Sveriges lantbruksuniversitet
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H and O stable isotope compositions of different soil water types
– effect of soil properties

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Master's Thesis in Soil Science
Soil and Water Management – Master's Programme

Examensarbeten, Institutionen för mark och miljö, SLU
2014:11

Uppsala 2014

SLU, Swedish University of Agricultural Sciences
Faculty of Natural Resources and Agricultural Sciences
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EX0788, Independent project/degree project in Soil Science – Master´s thesis, 45 credits, Advanced level, A2E
Soil and Water Management – Master's Programme 120 credits

Series title: Examensarbeten, Institutionen för mark och miljö, SLU
2014:11

Uppsala 2014

Keywords: soil water, centrifugation, cryogenic vacuum distillation, H and O stable isotopes, soil processes

Online publication: http://stud.epsilon.slu.se

Cover: Centrifuge with soil filled containers. Photo: Martine Stoll, 2014.

Acknowledgements

First of all, I would like to thank my supervisors Dr Christophe Hissler, Dr Arnaud Legout and Dr Mats Larsbo for their continuous support and advice. I also would like to express my thanks to the Public Research Centre – Gabriel Lippmann (CRP-GL) in Luxembourg who offered me this project. From there I would like to especially thank Jérôme Juilleret, who helped with the field sampling and provided the soil classifications, and François Barnich, who carried out the pH analysis. Furthermore, I very much appreciated the collaboration with the National Institute for Agricultural Research (INRA), Nancy, France, which offered their soil laboratory for centrifugation. Moreover, from INRA I would like to thank Christian Hossann for carrying out the cryogenic vacuum distillations and Claude Bréchet for the large amount of isotope analyses. I also would like to acknowledge apl. Prof Dr Christoph Emmerling from the University of Trier for providing his facilities for the measurements of microbial soil respiration. Lastly, I would like to express my gratitude towards my colleagues from the CRP-GL and my family for their moral support.

Abstract

Stable isotope compositions of water are usually investigated to trace the flow of meteoric water from precipitation through the soil matrix to ground water, stream water and plants. This tracer data can much reduce model uncertainty and give further details on water and solute movement. Still, the distribution of hydrogen and oxygen isotopes through the soil matrix and the isotope exchanges between the soil water and other soil compartments (living organisms, minerals, exchange capacity, organic matter) are still poorly studied. In this study different soil water types were extracted through gravity, centrifugation and cryogenic vacuum distillation in a laboratory experiment. The drainage water, capillary water and tightly bound water, *i.e*. soil water below the pressure of $pF_{4.2}$, were analysed for H and O stable isotopes. The isotopic signatures of deuterium (δD) and oxygen-18 (δ^{18}O) were used to determine differences between water types and between soils from 5 sampling sites in Luxembourg and northern France. Furthermore, the water extraction methods were tested for the suitability to separate water types efficiently. The H and O fractionation of drainage water was completely attributed to evaporation from the collection bottles. Centrifugation was found inadequate to separate capillary water into weakly and moderately bound waters for the soil types used in this study. Moreover, the isotopic fractionation of tightly bound water from the reference water was largely caused by inefficient cryogenic vacuum distillation but not exclusively. Despite these problems it was shown that the capillary and tightly bound water generally did not mix. The observed significant differences between sampling sites were most likely caused by clay content, total organic carbon content (TOC) and microbial soil respiration (MSR). On the other hand, no differences in isotope compositions were observed between soil horizons, despite the fact the TOC and MSR largely differ between horizons. In conclusion, extraction techniques currently used to separate the soil solution from the soil matrix need to be improved for the study of the interactions between the infiltrated water in the soil and the different soil compartments.

Keywords: soil water, centrifugation, cryogenic vacuum distillation, H and O stable isotopes, soil processes

Popular science summary

Soil is the foundation of life on land. Many of its functions are provided through the interaction with water moving through the soil. For instance, soil water ensures the transport of nutrients from the soil to the vegetation and connected water bodies. Much is already known about how water moves through the soil. For example, three different water types have been accepted by the scientific community: 1) water draining freely through large pores by gravity, 2) plant available water which is trapped in small soil pores, and 3) tightly bound water, which not even plants manage to draw out of the tiny pores it is trapped in. However, the way these water types mix and interact is not well-studied yet.

There are several reasons why it is important to know the movement of each water type through the soil in detail. Firstly, water has a big impact on the development of soils. Therefore, knowing the water movement can give insight into the past and future development of soils. Also the movement of nutrients contained in the soil water helps plan which methods need to be put into practice to maintain a sustainable forestry and agriculture. For example, to determine when and how much fertilizer needs to be applied to avoid that it is flushed out of the soil without taking effect first. Adding on to this, the movement of soil water is also taken into account during the assessment of pollutants such as pesticides and heavy metals. Primarily, to identify the impact pollutants can have on the environment, but also to assess how long it may take for this impact to be reverted.

Stable isotope compositions of water are usually investigated to trace the flow of water from rainfall throughout the soil matrix to groundwater, streams and plants. Isotopes are atoms of the same element but with different weights. Non-radioactive isotopes are considered stable. Hydrogen has two stable isotopes: the lighter hydrogen (^{1}H) and the heavier deuterium (^{2}H or D). Oxygen has three stable isotopes: ^{16}O, ^{17}O and ^{18}O. Water molecules (H_2O) can be made up of any combination of these isotopes. Researchers usually only consider the ratio of D to ^{1}H and of ^{18}O to ^{16}O to determine water mixing. Hence, these were the isotopes used in this study to gain fundamental knowledge about water mixing and to identify the influence processes taking place in the soil can have on the isotope composition of soil waters.

Certain chemical reactions and microbial processes preferentially use the lighter isotope compared to the heavier one because it requires less energy. However, thus far, the influence of soil processes on isotope compositions of different water types has not been quantified. To be able to analyse samples of all water types, water was extracted from various soil types by different methods in a laboratory experiment. The methods included gravity, centrifugation and cryogenic vacuum distillation.

Drainage water seemed to have a similar hydrogen and oxygen isotope composition than plant available water. On the other hand, the results of this study showed that plant available water and tightly bound water generally had differing isotopic compositions and therefore could not have mixed. One factor having an impact on the isotope composition of plant available water and tightly bound water was likely microbial soil respiration. Furthermore, the amount of clay and organic carbon present in the soil was linked to differences in isotopic composition of plant available water and tightly bound water between different soils. These

results indicate preferential use of lighter isotopes during microbial processes and water adsorption to clay particles.

The used water extraction methods presented strong limitations to the interpretation of the results. In conclusion, improved water extraction methods are needed before the gained knowledge from this study can be used to improve other fields of study which require a deeper understanding of water mixing and soil water interactions, for example in hydrological models and environmental impact assessments.

Abbreviations

^{18}O	Oxygen-18 isotope
ANOVA	Analysis of variance
B	B horizon or Breuil sampling site
C	Control or carbon
CEC	Cation exchange capacity, cmol+ kg^{-1}
CI	Confidence interval
CPE	Ceramic plate extraction
D	Deuterium
DM	Dry matter
DWe	Final drainage water
DWs	Initial drainage water
E	Ell sampling site
ES	Experimental soil samples
F	Matric potential
FC	Field capacity
FS	Fresh soil samples
GMWL	Global meteoric water line, $\delta D = 8 \times \delta^{18}O + 10$
GWC	Gravimetric water content, %
H	Hydrogen or Huewelerbach sampling site
I	Isotope
IRGA	Automated infrared gas analyser
IRMS	Isotope-ratio mass spectrometry
MSR	Microbial soil respiration, µg CO_2-C/ g DM / h
N	Nitrogen
O	Oxygen
PC	Principal component
PCA	Principal component analysis
pF	Logarithm base 10 of the absolute value of the matric potential, F, in cm
R	Rumelange sampling site
rpm	Revolutions per minute
TC	Total carbon, %
TOC	Total organic carbon, %
UP	Ultrapure water (18.2 MΩcm)
W	Weierbach sampling site
wt	Weight

Words used interchangeably

Isotopic signature and isotope composition

Drainage water and gravitational water

Weakly & moderately bound water and capillary water

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1. Introduction

Soil water ensures the transport of matter, e.g. nutrients, from the soil to the vegetation and connected water bodies. Three types of soil water have been acknowledged according to their retention strength and plant availability: 1) gravitational water, which moves downward through soil macropores eventually contributing to groundwater recharge and streamflow, 2) capillary water, which resides in the soil micropores and is mostly available to plants, and 3) tightly bound water below the wilting point (-1500 kPa), which remains unavailable to plants (Gobat, 1998; Cosandey & Robinson, 2000). The water tension is directly linked to the soil water content. Three main groups of forces work to maintain the water in the soil against the force of gravity: 1) capillary forces, 2) absorption by solids (F), and 3) osmotic forces, *i.e*. suction exerted by plant roots. Furthermore, soil water is influenced by many factors including chemical and physical soil characteristics, soil depth, vegetation type and evaporation. Among the soil properties, three influence the water regime the most: 1) the soil texture regulates the water retention strength, 2) the soil structure regulates the water conductivity and 3) the soil porosity limits the water storage capacity (Gobat, 1998; Cosandey & Robinson, 2000).

Recent hydrological studies already consider these three different water types in the understanding of hydrological processes. Nevertheless, the dynamics of the mixing processes that occur between the mobile and the bound waters during and after rain events is still poorly studied. Stable isotope compositions of water are usually investigated to trace the flow of meteoric water from precipitation throughout the soil matrix to groundwater, stream water and plants (Araguás-Araguás *et al.*, 1995; Goller *et al.*, 2005; Kværner & Kløve, 2006; Machavaram *et al.*, 2006; Li *et al.*, 2007; Klaus *et al.*, 2013; van der Heijden *et al.*, 2013). In addition, hydrogen and oxygen stable isotopes can be used to determine the waters residence times within the soil compartment and to estimate the mixing of different waters in the soil (McGuire *et al.*, 2002; Brooks *et al.*, 2010). This tracer data can much reduce model uncertainty and give further details on water and solute movement (McGuire *et al.*, 2007; van der Heijden *et al.*, 2013). Moreover, the investigation of different soil water types is important for pedological studies, environmental quality assessment (e.g. pesticide and heavy metal leaching), nutrient cycling analysis and nutrient budgets (McGuire *et al.*, 2002; van der Heijden *et al.*, 2013).

Still, the distribution of O and H isotopes throughout the soil matrix needs to be more clearly understood. So far the perception is that the isotope profile of water observed in soils is solely due to evaporative fractionation and its shape is dependent on climate and soil parameters (Araguás-Araguás *et al.*, 1995). Until today the influence of biogeochemical processes on the spatio-temporal variability of $\delta^{18}O$ and δD of the soil solutions was rarely quantified. The hydrogen and oxygen exchanges between the soil water and the other soil compartments (living organisms, minerals, exchange capacity, organic matter) are still poorly studied and require deeper investigations. For instance, the weathering of silicate minerals produces O^{2+} in the soil solution, exchange capacity in acidic soils releases and stores large quantities of H^+, and the degradation of the organic matter could also impact the oxygen and hydrogen isotope ratios of the soil water. Plants also release H^+ and OH^- when they take up nutrients from the soil solution. Yet, are we able to quantify the contribution of these different processes to the hydrogen and oxygen isotopic composition of soil water?

1.1. Aim

The aim of this study was to identify differences in the hydrogen and oxygen isotopic composition of 4 different types of soil water: drainage water (< ~$pF_{1.8}$), weakly bound water (< $pF_{2.5}$), moderately bound water ($pF_{2.5}$ - $pF_{4.2}$) and tightly bound water (> $pF_{4.2}$). Moreover, the study aims to determine whether relationships between the isotope composition of those soil water types and soil properties in forest ecosystems can be quantified.

The aim was not to obtain results representative of field conditions but, in a first instance, to observe the behaviour of specific soils and water types using laboratory experiments. Furthermore, the impact of plant activity on water isotopic signatures was not included.

1.2. Objectives

The 4 detailed objectives of this study were to:

- determine whether different types of soil water, *i.e.* drainage, weakly bound, moderately bound and tightly bound water, present distinct hydrogen and oxygen isotopic signatures,
- determine whether hydrogen and oxygen isotope signatures of the different water types are related to biological, physical or chemical soil properties,
- study the soil water balance in respect to the hydrogen and oxygen isotope composition between mixtures of the above mentioned types of soil water,
- test the suitability of two extraction methods, centrifugation and cryogenic vacuum distillation, commonly used for separating different soil water types. This objective will determine whether the above mentioned objectives can be addressed with confidence.

1.3. Hypothesis

Null Hypothesis, H_0: The hydrogen and oxygen isotopic signatures do not differ significantly between the different water types: drainage water, weakly bound water, moderately bound water and tightly bound water.

Alternative Hypothesis, H_1: The hydrogen and oxygen isotopic signatures differ significantly between the different water types: drainage water, weakly bound water, moderately bound water and tightly bound water.

Null Hypothesis, H_0': The biological, physical or chemical soil properties in forest soils do not directly influence the hydrogen and oxygen isotopic signatures of soil water.

Alternative Hypothesis, H_1': The biological, physical or chemical soil properties in forest soils directly influence the hydrogen and oxygen isotope signatures of soil water.

It is difficult to make predictions about the direction and degree of differences in isotopic compositions in soil water because the interactions between water, soil and living organisms are very complex. Therefore, no specific relationships were stated for the alternative hypotheses.

2. Literature review

2.1. H and O stable isotopes

Isotopes are elements with the same number of protons but differing numbers of neutrons in each atom. Due to additional neutrons in the nucleus some isotopes are heavier than others, *i.e.* they have a higher mass number. Non-radioactive isotopes are considered stable. Hydrogen, for example, has two stable isotopes: hydrogen (^{1}H) and deuterium (^{2}H or D). ^{1}H (> 99.9 %) is much more abundant than D (< 0.02 %). Oxygen has three stable isotopes with large differences in their approximate abundances: ^{16}O (99.63 %), ^{17}O (0.04 %) and ^{18}O (0.20 %). Together, hydrogen and oxygen isotopes can form 9 different isotopic configurations of water molecules (H_2O) (Faure, 1986). However, in hydrological studies of water oxygen-17 is usually not considered.

Isotope compositions of substances are expressed in ratios of the heavier isotope (I_h) to the lighter one (I_l) relative to the internationally accepted Vienna Standard Mean Ocean Water (VSMOW) as seen in **Equation 1** (Kendall & Caldwell, 1998).

$$\boldsymbol{\delta I_h}\ (‰) = \left[\frac{(I_h/I_l)sample}{(I_h/I_l)VSMOW} - \mathbf{1}\right] \times \mathbf{10^3} \qquad \textbf{Equation 1}$$

Delta (δ) is always expressed for the heavier isotope and given in units of per mil, ‰ (**Equation 1**). Positive values of δ indicate an enrichment of the substance in the heavier isotope relative to the standard while negative values indicate that the substance is depleted in the heavier isotope. During some processes the lighter isotope is preferentially used. For example, ^{16}O and ^{1}H are preferentially evaporated due to their higher vapour pressure compared to ^{18}O and D, which remain in the liquid phase. This fractionation of the isotope composition of a substance is conveyed by a change in δI_h (Faure, 1986). Furthermore, the relationship between δD and δ^{18}O in precipitation and surface waters, which usually follows the global meteoric water line (GMWL: $\delta D = 8 \times \delta^{18}O + 10$), will deviate from the GMWL as a result of evaporation (Gibson *et al.*, 2008). Moreover, evaporative fractionation depends on the atmospheric humidity and decreases with increasing temperature. This dependency means that above a specific humidity and temperature there is no more fractionation (Barnes & Turner, 1998).

2.2. Isotopic fractionation in soil

Each process in the soil matrix involving hydrogen and oxygen atoms and which preferentially uses the lighter (or heavier) isotopes leads to fractionation in soil water. For example, the preferential isotope exchange reactions between minerals, e.g. clay and sedimentary rock, and the soil water causes fractionation of both substrates (Faure, 1986). There are many more processes taking place in the soil which could influence the isotopic signature of H and O in soil water. It is likely microbes would prefer the lighter isotopes because they form weaker bonds than their heavier counterparts (Faure, 1986). A weaker bond means that microbes need less energy to break those bonds. For example, a study by Dijkstra *et al.* (2006) concluded that fractionation occurs during microbial processing of

nitrogen (N) and carbon (C) as microbes become enriched in ^{15}N and ^{13}C relative to the extractable N and C pools in soil organic matter (SOM). The enrichment in ^{15}N and ^{13}C in microbes does not occur because they preferentially take up organic matter enriched in these heavy isotopes (Lerch *et al.*, 2011). It is likely that the microbes only process the lighter isotopes. Furthermore, research showed that O-exchange occurring between water molecules and phosphates when mediated by microbes left the phosphate enriched in ^{18}O (Blake *et al.*, 1997). Similarly, Kool *et al.* (2009, 2011) showed that water may exchange oxygen atoms with nitrogen oxides (NOx) through biochemical reactions during nitrification and denitrification pathways. Yet, the changes in isotopic signatures of the resulting water and NOx molecules were not presented. Moreover, denitrifiers usually leave the remaining substrates higher in heavier isotopes than the product they release, *i.e*. the $\delta^{15}N$ is higher in the substrate NO_3^- then the resulting N_2O, and similarly $\delta^{15}N$ and $\delta^{18}O$ are increased in the remaining N_2O when N_2 is produced. Conversely, occasionally the product was more enriched in the heavy isotope than the remaining substrate. Nonetheless, if generally lighter oxygen atoms (^{16}O) are preferentially cleaved from the substrates, water may become lighter as mainly ^{16}O recombines with H^+ (Snider *et al.*, 2009).

Preferential behaviour in microbes may be restricted to certain circumstances, e.g. slow reactions under non-stress conditions. Overall, it is uncertain whether the net fractionation of H and O isotopes through microbial reactions would enhance or dampen the evaporative fractionation effect, e.g. some processes remove the lighter isotopes from the soil water while others may add some. Also, the net fractionation effect is likely dependent on soil conditions such as the level of saturation, microbial community composition and the water's mean residence time. For instance, in an unsaturated soil aerobic reactions dominate while in saturated soil anaerobic reactions take over. Also, the longer the water is in contact with the soil matrix the more influence the soils can have on the H and O isotope composition of soil water.

2.3. H and O isotopic signatures of different soil water types

Marques *et al.* (1996) observed that *in situ* soil water extracted by zero-tension lysimeters (ZTL) differed in their chemical composition from soil solutions extracted by tension lysimeters (TL) at 60 kPa (~$pF_{2.8}$). Other studies found similar differences in isotopic signatures between the TL water, ZTL water and stream water (Taylor *et al.*, 1991; McGuire *et al.*, 2002; Penna *et al.*, 2013). On the other hand, O'Driscoll *et al.* (2005) observed that TL water had similar isotope compositions to ZTL water. Not all studies indicated at which pressure the soil water was sampled with the tension lysimeters, which makes it difficult to compare studies. The fact that some studies found a difference in H and O isotopic signatures between soil water types may be because the waters bound to soil particles at different tensions behave differently; mainly they do not mix. Brooks *et al.* (2010) went further and directly challenged the concept of translatory flow still used as an assumption in hydrological studies by studying the different H and O isotopic signatures in many different water types: precipitation, stream water, two soil water types, groundwater and tree water. During translatory flow, water infiltrating the soil profile pushes down the "old" water until it eventually reaches the stream. This concept also assumes that soil water at any depth is well mixed. Hence, the water trees take up should be the same as the water reaching the stream from below ground. However, the data collected by Brooks *et al.* (2010) showed that

TL soil water, extracted with a tension of 60 kPa, has a different isotope composition than more weakly bound soil water in a Mediterranean climate and their signatures differed from stream water. Furthermore, the sampled tree water was also characterised by a different isotopic signature than the stream water. As the isotope signatures of TL soil and tree water were similar and both differed from stream water, Brooks *et al.* (2010) concluded that trees take up TL water from pools which do not noticeably contribute to stream water. The study by Penna *et al.* (2013) also found that TL water and tree sap presented the same isotopic composition but different from stream water. Importantly, in all studies the assumption was made that trees do not fractionate water during uptake (Kendall & McDonnell, 1998).

Additionally, soil water isotope ratios for ^{18}O and D decreased consistently with soil depth. The reason for this difference in isotope ratios with depth is uncertain. Yet again, the increased depletion with depth of heavy isotopes suggests that translatory flow is not occurring. As this pattern stays the same over the years, the TL water in small pores does not mix with gravitational soil water. In fact, Brooks *et al.* (2010) found that TL water was more depleted in heavy isotopes than gravitational water on each sampling day. Samples for both types of water were collected at the same depth and location. Small pores with a small neck drain last, meaning that during the summer months the large pores are empty while the smallest pores still contain the water from the autumn rain which couldn't be drained by gravity (Brooks *et al.*, 2010). Evaporation and suction applied by plant roots are two ways to drain small soil pores to the wilting point with only the latter being notable at greater depth. Still, it remains questionable whether the results found in the case study of Brooks *et al.* (2010) can be generalized and whether the processes they suggest would occur under different climate conditions and for different soil types.

2.4. Soil water extraction methods

This study focuses on two methods to extract soil water in the laboratory: centrifugation and cryogenic vacuum distillation. However, there are many alternatives which are not discussed including azeotropic distillation with kerosene or toluene, micro-distillation with zinc, $H_2O_{(liquid)}$ – $H_2O_{(vapour)}$ equilibration laser spectroscopy and mechanical pressing. Centrifugation separates the liquid from the solid phase by applying pressure through acceleration. During cryogenic vacuum distillation soil water is evaporated through a hot water bath and forced to condensate in a small collection tube by an extremely cold phase, usually liquid nitrogen. The purpose of the vacuum is to remove all pre-existing water from the pipes and connected tubes. In addition, the vacuum decreases the necessary temperature for evaporation. Cryogenic vacuum distillation and water vapour equilibration seem to be among the most commonly used lab extraction methods in hydrology. Yet, these methods can only reflect the total soil water and cannot divide it into different water types. Note that using gravity in the lab provided similar isotopic signatures to the use of zero-tension lysimeters and centrifugation showed similar results to using tension lysimeters (Marques *et al.*, 1996).

A study by Zabowski & Ugolini (1990) used centrifugation at a low speed of 1000 revolutions per minute (rpm) to extract soil water held between ~0 - 30 kPa and at a high speed of 10 000 rpm to remove water between 30 - 3000 kPa. Waters extracted by low and

high centrifugation speeds did not differ in their soil solution regarding cation, anion and carbon concentrations or pH. Zabowski & Ugolini (1990) suggest that the results did not differ because the soil water equilibrated among different pore sizes during the lag period between sampling and analysis. Element concentrations measured in centrifugation solutions were generally higher than in low-tension lysimeter (10 kPa/~$pF_{2.0}$) solutions. Also, there was a greater seasonal variation of parameters in centrifugation water compared to lysimeter water. These differences probably occurred because of a shorter mean residence time of the weakly bound lysimeter water. This limited interaction period leaves little time for exudates and nutrient uptake by roots and microbes to have a considerable control over the soil water composition (Zabowski & Ugolini, 1990). These results suggest that H and O isotope compositions of tightly bound water are even more influenced by the soil matrix as the mean residence time is relatively long. Moreover, the study by Zabowski & Ugolini (1990) concluded that sampling with centrifugation leads to much higher soil disturbance than the in-field collection with lysimeters.

A study conducted by 14 laboratories compared several methods of soil water extraction including cryogenic vacuum distillation and centrifugation (Walker *et al.*, 1994). Overall there were large differences in the isotope results between labs: up to 30 ‰ for D and 3.4 ‰ for ^{18}O. The variation in isotopic composition of the extracted water through various methods was greater for clays than sands and decreased with water content. Incomplete extraction was the most likely cause for the variations. The study highlights the need to develop standard protocols for the extraction of water from soils for isotopic analysis, which even today are not yet in place (Walker *et al.*, 1994).

Generally, incomplete distillation of soil water leads to fractionation of hydrogen and oxygen isotopes (Araguás-Araguás *et al.*, 1995; West *et al.*, 2006; Koeniger *et al.*, 2011). However, a very small amount of water may remain in pure sands (< 2 %) without causing fractionation (Araguás-Araguás *et al.*, 1995; West *et al.*, 2006). Also, the initial water content does not influence the precision or accuracy of the H and O isotope measurements (Koeniger *et al.*, 2011). Still, various papers mention very different times needed for cryogenic vacuum distillation to avoid fractionation of H and O isotopes. Sandy soils have been found to need a distillation time of 30 minutes to avoid fractionation (West *et al.*, 2006). This is more than twice as long as the 7.5 minutes to 15 minutes reported by Koeniger *et al*. (2011) and much less than the 7 h reported by Araguás-Araguás *et al*. (1995). Importantly, distillation times vary between soil types and generally increase with clay content (Araguás-Araguás *et al.*, 1995; West *et al.*, 2006). West *et al.* (2006) observed that soil water distillations of clayey soils needed to run for 40 minutes to avoid fractionation. On the other hand, Koeniger *et al*. (2011) states that their modified extraction protocol cannot be used for soils with high silt or clay content, e.g. silt sand and silt clay, because the precision of the isotopic analysis largely decreased for distillations of soil with increasing silt and clay content. Regrettably not all studies indicated all parameters of their cryogenic distillation method. For instance, some studies do not mention how the soils were prepared while others are missing the temperature and vacuum settings of the distillation set-up. This lack of information makes it difficult to explain the large differences in the results between various cryogenic distillation studies.

It is important to note that nowadays the soil water extraction causes higher inaccuracies in isotope signatures than the isotope analysis itself. New technologies are required to completely eliminate the need for soil water extractions for isotope analysis. This type of technology already exists for leaf water (West *et al.*, 2006). Hence, it also seems plausible to achieve for soil water.

3. Materials and Methods

3.1. Site and soil descriptions

For the purpose of this study, 4 soils in Luxembourg (sites W, R, H, E) and 1 soil in Burgundy, north-eastern France (site B), were sampled in forest ecosystems (**Figure 1**). The forests growing on the sampling sites ranged from coniferous to mixed to deciduous tree stands (**Table 1**). All sites lie in a maritime temperate climate according to the Köppen-Geiger climate classification system (Peel *et al.*, 2007). Among the soils were 3 Cambisols, 1 Luvisol and 1 Planosol (FAO, 2014). The soils were chosen to cover a large range of soil organic matter content and soil textures: sand, sandy loam, loam, and clay loam (**Figure 2**). All pedological characteristics of the soils are presented in **Table 2**.

Figure 1. Map of soil sampling sites (W = blue, R = red, H = green, E = orange and B = purple). Photo: Map data ©2014 GeoBasis-DE/BKG (©2009), Google

Table 1. Sampling site and soil descriptions.

	Weierbach W	Rumelange R	Huewelerbach H	Ell E	Breuil B
Location	Weierbësch forest, LU	Origerbësch forest, LU	Heischel forest, LU	Stiefeschbësch forest, LU	Breuil-Chenue state forest, FR
UTM Coordinates	Lon: 53013 Lat: 99699	Lon: 66954 Lat: 57873	Lon: 59822 Lat: 87097	Lon: 58346 Lat: 90315	Lon: 576918 Lat: 5239094
Altitude (m)	499	428	412	286	650
Forest type	Douglas-fir, spruce	beech, oak	beech	mixed deciduous	beech, oak, Douglas fir
WRB soil classification	Leptic Cambisol	Ferralic Cambisol	Chromic Luvisol	Dystric Endodolomitic Planosol	Alumic Cambisol
Soil texture	silty clay (A) & loam (B)	clay loam	sand	silt loam (A) to loam (B)	sandy loam
Rock fragments	many (15-40 %)	very few (0 - 2 %)	none (0 %)	very few (0 - 2 %)	many (15-40 %)
Soil depth (cm)	110	100	140	110	110
Parent material	loam	limestone	sandstone	marl	granite
Site drainage	ideal	ideal	ideal	fairly week without reducing conditions	moderate to ideal

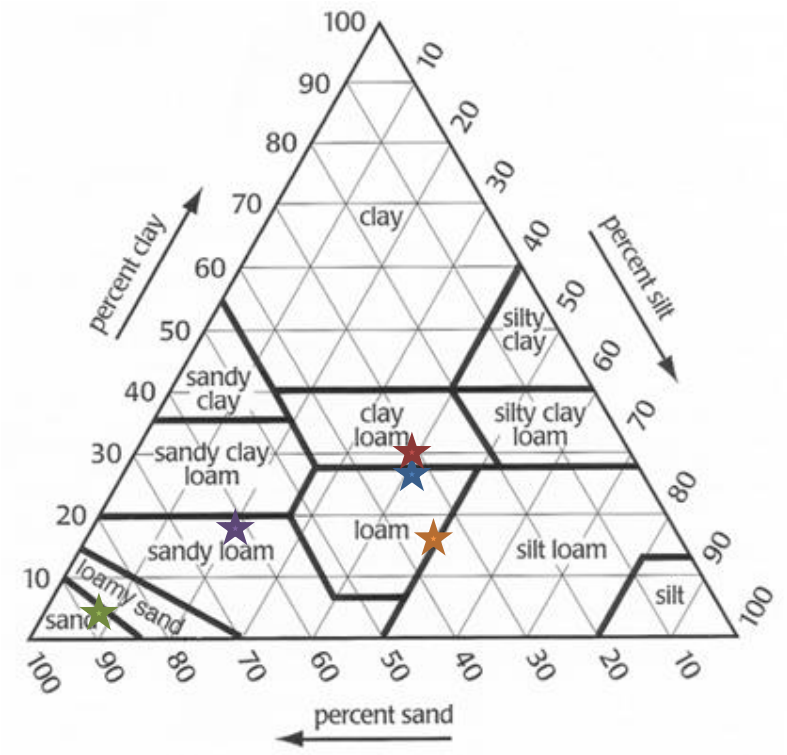

Figure 2. Texture classification of the five soil types used in this study (W = blue, R = red, H = green, E = orange and B = purple). The classification is made according to the Food and Agriculture Organization (FAO) texture triangle.

Table 2. Soil properties of the fine earth fraction (< 2mm). The first letter of the soil type indicates the sampling site while the second one indicates the soil horizon. Properties include the cation exchange capacity (CEC) and the total organic carbon content (TOC). Brackets designate estimated values.

Soil	Symbol	Sand	Silt	Clay	CEC	TOC	pH(H_2O)	pH(KCl)
		%	%	%	cmol+ kg^{-1}	%		
W-A	◆	5.6	45.6	48.7	12.8	35.2	3.3	2.7
W-B	■	31.5	41.7	26.7	2.0	3.9	4.3	3.8
R-A	◆	29.5	42.6	27.9	-	3.4	5.6	-
R-B	■	30.1	40.8	29.1	5.0	1.2	5.4	-
H-A	◆	87.7	7.4	4.9	2.2	2.5	4.1	3.2
H-B	■	87.8	7.5	4.7	1.0	1.0	4.1	3.6
E-A	◆	36.4	51.1	12.5	2.9	2.2	4.7	3.6
E-B	■	34.6	47.5	17.9	3.1	0.7	5.2	4.2
B-A	◆	60.8	20.4	18.9	10.0	[10.9]	4.1	3.2
B-B	■	63.4	21.8	14.8	4.8	[3.3]	4.8	4.1
Control	●	[100]	[0.0]	[0.0]	-	[<0.1]	-	-

3.2. Field sampling

At each site, sampling locations were selected close to an old soil pit which had already been described in detail (**Table 2**). The data from these classifications were used to help interpret the results of the experiments carried out for this study. After the O horizon had been removed, the A horizon and the underlying B horizon were sampled. The samples were sieved in the field using 6.3 mm mesh to remove stones and roots. At the sampling locations of Weierbach, Huewelerbach and Breuil some of the O horizon may have been added to the sample because it was difficult to distinguish the lower humus layer from the dark, A organo-mineral horizon. The frontier between O and A horizon was not clear (continuum). Two sample pits were dug and mixed per site in order to obtain a composite sample for each horizon of each site. The combinations of site and horizon are from now on referred to as soil types.

3.3. Soil moisture comparisons

Equation 2 was used to determine the gravimetric water content (GWC) in the soil at every processing step. For this purpose, small sub-samples were oven-dried to a constant weight at 105°C (Gobat, 1998; Cosandey & Robinson, 2000). Note that the GWC can exceed 100 % because the amount of water in the soil can be heavier than the soil itself (e.g. in case of a very light soil with high porosity and high water retention capacity).

$$\boldsymbol{GWC}\ (\%) = \frac{\boldsymbol{weight\ of\ fresh\ soil}\ (\mathrm{g}) - \boldsymbol{weight\ of\ dry\ soil}\ (\mathrm{g})}{\boldsymbol{weight\ of\ dry\ soil}\ (\mathrm{g})} \times \mathbf{100} \qquad \textbf{Equation 2}$$

At the laboratory, the soil was mixed by hand and a sub-sample of 3 kg was transferred into a plastic bag. The bags were stored in a cold room at 4-5°C to avoid evaporation until the soil water of the sample was extracted (see '3.4.1 Experiment 1' below). Before setting up the experiments, the remaining samples of each soil type were sieved with 2 mm mesh to retain only the fine earth fraction (FAO, 2014). Afterwards, the samples were air-dried and

homogenized. When the remaining GWC in the soil did no longer decrease through continued air-drying, the samples were considered dry enough.

To assess soil moisture levels after centrifugation, a standard measure of soil moisture at metric pressures of $pF_{2.5}$ and $pF_{4.2}$ was also carried out using a ceramic plate extractor. For this purpose, 3 replicates per sample of sieved and air-dried soil were left to saturate by capillarity in metal cylinders of 100 cm^3 for $pF_{2.5}$ and in thick rubber bands for $pF_{4.2}$ (**Figure 3a**). The use of smaller samples for $pF_{4.2}$ sped up the measurement without affecting the result as only the microporosity in soil remain important at this pressure. Once the soils were completely saturated, the samples were placed into a ceramic plate extractor (**Figure 3b**). Pressures of 0.33 bar ($pF_{2.5}$) were applied to the samples in the cylinders and 15 bar ($pF_{4.2}$) to the samples in the rubber bands until no more water drained out of the extractor. Then, the soils were oven-dried to a constant weight at 105°C. In the end, soil weight measures after extraction were related to the oven-dried soil weight to obtain the respective soil moistures at specific pF values (**Equation 2**). Moreover, the volume of the cylinder and the weight of the oven-dried soil allowed for bulk density calculations and porosity estimates. The porosities were calculated using standard mean densities of mineral and organic phases.

Figure 3. a) Soil filled cylinder and b) 15 bar ceramic plate extractor (Soilmoisture Equipment Corp., California, U.S.A). Photos: Martine Stoll, 2014

3.4. Experimental set-up

To address the objectives of the study, the following two experiments were carried out. First, it is essential to understand the classification of soil water types used in this study (**Table 3**). Drainage water is gravitational water present in the soil between saturation and field capacity. The pressure at which field capacity is reached depends on the soil type but is accepted to start at $pF_{1.8}$ (Gobat, 1998). The value of $pF_{2.5}$ was selected to separate the

weakly from the moderately bound water because this represents the lower end of the field capacity range. The value of $pF_{4.2}$ represents the water tension present in the soil at the permanent wilting point, a common measure used for all soil types (Cosandey & Robinson, 2000). This pressure separates the tightly bound water, which is not plant available, from the moderately bound water. The capillary water, *i.e.* the plant available water, is in fact a combination of weakly and moderately bound water.

Table 3. The classification of soil water types.

tightly bound water	moderately bound water	weakly bound water	drainage water

$pF_{4.2}$ = permanent wilting point

$pF_{2.5}$

$pF_{1.8}$ = field capacity start

Tap water was used as reference water for the experiments. It had previously been stored (5°C) in large plastic containers and sub-samples of it were taken for isotopic analysis. The water was filled in at once to ensure that all water needed in the experiment had roughly the same isotope composition (δD = -52.72 ‰ ± 0.34 and δ^{18}O = -8.34 ‰ ± 0.01). During the final drainage period (see '3.4.1 Experiment 1' below), two additional collection bottles with roughly 75 ml and 15 ml of tap water were set up in the laboratory. The bottles were weighed every hour and the room temperature and humidity were measured during drainage. A linear regression was fitted to the hourly weight measurements. Its linear equation was then used to estimate the amount of evaporation loss from the drainage waters for the time during which they were exposed to the atmosphere (~ 13 h). As the temperature and humidity during the final drainage were similar to the measurements during the initial drainage, the same equation was used for both drainage periods.

3.4.1. Experiment 1: isotopic differences between water and soil types

The aim of the first experiment was to quantify the differences in hydrogen and oxygen isotopic signatures between the 4 types of soil water presented in **Table 3** and to determine the impact of the soil type on these isotopic signatures. In addition, the data were used to determine the performance of the water extraction methods.

Step1: Preparation of the reactors

For the experiment, three 1 L plastic bottles (HDPE) per soil type were filled with air-dried soil. In addition, two control bottles were filled with pure sand. Hence, a total of 32 soil bottles were set up for experiment 1 (5 sampling sites x 2 horizons x 3 replicates + 2 controls). A control of pure sand was used because of its very low reactivity. Additionally, the control sand was left in an autoclave (Laboklav, SHP Steriltechnik AG) for 20 minutes at 134°C to minimize microbial activity. Two holes were drilled next to each other into the bottom rim of the bottles with a diameter of 2 mm for sandy soils and 3 mm for clay and loamy soils.

Step 2: Saturation and drainage

Next, the samples were saturated through capillarity by placing the bottles into a large bucket with tap water. Once the soils were completely saturated, the bottles were left to drain by gravity by placing them diagonally on a metal rack (**Figure 4a**). All drainage water was collected and weighed. The soil bottles were then made air-tight by sealing the holes with sealing clay, putting a lid on the bottles and wrapping paraffin tape around the lid (**Figure 4b**). At each step of the preparation the bottles were weighed to be able to calculate the different soil moistures.

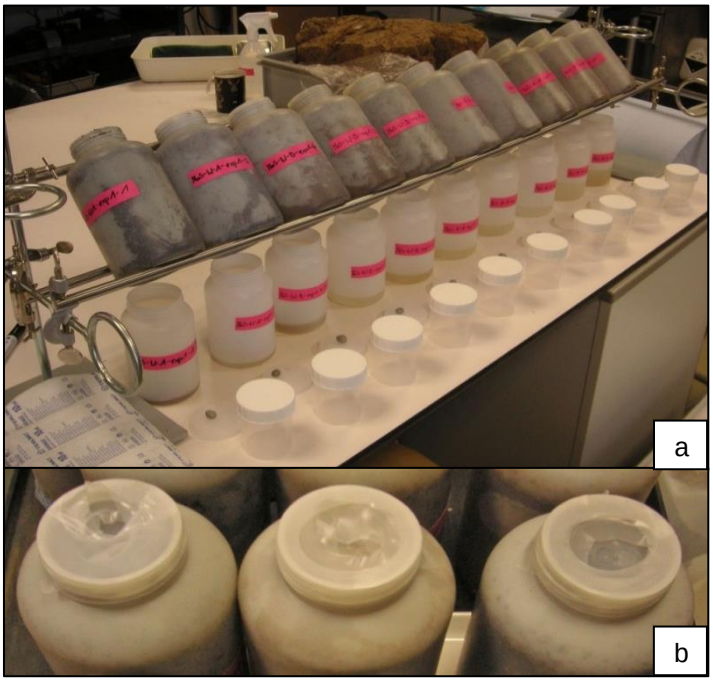

Figure 4. a) Drainage setup and b) lid and paraffin seal on soil bottles. Photos: Martine Stoll, 2014

Step 3: 5-week incubation

Subsequently, all bottles were stored in an incubator for 5 weeks to allow the water to interact with the solid phase. An incubation temperature of 13°C was chosen to represent field conditions. During this period, the bottles were weighed three times a week to register any potential losses to the atmosphere. After incubation, the soils were re-saturated and drained again, which was repeated the same way as during the initial phase.

Step 4: Water extraction

After the final drainage, all experimental soil samples (ES) were centrifuged twice to separate the bound waters: at $pF_{2.5}$ to remove the weakly bound water and $pF_{4.2}$ to extract all water between $pF_{2.5}$ and the wilting point (**Figure 5**). The fresh soil samples (FS) put aside for *in situ* water analysis were only sieved to 6.3 mm in the field and homogenized just before being centrifuged in the same way as ES. After centrifugation, the tightly bound water was extracted from the residual experimental soil through static cryogenic vacuum distillation (see '3.4.4 Cryogenic vacuum distillation' below).

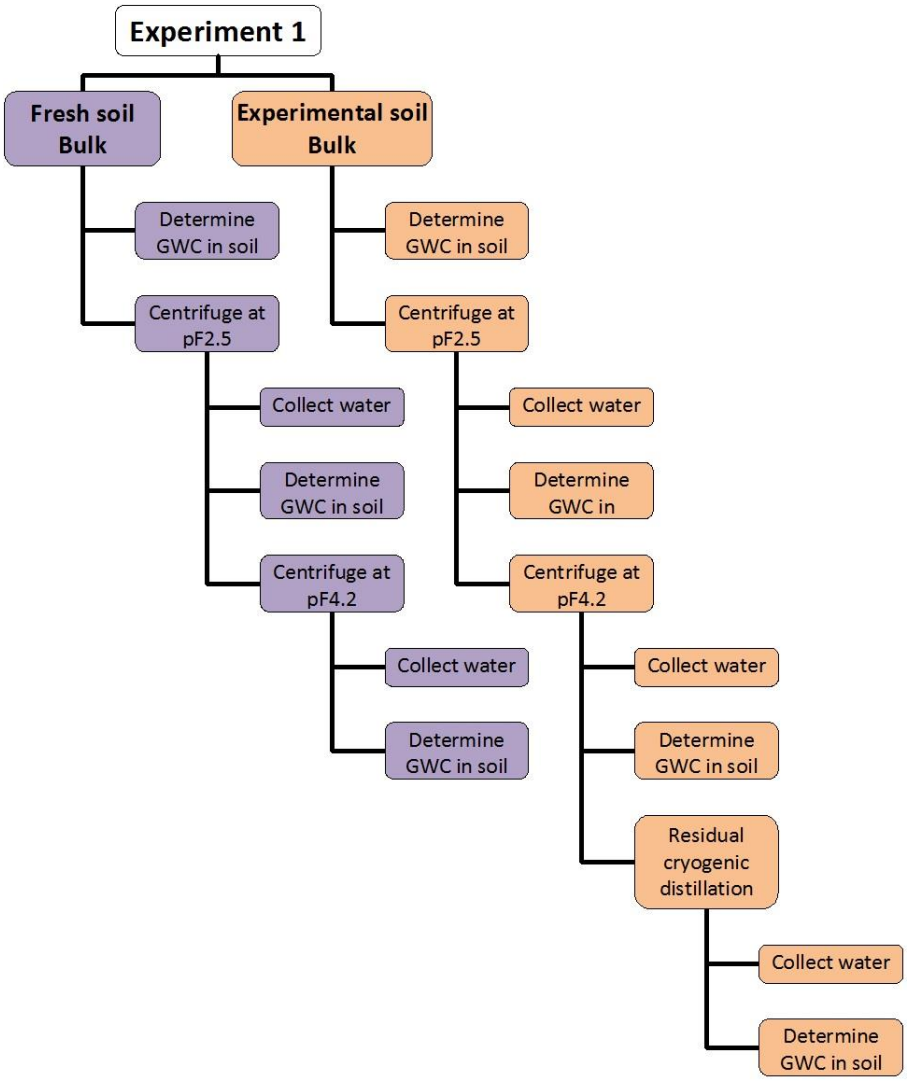

Figure 5. Diagram of the water extraction steps for experiment 1. GWC = gravimetric water content.

3.4.2. Experiment 2: isotopic mass balance

The aim of the second experiment was to study the isotopic mass balance of isotope signatures between different mixtures of water types.

Only the Weierbach B horizon was used for this experiment, thus, 5 additional bottles of this soil type were prepare in the same way as for experiment 1. There were 3 extraction components to this experiment (**Figure 6**):

S1. a subsample of the bulk soil at field capacity was used in cryogenic vacuum distillation to extract all water,

S2. centrifugation of a subsample of the bulk soil to $pF_{4.2}$ and residual cryogenic vacuum distillation,

S3. centrifugation of a subsample of the bulk soil to $pF_{2.5}$, then to $pF_{4.2}$ and residual cryogenic extraction.

The weights of the extracted waters were measured and the water samples were analysed as described below ('3.5.1 Laboratory'). The weighted isotopic ratios (wt x δI) of mixtures of water samples were compared to the weighted isotopic ratios of mixtures of water samples which were extracted differently but represented the same combination of water types (**Equation 3**). A difference in the mass balances above 5 % between comparisons was arbitrarily chosen as the significance level.

$$\frac{\sum_{i=1}^{n}(\boldsymbol{wt_i}\ (\mathrm{g}) \times \boldsymbol{\delta I_i}\ (‰))}{\boldsymbol{total\ weight}\ (\mathrm{g})} = \frac{\sum_{j=1}^{n}(\boldsymbol{wt_j}\ (\mathrm{g}) \times \boldsymbol{\delta I_j}\ (‰))}{\boldsymbol{total\ weight}\ (\mathrm{g})} \qquad \textbf{Equation 3}$$

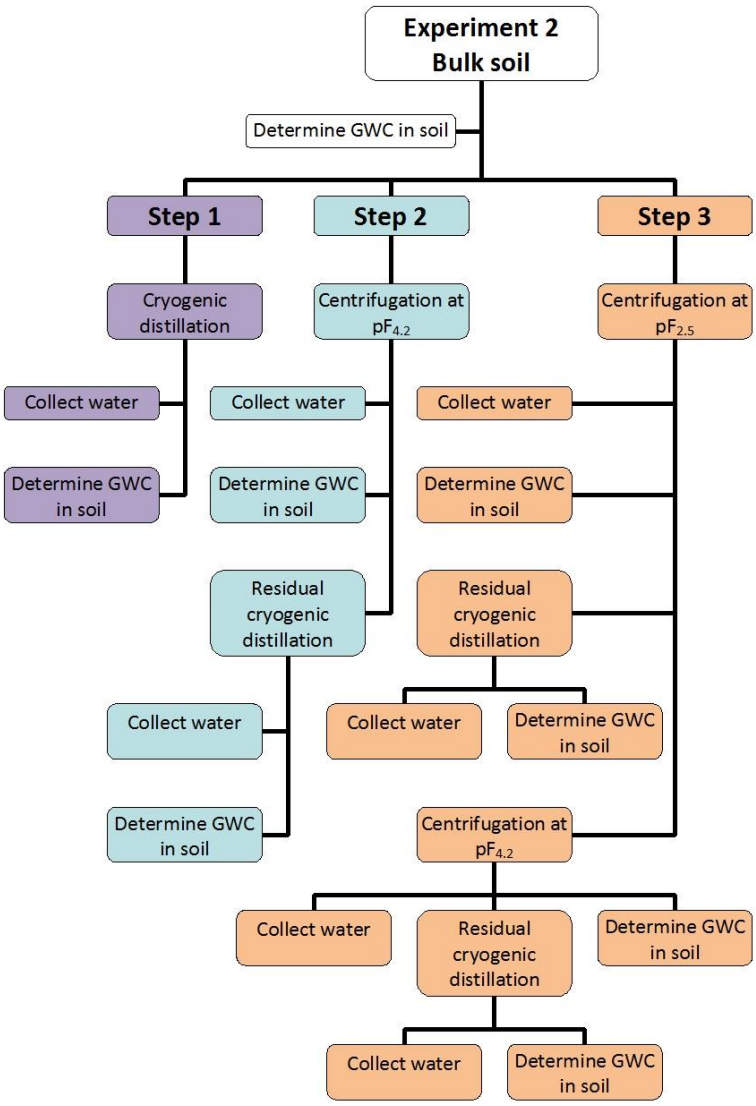

Figure 6. Diagram of the water extraction steps for experiment 2. GWC = gravimetric water content. The bulk soil is made up of all 5 W-B soil bottles set up for experiment 2.

3.4.3. Centrifugation

Centrifugations were run at 20°C for 20 minutes at 540 revolutions per minute (rpm), which corresponds to $pF_{2.5}$, and 3000 rpm, which corresponds to $pF_{4.2}$ (Jeffrain, 2006). Two centrifuges were used in this study: Jouan KR422 and Jouan KR4i ThermoFisher Scientific.

Each replicate of the fresh soil samples and the experimental soil samples was homogenized before centrifugation. The centrifuge containers were cleaned, first with de-ionised water then with ultra-pure (UP) water (18.2 MΩcm). The centrifuge containers were put together and a 2 µm filter (qualitative filter paper, 410) was added (**Figure 7**). Then, the soil was transferred into the containers which had previously been weighed. Before and after each centrifugation the containers were weighed to determine any water losses during the procedure. After centrifugation, the water which accumulated in the bottom container was sucked up with a syringe that had been rinsed twice with UP water. The collection water was filtered, weighed and a sample was prepared for isotope analysis. When a subsequent centrifugation was carried out, the above described procedure of water collection was repeated. Also, at each centrifugation step a small subsample of the soil was oven-dried to determine the gravimetric moisture content of the remaining soil.

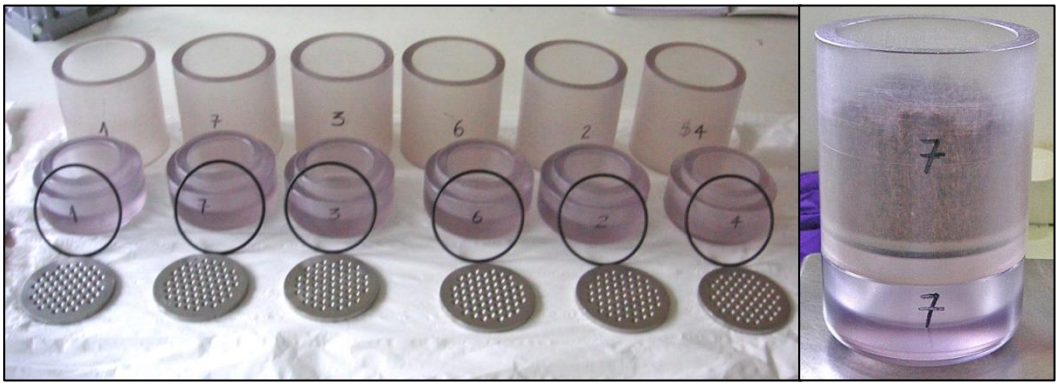

Figure 7. Centrifuge containers. Photos: Martine Stoll, 2014

3.4.4. Cryogenic vacuum distillation

During the used static vacuum distillation the vacuum is created once at the beginning of the distillation. In contrast, during a dynamic vacuum distillation the vacuum is continuously renewed.

A soil sample of about 5-10 g was filled into a large glass test tube which had previously been weighed. Quartz wool was stuck into the tube to prevent the soil from being sucked out during distillation. The tube was weighed again and then fixed to the distillation apparatus which was built according to West *et al.* (2006) (**Figure 8**). The air in all the pipes of the apparatus was removed and a static vacuum of 10^{-3} mbar was created.

The test tube containing soil was then shortly dipped into liquid nitrogen (-210°C to -196°C) before being placed into a hot water bath of 65°C. The small collection test tube was dipped into a container filled with liquid nitrogen for the duration of the distillation. The small connecting pipe between the large and small test tube was heated to between 70-75°C. The

heat together with insulation around the pipe allowed the water from the soil to be evaporated and completely transported to the collection tube without condensation being retained in the connecting pipe. The total duration of the distillation depended on the soil type. For soils with higher clay content the distillation usually took longer time to complete because water is more tightly bound.

When the distillation was complete, the test tube with the collection water was taken out of the liquid nitrogen, removed from the apparatus and immediately sealed with paraffin tape (Parafilm ®). The water was left to thaw before being weighed and prepared for isotopic analysis. Moreover, the soil test tubes were weighed again and then oven-dried to determine the residual GWC.

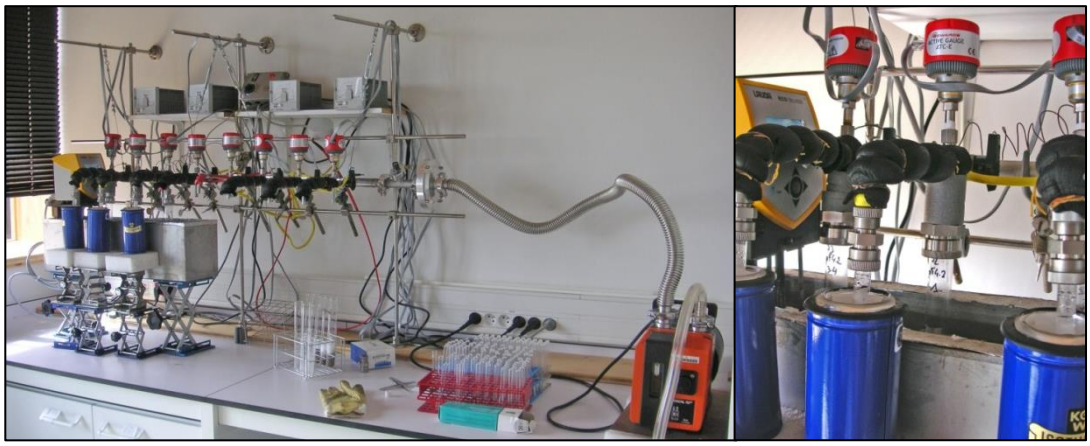

Figure 8. Cryogenic vacuum distillation apparatus. Photos: Martine Stoll, 2014

3.4.5. Microbial soil respiration

For experiment 1, two replicates for each soil type were analysed for microbial soil respiration (MSR) using an air circulation system, built according to Heinemeyer *et al.* (1989), connected to an automated infrared gas analyser (IRGA) (**Figure 9a**). The IRGA measures the difference in CO_2 absorption between the outside air and the air flushed through the soil samples. When microbial soil respiration occurs, the through-flow air becomes enriched in CO_2. To take MSR measurements, sub-samples of air-dried soil were progressively re-wetted until approximately field capacity and incubated for 8 days to revive aerobic microbial respiration. The incubation and the following MSR analysis were carried out at 22°C. Incubated soil samples of 30 g dry matter equivalent were placed into the plastic cylinders (**Figure 9b**) of the apparatus through which air was pumped. Measures were taken once an hour for 24 hours and were given in µg CO_2-C / g DM / h.

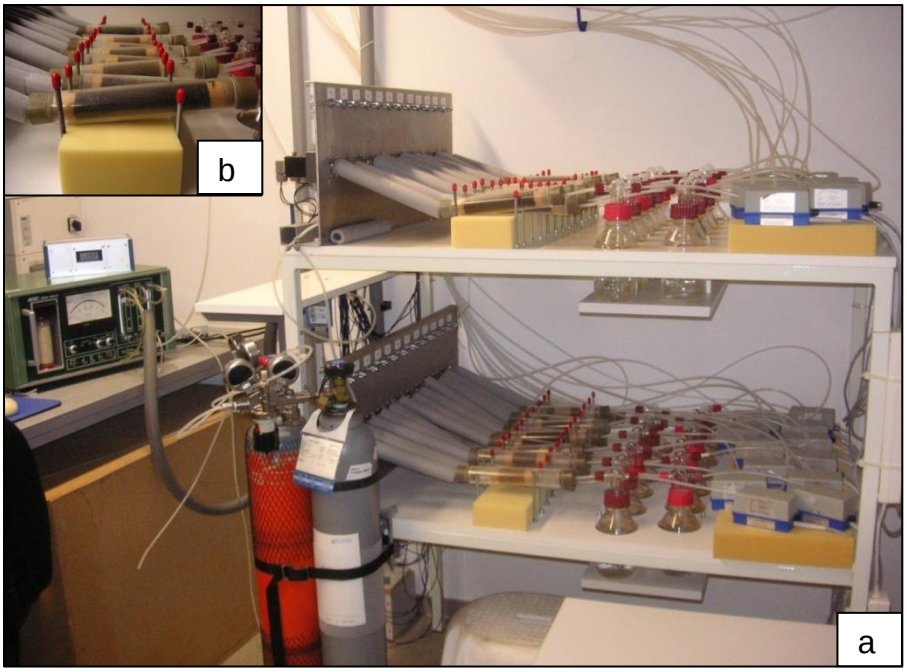

Figure 9. a) Air circulation system connected to an automated infrared gas analyser (IRGA) for the measurement of microbial soil respiration. b) The soil samples are inserted into plastic cylinders. Photos: Martine Stoll, 2014

3.5. Analyses

The soil water contents were converted to g of water per 100 g of dry soil for easy comparison between water types. The saturation water is the amount of water present in the bottles after complete saturation of the soil. Also, all drainage waters (DW) used in calculations and graphs are totals, *i.e.* total DW = collected DW + estimated evaporation loss.

3.5.1. Laboratory

Water samples were filtered with 0.45 µm acetate paper and stored in the cold room at 4-5°C until analysis. All water samples were analysed for hydrogen and oxygen isotope signatures. For this purpose, glass vials of 1.5 ml were filled to the top with water samples and closed with a special lid to avoid evaporation. An isotope-ratio mass spectrometer (IRMS) (GV Instruments, Isoprime) was used for isotope analysis. The IRMS instrument precision is 1.0 ‰ for deuterium and 0.1 ‰ for oxygen-18 (Araguás-Araguás *et al.*, 1995). The analyses for drainage and tap water also included pH(H_2O) measurements.

3.5.2. Statistics

The statistical analyses were carried out using Minitab 16. Principal component analysis (PCA) was used to identify the main characteristics differentiating the soil types, not including the control. Cluster analysis was then used to group the soil types by similarity. Moreover, one-way and two-way univariate ANOVA were used to analyse differences in hydrogen and oxygen isotopic data between sites, horizons and water types. When the

assumptions for parametric tests were not met, a non-parametric alternative was used, where appropriate. The significance level for all statistical tests was chosen to be 5 %.

PCA takes into account correlations between multiple variables at the same time to compute scores. These scores are calculated from a single variable (v) or are made up of several variables, e.g. (0.5 x v1) – (0.4 x v2) – (0.1 x v3). These combinations of variables, also called principal components (PC), explain a certain percentage of the variability in the data set, with PC1 explaining the most variation between individuals, PC2 the second most and so forth. However, the soil characteristics represented by principal components need to be defined subjectively (Townend, 2002). For example, a score made up of sand, silt and clay content could be said to represent soil texture. The PCA analysis of the 10 soil types, not including the control due to lack of data, included the following soil parameters: sand, silt and clay content, bulk density, porosity, GWC as obtained through ceramic plate extraction at $pF_{2.5}$ and $pF_{4.2}$, pH, TOC, total N, and MSR.

Cluster analysis is another multivariate analysis which takes into account all variables at once to determine how similar a number of individuals, e.g. soil types, are. The analysis starts by grouping the two individuals that are most similar and then adds individuals or groups of individuals which are next most similar. The similarity of individuals is determined by the distance between them in a multi-dimensional space due to the use of multiple variables. In this case, average linkage and the Euclidian distance between points/groups are used to determine distances. Also, variables were standardized to be weighted equally (Townend, 2002).

4. Results

4.1. Soil sample characteristics

Soil properties that were measured during the study can be found in **Table 4**. The mean gravimetric water content (GWC) obtained through air-drying was 2.9 % with the highest GWC measured for W-A (7.9 %). The bulk density of the fine earth fraction was lower in A horizons compared to B horizons. Also, its negative correlation with the gravimetric water content, both at $pF_{2.5}$ and $pF_{4.2}$, was strong (**Figure 10**). The porosity was inversely related to bulk density, hence it was always higher in the A horizon compared to the corresponding B horizon. Porosity increased with TOC and with clay content (**Figure 11**). Note that A horizons contained more moisture compared to the corresponding B horizons, both at pressures of $pF_{2.5}$ and $pF_{4.2}$ applied using ceramic plate extraction (CPE). There was a linear relationship between the GWC at $pF_{2.5}$ and the GWC at $pF_{4.2}$. Furthermore, CPE confirmed that sands (Control and H) had the lowest gravimetric water contents at these pressures, followed by soils which form large aggregates such as from E and R sites. MSR of different soil types ranged from very low to very high and increase with TOC. The variability between replicates of MSR measurements was on average 12 %. Moreover, A horizons have soil respirations that were one or two categories higher than for their corresponding B horizon. In addition, the MSR measurement confirmed that the sterilization of the control sand was effective.

Table 4. Soil properties of the fine earth fraction (< 2 mm) for all soil type samples. The first letter of the soil type indicates the sampling site while the second one indicates the soil horizon. Note that C stands for the control sand. The represented gravimetric water content (GWC) was estimated using a ceramic plate extractor.

Soil	Symbol	Bulk density	Porosity	GWC at $pF_{2.5}$	GWC at $pF_{4.2}$	MSR
		g cm^{-3}	*%*	*%*	*%*	*µg CO_2-C/g DM/h*
W-A	◆	0.39	78.1	85.2	44.0	1.98
W-B	■	0.72	71.0	61.4	22.5	0.53
R-A	◆	0.98	60.6	30.7	17.3	0.70
R-B	■	1.06	58.7	22.5	14.2	0.26
H-A	◆	0.96	61.9	19.7	10.3	0.92
H-B	■	1.34	47.7	6.2	2.5	0.15
E-A	◆	0.98	61.1	21.5	8.0	0.32
E-B	■	1.15	55.6	18.0	8.7	0.18
B-A	◆	0.72	68.6	50.3	29.0	1.68
B-B	■	0.99	60.4	27.5	18.9	0.34
C	●	1.52	41.6	3.2	0.5	0.02

According to PCA, principal component 1 (PC1) explained 70 % of the differences between soil types and was mainly controlled by bulk density, porosity, TOC, and the GWC at $pF_{2.5}$ and $pF_{4.2}$ as obtained by ceramic plate extraction. Hence, PC1 was determined to represent the soil water retention. The second principal component (PC2) represented soil texture (sand and silt content) and pH, explaining 20 % of the differences between soil types. Note that even though soil texture and porosity were related in this study they correspond to different principal components in PCA. Porosity describes the total pore space, while soil texture influences the pore size distribution and, hence, the water retention curve (Cosandey

& Robinson, 2000). According to cluster analysis, 3 groups of soils can be distinguished: x) loamy soils with intermediate water retention capacity and relatively high pH, y) sandy soils with intermediate to low water retention capacity and low pH, and z) soils with high water retention capacity of different soil textures and low pH (**Figure 12**). The W-A soil type stood on its own. It has a very high water retention capacity and very low pH as well as much higher clay content than all other soil types.

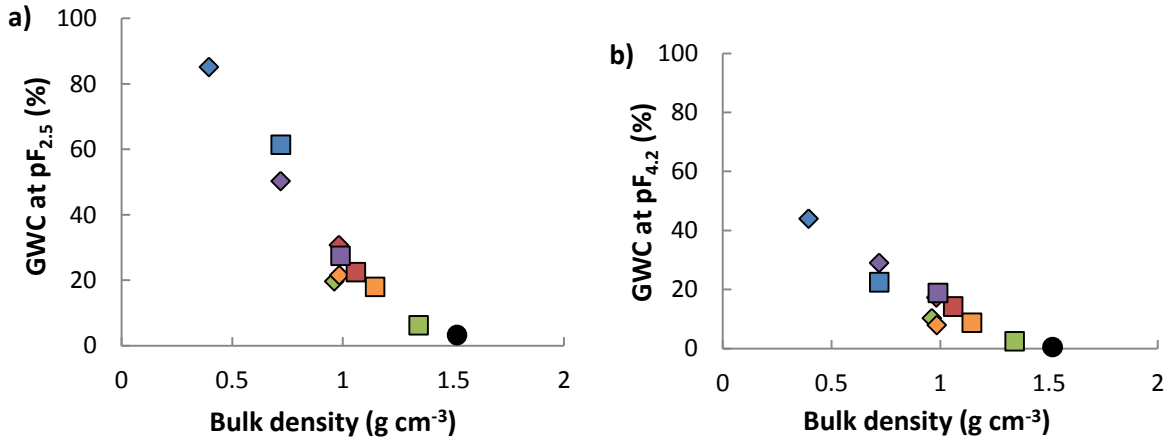

Figure 10. Relationship between bulk density and the gravimetric water content (GWC) obtained through ceramic plate extraction at a) $pF_{2.5}$ and b) $pF_{4.2}$. The symbols are explained in Table 4.

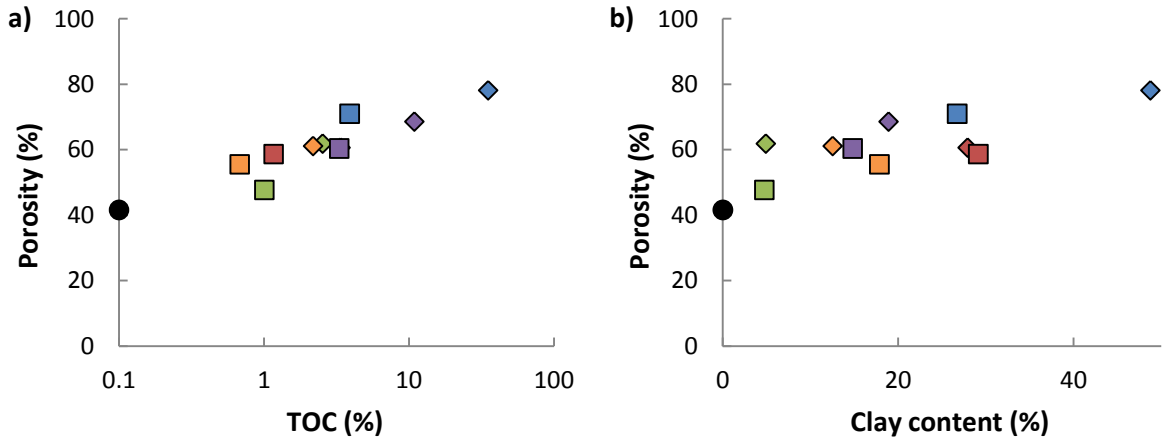

Figure 11. Relationship between the estimated porosity and a) total organic carbon (TOC, %) on a log scale, b) clay content (%). The standard deviations are too small for the error bars to be visible. The symbols are explained in Table 4.

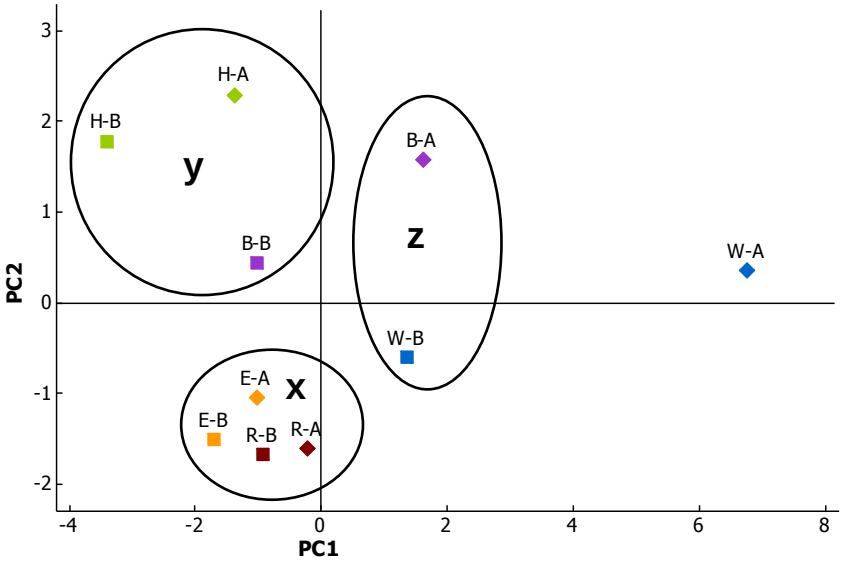

Figure 12. Scoreplot of soil water retention (PC1) against soil texture and pH (PC2) as obtained through principal component analysis. Three soil groups marked with x, y and z were delineated according to cluster analysis (average linkage, Euclidian distance).

4.2. Extraction of different water types

4.2.1. Drainage water

The amount of water needed for the first saturation of the soils was very similar to the amount needed for the re-saturation at the end of the incubation period. Likewise, the amount of water left in the bottles after the initial drainage, *i.e.* at field capacity, was very similar to the amount of water left in the bottles after the final drainage (data not shown).

For most of the samples the relationship between the amount of initial and final drainage water (DW) follows the line 'y = x' (**Figure 13**). The Ell soil presented the largest difference between drainage waters. Its final drainage was low in spite of the initial drainage being large compared to most soil types. The amount of drainage water collected differed between soil types but did not correlate with the amount of saturation water present in the bottles. Thus, other soil properties must have been responsible for the observed differences, though no correlations with available soil property measurements could be confirmed.

For the soil water at saturation and at field capacity (FC) the variation between replicates of the same soil type was very low. Also, the mean difference between the 'saturation water' and the 'water at FC + total drainage water' was ≤ 0.6 g per 100 g of dry soil at the start and ≤ 1.3 g per 100 g of dry soil at the end of experiment 1. The GWC reached at field capacity after both the initial and final drainage were much higher than the GWC of the fine earth fraction of the soils at CPE $pF_{2.5}$.

The initial (DWs) and final (DWe) drainage waters generally had pH values lower than the pH of the reference tap water used for saturation and varied around 7.5 (**Figure 14**). Nevertheless, the DWe of W-A and B-A were very acidic compared to the reference water. The pH changes between the initial and final drainage water were not one-directional. Moreover, both drainage waters did not visibly correlate with soil pH, not even when dividing

the data into A and B horizons (**Figure 14**). Yet, TOC, CEC, the saturated hydraulic conductivity (Ks) nor the sand, silt or clay contents correlated with the pH of the various drainage waters either (data not shown). Note that Ks is only available for 6 out of the 10 soil types, therefore it is difficult to assess its influence on soil water.

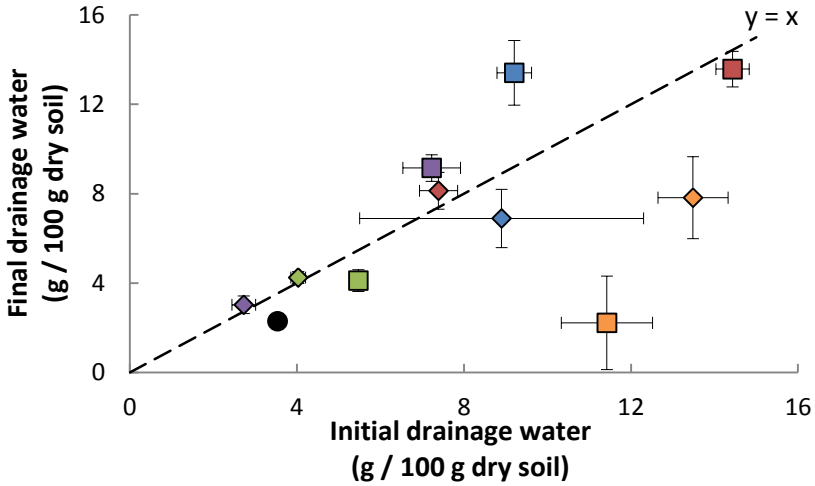

Figure 13. Comparison of the initial and final drainage waters. The drainage water is the weight of the collected drainage water with the estimated evaporation loss added. The error bars are the standard deviations of the water amount among the three bottled soil type replicates. The symbols are explained in Table 4.

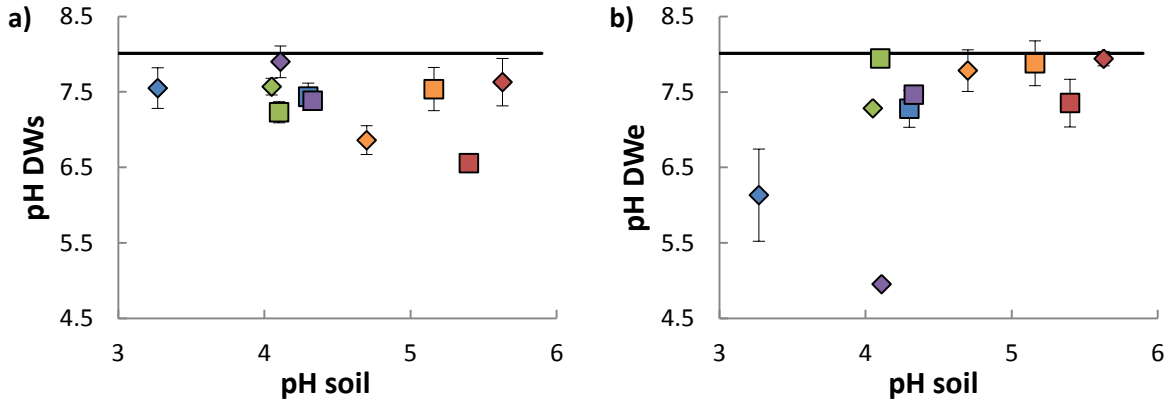

Figure 14. The pH of a) the initial (DWs) and b) the final drainage water (DWe) compared to the soil pH at field conditions. The symbols are explained in Table 4. The black line represents the pH of the reference tap water.

The experimental setup led to evaporation of the drainage water. The estimations of total evaporation from the drainage waters varied between 2.8 and 3.3 g. **Figure 15** indicates that H and O isotopic ratios of the drainage water deviated more from the isotopic signatures of the reference tap water when a higher percentage of drainage water was evaporated. Linear regression lines fitted to **Figure 15a and 15b** explained 86 % of hydrogen and 71 % of oxygen isotopic fractionation of the final drainage water. Even so, logarithmic regression lines also presented a good fit; therefore, the type of correlation is uncertain.

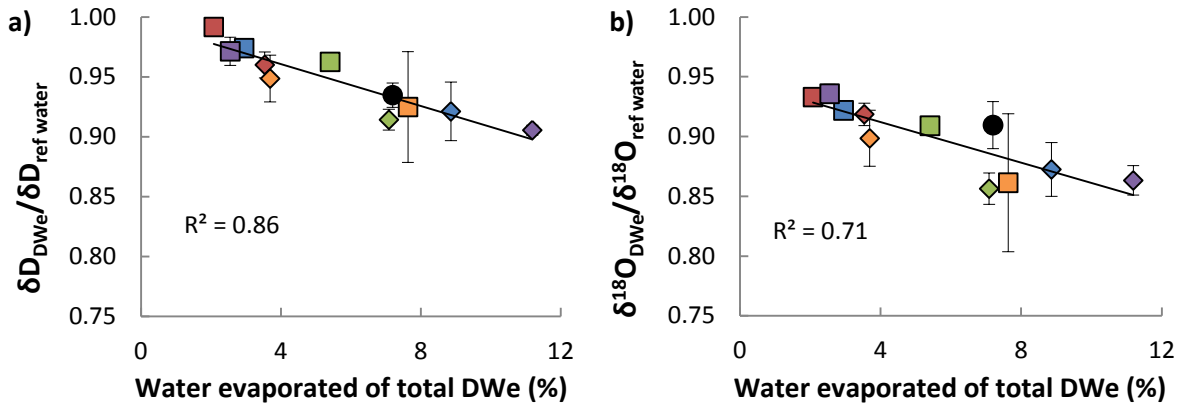

Figure 15. The deviations of a) the δD values (‰) and b) the δ^{18}O values (‰) of the final drainage water (DWe) from the reference water against the percentage of evaporated water. Means were calculated from 3 replicates. The symbols are explained in Table 4. In addition, linear regression lines were fitted to the data.

4.2.2. Incubation period

Some weight loss from the soil bottles was measured during the incubation period. When large changes in weight occurred, usually the clay seal was broken or water was observed below the paraffin tape at the top of the bottle. However, sometimes no liquid water was detected. Also, it was not possible to tell whether the water trapped below the paraffin tape escaped in liquid or gaseous form. This would be important to know when assessing the difference in isotopic signatures between the weakly bound water and the reference water as evaporation loss causes fractionation. Total weight loss was generally very small relative to the total water contents at field capacity of 370 g to 690 g (**Figure 16**). Still, A horizons lost noticeably more water than B horizons. Also, by the end of the incubation period all soil types had lost more weight than the control. Furthermore, total weight loss increased with microbial soil respiration (MSR) (**Figure 17**).

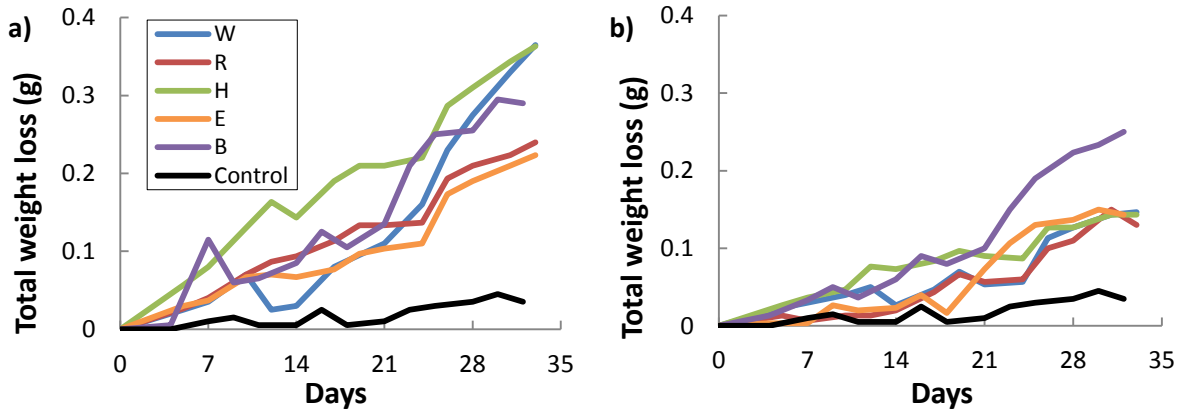

Figure 16. The cumulative weight loss (g) from the experimental bottles over the entire incubation period (days). The data are separated into a) A horizons and b) B horizons of the 5 sites. Two outliers (W-A-1 and B-A-27) were removed from the data set.

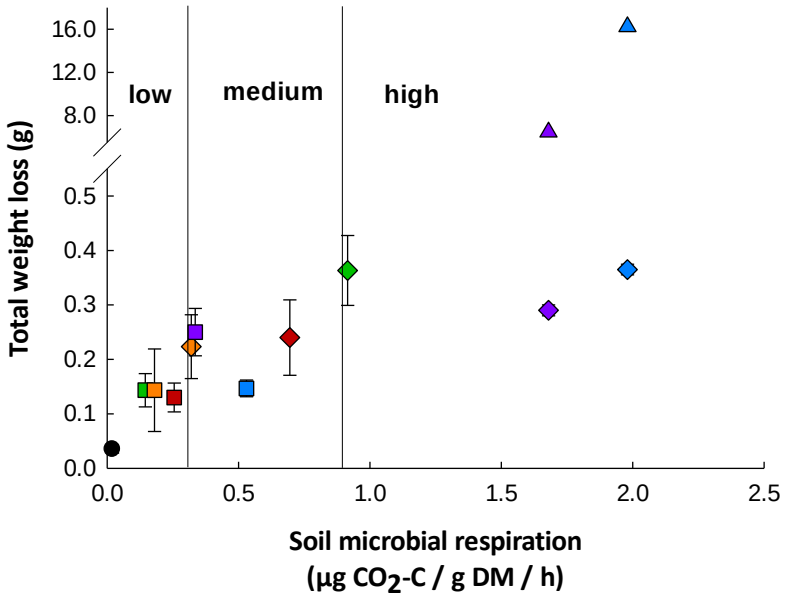

Figure 17. The total weight loss (g) from soil bottles at the end of the incubation period against microbial soil respiration (MSR, µg CO_2-C / g DM / h) for 11 soil types. Two outliers (W-A-1 and B-A-27) were removed from the mean total values and displayed individually as triangles. The error bars are the standard deviations of the total weight loss from the three bottled soil replicates, except for W-A and B-A. The symbols are explained in Table 4. The black lines divide the MSR into categories of magnitude: low, medium and high (Emmerling, 2014).

4.2.3. Weakly and moderately bound waters

The relationship between GWC after centrifugation to $pF_{2.5}$ and $pF_{4.2}$ was linear. However, the GWC after centrifugation was higher than the GWC after ceramic plate extraction when the same theoretical pressure was applied (**Figure 18**). Important to note is the linear relationship between the soil moisture obtained through ceramic plate extraction and centrifugation, indicating a systematic bias between the two methods. This difference in GWC between the two extraction methods did not correlate with any available soil characteristics, such as TOC, clay, silt and sand content. Moreover, for many soils the water amount left in the soil after centrifugation to $pF_{4.2}$ was the same or even higher than the soil moisture after CPE to $pF_{2.5}$. In **Figure 19**, values above the line 'y = x' indicate that the moisture at centrifugation $pF_{4.2}$ was even higher than the CPE moisture at $pF_{2.5}$. Oppositely, values below the line indicate that the moisture at centrifugation $pF_{4.2}$ was lower than the moisture for CPE $pF_{2.5}$. Note that centrifugation to $pF_{2.5}$ and $pF_{4.2}$ for experiment 2 did not extract as much water as the equivalent CPE either. Still, some water between $pF_{2.5}$ and $pF_{4.2}$ was extracted as the GWC of the soil after centrifugation at $pF_{4.2}$ (S3 = 41 %; S2 = 50 %) was lower than after CPE $pF_{2.5}$ (61 %). Nonetheless, a lot more water above $pF_{4.2}$ was not extracted as the GWC at CPE $pF_{4.2}$ was 22.5 %.

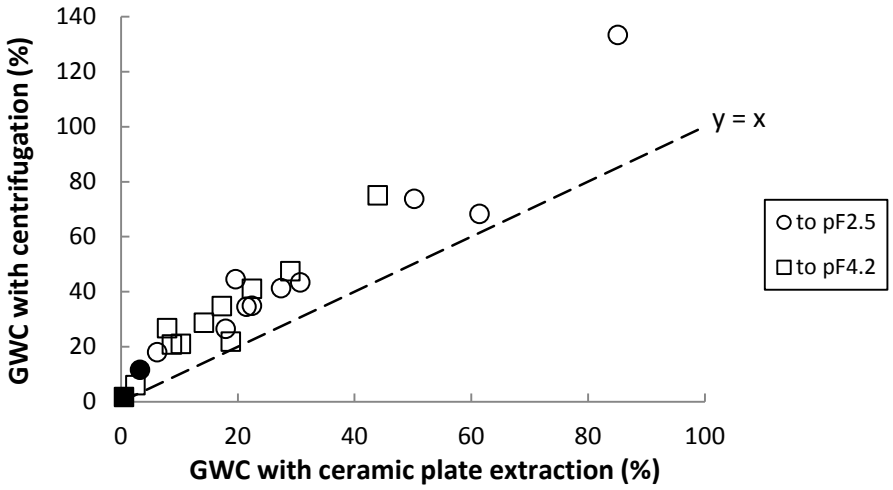

Figure 18. The gravimetric water content (GWC, %) in the soil after ceramic plate extraction and centrifugation at two pF values. The standard deviations are too small for the error bars to show from underneath the data markers. The filled markers represent the control sand.

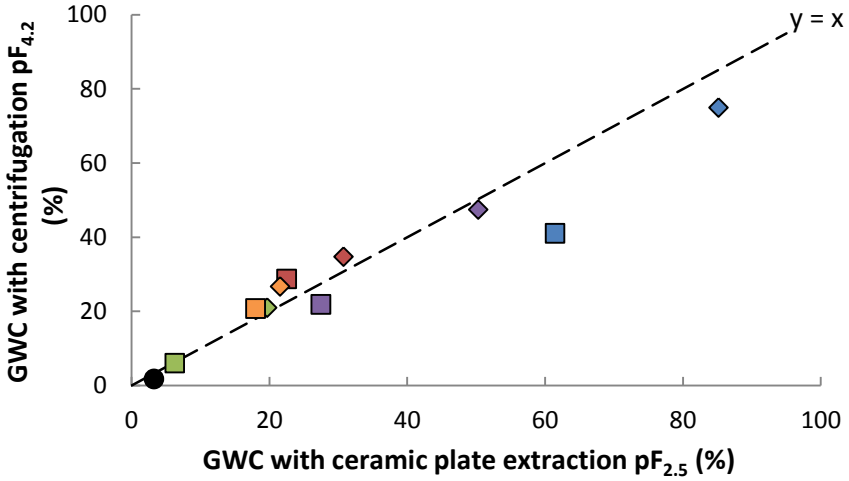

Figure 19. The gravimetric water content (GWC, %) in the soil after ceramic plate extraction at $pF_{2.5}$ and centrifugation at $pF_{4.2}$. The standard deviations are too small for the error bars to show from underneath the data markers. The symbols are explained in Table 4.

When the soil porosity was higher, the moisture in the soils after final drainage was higher too. In addition, **Figure 20** indicates that this strong positive correlation between remaining soil water and porosity prevails after centrifugation. On the other hand, the amount of extracted water by centrifugation was not correlated with porosity but was dependent on soil texture (data not shown). The soil texture seemed to be a more important factor regarding the amount of extracted water.

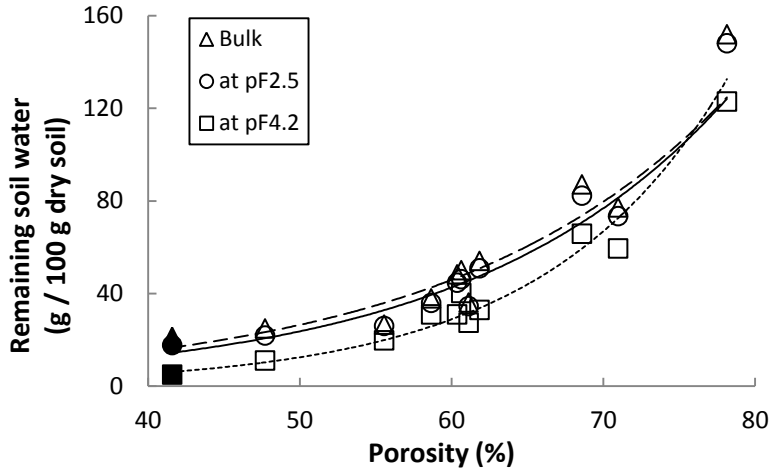

Figure 20. The estimated porosity of the soil types against the water amount (g / 100 g dry soil) left in the soil bulk after the final drainage and after centrifugation to $pF_{2.5}$ and $pF_{4.2}$. The filled markers represent the control sand. The black lines are exponential regression lines fitted to the 3 data sets (bulk = dashed, at $pF_{2.5}$ = smooth and at $pF_{4.2}$ = dotted). The fit of the regression lines is very good (R^2 = 90 % - 97 %).

4.2.4. Tightly bound water

Cryogenic vacuum distillation removed varying percentages of the remaining soil moisture depending on the soil type. The extraction yield varied between 80 % and 100 % with soil type (**Figure 21**), but the yield did not correlate with any available soil properties or the GWC in the soil before extraction or the weight of soil used. Importantly, the cryogenic extraction yield and the isotopic signatures of the tightly bound water were correlated (**Figure 22**). Nonetheless, soils with a yield close to 100 % (E-A, Control, H-A) had different isotopic signatures of the cryogenically extracted water compared to the reference tap water.

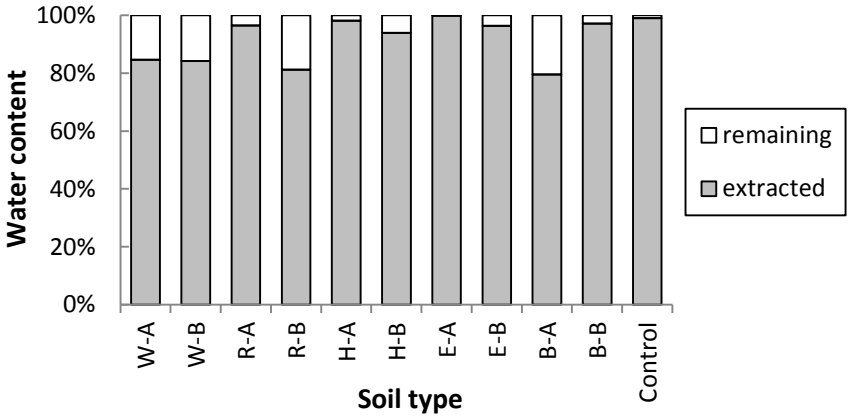

Figure 21. Cryogenic extraction yield, *i.e.* the percentage of water extracted from the experimental soil previously centrifuged to $pF_{4.2}$ and the water remaining in the soils for the method of cryogenic vacuum distillation. The maximum of 100 % of water extracted from the soil by cryogenic distillation equals to the amount of water extractable by oven-drying at 105°C. Only 1 replicate per soil type was used.

Note that cryogenic vacuum distillation extracted some of the *in situ* water left in the soil after air-drying (data not shown). This was the case for the soil types R-A, H-A, H-B, E-A, E-B, B-B and the control. Importantly, these soil types, excluding the control, also represented

one of the two distinct groups which can be observed on top of the correlation between isotopic signature and extraction yield in **Figure 22**. Moreover, the groups were very similar to the groups formed on either side of the PC1 axis in **Figure 12**. Only R-B does not keep to the same group as defined by cluster analysis.

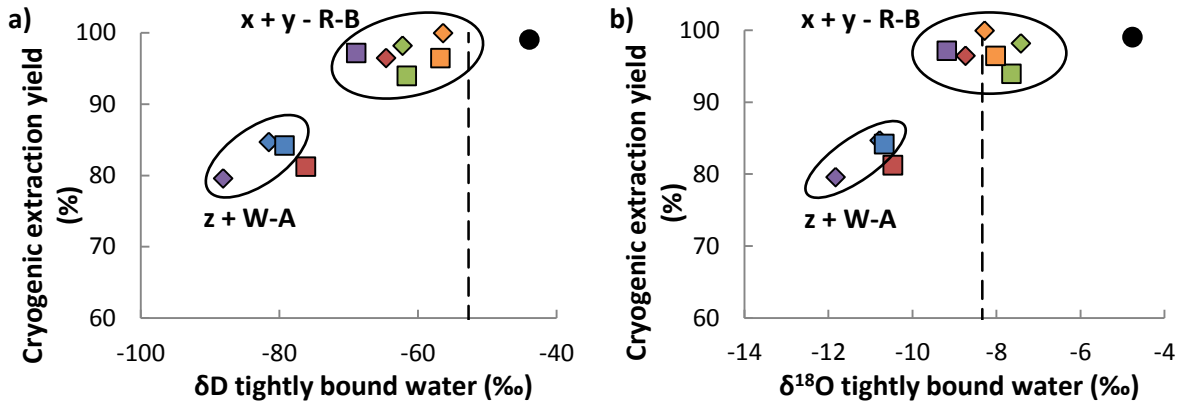

Figure 22. The percentage of water extracted from the experimental soil samples against a) δD values (‰) and b) δ^{18}O values (‰) of the water extracted through cryogenic vacuum distillation from soil samples which had previously been centrifuged to $pF_{4.2}$. The symbols are explained in Table 4 and the groups refer to Figure 12. The dashed line is the isotopic signature of the reference tap water used to saturate the soils.

4.2.5. Water type contributions to different soil samples

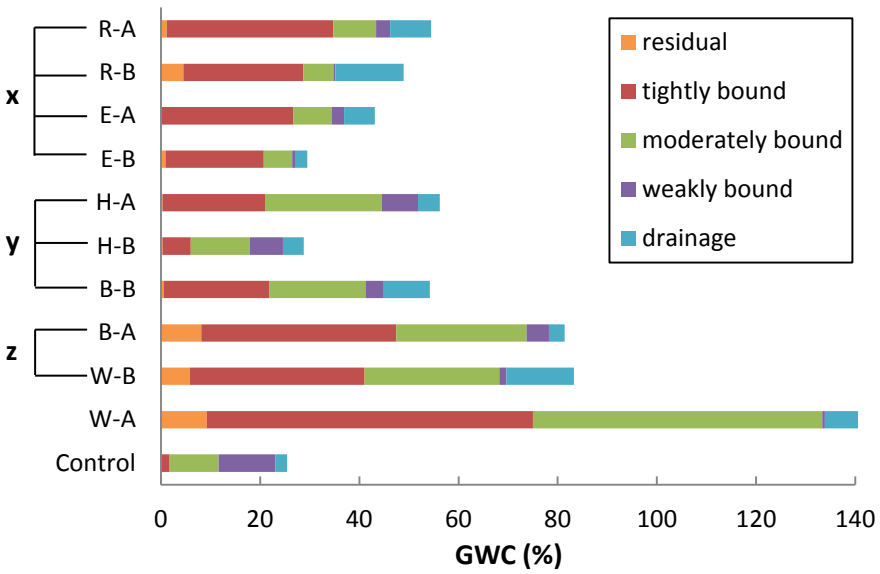

Figure 23. Contribution of the different water types to the total water content of the saturated soil samples. The soil moisture is expressed as gravimetric water content (GWC). Weakly bound (< $pF_{2.5}$) and moderately bound ($pF_{2.5}$ - $pF_{4.2}$) waters were extracted using centrifugation. Tightly bound (> $pF_{4.2}$) water was removed using cryogenic vacuum distillation. The residual water is the amount of soil water left after cryogenic extraction. The groups refer to Figure 12.

The gravimetric water content at saturation varied from 26 % to 139 % between soil types (**Figure 23**). Moreover, the soil moisture content increased with TOC. Also, the weight of collected drainage water was generally lower from sandy soils than from clayey or loamy soils. Yet when looking at the contributions of the different water types to the total water content of the saturated soil samples the generally lower drainage of sandy soils was not reflected as strongly. The percentages of GWC that the bound water types represent of the total soil water were highly variable between soil types as well. These differences were best explained with the results of PCA. W-A, W-B and B-B which contained the highest TOC contents and porosity retained the most water. For the other soil types with lower maximum water retention, a combination of TOC (PC1) and soil texture (PC2) determined the water retention at different pressures. For example, sandy soils generally held more weakly to moderately bound water than tightly bound water, except for B-B which has a relatively high TOC content compared to other B horizons. In contrast, the loamy soils held more tightly bound water than capillary water. Soil types belonging to group z + W-A had a lot of residual water, soil types belonging to group y contained almost no residual water and soils in group x had intermediate levels.

4.3. Isotopic signatures of soil water

4.3.1. *In situ* water

Centrifugation did not extract any weakly bound water (< $pF_{2.5}$) from the fresh soil samples. The H and O isotopic signatures of the moderately bound water extracted from fresh soil fit well along the global meteoric water line (GMWL). Also, the isotopic signatures of the moderately bound waters were similar to the isotope composition of rainfall in Luxembourg (δD = -58.8 and δ^{18}O = -7.7), except for the soil from the French site (**Figure 24**). The average H and O isotope signatures of rainfall were not available for Burgundy, France. Very similar patterns of isotope compositions between soil types were observed for deuterium and oxygen-18.

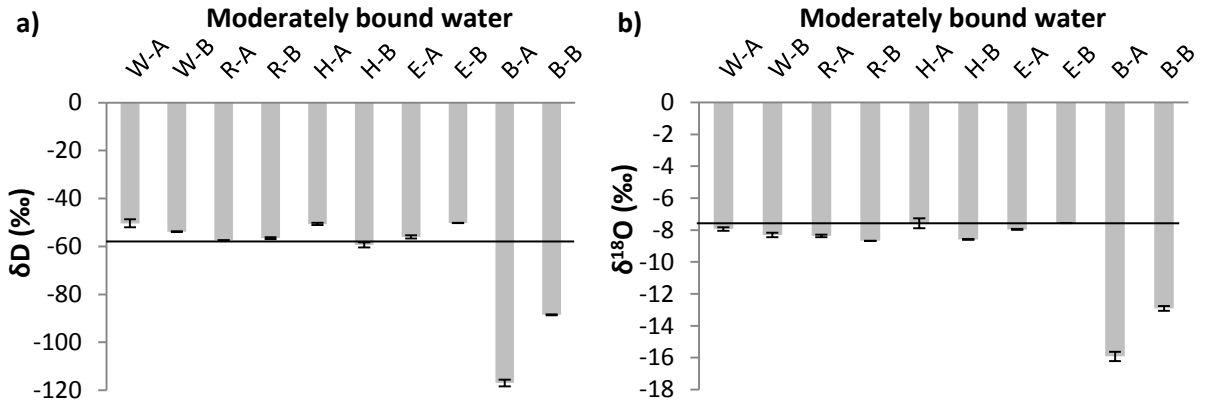

Figure 24. The a) δD and b) δ^{18}O values of the water extracted from the fresh soil through centrifugation at $pF_{4.2}$. Only one water sample per soil type was analysed. The error bars are the standard deviations from three isotope-ratio mass spectrometry (IRMS) measurements. The black lines indicate the mean isotopic signature of rainfall in Luxembourg for January, which is when the soil samples were taken (2011-2013: δD = -58.8 ‰ and δ^{18}O = -7.7 ‰).

4.3.2. Added water

The H and O isotopic signatures of the reference tap water were as follows: δD = -52.72 ‰ ± 0.34 and δ^{18}O = -8.34 ‰ ± 0.01. Overall there was small variation in δD and δ^{18}O values within the 3 water replicates of the same soil type which were drained or extracted through centrifugation: the mean coefficient of variances of the different water types varied between 0.9 % and 1.9 %. As there was only 1 cryogenic distillation carried out per soil type, there was no indication of variability for the isotopic signature in cryogenically extracted water.

The combined δD- δ^{18}O isotopic signatures of all water types extracted from experimental soil deviated from the initial signature of the reference tap water (**Figure 25**). Three groups of water samples can be easily distinguished according to their O and H isotopic signature: the drainage waters, the weakly and moderately bound waters, *i.e*. the capillary water, and the tightly bound waters. Differences in isotopic compositions of the different waters between sites and horizons were large for drainage and tightly bound waters while they were much less pronounced for the weakly and moderately bound waters.

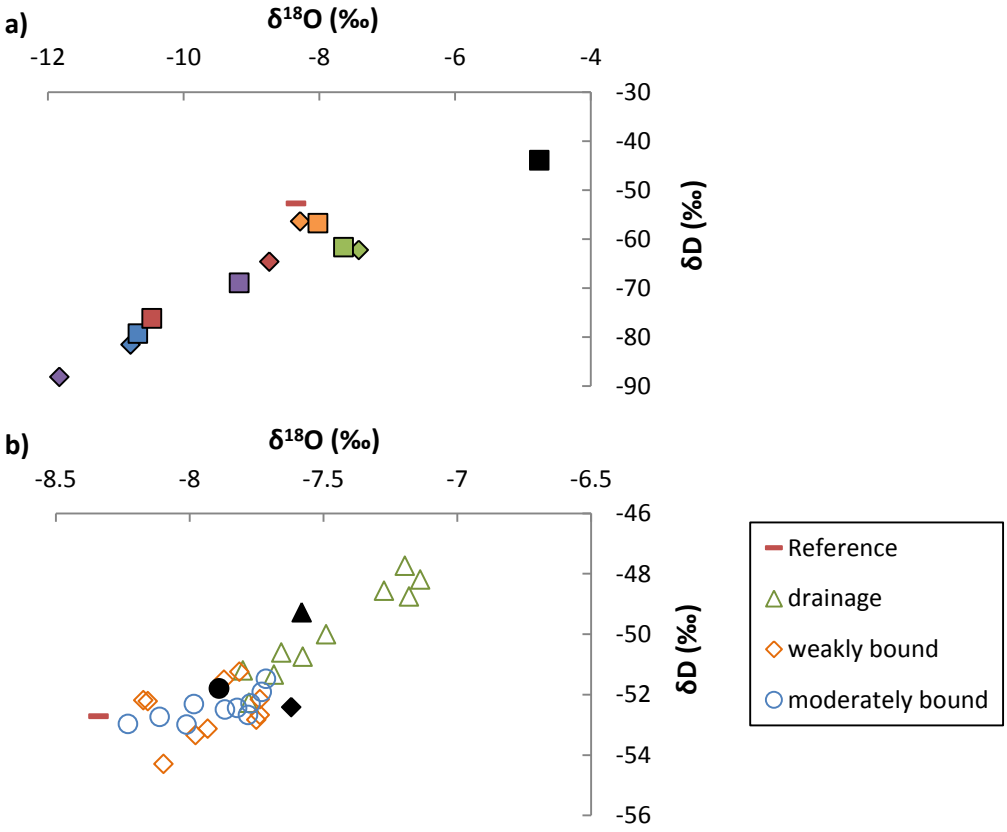

Figure 25. The relationship between deuterium and oxygen-18 signatures for a) the tightly bound water and b) all other water types. The black symbols represent the control of the respective water types and the red bar is the isotopic signature of the reference tap water. The symbols used under a) are explained in Table 4.

The drainage water was significantly more enriched in deuterium and oxygen-18 than the reference tap water ($p < 0.0005$). These water samples present an increase of δD (-52.3 ‰

to -47.7 ‰) with increasing $\delta^{18}O$ (-7.8 ‰ to -7.1 ‰) with the R^2 value of a linear regression line being 84 % (**Figure 26**). The drainage water showed significant differences in δD between horizons ($p < 0.0005$) and sites ($p < 0.0005$) as well as an interaction of the two factors ($p = 0.003$). In general, soils with lower pH had a more deuterium rich drainage water. The difference in isotopic signatures between different pH levels was even larger in A horizons compared to B horizons. The same two-way ANOVA for oxygen-18 could not be carried out because the assumptions of this parametric test could not be met. A non-parametric test was not considered due to very low sample sizes. However, a paired T-test confirmed that there was no significant difference in $\delta^{18}O$ between horizons ($p = 0.19$).

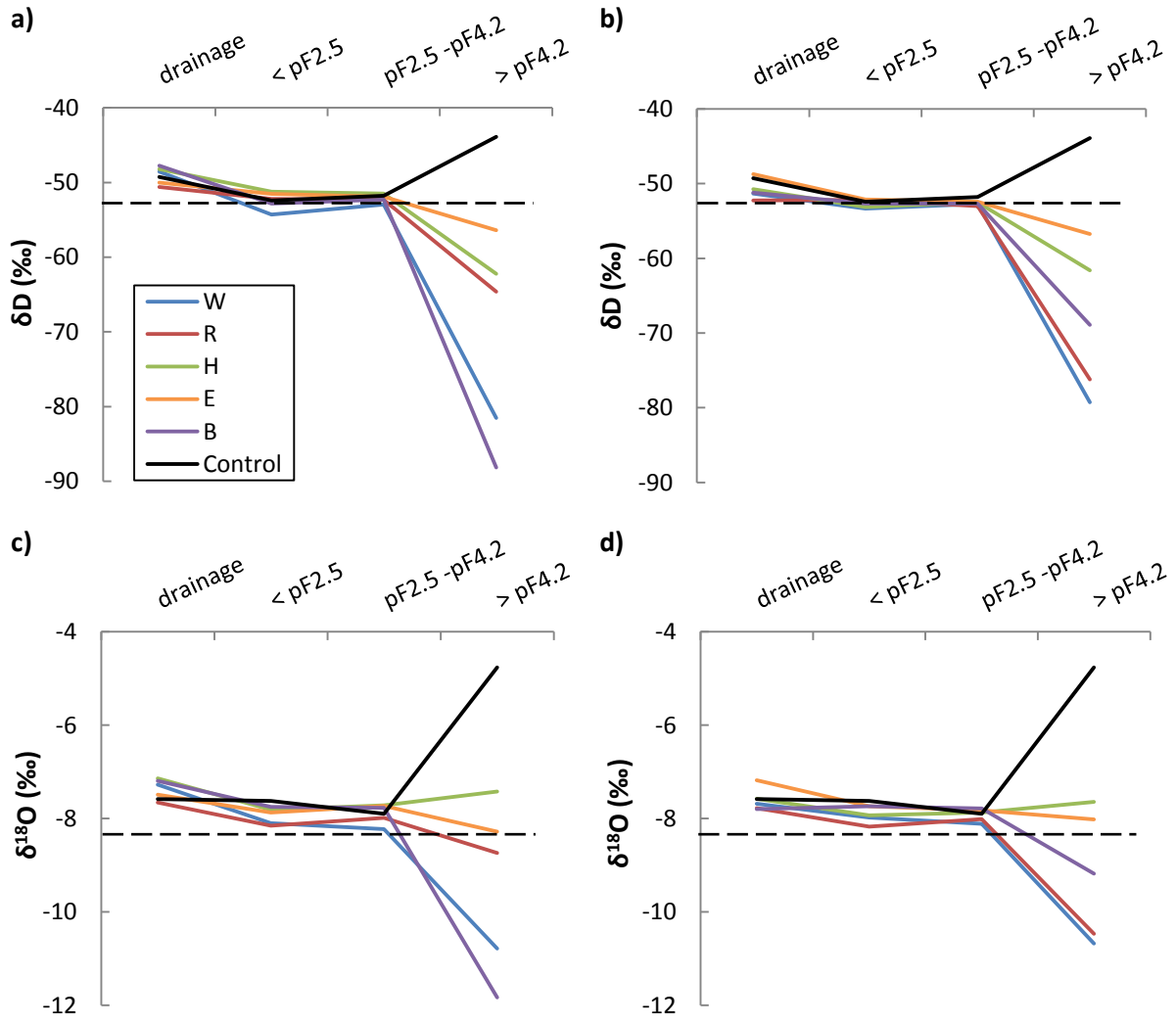

Figure 26. The δD values (‰) and δ^{18}O values (‰) of the four water types which were extracted from the A horizons (a, c) and the B horizons (b, d) of 5 sites. The dashed lines represent the isotopic signature of the reference tap water. The drainage water represents the final drainage. The weakly bound (< pF2.5) and the moderately bound (pF2.5 - pF4.2) waters were extracted using centrifugation while the tightly bound water (> pF4.2) was extracted using cryogenic distillation.

The isotopic signatures of the weakly and moderately bound waters were mostly scattered, showing a clear one-directional change in oxygen-18 from the reference water. The δD values of the weakly and the moderately bound waters did not differ significantly

from the reference tap water (p = 0.44 and p = 0.118 respectively). In contrast, the δ^{18}O values of the weakly and the moderately bound waters were significantly different from the reference tap water (p < 0.0005). The similarity in δD of the weakly and moderately bound waters to the reference water implied that these two bound waters had different isotope compositions than the drainage water. A paired T-test confirmed that the δ^{18}O values of the weakly and moderately bound waters also differed significantly from the drainage water (p < 0.005). Also, the isotopic signatures of the weakly bound water did not differ significantly from the moderately bound ones (δD: p = 0.38, δ^{18}O: p = 0.91).

The weakly bound water, did not present any significant differences in δD and δ^{18}O values when collected from different horizons (p = 0.40, p = 0.49), nor was there an interaction between factors of horizon and site (p = 0.109, p = 0.22). However, the weakly bound water from the W site was significantly more depleted in deuterium than from the R (p = 0.035), H (p = 0.033) and E (p = 0.007) sites. For oxygen-18, weakly bound water from the W site was significantly more depleted than from the E (p = 0.007) and B (p = 0.0007) sites. Also, the weakly bound water from the R site was significantly more depleted in oxygen-18 than from the H (p = 0.0007), E (p = 0.0001) and B (p < 0.0001) sites. The moderately bound water did not present any significant differences in δD values when collected from different soil horizons (p = 0.078) nor from different sites (p = 0.24). Also, there did not appear to be any interaction between the two factors of horizon and site for δD values in moderately bound water (p = 0.633). Similarly, the moderately bound water did not present any significant differences in δ^{18}O values when collected from different soil horizons (p = 0.36) nor was there an interaction between factors (p = 0.20). However, the moderately bound water from the W site was significantly more depleted in oxygen-18 than for all other sites (R: p = 0.038, H: p < 0.0001, E: p < 0.0001, B: p < 0.0001). Moreover, the moderately bound water from the R site was also significantly more depleted in oxygen-18 than for H (p = 0.011), E (p = 0.006) and B (p = 0.006).

A full statistical analysis could not be carried out for tightly bound water as only 1 replicate per soil type was available. The isotope compositions of the tightly bound water deviated noticeably from the reference tap water and the other soil waters in multiple directions (**Figure 25**). The control was more enriched in heavy isotopes than all other water types, while the tightly bound water of all other soil samples were generally more depleted in heavy isotopes. Two exceptions were the δD ratios of the E and H sites which were closer to the weakly and moderately bound waters and hence were also enriched in oxygen-18 compared to the reference water. The H and O isotopic fractionations of the tightly bound water compared to the reference water correlated positively with microbial soil respiration; the R^2 value of both logarithmic regression lines was 62 % (data not shown).

4.4. Isotopic mass balance between different water types

After centrifugation, the hydrogen isotopic signatures of the different water types extracted for experiment 2 were equal to the δD of reference tap water represented by the black line (**Figure 27a**). Also, there was no difference in isotopic signatures between centrifugation steps; not between $pF_{2.5}$ and $pF_{4.2}$ or between centrifuging straight to $pF_{4.2}$ and having $pF_{4.2}$ as a second centrifugation step. Note that more water was extracted when

centrifugation to $pF_{4.2}$ was carried out in two steps instead of one. In contrast to hydrogen, the water samples extracted through centrifugation were more enriched in oxygen-18 than the reference water and differed slightly among water types (**Figure 27b**). Furthermore, the δD values of the cryogenically extracted water did not change between various manipulations carried out on the combined W-B soil samples before cryogenic extraction. However, the cryogenically extracted water was much more depleted in deuterium than the reference water. Again on the contrary, small differences in oxygen-18 between water types were observed. The tightly bound water was more depleted in oxygen-18 than both the water above the pressure of $pF_{2.5}$ (> $pF_{2.5}$) and a mix of all soil water below field capacity (all). Also, the depletion in oxygen-18 compared to the reference water was much less pronounced. Interestingly, for all manipulations the water extracted by centrifugation was much more enriched in heavy isotopes than the cryogenically extracted water independent of which water types were mixed.

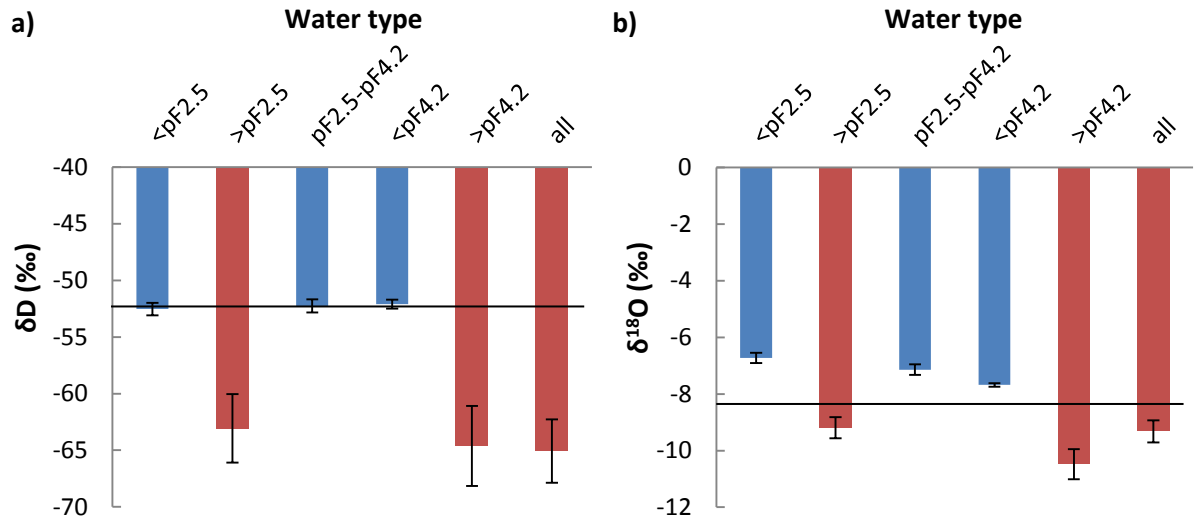

Figure 27. a) The δD values (‰) and b) the δ^{18}O values (‰) of different types of water which were extracted from Weierbach B horizon samples during experiment 2. Extractions were carried out either through centrifugation (blue: weakly bound water (< $pF_{2.5}$), moderately bound water ($pF_{2.5}$ – $pF_{4.2}$) and weakly plus moderately bound water (<$pF_{4.2}$)) or through cryogenic vacuum distillation (red: moderately and tightly bound water (> $pF_{2.5}$), tightly bound water (> $pF_{4.2}$) and a complete mix of all water below field capacity (all)).The error bars are the standard deviations obtained from 2-4 soil sample replicates. The black lines represent the isotopic signature of the reference tap water used to saturate the soil.

It is important to note that the cryogenic extraction yield differed for different mixes of water types (S1 to S3, **Figure 28**). S1, S2 and S3-2.5 had similar amounts of water left in the soil after cryogenic vacuum distillation. Nevertheless, because of the different initial moisture levels, the percentage of extracted water from the total amount differed. For S3-4.2 much more water was left in the soil after cryogenic extraction compared to the other 3 samples. Furthermore, the S3-4.2 sample had undergone the same manipulations as W-B of the experimental soil before cryogenic extraction and both samples showed a comparable extraction yield, although there was still a 5 % difference between their two yields.

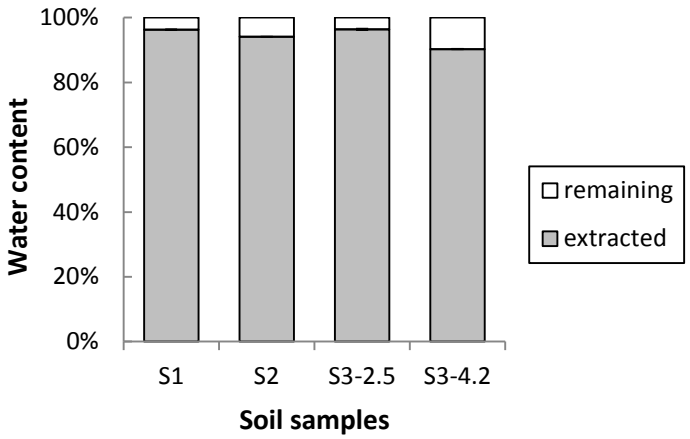

Figure 28. The percentage of water extracted from Weierbach B horizon samples during experiment 2 and the water remaining in the soils for the method of cryogenic vacuum distillation. The maximum of 100 % of water extracted from the soil by cryogenic distillation equals to the amount of water extractable by oven-drying at 105°C. Residual extractions were carried out for S1 on soil at field capacity, for S2 on soil previously centrifuged to $pF_{4.2}$ and for S3 on soil previously centrifuged to $pF_{2.5}$ and $pF_{4.2}$. Two to four replicates per soil type were used for extraction. The standard deviations are too small for the error bars to be visible.

The total weights of the same soil water mixtures extracted through various method combinations differed for 5 out of 6 mass balance comparisons (**Table 5**)**.** Two significant differences out of 6 water mixture comparisons were identified for δD and δ^{18}O, albeit they were two different ones for the two elements. For example, the two tightly bound water samples S2 and S3 had similar isotopic signatures for deuterium but the difference in δ^{18}O between samples was greater than 5 % (vi). Likewise, the capillary water presented a significant difference in the oxygen-18 isotopic signature when either sampled in one centrifugation step (weak & moderate S2) or in two steps (weak S3 + moderate S3) but no difference was observed for the isotopic signature of deuterium (iv). Despite the fact that these two comparisons (iv, vi) presented significant isotopic differences for ^{18}O, the comparison between the two mixtures of capillary and tightly bound water did not differ significantly (iii). Furthermore, the δD of these two mixtures of capillary and tightly bound water were significantly different from the water below field capacity being extracted through cryogenic vacuum distillation only (i and ii).

Table 5. Mass balances for the amount of extracted water and the isotopic signatures compared to VSMOW (δD, δ^{18}O) of water extracted from the experimental W-B soil as part of experiment 2 (S1, S2, S3). A difference above 5 % between comparisons was chosen as the significance level. The '+' sign indicates a combination of water types collected through different extraction methods or steps. The '&' sign indicates the joint extraction of different water types with one method.

Comparison	Water mixtures	Extracted water	δD	δ^{18}O
		g / 100 g dry soil	‰	‰
	weak & moderate (S2) + tight (S2)	60.4	-60.4	-9.4
i	=	≠	≠	=
	below field capacity (S1)	70.6	-65.1	-9.3
	weak (S3) + moderate (S3) + tight (S3)	58.4	-60.8	-9.4
ii	=	≠	≠	=
	below field capacity (S1)	70.6	-65.1	-9.3
	weak & moderate (S2) + tight (S2)	58.44	-60.80	-9.42
iii	=	=	=	=
	weak (S3) + moderate (S3) + tight (S3)	60.38	-60.43	-9.38
	weak (S3) + moderate (S3)	18.1	-52.3	-7.1
iv	=	≠	=	≠
	weak & moderate (S2)	15.1	-52.1	-7.7
	moderate (S3) + tight (S3)	54.8	-61.4	-9.6
v	=	≠	=	=
	moderate & tight (S3)	69.1	-63.1	-9.2
	tight (S3)	40.29	-64.62	-10.5
vi	=	≠	=	≠
	tight (S2)	45.31	-63.20	-9.9

5. Discussion

5.1. Method performance

5.1.1. Drainage water

At saturation, the Weierbach A horizon reached a GWC above 100 % due to its very low bulk density (high porosity and high organic matter content). In this case, the very high organic matter content largely increases the soil's water retention capacity (Cosandey & Robinson, 2000). These two factors allow the soil to trap a weight of water slightly higher than the weight of dry soil that contains this water. Additionally, W-A, B-A and to a lesser extend H-A were observed expanding in volume at saturation, possibly because of a slight hydrophobicity of organic compounds (repulsion forces) or swelling of clay. The H-A soil sample has fairly low TOC content but sometimes behaves like an organic rich soil, e.g. swelling when wetted. This behaviour may be due to presence of O horizon material in the A horizon.

Water losses due to handling of the bottles during saturation and drainage are negligible because the mean difference between the 'saturation water' and the 'water at FC + total drainage water' was very small. Also, the GWC after both drainage periods was similar meaning that the soil moisture conditions for centrifugation were similar to the conditions during incubation. Furthermore, the comparison of initial and final drainage water deviates from the line 'y = x' mainly because of the Ell soil (**Figure 13**). The 5-week incubation at field capacity of the Ell soil lead to a massive soil structure. This structure drastically reduced the soil porosity and hence its final drainage capacity, particularly in the B horizon. This oberved massive structure may be caused by the wetting of the clay (~15 %) and the very low TOC content (~1.5 %) in these soils in combination with the frequent handling of the bottles to measure their weight three times a week.

Generally, the amount of drainage water was lower from sandy soils than from clay or loamy soil. This lower drainage was probably obtained due to the fact that a fine sandy soil (H) and fine pure sand for the control were selected. The size of the sand particles was probably at the lower end of the spectrum, *i.e*. closer to 50 µm than 2 mm. In the bottles, the fine sand was in fact assumed to have had a lower macroporosity than the clay and loamy soils as the latter mainly formed aggregates of 2 mm in diameter after sieving (R and E soil samples). Instead of looking at the amount of drainage water, it is possible to look at it from the perspective of water remaining in the soil after drainage. Overall, when the soil porosity was higher the moisture in the soils after final drainage was higher too. The rather strong positve relationship between porosity and TOC as well as clay content explain why soils with high porosity retain more water at field capacity. This dominance of TOC controlling the soil water retention capacity in the sampled soil types was confirmed by PCA.

When tap water comes in contact with soil an immediate exchange between the water and the soil bases was expected. In more acid soil, like the used soil samples, mostly protons would enter the solution instead of base cations. Hence a positive correlation between soil pH and the pH of drainage water was expected. However, **Figure 14** could not confirm any direct correlation. It is likely that the tap water had a high acid neutralizing capacity. Therefore, the water pH would be buffered despite the addition of a high amount of

protons to the water. The pH of the final drainage waters for the very acid W-A and B-A soil samples probably differed largely from the pH of the reference tap water because not only re-saturation water was drained. It is plausible that some water that was in the bottle during incubation was also drained because there is a continuum between small and big pores in soils. The collected drainage water could have been a mix of newly added tap water and older tap water present in the soil samples during incubation. The degree of mixing depends on the hydrological properties of the soils in the context of soils close to saturation. The new infiltrating water may have created a piston flow which displaced old water from the soil matrix into large, freely draining pores (Jardine *et al.*, 1990; Luxmoore *et al.*, 1990).

Evaporation was found to cause H and O isotope fractionation of the drainage water. The regression line fitted to the evaporation data in **Figure 15** explained up to 86 % of the fractionation. Hence, there may be another factor causing additional fractionation. However, when the standard deviations of fractionation of δD and δ^{18}O from the reference water were taken into account, almost all data points touched the regression line. This overlap indicates that the correlation may be stronger than specified by the R^2 value. The amount of evaporation and, hence, isotopic fractionation, of the drainage water could be limited through changes in the method. For example, the extraction could be carried out in colder conditions with high relative humidity. Furthermore, the gravitational water could be extracted by suction corresponding to field capacity, using either a mean or soil specific pF value. This way the drainage water would not be exposed to the atmosphere for hours.

5.1.2. Incubation period

The weight loss from the bottles compared to the total amount of soil water was negligible. Still, could the amount of weight loss seen in **Figure 16** actually have a significant effect on isotopic signatures? The isotopic fractionation depends on how the weight loss occurred and whether the process involves isotopic preferences. Potential reasons for minor weight loss include drying of sealing clay, water exerting pressure due to gravity and microbial soil respiration. The main theory was that microbial respiration increased the CO_2 concentration in the bottle and hence the pressure, therefore water was pushed out of the bottle through a broken clay seal. Furthermore, microbial respiration releases water vapour which could escape through the top and would accumulate as condensation on the paraffin tape. This assumption could also explain the clear difference in weight loss between A and B horizons as B horizons generally have a lower MSR and the weight loss in the control bottles was almost zero. Moreover, as previous studies have shown, microbial processes can lead to isotopic fractionation of many elements (Blake *et al.*, 1997; Dijkstra *et al.*, 2006; Kool *et al.*, 2009, 2011; Snider *et al.*, 2009; Lerch *et al.*, 2011).

Note that Lerch *et al.* (2011) states that a prolonged incubation period over 30 days is necessary to obtain stable results when considering effects of microbial respiration. During a shorter incubation, variables do not have time to stabilise. High fluctuations in the variables due to soil disturbance during the set-up of the experiment would make it difficult to give clear statements. The incubation period of this study was longer than the limit specified by Lerch *et al.* (2011), therefore variables were assumed to be stable.

5.1.3. Centrifugation

Centrifugation was not as efficient as the ceramic plate extraction method because the GWC after centrifugation was higher than the GWC after CPE when the supposed same pressure was applied (**Figure 18**). In fact centrifugation did not extract any moderately bound water, *i.e.* any water between $pF_{2.5}$ and $pF_{4.2}$, for most soils as the water amount left in the soil after centrifugation to $pF_{4.2}$ was the same or even higher than the soil moisture after CPE to $pF_{2.5}$ (**Figure 19**). For example, after the first centrifugation, the W-A soils still had a GWC high above 100 % even though a soil moisture of 85 % was reached with CPE $pF_{2.5}$. In addition, **Figure 20** indicates that the strong positive correlation between remaining soil water and porosity prevails after each centrifugation. Again, the high OM content can trap more water than soil particles and form a hydrophobic layer which prevents intensive draining, even though there is more pore space in A horizons (Gobat, 1998; Cosandey & Robinson, 2000). Sandy soils (Control, H-B and B-B) were among the soils with the lowest difference in moisture levels between the two methods, which suggests that some soil characteristics were responsible for the difference between methods. Yet, there were no correlations found for the moisture difference between the two extraction methods and available soil properties.

For most soil samples centrifugation was not suitable to separate the weakly from the moderately bound water. Soils with higher silt and clay content occasionally even had standing water on top of the soil sample after centrifugation. It is therefore possible that soil structure changes occurred during centrifugation which led to the discrepancy in the amount of water extracted compared to ceramic plate extraction. The water extraction yield through centrifugation may be improved by adding drainage channels into the soil, e.g. with narrow plastic pipes. This alteration may not have any negative impact on the results as the original soil structure was already destroyed through sieving and air-drying. Although most fresh soil samples still had a better structure at the time of centrifugation than the experimental soils, it is possible that not all water up to $pF_{4.2}$ was extracted for FS either. As the fresh soil samples were not sieved to 2 mm, the CPE measurements could not be used as an indicator of centrifugation efficiency.

As the impact for the weakly and moderately bound waters were similar for sites and horizons, it may be best to say that the effects were observed for capillary water in general.

5.1.4. Cryogenic vacuum distillation

Though cryogenic vacuum distillation extracted water well above $pF_{4.2}$, the tightly bound water is also mixed with moderately bound water since centrifugation to $pF_{4.2}$ was not efficient. This raises the question whether the large variability in the amount of remaining water after cryogenic vacuum extraction and in the H and O isotopic signature of the cryogenically collected water for experiment 2 could be due to an unrepeatability of centrifugation. The answer is ‘No’. The cryogenic extraction step S1 also shows large variances in those variables and no centrifugation was carried out beforehand. Also centrifugation generally has low variability of the amount of water collected and H and O isotopic signatures between replicates. Furthermore, after both centrifugation steps the different soil replicates had similar gravimetric water contents.

The water extraction yield of cryogenic vacuum distillation and the isotopic signatures of the tightly bound water were correlated (**Figure 22**). This means that lower cryogenic extraction yields lead to depletion of heavier isotopes in the extracted water compared to the reference water. The depletion was likely caused by incomplete evaporation of the soil water. During evaporation the heavier isotope, e.g. deuterium, evaporates more slowly than the lighter hydrogen isotope, ^{1}H, *i.e*. the water vapour becomes depleted in heavy isotopes while the liquid phase staying in the soil becomes enriched. The method of cryogenic vacuum distillation was assumed to not cause any fractionation due to the successful use in previous studies (Araguás-Araguás *et al.*, 1995; West *et al.*, 2006). However the fractionation was not only attributable to the incomplete cryogenic extraction yield because soils with a yield close to 100 % still showed considerable fractionation of the extracted water compared to the reference water. Thus, it is possible that there are other factors that caused fractionation.

Note that not all *in situ* water could be removed from the soil samples before the start of the experiment without damaging the soil, e.g. destroying the organic matter by oven-drying the soil above 105°C. A water extraction close to 100 % indicates that cryogenic vacuum distillation removed *in situ* water from the soil sample because air-dried soils still retained a GWC of 0.2 % to 7.9 % after oven-drying at 105°C. Nevertheless, it was possible to determine that the fractionation was not due to the extraction of remaining *in situ* water. Interestingly, the soil types from which cryogenic distillation extracted *in situ* water (R-A, H-A, H-B, E-A, E-B, B-B = x + y - R-B) also represented one of the two distinct groups which can be observed on top of the correlation between the isotopic signature of tightly bound water and cryogenic extraction yield in **Figure 22**. The higher extraction yields compared to group z and W-A can be explained by the lower water retention capacity of these soil types (x + y) according to PCA. However, it is unclear why R-B achieved such a low extraction yield.

5.1.5. Isotopic mass balance

During experiment 2 the isotopic signatures did not differ between the various soil manipulations that were carried out using centrifugation, *i.e*. the number of centrifugations used to extract different capillary water types (**Figure 27**: blue columns). Though, there was a very large difference in H and O isotopic signatures between water extracted through centrifugation and cryogenically extracted water no matter which water types were mixed in one extraction sample (**Figure 27**: blue vs red). For example the δD of the water below $pF_{2.5}$ (S3-$pF_{2.5}$ blue) was the same as the δD of the water between $pF_{2.5}$ and $pF_{4.2}$ (S3-$pF_{4.2}$ blue) when extracted through centrifugation but the δD of the water below $pF_{2.5}$ (S3-$pF_{2.5}$ blue) was different from the δD of the water above $pF_{2.5}$ (S3-$pF_{2.5}$ red) extracted through cryogenic vacuum distillation. It seems that whenever tightly bound water was included in a soil water mixture, the H and O isotopic signatures were much more depleted in heavy isotopes compared to mixtures of water not containing tightly bound water. The strong depletion in heavy isotopes and the larger amount extracted of tightly bound water compared to capillary water must be the reason for the very large difference in isotopic signatures between water mixtures containing tightly bound waters and those which did not.

S1, S2 and S3-2.5 retained similar amounts of water in the soil after cryogenic vacuum distillation, which suggests that there is a limit to the water that cryogenic distillation may extract from this soil type. For S3-4.2 this limit was not reached as much more water was left in the soil relative to the other 3 samples. Interestingly, the S3-4.2 sample had undergone the same manipulations as the experimental W-B soil in experiment 1 before cryogenic extraction and both samples retained a very similar GWC after cryogenic extraction. It is possible that two consecutive centrifugations before cryogenic vacuum distillation alter the soil structure too much and significantly decrease water extraction. "Open" porosity may become "closed" porosity and water may be trapped. The only way to extract water from this "closed" porosity is diffusion through the solid phase.

The total weights of the same soil water mixtures extracted through various method combinations largely differed for 5 out of 6 mass balance comparisons (**Table 5**). This observation shows that the combination of extraction methods clearly influences how much water can be extracted. Hence, it is not surprising that some significant differences between water mixture comparisons were identified for δD and δ^{18}O. The fact that these differences were significant for different sample comparisons for both δD and δ^{18}O indicates that both elements vary independently. The distribution of comparisons that are similar or different did not give any detail about which method combinations were causing the differences in isotopic signatures, e.g. having one or two centrifugations before cryogenic vacuum distillation.

W-B was chosen for experiment 2 because it is a well-studied loamy soil with roughly equal proportions of sand, silt and clay contents. The mass balance analysis indicates that water type mixtures can only be compared with confidence when they were extracted in the same way. Note that soils with different textures or with the same texture but varying OM contents may behave differently.

5.2. Isotopic composition of the different soil water types

The moderately bound *in situ* water of the fresh soil samples followed the global meteoric water line, indicating that no fractionation occurred in the soil between the last rain events and the sampling date. The *in situ* water in the French fresh soil (B) most likely had a very different isotopic signature for deuterium and oxygen-18 than the waters of the Luxembourgish fresh soils certainly because of differences in isotope compositions in rainfall between Luxembourg and Burgundy, France. This should be checked in future inter-comparisons between French and Luxembourgish study sites.

A deviation of the H and O isotopic signature of all water types from the reference tap water indicates fractionation. Evaporation causes a linear deviation from the reference water with a stronger kinetic effect for oxygen-18 than for deuterium (Gibson *et al.*, 2008). The linear deviation of drainage water δD-δ^{18}O from the reference tap water had an R^2 value of 84 % with the isotope composition of the reference water being the most depleted in heavy isotopes (**Figure 25**). This presents more indications that the significant fractionation from the reference water was solely due to evaporation.

After cryogenic vacuum distillation, the evaporated (and condensated) water was isotopically analysed and not the remaining water in the soil, therefore the reference water should be the most enriched in heavy isotopes compared to the tightly bound soil waters if only incomplete evaporation had caused fractionation. Most of the soil types, especially those with high water retention capacity, showed a strong linear deviation in isotopic signatures from the reference tap water, indicating evaporative fractionation. However, the E and H soil samples and the control were more enriched in oxygen-18 than the reference tap water. Moreover, the control was also more enriched in deuterium compared to the reference. Hence, incomplete evaporation could not have caused the fractionation of these 5 soil types.

On the other hand, the isotopic signatures of the weakly and moderately bound waters did not deviate in a straight line from the reference tap water but were mostly scattered, indicating that no evaporative fractionation occurred (**Figure 25b**). The scatter was observed because the signature of deuterium was very similar to the reference tap water while the oxygen signature changed. Depending on the processes that cause isotopic fractionation of a specific water type it is not surprising that hydrogen and oxygen atoms may fractionate in a different way. For instance, the δ^{18}O values of the weakly and the moderately bound waters were significantly higher than the signatures of the reference tap water. In addition, the δ^{18}O of the final drainage water was significantly higher than the signatures of the weakly and the moderately bound waters. However, the differences to both of these bound waters were very small and identical (0.24 ‰ to 0.59 ‰) and are therefore unlikely to be hydrologically relevant. Also, since the fractionation of the drainage water was attributed to evaporation, the δ^{18}O of the drainage water may indeed be lower than the δ^{18}O of the capillary waters.

The isotopic signatures of the two capillary waters (weakly and moderately bound) were very similar (**Figure 26**). Centrifugation may create mixing of water types from distinct pore sizes. Alternatively, Zabowski & Ugolini (1990) suggest that isotopic similarities between water extracted at two centrifugation speeds occur because the soil water equilibrated among different pore sizes during the lag period between sampling and analysing or here between the incubation period and water extractions. In this study, the similarity in isotopic signatures between weakly and moderately bound waters was probably mainly because centrifugation did not in fact extract much moderately bound water as the comparison with ceramic plate extraction indicated.

The isotope composition of the capillary water and the tightly bound water differed noticeably. Though a large part of the H and O isotopic fractionation was attributed to inefficient cryogenic vacuum distillation not all of it can be explained this way. Hence, the difference in isotopic signatures between the two water types indicates that they did not mix.

In conclusion, the null hypothesis (H_0) that the H and O isotopic signatures do not differ significantly between the different water types (drainage, weakly bound, moderately bound and tightly bound water) was rejected.

5.3. Biogeochemical effects on isotopic fractionation of soil water during the experiments

The drainage water showed significant differences in isotope compositions between horizons and sites as well as an interaction of the two factors. In general, drainage water rich in deuterium was obtained from soils with lower pH. The difference in isotopic signatures between different pH levels was even larger in A horizons compared to B horizons. These differences between horizons were likely caused by the fact that A horizons usually drained less water. When little drainage water was collected, the percentage of evaporation from the total water was much higher because the surface area of evaporation was always the same, meaning that there was a higher potential for fractionation. Furthermore, A horizons and sandy soils were in general more acidic. It seems that this relationship caused a significant difference between soil types and an interaction between factors. The total organic carbon (TOC) content appears to be the main link between factors. A high TOC lowers the pH and traps water efficiently, thus reducing the drainage capacity of the soil. Less drainage water collected from soil types means that evaporation has a higher fractionation effect, thus, causing differences in H and O isotopic signatures between soil types. Therefore, the differences in the isotopic signatures of drainage water between sampling sites and horizons cannot be explained by a preferential use of one isotope during biogeochemical processes.

Nonetheless, differences in drainage water pH were observed between the initial (DWs) and final (DWe) collections; particularly a large decrease in pH was observed for W-A and B-A. The new infiltrating tap water may have created a matrix flow which displaced old water (with high residence time and low pH) from the soil matrix into large pores. In combination or alternatively, the connectivity and mixing between small and large pores was good in W-A and B-A soil samples. The high amount of bound water in these soil types could largely influence the pH of the comparatively low amount of drainage water (**Figure 23**). However, some soil types provoked a clear increase in pH from the initial to the final drainage water in spite of the soil pH being low (R-B, H-A, H-B and E-A). The evolution of the soil structure over 5 weeks may explain these observed differences in pH between the initial and final drainage waters.

The weakly bound water from the W site showed a significant depletion in deuterium relative to other sites (R, H and E). Their H isotopic signature seem to correlate with the mean TOC content (A & B horizon) at the different sites. Therefore it is possible that certain microbial processes or interactions with the organic matter caused the differences in the isotopic signatures of deuterium between sampling sites. There were significant differences in O isotopic signatures between sampling sites for weakly and moderately bound waters. Also, the soil type groupings as formed by ANOVA for both water types were similar to each other but could not be explained by a TOC gradient. The groupings for ^{18}O seem to follow a gradient of clay content. This may suggest that adsorption of water to soil particles has an influence on the fractionation of ^{18}O in the capillary water between soil types. Alternatively, weathering of clay particles may have an effect but it is unlikely that such processes would have influenced the isotopic signatures on such a short time-scale.

The $\delta^{18}O$ values of the weakly and the moderately bound waters may have become significantly higher than the signatures of the reference tap water through microbial soil

respiration as indicated by the weight loss from the soil bottles. During respiration, microbes take up O_2 and organic carbon ($C_nH_{2n}O_n$) to produce energy while releasing CO_2 and H_2O. It is possible that preferential processing of isotopes in this citric acid cycle caused the microbes to release water molecules with oxygen-18 which in turn enriched the soil water in oxygen-18 compared to the reference tap water. The intricacy of the citric acid cycle only leaves speculation as to the pathway through which the soil water would become enriched in heavy oxygen without influencing the isotopic ratio of hydrogen. Also, despite having large differences in TOC and MSR, no significant differences in H and O isotopic signatures were observed between horizons. The differences may not have been detected statistically because the TOC and MSR of the B horizons from the W and B sites were higher than the values of the A horizons from 2-3 other sites. However, if that were the only reason, the ANOVA test should have detected an interaction between sampling site and horizon.

The H and O isotopic signatures of the tightly bound water could not be evaluated for statistical differences between sampling sites and horizons due to a limiting sampling size. However, the fractionation of the tightly bound water compared to the reference water correlated positively with microbial soil respiration, meaning that MSR could explain part of the fractionation that is not due to the extraction method. Furthermore, could part of the fractionation effect in tightly bound water be an effect of preferential H and O retention of water in soil during re-wetting of the air-dried soil? Especially in the control this would be a likely explanation as many other soil parameters thought to influence H and O isotopic signatures were missing from the pure sand, e.g. organic matter, clay particles, microbial activity.

To conclude, the null hypothesis (H_0') that the biological, physical or chemical soil properties in forest soils do not directly influence the hydrogen and oxygen isotopic signatures of soil water was tentatively rejected.

6. Conclusion

The results of this study show that the choice of methods for water extraction are important when analysing hydrogen and oxygen isotopes of different water types.

The H and O fractionation of drainage water extracted through gravity was completely attributed to evaporation from the collection bottles. Furthermore, the centrifugation method that was used in this study is inadequate to separate weakly and moderately bound waters of the studied soil types. Moreover, the isotopic fractionation of tightly bound water from the reference water was largely caused by inefficient cryogenic vacuum distillation but not exclusively. The results indicate that cryogenic vacuum distillation may be suitable for soil types with low water retention capacity. Also, the mass balance analysis shows that water type mixtures can only be compared with confidence when they were extracted in the same manner.

The H and O isotope composition of capillary and tightly bound water generally differ from one another even when taking into account the high uncertainty of the isotopic analysis due to poor method performance. The capillary water and tightly bound water generally did not mix. One factor having an impact on the isotopic composition of capillary water and tightly bound water is likely microbial soil respiration (MSR). However, the degree and direction of change is not necessarily similar for deuterium and oxygen-18 stable isotopes. Furthermore, clay content, total organic carbon content (TOC) and probably the related microbial soil respiration (MSR) are important soil parameters which cause differences in isotopic composition in water between soil sites. In addition, parameters such as soil structure and the connectivity between large and small pores may contribute to differences in isotopic ratios of soil water between sampling sites. These results indicate preferential use of isotopes during microbial and adsorption-desorption processes. However, despite having large differences in TOC and MSR there were no significant differences between horizons.

This study needs to be built on with amended water extraction methods before the results can be used to improve pedological studies, environmental impact assessments, nutrient cycles, etc.

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