amended with a plant growing promoting bacteria consortium (PGPR) obtained from the Ronneburg site. This consortium consists of different strains of *Enterobacter sp.*, *Streptomyces sp.*, and *Bacillus sp*. Features and sequences can be found elsewhere (Kothe and Schmidt, 2006; Sineriz et al., 2005). Sunflower plants grew for 12 weeks under controlled conditions of temperature and watering, when they were harvested (Fig. 5). Above and underground plant material was washed with distilled water, and dried at 38ºC for circa 6 days. Undisturbed soil material from the pots were also collected.

The second experiment was a field trial, set up in the test site Gessenwiese where several plots with different amendments were also seeded with untreated *Helianthus annuus* (Fig. 6).

Plot 1 (BIO): Topsoil mixed with domestic compost (added in 2004).

Plot 2 (MBM): Top soil mixed with PGPR, mychorrizal *Glomus intraradices* pellets (provided by Prof. K. Turnau, Jagiellonian University, Krakow) and domestic compost (added in 2004).

Plot 3. (MS): Top soil mixed with *Streptomyces* from the site, and mychorrizal fungi pellets.

Plants were harvested, washed with distilled water, dried at room temperature and separated into roots, stems, and leaves/flowers. Soil material was also collected on site.

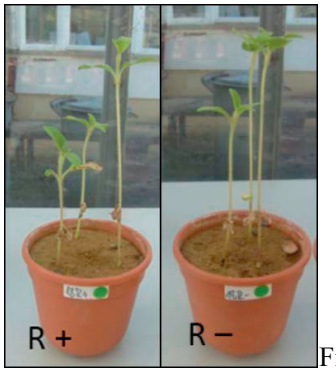

Fig. 5 Pot experiments with Gessenwiesen soil substrate and *Helianthus annus*. R+, amended with PGPR. R- Not amended. Photo: courtesy of F. Langella.

4.2. Analytical methods

4.2.1. Elemental Analysis

Tailings and soil samples were digested by alkali-fusion or microwave assisted acid digestion in closed vessels. Elemental analysis was obtained for Cd, Co, Hg, Ni and Pb using ICP-SFMS and for SI, Al, Ca, Fe, K, Mg, Mn, Na, P, Ti, As, Ba, Be, Cr, Cu;Mo, Nb, S, Sc, Sr, V, W, Y, Zn and Zr using ICP-AES, according to US EPA methods 200.8 (modified) and 200.7 (modified), respectively.

Dried plant material was milled and ashed at 550 ºC overnight followed by digestion of the residue with hot 14M HNO_3. Digests were evaporated to dryness and re-dissolved in 7M HCl. Elemental analysis was done in the same way as tailings and soil samples.

4.2.2. Cu and Fe purification

Cu and Fe from sample digests were purified using anion exchange chromatography following the procedure described by Marechal et al. (1999) and Mason et al. (2005) with calibration of the elution profiles as recommended by Borrok et al. (2007). Materials, eluents, final volumes used and the preparation of the fractions are described in papers I and II.

4.2.3. Cu and Fe isotope analysis

To assure the absence of interfering elements and a recovery of more than 90% fo the metals to avoid artificial isotope fractionation (Anbar et al., 2000), the purified fractions were analysed by single-collector ICP-SFMS. Cu and Fe isotope analyses were performed by multi-collector inductively coupled plasma-sector field mass spectrometry (MC-ICP-MS) using a Neptune (Thermo Fischer Scientific, Bremen, Germany) operated in medium resolution mode. Standards and equations are described in section 2.1 and detailed operational procedures in papers I and II.

5. Summary of findings

5.1. Cu and Fe isotope fractionation in an oxidising environment. (paper I)

The oxidizing tailings of the Laver mine have a chemical zonation: an oxidized zone, an unoxidised zone and a secondary enrichment zone, as reported by Holmström et al. (1999). In the unoxidised zone, Cu is partly retained and partly lost as a dissolved fraction and this retained Cu is present in form of sulphur-bound minerals such as pyrrhotite and chalcopyrite. The δ^{65}Cu value in the non-oxidised tailings at Laver is 1.31±0.03‰ and it is considerably lower at the limit between the oxidised and unoxidised zone reaching a δ^{65}Cu value of -4.35±0.02‰, and a Cu concentration of 3300 mg/kg, where the secondary enrichment zone is located. In this secondary enrichment zone, covellite precipitated due to a change in redox conditions and the replacement of Fe(II) for Cu(II) at the pyrrhotite surfaces through an ion exchange reaction, but also as separate grains (Holmström et al., 1999). In the oxidised zone little or no chalcopyrite is left (Holmström et al. 1999) and Cu is present mostly in Fe-(oxy)hydroxides. In this zone the average δ^{65}Cu value is -0.16 ‰, and there is an abrupt change in those values from the one at the oxidation front. A profile of δ^{65}Cu in the tailings at Laver is shown in the figure 6.

The relation between the observed Cu isotope fractionation values in the tailings at Laver and the geochemical processes involved can be summarised as:

1. The original value of the unoxidised tailings it is assumed to be 1.31±0.01 ‰, corresponding to the deepest sample of the profile. This value is close to the range of δ^{65}Cu -1.00 to 1.00 ‰, expected for terrestrial primary Cu sulphides (Kimball et al., 2009)
2. Oxidative dissolution of primary sulphide minerals, such as chalcopyrite when the infiltrated water reaches the unoxidised tailings. This process is known for the release of isotopically heavy Cu into the solution (Mathur et al., 2005).
3. Covellite precipitation, which involves the reduction of Cu from $Cu(II)_{aq}$ to $Cu(I)_{CuS}$. In this reaction the lighter Cu is preferentially transferred from the solution to the newly formed mineral (Ehrlich et al., 2004; Pekala et al., 2011), as seen in the oxidation front of the tailings.
4. Sorption of Cu(II) into Fe-(oxy)hydroxides, occurring after the downward movement of the oxidation front and later dissolution of the precipitated covellite. The process of sorption of Cu(II) into ferrihydrite preferentially preferentially takes the heavier Cu isotope (Balistrieri et al., 2008), and the samples from the oxidised zone are enriched in the heavier isotope compared to the values registered at the oxidation front.

A similar Cu isotope profile is seen at the Kristineberg test site. Here, the δ^{65}Cu value obtained at the oxidation front is 0.17±0.01‰, slightly lower than the values at both unoxidised and oxidized zones. This enrichment of the lighter Cu isotope in the oxidation front is caused by the oxidation of primary sulphides that release the preferentially the heavier Cu isotope and thereby, the remanent solid in the oxidised zone is enriched in the lighter one. The Cu isotope profile is shown on Fig. 7a. The difference between the δ^{65}Cu values in Laver and Kristineberg are assumed to be because of variations of initial Cu isotope ratio values in both tailings, and age effects. The age effects are evident in the

deeper oxidation front in Laver, which has a higher water saturation and thereby a more reduced environment than in the shallower oxidation front in Kristineberg

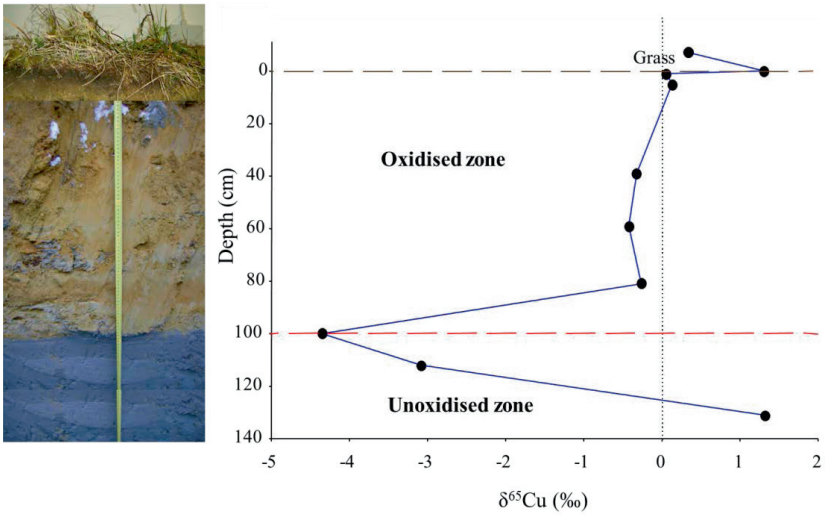

Fig. 6. Photo and Cu isotope fractionation profile in the tailings at Laver. Dashed line at 100 cm depth indicates the oxidation front.

The variation of the $\delta^{56}Fe$ values along the profile in the Kristineberg test site is shown in the figure 7b. The processes involved in this variation are:

1. The original $\delta^{56}Fe$ value of the tailings can be considered as the average of the $\delta^{56}Fe$ values along the unoxidised zone (-0.49‰).
2. Pyrite oxidation (as summarised in reactions 1 and 2) occurs at the oxidation front. At the surface of the pyrite, the lighter Fe isotope goes into the solution caused by the electron exchange reactions (Fernandez and Borrok, 2009). In the Kristineberg profile the solid phase is enriched in the heavier Fe isotope, and it is expected that the leached fluid is enriched in the lighter Fe isotope.
3. Precipitation of Fe-(oxy)hydroxides as single grains or at the surface of pyrite favours a enrichment of the heavier Fe isotope in the solid phase. Preferential partitioning of the isotopes in the $Fe(III)_{aq}$ species, product of a redox transformation, is considered the main cause of this behaviour (Bullen et al., 2001).
4. Other factors involving the presence of Fe-bearing silicates in the bulk material from the oxidised zone may reduce the $\delta^{56}Fe$ values, as reported by (Herbert and Schippers, 2008)

As an overall view, principally the process of pyrite oxidation in the test cell causes a slight depletion of the heavier Fe isotope from the unoxidised to the oxidised zone.

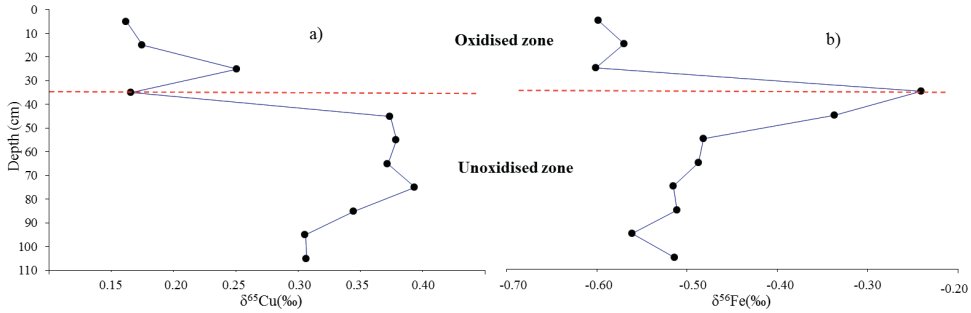

Fig. 7. a)Cu and b) Fe isotope fractionation profile in the tailings at Kristineberg. Dashed line indicates the oxidation front.

5.2. Cu isotope fractionation in plants and organic material (papers I -II)

As a part to obtain a Cu isotope profile in the tailings at Laver, wild grass samples growing on top of the analysed profile, had a δ^{65}Cu value of 0.34±0.01‰, lower than the one for humus (1.45±0.08‰), but heavier than the average oxidised zone (-0.22±0.05‰). Humus values confirm in the field the results reported by Bigalke et al. (2010b) who shows during a lab experiment that the process of Cu complexation in insoluble humic substances tends to enrich the the heavier isotope.

Plant samples (*Helianthus annuus*) that grew in pots under controlled conditions (Gessenwiese top soil) and plots in the field (Gessenwiese test site), regardless the amount of bacteria consortium, mychorriza or compost present, also show an overall enrichment in the heavier Cu isotope from soil to leaves. However, the enrichment of the heavier Cu isotope in the plants compared to its substrate it is contradictory to the results of Jouvin et al. (2012) and Weinstein et al. (2011). Several factors such as bacterial activity and weathering processes occurring in the field may shift the Cu isotope fractionation in the plants. This topic will be detailed in the section 5.3.

5.3. Effect of organic amendments in the uptake of Cu and Fe isotopes by plants. (paper II)

The effect of the presence of bacteria in the substrate were plants grow was studied regarding the way that Cu and Fe are fractionated by plants as part of a metabolic process. The addition of the PGPR (detailed in section 4.1.2) to sunflowers (*H. annus*) growing in pots under controlled conditions causes little variation in the Cu fractionation from soils to roots and plants, compared to the non-amended pots. Similar results are shown for the Fe isotope fractionation (Figures 8a and 8b). The best way to compare the bacteria effect is to relate these results to previous work, where the bacterial effect was minimal to none. Jouvin et al. (2012) and Weinstein et al. (2011) performed this kind of experiments obtaining different results to this investigation. In the literature, the authors reported that Cu is fractionated towards the lighter isotope from the soil to the shoots. The most reasonable explanation for this discrepancy is the presence of *Strptomyces* in PGPR which are known for releasing siderophores which can increase the metal

availability in the soil, changing the preferential adsorption of the Cu isotope in the siderophore structure and therefore in the roots/plant.

The translocation process in the sunflowers follow the observations by Jouvin et al. (2012), because the shoots are enriched in the lighter Cu isotope compared to the roots. Transport and diffusion through the cell membranes is the main cause of this fractionation.

The isotopic fractionation of Fe in the pot experiments follows a trend towards the enrichment of the lighter isotope from soils to roots and to shoots, regardless if the substrate is inoculated or not. A general explanation is that bacteria such as *Streptomyces sp.* present in PGPR, can take up the heavier Fe isotope (Brantley et al., 2001) releasing the lighter into the substrate and later taken up by the roots. In addition, the presence of an available and appropriate Fe pool in the substrate can restrict the complexation of Fe in the rhizosphere, favouring the uptake of the metal via reduction processes (Jouvin et al., 2008).

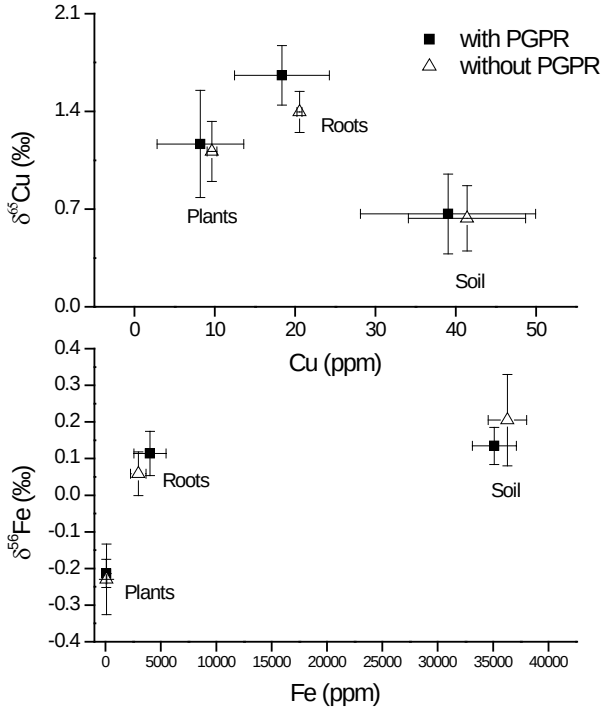

Fig 8. a) Cu and b) Fe isotopic composition in soils, roots and plants from pots experiments (*Helianthus annuus* in Ronneburg soil and amended or not with PGPR). Bars represent 2 δ.

Other organic amendments such as mychorriza and compost were tested in the field trials. Contrary to previous research (Jouvin et al., 2008; Weinstein et al., 2011), there is a general increase in the δ^{65}Cu values from soils to leaves, observed in the studied 3 plots (figure 9a). It can be seen that the influence of the addition of bacteria or compost in the

Cu isotope fractionation is limited when it comes to the general result (soil to leaves, compared to BIO). However, the uptake of this metal by the roots differs according to the addition of those amendments, with a Cu isotope signature more enriched in the heavier isotope compared to the BIO plot. Degrading organic material is acting as a sink for the heavier Cu isotope (Bigalke et al., 2010b) leaving a soil pool enriched in the lighter Cu isotope to be available for the plants.

The general trend of the Fe isotope fractionation of the sunflower plants in all plots is towards a lighter isotope signature as they go up in the plant (figure 9b). This is in accordance to the work of Kiczka et al. (2010b) and described previously. The addition of mychorriza and increased *Streptomyces* activity can act as a sink for the heavy Fe isotope (Brantley et al., 2001) favouring the selection of the lighter Fe isotope in the soil pool and therefore influencing the uptake of Fe by the roots.

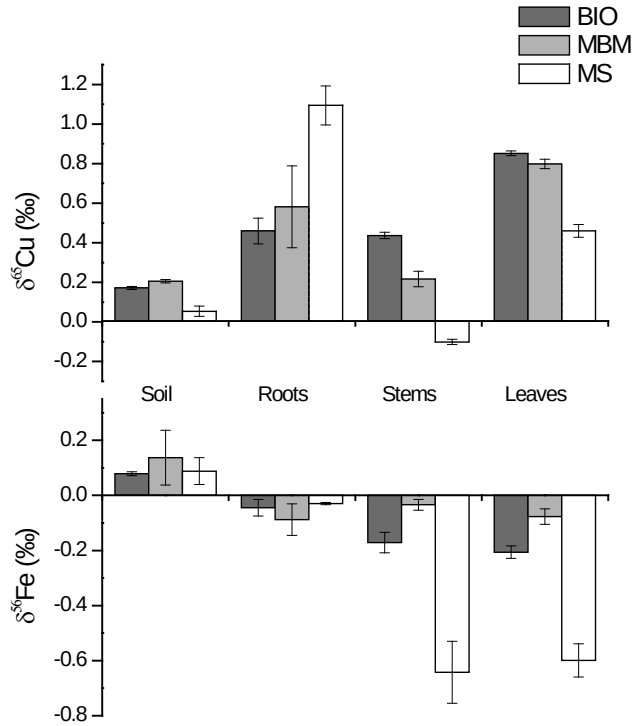

Fig 9. a) Cu and b) Fe isotopic composition in soils, roots, stems and leaves from the field trial (*Helianthus annuus* in the Gessenwiese test site). BIO, MBM and MS refer to the plots used in the field. Bars represent 2 δ.

5.4. Environmental considerations. (papers I-II)

The use of metal stable isotopes as indicators of the transport and fate of heavy metals that can be potentially harmful to the ecosystem could bring new insights to the evaluation of remediated metal polluted sites. The understanding of how the metals

behave during processes such as weathering (oxidation) of sulphide minerals and the implications of the isotope fractionation of heavy metals during their biogeochemical cycle would improve.

The biological cycle of metals has been object of profound analysis over the years. In this study the Cu and Fe isotope fractionation by plants, offers a limited but important view on the factors that might affect the cycle of those metals across the Earth's surface, and also important on the way that they could enter and fractionate into higher organisms and eventually in the human beings, offering an aspect that should be taken into account for health issues.

As a way to evaluate different study scales, Cu and Fe isotopes showed the differences between pot and field experiments, especially in the case of the multiple variables that can affect the field system. The results obtained confirm once more the care that should be taken when extrapolating conclusions from simplified systems to natural environments.

6. Future research

In order to analyse the fate of the metals in mine affected areas, the study of biofilms that are adapted to survive in high metal concentration areas (Cu, Fe and Zn) could shed a light on the mobilization of the heavy metals. Basically, we will study if the bacterial organisms present in biofilms are able to act as a sink for heavy metals or if they are purely a step in the remobilization of the metals. The results obtained will not only increase the knowledge of the behaviour of the metals in the supergene environment, but would also suggest or not the use of bacterial material to remediate metal-polluted sites.

Following the study of diverse scales for testing plants and bacteria consortia as a tool-box, samples from lysimeter experiments will be evaluated. Cu and Fe isotopes will be measured in the provided soil and biomass samples to establish conclusions regarding the fate of these metals. This is a joint effort with our partners in the Center of Ecological Services at the University of Bucharest.

The differences of isotope fractionation of Cu and Fe during the bioleaching of minerals such as chalcopyrite will be studied as a step towards the understanding of the processes in the cycle of the metals in the environment. Extraction of metals such as Cu from ores using bioleaching techniques is already used in the mining industry, and the results obtained could help in the evaluation of the industrial process and its waste. This will be a joint cooperation with the Division of Sustainable Process Engineering at Luleå University of Technology.

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Paper I

Copper and iron isotope fractionation in mine tailings at the Laver and Kristineberg mines, northern Sweden

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Copper and iron isotope fractionation in mine tailings at the Laver and Kristineberg mines, northern Sweden.

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Abstract

Previous research has shown that Cu and Fe isotopes are fractionated by dissolution and precipitation reactions driven by changing redox conditions. In this study, Cu isotope composition ($^{65}Cu/^{63}Cu$ ratios) was studied in profiles through sulphide-bearing tailings at the former Cu mine at Laver and in a pilot-scale test cell at the Kristineberg mine, both in northern Sweden. The profile at Kristineberg was analysed also for Fe isotope composition ($^{56}Fe/^{54}Fe$ ratios). At both sites sulphide oxidation resulted in an enrichment of the lighter Cu isotope in the oxidised zone of the tailings compared to the original isotope ratio, probably due to preferential losses of heavier Cu isotope into the liquid phase during oxidation of sulphides. In a zone with secondary enrichment of Cu, located just below the oxidation front at Laver, $\delta^{65}Cu$ (compared to the ERM-AE633) was as low as -4.35 $\pm$ 0.02‰, which can be compared to the original value of 1.31 $\pm$ 0.03‰ from the unoxidised tailings. Precipitation of covellite in the secondary copper enrichment zone explains this fractionation. The Fe isotopic composition in the Kristineberg profile is similar in the oxidised zone and in the unoxidised zone, with average $\delta^{56}Fe$ values (relative to the IRMM-014) of -0.58$\pm$ 0.06‰ and -0.49 $\pm$ 0.05‰, respectively. At the well-defined oxidation front, $\delta^{56}Fe$ was less negative, -0.24 $\pm$ 0.01 ‰. Events such as Fe(II)-Fe(III) equilibrium and precipitation of Fe-(oxy)hydroxides at the oxidation front are assumed to cause this Fe isotope fractionation. This field study confirms that geochemical redox processes are important for the isotopic composition of Cu and Fe in natural systems.

Keywords: Cu isotopes, Fe isotopes, Cu secondary enrichment, weathering, Cu oxidation, Fe oxidation, covellite formation.

1. Introduction

During and after mining activities the milled ore material, or mine tailings, is often left exposed to natural weathering processes. Tailings are composed mostly of non-valuable minerals, but since the recovery of target minerals is never 100 %, some of the ore minerals could remain in the tailings. The presence of Cu and Fe sulphide minerals such as chalcopyrite ($CuFeS_2$) and pyrite (FeS_2) in the tailings and their eventual weathering through oxidation mechanisms may release acid and metals in high concentrations that might leave the impoundment with the seepage, or be retained within the tailings via precipitation of supergene minerals (Lottermoser, 2007).

Copper has two oxidation states: Cu^{+} present in minerals such as chalcopyrite ($CuFeS_2$), chalcocite (CuS) and covellite (CuS_2); and Cu^{2+} which is highly soluble and present in aqueous solutions. Cu is a micronutrient in plants, involved in enzymatic functions during redox reactions, but in high concentrations can cause damage to the plasma membrane of root cells (Marschner, 1995). Depending on parameters such as pH, soil type and redox conditions, Cu can be adsorbed to different surfaces that includes organic matter, Fe-oxides and clays (Bradl, 2004). Copper has two stable isotopes, ^{63}Cu and ^{65}Cu, with relative isotopic abundances of 69.15% and 30.85%, respectively (De Laeter et al., 2003).

Iron is an element widely present in the Earth's surface, being the fourth most common element after O, Si and Al (Wedepohl, 1995). The pathways by which Fe is weathered are controlled by the surface conditions, especially oxygen content, and it is in the oxic and circumneutral environment where the species Fe (III) is favoured, compared to Fe (II). However, iron sulphides are stable under oxygen free (anoxic) conditions. Iron is also one of the most important trace nutrients in biological systems (Stumm and Morgan, 1996), has four stable isotopes ^{54}Fe, ^{56}Fe, ^{57}Fe and ^{58}Fe. Of these, ^{56}Fe is the most abundant (91.75%) followed by ^{54}Fe (5.84%), ^{57}Fe (2.12%) and ^{58}Fe (0.28%) (De Laeter et al., 2003).

In recent years there has been an increased interest in the study of Cu and Fe isotopes due to the development of more precise mass spectrometers and successful techniques that separate Cu and Fe from a complex matrix (Borrok et al., 2007; Marechal et al., 1999; Zhu et al., 2000). Measurement of Cu and Fe isotope ratios ($^{65}Cu/^{63}Cu$ and

$^{56}Fe/^{54}Fe$) in samples from laboratory experiments and nature will help to have hindsight about the isotopic processes that occur in the environment. Several reaction mechanisms can cause isotope fractionation in the above mentioned metals. In the case of Cu the following can be described: (1) Redox processes. Cu isotope fractionation is influenced by the reactions occurring during the transition between both anoxic and oxic environments, being this fact confirmed by several studies. Some examples are given by Ehrlich et al (2004) showing a mean Cu isotope fractionation ($\delta^{65}Cu$) of 3.06 ± 0.14‰ for the relation between $Cu(II)_{aq}$ and covellite (CuS, with Cu(I)) in the precipitation of the mineral under anoxic conditions, where $Cu(II)_{aq}$ is reduced to Cu(I) during covellite formation. The reduction step from Cu(II) to Cu(I) is the main factor that controls the isotope fractionation between $Cu(II)_{aq}$ and the reacting Cu sulphides for the alteration of minerals such as chalcocite, where the unidirectional transfer of ^{63}Cu from the solution to mineral enriches the solution in ^{65}Cu, as indicated by Pekala et al. (2011). Fernández and Borrok (2009) related the Cu isotopic change during the weathering of chalcopyrite-rich rocks to a redox transformation of Cu on the mineral surface. Redox processes induced by biological organisms can also cause Cu isotope fractionation, reduction of Cu(II) to Cu(I) by proteins in live cells preferentially incorporates the lighter Cu into the cell (Navarrete et al., 2011a). (2) Mineral dissolution. Abiotic dissolution of Cu sulphide minerals such as chalcopyrite, chalcocite and bornite has been previously studied (Fernandez and Borrok, 2009; Kimball et al., 2009; Maher et al., 2011; Mathur et al., 2005; Wall et al., 2011) suggesting a redox isotope effect by the oxidation of the Cu(I) present in the minerals, releasing the heavier Cu isotope into the solution. In a second stage of the dissolution there is a decrease in the apparent isotope fractionation of Cu, where kinetic isotope effects are the main influence for this behaviour. Dissolution of sulphide minerals in the presence of microorganisms can cause Cu isotope fractionation, but in less extent compared to the abiotic dissolution (Kimball et al., 2009; Mathur et al., 2005). (3) Sorption onto minerals and organic matter. Fe-(oxy)hydroxides are commonly formed during sulphide dissolution and are able to retain trace elements such as Cu by adsorption or coprecipitation. Several studies have shown that Cu(II) sorbed onto minerals such as ferryhydrite (Balistrieri et al., 2008) and goethite and gibbsite (Pokrovsky et al., 2008) is more enriched in the heavy Cu isotope than Cu_{aq}. The Cu

isotope fractionation due to sorption onto bacteria differs according to the pH of the solutions where it is occurring. At circumneutral pH no isotope fractionation was observed by Pokrovsky et al (2008) during the adsorption of Cu by soil bacteria and diatoms. However, at acidic environments the Cu adsorbed to *Pseudomonas aureofaciens* was enriched in the lighter Cu isotope relative to the solution medium (Pokrovsky et al., 2008). The heavier Cu isotope is preferentially bounded to insolubilized humic acid during complexation reactions (Bigalke et al., 2010b), (4). Biological processes. Examples are given by bacterial incorporation of the lighter Cu into bacterial cells and proteins (Navarrete et al., 2011a; Zhu et al., 2002), and the uptake of the lighter isotope in plants, from the soils to the roots and shoots (Jouvin et al., 2012; Navarrete et al., 2011b; Weinstein et al., 2011).

Copper isotope ratios have been measured in several matrices, such as ore minerals (Maher, 2007), sediments (Marechal et al., 1999), Acid Mine drainage (AMD) (Kimball et al., 2009) among others. Cu isotope fractionation studies in soil samples have been carried out, such as in hydromorphic soil profiles showing oxic soil weathering that present an enrichment in the lighter Cu isotope in the organic layers, which is explained either by plant transfer cycling or atmospheric deposition (Bigalke et al., 2010c; Bigalke et al., 2011). Weathering of black shales causes a depletion of the heavier Cu isotope in the soil material and an enrichment in the pore water, compared to the parent material (Mathur et al., 2012). An application of the study of Cu isotopes is its use as a tracer of anthropogenic contamination sources in soils and lake sediments (Bigalke et al., 2010a; Thapalia et al., 2010).

Three main pathways for stable Fe isotope fractionation can be enounced: (1) Redox transformations. Since Fe is a redox sensitive element, it is expected that Fe isotope variations will occur along the redox interfaces in natural systems either inorganic or microbially mediated. A well-studied example is given by the abiotic oxidation of $Fe(II)_{aq}$ to Fe(III) followed by its precipitation to ferric oxide or hydroxide which results in enrichment of the heavier Fe isotope in the precipitate, to the same extent as biological processes (Bullen et al., 2001). (2) Dissolution of Fe minerals. During the abiotic dissolution of goethite by proton-promoted mechanisms and dissolution of hematite no isotopic fractionation of Fe was reported (Skulan et al., 2002; Wiederhold et al., 2006).

However, during ligand controlled and reductive dissolution of goethite kinetic effects dominated the Fe fractionation at the early stages where the solution was enriched in the lighter isotope. In the late stages an equilibrium isotope effect was proposed after a depletion of the light fraction in the solution (Wiederhold et al., 2006). The oxidative weathering of sulphide-rich rocks results in the enrichment of the fluid phase in the heavier Fe isotopes under acidic conditions, and in circumneutral conditions the precipitation of Fe(III)-oxide phases leads to an enrichment of the lighter isotopes in the solution (Fernandez and Borrok, 2009). Fe isotope fractionation has also been observed during the dissolution of phyllosilicates (Kiczka et al., 2010a).(3) Biological uptake. Fe isotope fractionation by plant uptake has been recently studied, with variation from the heavier signatures in soils/roots towards lighter ones in leaves and flowers (Guelke and von Blackenburg, 2007; Kiczka et al., 2010b). The largest Fe isotope fractionation values in natural samples are registered for hydrothermal spring deposits, Fe-Mn nodules and crusts and Banded Iron Formations (BIF), but the terrestrial igneous rocks do not show large variations (Beard et al., 2003). However, the Fe isotopic fractionation (δ^{56}Fe) in natural samples is diverse and varies by ~5‰ (Bullen, 2011). Diverse field studies have been published regarding field characterisation to interpret geochemical processes and cycles using Fe isotopes (Kiczka et al., 2011; Teutsch et al., 2009). Special mention should be given to the study of the Fe isotope fractionation in mine tailings done by Herbert and Schippers (2008), where processes such as aqueous Fe(II)-Fe(III) equilibrium, microbial Fe(II) oxidation and Fe-(oxy)hydroxide precipitation are the main pathways that describe slightly higher δ^{56}Fe values below the oxidation front, compared to the values above it.

Since sulphide mine tailings often have high concentrations of Cu and Fe, it is possible that isotope fractionation of these metals due to effects of surface geochemical processes such as dissolution and reduction/oxidation of metals could be traceable, as shown in Fernandez and Borrok (2009) and Johnson et al (2008), for example. The aim of this study was to investigate the fractionation of Cu and Fe stable isotopes occurring during the oxidation of primary sulphides and formation of secondary Cu minerals below the oxidation front in tailings material. For this purpose a pyrrothite-containing tailings profile from the Laver Mine (northern Sweden) and plant samples of grass from the same

area were characterised using chemical elemental data and copper isotopic composition. The tailings at Laver are well studied. They have a distinctive boundary between the oxidised zone, Cu enriched zone and unoxidised zone (Alakangas et al., 2009; Holmström et al., 1999), and are therefore well suited to study Cu isotope fractionation during sulphide oxidation. In addition, Cu and Fe isotope composition in a profile through relatively fresh pyrite-rich tailings from the Kristineberg test site in northern Sweden was studied to account for aspects of different composition, ageing and other variables. This is one of the first studies of copper and iron isotopes performed on samples collected directly from oxidising mine waste.

2. Site Description

2.1 Laver Mine

Laver Mine is an abandoned copper mine located in northern Sweden (Figure 1). Approximately 1.537 million tons of ore with Cu, Ag and Au were extracted under the operation of Boliden AB. The operational period of the mine was 10 years, ending in 1946, when approximately 1.2 million tons of tailings were deposited in a valley nearby to the mine area. Approximately 25% of the tailings were washed away due to a snowmelt runoff in 1951 and 1952. It was then necessary to construct a second clarification pond, 3 km downstream of the tailings (Ljungberg and Öhlander, 2001). In 1974, the eroded parts of the tailings were limed, fertilized and seeded with a grass mixture (Lindgren, 1975), in order to neutralise the acidic water derived from the oxidation of sulphide minerals and slow down further sulphide mineral oxidation. The vegetation in the area could be classified as a coniferous forest with some areas of deciduous forest and bog lands and the major soil type is a podzol weathered till (Fromm, 1965). The area is drained by the small brook Gråbergsbäcken that flows on the side of the impoundment. Currently, the tailings have a vegetation cover and in the lower zones where the groundwater reaches the surface, layers of metal precipitates such as Fe-(oxy)hydroxides can be seen.

It was confirmed by Holmström et al. (1999) that the tailings deposit has a chemical zonation, with two distinct zones. As a top layer there is a strongly oxidised zone and below it, a non-oxidised zone. The main minerals present in the non-oxidised

tailings are quartz, plagioclase, pyrrhotite, chalcopyrite, pyrite and sphalerite, while in the oxidised tailings only remnants of sulphides are left and the minerals have a coating of Fe-hydroxides. The chemical analysis of these two zones show that the oxidised zone is depleted in sulphide bound metals, compared to the non-oxidised zone. The Laver tailings are relatively poor in sulphides, with a S content in the non-oxidised tailings of 7300 ppm and in the oxidised 2142 ppm. In the same study by Holmström et al. (1999) it was determined that there is also a secondary copper enrichment zone between the oxidised and non-oxidised zones, where the content of Cu and S is higher than in the other two described zones due to the ongoing process of covellite formation.

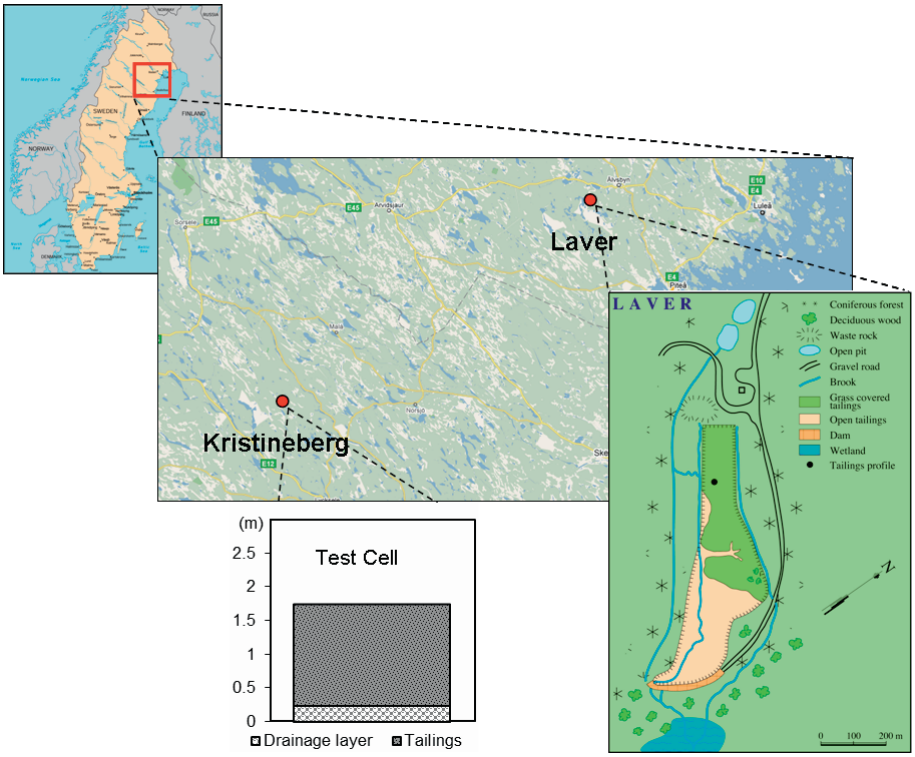

Figure 1. Location of the Kristineberg test site and Laver mine and their surroundings.

2.2 Kristineberg test cells

In 2001, six test cells were constructed at the Kristineberg mine site in Northern Swede, operated by Boliden AB. The test cells consisted of concrete cells (5x5x3 m^3), with an isolation consisting of an inert high density polyethylene liner, and containing fresh unoxidised tailings from the Kristineberg Pb-Zn-Cu mine. Five of these cells were covered with diverse materials with the purpose to evaluate different dry covers that will help to decrease sulphide oxidation in tailings materials; one cell (cell 6) was not filled up completely with tailings material and left uncovered for reference use. The fresh tailings consisted of sandy-silt particles where the main sulphide minerals were pyrite (FeS_2) 48%, and pyrrhotite ($Fe_{1-x}S$) 4.8%. The concentration of As, Cu, Cd, Pb and Zn was high in the tailings (Table 1).

Five years after construction, the uncovered test cell already showed evidence of on-going oxidation of minerals such as pyrite, pyrrhotite and sphalerite. Fe and S were released in the leachate waters along with Mn, Co, Ni and Zn in high concentrations. Dissolved Cu and Pb concentrations in the leachate waters were low because both elements were removed from the solution by co-precipitation or sorption due to relatively high pH values. The buffering capacity of the alkaline process water deposited together with the tailings was not yet exhausted (Alakangas and Öhlander, 2006).

Table 1. Average concentration of selected metals in the fresh tailings at Kristineberg mine site. (Alakangas and Öhlander., 2006).

Element	Concentration (ppm)
As	3290
Cu	1430
Cd	11.7
Pb	1270
S	141000
Zn	6080

3. Material and Methods

3.1 Sampling

Tailings and humus samples from Laver were taken in October 2009 from a pit dug using a mechanical excavator and collected manually using metal-free tools. The coordinates of the dug pit is N 65° 46.183' E 20° 14.958' (Figure 1). The tailings samples

were collected at 20 cm intervals until a depth of 150 cm and a humus sample composed mostly by degrading litterfall material (O-horizon) was also collected. All samples were stored in plastic non diffusive bags that were kept refrigerated until later preparation and analysis was performed. Grass samples from the *Festuca* genus were taken in the surrounding areas where the pit was dug, and the white cottongrass samples (*Eriophorum scheuchzeri)* were taken from a boggy area where the groundwater from the tailings reaches the surface, and crusts of goethite and lepidocrocite (Holmström et al., 1999) can be seen on the surface. The data for the pH of the soil samples is the average of 5 replicates, using a sample-water ratio of 3:5.

Tailings samples in a profile from the reference (uncovered) cell at Kristineberg were taken during the summer of 2009. Samples were collected every 10 cm to a depth of 1.1 m. The samples were stored in closed plastic cups and kept refrigerated until further analysis. For more details, see Nason (2012).

3.2 Chemical Analysis

All the chemical analysis for tailing samples in both study sites and plants samples in Laver mine were performed by a certified analytical laboratory, ALS Scandinavia AB in Luleå.

3.2.1 Tailing and humus samples

3.2.1.1 Elemental Analysis

The tailings and humus samples were analysed for Cd, Co, Hg, Ni, Pb with ICP-SFMS SFMS (Inductively Coupled Plasma – Sector Field Mass Spectrometry) and Al, Ca, Fe, K, Mg, Mn, Na, P, Ti, As, Ba, Be, Cr, Cu, Mo, Nb, S, Sc, Sr, V, W, Y, Zn, Zr using ICP-AES (Inductively Coupled Plasma – Atomic Emission Spectroscopy) according to US EP methods 200.8 (modified) and 200.7 (modified). Previously to these analyses, samples were digested by alkali fusion or microwaves-assisted acid digestion in closed vessels. The instrumental precision was better than 5%.

3.2.1.2 Cu and Fe purification.

In this study, Cu from sample digests was purified using anion exchange chromatography following the procedure described by Marechal et al. (1999) and Mason

et al. (2005) with calibration of the elution profiles for individual columns, as recommended by Borrok et al. (2007). The anion-exchange columns were made of polyethylene, had a height of 5cm and were filled with 1g of AGMP-1 resine, mesh 100-200 (Bio-Rad Laboratories). Due to differences in Cu and Fe concentrations in the two tailings sites, the load and the eluent volume used in the separation procedure varied. For some samples, Cu was partially co-eluted with Fe resulting in incomplete separation of analytes. Two or even three separations were then required in order to achieve quantitative Cu recovery (> 95%). The final volumes and eluent used during the anion exchange chromatography are shown in Table 2. The purified fractions were evaporated to dryness and re-dissolved in 5 ml 0.7M HNO_3 for the Cu fractions and 0.3M HNO_3 for the Fe fractions.

Table 2. Eluent and volume used in the anion exchange chromatography.

Separation steps	Laver tailings	Kristineberg tailings
Sample load	3 ml digested sample in 7M HCl + 0.001% H_2O_2	2 ml digested sample in 8M HCl + 0.001% H_2O_2
Matrix elution	10 ml of 7M HCl + 0.001% H_2O_2	10 ml of 8M HCl + 0.001% H_2O_2
Cu elution	24 ml of 5M HCl + 0.001% H_2O_2	12 ml of 5M HCl + 0.001% H_2O_2
Fe elution	Not performed	10 ml of 5% HCl + 0.001% H_2O_2

3.2.1.3 Cu and Fe isotope analysis

The purified Cu and Fe fractions were analysed by single-collector ICP-SFMS (Element2, Thermo Fisher Scientific, Bremen, Germany) prior to isotope analyses in order to obtain accurate analyte concentrations, as well as to ensure the absence of interfering elements. Typical operational conditions and measurement parameters can be found elsewhere (Axelsson et al., 2002; Engström et al., 2004).

Prior to Cu isotope analysis samples, working-and isotope standards were diluted to a Cu concentration of 1 mg/l and matrix matched to 0.34 M HNO_3 (sp) followed by addition of Zn at 2 mg l^{-1}, for on-line mass bias correction. In this study, the ERM-AE633 was used as delta zero reference material for Cu. According to personal communication with the supplier and the material certificate, the isotopic composition of the ERM-AE633 is traceable and identical to the NIST Cu reference standard SRM 976 since both materials have identical Cu isotopic abundance ratio(IRMM and ERM, 2002; NIST, 1994).

For Fe isotope analyses, the purified Fe fractions, working standards and isotope standard IRMM-014 (delta zero reference material) were diluted to a concentration of 2 mgl^{-1} and matrix matched to 0.34 M HNO_3 (sp). Additionally, the samples and standards were spiked with Ni (internal standard) at 4 mg l^{-1} for on-line mass bias correction.

Cu and Fe isotope analyses were performed by multi-collector inductively coupled plasma-sector field mass spectrometry (MC-ICP-MS) using a Neptune (Thermo Fischer Scientific, Bremen, Germany) operated in medium resolution mode. A Pt guard electrode was used to maximise the ion transmission. Typical operating conditions and measurement are detailed elsewhere (Stenberg et al., 2004; Stenberg et al., 2003). The isotopic analyses for all the samples and standards were performed in duplicate, using Ni for on-line mass discrimination correction in combination with the sample-standard bracketing technique, as explained by Malinovsky et al. (2003). The reported error is 2σ of the standard deviation on instrumental long-term reproducibility, based in both on-line data processing that included baseline subtraction, calculation of ion beam intensity ratios and filtering of outliers and further off-line statistical treatment. The equations 1 and 2 were used to calculate Cu and Fe delta values in per mil where the measured ratios are corrected for mass discrimination following the procedure by Baxter et al. (2006).

$$\delta^{65}Cu(‰) = \left[\frac{\left({}^{65}Cu/{}^{63}Cu\right)_{sample}}{\left({}^{65}Cu/{}^{63}Cu\right)_{ERM-AE633}} - 1 \right] \times 1000 \qquad \text{(eq. 1)}$$

$$\delta^{56}Fe(‰) = \left[\frac{\left({}^{56}Fe/{}^{54}Fe\right)_{sample}}{\left({}^{56}Fe/{}^{54}Fe\right)_{IRMM-014}} - 1 \right] \times 1000 \qquad \text{(eq. 2)}$$

3.2.2. Plant samples

Plant samples were prepared according to the same procedure prior to chemical analysis. First, the roots and the withered leaves were removed and a filter paper was used to rub the rest of the plant and therefore eliminate the superficial impurities on the plants. The samples were rinsed with deionised water to remove additional external impurities and then dried at room temperature. 10-30 g of the dried material was ashed at 550 °C overnight followed by digestion of the residue with 10 ml of hot 14 M HNO_3. Digests were evaporated to dryness and re-dissolved in 4 ml 7 M HCl. An internal standard (In) was added to a final concentration of 25 µg/l. These digests were analysed

for metal concentration, according to chapter 3.2.1.1. The method for Cu separation for isotope analysis was the same as the one reported previously in this study for the tailings and humus samples.

4. Results

4.1 Description of the samples

The visual appearance of the profile of the pit dug at Laver consisted of two distinct zones with characteristic colours. The uppermost 100 cm layer had a yellow-brown colour. Beneath this layer, the tailings had a distinctly blue-grey colour. These observations are consistent with the ones reported by Holmström et al. (1999) where the top layer had a yellow-brown colour to a depth of 50-150 cm, classifying this layer as the oxidised zone and the bottom layer as the unoxidised zone . The tailings samples at Kristineberg did not show a striking change of colour between the layers as in Laver. However, the deeper samples were unoxidised, similar to the original material used to fill up the test cell.

4.2 Profile characterisation

4.2.1 Laver Mine

The pH from the tailings samples ranged from 3.99 to 7.35 through the profile, with a pronounced increase in the most superficial samples as seen in figure 2a. In the profile, the Cu concentration (figure 2b) goes from 18.4 mg/kg in grass samples, to an average of 260.8 mg/kg in the oxidised zone and 2943 mg/kg in the unoxidised zone. The highest concentration of Cu (3330 mg/kg) was found at a depth of 100 cm, at the limit between the oxidised and unoxidised zones. Sulphur (figure 2d) had a similar trend as Cu; For Fe, the concentration profile showed a distribution fairly stable along the oxidised and unoxidised zones (figure 2c). Average chemical composition of the zones of the tailings for the whole impoundment is shown in table 3.

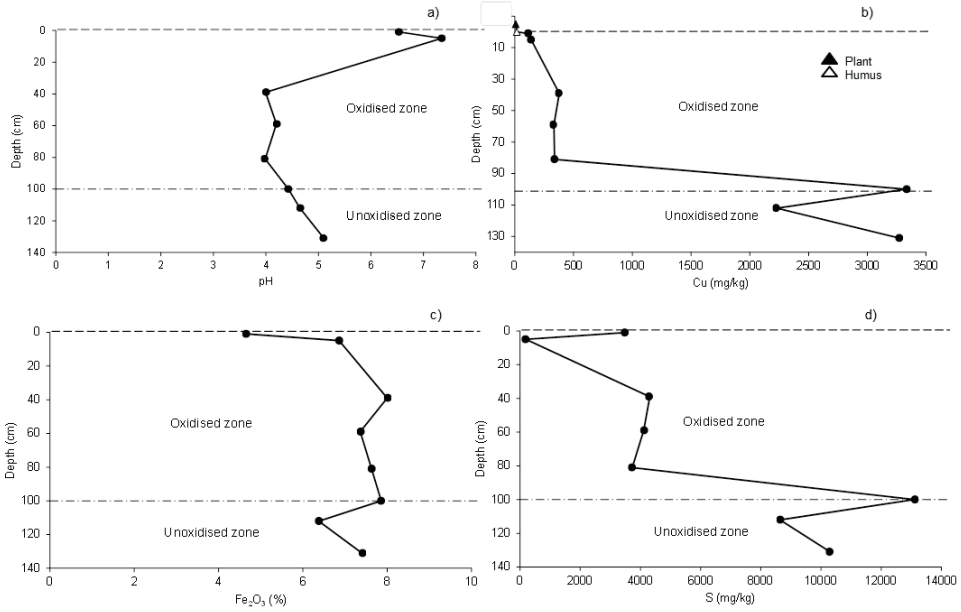

Figure 2. Profiles for pH, and concentration of Cu, Fe, and S in the tailings of the Laver Mine. The dashed line indicates the interface air-soil and the dashed-dotted line represents the oxidation front.

4.2.2 Kristineberg

The sulphide tailings in the test cell in Kristineberg have been oxidised for approximately 8 years before the sampling. An oxidation front was indicated by the low pH value (Figure 3a) and this is the feature that stands out in the pH profile. High Fe and S concentrations occurred at the oxidation front (S=324000 mg/kg; Fe_2O_3=38.4%). A marked decrease in the Fe and S concentrations from the oxidation front to the surface of the tailings is seen in the profiles shown in the figures 3c and 3d. However, the Cu concentration profile showed fewer differences between the oxidised and unoxidised zones (figure 3b).

4.3. Metals in plant samples

Two sets of plants from Laver were analysed for metal composition (Cd, Co, Cr, Cu, Ni, Pb, Zn). The first set consisted of a selection of wild grass species that were located in the area where the soil profile was taken. The second set consisted in the

specimens of cottongrass. In the table 4 the metal concentrations in the organic layer and *Festuca* samples are shown. The white cottongrass samples showed a higher concentration of Cu than the grass samples

Table 3. Average chemical composition of oxidised and unoxidised zones of the tailings at Laver Mine.

Element	**Unoxidised zone** [a] (100-130cm depth) (%TS ± s.d.)	**Oxidised zone** [b] (0-99 cm depth) (%TS ± s.d.)
SiO_2	71.1 ± 1.3	64.5 ± 12.1
Al_2O_3	12.8 ± 0.3	11. 1 ± 2.5
CaO	2.1 ± 0.4	2.11 ± 0.3
Fe_2O_3	7.2 ± 0.8	6.90 ± 1.3
K_2O	2.1 ± 0.2	1.95 ± 0.4
MgO	1.3 ± 0.2	1.22 ± 0.3
Na_2O	2.6 ± 0.2	2.2 ± 0.6
P_2O_5	0.08 ± 0.02	0.11 ± 0.09
TiO2	0.19 ± 0.04	0.16 ± 0.03
LOI	1.4 ± 0.3	2.6 ± 0.6
	(mg/kg ± s.d.)	(mg/kg ± s.d.)
As	139 ± 37	75.7 ± 21.6
Ba	295 ± 41	273 ± 49
Be	1.7 ± 0.1	1.5 ± 0.6
Cd	8.8 ±1.6	0.41 ± 0.26
Co	14.5 ± 2.7	2.3 ±1.1
Cr	68.8 ± 10.8	63.4 ± 16.6
Cu	2943 ± 618	260 ± 124
Hg	c. 0.05	c. 0.06
Mo	24.3 ± 9.6	38.3 ± 14.2
Ni	18.3 ± 5.3	6.7 ± 3.2
Pb	26.8 ± 4.8	34.1 ± 8.9
S	10680 ± 2250	3166 ± 1693
Sc	6.7 ± 1.3	5.9 ± 1.1
Sr	137 ± 56	123 ± 11
V	35.5 ± 8.1	36.1 ± 3.9
Y	17.9 ± 1.9	13.4 ± 1.0
Zn	1400 ± 243	242 ± 7.1
Zr	136 ±8	122 ± 4

[a] Includes the sample from the oxidation front

[b] Excludes samples containing organic material, such as humus.

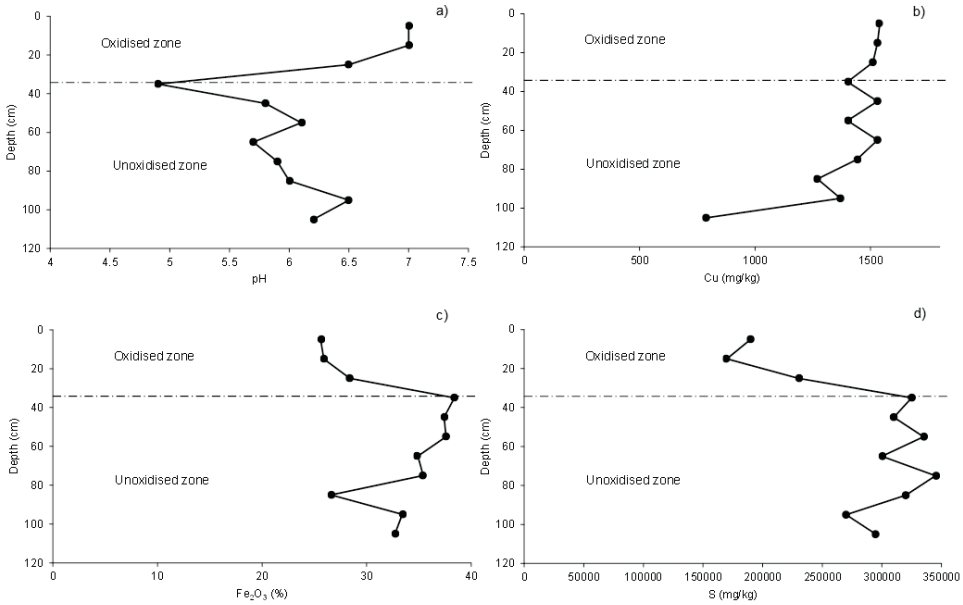

Figure 3. Profiles for pH and concentration of Cu, Fe and S in the test cell of the tailings in the Kristineberg site. The dashed line indicates the oxidation front.

Table 4. Metal concentration in humus and plants at Laver (mg/kg)

Metal	Humus	*Festuca sp.*	*E. scheuchzeri*
Cd	<0.06	<0.02	0.11
Co	0.29	0.03	0.18
Cr	2.43	0.47	0.38
Cu	16.6	7.47	18.4
Fe	2871	455	1170
K	13600	14100	31900
Ni	1.52	0.31	0.33
Pb	7.28	0.78	0.76
Zn	65	23	13

95% confidence level

4.4 Copper and Fe isotopic composition in the profiles

4.4.2 Laver Mine

The Cu isotopic composition along a profile in the tailings at Laver mine is shown in the Figure 4. The δ^{65}Cu values ranged from -4.35±0.02 to 1.31±0.03‰. The lowest value occurs in the transition from the oxidised to the unoxidised zone, where the

secondary Cu enrichment zone is located, and the highest value is measured for the deepest sample in the unoxidised zone. In the oxidised zone most values are close to zero.

4.4.3 Kristineberg test-cell

The δ ^{65}Cu profile for the test cell at Kristineberg had a range that goes from 0.16±0.01‰ to 0.39±0.03 ‰, with a clear shift in the values from the unoxidised zone with an average of 0.35±0.04 ‰ to the oxidation front with a value of 0.17±0.04‰ (Figure 5a). In the case of the δ ^{56}Fe profile at this site, the highest value - 0.24±0.01‰ was found at the oxidation front and two specific areas can be observed according to their values of δ ^{56}Fe: An unoxidised zone with an average value of -0.49±0.05 ‰, and an oxidised zone withan average value of -0.58±0.06‰ (figure 5b).

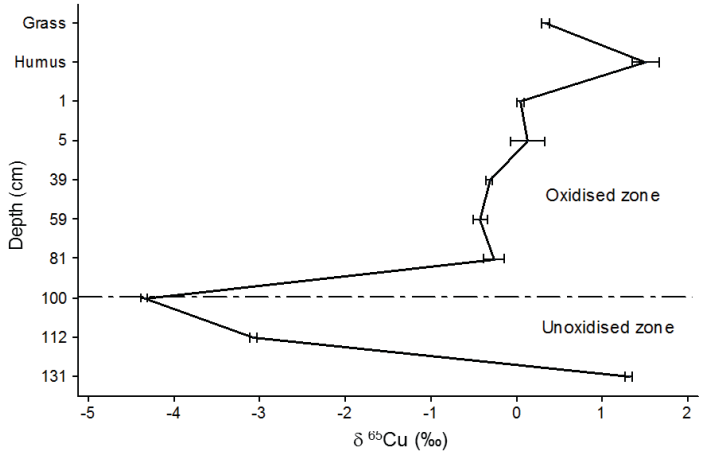

Figure 4. Vertical distribution of the copper isotopic composition in the tailings of Laver mine. The dashed-dotted line indicates the oxidation front. Bars represent 2σ.

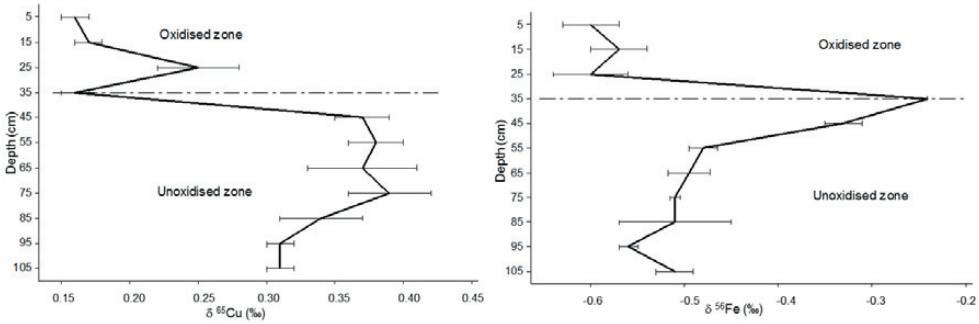

Figure 5. Vertical distribution of the copper and iron isotopic composition in the test cell in Kristineberg. The dashed-dotted line indicates the oxidation front. Bars represent 2σ.

5. Discussion

5.1 Metal and S concentration in the tailings zones

The upper oxidised zone at Laver mine showed an increase in the pH values, probably as a product of the remaining liming process back in 1974, but other causes such as a high buffering capacity due to the presence of organic matter could also explain this increase. In the layers of the profile where the pH is below 5.5, such as the lower oxidised zone, the release of acidity caused by sulphide oxidation in aerobic conditions and in lack of anionic ligands, enhances Cu^{2+} mobility, biological availability and toxicity (Martínez and Motto, 2000). In the presence of H_2S in anoxic conditions, a pH close to 4 favours the precipitation of Cu sulphides (Morse, 1987), which could cause an accumulation of Cu in the boundary between the oxidised and unoxidised zones and it is in the latest where reduced conditions prevails (Holmström et al., 1999). A distinctive Cu enrichment zone that was found by Ljungberg and Öhlander (2001) below the oxidation front could not be differentiated from the copper concentration profile, since the samples at the oxidation front and the deepest one at the unoxidised zone have roughly the same Cu concentration. Therefore, it could be assumed that the copper enrichment zone extends for over 30 cm below the oxidation front. This idea is supported by the fact that in the previous work by Holmström et al. (1999), the Cu concentration in the copper enrichment zone varied from 2000 to 3000 mg/kg, in layers that could be more than 100 cm wide. In the oxidised zone Fe is mainly bound in (oxy)hydroxides and in the non-

oxidised zone mainly to sulphides. There is a marked decrease in the Fe concentration towards the surface, product of the presence of organic matter through litter input that could be playing an important function in the possible dilution effect on the concentration of Fe in the upper areas of the profile. The elemental profile for S shows the same pattern as the Cu profile, with a good correlation (r^2=0.904), which supports the idea that this metal is present in form of sulphur-bound minerals, such as chalcopyrite in the unoxidised zone and also covellite in the zone with secondary Cu-enrichment (Ljungberg and Öhlander, 2001).

The elemental profiles in the Kristineberg test cells do not show a clear view of concentration variation of the elements along the profile, especially not in the case of Cu. However, the decrease in the concentrations of Fe and S in the oxidised zone support that oxidation processes occur, which are promoting the leaching of SO_4^{2-} and immobilisation of Fe in the oxidation front caused by its precipitation as Fe-(oxy)hydroxides (Figure 4). Due to buffering by the carbonates and $Ca(OH)_2$ added to process water and mixed with the tailings, pH has not been sufficiently low for the transport of soluble Cu downwards in the profile. Instead it has been sorbed to minerals in the oxidised zone (Alakangas and Öhlander, 2006).

As a way to examine the transport and redistribution of Cu, Fe and S within the Laver and Kristineberg profiles, the elemental mass change compared to an immobile element was calculated using the equation 3, where C^i_n and C^f_n are the initial and final concentrations of the immobile element, respectively (Zr in this case), and C^i_e and C^f_e are the initial and final concentrations of the normalised element, respectively (Cu, Fe and S in this study). The results obtained are shown in the figure 6. In Laver a similar trend for Cu and S is seen, being both enriched at the oxidation front and depleted in the oxidised zone. The presence of possible copper sulphide minerals in the oxidised zone is limited (Holmström et al., 1999). Therefore Cu is partly retained below the oxidation front and partly lost as a dissolved fraction. Through all the profile Fe is enriched in both oxidised and unoxidised zones, present not only in primary sulphide but also as (oxy)hydroxides species to different extent as reported previously by Holmström et al. (1999).

$$\text{Elemental mass change (\%)} = \left[\left(\frac{C_e^f}{C_e^i} \times \frac{C_n^i}{C_n^f} \right) - 1 \right] \times 100 \qquad \text{Eq. 3}$$

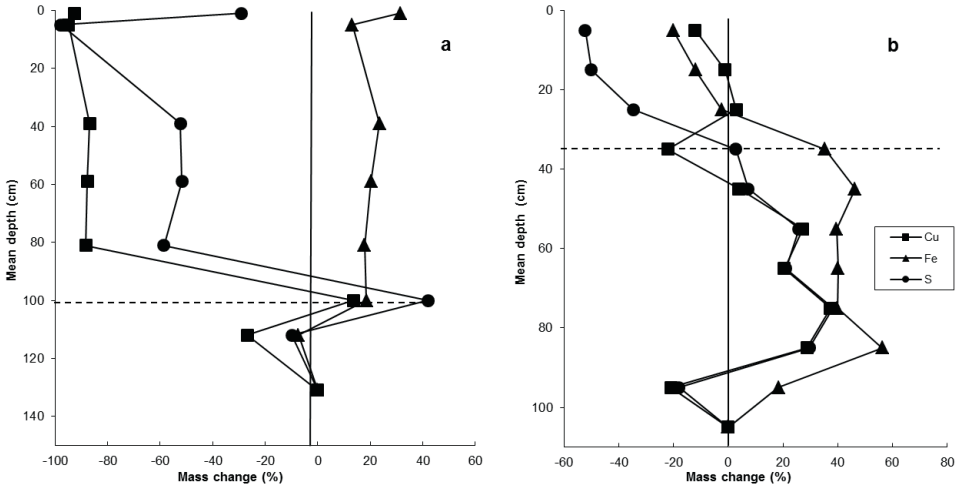

Figure 6. Mass balance plots of the profiles. (a) Laver (b) Kristineberg, modified from Nason (2012). Zr was used as the immobile element. Original materials were the samples at 130 cm (Laver) and 110 cm depth (Kristineberg). Dotted lines indicates the oxidation front

The mass change profile in Kristineberg shows a slight release of Cu in the oxidation front where the sulphide oxidation is prevalent due to the overall reaction of pyrite (Nason, 2012). The mass change results at both sites should be interpreted with caution, because of the marked heterogeneities in the samples form the oxidation to the unoxidised zone, a possible input of materials and elements via airborne transportation, illuviation, lateral flow and/or capillary rise that could eventually be adsorbed in the Fe-(oxy)hydroxides surfaces modifying the mass balance. In the case of Laver, the sample at 130 cm used as original material could be as well be part of the secondary enrichment zone of the tailings based on its high Cu concentration value.

5.3 Cu bioaccumulation in plants and humus

The Cu bioaccumulation coefficient, which is the ratio between the total metal content in plants and the total metals in soil (Alloway, 1995), for the *Festuca* sample in Laver is roughly 6%, a moderate value compared to other autochthonous plants in different copper contaminated sites that could reach values of 14% (Guo et al., 2009).

The white cottongrass samples showed a higher concentration of Cu than the grass samples. Specific metabolic processes in the grass and cottongrass plants can cause

this variation in Cu concentration to some extent. Nevertheless, the acidic pH values from the nearby Gråbergsbäcken creek (Alakangas et al., 2009) can offer a view of the pH conditions in the water saturated area where the cottongrass grows. This acidic conditions can allow a higher mobility and bioavailability of Cu (Tyler and Olsson, 2001), that can also explain the larger Cu uptake by the cottongrass. The Cu bioaccumulation coefficient of the white cottongrass at Laver (12.1%) is similar to the one in plants of the same genus grown in wetlands (11.8%) (Nyquist and Greger, 2009)

5.4 Cu isotope fractionation in the tailings

The original value of $\delta^{65}Cu$ of the fresh tailings before the oxidation of sulphide minerals started could be assumed to be 1.31 ± 0.03‰. This assumed value corresponds to the deepest sample in the unoxidised zone. While the oxidation process continues, the values of the Cu isotope ratio will change according to the geochemical processes that are occurring in the tailings. In the early stages of oxidation of sulphide bearing rocks, there is a preferential partitioning of the heavier Cu isotope into the solution phase, leaving the solid phase (chalcopyrite) enriched in the lighter Cu isotope, as pointed out by Fernandez and Borrok (2009) and in a chalcopyrite leaching study done by Kimball et al. (2009).

The difference in the Cu isotope fractionation values between the fresh unoxidised tailings and the oxidation front is $\Delta^{65}Cu_{(\text{fresh tailings-oxidation front})} = 5.66$‰. This large value is caused by several reactions, starting with the oxidative dissolution of minerals such as chalcopyrite when the infiltrated water reaches the unoxidised tailings. This process is known for the release of isotopically heavy Cu into the solution (Mathur et al., 2005). The process following this dissolution is the covellite formation, that after the release of Cu(II) into the solution by the oxidation of Cu-bearing sulphides, Fe(II) is replaced by Cu(II) at the pyrrhotite surfaces through an ion exchange reaction, as reported by Holmström et al.(1999). The formation of covellite involves the reduction of Cu, from $Cu(II)_{aq}$ to $Cu(I)_{CuS}$ in which the lighter Cu isotope is preferentially transferred from the solution to the newly formed mineral (Ehrlich et al., 2004; Pekala et al., 2011), causing in this way an enrichment of ^{63}Cu in the covellite. Since this is an open system it is expected that the kinetic transfer of ^{63}Cu to the covellite continues without reaching an equilibrium fractionation. The samples taken along the profile to obtain the $\delta^{65}Cu$ value

are bulk samples, and it is probable that in the oxidation front sample not only covellite, but also primary Cu sulphide minerals such as chalcopyrite contribute to the observed value. In this case, the δ^{65}Cu value for the precipitated covellite could be lower than -4.35‰ because the higher Cu isotopic value of minerals such as chalcopyrite present in the unoxidised and the enrichment zone, contribute to some extent to the Cu isotopic fractionation value. In the oxidation zone there is an abrupt enrichment in the heavy Cu isotope compared to the observed in the oxidation front. In this layer of the tailings the weathering processes of minerals such as pyrrhotite and chalcopyrite leave the area depleted in such sulphide minerals and its associated metals. Cu has been mobilised to the deeper parts of the tailings, released in the groundwater flow or adsorbed/coprecipitated with Fe-(oxy)hydroxides. The process of sorption of Cu(II) onto ferrihydrite preferentially takes the heavier Cu isotope (Balistrieri et al., 2008) and it is achieved mainly under equilibrium conditions. As the oxidation front moves downwards in the Laver tailings, the dissolution of the covellite previously precipitated could be a main source for Cu (II). Some of this dissolved Cu (II) might be transported upwards by capillarity through the clay/silt material and sorbed or coprecipitated with the Fe-(oxy)hydroxides. During the latter process, the fractionation occurs binding preferentially the heavier Cu isotope.

The vegetation growing on the fertilised surface of the tailings may also play a role in the fractionation of Cu isotopes in the area. The humus, a product of the decomposition of the vegetal layer, is enriched in the heavier isotope compared to the soil samples in the oxidised zone, because of the complexation of insoluble humic substances with Cu in solution. The heavier isotope forms the stronger bonds with the organic compound, as reported by Bigalke et al. (2010b). The bacteria present in the upper layers of the tailings is a sink for the heavy Cu isotope, due to the probable precipitation of amorphous nanoparticles of Cu and Fe oxides around cell membranes, as described by Mathur et al. (2005). Annual plant recycling and soil-humus mixing could contribute to the enrichment of ^{65}Cu in the upper soil layer and organic layer compared to the oxidised zone, even when there is hardly a Cu mass change between the oxidised layers and the organic layer.

At the surface, a decrease in the concentration of the heavier isotope in the grass sample compared to the one in the O-horizon (humus) sample is seen. The latter is an indication of the complexation of Cu with organic ligands which evolve to isotopically heavier species (Bigalke et al., 2010b), and that the lighter isotopes are taken up preferentially by the plants (Weinstein et al., 2011). According to the model proposed by Jouvin et al. (2012), speciation and diffusion of Cu in the solution leads to an increase in the light Cu isotope pool to be taken up by the roots of the plants. Due to the limited amount of data in our study, we cannot relate the referenced model to the tailings profile. However, we can infer that the isotopically Cu light pool from the litterfall is reutilised during plant uptake enriching the organic layers of the profile in the heavier Cu isotope to some extent.

The graph for the Cu isotope composition in the test cell in Kristineberg (Fig. 5) has similarities to the one in Laver, with a marked enrichment of the lighter isotope (^{63}Cu) in the oxidation front, caused by the oxidative dissolution of Cu-sulphide minerals and later covellite formation occurring in this zone. Holmström et al. (2001) reported a Cu enrichment zone below the oxidation front in a tailings impoundment at Kristineberg caused by the formation of covellite via transformation of pyrrhotite, chalcopyrite, galena and pyrite.

The values of the δ ^{65}Cu at Laver and Kristineberg differ, caused probably by two main reasons. The first is that there is no major Cu concentration change in Kristineberg, but at Laver there is over an 85% loss of Cu in the oxidised zone (figure 6a). In the Laver case, ^{65}Cu has preferentially left the system in the fluid phase leaving the remaining minerals enriched in ^{63}Cu. This difference in Cu loss between the two tailings is a consequence of the amount of time on which the tailings have been exposed to weathering. This time is longer at Laver than at Kristineberg, while at the later site the heavier Cu isotope has still not left the system to the same magnitude. The difference in the δ ^{65}Cu values between the oxidised and unoxidised zones in both sites is caused by different factors: initial Cu isotope ratio values that will depend on the amount and nature of the Cu-bearing minerals present in the tailings and ageing, which evidences the key effect of kinetic factors in the Cu isotope fractionation within the tailings.

5.5 Species contribution to the Cu isotopic fractionation at Laver

Biotite and muscovite contribution to the Cu isotope fractionation in the unoxidised zone could be neglected in this case. Both minerals were found as gangue minerals in the Laver tailings and their Cu content can be considered low compared to the one in chalcopyrite (Ljungberg and Öhlander, 2001). Chalcopyrite is the main mineral regulating the Cu isotope composition in the unoxidised tailings at Laver (Holmström et al., 1999).

The Cu isotope composition in the Cu enrichment zone in Laver is mainly regulated by the presence of chalcopyrite and especially the newly precipitated covellite. To the extent that the covellite is precipitated, the tailings is enriched in the lighter Cu isotope reaching a point where chalcopyrite contribution is low (i.e. 100 cm depth sample). The sample at 112 cm depth can be considered as an example of mixture of both chalcopyrite and covellite contributing to the Cu isotopic composition, even though there is no information of the mineral content in the sample.

The presence of Cu minerals in the oxidised zone is limited. Most of the Cu present in this zone is sorbed onto the Fe-(oxy)hydroxides existing in there and they mainly determine the Cu isotope composition. However, in the deeper layers of the oxidised zone some covellite might still be present since the $\delta\ ^{65}Cu$ values are slightly lighter than the ones registered in the upper layers.

5.6 Fe isotope fractionation in the Kristineberg test cell

The original $\delta\ ^{56}Fe$ values in the tailings can be considered the average of the results obtained in the unoxidised zone, where the fresh tailings is present. In the case of the test cell in Kristineberg, Fe-sulphide oxidation is the major process that occurs there (Alakangas and Öhlander, 2006). Pyrite is the main sulphide mineral in the unoxidised zone, and along with others such as chalcopyrite are oxidised as soon as the oxidation front reaches the unoxidised zone. Oxidation of pyrite is regulated by the oxidation of Fe(II) via O_2 or Fe(III) (Reactions 4 and 5). For the weathering via Fe(III) to be continued, Fe(II) has to be oxidised, according to the reaction 6.

$$FeS_2 + 7/2O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \quad \text{React. 4}$$

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \quad \text{React. 5}$$

$$Fe^{2+} + 1/4H_2O + H^+ \rightarrow Fe^{3+} + 1/2H_2O \quad \text{React. 6}$$

Since the test cell has a pH>5, it is expected that Fe-(oxy)hydroxides precipitates when the newly oxidised Fe(III) is in contact with O_2 and water. Abiotic $Fe(II)_{aq}$ oxidation and subsequent ferrihydrite precipitation favours an enrichment of the heavier Fe isotope in the solid phase as a product of a prior preferential partitioning of the isotopes in the coexisting $Fe(II)_{aq}$ species (Bullen et al., 2001). Fe-(oxy)hydroxides can also precipitate at the surfaces of pyrite, which contributes to increase of the $\delta^{56}Fe$ values at the oxidation front. It is also noted that the oxidation of pyrite bearing rocks leads to an enrichment on ^{56}Fe in the solid phase under circumneutral pH conditions (Fernandez and Borrok, 2009). The latest two cited studies provide an explanation for the increase of $\delta^{56}Fe$ around the oxidation front where Fe oxide phases are precipitating. The leached fraction containing low concentration of Fe (Alakangas and Öhlander, 2006), might be enriched in the lighter Fe isotope needed to establish an isotopic balance in the system. Leachate water was not analysed in this study, but this inference is based in the results obtained by Herbert and Schippers (2008).

As an overall view, mainly the process of pyrite oxidation in the test cell causes a slight depletion of the heavier Fe isotope from the unoxidised to the oxidised zone. This change cannot be explained by the precipitation of Fe-(oxy)hydroxides alone. Previous steps such as Fe(II) oxidation (Bullen et al., 2001) and the relationship between dissolved Fe^{2+} and Fe^{3+} (Bullen et al., 2001; Johnson et al., 2002) can cause Fe isotope fractionation along the oxic/anoxic interphase and in the oxidised zone. However, other factors such as the presence of Fe bearing silicates in the bulk material may reduce the $\delta^{56}Fe$ values, as reported by Herbert and Schippers (2008). The data set did not include information about pore water in the tailings, and for that reason it is not possible to establish the extent of a biological fractionation of Fe in the tailings, based in a mass balance calculation.

The studied profile shows a relation between the oxidation of pyrite and Fe isotope variation along the profile. The $\Delta^{56}Fe_{unoxidised\text{-}oxidised}$ in the profile is approximately

0.1 and it could be expected that this variation may increase as long as the pyrite in the test cell is still oxidising and more Fe(oxy)hydroxide precipitates as a product of such an event.

6. Conclusions

Cu isotopes were fractionated along the tailings in Laver due to the different redox-driven reactions that occur on the site. Events such as the reduction of Cu(II) to Cu(I), and subsequent covellite precipitation in a redox-boundary zone at the tailings site can preferentially enrich the precipitated mineral in the lighter isotope. Following dissolution of covellite as the result of the downward movement of the oxidation front leads to the sorption of Cu (II) onto Fe-(oxy)hydroxides. This sorption process preferentially takes the heavier Cu isotope (Balistrieri et al., 2008) from the dissolved Cu (II) pool in the oxidised zone of the tailings. Humic substances contained in the uppermost organic layer of the tailings can complexate the Cu in solution, taking preferentially the heavier isotope, which forms the stronger bonds (Bigalke et al., 2010b). This observation in a natural system has not being reported before. The Cu fractionation profile along the tailings material in the Kristineberg test site has a similar pattern as in Laver, with an enrichment of the lighter isotope at the oxidation front. The processes that cause Cu fractionation in the sulphide-rich tailings in Kristineberg are related to the ones in Laver, especially the reduction of Cu(II) to Cu(I). Differences such as the mineral composition of the tailings and time since the weathering started, results in different levels of Cu isotope ratios at the oxidation front and in the oxidised and unoxidised zones.

Fe isotope ratios in the Kristineberg test cell ranged from -0.24 to -0.60‰ with a small enrichment in the heavier isotope at the oxidation front, linked to the pyrite oxidation under circumneutral conditions occurring in that zone. Cu and Fe isotopes are both isotopically fractionated by redox processes, not only under lab conditions but also in natural systems where geochemical processes can be traceable using the variation of the fractionation of those metals

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Paper II

The role of bacteria and organic amendments in the Cu and Fe isotope fractionation in plants studied in phytoremediation of mine contaminated sites.

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Manuscript

The role of bacteria and organic amendments in the Cu and Fe isotope fractionation in plants studied in phytoremediation of mine contaminated sites.

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Abstract

Cu and Fe isotope fractionation by plant uptake and translocation is a matter of recent studies. As a way to apply the use of Cu and Fe stable isotopes in the (phyto)remediation of contaminated sites, we studied the effect of different organic amendments in a mine spoiled soil seeded with *Helianthus annuus* in pot experiments and field trials. Our results show that the addition of the microbial tool-box has an influence on the isotope fractionation of Cu and Fe by uptake and translocation in pot experiments, with an increase in the δ^{65}Cu values from soil to roots of 0.99‰, in average. In the field trials we found that the amendment with site specific bacteria and mychorriza enriches the leaves in ^{65}Cu compared to the soil and δ^{56}Fe values in the leaves are lower than those from the bulk soil, although some differences are seen according to the amendment used. Siderophores possibly released by the bacterial consortium can be the responsible of this change in the Cu and Fe fractionation. There are differences regarding the δ^{65}Cu and δ^{56}Fe values obtained in the field and pot experiments, especially in the bacteria amended samples the presence of metal-organic complex and weathering processes are believed to be the factors causing this variation. The isotope results for Cu and Fe show that pot experiments are not directly comparable to field tests.

Keywords: *Cu isotopes, Fe isotopes, phytoremediation, siderophores, mychorriza, mine spoiled soil.*

1. Introduction

Iron is essential for plant development, participating in processes such as photosynthesis and chloroplast production. It is also a major constituent of the cell redox system [1]. Despite Fe being one of the most abundant element in soils, the lack of Fe is a common cause that restricts the growth of plants because it is present in its ferric state, while Fe(II) is taken up preferentially, depending on the plant species [2]. An excessive uptake of Fe can happen in water saturated soils where the mobile Fe^{+2} is present, causing damages in the cellular structure, DNA and proteins [1, 3]. Copper is also present as a micronutrient in plants, playing an important role in CO_2 assimilation and ATP synthesis. Nevertheless, the exposure to a high Cu concentration medium induces stress and can cause injury to plants leading to plant growth retardation and leaf chlorosis [1].

The isotopic fractionation of metals as a product of higher plants uptake is a field that has been recently studied. However, those investigations have been focused mostly on the isotopic discrimination of heavy metals such as Fe, Cu and Zn by plant translocation [4-7]. Cu has two naturally occurring isotopes ^{63}Cu (abundance 69.15%) and ^{65}Cu (30.85%), Fe has three stable isotopes ^{56}Fe (91.75%), ^{57}Fe (2.12%) and ^{58}Fe(5.84%) [8]. When there is a change in the isotopic ratio between a sample and a given standard or between two reservoirs, it is defined as isotope fractionation. It has been demonstrated that there is a shifting in the Fe and Cu isotope composition from the substrate to the plants, being this change as important as the one occurring during redox reactions in soils [9, 10]. However, there are also changes in the Cu and Fe isotope composition related to the plant translocation of those metals, with an enrichment of the light isotopes in the newer leaves, as a result of cell membrane transport [6, 7]. Processes such as speciation, diffusion and reduction among others can cause Cu isotope fractionation during plant uptake and translocation [5].

The models explaining the fractionation of Fe in plants are different according to the strategy of the plants. Strategy I plants were enriched in the lighter Fe isotope and strategy II plants slightly in the heavier [4]. Different redox transition processes within the plants are associated to Fe isotope fractionation with an enrichment in the lighter Fe isotope from root to flowers [6]. Other factors that can regulate the fractionation of metals in the plant environment are the soil type on which they grow and the bacterial activity

present in the environment. In the case of Fe dissolution by siderophore-producing soil bacteria (such as *Streptomyces sp*.), the observed Fe fractionation shows that the dissolved Fe is lighter than the bulk Fe in the mineral [11] and the uptake by bacterial cells of the heavy ferric ion leaves the solution depleted in ^{56}Fe [12]. Moreover, the precipitation of amorphous and crystalline Fe minerals in the cell surface [13] might cause a change in the Fe isotopic composition of the system. The study of the fractionation of Cu by bacteria has been documented, with a preferential association of ^{65}Cu with *A. ferroxidans* cells during oxidative sulphide leaching [14], and an uptake of the lighter Cu isotope in live cell bacteria (*B. subtilis, E. coli*) product of metabolic reactions [15].

An interesting substrate to analyse the uptake of metals by higher plants and subsequent isotope fractionation, is the disposal sites of abandoned mines in which the metal content is higher than in natural conditions. Following this idea and in the framework of the interdisciplinary Umbrella Project, pots and field experiments were carried out using soil substrate from the Gessenwiese test site (Thuringia, Germany), sunflower (*Helianthus annuus*) and a specific tool-box of plant growth promoting bacteria (PGPR). In field experiments mychorriza was also used.

Having in mind that bacterial activity can change the Cu and Fe isotope composition of systems such as soil, and being this substrate essential for plant development, it could be assumed that a variation in the Cu and Fe isotope fractionation during plant translocation might occur. In this study we aim to: i) study Cu and Fe isotope fractionation by uptake and translocation of *Helianthus annuus* in a mine spoiled soil. ii) Evaluate the importance of organic soil amendments in the Cu and Fe fractionation in plants. iii) Determine the potential of pot experiments compared to field trials in metal fractionation studies. iv) Identify key (bio)geochemical process in the redistribution of Cu and Fe in the environment.

2. Materials and methods

Soil and plant material were obtained from pot and field experiments where soil from the Gessenwiese test site was used. The Gessenwiese test site is traditionally used to test decontamination technologies for heavy metals, such as bioremediation [16]. This

site is located in the former mining district of Ronneburg (Thuringia, Germany) where U was mined from 1952 to 1990 [17]. More information about the site can be found elsewhere [16, 18-21]. One interesting observation of the past studies in this area is the finding of two autochthonous *Streptomyces mirabilis* strains that are resistant to high concentrations of Ni and Zn. Yet, resistance to Cu could not be detected [21]. Pot experiments were carried out by planting directly 3 seeds of sunflower (Revierberatung Wolmensdorf, Germany) in pots filled with 800 g of the top soil from the Ronneburg mine. Five replicates were prepared, without bacteria consortium and with inoculum of PGPR. PGPR consists of ten different strains that belong to the *Enterobacter*, *Streptomyces* and *Bacilus* genus. The 16S rDNA sequences were deposited on the NCBI database under the serial accession numbers JX133221 to JX133227 (Langella and Kothe, unpublished) and according to[22]. The plants grew under controlled conditions in a greenhouse, with temperatures oscillating between 12 and 16 °C and watered with distilled water. After a period of 12 weeks the plants were harvested, separated into roots, stems and leaves. Each section was washed with distilled water, dried at 38 °C for 6 days and then milled. Homogenised undisturbed soil material from the pots was also collected.

The field trial under investigation of this study was carried out on the compost plot from June to September 2011 in the framework of the Umbrella Project. The test site in Gessenwiese was divided into several plots with different amendments: Plot 1 (BIO): Top soil with domestic compost (added in 2004). Plot 2 (MBM): Top soil mixed with PGPR, mychorrizal *Glomus intraradices* pellets (provided by Prof. K. Turnau, Jagiellonian University, Krakow) and domestic compost (added in 2004). Plot 3 (MS): Top soil mixed with *Streptomyces* from the site, and mychorrizal fungi pellets. Samples from above and underground plant material were collected after 12 weeks and separated into roots, stems and leaves. Each section was washed with distilled water and dried at 38 °C for circa 3 days and milled. Undisturbed soil material was collected and homogenized.

3. Chemical Analysis

Plant material was ashed at 550°C overnight and then digested with 10 ml of hot 14M HNO_3. After this procedure the digests were evaporated to dryness and re-dissolved in 4ml 7M HCl. This solution was analysed for Cu and Fe concentration using ICP-AES

(Inductively Coupled Plasma – Atomic Emission Spectrometry) according to US EPA method 200.8 (modified). Soil material was prepared by alkali fusion or microwaves-assisted acid digestion in closed vessels and analysed in the same manner as the plant material.

3.2. Copper and Iron purification and mass spectrometry

Solutions aliquots containing 5 μg of Cu were purified using anion exchange chromatography (AGMP-1 resin, mesh 100-200, Bio-Rad Laboratories) according to the methods described by [23] and [24]. Cu was eluted with 5M HCl and Fe with 5%HCl+0.001%H_2O_2. These eluted solutions were evaporated to dryness and re-dissolved in 5 ml 0.7M HNO_3. The samples reached a minimal recovery of 93% for Cu and 95% for Fe.

Copper and Fe isotopic compositions were measured using a Neptune MC-ICP-MS (Thermo Fischer Scientific, Bremen, Germany). Samples were diluted to Cu concentration of 0.5 mg/l and matrix matched to 0.5 M HNO_3 (sp) followed by addition of Zn at 1 mg/l, for on-line mass bias correction in the case of Cu isotope measurements. For Fe isotope measurements samples were diluted to Fe concentration of 2 mg/l and matrix to 0.5 M HNO_3, adding Ni at 4mg/l for on-line mass correction. Mass discrimination was corrected with the sample-standard bracketing method [25]. The absence of molecular or elemental interferences was demonstrated when the samples followed a mass-dependant fractionation when δ^{57}Fe and δ^{56}Fe were plotted against each other. The instrument was operated in medium resolution mode for both isotopic measurements. Typical operating conditions and measurement are detailed elsewhere [26, 27]. Errors are reported as 2σ of the standard error based in on-line data processing.

The standard ERM-AE633 was used as delta zero reference for Cu isotope measurements (traceable to NIST SRM 976 [10]) and IRMM-014 as a delta zero reference for Fe isotope measurements. Isotope ratios are expressed as parts per 1000 relative to a standard, according to equations 1 and 2.

$$\delta^{65}Cu(‰) = \left[\frac{\left({}^{65}Cu/{}^{63}Cu\right)_{sample}}{\left({}^{65}Cu/{}^{63}Cu\right)_{ERM-AE633}} - 1 \right] \times 1000 \qquad \text{Eq. 1}$$

$$\delta^{56}Fe(‰) = \left[\frac{\left({}^{56}Fe/{}^{54}Fe\right)_{sample}}{\left({}^{56}Fe/{}^{54}Fe\right)_{IRMM-014}} - 1 \right] \times 1000 \qquad \text{Eq. 2}$$

4. Results and Discussion

Effects of bacteria in the fractionation of Cu and Fe in plants under controlled conditions. To evaluate the role of the augmented bacteria in the fractionation of Fe and Cu in plants and how it is affected, the pot experiments described in the methods section were used. The results of Cu and Fe concentrations and their isotope signature are shown in figure 1. The Cu concentration and δ^{65}Cu values in bulk soils is similar whether it is amended or not with PGPR. Average δ^{65}Cu values in soils are 0.63±0.23‰ without PGPR and 0.67±0.29‰ with PGPR. A noticeable difference in the δ^{65}Cu values between both set ups is not registered for two main reasons: firstly, the time in which the experiment was performed (12 weeks) could have not been enough to propitiate a significant isotope fractionation in the whole bulk soil. Secondly, the process to homogenise the soil samples could have "masked" the isotope signature from the areas where and isotope effect is mainly expected to occur (i.e. rhizosphere). Future research that takes carefully the rhizosphere system into consideration will be needed. In the step of metal uptake from bulk soil to roots, the lower concentration of Cu in the roots compared to the soil is expected [2]. Comparing the δ^{65}Cu values of the roots grown on soil substrate with and without PGPR and the bulk soils, there is a enrichment in the heavy Cu isotope in the roots, which is different from the results of previous published studies. Jouvin et al[5] reports enrichment of the light Cu isotope in the roots of plants which is consistent with a reduction process that occurs at the root membrane during uptake. Still, this experiment was performed using hydroponic techniques, which can have little to none bacterial activity. In our study, soil with and without PGPR was used as a substrate. The PGPR is composed among others by two different strains of *Streptomyces*, which have the capacity to release siderophores *in vitro*. It has been reported that there is a significant effect on Cu uptake mediated by bacterial consortium able to synthetize siderophores, evidencing an increase of the Cu availability in the soil

and subsequent accumulation in the roots of *H. annuus* [28]. If we consider the effect of siderophores in the binding of Cu, the heavier Cu isotope should be bounded to this organic moieties around the root cell wall causing a retention of the metal in the roots. In this case the bulk outcome of the isotope measurement favours the isotopic signature of the root surface where the heavier Cu is adsorbed, instead of the isotopically lighter stele [5]. There is a slight enrichment of the heavy Cu isotope in the sample with PGPR, which can be considered as a response of the sorption of this metal into siderophores compounds released by the augmented bacteria.

The $\delta^{65}Cu$ values in our study show that shoots are enriched in the lighter Cu isotope compared to the roots (fig. 1), regardless the increased presence of bacterial consortium in the substrate ($\Delta^{65}Cu_{shoot\text{-}roots}$ -0.28±0.25‰ for non-inoculated pots, n=3 and -0.42±0.45‰ for inoculated pots, n=2). This observation is in accordance with previous research that suggest that this fractionation toward lighter isotopes in the plant is caused by a combination of diffusion and transport through the cell membranes [7]. Translocation of Cu from root to shoot involves its complexation with proteins but it does not involve a redox transformation. This Cu fractionation toward the lighter isotope is originated during xylem and phloem transport from the reduction step that takes up the light Cu isotope pool. It has been reported that plants are enriched in the light Cu isotope compared to the soil where they grew [7], but in that case soil bacterial activity is not described. In our study an enrichment in the heavier isotope compared to the substrate is seen, and it can be inferred that the presence of microbial activity at any extent could be a key factor that causes this shift in the results. In an overall view, the presence or not of PGPR has no significant influence on the isotopic signature of the shoots. Our results help to understand the complex nature of isotopic traces and pathways.

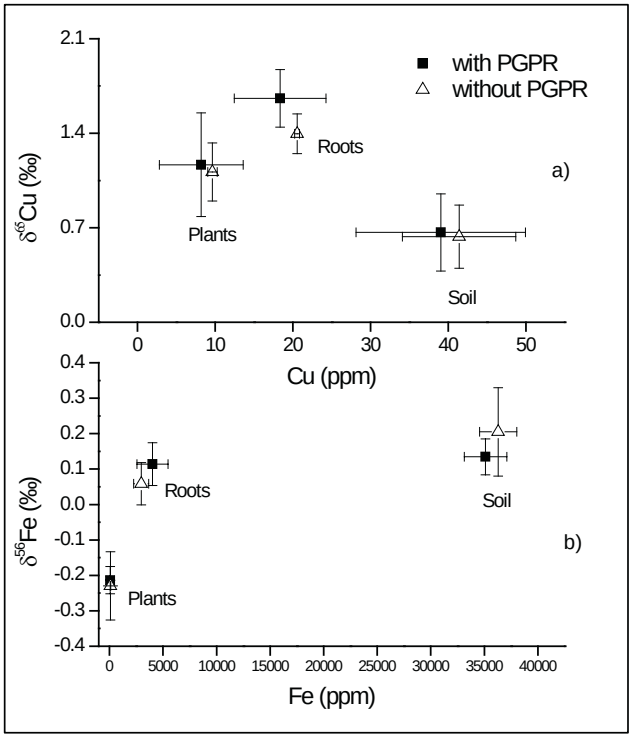

Fig 1. Cu and Fe concentration against Cu and Fe isotopic composition in soils, roots and plants from pots experiments (*Helianthus annuus*). Bars are 5% error for Cu and Fe concentrations and 2σ for Cu and Fe isotopic composition.

The δ^{56}Fe values from the pots experiments shown in figure 1 describe a trend for both inoculated and non-inoculated experiments towards the enrichment of the lighter Fe isotope from soils to roots and to plants. The concentration of Fe from soil to roots and plants decreases noticeably. The addition of the bacterial consortium to the soil in the pot experiment has a slight effect in the isotopic fractionation of Fe, being the non-inoculated soil enriched in the heavier isotope compared to the inoculated soil. *Streptomyces sp* can release the lighter Fe isotope into the medium where they live, taking up the heavier[11] and this bacteria is present in the consortium used. This difference could be larger or different in the area close to the roots, as a product of the effects of root exhudates and rhizosphere-produced compounds. However, similar masking effect as the one described in Cu isotopes can occur also in the case of Fe isotopes. There is a enrichment in the lighter Fe isotope from soil to roots in accordance with the uptake mechanisms proposed by [6, 9]. Previous work by Guelke and von Blanckenburg[4] propose that the method for

strategy II plants (such as *H. annuus*) to take up Fe from soils to roots (via Fe(III) complexation with phytosiderophores) has little no none effect on the isotope fractionation of Fe. Our work does not follow the pattern proposed by Guelke and von Blanckenburg[4]. Instead we observed isotope fractionation of Fe from soil to roots in a similar trend to those reported by Kiczka et al[6] and later confirmed by Guelke-Stellin and von Blanckenburg[9].

The presence of an appropriate Fe pool in the soils is the main reason that can cause a containment of phytosiderophores by the roots of the plants, restringing the complexation of Fe in the rhizosphere and uptaking the Fe from the soil by the roots *via* reduction processes, as strategy I plants would do [6]. The results obtained support this hypothesis, because the soils used in our experiments have a high Fe concentration (3.4%). In the same fashion, the hypothesis of the possible release of siderophores by some of the bacteria from the PGPR can also be considered as a reason of why the root samples with PGPR are more enriched in the heavier Fe isotope compared to the roots with no PGPR. According to previous studies, the complexation of Fe with siderophores could preferentially bind the heavier Fe isotope because of their stronger bond [29, 30] The uptake of Fe from bacteria-released siderophores by the roots might cause this enrichment. In any circumstance, the plant-available Fe pool which could be a combination of dissolved-reduced metal, siderophore and phytosiderophore bound metal is the one that dictates the isotopic Fe concentration in the roots, as a first step.

Translocation from roots to shoots has an effect in the Fe isotope composition of the above ground plant material. Enrichment in the lighter Fe isotope is reported in the plants compared to roots and bulk soil. This translocation process comprises Fe reduction along the transport from the xylem to the cytoplasms of the leaves, possibly of Fe(III)-citrate and the membrane transfer of Fe(II)-nicotiamide [6]. There is no noticeable difference between leaves from pots with or without PGPR. Overall $\Delta^{56}Fe_{soil\text{-}plants}$ values for non-inoculated and inoculated experiments are -0.41±0.06‰ and -0.37±0.12‰, respectively.

Bacterial effects seemed not to have caused any particular effect on the Fe isotope fractionation on the above ground biomass.

Addition of soil amendments in the field: possible processes affecting the fractionation. When comparing the results obtained from the compost plot (BIO), the compost+mychorryza+PGPR (MBM) and *Streptomyces*+mychorryza (MS) plots, in the Cu isotopic signature of the bulk soils there is a visible difference between MS and the remaining plots with the first one less enriched in the heavier isotope (fig 2a). In the root samples, the Cu isotopic signature from the MS plot is also different, but in this case it is more enriched in the heavier isotope compared to the other plots. In a natural environment the addition of bacteria and mychorriza, as well as compost has an effect in the uptake process of Cu isotopes. The bacterial function in the rizosphere can cause depletion on the lighter isotope in the surroundings of the roots [31], forcing the roots to uptake and adsorb a isotopically heavy Cu pool. The fractionation between roots and stem in all plots show that the lighter Cu isotope is preferentially taken up during this translocation process, just as explained in the previous section of this study and reported by others [5, 7]. However, the Cu isotope signature in the studied plots tends to the heavier isotope in leaves compared to stems, contrary to what it is expected, according to the work by Weinstein et al[7] that reports that when you go higher in a plant, this one becomes more enriched in the lighter isotopes. This difference could be assumed to be caused by the several environmental variables that cannot be controlled in the field, such as the introduction of atmospheric particles into the tissue of the leaves. So far we cannot confirm the cause of this result.

In the soil samples from the plot experiments, the Fe isotope signature of the three soil samples are similar. However, the value for the BIO plot is slightly lower than the one of the other soil samples (figure 2b). The compost used in this plot may have had a strong influence in the δ^{56}Fe value observed. It is known that organic material and its derivate are a sink for the lighter Fe isotope [6, 32]. A reasonable assumption is that when increasing the organic material in the soil, its Fe isotopic composition would be enriched in the lighter Fe isotope.

The general trend of the Fe isotope fractionation of the sunflower plants in all three plots is towards a lighter isotope signature as they go up in the plant (figure 2). The plants in the BIO plot are in accordance with the work of Kiczka et al[6] that describes a

fractionation towards the lighter isotope from soil to, roots, stems and leaves, as explained in the previous section of this article.

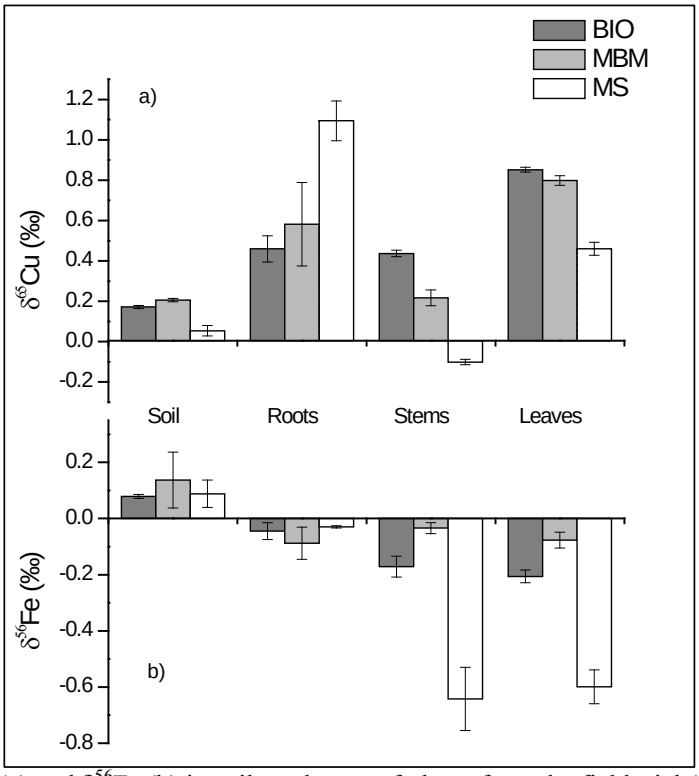

Fig. 2 δ^{65}Cu (a) and δ^{56}Fe (b) in soils and parts of plants from the field trial (*Helianthus annuus* in the Gessenwiese test site). BIO, MBM and MS correspond to the used amendments in the soil. Bars represent 2σ.

The addition of amendments into the top soil of the test site has a clear influence on the Fe isotope fractionation in the plants, with considerable differences in their δ^{56}Fe values compared to the non-amended plot. Increased presence of mychorriza and *Streptomyces* activity could help the roots to uptake the lighter Fe isotope available in the soil pool by acting as a sink of the heavier Fe [11], facilitating the selection of the lighter Fe isotope throughout the translocation processes in the plant. Another factor to take into account is the possible presence of Fe-siderophore complexes in the amended plot, which preferentially binds the heavier Fe isotope into their structure [30].

Implications for remediation It is common practice to first try out new techniques and method to remediate polluted sites in a reduced scale to then implement them in a medium or large scale. This study shows that there is a distinct difference between the results obtained in pot experiments and field trials. There is no doubt that uncontrollable variables in the field have an influence in the way that Cu and Fe are fractionated by the plants. In the top soil non-amended samples from pots experiments and field trials (BIO plot) there is a visible difference in the Cu isotope fractionation by the bulk soil and plant material, with the pot samples being more enriched in the heavier Cu isotope (fig 3a). In the field trials, the Cu isotope signature in the soil, roots and plants is enriched in the lighter Cu isotope compared to the pot experiment. It would look as the effect of the amendment of the soil could have a bigger effect on the pot experiments because the bacterial consortium can face up less competition than in the field.

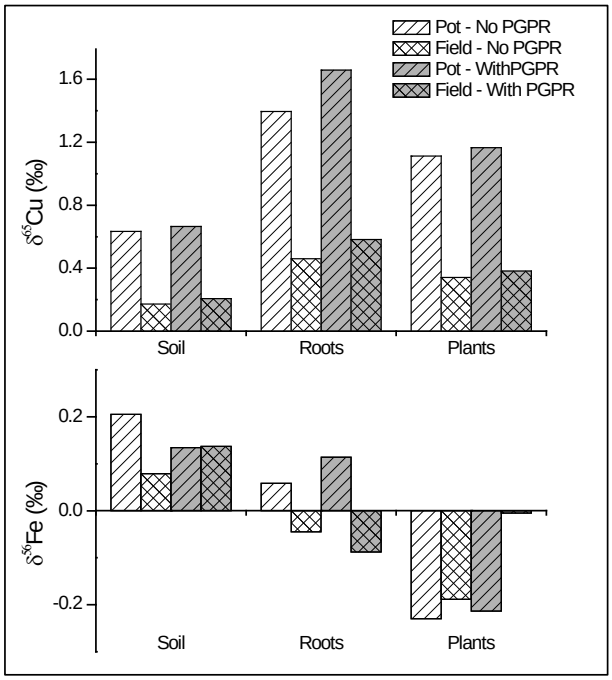

Fig. 3 Cu (a) and Fe (b) isotope composition of soil roots and aboveground plant material from pot and field experiments, with and without the addition of PGPR. Values are the average from the total of samples measured.

In the soil material, the Fe isotope signature shows slight differences between pot and field samples without PGPR (fig. 3b). This indicates that diverse factors such as weathering and biological activities can change this signature in natural samples. It is important to point out that the presence of the amendment in the field trial enriches the roots in the lighter isotope in a visible way, highlighting that the metabolic mechanism of *Streptomyces sp*. that can fractionate Fe could be the major influence in the uptake of this metal by the roots. The Fe isotope composition from soil to plants is towards an isotopically lighter material. However, the field sample with PGPR is noticeably less enriched in the lighter Fe isotope than the pot sample. Inorganic processes such as weathering and subsequent formation of Fe-(oxy)hydroxides [33]can be a cause of a depletion of the available lighter Fe isotope for plants in the field trial. Atmospheric deposition of particles in the stems and leaves can also play a function in the obtained δ^{56}Fe value, despite the rinsing of the samples with distilled water.

Great care should be taken in extrapolating results from experiments performed in the lab, at controlled conditions, to field observations. It is demonstrated that the overall fractionation could not vary (i.e. isotope fractionation from soil to plants), but steps that involve specific metabolic pathways and reactions could vary for reasons such as metal complexation with organic compounds and weathering processes. However, for phytoremediation purposes the overall result attained is the main goal, and in this case pots and field trials have similarities between them regarding the isotope fractionation of Cu and Fe.

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