Locally Produced Leaf Wax Biomarkers in the High-Altitude Areguni Mountains Outweigh Downstream Transport

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Abstract: Sedimentary records of lipid biomarkers such as leaf wax n-alkanes are not only influenced by ecosystem turnover and physiological changes in plants, they are also influenced by earth surface processes integrating these signals into the sedimentary record, though the effect of these integration processes are not fully understood. To determine the depositional constraints on biomarker records in a high-altitude small catchment system, we collected both soil and stream sediments along a 1000 m altitude transect (1500 – 2500 masl) in the Areguni Mountains, a subrange of the Lesser Caucasus Mountains in Armenia. We utilize a treeline at ~ 2000 masl, which separates alpine meadow above from deciduous forest below, to assess the relative contribution of upstream biomarker transport to local vegetation input in the stream. We find that average chain length (ACL), hydrogen isotope (δD) and carbon isotope ($\delta^{13}C$) values of *n*-alkanes are significantly different in soils collected above and below the treeline. However, samples collected from the stream sediments do not integrate these signals quantitively. As the stream drops below the treeline, the ACL, δD and $\delta^{13}C$ values of *n*-alkanes preserved in streambed sediments reflect a bias toward *n*-alkanes sourced from trees. This suggests that there is either 1) minimal transportation of organic matter from the more open vegetation in higher elevations, or 2) greater production of target biomarkers by trees and shrubs found at lower elevations results in overprinting of stream signals by local vegetation. Though these observations may preclude using *n*-alkanes to measure past treeline movement in these mountains, δD values of biomarkers in fluvial deposits in these settings are more likely to record local hydrological changes rather than reflect fractionation changes due to turnover in upstream vegetation structure.

1. Introduction

Mountain regions are important hubs for biodiversity and can provide refuge for a number of endemic species of flora and fauna (Antonelli et al., 2018). However, these high-altitude environments are often particularly vulnerable to climate change (Guisan and Theurillat, 2000). Therefore, gaining an understanding of sensitivity of these regions to past climate change is important for projecting the effects of future climate change on fragile ecosystems. The Caucasus Region in particular has been identified as a biodiversity hotspot covering the Republics of Armenia, Georgia, Azerbaijan, and parts of the Russian Federation, Türkiye, and Iran, that supports a wide variety of plant and animal species (Zazanashvili, 2009; Gasparyan and Glauberman, 2022). To better understand climate and environmental change in both the past and the present, it is necessary to refine our understanding and interpretation of paleoclimate records in this region. Plant wax biomarkers have been used in this region in both geological and archaeological contexts to reconstruct past climates, therefore understanding modern variability and transport processes will help refine these interpretations (Brittingham et al., 2019; Glauberman et al., 2020; Malinsky-Buller et al., 2021, 2024; Trigui et al., 2019). Specifically, we are interested in understanding the sedimentary processes involved in the formation, transport, recycling, and accumulation of organic biomarkers in sedimentary archives and assessing whether these archives record a local environmental signal or are a mix of local and transported organic material.

Normal alkanes (*n*-alkanes) are an important component of the epicuticular wax in terrestrial plants. This waxy coating on plants protects against ultraviolet damage, water loss and predation (Jetter et al., 2006). Specific compounds in this wax, such as *n*-alkanes, are a useful tool for reconstructing past environmental changes through the analysis of the distribution of alkane homologues as well as their stable hydrogen (δD) and carbon ($\delta^{13}C$) isotope values. Previous

research in the Greater and Lesser Caucasus Mountains has documented the applicability of the average chain length (ACL) of leaf wax biomarkers as a tool for differentiating between grassy and deciduous vegetation (Bliedtner et al., 2018; Trigui et al., 2019a), although on a global scale ACL does not differentiate well between vegetation types (Bush and McInerney, 2013a).

The carbon isotope ($\delta^{13}C$) values of plant tissue is primarily determined by the photosynthetic pathway of the plant (Diefendorf and Freimuth, 2017). C_3 plants, which thrive in areas with cooler growing season temperatures, have more negative $\delta^{13}C$ values than do C_4 plants, which thrive in warmer growing season temperatures (Ehleringer et al., 1977). C_3 vegetation is further influenced by water use efficiency, as water stress influences the c_i/c_a ratio of plants (Farquhar et al., 1982). $\delta^{13}C$ values in lipids generally follow the same trends, and C_3 plants have more negative $\delta^{13}C$ lipid values than C_4 plants (Diefendorf and Freimuth, 2017). However, carbon fractionation of lipids is not consistent in different classes of plants (Diefendorf et al., 2011; Pedentchouk et al., 2008; Sikes et al., 2013). Currently, C_4 vegetation makes up around 3% of plant species in Armenia (Rudov et al., 2020), and was present in the Kalavan region during the Holocene (Tornero et al., 2016).

The hydrogen isotope (δD) values of *n*-alkanes in terrestrial plants record the δD values of environmental water (Sachse et al., 2012). This is typically reflective of δD values in precipitation, though precipitation δD values can also undergo positive shifts due to soil evaporation. The δD values of plant waxes are also influenced by fractionation during biological synthesis of lipids, which imparts a strong negative fractionation on δD values, as well as transpiration of leaf water (Gamarra et al., 2016).The fractionation between meteoric water and lipids is typically larger in gymnosperms than in angiosperms (Oakes and Hren, 2016; Pedentchouk et al., 2008).

Despite the benefits in measuring δD and $\delta^{13}C$ values in *n*-alkanes for understanding environmental and hydrological processes, not all the processes modifying isotope values from plant to *n*-alkane deposition are well understood. Sedimentary integration is one of the most poorly understood aspects of this process (Sachse et al., 2012). A number of studies on the integration of leaf waxes in catchments have been published in recent years which help clarify these processes (Alewell et al., 2016; Feakins et al., 2018; Häggi et al., 2016; Hemingway et al., 2016; Ponton et al., 2014; Suh et al., 2019). Previous studies on the integration of organic biomarkers have produced mix results, with some demonstrating spatial integration of catchment signals (Alewell et al., 2016; Feakins et al., 2018; Hemingway et al., 2016), whereas others did not observe this (Häggi et al., 2016; Ponton et al., 2014). However, these previous studies typically focused on very large river systems, which will undergo different transport processes than the first-order streams analyzed in this study. A number of these studies (Alewell et al., 2016; Feakins et al., 2018; Hemingway et al., 2016; Ponton et al., 2014) also observed seasonal differences in biomarker load in river sediments.

Thus, the sedimentary processes involved in the formation, transport, recycling, and accumulation of organic biomarkers in first and second order streams are not well understood. One challenge in assessing these processes in small streams is that the environment and plant communities are often homogenous, and thus it is not possible to differentiate between local and upstream transported organic material. To better understand transport processes affecting organic material in small catchments, we studied a set of streams in the Dany River, a tributary of the Barepat River, located in the Areguni Mountains in the Lesser Caucasus Range. This stream system is divided into two distinct ecological regions by the treeline (at ~ 2000 masl), which separates alpine meadow above the tree line (2000 – 2500 masl) from deciduous forest below

(1500 – 2000 masl). To evaluate the input of *n*-alkanes from upstream transported organic material relative to vegetation near the stream, we collected soil samples on the slopes of the mountains from both above- and below the treeline throughout the watershed and sediments deposited in the streambed along an elevation transect. Comparison of the hillside and streambed sedimentary *n*-alkanes allows assessment of the input of *n*-alkanes locally produced by vegetation compared to those transported in stream sediments within the catchment.

An additional motivation of this research is that treelines are a vulnerable feature of higher altitude environments. Previous research in the Areguni Mountains study area has assessed the relationship between treeline dynamics and climate forcing in the past (Ghukasyan et al., 2010; Malinsky-Buller et al., 2021; Montoya et al., 2013; Tornero et al., 2016). Pleistocene sediments uncovered at archaeological sites at Kalavan village within this area have the potential to reconstruct this relationship through the analysis of plant wax biomarkers deposited in fluvial sediments. However, in order to reconstruct these systems in the past it is important to understand modern biomarkers integration processes in the first and second order streams and their potential effects on the sedimentary archives of the Areguni Mountains.

2. Methods

2.1 Sample Collection and Extraction

Hillslope soil samples were collected in September 2018 along an altitude transect (1500 – 2500 masl) above the Dany River watershed, a first order tributary of the Barepat River in the Areguni Mountains, Armenia (Fig 1), which traverses the treeline at ~2000 masl. Forest vegetation is predominantly oak (*Quercus macranthera*), beech (*Fagus orientalis*) and hornbeam (*Carpinus orientalis*), while above treeline alpine meadow is comprised of *Hercleum sp.* and *Senecio sp.* (Joannin et al., 2022; Volodicheva, 2002). Soil samples were collected by first clearing the top ~10

cm of soil to remove roots. Stream bed sediment samples were collected from the Dany River throughout the altitude transect at intervals of ~100 m in altitude. In all cases, roughly 100 g of sediment were collected for extraction of *n*-alkanes. Samples were extracted using a Soxhlet apparatus with 2:1 dichloromethane:methanol for 48 hours. Following lipid extraction, n-alkanes were separated from total liquid extract by passing samples through a column of activated silica gel (1.25 g) in baked Pasteur pipettes with 2 mL hexane (non-polar fraction), 4 mL dichloromethane (slightly polar fraction) and 4 mL methanol (polar fraction). n-alkanes were quantified through the analysis of the hexane fraction. We quantified n-alkanes using a BP-5 column (30 m × 0.25 mm i.d., 0.25 μm film thickness) with He as the carrier (1.5 ml/min). Oven temperature was set at 50 °C for 1 min, ramped to 180 °C at 12 °C/min, then ramped to 320 °C at 6 °C/min and held for 4 min. (Brittingham et al., 2017; Smolen and Hren, 2023). We measured a standard mixture of *n*-alkanes (C_{20}-C_{33}) of known concentration to correct for mass dependent response decreases in longer chain *n*-alkanes. Odd over even predominance (OEP) (Eq. 1) and average chain length (ACL) (Eq. 2) were used to evaluate distributions of *n*-alkanes (Bush and McInerney, 2013b). We also calculated P_{aq}, an *n*-alkane proxy to evaluate the possible biomarker contribution of aquatic and emergent plants (Eq. 3) (Ficken et al., 2000).

$$\mathrm{OEP} = \frac{C_{25} + C_{27} + C_{29} + C_{31} + C_{33}}{C_{24} + C_{26} + C_{28} + C_{30} + C_{32}}$$

$$\mathrm{ACL} = \frac{25 * C_{25} + 27 * C_{27} + 29 * C_{29} + 31 * C_{31} + 33 * C_{33}}{C_{25} + C_{27} + C_{29} + C_{31} + C_{33}}$$

$$\mathrm{Paq} = \frac{C_{23} + C_{25}}{C_{23} + C_{25} + C_{29} + C_{31}}$$

2.3 Stable Isotope Analysis

δD and $\delta^{13}C$ values of individual *n*-alkanes were measured with a Thermo GC-Isolink coupled with a Thermo Scientific MAT 253 (manufacturer) isotope ratio mass spectrometer with a BP-5 column (30 m × 0.25 mm i.d., 0.25 μm film thickness). Oven temperature was set at 50°C for 1 min, ramped to 180°C at 12°C/min, then ramped to 320°C at 6°C/min and held for 4 min. Internal standards (Mix A5 from A. Schimmelman) were run every four samples across a range of concentrations (5-30 V/s) to correct for size effects. Standard errors were 0.4‰ for $\delta^{13}C$ and 3‰ for δD. Isotope ratios (R) were converted to δX ($\delta^{13}C$ and δD) values (Eq. 3) and are expressed in permill (‰).

$$\delta X = \left(\frac{R_{Sample}}{R_{Standard}} - 1\right) * 1000$$

3. Results

3.1. Alkane abundances

The most abundant alkane homolog in samples collected in the Areguni Mountains is the C_{29} or C_{31} alkane, which is typical for terrestrial plants (see S1-S4 for illustrative chromatograms). Odd numbered alkanes are significantly more abundant than even numbered alkanes, and the OEP of all samples is 11.2, with a range from 7.4-18.4. There is no significant difference between the mean OEP of soil (11.1) and stream (11.3) samples in the watershed. These values are similar to those previously measured in the Greater and Lesser Caucasus Mountains (Bliedtner et al., 2018; Trigui et al., 2019).

The mean average chain length (ACL) of all samples averages 29.7, with a range from 28.4 to 31.8 (Fig 3). In soils above the treeline, the mean ACL value is 30.6 (range of 29.8-31.8). In soils below the treeline, the mean ACL value is 29.5 (range of 28.4-30.4). There is a significant

difference between the average ACL values of the *n*-alkanes in above treeline and below treeline soils (Student's t-test, $p<0.001$, n=30). Stream sediment above the treeline have an average ACL value of 29.7 (range of 29.1-30.2) and stream sediments below the treeline have an average ACL value of 29.3 (range of 28.6 -30.0). The stream sediments from below the treeline have a significantly (Student's t-test, $p<0.001$, n=21) lower average ACL value than those above the treeline.

The P_{aq} values of *n*-alkanes in these samples suggests a mostly terrestrial origin of the organic matter. Higher P_{aq} values indicate contributions of floating and emergent macrophytes. However, we do not find a difference between the P_{aq} values in the stream sediments when compared to the soil samples, indicating that the organic load of the stream sediments is mostly of terrestrial origin. Terrestrial plants have average P_{aq}values of 0.09, with emergent plants averaging 0.25 (Ficken et al., 2000). Only eight of the 51 samples in this study had P_{aq}values above 0.20, four stream and four soil samples. This indicates that there was not a significant contribution of aquatic plants in the Dany stream sediments, and the biomarker load is primarily terrestrial in origin.

3.2. δD and $\delta^{13}C$ values

The $\delta^{13}C$ values in soils and stream sediments collected from the Areguni Mountains reflect a C_3 landscape, which is typical in Armenia. $\delta^{13}C$ values in all samples ranged from -36.0 to -32.3‰ (Fig 4). The range is similar for both soil samples (-35.9 to -32.3‰) and stream samples (-36.0 to –32.5‰). However, there is a significant difference in the $\delta^{13}C$ values of above and below treeline samples, both in the stream and soil samples collected. Above the treeline, $\delta^{13}C$ values in soils average -34.9‰, and below the treeline soil alkanes average -33.3‰ ($p<0.0001$, student's t-test, n=30). Stream sediment $\delta^{13}C$ values average -35.0‰ above the treeline and -33.6‰ below

the treeline ($p<0.0001$, student's t-test, n=21). $\delta^{13}C$ values in stream samples exhibit a step-like behavior, with ~2‰ shift to more negative values as the stream drops below the treeline.

The δD values measured in soil samples collected in the catchment ranged from -144 to -185‰ (Fig 5). These values were significantly more negative in above treeline sediments (-175‰) than in below treeline sediments (-156‰) ($p<0.001$, student's t-test, n=30). δD values were also more negative in stream sediment samples collected above the treeline (-175‰) than below the treeline (-158‰) ($p<0.001$, student's t-test, n=21). As with the $\delta^{13}C$ values, the δD values of stream sediment samples show sudden change as the stream drops below the treeline.

4. Discussion

4.1 Integration of local and upstream soil *n*-alkanes into the river sediments

The hillslope soil leaf wax ACL (Fig 3), $\delta^{13}C$ (Fig. 4) and δD (Fig. 5) show a step-like change at the treeline, indicating a significant separation between upstream (above treeline) and downstream (below treeline) soils. Using this separation, it is possible to assess the contributions and integration of upstream vs. downstream soils to the streambed sediments along the altitude transect. The step-like transition in streambed δD and $\delta^{13}C$ values indicates an over-printing of upstream alkane isotope values by input from deciduous vegetation. Thus, local production largely outweighs upstream transport in this setting. However, to firmly evaluate the upstream and downstream hillslope soil contribution to streambed sediments, there is a need to quantitatively evaluate the area-weighted production of *n*-alkanes above and below the treeline.

4.2. Modeling *n*-alkane production and estimating upstream transport and integration

To further evaluate the integration of *n*-alkanes above and below the treeline, we created a mixing model that calculates the expected δD, $\delta^{13}C$ and ACL values at each one of the sampling locations based on the *n*-alkane production of hillslope sediments above each streambed sampling

point (Fig. 6). Our mixing model assumes that the *n*-alkanes in the river are a function of the weighted *n*-alkane production above the sampling location.

The parameters we used for our mixing model are: 1. Satellite images (Google Earth) to map the areas covered by alpine meadow and forest vegetation throughout the Dany River catchment. 2. An estimate of net primary productivity of organic material production in grasses and trees (grams per area) (Brun et al., 2022). 3. Estimates of *n*-alkane production in grasses and trees in the Greater and Lesser Caucasus Mountains (grams of *n*-alkane per gram of organic material) (Bliedtner et al., 2018; Trigui et al., 2019). 4. End member values of δD, $\delta^{13}C$ and ACL derived from the average hillslope soils above and below the treeline. At each sample point within the catchment, we first calculated the upstream area covered by the two dominant vegetation types within the catchment (deciduous forest and alpine meadow) (Figure 6). This area was then multiplied by the previously mentioned constants (Table 1). By multiplying these terms (area x organic mass production x *n*-alkane production x end member soils value), we created an *n*-alkane production map for the Dany River catchment. Using this method, we calculated, the amount of grass and tree *n*-alkanes produced on the hillslopes above the sampling locations and the expected δD, $\delta^{13}C$ and ACL values for each stream sampling location (Figure 7a, 7c, 7e).

We compared the results of our mixing model with the measured δD, $\delta^{13}C$ and ACL in the streams. Stream sediment samples collected above the treeline (from ~2000-2600 masl) fall within the range of expected values, however, samples below the treeline consistently over-sample *n*-alkanes sourced from below treeline vegetation. Measured δD, $\delta^{13}C$ and ACL values do not have a linear relationship with the expected values based on vegetation area (Fig 7b, 7d, 7f). These measured values would produce under-estimates of the upstream area of alpine grasses, yielding incorrect reconstructions of paleo-vegetation in sedimentary records. Comparing the mixing model

with the observations indicates that an area-weighted mixing process is not an adequate model for explaining the *n*-alkanes signal in the streambed sediments. A simple and straightforward way to interpret this discrepancy is that an area-weighted quantitative integration of *n*-alkanes is not a good model for describing this catchment system, and that local production is much larger than transported organic material.

However, there are still other factors that may be driving this process that our mixing model does not account for. First, the average slope of forested areas in the Dany watershed is higher than those in grassy areas. These steeper slopes would cause more sediment transport into the stream bed. Second, although production of *n*-alkanes in grasses and trees is not significantly different in the Greater and Lesser Caucasus Mountains, concentrations are higher in soils in deciduous areas (Bliedtner et al., 2018; Trigui et al., 2019). This retention of more biomarkers in forest soils would also increase the contribution of deciduous alkanes into the stream bed. Third, stream downcutting into older sediments has the potential to re-mobilize stored organic carbon, which may contain a greater load of deciduous *n*-alkanes. However, analysis of pollen from a lake core nearby (~ 5km from the Dany catchment) in the Areguni Mountains shows a gradual shift over the last 4000 years from a grass-dominated landscape to the deciduous forest present today (Joannin et al., 2022b). Therefore, stored biomarkers are more likely to be grass-dominant, and this is unlikely to explain the measured bias to deciduous alkanes.

Since *n*-alkanes in the first order stream in this study do not quantitively integrate *n*-alkanes based on the upstream area of different vegetation types, this likely precludes the use of *n*-alkanes as a tool to reconstruct vertical treeline movement in this setting. However, this is a benefit for attempts to reconstruct hydrological changes through the analysis of δD values in *n*-alkanes. Given the ~20‰ difference in apparent fractionation (ε) values for above and below treeline sediments,

changes in upstream vegetation cover would alter measured δD values in *n*-alkanes in sedimentary archives. Without this quantitative integration, *n*-alkanes measured in the Pleistocene sediments found in this watershed are more likely to reflect changes in δD values of precipitation, and therefore would serve to reconstruct hydrological cycles, rather than changes in upstream vegetation cover. Since $\delta^{13}C$ and ACL of *n*-alkanes are also different in above and below treeline sediments, these other analyses would also be useful to identify periods with large changes in treeline that might complicate interpretation of δD values.

In order to illustrate this point, we present hypothetical records of biomarker δD values from three points in the Dany watershed (Fig. 7) documenting 20‰ and 30‰ shifts in precipitation δD values. We use δD values of precipitation from water samples collected at the nearest meteorological station with isotope data in Armenia (Dilijan, Brittingham et al., 2019b) Given the lack of quantitative integration in the catchment, a paleoclimate record from either above (A) or below (C) treeline would record the shift in precipitation δD values. Below treeline sedimentary records, with stream organic biomarker load overprinted by local vegetation production, would likely provide a means to reconstruct the δD precipitation values. However, records near the treeline (B) would be influenced by changes in apparent fractionation values associated with changes in vegetation around the stream. Co-occurring climate forcing of shifts in δD values of precipitation and changes in treeline altitude would cause paleoclimate records in this zone to over-estimate the magnitude of precipitation δD value shifts.

5. Conclusion

Sediment and stream samples from the Areguni Mountains, a subrange of the Lesser Caucasus Mountains in Armenia, demonstrate that there is a significant difference in hillslope soil δD, $\delta^{13}C$

and ACL values above and below treeline. *n*-alkanes in sediments in the Areguni Mountains can be used to differentiate between the above and below treeline sediments. However, *n*-alkanes extracted from stream sediments reflect their local area, rather than demonstrating transport from the higher-altitude alpine meadow. These results provide a complication for attempts to reconstruct changes in past treeline in this mountain range, given that the biomarker load in stream does not reflect the relative area of different upstream vegetation types. However, these results simplify interpretation of past *n*-alkane δD values, as apparent fractionation differences between grasses and trees are less likely to impart a significant influence on δD values in stream bed *n*-alkanes.

6. Competing interests

The contact author has declared that none of the authors has any competing interests

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	Conc[1]	NPP[2]	ACL[3]	$\delta^{13}C$[3]	δD[3]
Forest	7.69	1099.4	29.5	-33.3	-156
Alpine Meadow	3.03	719.1	30.6	-34.9	-175

Table 1: Constants used for mixing model. 1: *n*-alkane concentration in grasses and trees in the Caucasus Mountains (grams of *n*-alkane/gram of organic material) (Bliedtner et al., 2018; Trigui et al., 2019). 2: NPP for forest and grassland areas (grams per area) (Brun et al., 2022). 3: mean values for ACL, $\delta^{13}C$ and δD (this study)

Figure Captions

Figure 1: (Left) Topographic map (ASTER Global DEM) of Armenia with inset map of sampling location (black box) (Right) Inset map of soil (yellow circles) and stream (blue circles) samples collected in the Areguni Mountains, along with the limit of the Barepat (dashed line) and Dany watersheds (solid line)

Figure 2: The δD and $\delta^{13}C$ values of *n*-alkanes extracted from above treeline (green squares) and below treeline (red triangles) sediments

Figure 3: The average chain length (ACL) values of *n*-alkanes extracted from above treeline (yellow squares) and below treeline (green squares) sediment across the sampling elevation gradient

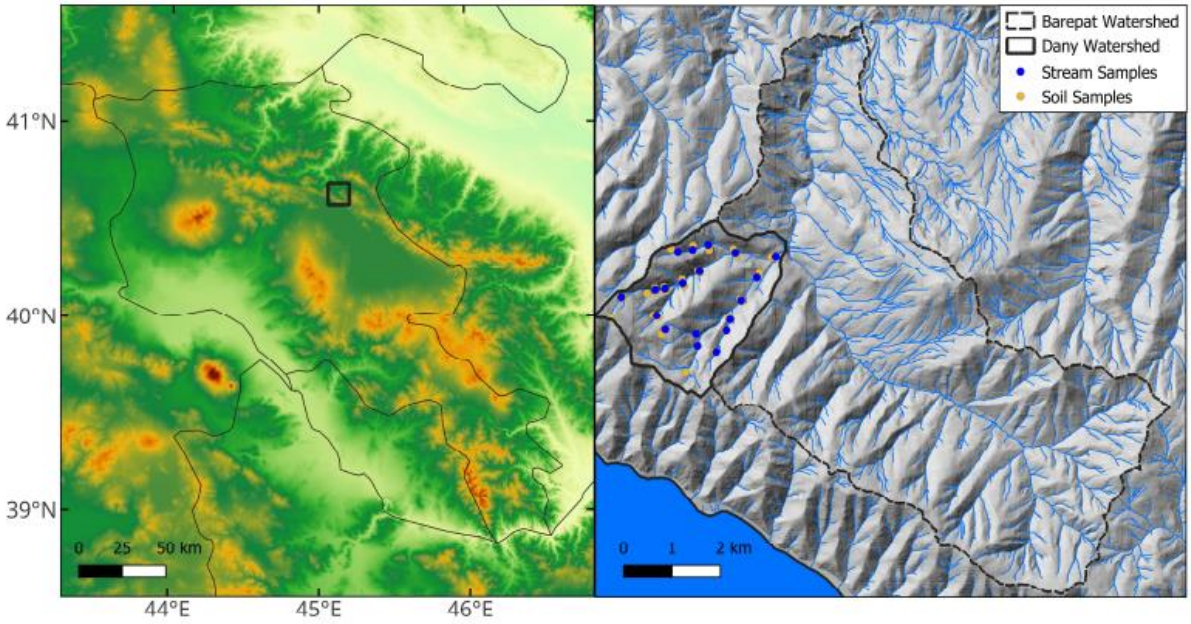
Figure 4: The $\delta^{13}C$ values of *n*-alkanes extracted from above treeline (yellow squares) and below treeline (green squares) sediment across the sampling elevation gradient

Figure 5: The δD values of *n*-alkanes extracted from above treeline (yellow squares) and below treeline (green squares) sediment across the sampling elevation gradient

Figure 6: Mixing model used to calculate expected values of stream sample points. Upstream watershed area covered by deciduous forest (green) and alpine meadow (yellow) was calculated at each sample location (blue dots). Point watershed for sample 24 (dashed line) is shown here as an example.

Figure 7: Comparison of the measured ACL, δD and $\delta^{13}C$ values against expected values of stream sediments. Dashed line represents the range of expected values from stream sediments if vegetation was integrated equally by area

Figure 8: A photograph of the Dany watershed with hypothetical paleoclimate record from three locations: (A, dashed line) Below treeline, (B, solid line) near treeline with fluctuations in treeline altitude, and (C, dotted line) above treeline



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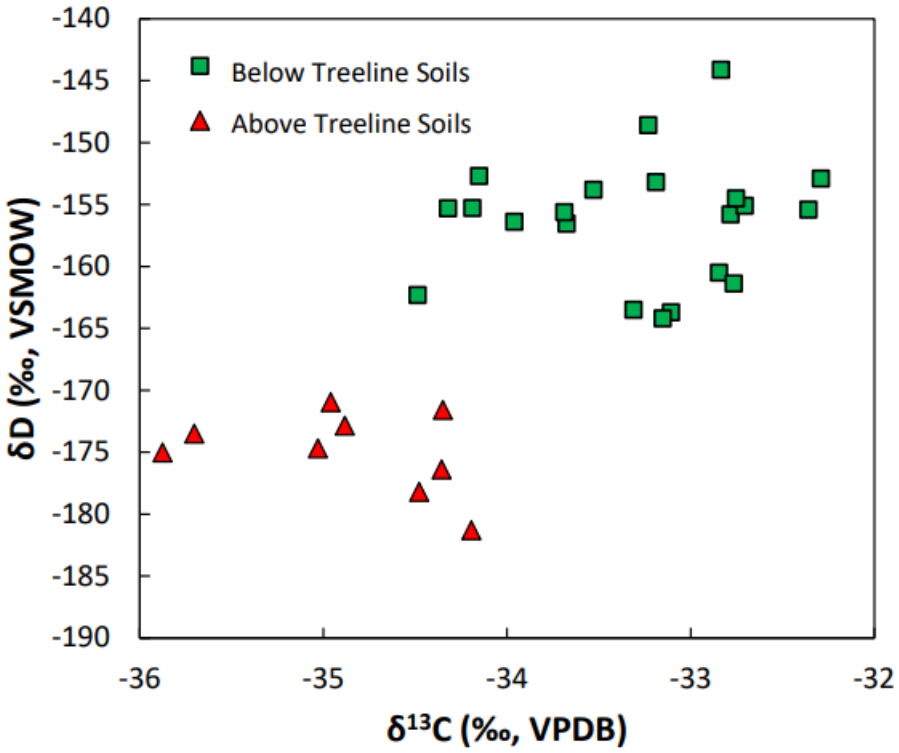

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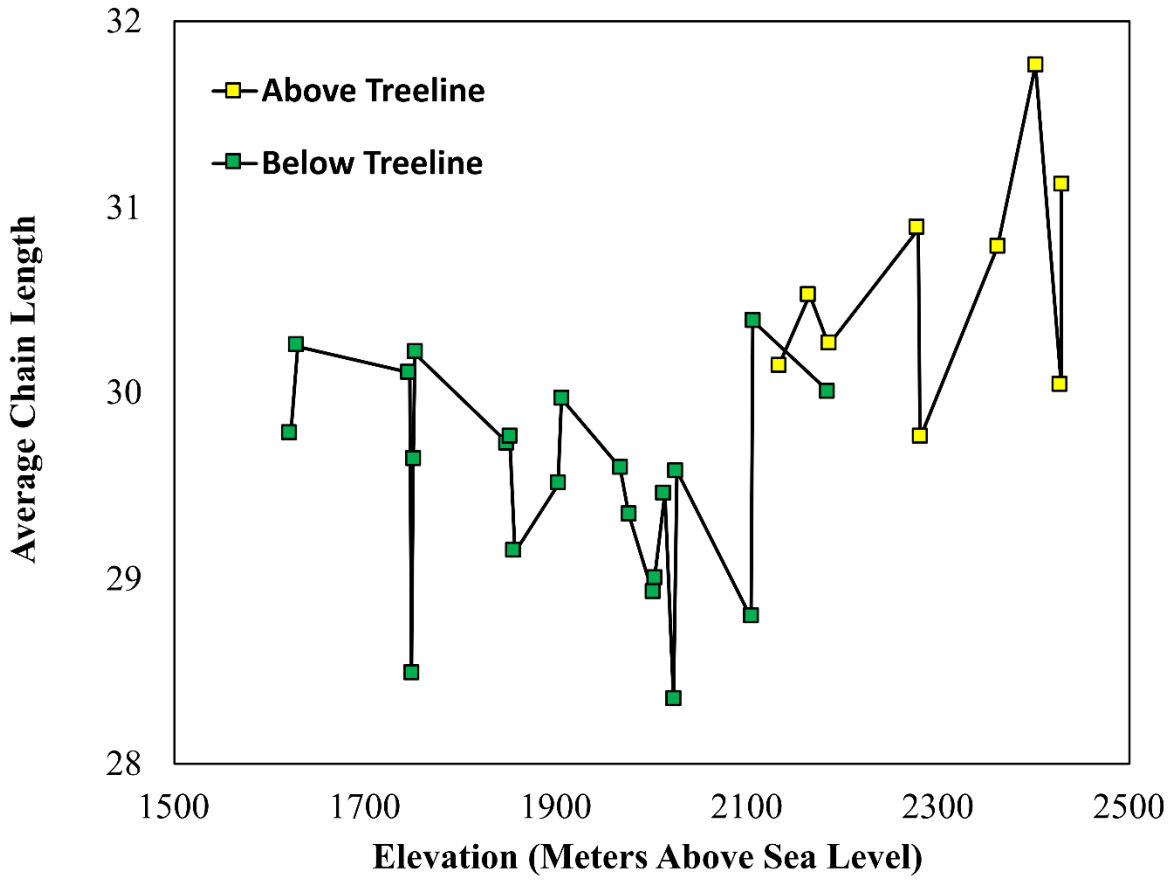

Figure 3: The average chain length (ACL) values of *n*-alkanes extracted from above treeline (yellow squares) and below treeline (green squares) sediment across the sampling elevation gradient

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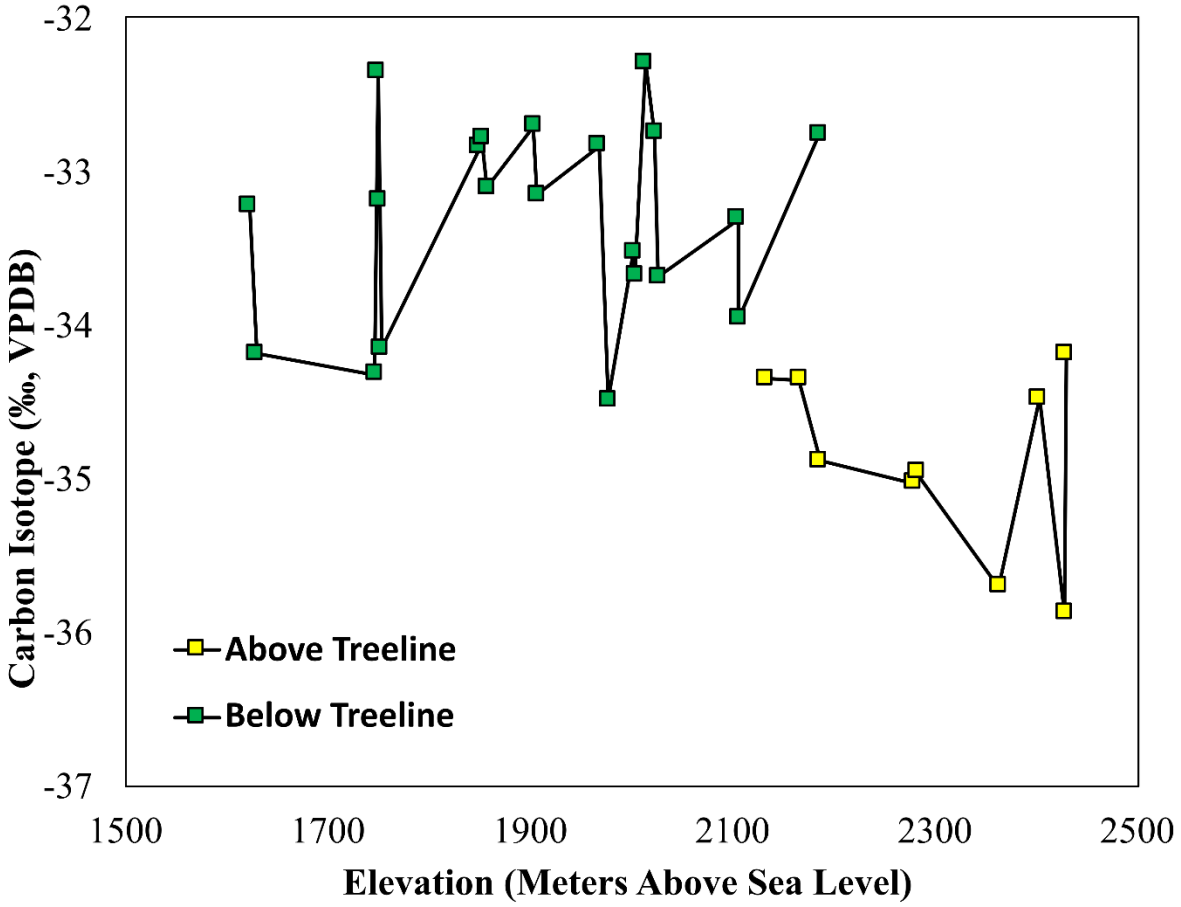

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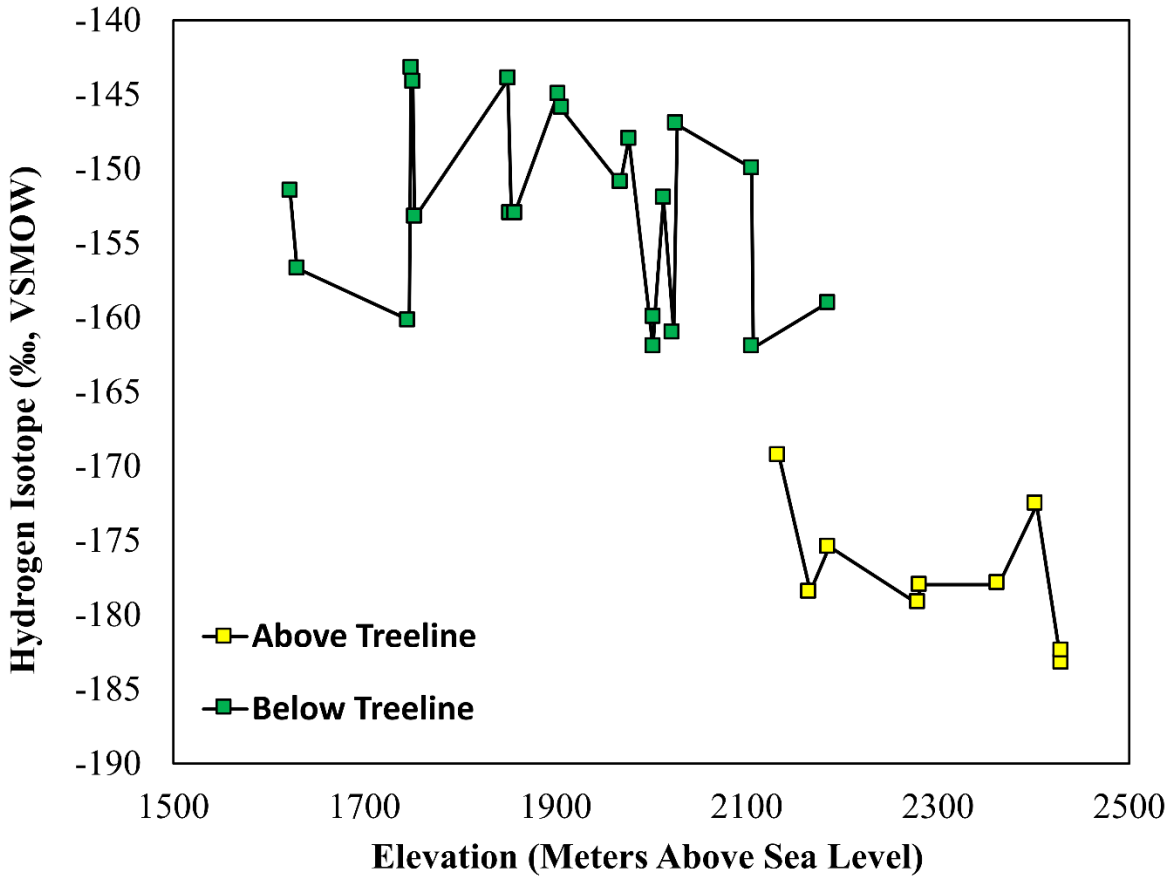

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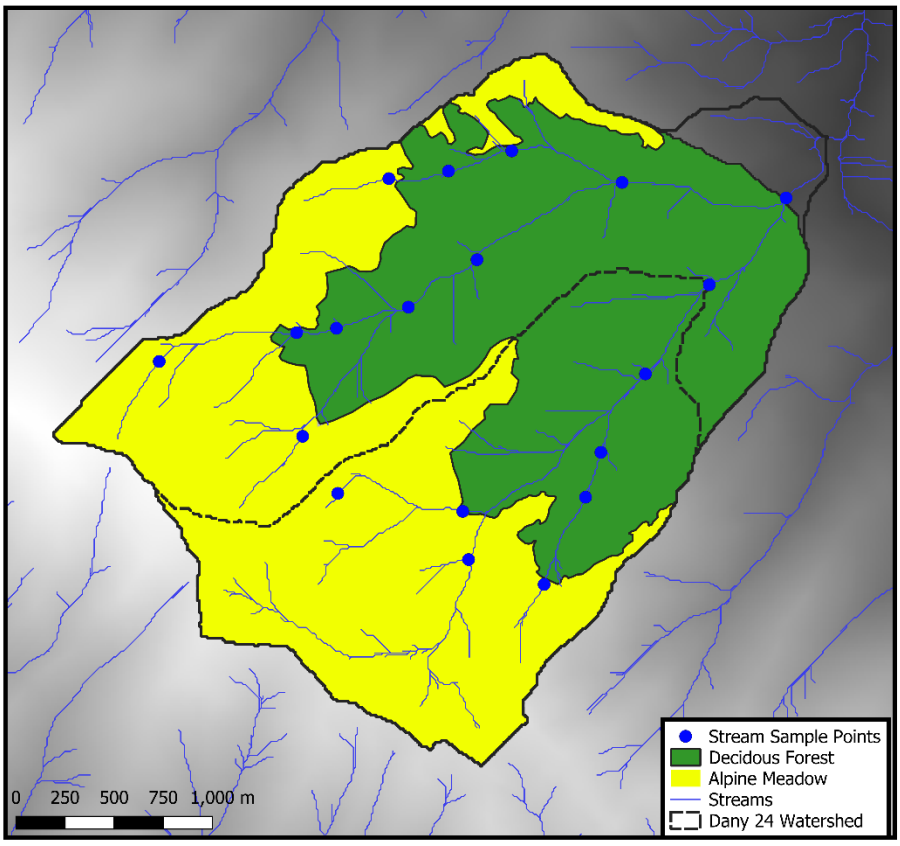

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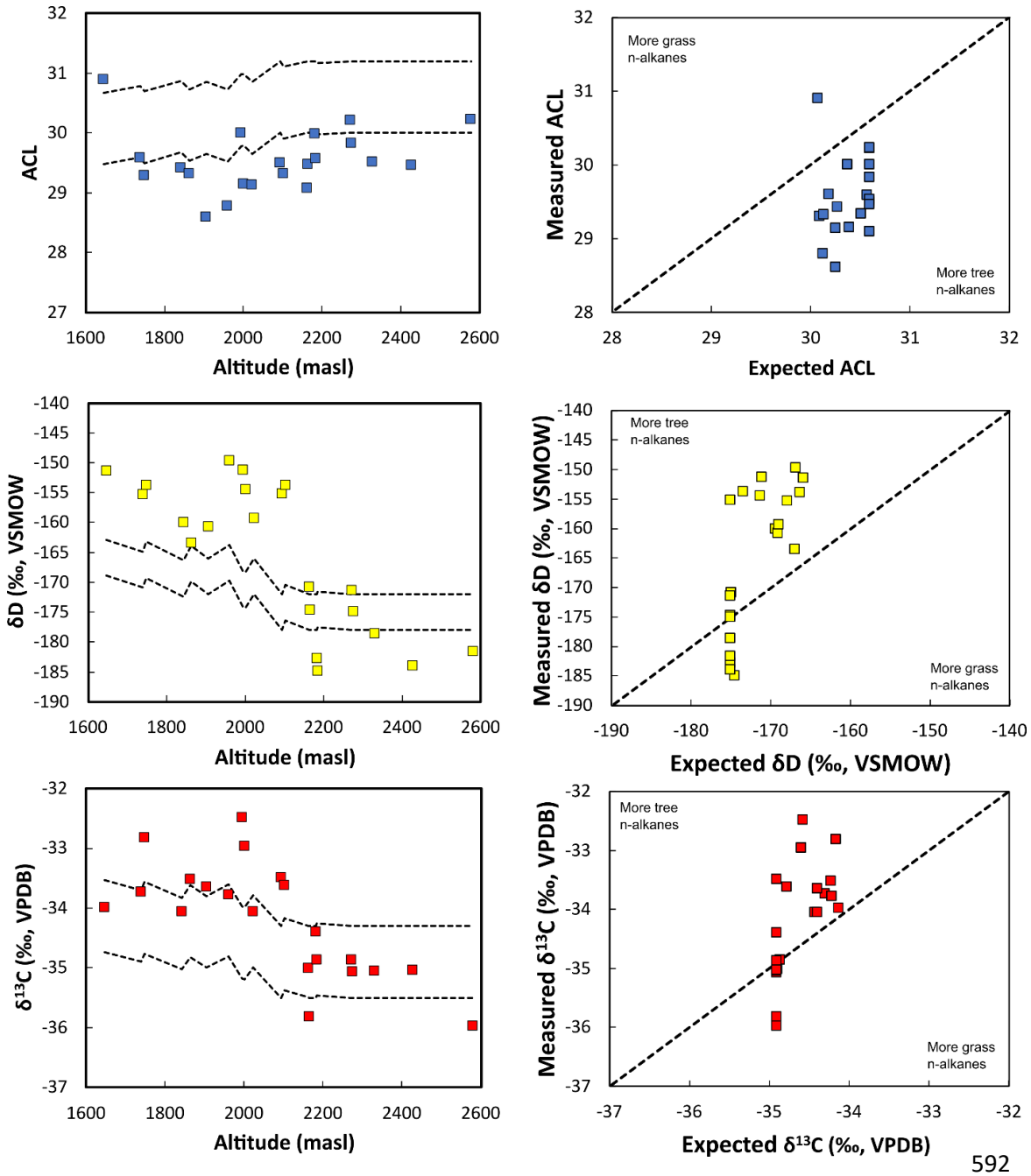

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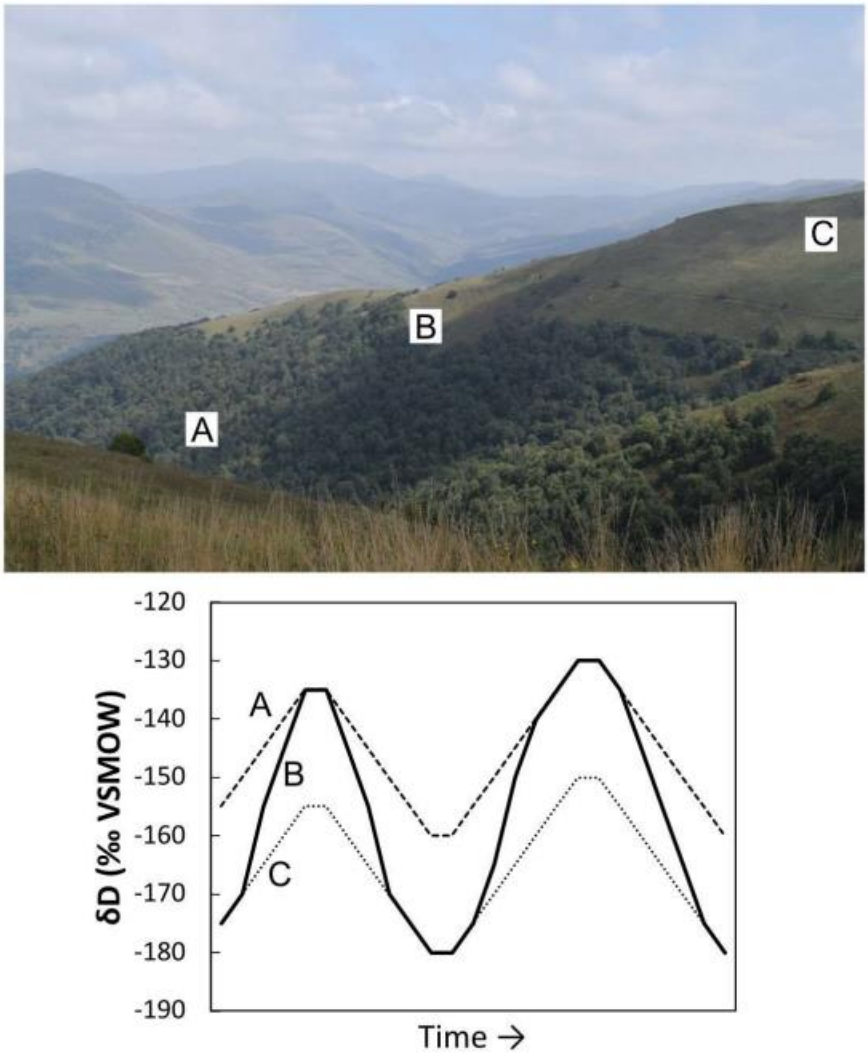

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