June 4th, 2020

Dear Dr. Josie Geris,

Please find our revised manuscript, point-by-point reply to comments, and marked-up manuscript version for the manuscript *Combination of soil water extraction methods quantifies isotopic mixing of water held at separate tensions in soil.*

We addressed all comments by you and reviewers on the original manuscript by revising and adding clarifications to six main areas/topics within manuscript:

- Time-dependent mixing model
- Soil wetting procedure
- Fractionation effects and offsets in isotope data
- Two Water Worlds hypothesis background and relation to study
- Background on soil physical processes relative to study
- Suggestions for future studies

Alongside addressing each detailed comment, revisions were made throughout the manuscript to maintain consistency and help simplify the main areas/topics listed above. For example, we now define and use more common verbiage for soil water pools (e.g. gravitationally drained water, capillary water, and hygroscopic water) throughout the paper to help relate our approach, methods, and results to other ecohydrological studies. We followed your suggestion to make the conceptual diagram that will help convey these topics and their relation to our analyses and interpretations of results.

During the revision process we found that some of our planned changes were redundant if fully implemented. However, we have highlighted exact changes point-by-point below that address each reviewer comment sufficiently without adding unnecessary redundancies.

Lastly, thank you for the time extension for our minor revisions as the extra time greatly aided in addressing all comments as best as possible.

Sincerely,

William Bowers, Jason Mercer, Mark Pleasants, and David Williams

Point-by-point reply to reviewer comments in revised manuscript submitted to HESS, *hess-2019-687*

By William Bowers and co-authors

This document combines all comments and replies by each reviewer, relative changes in revised manuscript, and marked-up revised manuscript. A detailed reply to each reviewer comment can be found at https://www.hydrol-earth-syst-sci-discuss.net/hess-2019-687/.

For ease of reviewing, we repeat all reviewer comments in **bold font** followed by our changes to the manuscript in blue font noting section in the manuscript where the changes are made. All changes in the marked-up revised manuscript are in underlined dark red font.

Detailed replies:

Reviewer 1: (1) The manuscript presents the results of an experiment designed to estimate the rate of isotopic mixing in a soil between two waters that differ in their H and O isotope ratios added to soils sequentially following oven-drying. They do show what appears to be a time-dependent process and argue that the time to equilibration is on the order of days (>4 for this soil). I think the manuscript is a contribution to the ongoing and needed effort to better understand the underlying processes that control soil water isotope ratio variation. However, I have what I think are important concerns with the current version.

A key, underlying assumption (that the authors acknowledge) is the absence of fractionation effects associated with water addition after oven-drying or with the extraction process. While this may be a valid assumption, there is evidence in their results that it's false, particularly for d18O. The authors assess the potential for enrichment as a function of evaporation by mass balance (comparing mass loss with effluent captures) but this does not account for any fractionation effects associated with clay mineral interactions and is itself subject to errors. It is notable that the quantities used in the mass balance calculations were not the isotope ratios of the added waters, but the value of the isotope ratio of the water extracted by CVD immediately after adding the second water. The authors refer to a "slight" offset, but looking at the data in figure 3, there is apparently as much as a 2‰ difference between the "light" water added and the measured CVD-extracted water. This is not a small difference in my view.

> Reply: The reviewer referred to fractionation effects present in our study, specifically highlighting that interaction with clay minerals needs to be discussed. Additionally, the reviewer required more justification for using bulk water extracted after "light" water was applied (BSE_{light}) for mass balance rather than applied "light" water. We explained the rationale for using BSE_{light} extracted water for mass balance calculations in methods in relation to fractionation offsets reported in previous studies. In addition, the offset is mentioned in results and how light water applied and BSE_{light} extracted water are not statistically different (MANOVA). Lastly, use of BSE_{light} extracted water is covered more in discussion.
>
> We revised the text with the following statements:
>
> *L206-213*: The δ values determined for BSE_{light} samples were used in the mass balance model rather than that of the light water added to accommodate for the slight δ offset between these waters. This slight offset may have developed from evaporative fractionation (Allison et al., 1983) that likely occurred when applying the light water to the recently oven-dried soil within the dry local

atmosphere within our lab, or from a small amount of hygroscopic water adsorbed from local atmosphere once soil was removed from the oven (Hillel, 2003). The direction of this slight offset was not consistent with previous observations of isotope effects associated with interactions with clay minerals (Gaj et al., 2017) or carbonates (Meißner et al., 2014).

L261-265: The isotope ratio of water recovered using CVD of $\mathrm{BSE}_{\mathrm{light}}$ samples (bulk sample extraction after light water applied) indicates that potentially the water in the sample at this step was altered slightly by evaporative enrichment of heavy isotopes mixed into the oven dried soil, which had a high amount of surface area exposed to dry local atmosphere. Although this changed the isotopic value of water in soil before application of the heavy water, the light waters applied and $\mathrm{BSE}_{\mathrm{light}}$ extracted waters were not significantly different ($p > 0.05$).

L332-336: We chose to use the isotope value of the bulk water extracted after the light water was applied ($\mathrm{BSE}_{\mathrm{light}}$) as the end-member in the mass balance model rather than the isotope ratio value of the light water itself. We felt this was justified for the objective of our study, which was to demonstrate the capability of the combined centrifuge-CVD method to evaluate mixing dynamics among different soil water pools.

Reviewer 1: (2) I am also curious about the method used to add water. The sequence was: oven dry 350g of soil, add 20 ml of "light" water and mix, subsample into centrifuge inserts and immerse in "heavy" water (presumably completely?). These soils were then presumably saturated. Were they allowed to drain at all before centrifugation, etc.? What is the field capacity of this soil and how does it compare to the amount of water added in the first step? I think it would be useful to know if freely-draining water was part of the pool extracted in the first step.

Reply: More detail has been added to the methods section for the wetting procedure addressing the wetness of samples, drainage during wetting that precludes samples from being completely saturated, and whether low tension extraction contains gravitationally drained water. In addition, we clearly and more broadly applied definitions for common soil water pools in soil near saturation that are incorporated throughout manuscript and highlighted in the updated Fig. 1 and the new conceptual diagram, Fig. 3.

We revised the text with the following statements:

L130-132: After the soil cooled from the drying procedure we applied 20ml of the light water with a spray bottle to the 350 g sub-sample and mixed by gloved hands to ensure homogenous application. 18-30g of this slightly wetted soil was gently packed to form soil columns in each of six custom made centrifuge inserts

L141-143: the packed inserts were then wetted from the bottom up by immersing in a container with heavy water at a level just below the soil level in each insert. This ensured the soil samples were wetted to near saturation by reducing the chance of air being trapped within the soil matrix.

L145-147: Complete saturation was not possible as some water was lost from perforations at the bottom of the inserts when they were removed from the container of heavy water.

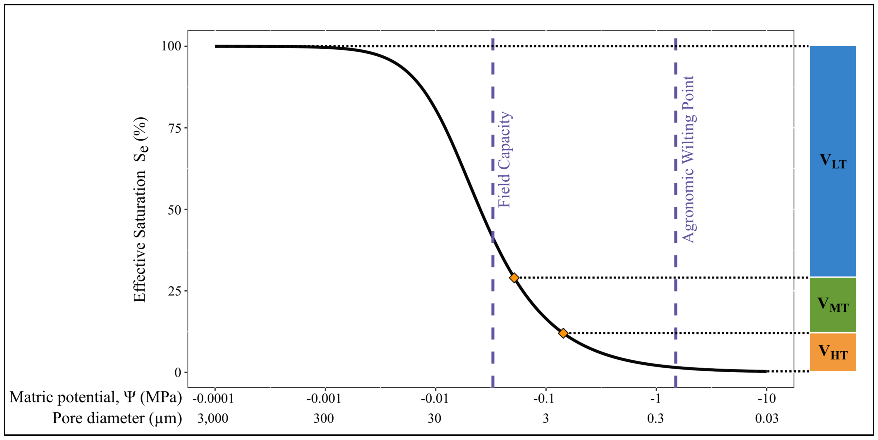

Figure 1: Soil retention curve for a sandy loam soil using van Genuchten parameters for a general sandy loam (Kosugi et al., 2002). Average volumes (V) from each extraction step of the experiment are illustrated on the right with LT for Low Tension, MT for Mid Tension, and HT for High Tension. Vertical lines are matric potential points of interest: field capacity of -0.033 MPa and agronomic wilting point of -1.5 MPa. The y-axis is effective saturation, a standardized form of volumetric water content. The x-axis has two scales: the top scale is matric potential in MPa and bottom is relative maximum pore size filled at the respective matric potentials (Schjonning, 1992). Samples wetted with both light and heavy waters were near but not at 100% effective saturation.

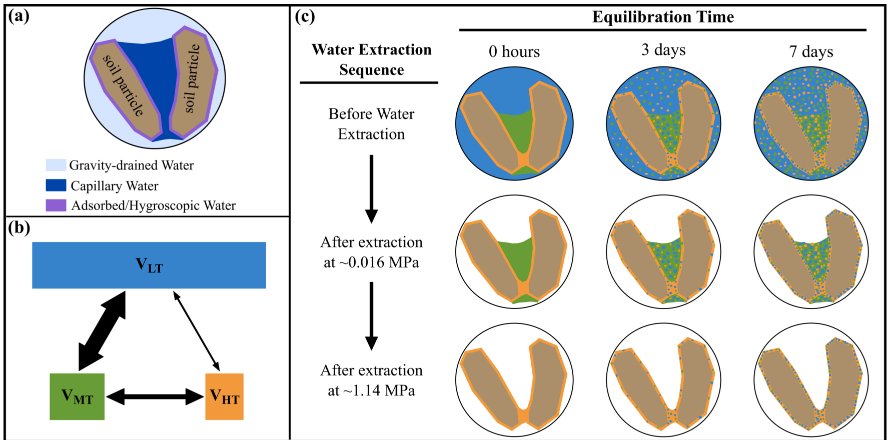

Figure 3: (a) Spatial relationship of the three most commonly discussed water pools that make up the bulk water pool in soil near saturation. Absorbed/hygroscopic water, capillary water and gravity-drained water are depicted in hypothetical cross-section view of two soil particles within the soil matrix. (b) Relative volumes (V) of soil water pools in this study based on Fig. 1 (LT= low tension, MT= mid tension, and HT= high tension) and the relative amount of interactions (size of black arrows) between pools as equilibration time proceeds. (c) Three soil water pools for this study in hypothetical pore space, as diagramed in the first panel, at three equilibration timepoints and various points in the water extraction sequence. Based off of Fig. 1 water extracted at low tension is comprised of gravity-drained water and capillary water, that extracted at mid tension is composed of capillary water, and water extracted at high tension is comprised of capillary water and hygroscopic water. As equilibration time increases, each pool moves closer towards a well-mixed state (i.e. equilibrium).

Reviewer 1: (3) I think the authors need to more clearly explain their rationale in using the "time-dependent isotope mixing equations." While I see that an exponential fit to the observed data makes sense (at least for d2H) and that there is a tendency for them to converge, I am not sure the logic holds and I think the fitting approach used might obscure the lack of convergence between the CVD data and the centrifuge data (the CVD data plot well below the fitted line in Fig. 4 for d2H on day 7). The idea is that the low –> mid –> CVD represent a gradient from more to less of the recently added "light" water and capture the mixing process as it proceeds. I don't think this approach captures processes that might involve water interacting with clay and I am not convinced that the mixing is "complete" after 4 days based on the results presented in Fig. 4. The authors also acknowledge but do not attempt to explain the very different patterns observed for d18O. I think there's more to these patterns than it taking longer for H218O and H216O isotopologues to mix than those of H. If this were a simple mixing process, shouldn't both H & O behave similarly in terms of trajectory? I think more careful thought needs to go into interpreting these results. I also think the authors should report the clay mineralogy since multiple authors have suggested potential impacts of clay type on extracted water isotope ratios.

Reply: The reviewer is concerned about the time-dependent mixing model used to evaluate data. We now provide rationale within the methods section for applying the model. The assumption and limitations of the model are now included in the results section and we provide more details about the δ^{18}O data offset that was highlighted by the reviewer. We expanded our description of the model results in the discussion section relating to the δ^{18}O values observed in waters extracted at high tension after 7 days of mixing and highlight and explain differences between the time-dependent mixing model and MANOVA.
We revised the text with the following statements:
L227-228: We further used a time-dependent isotope mixing equation to approximate the time required for soils to completely mix (i.e. reach equilibrium).

L299-302: δ^{18}O values indicate possible fractionation expressed at day 3 and 7 equilibration timepoints with offsets towards heavier values. Due to these offsets, probability densities were not evaluated with δ^{18}O data since our time-dependent mixing model did not account for fractionation offsets occurring during equilibration.

L321-329: The time-dependent mixing model indicated that complete mixing was achieved at ~4.33 days and this timeframe was consistent with the MANOVA results between the waters recovered at the three tensions. However, at 7 days the waters extracted under high tension were significantly different than those of the $BSE_{light+heavy}$ samples (MANOVA), but were within the 90% credible interval for δ^2H of δ_e according to the time-dependent mixing model. This highlights a key difference between the statistical methods of comparison: while the MANOVA compares the multivariate normal means across isotopes, our mixing model analysis ignored the δ^{18}O values due to yet unexplained (see below for further discussion) deviations in the mixing model. Nonetheless, while these methods highlight slight differences in their estimate of when the two added waters were completely mixed across all extracted fractions, they both highlight the long time lags in mixing.

L337-346:We observed slightly higher d^{18}O values of the extracted water pools at days 3 and 7 than predicted based on simple mixing of the two waters added to the dry soil (Fig. 5). Because we recovered the expected mass of water (>99%) for these samples, we do not feel the observed ^{18}O enrichment was a result of evaporation. Water interactions with clay minerals (Gaj et al., 2017) and carbonates (Meißner et al., 2014), in contrast, typically result in depletion of ^{18}O in matrix water. The positive shift in δ^{18}O of soil water observed in our study however is consistent with observations reported by Oerter et al. (2014) who found that at low water content d^{18}O of matrix water increased in the presence of clays enriched with potassium. We cannot discount the possibility of such ionic interactions in our study. The time course for ionic exchanges with clays that influence the oxygen isotope composition of matrix water might explain why the mixing dynamics observed in our study differed between H and O isotopes. Identifying and analyzing such effects require more thorough analysis.

Reviewer 1: (4) I was surprised to see the "wilting point" value of -1.5 MPa used. I know the authors are aware that this value is quite high (less negative) compared to values many plants adapted to low-water environments can achieve and experience no damage.

Reply: We placed into context the term “wilting point” and that this useful reference point is derived for agronomic applications. We now fully explain in the revised methods section our rationale with caveats for use of this reference point in our study.
We revised the text with the following statements:
L163-168: We focused on extracting waters near two ecohydrologically relevant pressures for the waters recovered at “low” and “mid” tension: field capacity (i.e., the point at which no more water drains freely under force of gravity) and agronomic wilting point. While field capacity and wilting point varies among different soil types and plants, reference values of 0.033 MPa and 1.5 MPa for field capacity and agronomic wilting point are useful as guidelines for understanding potential boundaries on ecohydrologically separate water pools.

Reviewer 1: (5) I think the authors should clarify what they mean by "precision" and "accuracy" in the isotope analysis section. Presumably the "accuracy" is some measure of how different the measured/corrected values of an internal reference material were relative to a consensus value, but I think this should be explicitly stated. Similarly, the "precision" is presumably some estimate of variance of the reference material (1 standard deviation of how many replicates?), but again this should be stated.

Reply: We clarified what we mean by precision and accuracy in the methods section.
We revised the text with the following statements:
L192-194: We report the accuracy as the absolute difference between the mean of analyzed lab reference water samples (n=15) and the calibrated value of lab reference water. We report precision as the standard deviation of all lab reference water samples analyzed (n=15).

Reviewer 1: (6) In line 169 the authors refer to "atomic fraction" when I think they mean "isotope ratio" (e.g., 18O/16O).

Reply: “Atomic fraction” was changed to “isotope ratio” in the methods section.
We revised the text with the following statements:
L200-202:

$$m_{\mathrm{LW}} R_{\mathrm{LW}} + m_{\mathrm{HW}} R_{\mathrm{HW}} = m_{\mathrm{LT}} R_{\mathrm{LT}} + m_{\mathrm{MT}} R_{\mathrm{MT}} + m_{\mathrm{HT}} R_{\mathrm{HT}} \tag{2}$$

m is mass of water in kg and R is isotope ratio calculated from either δ^2H or δ^{18}O values for the particular water component.

Reviewer 2: (1) I found the analysis and discussion of the results to be quite “thin”. By that, I mean that there is not an especially in depth or nuanced explanation and discussion of many components

throughout. Specific examples follow, but in general, I suggest that the senior authors of the manuscript return to it with a more discriminating eye and identify where it can be "deepened".

The authors base the rational for conducting the study on making progress on identifying the potential soil water reservoirs (isotopic or otherwise) that underpin the Ecohydrologic separation, or "Two Water Worlds" (TWW) hypothesis. However, there is only the most minor discussion of this concept in the introduction, and then the authors return to it throughout the results and discussion citing how their findings apply to TWW. This is problematic because the reader doesn't have any firm understating of TWW or how the authors are interpreting TWW (because interps vary). I suggest there be a fuller discussion of TWW and how this study specifically contributes to investigating it in the introduction. Because HESS has an open review process, subsequent reviewers have the advantage of seeing previous reviewer's comments. That is the case here, and while I do not intend to "pile on" the authors, I do support Reviewer #1's comments, especially in regards to the mixing analysis (see my specific comments below).

Reply: More detailed description of the Two Water Worlds (TWW) hypothesis and the proposed mechanism, ecohydrological separation, to explain the TWW hypothesis have been added to the introduction section. In addition, we have included a more thorough discussion within the introduction section about common soil water pools referred to in ecohydrological separation studies and their relation to the soil water retention curve. The revised manuscript has been adjusted throughout to be consistent with reference to these pools. The relation of soil water pools examined in our study to previous work is also highlighted in a new conceptual diagram, Fig. 3.
We revised the text with the following statements:
L35-43: The Two Water Worlds (TWW) hypothesis (McDonnell, 2014) considers that transpiration and runoff to streams derive from separate pools of water that are incompletely mixed in time or across pore regions in the soil. Brooks et al. (2010) presented stable isotope evidence of ecohydrologic separation between plant available water in smaller pore regions and mobile water passing through preferential flow paths when smaller pores were filled, challenging the hypothesis of translatory flow and establishing a mechanism to explain the TWW hypothesis. Yet, most studies examining ecohydrologic separation and the TWW hypothesis fail to differentiate isotopic signatures beyond that of mobile water and bulk soil water. More comprehensive evaluation of soil water isotopes across multiple pore sizes and soil regions is needed to examine recharge processes explaining the TWW hypothesis (Berry et al., 2018; Brantley et al., 2017; Brooks et al., 2010; McDonnell, 2014; Sprenger et al., 2019).

L47-59: Characterization of water isotope ratios in soils involves careful consideration of methods used to recover soil water. 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, the relationship between volumetric water content and matric potential (negative equivalent of matric tension) (Sprenger et al., 2015). Terminology for water pools recovered at different applied energies has been debated. For the purposes of relating our study to ecohydrologic separation studies, we define two commonly defined pools, gravity-drained water and matrix water, consistent with recent terminology used by Brantley et al. (2017). Gravity-drained water is the most mobile pool of water within soil that freely drains through large pores under the force of gravity. Whereas matrix water consists of capillary and hygroscopic water that does not drain freely under force of gravity but is held across a broad range of tensions by smaller pores that may or may not be accessible to plants. There is likely a continuum water mobility in soil from the largest pores to the smallest pores with progressively less water mobility as pore size decreases (Sprenger et al., 2018). However, we currently lack methodology to infer the degree of connectivity and dynamics of mixing over time between separate soil water pools extracted at different applied energies.

Reviewer 2: L44: Need a brief explanation of what in situ equilibration is and some references of papers using either of these methods.

Reply: We added a reference and more details here.
We revised the text with the following statements:
L60-62: Methods to characterize soil water pools in situ include water vapor laser spectroscopy that assumes most mobile soil water is in equilibrium with soil water vapor (Oerter and Bowen, 2017) or field extraction using suction lysimeters (Sprenger et al., 2015).

Reviewer 2: L100: These waters aren't all that different in isotope compositions. Nota Bene: Kona Deep drinking water is about 0 ‰ in _18O and _2H and is available on Amazon.

Reply: We included in the methods section our rationale for the selection of the waters used for wetting the soil samples and suggested in the discussion section how future studies should consider selecting waters with greater differences in isotope values.
We revised the text with the following statements:
L127-129: We selected these waters because of their contrasting isotopic values representing the natural range expected for cold season (light water) and warm season (heavy water) precipitation in temperate continental interior regions.

L366-369: Additional improvements and expanded applications of the combination approach we present should be considered. For example, use of waters with a greater isotopic difference for experimentally wetting dry soil and reversing the order of the addition of the heavy and light waters would better resolve rates of mixing and possible fractionation effects.

Reviewer 2: L107: The abstract claims that the light water was enough volume to fill only the smallest pores. The procedure described here seems very arbitrary. How do you have any confidence or measure of what soil pores where filled and to what extent?

Reply: We revised the statement in the abstract to clarify that the first applied water was held at high matric tension. In addition, the relationship between matric tension and pore size is highlighted within revised introduction section (covered above in reply to "Reviewer 2: (1)") and by revised scales on x-axis of Fig. 1.
We revised the text with the following statements:
L10-13: We wetted oven-dried, homogenized sandy loam soil first with isotopically "light" water (δ^2H = -130‰; δ^{18}O = -17.6‰) to represent antecedent moisture held at high matric tension, and then brought the soil to near saturation with "heavy" water (δ^2H = -44‰; δ^{18}O = -7.8‰) representing new input water.

Reviewer 2: L118: Are you sure this was the extraction temp? Did you use boiling water? Laramie is pretty high elevation and thus water has a low boiling point.

Reply: We added more clarification in the methods section about the extraction method and equipment for cryogenic vacuum distillation at Stable Isotope Facility of University of Wyoming.
We revised the text with the following statements:
L152-154: We performed the CVD procedure at 102ºC and <0.1-2.7 Pa vacuum pressure, which were controlled and monitored using heating coils, thermistors, and vacuum gauges. The vacuum pressure used during CVD is not the same as the estimated tension applied using CVD described in section 2.3.

Reviewer 2: L122: 95% is still not ALL of the water.

Reply: We adjusted this statement to more accurately reflect the extraction efficiency achieved in our study, rather than simply stating how our efficiencies related to guidelines presented in West et al., 2006. In addition, we highlighted recent work on this topic.
We revised the text with the following statements:
L154-159: The final sample masses after extraction were compared to oven-dried masses to determine the recovery of extracted water; every sample processed in our experiment had greater than 99% of water extracted at this step. Recent work has highlighted that CVD near 100ºC or oven drying soil near 105ºC do not extract all of the water from soil (Adams et al., 2019). The amount of water not recovered using CVD in the current study was assumed to be negligible with minimal impact on the isotopic values of extracted water.

Reviewer 2: L127: You use the Two Water Worlds terminology here, but you haven't ever really discussed it in any detail in the introduction. I suggest you do so, to help contextualize the rest of the paper.

Reply: We have revised this part of the methods section to be consistent with other changes we have made to the introduction section (covered above in reply to "Reviewer 2: (1)").
We revised the text with the following statements:
L166-168: While field capacity and wilting point varies among different soil types and plants, reference values of 0.033 MPa and 1.5 MPa for field capacity and agronomic wilting point are useful as guidelines for understanding potential boundaries on ecohydrologically separate water pools.

Reviewer 2: L136: Three and four hours seems like a long time! On what basis did you choose these times?

Reply: We agree these centrifuge extraction times are long and can be shortened. Our statement addressing this issue (acknowledged in specific comment "Reviewer 2: L290" below) remains in the discussion section for guidance to others who wish to apply the centrifuge method.
The text that is related to this comment:
L372-374: 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.

Reviewer 2: L138: You never really discuss what is tightly or highly bound, or what the potential mechanisms for this soil water are. There are many aspects to this, from soil pore size, to soil mineralogy, etc. This is a main concept of your paper, but you never give readers any background or basis of understanding how you are using this terminology and "boundness" concepts.

Response: We added more detail and clarification to the introduction section about matric tension and other commonly defined soil water pools (covered above in reply to "Reviewer 2: (1)"). In addition, we modified the methods section with the following text to be consistent with how we define and use these concepts and definitions relating to different pools of soil moisture.
We revised the text with the following statements:
L178-181: Afterward, the remining water in in each sample was extracted using CVD and is referenced here as "high tension" extraction; this is a fraction of water held under high tension that is rarely directly compared to more mobile waters within soils that have sufficient volumetric water content to permit sampling with methods like suction lysimeters.

Reviewer 2: L140: Again, are you sure it was ALL of the water left. Or was it 95%? I dont mean to be tedious here, and there are limits to CVD, but that is prwcisely my point. Even CVD at 100 C wont get all the water out that is in interlayers spaces in clays, etc. Are more nuanced discussion is needed (maybe it comes later in the discussion), and at least some acknowledgement of the study's potential limitations is needed. I will look for that as I read…

> Reply: We added more clarification and detail to the methods section to address this comment as noted in the reply to "Reviewer 2: L122" above.

Reviewer 2: L142: I think you should move up the details about the centrifuge and inserts. It hard to envision what you did until you tell us about the inserts.

> Reply: Details on the centrifuge inserts have been moved up in the methods section as requested by the reviewer.
> We revised the text with the following statements:
> *L130-139*: After the soil cooled from the drying procedure we applied 20ml of the light water with a spray bottle to the 350 g sub-sample and mixed by gloved hands to ensure homogenous application. 18-30g of this slightly wetted soil was gently packed to form soil columns in each of six custom made centrifuge inserts (Fig. 2). The custom steel tube inserts were perforated with small drilled holes at the bottom and fitted with a collar at the top. The collar secured the position of the insert within the sleeve at roughly 19mm above the bottom to establish a reservoir for collecting extracted water through the perforated bottom during extraction by centrifugation (below). We placed four steel screens secured by rubber o-rings at the bottom of each insert to reduce loss of soil yet permit water flow during centrifugation. In addition, we placed a small gravity secured cap on top of each insert to reduce evaporation from soil samples in inserts during equilibration and centrifugation. The caps were loose enough to not generate vacuum within the sample as water was eluted during centrifugation.

Reviewer 2: L149: Good that you accounted for evap during the procedure. I assume it was done at room temp, but I could easily see temp being higher inside the centrifuge, especially for 3 to 4 hours. Did you measure this?

> Reply: Temperature during centrifugation was controlled and details have now been added to the methods section.
> We revised the text with the following statements:
> *L162-163*: Centrifugation was performed with the cooling function activated; the internal temperature during centrifugation never exceeded 25ºC.

Reviewer 2: L167: What is atomic fraction? Do you mean isotope ratios (not in delta format)? Or do you mean mixing fraction?

> Reply: Changes to manuscript in response to this comment are covered above in reply to "Reviewer 1: (6)".

Reviewer 2: L178: Is this 1% the total mass (water + soil) or just water? If it was total mass, then a decent amount of water lost to evap (and a big shift in isotope ratios) could be contained in the 1% number. I suggest a sensitivity analysis be done to quantify (in isotope terms) what the effects of this much water loss would actually be. It may seem tedious and unnecessary, but with this much handllng of the wet soil, I could easily see evaporation being a bigger factor in isotope results than a casual view would expect.

Reply: Clarification was added to the methods section to highlight that 1% mass uncertainty was of water, not water and soil.
We revised the text with the following statements:
L221-222: We observed differences of only less than 1% of the mass of the extracted water in all cases, and therefore discounted the impacts of evaporative fractionation on our results and interpretations.

Reviewer 2: L217: You state that the BSE waters were not significantly different from the applied waters, but in Figure 3 upper left panel they sure look different to me. It seems that you were not getting back what you put in. This seems problematic.

Reply: We covered the revised changes in response to this comment above in reply to “Reviewer 1: (1)”.

Reviewer 2: L218 / Figure 3: Suggest adding A -F labels to the panels in Figure 3. Also, the x-axis labels and ticks seem inadequate.

Reply: We agree that adding labels would help. We have updated this figure, which is now Fig. 4 in the revised manuscript.

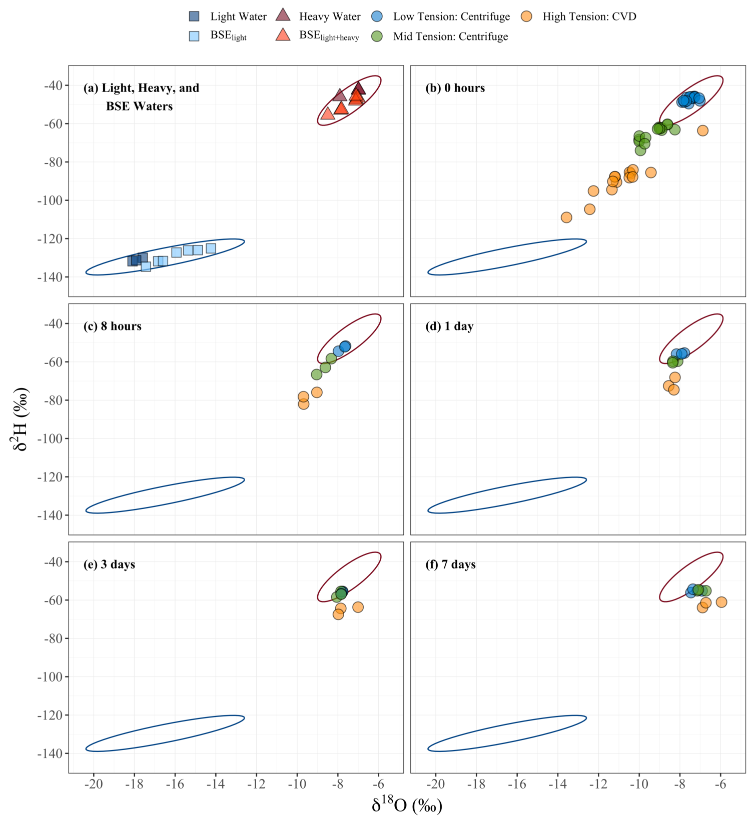

Figure 4: Isotopic values of water samples in dual-isotope space, δ^2H_{VSMOW} (‰) vs. $\delta^{18}O_{VSMOW}$ (‰). (a) Light, Heavy, and BSE(bulk sample extraction) waters with 95% confidence interval ellipses generated by pooled data of Light, Heavy, and BSE waters since the pooled groups were found to be not significantly different with pairwise MANOVA (Table A1 in Appendix) (blue ellipse =

BSE$_{light}$ and Light Water, red ellipse = BSE$_{light+heavy}$ and Heavy Water). (b-f) Waters extracted at low, mid, and high tension for each equilibration timepoint. The 95% confidence interval ellipses from (a) are included in (b-f) for reference.

Reviewer 2: L222: I suggest keeping the applied water points in all panels in Figure 3 for easier comparison. Perhaps make them dashed outline or ghosted or something to show them but not distract form the time series data.

Reply: This has been addressed above in reply to “Reviewer 2: L218”.

Reviewer 2: L227: So basically, after enough time, all the waters extracted by any means all converged upon the Heavy water signature. And the heavy water signature is the one that you soaked the sample in, but only put a little of the light water in the same samples?

Reply: We added clarification in the results section in response to this comment.
We revised the text with the following statements:
L277-279: Over time the isotopic ratio values for waters recovered from all three tensions converged upon the expected equilibrium value based on mass balance mixing of the two applied waters, predominantly weighted by the heavy water due to the proportionally much larger amount of heavy water applied.

Reviewer 2: L231: So, the conclusion is that the samples were all well mixed? OR something else? Because the dont look well mixed to me, especially not until dy 3 or later. Am I missing the point? If so, please explain better.

Reply: We made some changes to make this section of the results more explicit.
We revised the text with the following statements:
L286-287: These values suggest all water applied was accounted for in extraction processes and that minimal, if any, fractionation occurred due to evaporation.

Reviewer 2: L243: How do you evaluate the mixing results if you dont actually known how much of each type of water you put into the soil? Seems to me that with so much more heavy water than light, you are not really evaluating mixing, but more like the time to equilibrium, wherein the heavy water signal just overwhelmed the light because there was so much more of it.

Reply: We have clarified within methods section how we performed the calculations that permitted approximation of light water applied and direct measurement of heavy water infused in each sample. In addition, we have incorporated the term equilibrium more throughout the revised manuscript as we agree that two applied waters are mixing overtime to an equilibrium state. This equilibrium state is heavily weighted isotopically by the heavy water since a much larger proportion of heavy water was initially applied to the wetted soil. Please see reply above to “Reviewer 2: L227” for changes to results in relation to this comment.
We revised the text with the following statements:
L147-148: Wetted samples were weighed prior to the centrifuge extraction process to determine total wetted weight and amount of heavy water infused in each sample.

L213-218:The mass of water remaining in soil samples before high-tension extraction was calculated using gravimetric water contents and the mass of the soil samples after the mid tension centrifuge step. The mass of total water applied to each sample was determined by adding the masses of water remaining in the soil before high tension extraction and water extracted from both centrifuge steps. The mass of light water applied was determined by subtracting the amount of heavy water infused in the sample (covered in section 2.2) from the mass of total water applied.

Reviewer 2: L248: This section reads more like a conclusions paragraph than the start of a discussion. You haven't really supported any of these statements, yet.

Reply: We intended here to lay out the structure and direction for the discussion section by highlighting what we feel are the most important take-home points. No changes were made to the revised manuscript in response to this comment.

Reviewer 2: L252: What are the proposed mechanisms of mixing? This is hard to determine, because you haven't ever discussed where in soil water is actually held. Is the "mixing" done via diffusion? if so, water self- diffusion in soils is fairly well studied and you could greatly increase the impact of your findings by bringing in some discussion of that work. This seems like an over simplistic analysis of your results, which are a bit fast and loose as it is. No offense intended, just that I am seeking more detail and justification in your measurements and results.

Reply: The proposed mechanism of mixing, self-diffusion, remains briefly introduced in the abstract with more explicit reference to equilibrium and is now highlighted in more detail in the discussion section. We have added literature references for mechanisms controlling liquid water diffusion rate in soil. We also provide more discussion about how we expect mixing to differ in finer textured and unsaturated soils.
We revised the text with the following statements:
L16-18: We assessed differences in the isotopic composition of extracted water over the 7 d equilibration period with a MANOVA and a model quantifying time-dependent isotopic mixing of water towards equilibrium via self-diffusion.

L347-357: Since we limited vapor transport and advection in the current study by holding samples in a closed, isothermal vessel near saturation, we assume the isotope mixing among soil pore waters was dominated primarily by self-diffusion of isotopologues by Brownian motion. This mixing towards equilibrium by self-diffusion in hypothetical pore space is shown in Fig. 3. Diffusion rate in soil solution is a function of the diffusion coefficient for the solute of interest, a tortuosity factor, volumetric water content (θ) and the solute effective concentration gradient (Chou et al., 2012). We did not measure these variables in our study; rather we simplified the analysis by lumping these processes into a single empirical parameter (k) in our time-dependent mixing model (Eq. (3)). However, we expect soil water content as well as other features that determine tortuosity, like aggregate structure and pore size distribution will have strong influences on the isotopic mixing times of soil water pools. For example, complete mixing in finer textured soils and unsaturated soils will be much longer than those reported here because of these effects, but can be assessed using the general approach we describe.

Reviewer 2: L266: Are there carbonates in your soil? Easy test with HCl.

Reply: We did not detect carbonate with 1N HCl and this has been added to the methods section at the point where we introduce the soil used in the experiment.
We revised the text with the following statements:
L120-121: We did not detect carbonates in the soil using tests with 1N HCl (Schoeneberger et al., 2012).

Reviewer 2: L270: This is the first time you have acknowledged that your samples have perturbed soil structure and thus pore sizes. This may be the biggest reason for any isotope effect of any discussed.

> Reply: In response to this comment we have noted in abstract, methods and discussion sections that the structure of the soil used in our study was disturbed and likely lacked complex aggregates. We revised the text with the following statements:
> *L18-20*: The simplified and homogenous soil structure and nearly saturated moisture conditions used in our experiment likely facilitated rapid isotope mixing and equilibration among antecedent and new input water.
>
> *L112-115*: 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. Our experimental soil therefore was highly homogenized and lacked natural physical structure with complex soil aggregates.
>
> *L318-321*: Complete mixing would likely take longer for undisturbed soil samples with complex aggregate structure compared to our homogenized and disturbed soil samples. The connectivity of water pools within and between soil aggregates and other pore regions for undisturbed soil is likely much lower than in disturbed soils where this complex structure has been reduced.

Reviewer 2: L277: Finally, the discussion I was yearning for. Can you expand by making some calculations that support these arm waving statements?

> Reply: We added more details to the discussion section about diffusion rate in soils as mentioned above in reply to "Reviewer 2: L252". We found it was more appropriate to reference related work on diffusion rate in soils since we did not take all the necessary measurements to parameterize a more mechanistic model of liquid water diffusion and transport.

Reviewer 2: L288: Good point on the pore size changing during the spinning.

> Reply: No changes were made in response to this comment.

Reviewer 2: L290: Yes, shorter spin times!

> Reply: We agree that this would make the method more realistic for wider applications. No changes were made in response to this comment.

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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 separate and potentially interacting pools of soil water provide a powerful means to precisely resolve plant water sources and quantify water residence time and connectivity among soil water regions during recharge events. Here we present an approach for quantifying the time-dependent isotopic mixing of water recovered at separate suction pressures or tensions in soil over an entire moisture release curve. We wetted oven-dried, homogenized sandy loam soil first with isotopically “light” water (δ^2H = -130‰; $\delta^{18}O$ = -17.6‰) to represent antecedent moisture held at high matric tension, and then brought the soil to near saturation with “heavy” water (δ^2H = -44‰; $\delta^{18}O$ = -7.8‰) representing new input water. Soil water samples were then sequentially extracted at three tensions (“low tension”, centrifugation ≈ 0.016 MPa; “mid tension”, centrifugation ≈ 1.14 MPa; and “high tension”, 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 water over the 7 d equilibration period with a MANOVA and a model quantifying time-dependent isotopic mixing of water towards equilibrium via self-diffusion. The simplified and homogenous soil structure and nearly saturated moisture conditions used in our experiment likely facilitated rapid isotope mixing and equilibration among antecedent and new input water. Despite this, the isotope composition of waters extracted at mid compared to high tension remained significantly different for up to 1 day, and that for waters extracted at low compared to high tension remained significantly different for greater than 3 days. Complete mixing (assuming no fractionation) for the pool of water extracted at high tension occurred after approximately 4.33 days. Our combination approach involving extraction of water over different domains of the moisture release curve will be useful for 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 of soil water requires methods that differentiate the isotopic signature of water pools held across different sized soil pores and ranges of matric tensions or suction pressures. A variety of field- and lab-based methods are typically employed for such analyses and each separately assesses different pools of water recovered at discrete ranges of tension (Oerter and Bowen, 2017; Orlowski et al., 2016b; Sprenger et al., 2015). These

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methods effectively recover and analyze water from different soil pore size ranges and only a few methods are capable of sampling hygroscopic water, i.e. the water that forms thin films around soil particles held at matric tensions greater than plants are able to extract. The Two Water Worlds (TWW) hypothesis (McDonnell, 2014) considers that transpiration and runoff to streams derive from separate pools of water that are incompletely mixed in time or across pore regions in the soil. Brooks et al. (2010) presented stable isotope evidence of ecohydrologic separation between plant available water in smaller pore regions and mobile water passing through preferential flow paths when smaller pores were filled, challenging the hypothesis of translatory flow and establishing a mechanism to explain the TWW hypothesis. Yet, most studies examining ecohydrologic separation and the TWW hypothesis fail to differentiate isotopic signatures beyond that of mobile water and bulk soil water. More comprehensive evaluation of soil water isotopes across multiple pore sizes and soil regions is needed to examine recharge processes explaining the TWW hypothesis (Berry et al., 2018; Brantley et al., 2017; Brooks et al., 2010; McDonnell, 2014; Sprenger et al., 2019). At a more fundamental level, such methods are needed to 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 and trace fluxes of nutrients via water transport in the critical zone.

Characterization of water isotope ratios in soils involves careful consideration of methods used to recover soil water. 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, the relationship between volumetric water content and matric potential (negative equivalent of matric tension) (Sprenger et al., 2015). Terminology for water pools recovered at different applied energies has been debated. For the purposes of relating our study to ecohydrologic separation studies, we define two commonly defined pools, gravity-drained water and matrix water, consistent with recent terminology used by Brantley et al. (2017). Gravity-drained water is the most mobile pool of water within soil that freely drains through large pores under the force of gravity. Whereas matrix water consists of capillary and hygroscopic water that does not drain freely under force of gravity but is held across a broad range of tensions by smaller pores that may or may not be accessible to plants. There is likely a continuum water mobility in soil from the largest pores to the smallest pores with progressively less water mobility as pore size decreases (Sprenger et al., 2018). However, we currently lack methodology to infer the degree of connectivity and dynamics of mixing over time between separate soil water pools extracted at different applied energies.

Methods to characterize soil water pools in situ include water vapor laser spectroscopy that assumes most mobile soil water is in equilibrium with soil water vapor (Oerter and Bowen, 2017) or field extraction using suction lysimeters (Sprenger et al., 2015). However, more often analysis of soil water isotopes involves water extraction in the lab of soil samples collected from the field. 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 (Sprenger et al., 2015). Suction cup lysimeters typically sample water held at low tension (0.05 to 0.10 MPa) and therefore are limited to analysis of only the highly mobile fraction of soil water, but application of much higher tensions using suction cup lysimeters is feasible (Li et al., 2007). Mechanical squeezing and centrifugation recovers water across much broader tension ranges and with no fractionation, but are unable to drain pores with diameters less than 0.03 μm (i.e. extract water held at tensions beyond 1 MPa) (Orlowski et al.,

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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 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 determine the isotopic composition of specific pools of water in the soil but are rarely employed in combination to understand the dynamics of soil water pools that make up the bulk water.

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 using centrifugation was consistently incompletely mixed after 72 hours of equilibration time. However, their experimental design precluded analysis of the time necessary for hygroscopic, capillary and gravitationally drained waters to completely mix. In addition to understanding mixing between water pools within soil, recent work has highlighted the importance of considering also fractionations that may affect the isotopic composition of extracted water. Isotope effects related to adhesion under various matric potentials, soil wettability, and solid interfacial chemistry of soil particles are important to consider (Gaj et al., 2019; Gaj and McDonnell, 2019).

Here we present and evaluate a step-wise procedure to recover and analyze the isotopic composition of different pools of soil water and characterize the dynamics of their interaction over time. To demonstrate the method, we confine our initial study to soil moisture conditions near saturation and investigate the time-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 within soil?

2 Methods

2.1 Experimental design

Our experiment involved sequentially wetting oven-dried soil using isotopically contrasting water inputs that then allowed us to quantify the degree that separate pools of soil water mixed over time. We used a novel combination of centrifuge extraction and cryogenic vacuum distillation to recover pools of soil water held at discrete ranges of tension, spanning gravitationally drained, capillary and hygroscopic water pools. We first applied a small amount of isotopically “light” water to oven-dried soil followed by nearly saturating the soil samples with an isotopically “heavy” water. Three pools of water were recovered from wetted soils after variable mixing times through a stepwise increase of applied energy using two centrifugation speed steps followed by distilling the remaining water in the soil samples using 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

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($\mathrm{BSE}_{\mathrm{light}}$) or immediately after adding both the isotopically light and heavy waters ($\mathrm{BSE}_{\mathrm{light+heavy}}$). Prior to step-wise extraction for the remainder of the soil samples, the light and heavy water applied were allowed to freely mix and equilibrate under nearly 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 recovered 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^{2}H and δ^{18}O).

2.2 Experimental soil and wetting procedure

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. Our experimental soil therefore was highly homogenized and lacked natural physical structure with complex soil aggregates. We employed the hydrometer method to determine soil particle size distribution using sodium hexametaphosphate as the chemical aid for dispersion (Black and Day, 1965). The particle size distribution defined by the U.S. Department of Agriculture classification system was 9% clay, 32% silt, and 59% sand. We constructed a soil retention curve (Fig. 1) using previously reported parameters for modeling water retention of sandy loam soil (van Genuchten, 1980; Kosugi et al., 2002), and highlight also the relative maximum pore size filled across the range of matric potentials as described by Schjonning (1992). We did not detect carbonates in the soil using tests with 1N HCl (Schoeneberger et al., 2012).

We prepared the homogenized soil material by oven drying a 350 g sub-sample at 105ºC for 48 hr. We then sequentially applied two isotopically distinct waters to bring the soil to near saturation. The isotopically light water used in the experiments was local tap water from the University of Wyoming campus in Laramie, and the heavy water was from multiple bottles of FIJI Water (FIJI Water LLC, Los Angeles, CA, USA). The isotope ratio value standardized to Vienna Standard Mean Ocean Water (VSMOW) for the light water was -130±2‰ for δ^{2}H and -17.6±0.5‰ for δ^{18}O (n=5) and for the heavy water was -44±2‰ for δ^{2}H and -7.3±0.4‰ for δ^{18}O (n=5). We selected these waters because of their contrasting isotopic values representing the natural range expected for cold season (light water) and warm season (heavy water) precipitation in temperate continental interior regions.

After the soil cooled from the drying procedure we applied 20ml of the light water with a spray bottle to the 350 g sub-sample and mixed by gloved hands to ensure homogenous application. 18-30g of this slightly wetted soil was gently packed to form soil columns in each of six custom made centrifuge inserts (Fig. 2). The custom steel tube inserts were perforated with small drilled holes at the bottom and fitted with a collar at the top. The collar secured the position of the insert within the sleeve at roughly 19mm above the bottom to establish a reservoir for collecting extracted water through the perforated bottom during extraction by centrifugation (below). We placed four steel screens secured by rubber o-rings at the bottom of each insert to reduce loss of soil yet permit water flow during centrifugation. In addition, we placed a small gravity secured cap on top of each insert to reduce evaporation from soil samples in inserts during equilibration and centrifugation. The caps were loose enough to not generate vacuum within the sample as water was eluted during centrifugation. We recorded weights of inserts and sleeves prior to adding the soil. Except for samples that were immediately taken for bulk sample extraction ($\mathrm{BSE}_{\mathrm{light}}$) using cryogenic vacuum distillation (CVD), the packed inserts were then wetted from the bottom up by immersing in a container with heavy

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water at a level just below the soil level in each insert. This ensured the soil samples were wetted to near saturation by reducing the chance of air being trapped within the soil matrix. We then removed a second set of samples for bulk sample extractions (BSE$_{light+heavy}$) using CVD. The remaining samples were transferred to storage in an airtight container at 20ºC in the lab until the desired equilibration timepoints were reached. Complete saturation was not possible as some water was lost from perforations at the bottom of the inserts when they were removed from the container of heavy water. Wetted samples were weighed prior to the centrifuge extraction process to determine total wetted weight and amount of heavy water infused in each sample.

After each centrifugation step, we recorded weights of sleeves and inserts, and we collected and filtered extracted water into plastic vials with silicon caps, ready for stable isotope analysis. Vials with Parafilm were stored in a 4ºC fridge until processed. The remaining water after centrifugation was extracted using CVD ~ 2 hours to ensure all water was removed (West et al., 2006). We performed the CVD procedure at 102ºC and <0.1-2.7 Pa vacuum pressure, which were controlled and monitored using heating coils, thermistors, and vacuum gauges. The vacuum pressure used during CVD is not the same as the estimated tension applied using CVD described in section 2.3. The final sample masses after extraction were compared to oven-dried masses to determine the recovery of extracted water; every sample processed in our experiment had greater than 99% of water extracted at this step. Recent work has highlighted that CVD near 100ºC or oven drying soil near 105ºC do not extract all of the water from soil (Adams et al., 2019). The amount of water not recovered using CVD in the current study was assumed to be negligible with minimal impact on the isotopic values of extracted water.

2.3 Soil water extractions

We extracted water from soil using a Sorvall RC 5B Plus centrifuge fitted with a Sorvall aluminum rotor with four stainless steel sleeves designed for 50 ml Falcon Tubes (Sorvall, Newton, CT, USA). Centrifugation was performed with the cooling function activated; the internal temperature during centrifugation never exceeded 25ºC. We focused on extracting waters near two ecohydrologically relevant pressures for the waters recovered at "low" and "mid" tension: field capacity (i.e., the point at which no more water drains freely under force of gravity) and agronomic wilting point. While field capacity and wilting point varies among different soil types and plants, reference values of 0.033 MPa and 1.5 MPa for field capacity and agronomic wilting point are useful as guidelines for understanding potential boundaries on ecohydrologically separate water pools. Rotations per minute (RPM) for the centrifuge extractions at field capacity and agronomic wilting point were calculated using an equation from Nimmo et al. (1987), which relates rotational velocity to matric potential and radii of a centrifuge set-up:

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

where Ψ is matric potential (Pa), ρ is density of water (kg/m^3), ω is rotational velocity (s^{-1}), r_1 is the radius (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 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 using Eq. (1) varies depending

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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. Afterward, the remining water in in each sample was extracted using CVD and is referenced here as "high tension" extraction; this is a fraction of water held under high tension that is rarely directly compared to more mobile waters within soils that have sufficient volumetric water content to permit sampling with methods like suction lysimeters. Applied tension using CVD is estimated to be greater than 100 MPa (Sprenger et al., 2015).

2.4 Stable isotope analysis

The stable isotope composition of water 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 those for the international standard, Vienna Standard Mean Oceanic Water (VSMOW). Our measurements on samples were corrected to the VSMOW scale using working reference waters calibrated to VSMOW and SLAP reference waters obtained from the IAEA. 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) at the University of Wyoming Stable Isotope Facility. 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 $\delta^{18}O$. We report the accuracy as the absolute difference between the mean of analyzed lab reference water samples (n=15) and the calibrated value of lab reference water. We report precision as the standard deviation of all lab reference water samples analyzed (n=15).

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 account for the light and heavy water applied to the oven-dry soil and determine the distribution of added water across extracted fractions. Using Eq. (2) below, all possible combinations of replicates in this study at each equilibration timepoint and for each isotope (δ^2H and $\delta^{18}O$) were assessed (n=54).

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

m is mass of water in kg and R is isotope ratio calculated from either δ^2H or $\delta^{18}O$ values for the particular water component. 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 was divided by sum of inputs and multiplied by one hundred. Subscripts $_{HW, LT, MT,}$ and $_{HT}$ refer to the heavy water added, low tension, mid tension and high tension extracted waters. 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 in the mass balance model rather than that of the light water added to accommodate for the slight δ offset between these waters. This slight offset may have developed from evaporative fractionation (Allison

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To assess fractionation associated with evaporation, we calculated the difference in mass for soil filled inserts and corresponding extracted waters. This was evaluated throughout the experiment between centrifuge steps as well as prior to and after equilibration periods. We observed differences of only less than 1% of the mass of the extracted water in all cases, and therefore discounted the impacts of evaporative fractionation on our results and interpretations.

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

We further used a time-dependent isotope mixing equation to approximate the time required for soils to completely mix (i.e. reach equilibrium). 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), δ(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 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 varied with different tensions, δ_0 and k were allowed to vary based on each extracted water pool (i.e. low tension, mid tension, and high tension). The interaction among the three pools of water in this study within an ecohydrological perspective is diagramed in Fig. 3.

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 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

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values and standard deviations used for δ_e were -57±5‰ for δ^2H (n=27) and -8.6±0.7‰ for δ^{18}O (n=27), which are heavily weighted towards the value of the heavy water reflecting the much larger proportion of this water in fully wetted samples.

We compared the distribution of the expected equilibrium value (δ_e) to those of the different extracted fractions to evaluate mixing times. We considered the system to be completely mixed when the median expected δ value of the different extracted fractions was within the 90th percent credible interval of δ_e.

All 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). The time-dependent mixing models were analyzed using the probabilistic programming language Stan (Carpenter et al., 2017), using 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 within each of the tension ranges was consistent across all samples. The low and mid tension centrifuge extractions removed 71±6 % and 17±6 % (n=27) of the soil water, and high tension CVD extraction recovered the remaining 12±1 % (n=27) of the soil water. Average volumes from the three extraction steps in the experiment are illustrated on Fig. 1 in relation to the soil water retention curve for sandy loam soil.

The isotope composition of waters extracted at the three tensions were clearly different at 0 h after soil wetting, but differences diminished with the amount of time the added light and heavy waters were allowed to interact (Fig. 4, Table 1). The isotope ratio of water recovered using CVD of BSE_{light} samples (bulk sample extraction after light water applied) indicates that potentially the water in the sample at this step was altered slightly by evaporative enrichment of heavy isotopes mixed into the oven dried soil, which had a high amount of surface area exposed to dry local atmosphere. Although this changed the isotopic value of water in soil before application of the heavy water, the light waters applied and BSE_{light} extracted waters were not significantly different ($p > 0.05$). The isotope ratio values of the $BSE_{light+heavy}$ samples were not significantly different from that of the heavy waters applied ($p > 0.05$). At 0 h the isotope ratio values of water extracted using centrifugation at low rotational velocity (water extracted at low tension) were not significantly different from those of either the heavy waters applied ($p > 0.05$) or the $BSE_{light+heavy}$ samples ($p > 0.05$). Yet for the samples assessed at 0 h the isotope ratio values among waters extracted across the three different tensions were significantly different from one another (p values < 0.01) (Table 2). After 8 h of mixing the isotope ratio values of water extracted at low tension were significantly different from that of the heavy water applied ($p < 0.05$) and these remained significantly different over the remaining equilibration times (p values < 0.01). After 1 d the isotope ratio values of the waters extracted at low tension were not significantly different from those extracted at mid tension ($p > 0.05$) while the isotope ratio values of water extracted at mid tension were significantly different from those extracted at high tension ($p < 0.01$). After 3 d of mixing the isotope ratio values of waters extracted at low and high tensions remained statistically different ($p = 0.05$), but even these were indistinguishable after 7 d of mixing ($p > 0.05$). Over time the isotopic ratio values for waters recovered from all three tensions converged upon the expected equilibrium value based on mass balance mixing of the two applied waters, predominantly weighted by the

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heavy water due to the proportionally much larger amount of heavy water applied. The isotope ratio values of water extracted at high tension were significantly different (p values < 0.01) than BSE_{light} samples, $BSE_{light+heavy}$ samples, heavy water samples and light water samples for all equilibration timepoints. 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

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 δ^{18}O data with a range of 99.88% to 100.25%. These values suggest all water applied was accounted for in extraction processes and that minimal, if any, fractionation occurred due to evaporation.

3.3 Time-dependent mixing model

Model estimates determined from the time-dependent mixing equation (Eq. (3)) are provided in Fig. 5 and Fig. 6. A 1:1 relationship between observed and predicted values indicates the model did reasonably well at predicting observed values and their uncertainty with only one value observed outside the given uncertainty bound for δ^2H (Fig. 7). Results were generally consistent between the two isotopes, however δ^{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. 6). It took about 5 hours for the isotope values of water extracted at low tension to become similar to the expected equilibrium (i.e., well-mixed) δ_e value. Water extracted at mid tension did not attain a thoroughly mixed value until 12 hours. It took ~104 hours for the water recovered at high tension by CVD to reach the expected equilibration value. These model results suggest it would have taken the sequentially added waters a little more than 4 days to completely mix and equilibrate across the pools of soil water. δ^{18}O values indicate possible fractionation expressed at day 3 and 7 equilibration timepoints with offsets towards heavier values. Due to these offsets, probability densities were not evaluated with δ^{18}O data since our time-dependent mixing model did not account for fractionation offsets occurring during equilibration.

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 extracted at different tensions within a single soil sample offering a method to assess the time-dependent isotopic exchange among soil pools. 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 occupying different pore spaces added sequentially to dry soil do not immediately and completely mix. Lags in isotopic mixing and equilibration have implications for studies focused on plant water sources, soil water age or residence times, water

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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 as well as possible minor gravitational downward movement of water during equilibration storage, these results reveal relatively long lag times for complete mixing. Complete mixing would likely take longer for undisturbed soil samples with complex aggregate structure compared to our homogenized and disturbed soil samples. The connectivity of water pools within and between soil aggregates and other pore regions for undisturbed soil is likely much lower than in disturbed soils where this complex structure has been reduced. The time-dependent mixing model indicated that complete mixing was achieved at ~4.33 days and this timeframe was consistent with the MANOVA results between the waters recovered at the three tensions. However, at 7 days the waters extracted under high tension were significantly different than those of the $BSE_{light+heavy}$ samples (MANOVA), but were within the 90% credible interval for δ^2H of δ_e according to the time-dependent mixing model. This highlights a key difference between the statistical methods of comparison: while the MANOVA compares the multivariate normal means across isotopes, our mixing model analysis ignored the $\delta^{18}O$ values due to yet unexplained (see below for further discussion) deviations in the mixing model. Nonetheless, while these methods highlight slight differences in their estimate of when the two added waters were completely mixed across all extracted fractions, they both highlight the long time lags in mixing.

The mass balance mixing model revealed that 99% of the water applied to the oven dry soil in our experiment was recovered over the sequence of centrifuge and CVD extractions suggesting minimal losses or isotopic fractionation with evaporation after the soils were completely wetted. We chose to use the isotope value of the bulk water extracted after the light water was applied (BSE_{light}) as the end-member in the mass balance model rather than the isotope ratio value of the light water itself. We felt this was justified for the objective of our study, which was to demonstrate the capability of the combined centrifuge-CVD method to evaluate mixing dynamics among different soil water pools.

We observed slightly higher $\delta^{18}O$ values of the extracted water pools at days 3 and 7 than predicted based on simple mixing of the two waters added to the dry soil (Fig. 5). Because we recovered the expected mass of water (>99%) for these samples, we do not feel the observed ^{18}O enrichment was a result of evaporation. Water interactions with clay minerals (Gaj et al., 2017) and carbonates (Meißner et al., 2014), in contrast, typically result in depletion of ^{18}O in matrix water. The positive shift in $\delta^{18}O$ of soil water observed in our study however is consistent with observations reported by Oerter et al. (2014) who found that at low water content $\delta^{18}O$ of matrix water increased in the presence of clays enriched with potassium. We cannot discount the possibility of such ionic interactions in our study. The time course for ionic exchanges with clays that influence the oxygen isotope composition of matrix water might explain why the mixing dynamics observed in our study differed between H and O isotopes. Identifying and analyzing such effects require more thorough analysis.

Since we limited vapor transport and advection in the current study by holding samples in a closed, isothermal vessel near saturation, we assume the isotope mixing among soil pore waters was dominated primarily by self-diffusion of isotopologues by Brownian motion. This mixing towards equilibrium by self-diffusion in hypothetical

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pore space is shown in Fig. 3. Diffusion rate in soil solution is a function of the diffusion coefficient for the solute of interest, a tortuosity factor, volumetric water content (θ) and the solute effective concentration gradient (Chou et al., 2012). We did not measure these variables in our study; rather we simplified the analysis by lumping these processes into a single empirical parameter (*k*) in our time-dependent mixing model (Eq. (3)). However, we expect soil water content as well as other features that determine tortuosity, like aggregate structure and pore size distribution will have strong influences on the isotopic mixing times of soil water pools. For example, complete mixing in finer textured soils and unsaturated soils will be much longer than those reported here because of these effects, 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 and CVD as a means to extract waters sequentially from a single sample across a range of tensions. First, the 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 tension 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 different tensions should be explored further.

Additional improvements and expanded applications of the combination approach we present should be considered. For example, use of waters with a greater isotopic difference for experimentally wetting dry soil and reversing the order of the addition of the heavy and light waters would better resolve rates of mixing and possible fractionation effects. Furthermore, applying this combination method to undisturbed soil would need to carefully consider how soil is sampled before placed in centrifuge inserts. Collecting field samples directly into inserts would minimize compaction and disturbance of aggregate structure. In addition, the oven-drying step could be eliminated, and equilibration could be assessed by using antecedent moisture within undisturbed soil samples. 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 mechanisms that create spatial and temporal heterogeneity in the isotopic composition of soil water (Dubbert et al., 2019).

5 Conclusion

We present a method for separately extracting water held at different tensions within soil for isotopic analysis and provide 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 and self-diffusion of water in soils with different soil textures, for undisturbed soil cores that retain complex structure, and under variably saturated conditions. Additional work is needed to refine the application of the centrifuge-CVD combination method 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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$J_d = D_o\theta\xi(dc/dx)$ →→→→→→→→→→ (5)¶

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Deleted: Therefore, careful consideration should be taken when incorporating time in future evaluation of isotopic equilibration among soil water pools. It is also important to note that the rate of diffusion is dependent on volumetric water content and not matric potential. SinceSince soil samples in our study were nearly saturated, the fractional volumetric water volume content did not restrict diffusion, and tortuosity for the sandy loam soil in our study was likely low compared to that of fine textured soilsfactor was also relatively high since it is positively related to volumetric water content (Chou et al., 2012).…For example, Hydraulic conductivity is also a function of volumetric water content and has a positive relationship that logically makes sense because as more pores are filled with water, the pores are ... [151]

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Code and data availability. The code and data used in this study can be accessed via Open Science Framework (doi:10.17605/OSF.IO/ET3G5).

Author contributions. WHB conducted the experiment, performed data analysis, developed figures, and drafted the paper. JJM helped conceive the experiments, prototyped and refined the centrifugation insert, performed data analysis, and developed the self-diffusion model. MSP provided ideas on experiment design and interpretation of experiment results. DGW helped conceive the experiment and write the paper. All authors edited the paper.

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

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Review statement.

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

Effluent	Timepoint	Mean δ^2H ‰	Mean $\delta^{18}O$ ‰	Range δ^2H ‰	Range $\delta^{18}O$ ‰	Number of samples
Low Tension: Centrifuge	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
Mid Tension: Centrifuge	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
High Tension: CVD	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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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	MANOVA Comparison	Timepoint p-values				
Group 1	Group 2	0 hours	8 hours	1 day	3 days	7 days
Mid Tension: Centrifuge	Low Tension: Centrifuge	**<0.0001**	**0.02**	0.7	1	1
Mid Tension: Centrifuge	High Tension: CVD	**<0.0001**	**0.0001**	**0.005**	0.1	0.2
Mid Tension: Centrifuge	$\text{BSE}_{\text{light+heavy}}$	**<0.0001**	**0.0002**	**0.002**	0.1	0.4
Low Tension: Centrifuge	High Tension: CVD	**<0.0001**	**<0.0001**	**0.0001**	**0.05**	0.3
Low Tension: Centrifuge	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 the time-dependent mixing model.

		δ_e (‰)	k (10^2 hr^{-1})	b_0 (10 ‰)	b_1 (10 ‰ hr)
Isotope	Effluent		Mean (SD)		
δ^2H	All	-57.4 (4.8)			
	Low Tension: Centrifuge		15.8 (3.2)	8.9 (2.0)	5.1 (3.4)
	Mid Tension: Centrifuge		3.1 (1.0)	17.7 (5.4)	29.7 (13.4)
	High Tension: CVD		1.6 (0.6)	40.6 (13.9)	85.7 (36.7)
δ^{18}O	All	-8.6 (0.7)			
	Low Tension: Centrifuge		0.5 (0.2)	2.9 (0.6)	1.1 (10)
	Mid Tension: Centrifuge		1.5 (0.5)	2.9 (0.9)	3.9 (1.8)
	High Tension: CVD		3.7 (1.4)	5.7 (1.7)	10.5 (3.7)

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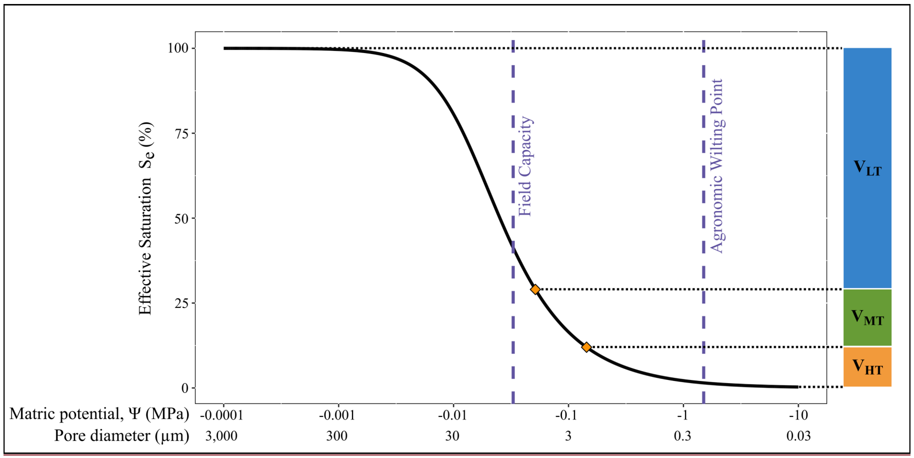

Figure 1: Soil retention curve for a sandy loam soil using van Genuchten parameters for a general sandy loam (Kosugi et al., 2002). Average volumes (V) from each extraction step of the experiment are illustrated on the right with LT for Low Tension, MT for Mid Tension, and HT for High Tension. Vertical lines are matric potential points of interest; field capacity of -0.033 MPa and agronomic wilting point of -1.5 MPa. The y-axis is effective saturation, a standardized form of volumetric water content. The x-axis has two scales; the top scale is matric potential in MPa and bottom is relative maximum pore size filled at the respective matric potentials (Schjonning, 1992). Samples wetted with both light and heavy waters were near but not at 100% effective saturation.

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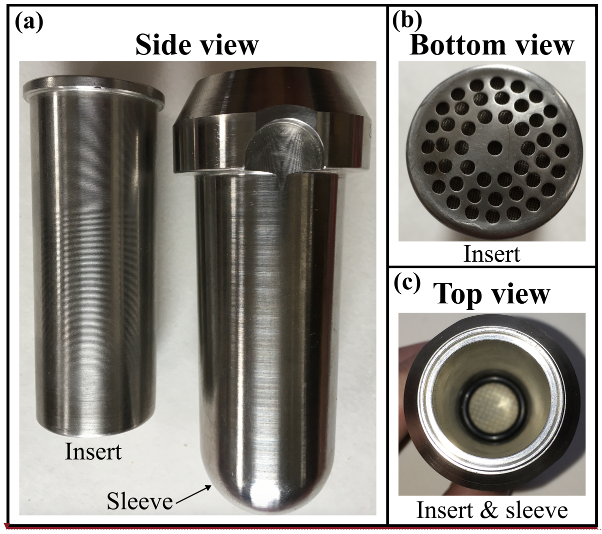

Figure 2: (a) Image of custom-made centrifuge insert and Sorval sleeve. (b) Bottom view of insert perforated with drilled holes to allow water movement during centrifugation. (c) Top view includes steel screens at bottom of insert secured with rubber o-ring to reduce soil loss during centrifugation. The steel tube inserts were fitted with a collar at the top that 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 screens and perforated bottom. Small gravity secured caps described in methods section 2.2 collars are not shown in this image.

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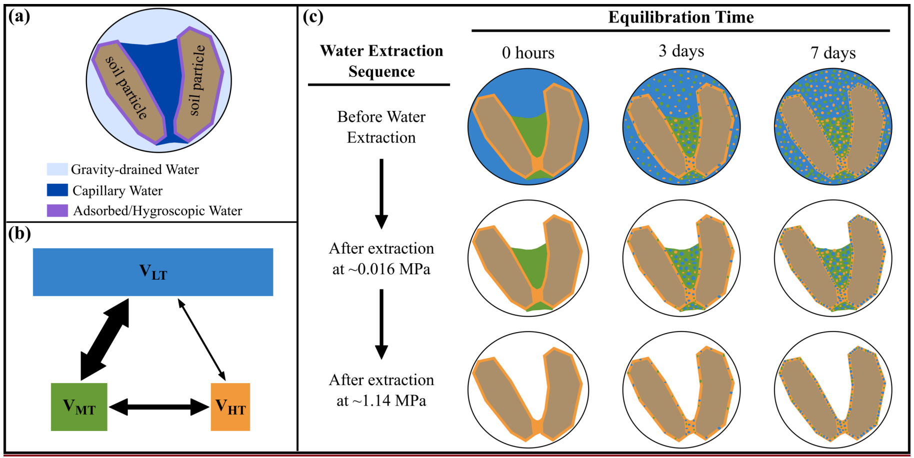

Figure 3: (a) Spatial relationship of the three most commonly discussed water pools that make up the bulk water pool in soil near saturation. Absorbed/hygroscopic water, capillary water, and gravity-drained water are depicted in hypothetical cross-section view of two soil particles within the soil matrix. (b) Relative volumes (V) of soil water pools in this study based on Fig. 1 (LT= low tension, MT= mid tension, and HT= high tension) and the relative amount of interactions (size of black arrows) between pools as equilibration time proceeds. (c) Three soil water pools for this study in hypothetical pore space, as diagramed in the first panel, at three equilibration timepoints and various points in the water extraction sequence. Based off of Fig. 1 water extracted at low tension is comprised of gravity-drained water and capillary water, that extracted at mid tension is composed of capillary water, and water extracted at high tension is comprised of capillary water and hygroscopic water. As equilibration time increases, each pool moves closer towards a well-mixed state (i.e. equilibrium).

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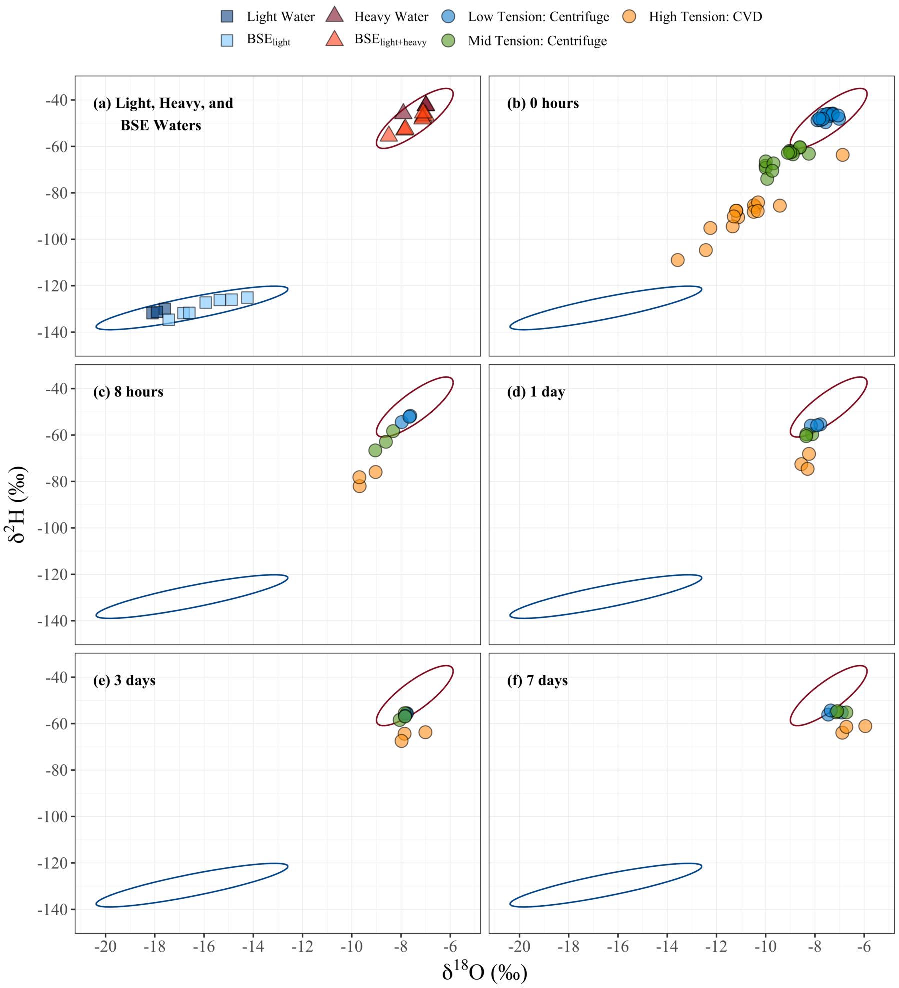
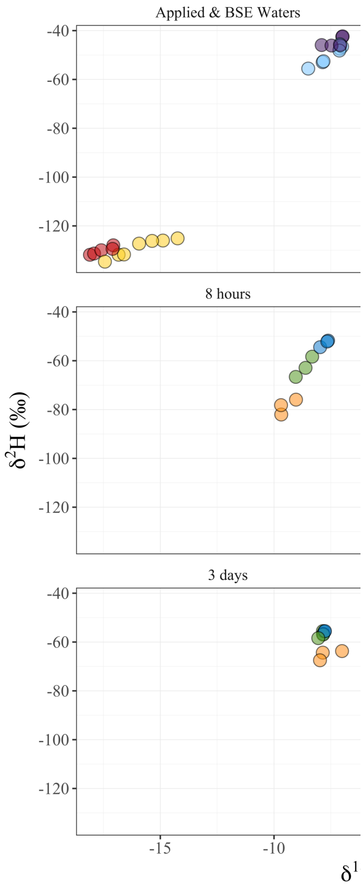


Figure 4: Isotopic values of water samples in dual-isotope space, δ^2H_{VSMOW} (‰) vs. $\delta^{18}O_{VSMOW}$ (‰). (a) Light, Heavy, and BSE(bulk sample extraction) waters with 95% confidence interval ellipses generated by pooled data of Light, Heavy, and BSE waters since the pooled groups were found to be not significantly different with pairwise MANOVA (Table A1 in Appendix) (blue ellipse = BSE$_{light}$ and Light Water, red ellipse = BSE$_{light+heavy}$ and Heavy Water). (b-f) Waters extracted at low, mid, and high tension for each equilibration timepoint. The 95% confidence interval ellipses from (a) are included in (b-f) for reference.



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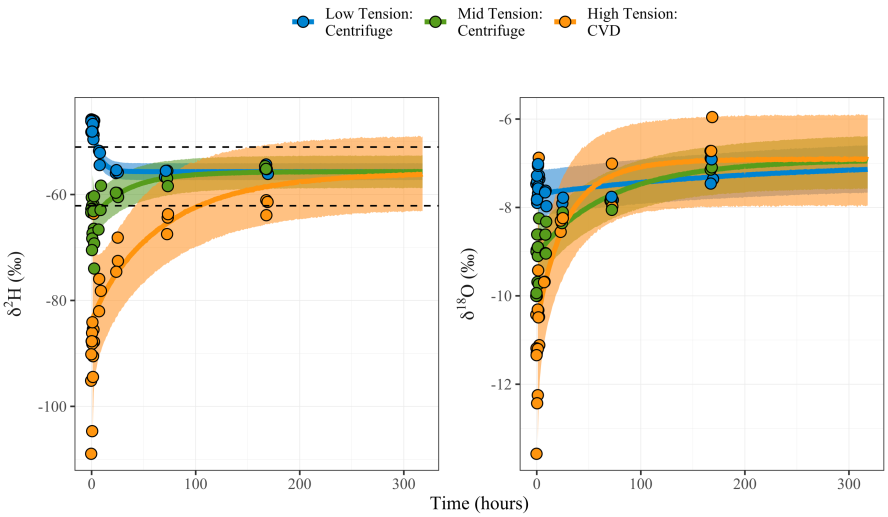
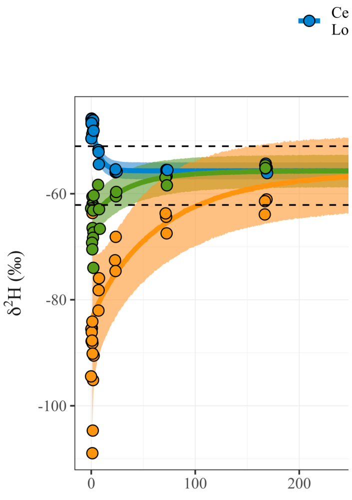


Figure 5: Time-dependent mixing model curves plotted for δ^{2}H and δ^{18}O (‰, VSMOW) for each extracted water fraction over time. Shaded regions are 90th credible intervals for each curve. The dashed lines are for the 90th credible interval for the equilibrium (δ$_e$) estimate of δ^{2}H. δ^{18}O measured values indicated possible fractionation offset near when equilibrium was achieved according to δ^{2}H values. Due to this offset, probability densities with δ^{18}O data were not evaluated similarly to the δ^{2}H values since the time dependent mixing model works under the assumption that there are no fractionation offsets occurring. Therefore, no dashed lines for right plot with δ^{18}O data.



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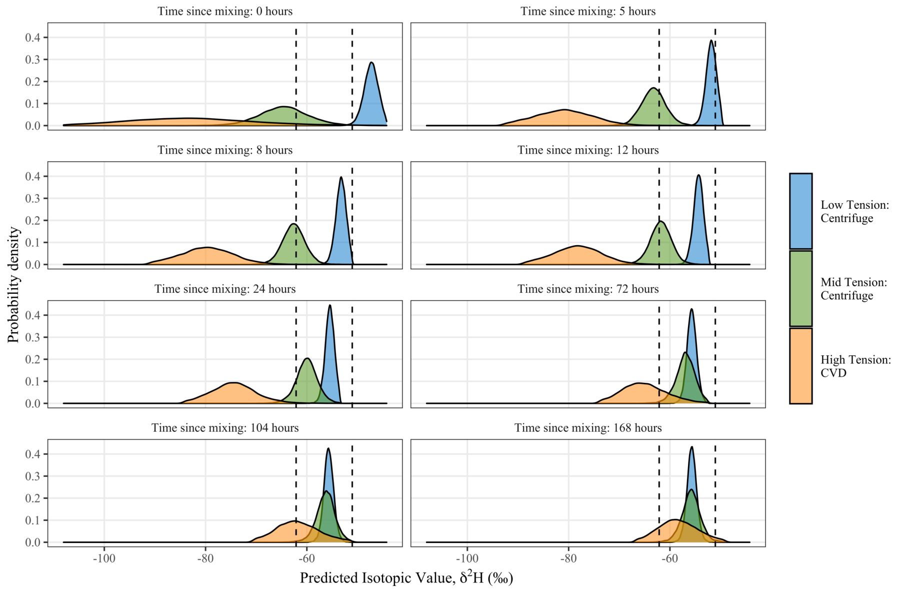

Figure 6: Time-dependent model for mixing time with distributions of δ^2H for each of the three extracted water fractions over time in relation to the 90th credible interval for equilibrium value (δ_e, dashed lines). Panels include extraction times for the experiment as well as important timepoints for mixing. At 5 hours the median low tension value was within the 90th credible interval of the equilibrium value. At 12 hours, the isotope composition of waters extracted at low and mid tension were similar to the the equilibrium value. It was not until 104 hours (~4.33 days) that the median isotopic value of the water extracted at high tension was also within the 90th credible interval of the equilibrium value.

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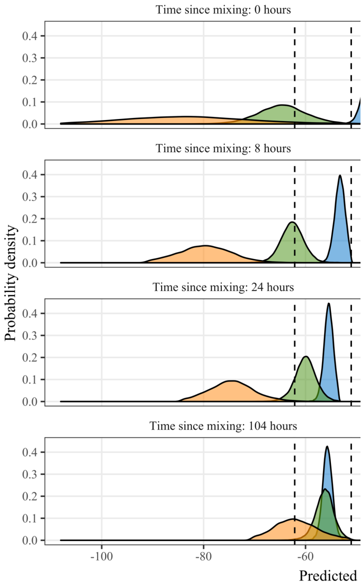

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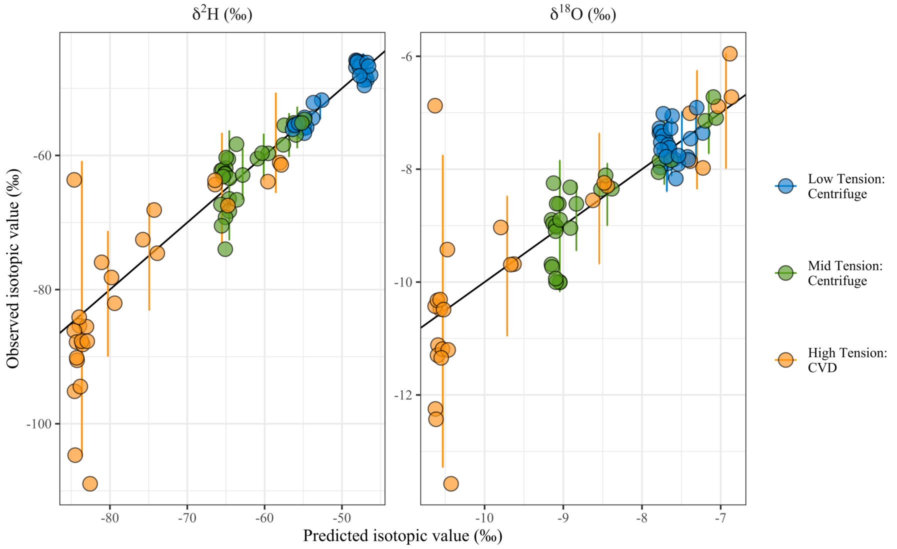
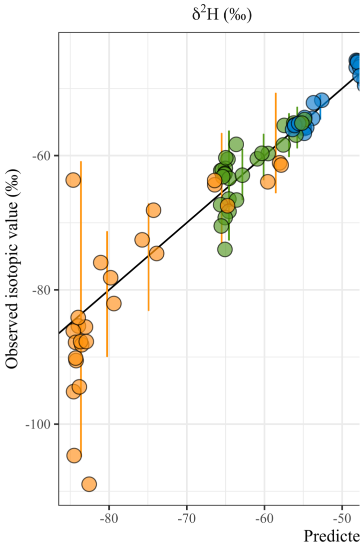


Figure 7: Comparison of predicted with Eq. (3) and observed values for waters extracted with different tensions. The 1:1 line is shown. Bars represent the credible interval (90%) of the predicted values by timepoint and tension. A slight jitter (3%) has been added to the predicted value (x-axis) in an effort to display the points.



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Appendix A:

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

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