Combination of soil water extraction methods quantifies isotopic mixing of waters held at separate tensions in soil

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Abstract. Measurements of the isotopic composition of water recovered from soil at different tensions provide a powerful means to identify potential plant water sources and quantify heterogeneity in residence time and connectivity among soil water regions. Yet incomplete understanding of mechanisms affecting isotopic composition of different
10   soil water pools and the interactions between antecedent and new event water hinders interpretation of the isotope composition of extracted soil and plant waters. Here we present an approach for quantifying the time-dependent isotopic mixing of water held at separate tensions in soil. We wetted oven-dried, homogenized sandy loam soil first with isotopically "light" water (δ^2H = -130‰; δ^{18}O = -17.6‰) using a sufficient volume to fill only the smallest soil pores, and then with "heavy" water (δ^2H = -44‰; δ^{18}O = -7.8‰) to fully saturate the remaining soil regions. Soil
15   water effluents were then sequentially extracted at three tensions ('low' centrifugation = 0.016 MPa; 'medium' centrifugation = 1.14 MPa; and 'high' cryogenic vacuum distillation at an estimated tension greater than 100 MPa) starting after variable equilibration periods of 0 h, 8 h, 1 d, 3 d and 7 d. We assessed differences in the isotopic composition of extracted effluents over the 7 d equilibration period with a MANOVA and a mixing model describing the time-dependent effects of isotope self-diffusion and exchange. The saturated moisture conditions used in our
20   experiment likely facilitated rapid isotope exchange and equilibration among different pools. Despite this, the isotope composition of waters extracted at medium compared to high tension remained significantly different (MANOVA) for up to 1 day, and that for waters extracted at low compared to high tension remained significantly different for greater than 3 days after soil wetting. Equilibration (assuming no fractionation) predicted from the time-dependent mixing model for water held at high tension occurred after approximately 4.33 days. Our approach will be useful for
25   assessing how soil texture and other physical and chemical properties influence isotope exchange and mixing times for studies aiming to properly characterize and interpret the isotopic composition of extracted soil and plant waters, especially under variably unsaturated conditions.

1 Introduction

Quantifying residence time and connectivity among discrete pools of soil water relies on methods that differentiate
30   the isotopic signature of soil water held across different sized soil pores and at a range of different matric tensions. A variety of field- and lab-based methods are typically employed for such analyses (Oerter and Bowen, 2017; Orlowski et al., 2016b; Sprenger et al., 2015), but each separately assess different pools of water within distinct ranges of tension,

or fail to differentiate isotopic signatures beyond that of bulk soil water. More comprehensive evaluation of soil water isotopes across multiple pore sizes and tensions is needed to reconcile observations suggesting plants take up matrix

35 water that is incompletely mixed with isotopically distinct mobile soil water (Brantley et al., 2017; Brooks et al., 2010; McDonnell, 2014; Sprenger et al., 2019)Questions remain about whether the available isotopic observations support ecohydrologic separation among soil water pools or simply represent expected patterns emerging from heterogeneous flow paths and differences in pore water residence times (Evaristo et al., 2019). As such, more thorough and discriminating approaches for evaluating soil water isotopes are needed to challenge these models (Berry et al., 2018).

40 At a fundamental level, such methods are needed to more thoroughly address dynamics of soil water movement, mixing, and isotopic fractionation (Barnes and Allison, 1988; Braud et al., 2005; Gaj and McDonnell, 2019) to improve quantification of the water budget, partitioning of water fluxes, and transport of nutrients via water fluxes in the critical zone.

Characterization of water isotope ratios in soils include in situ direct equilibration or field extraction using

45 suction lysimeters, but more often involves water extraction of soil samples collected from the field. Depending on the method employed, water is recovered at different energies and the proportion of water extracted is dependent on the volumetric water content of the sample and the soil water retention curve for the soil of interest (Sprenger et al., 2015). The most common of these extraction methods, in order of lowest to highest amount of energy applied to the soil sample, are suction cup lysimeters, mechanical squeezing, centrifugation, and cryogenic vacuum distillation

50 (Sprenger et al., 2015). Suction cup lysimeters typically sample water held at low tension (0.05 to 0.10 MPa) and are therefore limited to analysis of only the highly mobile fraction of soil water. Mechanical squeezing and centrifugation can recover water across much broader tension ranges and with no fractionation, but are unable to drain pores with diameters less than 0.03 μm (Orlowski et al., 2016b; Sprenger et al., 2015). Centrifugation is particularly useful because the rotational velocity and the centrifuge set-up are physically related to the energy applied to the soil sample

55 and therefore the pore size drained (Edmunds and Bath, 1976). Cryogenic vacuum distillation (CVD) recovers nearly all water from a soil sample, with the more clay- and more organic-rich soil samples requiring greater extraction times or temperatures (Orlowski et al., 2016a). Each method has been used to inform the community about the mobile or bulk water within soils. However, we lack approaches to assess separately different fractions of soil water and infer the degree of connectivity and dynamics of mixing between mobile and matrix water.

60 CVD has been separately compared to centrifugation with the assumption that water held across matric tensions is well-mixed (Tsuruta et al., 2019), but recent findings show that applying the two methods in combination has the potential to assess water isotope compartmentation and interactions that can inform proper characterization of soil water isotopic compositions for ecohydrological studies (Adams et al., 2019). Adams et al. (2019) concluded that soil water extracted via centrifugation was consistently incompletely mixed after 72 hours of equilibration time due

65 to statistically different isotopic compositions of centrifuge-obtained water and the remaining water extracted via CVD. However, their experimental design precluded analysis of the time necessary for bound and mobile waters to completely mix. In addition to understanding interactions between water pools within soil affecting the isotopic composition of extracted water, recent work has highlighted the importance of considering possible fractionations that may affect the isotopic composition. As new research suggests, isotope effects related to adhesion under various matric

70   potentials, soil wettability, and solid interfacial chemistry of soil particles is important to consider (Gaj et al., 2019; Gaj and McDonnell, 2019).

Here we present and evaluate a step-wise procedure to access and quantify isotopically different pools of water in the soil and characterize the dynamics of their interaction over time. In order to examine the largest extent of tension ranges in soil, we confine our initial study to soil moisture conditions at saturation and investigate the time-
75   course of mixing between waters applied sequentially to oven-dried soil. We addressed the following questions:

1. Can soil water held at different tensions be separately extracted from the same soil sample and analyzed for isotopic composition?
2. Do isotopically labeled fractions of water sequentially added to dry soil thoroughly mix?
3. Can the time-course for isotopic mixing be determined quantitatively for waters held at different tensions
80   within soil?

2 Methods

2.1 Experimental design

Our laboratory experiment involved applying a small amount of isotopically 'light' water to oven-dried soil followed by saturating the soil samples with an isotopically 'heavy' water. Three water effluents (low, mid, and high tension)
85   were then removed from saturated soil samples through a stepwise increase of applied energy using two centrifugation speed steps followed by distilling the remaining water in the soil samples via cryogenic vacuum distillation (CVD). Subsets of samples were extracted only using CVD (hereafter called 'bulk sample extraction' or 'BSE') either immediately after applying only the small amount of isotopically light water ('BSE$_{light}$') or immediately after adding both the isotopically light and heavy waters ('BSE$_{light+heavy}$'). Prior to step-wise extraction for the remainder of the soil
90   samples the light and heavy water applied were allowed to freely mix and equilibrate under saturated conditions for variable amounts of time: 0 hours (n=15), 8 hours (n=3), 1 day (n=3), 3 days (n=3), and 7 days (n=3). The water effluents from each soil sample, either from BSE or step-wise extractions, and from various timepoints were then analyzed for hydrogen and oxygen stable isotope ratios (δ^2H and δ^{18}O).

2.2 Experimental soil and wetting procedure

95   We used a sandy loam soil collected from the top 10 cm of the surface from prairie vegetation east of Laramie, WY. Soil was passed through a 2-mm sieve and all coarse litter was removed except for very fine fragments. Soil particle size was determined by the hydrometer method using sodium hexametaphosphate as the chemical aid in sample dispersion (Black and Day, 1965). Particle size distribution defined by the U.S. Department of Agriculture classification system was 9% clay, 32% silt, and 59% sand.

100   Two isotopically distinct waters were applied during the sequential soil wetting. The 'light' water was depleted in ^{2}H and ^{18}O relative to the 'heavy' water. Light water used in the experiments was local tap water and heavy water was from multiple bottles of FIJI Water® (FIJI Water LLC, Los Angeles, CA, USA). The isotope ratio value for the light water was -130±2‰ for δ^2H and -17.6±0.5‰ for δ^{18}O (n=5) and for the heavy water was -44±2‰ for δ^2H and -7.3±0.4‰ for δ^{18}O (n=5).

105   Samples were prepared by oven drying 350 g of soil at 105ºC for 48 hr. After a short period of cooling 20 ml of the light water was applied to the 350 g sample with a spray bottle and mixed by gloved hands to ensure homogenous application. 18-30 g of this slightly wetted soil was gently packed to form soil columns in each of six custom made centrifuge inserts, described below in section 2.3. Weights of inserts and sleeves prior to adding the soil were recorded. Except for sub-samples that were immediately collected for bulk sample extraction (BSE_light), the packed inserts were

110   wetted from the bottom up by immersing in the heavy water. This ensured the soil samples were fully saturated preventing air from being trapped within the soil matrix. Other sub-samples were then collected for additional bulk sample extractions (BSE_light+heavy). Fully wetted samples in the custom inserts were stored in airtight containers at 20 ºC in the lab until desired equilibration timepoints were reached.

    After each centrifugation step, weights of sleeves and inserts were recorded, and effluents were collected and

115   filtered into plastic vials with silicon caps, ready for stable isotope analysis. Vials with Parafilm were stored in a 4ºC fridge until processed. After the centrifuge mid tension extraction, soils from inserts were removed and subsampled to extract high tension effluent in soil matrix via CVD. Extraction of water from soil samples via CVD was performed using the University of Wyoming Stable Isotope Facility's water extraction line. All soil samples were extracted at 102ºC and <0.1-2.7 Pa vacuum pressure (not to be confused with estimated tension applied via CVD described in

120   section 2.3) for ~ 2 hours to ensure all water was removed (West et al., 2006). The final masses after extraction were compared to oven-dried masses to determine if all water was extracted; every sample processed in our experiment had greater than 95% of water extracted at this step.

2.3 Soil water extractions

We focused on extracting waters near two ecologically relevant pressures for the 'low' and 'mid' tension effluents:

125   field capacity and wilting point. While these points will vary from soil-to-soil and plant-to-plant, standard values of 0.033 MPa and 1.5 MPa for field capacity and wilting point are useful as guidelines for understanding potential boundaries on the water pools predicted by the two water worlds hypothesis. Rotations per minute (RPM) for the first two desired extractions at field capacity and permanent wilting point were calculated using an equation from Nimmo et al. (1987) below, which relates rotational velocity to matric potential and radii of a centrifuge set-up:

130    $$\Psi = \rho \frac{\omega^2}{2} (r_1{}^2 - r_2{}^2) \tag{1}$$

where Ψ is matric potential in Pa, ρ is density of water in kg/m^3, ω is rotational velocity in s^{-1}, r_1 is the radius in m from the center of the centrifuge rotor to a point of interest in the soil column during rotation, and r_2 is the radius (m) from the center of centrifuge rotor to the perforated bottom of the insert where the water drains. Due to difficulties in determining the precise force distribution (Zhang et al., 2018) and since force applied via Eq. (1) widely varies

135   depending on the r_1 value selected, we used the center of the soil column as the point of interest for r_1. The first centrifuge step ('low tension') at ~0.016 MPa was performed for three hours at 950 RPM. The second centrifuge step ('mid tension') at ~1.14 MPa was performed for 4 hours at 8000 RPM. The final effluent, extracted via CVD and referred to here as our 'high tension' extraction, is a tightly bound fraction of soil water that is rarely directly compared

to the more mobile waters. Applied tension via CVD is estimated to be greater than 100 MPa (Sprenger et al., 2015),
but the water extracted at this step in our experiment included all that remained in the sample following the mid tension
extraction at 1.14 MPa.

Centrifugation was performed using a Sorvall RC 5B Plus centrifuge and a Sorvall aluminum rotor with four
stainless steel sleeves designed for 50 ml Falcon Tubes (Sorvall, Newton, CT, USA). Six custom inserts for the sleeves
were made (Fig. 1). The steel tube inserts were fitted with a steel mesh plate at the bottom and a collar at the top. The
collar secured the position of the insert within the sleeve at roughly 19 mm above the bottom to establish a reservoir
for collecting extracted water through the steel mesh plate. Four fine mesh metal screens and a rubber ring were placed
at the bottom of the insert during centrifugation to reduce the loss of soil from the insert and allow water to elute. In
addition, a small gravity secured cap was placed on top to reduce evaporation from soil samples in inserts during
centrifugation and storage.

2.4 Stable isotope analysis

The stable isotope composition for samples is expressed as δ values in units of permil (‰), where $\delta = ((R_{sample}/R_{standard})$
$- 1) \times 1000$. R_{sample} and $R_{standard}$ are the isotope ratios of $^2H/^1H$ or $^{18}O/^{16}O$ for samples and standards. Reported δ^2H and
$\delta^{18}O$ permil values use known ratios for Vienna Standard Mean Oceanic Water (VSMOW) as $R_{standard}$ and all samples
were normalized with working reference waters with known delta values on the VSMOW scale determined from
calibrations against VSMOW and SLAP IAEA reference waters. Water isotope analysis was performed at the
University of Wyoming Stable Isotope Facility. Samples were analyzed on a Delta V isotope ratio mass spectrometer
(IRMS) using a Temperature Conversion/Elemental Analyzer (TC/EA) interface (Thermo Scientific Corporation,
Bremen, Germany). For all, the analytical accuracy for the quality assessment lab reference water was 0.33‰ for δ^2H
and 0.38‰ for $\delta^{18}O$, while the analytical precision for the quality assessment lab reference water was 0.98‰ for δ^2H
and 0.22‰ for $\delta^{18}O$.

2.5 Data analysis and mixing times

We fitted a two-part mass balance mixing model using δ^2H and $\delta^{18}O$ data to assess whether all water applied was
accounted for in the three effluents. Using Eq. (2) below, a total of 54 models were performed with the repetitions at
each equilibration timepoint and for each isotope (δ^2H and $\delta^{18}O$).

$$m_{LW} F_{LW} + m_{HW} F_{HW} = m_{LT} F_{LT} + m_{MT} F_{MT} + m_{HT} F_{HT} \qquad (2)$$

m is mass of water in kg and F is atomic fraction calculated from either δ^2H or $\delta^{18}O$ values for the particular water
component. Atomic fraction calculations for $\delta^{18}O$ did not include the ratio of ^{17}O due to simplicity and its minor
influence in this case. The left side of Eq. (2) represents water inputs to the soil samples while the right side represents
water components recovered using the step-wise extractions. To determine the percent of recovered water, the sum of
outputs were divided by sum of inputs and multiplied by one hundred. Subscripts $_{HW, LT, MT,}$ and $_{HT}$ refer to the heavy
water added, low tension effluent, mid tension effluent and high tension effluent. Subscript $_{LW}$ refers to light water

extracted from the bulk soil extraction after only the isotopically light water was applied (BSE$_{light}$). The δ values determined for BSE$_{light}$ samples were used due to slight offset from the δ value of the light water applied. Masses of high-tension effluents were calculated using gravimetric water contents and mass of wetted soil samples after the mid

175 tension centrifuge step. Mass of light water applied was calculated using mass differences in inserts between steps and gravimetric water contents of samples that went through CVD extraction for final effluent.

In order to assess the influence of possible fractionation associated with evaporation, we assessed the difference in mass throughout the experiment for soil filled inserts between centrifuge steps and corresponding effluent produced as well as prior to and post equilibration periods. We found that there was less than 1 % of mass uncertainty

180 in all cases and therefore discounted the impacts of evaporative fractionations on our results and interpretations.

We conducted a pairwise MANOVA between the paired mean δ^2H and $\delta^{18}O$ isotope values for each of the three effluents from the three tension ranges, the δ values of the two applied waters, and the δ values of waters from BSE$_{light}$ and BSE$_{light+heavy}$ samples. There was a total of seven groups compared against one another at each of the five timepoints.

185 We further used a time-dependent isotope mixing equation to approximate the time required for soils to completely mix. The model takes the general form:

$$\delta(t) = \delta_e + (\delta_0 - \delta_e)e^{-kt} \qquad (3)$$

where t is time since mixing (hour), $\delta(t)$ is the isotope ratio of water extracted at a particular tension by centrifugation or CVD at a particular time point, δ_e is the equilibrium isotopic ratio expected for the extracted water under perfectly

190 mixed conditions assuming no fractionation or other effects, δ_0 is the isotopic ratio of the extracted sample at time 0, and k is the time or proportionality constant (hour $^{-1}$). Because we were interested in how the isotopic values of waters vary with different tensions, δ_0 and k were allowed to vary based on tension.

We used data across all experiments to fit Eq. (3), which made initial conditions (δ_0) somewhat uncertain. To account for this error and the expectation that such uncertainties would converge as time went on, we applied a

195 heteroskedastic error term that depends on time since mixing:

$$\sigma = b_0 + \frac{b_1}{t} \qquad (4)$$

where b_0 and b_1 are slope and intercept terms that vary with the different extraction tensions. We determined δ_e from the mean value of fully mixed water inputs on the left side of Eq. (2) from every two-part mixing model. Mean δ values and standard deviations used for δ_e were -57±5‰ for δ^2H (n=27) and -8.6±0.7‰ for $\delta^{18}O$ (n=27).

200 We compared the distributions of the equilibrium parameter to the other tension distributions over time to evaluate mixing times. We considered the system to be thoroughly mixed when the median expected δ value of all tensions was within the 90th percent credible interval of δ_e.

All general statistical analyses were performed with the R v. 3.6.1 software (R Core Team, 2019). The emmeans R package was used to conduct the MANOVA analysis (Lenth, 2019). Time-dependent mixing modeling

205   was performed using the probabilistic programming language Stan (Carpenter et al., 2017), via the rstan programming interface (Stan Development Team, 2019).

3 Results

3.1 Isotope ratios of extracted waters and MANOVA

The amount of water removed from the soil for each of the tension ranges was consistent across all samples. The low
210   tension spins removed 71±6 % (n=27) of the water, the mid tension spins removed 17±6 % (n=27) of the water, and high tension effluent recovered using CVD was 12±1 % (n=27) of the water. A soil retention curve was made with previously reported parameters (van Genuchten, 1980) for modeling water retention of sandy loam (Kosugi et al., 2002). Average volumes reported above from the three extraction steps in the experiment are illustrated on Fig. 2.

The isotope composition of waters extracted at the three tensions were clearly different at 0 h from mixing,
215   but differences diminished with the amount of time the light and heavy waters were allowed to interact (Fig. 3). The isotope ratio for water removed from BSE_{light} samples (bulk sample extraction after light water applied) was not significantly different from that of the light water applied ($p > 0.05$), and the isotope ratio of the $BSE_{light+heavy}$ samples was not significantly different from that of the heavy water applied ($p > 0.05$). At 0 h the isotope ratio values of low tension effluents were not significantly different from that of either the heavy water applied ($p > 0.05$) or the
220    $BSE_{light+heavy}$ samples ($p > 0.05$). In addition, at 0 h the isotope ratio values of all three different tension effluents were significantly different from one another (p values < 0.01) (Table 2). After 8 h of mixing the isotope ratio values of the low tension effluents were significantly different from that of the heavy water applied ($p < 0.05$) and these remained significantly different over the remaining mixing times (p values < 0.01). After 1 d the isotope ratio values of the low tension effluents were not significantly different from those of the mid tension effluents ($p > 0.05$) while the isotope
225   ratio values of the mid tension effluents were significantly different from those of the high tension effluents ($p < 0.01$). After 3 d of mixing the isotope ratio values of waters extracted at low and high tensions remained statistically different, but even these were statistically indistinguishable after 7 d of mixing ($p > 0.05$). A shortened list of the comparisons between groups is presented in Table 2 and a complete list is found in Appendix A, Table A1.

3.2 Two-part isotope mass balance model

230   The results from the mixing model using Eq. (2) were uniform across soil samples. The mean percent recovered water was 100.2±0.4 % (n=27) based on δ^2H data with a range of 99.34% to 102.05%, and 100.1±0.1 % (n=27) based on $\delta^{18}O$ data with a range of 99.88% to 100.25%.

3.3 Mixing times

Model estimates determined from the time-dependent mixing equation, Eq. (3), are provided in Fig. 4 and 5. A 1:1
235   relationship between observed and predicted values indicates the model did reasonably well at predicting observed values and their uncertainty with only one value outside the given uncertainty bound (Fig. 6). Results were generally consistent between the two isotopes, however $\delta^{18}O$ expressed an upward shift in values as the mixing time proceeded. Mean values of parameters for the time-dependent mixing models are reported in Table 3.

δ^2H values at the beginning of the experiment, across tensions, were distinct from one another (Fig. 5). It
took about 5 hours for the low tension isotope values to become similar to the expected equilibrium (i.e., well-mixed)
value. Mid tension waters did not attain a thoroughly mixed value until 12 hours. In the case of the high tension water,
it took ~104 hours for the two waters to fully mix. This suggests it would have taken the system a little more than 4
days to completely mix. δ^{18}O measured values indicated possible fractionation offset near when equilibrium was
achieved according to δ^2H values. Due to this offset, probability densities with δ^{18}O data were not evaluated similarly
to the δ^2H values since the time dependent mixing model works under the assumption that there are no equilibrium
fractionation offsets occurring.

4 Discussion

Recent work by the ecohydrological community has emphasized the need to understand how the isotopic composition
of various pools of water held at a range of tensions interact and evolve over time (Adams et al., 2019; Oerter et al.,
2019; Poca et al., 2019). Our approach successfully permitted analysis of the isotopic composition of water held at
different tensions within a single soil sample offering a method to assess the time-dependent isotopic exchange among
soil regions. We believe our approach can be extended to investigate potential isotopic fractionations and chemical
exchanges that shape the isotopic and geochemical composition of water in different soil regions over time. Our
findings are consistent with those from other recent studies (Adams et al., 2019) suggesting that waters initially held
at different matric potentials do not immediately and completely mix. Lags in isotopic mixing have implications for
studies focused on plant water use, soil water age or residence times, water balance, and flux partitioning (Evaristo et
al., 2015, 2019; Evaristo and McDonnell, 2019; Good et al., 2015; He et al., 2019; Sprenger et al., 2018; Wang et al.,
2019).

The isotopically distinct waters applied to oven-dry soil in our proof-of-concept study required more than 3
days to fully mix and equilibrate. Even with some advection through and out of the soil matrix during centrifugation
steps, these results reveal long lag times for complete mixing. The time-dependent mixing model indicated that
complete mixing was achieved after 4.33 days and this timeframe was consistent with the MANOVA results. The
mass balance mixing model revealed that 99% of the water applied to the soil in our experiment was extracted.
However, there was a possible fractionation offset observed in the δ^{18}O data, notably for the 3 and 7 day samples (Fig.
4) that could not be explained by isotope effects due to interactions with ions (Oerter et al., 2014), clay minerals (Gaj
et al., 2017), or carbonates (Meißner et al., 2014) within the study soil. The offset also could not be attributed to
evaporation nor incomplete extraction via CVD since the offset was observed in both low tension and mid tension 7
day effluents. Our data indicate there is still much to know and explore in terms of how isotopic composition of soil
water may change over time once antecedent and new event water reaches a mixed equilibrium. In addition, there is
still much to understand in mixing time constraints by soil texture, especially field samples with native structure.

We assume the isotope mixing among soil pore waters held at different tensions in our study was driven
primarily by self-diffusion of isotopologues via Brownian motion. Isotopes are commonly treated as solutes when
modeling diffusion in soil. However, isotopes are more regularly used for understanding the redistribution of water
within the soil by diffusion and advection from regions of higher to lower volumetric water content and water potential

275   driven by vapor movement and evaporative flux near the soil surface (Barnes and Allison, 1988; Rothfuss et al., 2015). In the present study we limited vapor transport and advection by holding samples in a closed, isothermal vessel under saturation such that self-diffusion dominated the mixing and isotopic exchange among soil regions. Diffusion rates in unsaturated soil are a function of the diffusion coefficient for the solute of interest in bulk water multiplied by the tortuosity factor and fractional water volume. Since soil samples in our study were saturated, the fractional water

280   volume did not restrict diffusion, and tortuosity for the sandy loam soil in our study was likely low compared to that of fine textured soils. We predict therefore that times for complete mixing in unsaturated or finer textured soils will be much longer than those reported here but can be assessed using the general approach we describe.

Further development of the general approach we present should address potential artifacts related to centrifugation as a means to extract waters sequentially from a single sample across a range of pressures. First, the

285   pressure applied to the soil varies within the soil column at a single rotational velocity depending on distance from the center of the centrifuge rotor. This is unavoidable, but potential artifacts may be reduced or avoided by using low-profile centrifuge vessels. Second, the pressure enacted by the soil may change between or during centrifugation steps since the proportion of small pores within the soil column increases as pores get compacted to smaller diameters. This also is unavoidable, and the magnitude of this effect on the distribution of isotopically distinct waters recovered at

290   different pressures should be explored further. Finally, minimizing the time of centrifugation at each step (Fraters et al., 2017) would provide more highly resolved estimates of soil water mixing times and increase sample throughput. Higher sample throughput is needed since low temporal and spatial resolution of sampling from the field often limits our ability to thoroughly test predictions of the two water worlds hypothesis (Dubbert et al., 2019).

5 Conclusion

295   This work presents a method for separately extracting water held at different tensions within soil for isotopic analysis and provides a quantitative framework for evaluating time-dependent mixing of isotopically distinct waters within a soil sample. Our general approach could be extended to provide a means to evaluate the time-dependent interactions among pools of soil water in soils with different soil textures, for undisturbed soil cores that retain native structure, and under unsaturated conditions. Some additional work is needed to refine the application of the centrifuge method

300   for such studies, but embracing the general notion of a combination method will overcome perceived limitations unique to each separate extraction technique.

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Competing interests. The authors declare they have no conflict of interest.

Review statement.

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Table 1: Mean, standard deviation, and range of isotope values for each effluent from each timepoint

Effluent	Timepoint	Mean δ^2H ‰	Mean $\delta^{18}O$ ‰	Range δ^2H ‰	Range $\delta^{18}O$ ‰	Number of samples
Centrifuge: Low Tension	0 hours	-47 ± 1	-7.5 ± 0.3	-46 to -50	-7.0 to -7.9	15
	8 hours	-53 ± 1	-7.8 ± 0.2	-52 to -54	-7.6 to -8	3
	1 day	-56 ± 1	-8.0 ± 0.2	-55 to -56	-7.8 to -8.2	3
	3 days	-56 ± 1	-7.8 ± 0	-56 to -57	-7.8 to -7.8	3
	7 days	-55 ± 1	-7.3 ± 0.3	-54 to -56	-6.9 to -7.5	3
Centrifuge: Mid Tension	0 hours	-65 ± 4	-9.2 ± 0.6	-60 to -74	-8.2 to -10	15
	8 hours	-63 ± 5	-8.6 ± 0.4	-58 to -67	-8.3 to -9	3
	1 day	-60 ± 0	-8.3 ± 0.2	-60 to -60	-8.1 to -8.4	3
	3 days	-57 ± 1	-7.9 ± 0.2	-56 to -58	-7.8 to -8.1	3
	7 days	-55 ± 0	-7.0 ± 0.2	-55 to -55	-6.7 to -7.1	3
CVD: High Tension	0 hours	-89 ± 10	-10.8 ± 1.5	-64 to -109	-6.9 to -13.6	15
	8 hours	-79 ± 3	-9.5 ± 0.4	-76 to -82	-9.0 to -9.7	3
	1 day	-72 ± 4	-8.4 ± 0.2	-68 to -75	-8.2 to -8.6	3
	3 days	-65 ± 2	-7.6 ± 0.6	-64 to -67	-7.0 to -8	3
	7 days	-62 ± 2	-6.5 ± 0.5	-61 to -64	-6.0 to -6.9	3

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430 **Table 2: The results of pairwise MANOVA tests for the experiment with comparisons between groups of samples, group 1 compared to group 2 on respective rows. Significant values are highlighted in bold, p-value ≤ 0.05. Only showing comparisons that changed from significant to insignificant or vice versa throughout the experiment, while sixteen comparisons not shown stayed either significant or insignificant for all timepoints.**

MANOVA Comparison Group 1	MANOVA Comparison Group 2	Timepoint p-values				
		0 hours	8 hours	1 day	3 days	7 days
Centrifuge: Mid Tension	Centrifuge: Low Tension	**<0.0001**	**0.02**	0.7	1	1
Centrifuge: Mid Tension	CVD: High Tension	**<0.0001**	**0.0001**	**0.005**	0.1	0.2
Centrifuge: Mid Tension	$BSE_{light+heavy}$	**<0.0001**	**0.0002**	**0.002**	0.1	0.4
Centrifuge: Low Tension	CVD: High Tension	**<0.0001**	**<0.0001**	**0.0001**	**0.05**	0.3
Centrifuge: Low Tension	Heavy Water	1	**0.05**	**0.001**	**0.001**	**0.004**

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Table 3: Means and standard deviations of parameters used in time-dependent mixing model.

| Isotope	Effluent	δ_e (‰)	k (10^2 hr^{-1})	b_0 (10^1 ‰)	b_1 (10^1 ‰ hr)
			Mean (SD)		
δ^2H	All	-57.4 (4.8)			
	Centrifuge: Low Tension		15.8 (3.2)	8.9 (2.0)	5.1 (3.4)
	Centrifuge: Mid Tension		3.1 (1.0)	17.7 (5.4)	29.7 (13.4)
	CVD: High Tension		1.6 (0.6)	40.6 (13.9)	85.7 (36.7)
$\delta^{18}O$	All	-8.6 (0.7)			
	Centrifuge: Low Tension		0.5 (0.2)	2.9 (0.6)	1.1 (10)
	Centrifuge: Mid Tension		1.5 (0.5)	2.9 (0.9)	3.9 (1.8)
	CVD: High Tension		3.7 (1.4)	5.7 (1.7)	10.5 (3.7)
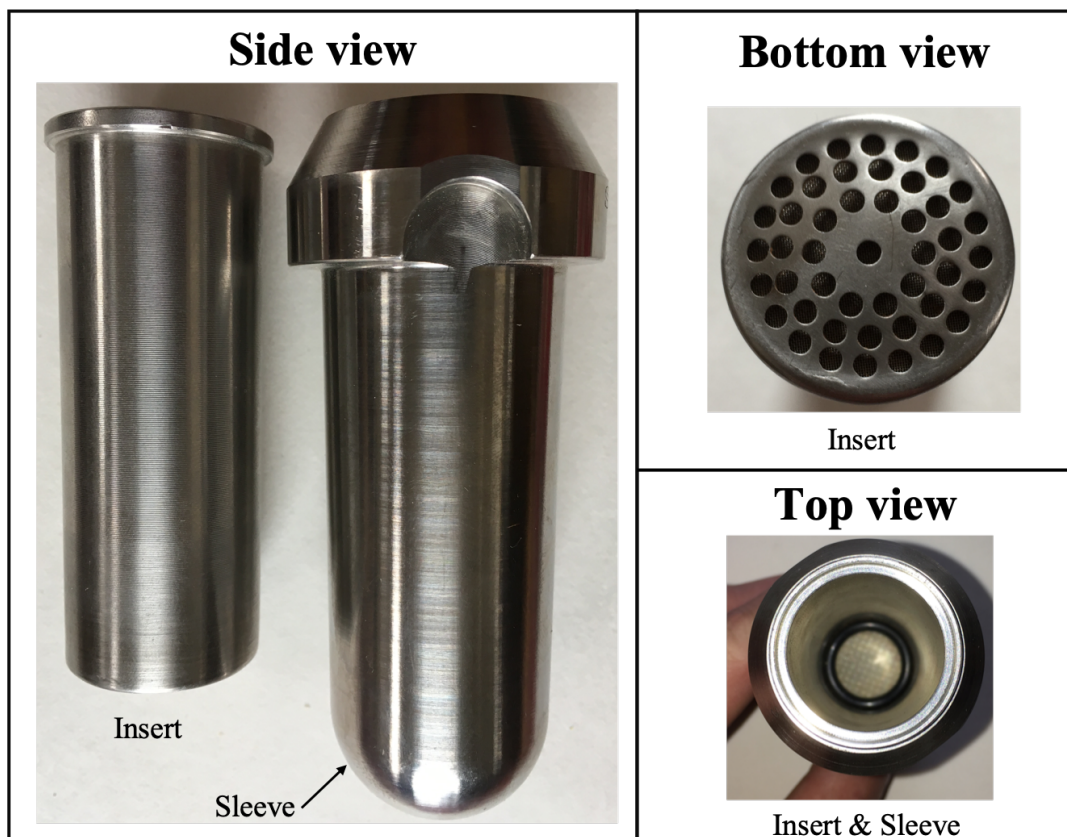
440 **Figure 1: Image of custom-made centrifuge inserts and Sorval sleeves. Top view includes screens at bottom of insert with rubber collar to hold screens in place.**
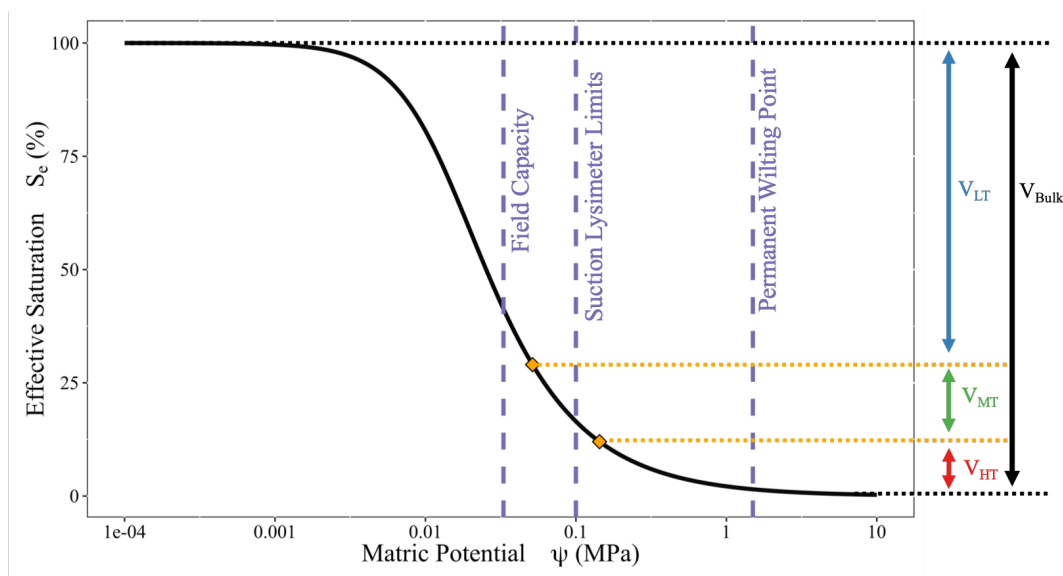
Figure 2: Soil retention curve for the sandy loam soil using van Genuchten parameters for a general sandy loam (Kosugi et al., 2002). Average volumes from each step of the mixing time experiment are illustrated on side with LT for Low Tension, MT for Mid Tension, HT for High Tension, and Bulk being volume if extracted via cryogenic vacuum distillation. Vertical lines are pressure points of interest. Effective saturation is the y-axis, a standardized form of theta or volumetric water content, and matric potential is the x-axis in MPa.

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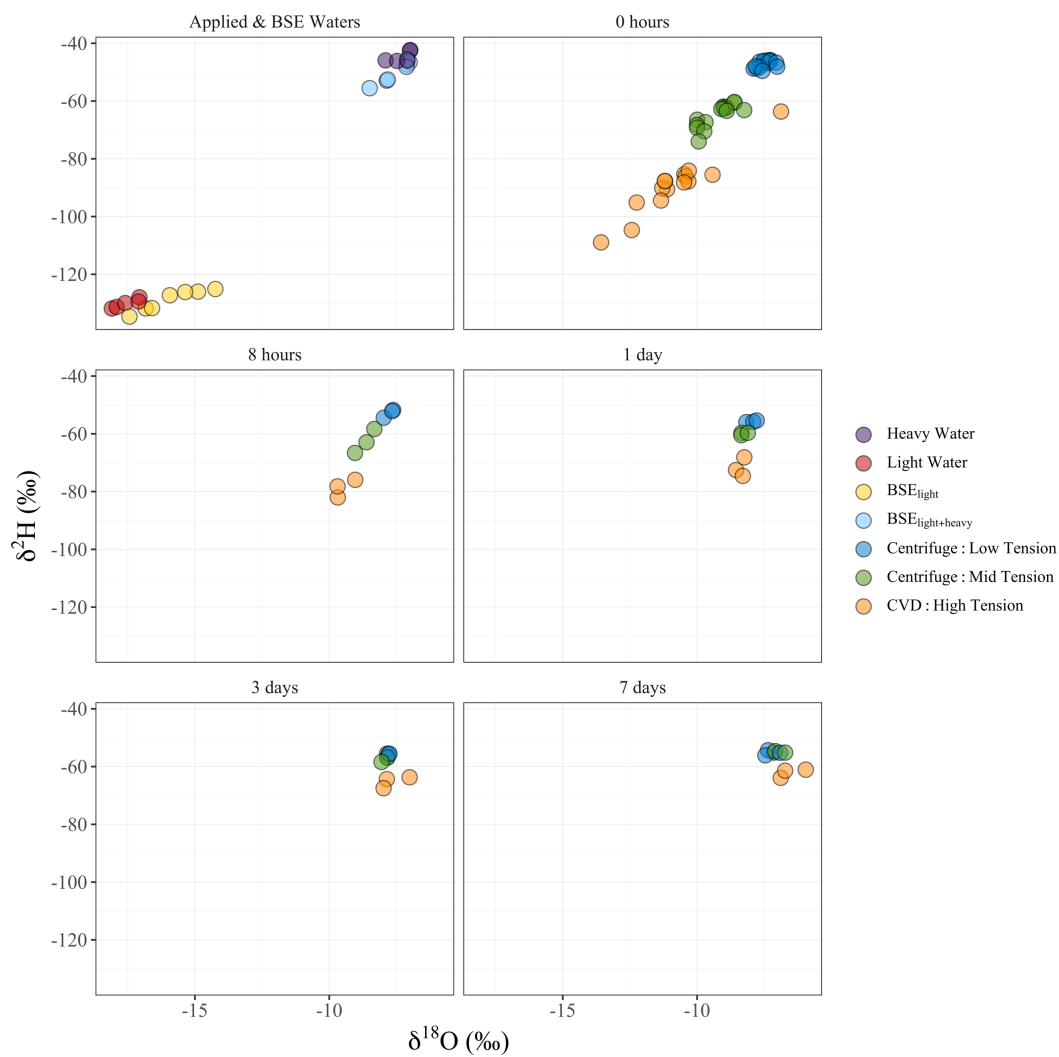
Figure 3: Isotopic values of water samples in dual-isotope space, δ²H (‰) vs. δ¹⁸O (‰). Each panel corresponds to timepoint samples were put through extraction method, except first panel includes the applied waters and BSE waters.

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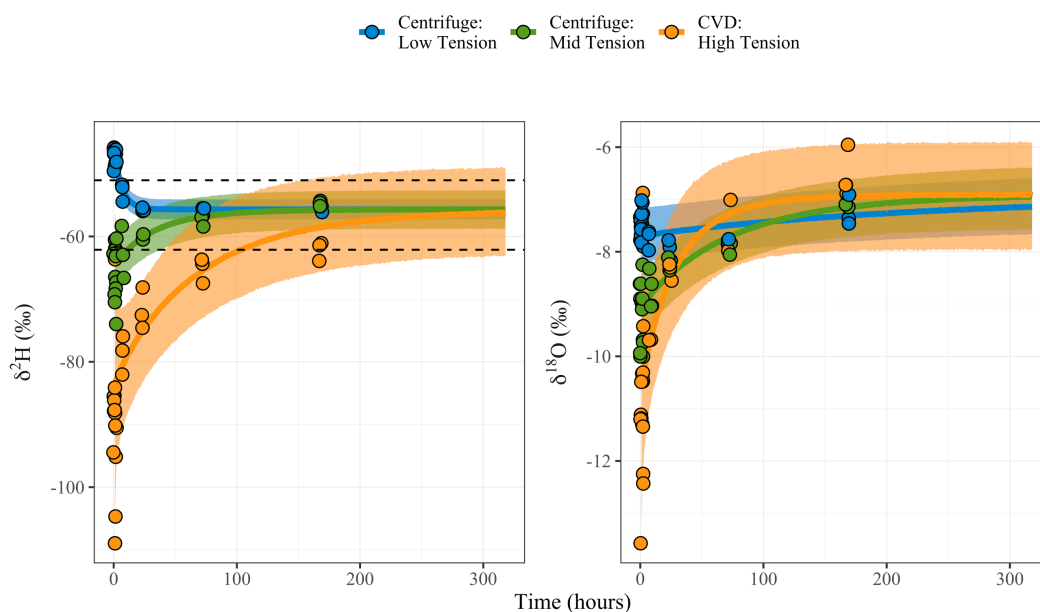
Figure 4: Time-dependent mixing model curves plotted for both δ^2H_{VSMOW} ‰ and $\delta^{18}O_{VSMOW}$ ‰ data for each effluent over time. Shaded regions are 90th credible intervals for each curve. The dashed lines are for the 90th credible interval for equilibrium, δ_e, estimate of δ^2H.
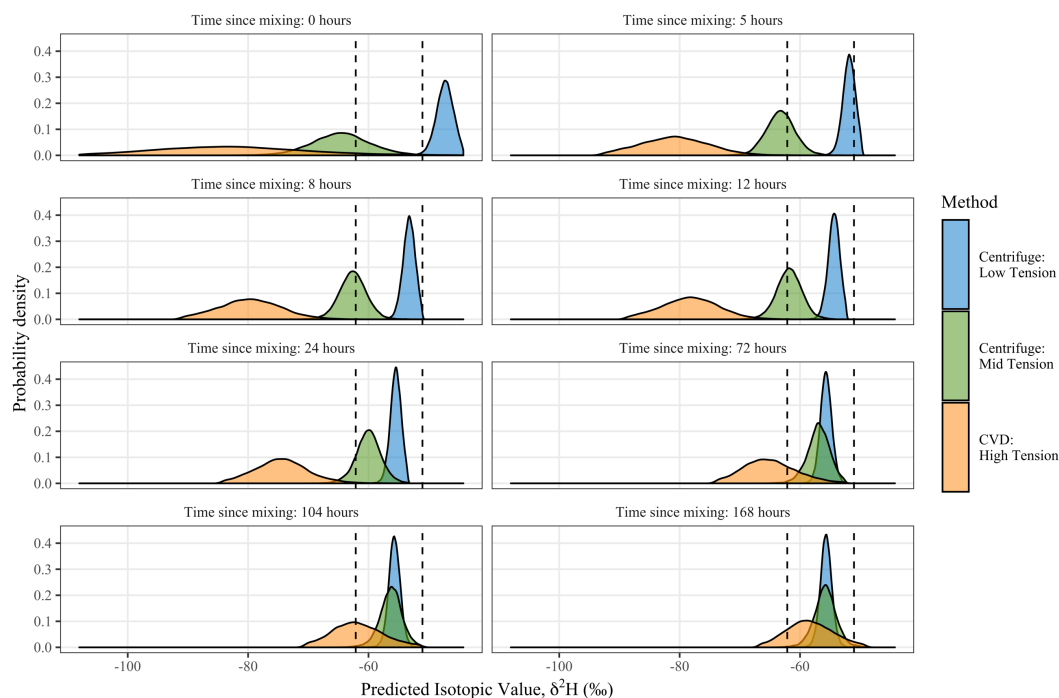
Figure 5: Time-dependent model for mixing time with distributions of δ²H data for each of the three effluents over time in relation to the 90th credible interval for equilibrium value (dashed lines). Panels include extraction times for experiment as well as important timepoints for mixing. At 5 hours the median low tension value is within the 90th credible interval of the equilibrium value. At 12 hours, low and mid tension effluent's values look like the equilibrium value. It is not until 104 hours (~4.33 days) that the median of the high tension effluent is also within the 90th credible interval of the equilibrium value.
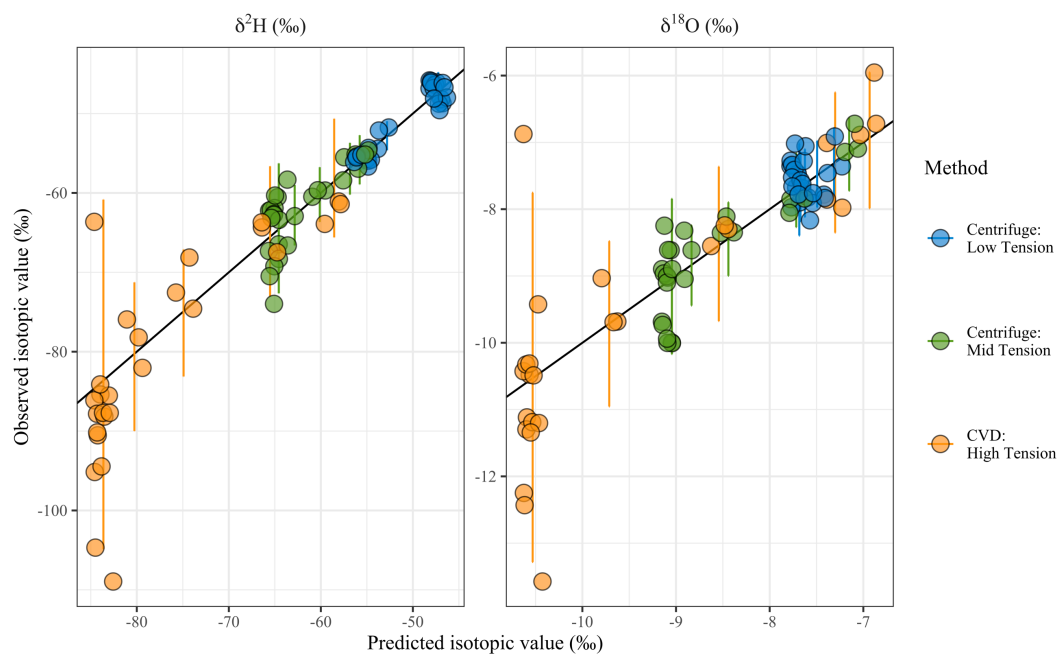
Figure 6: Comparison of predicted with Eq. (3) and observed value for given isotope and method. Black line represents a 1:1 line. Bars represent the credible interval (90%) of the predicted values by timepoint and tension. A slight jitter (3%) has been added to the predicted value in an effort to display points, as there is some clustering of the data.

480

Appendix A:

485   **Table A1: The p-value results of pairwise MANOVA tests for the experiment with comparisons between groups of samples, group 1 compared to group 2 on respective rows. Significant values are highlighted in bold, p-value ≤ 0.05. Showing comparisons not shown in Table 2.**

MANOVA Comparison Group 1	MANOVA Comparison Group 2	Timepoint p-values				
		0 hours	8 hours	1 day	3 days	7 days
Centrifuge: Mid Tension	BSE_{light}	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Centrifuge: Mid Tension	Light Water	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Centrifuge: Mid Tension	Heavy Water	**<0.0001**	**<0.0001**	**<0.0001**	**0.0004**	**0.006**
Centrifuge: Low Tension	BSE_{light}	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Centrifuge: Low Tension	$BSE_{light+heavy}$	1	0.9	0.2	0.1	0.3
Centrifuge: Low Tension	Light Water	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
CVD: High Tension	BSE_{light}	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
CVD: High Tension	$BSE_{light+heavy}$	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
CVD: High Tension	Light Water	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
CVD: High Tension	Heavy Water	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
BSE_{light}	$BSE_{light+heavy}$	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
BSE_{light}	Light Water	1	0.9	0.8	0.8	0.8
BSE_{light}	Heavy Water	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
$BSE_{light+heavy}$	Light Water	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
$BSE_{light+heavy}$	Heavy Water	0.8	0.2	0.1	0.1	0.1
Light Water	Heavy Water	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**