In this document, comments received by the author appear in blue. Line and page references made by the author refer to line and page numbers in the revised manuscript with tracked changes, which appears at the end of this Author's Response document.

Comments from Marcel van der Meer, Associate Editor

Dear Sara,

First of all I would like to thank both reviewers for reviewing your manuscript and you for your thorough reply. I think the reviewers made some good suggestions concerning your manuscript and I think you should definitely address these. I have the impressions that the spacing between paragraphs, although very clear, also let to some additional comments. I am not sure if this fits within the style of the journal. I have also tried to find "Line 4 methods", but have been unable to find it or any other place this comment could refer to. Perhaps we will find it in the next version. I had one very minor additional comment, I find it confusing to use enrichment (or depletion) for concentrations and amounts in an stable isotope paper (Page 2 line 9). I think if you address the reviewers comments as you have indicated your manuscript would be very suitable for publication in Biogeosciences.

Best regards,

Marcel

Author response to Marcel van der Meer, Associate Editor

Dear Marcel,

Thank you for your thoughtful comments. We have revised the manuscript according to your comments and those from two anonymous referees, as we detail in our responses to them below.

In regards to your comment on paragraph spacing, we looked but could not find guidance within the manuscript preparation instructions on paragraph spacing, so have not altered the spacing from what we previously submitted.

We did indeed find the missing "Line 4 methods" reference and changed this word to "factor" (page 5, line 16).

We deleted the reference to CO_2 enrichment you mention on Page 2 Line 9 when we deleted this entire paragraph (page 2, lines 10-24).

Thank you for this opportunity to contribute to Biogeosciences, and thank you again for your help improving our paper.

Best regards,

Sara

Comments from Anonymous Referee #1

1. *Upon initial reading of the paper I got a little confused trying to sort out exactly what "pheo-bulk meant. I think now I understand it depends on the material being discussed, for example it could be either: the difference between _15Npheo of leaves and bulk _15N of leaves or the difference between _15Npheo of soil and bulk _15N of soil organic matter. But this is different from comparing the _15Npheo in soil/litter with _15Npheo and _15Nbulk of leaves. I'm not sure if anything really needs to be changed, but maybe a sentence of clarification somewhere might help?*

We appreciated having this confusion pointed out and have provided clarification in the isotopic notation subsection of the methods section (Page 9 line 5). We only use "pheo-bulk" in the paper as a subscript for $^{15}\varepsilon_{pheo\text{-}bulk,}$ which we use to describe biosynthetic isotopic fractionation within whole leaf (bulk) tissue. To reduce confusion, we have changed this term to $^{15}\varepsilon_{pheo\text{-}leaf}$ and $^{15}\varepsilon_{chl\text{-}bulk}$ to $^{15}\varepsilon_{chl\text{-}leaf.}$ We use the symbol ε to provide a direct comparison with previously reported values of isotopic fractionation between chlorophyll and whole leaves (Chikaraishi et al., 2005). We do discuss isotope differences between isolated compounds and bulk samples for other materials, but not using the epsilon notation. In those cases, we describe a difference in isotope values by simple subtraction, which is abbreviated with delta notation in Table 4.

2. *The methods for HPLC should be more specific. For example what does "variable ratio" mean? (p. 6 line 24). What is the advantage of using two HPLC steps? Was the sample divided in half, then each part passed through each HPLC method/column? Or was it successive, i.e. sample goes through column A and into B?*

We expanded the methods section for HPLC to provide clarity on these points (Page 7, Lines 6-20). Variable ratio means that over the course of the run, the ratio of solvents flowing through the HPLC column changes. We have detailed how the percentages of solvents were varied throughout the run. We clarified that sample fractions corresponding to peaks collected from the first HPLC column run were subsequently run through a second column.

3. *I strongly suggest the authors consider depositing their data into a data repository where it can be easily accessed by anyone, in keeping with global scientific trends of making more data open access.*

We have submitted our data to the open access database PANGAEA (https://www.pangaea.de/, Data submission 2019-08-01T06:12:00Z), and provide this information on Page 15, Line 21.

4. *Do the authors have any theories for why trees exhibit more positive "pheo-bulk of leaves compared to herbaceous plants? Cellulose accumulation is mentioned in the paper but I'm unclear how it's connected to nitrogen isotope values?*

We agree that our findings invite further discussion of why trees in our study exhibit greater intra-leaf isotopic fractionation between pheophytin and whole leaf tissue ($^{15}\varepsilon_{pheo\text{-}bulk}$) than plants. Without going into speculation at great length within the paper, we have provided a suggestion on Page 11, Line 19 that the large fractionation in trees could be a systematic effect of their "greater size, longevity, and resulting N storage and redistribution requirements relative to herbaceous plants." We removed mention of cellulose accumulation; while a distinguishing character of trees, cellulose contains no nitrogen and would not directly affect $\delta^{15}N$ values.

We suggest this explanation because differences in rates of growth or pathways of N compound synthesis, breakdown, and redistribution are processes that would have potential for N isotope fractionation. Due to their size and longevity, trees have different N storage and redistribution requirements than herbaceous plants. Although bulk leaf $\delta^{15}N$ is not observed to change on abscission (Kolb and Evans, 2002), seasonal or within-season breakdown and redistribution of foliar N compounds could involve N isotope fractionation that results in partitioning of N isotopes within leaf compounds During the growing season, chlorophyll is continually broken down and replaced. As we mention in the paper, because the energy needed to break the Mg-N bond is substantially smaller than the covalent bond, Mg loss from Chl would theoretically be expected to have little or no N isotope fractionation. However, in perennial plants with long lives such as the trees in our study, it is possible that this process happens many times, compounding expression of isotopic fractionation from this process. It is worth noting that others have also observed intra-leaf patterns in N isotope fractionation among different plant types that are not well understood. Chikaraishi et al. (2005) noted that $^{15}\varepsilon_{chl\text{-}bulk}$ of chlorophylls from C4 plants show much greater discrimination against 15N than do C3 plants, despite biosynthesis via the same pathway. We will remove mention of cellulose accumulation; while a distinguishing character of trees, cellulose contains no nitrogen and would not directly affect $\delta^{15}N$ values.

5. *I read another paper recently (Wang et al 2019, GRL) that used bulk N isotope values of black carbon deposited in lake sediments as a proxy for regional N availability over the last 10,000 years. I wonder if the _15Npheo proxy in soil could be coupled or compared with that method, both to further validate both proxies but also to study changes in N availability in more detail.*

We appreciate the encouragement to expand our discussion of how the $\delta^{15}N_{pheo}$ proxy could be applied to investigate N dynamics. We have added a section to our discussion, "4.3 Implications of the $\delta^{15}N_{pheo}$ proxy" (page 14, line 28). In it, we suggest two key opportunities for application of a soil-based $\delta^{15}N_{pheo}$ proxy data to advance understanding of past terrestrial N dynamics. First, the ability to track changes in foliar $\delta^{15}N$ over time implies comparatively direct insight into factors affecting $\delta^{15}N$ of plants, notably the availability of nitrogen. Central questions concerning the timescale of N cycle responses to elevated CO_2 concentration in the atmosphere, and whether availability of this limiting nutrient increases or decreases with climate change, could be explored by selecting a time series that covers changes in atmospheric pCO_2 (Goulden, 2016).

Second, comparison of compound-specific $\delta^{15}N_{pheo}$ with other $\delta^{15}N$ proxies over the same time domain could provide insights into processes that cause them to deviate from one onother. Alternative sources of $\delta^{15}N$ proxies include subaqueous sediment deposits in lakes, ungulate tooth enamel, and bulk wood,

black carbon, or soil. Deviations in records of $\delta^{15}N$ of pheo and tooth enamel at a common site would highlight changes in factors affecting dietary isotope fractionations, such as animal growth rates. $\delta^{15}N_{pheo}$ records could provide information on terrestrial sources of $\delta^{15}N$ relevant to aquatic sediment $\delta^{15}N$ records, and allow aquatic and terrestrial signals to be distinguished. $\delta^{15}N_{pheo}$ could validate bulk proxy records, or highlight diagenetic limitations of the record. Within soil horizons, comparing $\delta^{15}N_{pheo}$ with $\delta^{15}N_{bulk}$ could provide information both on N availability to plants and dominant pathways of N loss, hydrologic or gaseous, at a site, allowing for comparison of multiple N cycle dynamics over time.

Technical corrections

1. *Line 4 methods should be "factor"*

Thank you; we have changed this (Page 5, Line 16).

2. *Figure 3: is there a blue triangle where there shouldn't be in the 2500 mm precipitation category?*

We have revised the figure legend to indicate that the blue trial corresponds to a sample from soil below 20 cm (Page 26).

3. *Figure 5: Color code here instead of label? Some of the labels overlap and can be hard to read.*

We have changed the dimensions of the figure to reduce label overlapping and improve appearance, and also manually separated the remaining overlapping labels (Page 29).

4. *Table 2: what is "Py Chl a"? I could not find a definition.*

Py Chl a is Pyrochlorophyll a. We now spell out the full word in the text (page 7 line 15 and Table 2).

References

Chikaraishi, Y., Matsumoto, K., Ogawa, N. O., Suga, H., Kitazato, H., and Ohkouchi, N.: Hydrogen, carbon and nitrogen isotopic fractionations during chlorophyll biosynthesis in C3 higher plants, Phytochemistry, 66, 911-920, 10.1016/j.phytochem.2005.03.004, 2005.

Kolb, K. J., and Evans, R. D.: Implications of leaf nitrogen recycling on the nitrogen isotope composition of deciduous plant tissues, New Phytologist, 156, 57-64, 2002.

Comments from Anonymous Referee #2

1. *- Structurally: there are some paragraphs with only one sentence - I am not sure this is within the journal template, I recommend the authors to structure the manuscript with more concise paragraphs and better connections between paragraphs. it will be an easier read for everyone. Related to that, there are many sections in the methods and results and none in the discussion.*

For instance, section 2.2 and 2.3 could be combined. Accordingly, subsections in the discussion also would be better and easier to follow the flow of the discussion as in results.

We appreciate these suggestions for improving readability and have implemented all that were specified. We have added subsections to the discussion, focusing fractionation within leaves, potential as a soil-based proxy, and possible applications of the proxy (Page 11, Lines 8 and 23, and Page 14, Line 28). We have combined sections 2.2 and 2.3 into a single section called "Sample collection and preparation" (Page 6, line 6).

Following our revisions, sections on isotopic notation and results are the locations in our paper where we have paragraphs that either have only one sentence or are very short, and our instinct is that these are appropriate levels of conciseness. We hope that the changes we've described above, in addition to the other clarifications and rewriting we have described, some of which lengthened or combined short paragraphs (e.g. page 6 line 2) or reduced large ones, will succeed in achieving an accessible and digestible manuscript.

2. *- it will be probably corrected during the post-review process but still, do not forget to format the citation within the text ex: page 2, line 14 (e.g. (Drake...))*

We agree and have edited several in-text citations, though the specific one referenced was deleted along with the rest of the paragraph.

3. *- I highly recommend authors to provide the data to databases where it is easily accessible upon publication. We should be supportive to open science and open data policies.*

We agree to do this and have uploaded our database to PANGAEA https://www.pangaea.de/.

4. *I am missing an introduction to compounds used. A nice introduction to pheophytin is only done in the discussion until I reached that point I did not really get why we are looking at pheo rather than chlorins (as the title say) and chl as it was introduced in the introduction. Overall the intro part gave a nice discussion on N dynamics in terrestrial environments, including the PNL where I was hoping to see this also in the discussion. how compound specific isotopic approaches would advance our understanding of N dynamics? what input 15Npheo will provide in terms of all the ongoing discussion? these could be implemented to discussion part in accordance with the introduction. Otherwise, the introduction could be (maybe should be) more technical and focus on more in compounds and isotopic fractionation for instance.*

We appreciate this critique of missing pieces from our introduction and discussion. In our introduction, we agree we should provide a better introduction to the compounds used and should avoid going into detail on PNL that is not relevant for this paper. In the discussion, we agree we should expand our discussion of how compound-specific isotopic approaches would advance understanding of N dynamics.

In the introduction section, we have explained that we examined chlorin fractions for the presence of individual compounds in sufficient abundance for isotopic analysis, and that pheophytin a (pheo *a*) is a

chlorin previously found in greater relative abundance than any other in organic soils and litter (Sanger, 1971a; Gorham and Sanger, 1967), and is therefore of particular interest (page 4, line 24

In the discussion section, we will discuss how the $\delta^{15}N_{pheo}$ proxy could be applied. We have added a section to our discussion, "4.3 Implications of the $\delta^{15}N_{pheo}$ proxy" (page 14, line 28). In it, we suggest two key opportunities for application of a soil-based $\delta^{15}N_{pheo}$ proxy data to advance understanding of past terrestrial N dynamics. First, the ability to track changes in foliar $\delta^{15}N$ over time implies comparatively direct insight into factors affecting $\delta^{15}N$ of plants, notably the availability of nitrogen. Central questions concerning the timescale of N cycle responses to elevated CO_2 concentration in the atmosphere, and whether availability of this limiting nutrient increases or decreases with climate change, could be explored by selecting a time series that covers changes in atmospheric pCO_2 (Goulden, 2016).

Second, comparison of compound-specific $\delta^{15}N_{pheo}$ with other $\delta^{15}N$ proxies over the same time domain could provide insights into processes that cause them to deviate from one onother. Alternative sources of $\delta^{15}N$ proxies include subaqueous sediment deposits in lakes, ungulate tooth enamel, and bulk wood, black carbon, or soil. Deviations in records of $\delta^{15}N$ of pheo and tooth enamel at a common site would highlight changes in factors affecting dietary isotope fractionations, such as animal growth rates. $\delta^{15}N_{pheo}$ records could provide information on terrestrial sources of $\delta^{15}N$ relevant to aquatic sediment $\delta^{15}N$ records, and allow aquatic and terrestrial signals to be distinguished. $\delta^{15}N_{pheo}$ could validate bulk proxy records, or highlight diagenetic limitations of the record. Within soil horizons, comparing $\delta^{15}N_{pheo}$ with $\delta^{15}N_{bulk}$ could provide information both on N availability to plants and dominant pathways of N loss, hydrologic or gaseous, at a site, allowing for comparison of multiple N cycle dynamics over time.

page 3 line 13:... terrestrial d15Nleaf : leaf subscript
Agreed, thank you, we have made this change.

page 4 line 8: is climate a landscape effect? maybe precipitation is a better word?

As we are studying impact of position on a "climosequence" along which both precipitation and temperature vary, referring to climate effects seems more appropriate than precipitation effects. We have replaced "landscape effects" with "environmental effects" to avoid confusion over whether the effects we are measuring are relevant to global climate change or only to the landscape scale (product of shading, aspect, precipitation patterns, etc.) (page 4, line 22).

page 4 paragraph starting from line 15 needs reconstruction, it is not an easy or maybe not well written paragraph.

We have rewritten this paragraph to improve parallel structure and shorten the final sentence (page 4, lines 24 through 32).

page 5 line 21: first sentence is a sampling strategy should be in the below section (2.2).

We agree. We have removed this sentence and moved the detail that the pits were dug in open, grassy areas with minimal slope (page 6 line 2) to Section 2.2 on sample collection and preparation. We have moved the second sentence to the preceding paragraph where grazing is discussed (page 5 line 27).

Page 5 lines 29-30: (and generally many more) can authors be more specific?
We have deleted the parenthetical phrase "(and generally many more)" from this sentence.

page 6 line 1: what depth is the deepest soil sample from?
We have added a phrase to say that the deepest pit was dug to a depth of 65 cm (page 6, line 16).

page 7 lines 13 and 15: JAMSTEC acronym should change places. line 15 should be in line 13
We agree and have made this correction.

page 9 line 31 d15Npheo - o is missing
We agree and have made this correction.

page 10 line 6: ...along the soil profile do (?) not deviate ....
We agree and have made this correction.

page 12 lines 1-2: citation needed at this sentence where pheo is introduced.
We have provided citations for both the biosynthetic and degradation pathways of pheophytin synthesis.

page 12 paragraph starting with line 22: Can authors provide more info on the ages presented here? where are mentioned other sites here? close by? this paragraph and information given here can be improved.
We have clarified that radiocarbon dates of 4130 and 8030 yBP were taken on soil organic carbon (SOC) deep within the soil profiles at climosequence sites A and D, respectively (Chadwick et al., 2007), as located in Figure 1. All soils along the climosequence have the Hawi volcanic flow as their parent material, which cooled around 150 ky ago, and so can be considered to be the same age, though differing climate and vegetation across the range of sites would be expected to result in different rates of organic matter production, decomposition, and preservation in soil. We have additionally clarified the key point here, which is that is that these radiocarbon dates indicate that our studied soils may reflect organic contributions from several thousands of years of soil development (page 13 line 30).

References: please double check the format some references are all in caps lock
We agree and have made this correction.

Figures: 1: would it be possible to indicate the vegetation somehow on these maps?
Yes: we have colored our map to indicate the four vegetation zones bounded by precipitation isoclines.

2 & 5: y axis title is cut, missing some part of 15N
We agree and have made this correction.

Table 1: please add any other info on the sites below the letter like elevation or precipitation.
We agree and have made this correction. We added precipitation values below the site letters so that the key site differences are evident at a glance.

Table 3: I think the names should be written italics
We agree and have made this correction.

References

Chadwick, O. A., Kelly, E. F., Hotchkiss, S. C., and Vitousek, P. M.: Precontact vegetation and soil nutrient status in the shadow of Kohala Volcano, Hawaii, Geomorphology, 89, 70-83, 10.1016/j.geomorph.2006.07.023, 2007.

Sanger, J. E.: Identification and Quantitative Measurement of Plant Pigments in Soil Humus Layers, Ecology, 52, 959-963, 1971.

Strong correspondence between nitrogen isotope composition of foliage and chlorin across a rainfall gradient: Implications for paleo-reconstruction of the nitrogen cycle

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Abstract. Nitrogen (N) availability influences patterns of terrestrial productivity and global carbon cycling, imparting strong but poorly resolved feedbacks on Earth's climate system. Central questions concern the timescale of N cycle response to elevated CO_2 concentration in the atmosphere, and whether availability of this limiting nutrient increases or decreases with climate change. Nitrogen isotopic composition of bulk plant leaves provides information on large-scale patterns of N availability in the modern environment. Here we examine the utility of chlorins, degradation products of chlorophylls, hypothesized to persist in soil subsequent to plant decay, as proxies for reconstructing past plant $\delta^{15}N$. Specifically, we test the hypothesis that $\delta^{15}N$ of plant leaves ($\delta^{15}N_{leaf}$) is recorded in $\delta^{15}N$ of pheophytin *a* ($\delta^{15}N_{pheo}$) along the leaf-litter-soil continuum across an array of ecosystem climate conditions and plant functional types (C3, C4, legumes, and woody plants). The $\delta^{15}N$ of live foliage and bulk soil display marked declines with increasing rainfall, consistent with past studies in Hawaii and patterns worldwide. We find measurable chlorin concentrations along soil-depth profiles at all sites, with pheophytin *a* present in amounts required for isotopic analysis (>10 nmol). $\delta^{15}N_{pheo}$ in leaves, litter, and soil track $\delta^{15}N_{leaf}$ of plant leaves. We find potential for $\delta^{15}N_{pheo}$ records from soil to provide proxy information on $\delta^{15}N_{leaf}$.

1. Introduction

A combination of high biological requirements, limited natural supply, and high chemical reactivity and mobility gives nitrogen (N) important controls over photosynthesis and respiration in the terrestrial biosphere ~~(Vitousek and Howarth, 1991).~~(Vitousek and Howarth, 1991). The response of the terrestrial carbon (C) cycle to climate change and increased atmospheric carbon dioxide (pCO_2) will depend largely on the availability of N (Hungate et al., 2003; Wang and Houlton,

2009; Ainsworth and Long, 2005; Denman, 2007; Thornton et al., 2009), raising the question of how N availability may also be expected to change.

Humans have modified the N cycle directly through production of reactive N ~~(Galloway et al., 1995)~~(Galloway et al., 1995) and likely indirectly through several climate-induced changes receiving ongoing investigation, including changes in biological demand (McLauchlan et al., 2013), changes in organic mineralization (Durán et al., 2016) and mineral weathering rates (Houlton et al., 2018), and changes in N-fixation rates. Experimental manipulations have yielded tremendous insights into mechanisms underlying complex carbon-climate-N cycle dynamics, yet limitations of geography, complexity, and time have left open questions about the overall N biogeochemical response to climate change at the landscape and global scales.

~~Of particular concern is the hypothesis of progressive nitrogen limitation (PNL) under CO_2 enrichment. This set of ideas predicts that under rising atmospheric pCO_2, feedbacks between plants, soil, and microbes result in diminishing quantities of available N, thereby constraining plant productivity over time. This hypothesis is supported by results from some annual-to-decadal-scale experiments, including free air CO_2 enrichment (FACE) studies ((Luo et al., 2004;Ainsworth and Long, 2005) and citations therein). Others have pointed to limitations in this model whereby plant-soil-microbe systems adjust to changes in C by N interactions in a way that allows for sustained plant CO_2 uptake (e.g. (Drake et al., 2011)). On decade to millennial timescales, with time and space for species shifts and turnover of soil resources, and with environmental conditions such as temperature and moisture concurrently changing, major N gain and loss pathways can be expected to change under a warming world in ways that could prevent PNL. For example, N_2 fixation may increase, either due to lower energetic barriers or increases in populations of N_2 fixers in plant/microbial communities. In terms of N losses, denitrification rates may increase, either directly or through respiration-driven changes in soil oxygen availability. Changes in moisture that accompany the changes in temperature may augment or counteract the effects of temperature on these pathways or on decomposition (Parton et al., 1996;Greaver et al., 2016).~~

Insight into actual outcomes on real landscapes over decadal-to-millennial timescales would be helpful to predicting how feedbacks between C and N cycles will play role in climate change this century (Luo et al., 2004). If we could observe how N availability to plants responded to past periods of change in climate and pCO_2, such as during transitions from glacial to interglacial periods, we could improve our projections for future integrative responses of the N cycles to a complex, changing world~~.~~ (Hungate et al., 2003; Houlton et al., 2015). Knowledge of past N cycle behaviour would provide both insight into long-term dynamics and important baselines from which we can better assess anthropogenic impacts.

Natural abundance stable isotopes of N ($\delta^{15}N$) are useful integrators of N cycle processes in modern environmental systems (Robinson, 2001), and are natural sources of evidence for N cycle behaviour in the past. Due to high reactivity and mobility of N, point-based concentration measurements give temporally limited views of dynamic plant-soil-microbial cycling of N.

$\delta^{15}N$ of plants and soils reflect a time-integrated signal of the N cycle. The gaseous loss processes (denitrification, ammonia volatilization, and anammox) which fractionate the light and heavy isotopes in terrestrial ecosystems are substrate-dependent, making $\delta^{15}N$ sensitive to changes in the availability of nitrogen (Houlton and Bai, 2009). Conversely, at low N availability, there is likely to be less isotopic expression of these pathways, as well as potential for greater dependence of plants on ectomycorrhizae, thereby reducing isotopic difference between plant foliage and N sources (Hobbie and Ouimette, 2009). As a result, higher $\delta^{15}N_{leaf}$ corresponds to higher N availability to plants on average. (e.g., ~~(~~Handley et al~~.,~~. (1999~~;~~); (Amundson et al., 2003; Houlton et al., 2007; Craine et al., 2009; Martinelli et al., 1999)). Reconstructions of $\delta^{15}N_{leaf}$ would accordingly provide information on availability of N to plants in the past.

The same reactivity of N that makes $\delta^{15}N_{leaf}$ a valuable proxy for N availability in modern landscapes makes obtaining a primary N isotope signal from dead, buried, and decomposed organic matter particularly challenging. Primary $\delta^{15}N$ signatures are altered by decomposition and diagenesis (Thackeray, 1998; Meyers and Ishiwatari, 1993; Hedges and Oades, 1997), and bulk interpretations are confounded by preferential preservation and accumulation of macromolecules with distinct $\delta^{15}N$ values ~~(Hobbie and Ouimette, 2009;Wedin et al., 1995)~~(Hobbie and Ouimette, 2009; Wedin et al., 1995). Because low-oxygen environments preserve organic matter, sediment accumulations in small lakes have been used as sources of terrestrial $\delta^{15}N_{leaf}$ (McLauchlan et al., 2013). ~~However, lake ecosystem processes can obscure the original $\delta^{15}N$ of terrestrial sources (Meyers and Ishiwatari, 1993).~~However, lake ecosystem processes can obscure the original $\delta^{15}N$ of terrestrial sources (Meyers and Ishiwatari, 1993). Land-based paleo-proxy $\delta^{15}N_{leaf}$ inferences have targeted bulk N protected structurally, in fossil faunal material (Stevens and Hedges, 2004), in tree wood (Hietz et al.), and~~, in one case,~~ pollen (Descolas-Gros and Scholzel, 2007). These approaches require controversial case-by-case defence of the primary nature of $\delta^{15}N$ due to diagenetic vulnerability (Harbeck et al., 2004; Thackeray, 1998) and redistribution of N (Gerhart and McLauchlan, 2014). In the case of faunal material, it is also necessary to consider species-specific trophic enrichment factors, age-effects, and dietary protein content (Sponheimer et al., 2003; Overman and Parrish, 2001). Many of these approaches are further limited for understanding landscape-level patterns by the poor spatial distribution of samples.

Compound-specific isotope analysis (CSIA) of sedimentary material offers advantages over these methods: Isotope ratios of individual compounds can be more resistant to diagenesis than those of bulk materials, and have the further advantages of deriving from more constrainable sources. This last characteristic permits analysis of material from integrative depositional environments. In marine N biogeochemistry, subaqueous sediments that collect and bury organic compounds in time series deposits are widely used for paleoenvironmental reconstruction (Junium et al., 2011; Meyers, 1997). Soils likewise accumulate organic material over time, though not perfectly analogously to subaqueous systems.

The ideal characteristic compound for CSIA reconstruction of $\delta^{15}N_{leaf}$ in soil is N-rich, produced only above-ground, and retains the primary $\delta^{15}N$ of plant leaves. We suggest that degradation products of the chlorophyll molecule (chlorins) meet all

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the above criteria. Chlorins derive from chlorophyll through processes of senescence, decomposition, diagenesis, and grazing (Keely, 2006; Treibs, 1936). They have been successfully extracted from plants, litter, organic soil layers, sediments, and coal deposits (Kennicutt et al., 1992; Sanger, ~~1971;~~1971a; Hodgson et al., 1968; Bidigare et al., 1991; Dilcher et al., 1970), but have not previously been sought in mineral soil horizons.

$\delta^{15}N$ of chlorophyll and chlorins derived from algal pigments deposited in sediments have been used to infer N cycling processes in aquatic systems (Higgins et al., 2010; Sachs and Repeta, 1999; Enders et al., 2008; Tyler et al., 2010), both as ~~the biological offset in algae is relatively well constrained (Higgins et al., 2011;Sachs et al., 1999).~~combined chloropigment fractions and individual pigment compounds (Higgins et al., 2010; Kusch et al., 2010). Several studies have shown that the $\delta^{15}N$ of plant chlorophyll ~~retains a~~reflects bulk foliar $\delta^{15}N$ ~~signature~~ with ~~an even~~a smaller offset than that between algal chlorophyll and bulk algal $\delta^{15}N$ (Chikaraishi et al., 2005; Bidigare et al., 1991; Kennicutt et al., 1992), but ~~landscape effects such as climate on this offset have not been explored.~~

$\delta^{15}N$ has not previously been measured on chlorins extracted from decomposed plants, terrestrial organic matter, or soil~~, and the~~. The behavior of chlorophyll *a* degradation products and their $\delta^{15}N$ values along the leaf-soil continuum ~~is yet to be explored. Understanding the fate of pheo *a* and $\delta^{15}N$ of pheo *a* is essential; this will determine whether systematic or non-systematic effects confound its utility as a paleo tool~~, and any environmental effects such as climate on isotope offsets, are also unknown.

Here we examine chlorin fractions for the presence of individual compounds in sufficient abundance for isotopic analysis. Pheophytin a (pheo *a*) is a chlorin previously found in greater relative abundance than any other in organic soils and litter (Sanger, 1971a; Gorham and Sanger, 1967), and is therefore of particular interest. We next examine $\delta^{15}N$ of ~~pheo *a* pools~~ abundant chlorins in leaves, litter, and soil and compare values to bulk foliar sources to evaluate ~~this~~potential use of the compound as a proxy for past terrestrial ~~N cycling~~foliar $\delta^{15}N$. For ~~chlorins~~a chlorin to be a useful foliar $\delta^{15}N$ proxy in the terrestrial environment, we hypothesize two conditions~~.~~: First, ~~they~~it must persist in quantities sufficient for isotopic analysis. Second, ~~they~~it must ~~retain~~record the $\delta^{15}N$ value of the leaves ~~they~~it derived from~~.~~. To meet this second condition, the chlorin $\delta^{15}N$ must retain foliar $\delta^{15}N$ with at most a constrainable isotopic offset, independent of environmental conditions and throughout the processes of biosynthesis, senescence, and decomposition ~~regardless of changes in environmental conditions~~.

We evaluate these hypotheses on the natural climate gradient of Kohala Mountain, on the Big Island of Hawaii. These ecosystems are well suited for testing questions about relationships between climate, biogeochemistry, and the preservation or degradation of organic compounds. Relatively low plant diversity and broad environmental niches permit comparison of the bulk and compound-specific $\delta^{15}N$ of leaves, litter, and soil, of similar grassland communities in sites ranging widely in climate and $\delta^{15}N$, to investigate patterns of deviation of compound-specific values from bulk values.

2. Methods

2.1 Site Description

The four sites of this study are located on Kohala Mountain, the oldest of five volcanoes that make up the Big Island of Hawaii (Fig. 1). The sites are a subset of a well characterized climosequence running from the crest of Kohala down the leeward side (see (Chadwick et al., 2003), (Kelly et al., 1998b), (Vitousek and Chadwick, 2013), (von Sperber et al., 2017)). Prevailing winds from the northwest create a dramatic gradient in precipitation, from 2500 mm falling annually at the uppermost site to 210 mm at the lowest site (Giambelluca et al., 1986). In contrast to the substantial shift in precipitation, the elevation of the mountain produces only a moderate gradient in air temperature, ranging from 17°C at the upper site to 23°C at the lowest site. Soils have been forming since emplacement of the Hawi lava flows, approximately 150,000 years ago (Chadwick et al., 2003; Spengler and Garcia, 1988; Wolfe and Morris, 1996). This gradient thereby brings into focus precipitation as the most substantial systematic ~~facto~~factor of change across sites.

Climate history for this region has been reconstructed from pollen assemblages in cores from bogs near the Kohala summit (Hotchkiss, 1998), and inferred from patterns of sea-level change (Ziegler et al., 2003) Aeolian deposits (Porter, 1997), and soil calcite deposits at the drier sites (Chadwick et al., 2003; Porter, 1997). During glacial periods the summit was cooler and drier, while the lower sites show less climate variability, having remained arid throughout soil development.

Vegetation across the gradient was altered by clearing of land for pasture in the last two hundred years (Cuddihy and Stone, 1990; Chadwick et al., 2007), resulting in the introduction of non-native species and grasses with the C4 carboxylation pathway. All of the sites in this study experience grazing by cows, with sites F and I grazed most heavily. Site C is lowland dry scrubland and grassland (150 to 500 mm annual rainfall), dominated by buffel grass (*Cenchrus ciliaria*) and the leguminous tree keawe (*Prosopis pallida*). Sites F (790 mm annual rainfall) and I (1260 mm annual rainfall) lie in lowland dry and mesic forest, woodland, and shrubland zones, which through conversion to pasture are dominated by the grass kikuyu (*Pennisetum clandestinum*). Site M lies in the wet forest and woodland zone (2500 mm annual rainfall), in which ohia (*Metrosideros polymorpha*) is the dominant tree and the native understory was previously dominated by tree fern (*Cibotium spp.*) and uluhe (*Dicranopteris linearis*), but conversion of land to pasture has introduced kikuyu and other species. The herbs clover (*Desmodium incanum*) and Madagascar ragwort (*Senecio madagascariensis*) were additionally prevalent at sites I and M.

~~All pits were dug to at least 50 cm depth in open, grassy areas, with minimal slope. All of the sites in this study experience grazing by cows, with sites F and I grazed most heavily.~~

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2.2 Sample collection and preparation

Leaves were sampled across sites from six plant species; from the upper 1/3 of the canopy in the case of the trees: Two grasses, *P. clandestinum* and *C. ciliaria*; two herbs, *D. incanum* and *S. madagascariensis*; and two trees, *P. pallida* and *M. polymorpha*. Two of these species are N fixers (*D. incanum and P. pallida*), and two have a C4 carboxylation pathway (*P. clandestinum and C. ciliaria*) while the others have C3. Each species was collected wherever present within a radius of ~50 m from the soil pit dug at each site, but not all species were present at all sites. For grasses and herbs, at least three individuals ~~(and generally many more)~~ were collected at each site and bulked for processing as a single sample. For trees, leaves were collected from at least 3 branches per tree and bulked for processing.

Soils were sampled from pits dug in open, grassy areas with minimal slope. Pits were dug to a depth of greater than 50 cm, with the deepest dug to 65 cm. At these sites, litter and organic horizon layers were sampled as a single genetic horizon termed "litter," consisting of organic materials with fibrous through humic composition and which had a total thickness of less than 2 cm. Mineral horizons were sampled at regular depth intervals of ~10 cm to a depth of 50 cm. Samples were bagged in Whirlpak bags and kept dark and chilled on ice in coolers until processing.

~~**2.3 Sample preparation**~~

Live foliage was rinsed with deionized (DI) water to remove dust or other contaminants and then freeze-dried for preservation. Soils and litter were likewise freeze-dried. Dried samples were ground using either a carbide-steel shatter box (SPEX SamplePrep, University of California, Davis) or a mortar and pestle. Samples were stored in a dark freezer.

2.43 Compound extraction and purification

Samples were handled under very limited light conditions to minimize photo-degradation. Pigments were extracted by sonication in triplicate using ~100 mL of acetone (300 ml total) for 1 to 80 g of subsamples. Extracts were concentrated by evaporation to near dryness under nitrogen or argon. The condensed acetone extract was partitioned between 10 ml hexane and 30 ml Milli-Q water via liquid-liquid extraction to remove more polar compounds. The hexane fraction was retrieved, and dried under gentle flow of argon gas, and this crude chlorophyll extract was dissolved in dimethylformamide, and passed through a syringe filter (0.4µm) to remove particulates prior to High Pressure Liquid Chromatography (HPLC) chromatography.

Two-dimensional HPLC was used to separate and purify chlorophyll *a* and *b* and their photo-reactive degradation products. ~~Each individual compound was collected following separation with a fraction collector system of the HPLC~~ (Agilent 1200

Series with a diode-array detector (DAD), and a fraction collector). Sample fractions corresponding to peaks collected from the first HPLC column run were subsequently run through a second column for additional purification of individual compounds. For the first HPLC separation step using reversed phase, the sample was passed through a Zorbax Eclipse XDB C18 column (9.4 x 250 mm; 5 µm) with a liquid phase consisting of acetonitrile~~, /~~pyridine (100:0.5) and ethyl acetate~~, and /~~pyridine (100:0.5) in variable ratio, for 35 minutes at 4.2 ml/min and 30°C, after (Kusch et al., 2010). For the first five minutes of the run, acetonitrile/pyridine was 75% of the eluent, and this percentage was linearly reduced to 50% over the subsequent 30 minutes of the run. For all samples, pheophytin *a* (pheo *a*) was collected upon elution at ~19 minutes. For five plant samples, chlorophyll *a* ~~(chl~~and pyrochlorophyll *a*~~) was~~ were collected upon elution at ~12 minutes. Spectra were checked for purity across wavelengths 200-900 nm. In the second HPLC separation step, also using reverse phase, the sample was passed through an Agilent Zorbax Eclipse PAH column (4.6 x 250 mm; 5 µm), with a liquid phase consisting of acetonitrile~~, /~~pyridine (100:0.5) and ethyl acetate~~, and /~~pyridine (100:0.5) in variable ratio~~,~~ for 35 ~~min~~minutes at a flow rate of 1 ml/min and 15°C. For the first five minutes of the run, acetonitrile/pyridine was 80% of the eluent; between 5 and 30 minutes this percentage was linearly reduced from 80% to 40%; and for the last 5 minutes the column was flushed with 100% ethyl acetate/pyridine.

2.~~5~~4 Analytical methods

Nitrogen isotopic composition, total N, and total C analysis of bulk soil (including litter) and leaf samples was performed using an elemental analyser interfaced to a continuous-flow isotope ratio mass spectrometer (EA-IRMS) at the University of California, Davis Stable Isotope Facility. The mean value of analytical precisions obtained for standard materials is 0.3‰ for $\delta^{15}N$. C/N ratios are reported in mass units. In the case of soil, analysis was performed on ~20 mg of material using an Elementar Vario EL Cube or Micro Cube elemental analyser (Elementar Analysensysteme GmbH, Hanau, Germany) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). In the case of leaves, analysis was performed on ~4 mg of material using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Samples are combusted at 1000°C in a reactor packed with copper oxide and lead chromate. Following combustion, oxides were removed in a reduction reactor (reduced copper at 650°C). The helium carrier then flowed through a water trap (magnesium perchlorate). N_2 and CO_2 were separated using a molecular sieve adsorption trap (for soil) or Carbosieve GC column (65°C, 65 mL/min) (for leaves) before entering the IRMS. Bulk isotopes of two plants (Koh F buffel/kikuyu) and one soil core (4 layers, fresh and litter1) were analysed at ~~JAMSTEC.~~the Japan Agency for Marine Earth-Science and Technology (JAMSTEC).

$\delta^{15}N$, total N, and total C analysis of isolated pheo *a* fraction was performed on a Nano-EA-IRMS at ~~the Japan Agency for Marine Earth-Science and Technology (JAMSTEC).~~JAMSTEC. This is a modification of an EA-IRMS system to achieve ultra-sensitive analysis (Ogawa et al., 2010). All samples contained at minimum 10 nmol N, resulting in $\delta^{15}N$ precision of <

±0.4‰. To investigate the possibility of N contamination, nitrogenous volatile was analysed for the ~~pheophytin~~pheo *a* fraction by gas chromatography/nitrogen-phosphorus detector (GC/NPD) with trimethylsilyl derivatization (BSTFA, Agilent Technologies, Palo Alto, CA) at JAMSTEC. C/N ratios from EA/IRMS were used as the "purity indicator" of each chlorophyll compound. As is described below, if a sample showed the clean spectrum pattern of a chlorin but had significantly large C/N ratio, it is likely that it contained C-containing contaminants, such as carbon hydrates, which are not detectable by DAD.

2.~~6~~5 Purity of isolated compounds

While absorption spectra in UV/visible wavelengths showed no evidence for other absorptive components in the pheo *a* extracts, C/N weight ratios greater than 11.8 revealed inclusion of non-pheo *a* carbon in the extracts. C/N ratios were higher in pheo *a* extracted from litter (31) than plant samples (14.5), and even higher in pheo *a* samples from soils (113). Lack of relationship between C/N and bulk-pheo $\delta^{15}N$ offset suggests that impurities do not contribute significantly to measured $\delta^{15}N$ values, however (Fig. 2). This supposition was confirmed by results from the GC-NPD), which showed a lack of nitrogenous compounds (e.g. containing amino groups) in the pheo *a* extracts. Although we caution that polar and less-volatile compounds cannot be detected by this method, the extraction methods make amino acids and other nitrogenous contaminants unlikely. The analytical protocol is designed to remove as many of such contaminating compounds as possible; e.g. amino acids are not soluble in acetone.

2.~~7~~6 Isotopic notation

Nitrogen isotopic compositions are reported using conventional delta (δ) notation:

$$\delta\ (‰) = (R_{sample}/R_{standard} - 1) \times 1000 \qquad \text{(Equation 1)}$$

where R represents the $^{15}N/^{14}N$ ratio and subscripts indicate the sample or isotopic reference. The sample isotopic composition is measured directly relative to the N_2 laboratory reference gas (δS_{Ref}), and the composition of the sample relative to the internationally recognized $\delta^{15}N$ reference, AIR, is calculated by:

$$\delta S_{AIR} = \delta S_{Ref} + \delta Ref_{AIR} + 10^{-3} \delta S_{Ref}\, \delta Ref_{AIR} \qquad \text{(Equation 2).}$$

Epsilon notation is used to describe biosynthetic isotopic fractionation within whole leaf (bulk) tissue. Nitrogen isotopic fractionation of pheo *a* relative to bulk leaf tissue ($^{15}\varepsilon_{pheo\text{-}bulk\text{-}leaf}$) and chlorophyll *a* relative to bulk leaf tissue ($^{15}\varepsilon_{chl\text{-}bulk\text{-}leaf}$) are defined according to Equation 3:

$$^{15}\varepsilon_{compound\text{-}bulk\text{-}leaf} = 1000[\frac{\delta 15Ncompound+1000}{\delta 15Nbulk+1000}\frac{\delta 15Ncompound+1000}{\delta 15Nleaf+1000} - 1] \quad \text{(Equation 3).}$$

3. Results

3.1 Bulk isotope and C and N concentration data

Site-averaged bulk $\delta^{15}N$ of all soil samples decreased with increasing precipitation (and elevation) across all sites, from an average of 12.4‰ at site C to 5.1‰ at site M (Table 1). Site-averaged $\delta^{15}N$ of litter decreased between sites C and M (4.3‰ to 2.8‰), but sites F and I were higher than either of these values (9.0‰ and 7.2‰). Average foliar $\delta^{15}N$ likewise decreased between sites C and M (4.2‰ to 0.5‰), but site F (9.1‰) was higher than C (Fig. 3). Site-averaged bulk soil $\delta^{15}N$ values for those samples on which $\delta^{15}N_{pheo}$ was measured follow similar trends (Table 2), though on average site values are slightly lower (8.4 vs. 8.9‰), reflecting the shallower average depth of the soil samples.

Soil %N increased from site C (0.2%) to site M (1.2%), and %C increased across these sites from 1.9% to 17.2%. C/N increased between these sites from 11.5 to 16.3 (Table 1). Litter %C and C/N was notably higher at site M (36.3% and 25.2) than at the other sites, although litter %N was relatively flat across all sites. Vegetation %C was likewise highest at site M (43.1%), though this value was less exceptional compared with vegetation at other sites.

Vegetation had the highest C/N (average of 19.9), followed by litter (17.2) and soil (12.4). At sites C, F, and M, C/N of litter is closer to that of vegetation than to soil, while at site I C/N of litter is closer to that of soil than to vegetation.

3.2 Chlorin compound detection

Chlorins were detected in leaf, litter, and soil samples, including in soil mineral horizons up to a depth of 32 cm (Table 2). As chlorophyll *a* has greater absorbance at 660 nm than ~~Pheo~~pheo *a,* direct proportionality between relative HPLC peak areas and relative compound abundance in the sample should not be assumed. In plant leaves, chlorophyll *a* was the dominant chlorin. Chlorophyll *a* was also found in smaller to trace amounts in litter and some soils. In litter, pheo *a* was the dominant pigment, with the exception of site M, where chlorophyll *a* was more abundant in the litter sample. In site C, chlorophyll *a* was absent from litter and soil. In soils, ~~pheophytin~~pheo *a* was the most abundant degradation product. Pheo *a*, targeted for isotopic

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analysis due to its superlative abundance, was present in sufficient concentration for isotopic analysis above ~20 cm in soils at most sites.

3.3 Pheophytin *a* N isotope data of leaves, litter, and soil

Intra-leaf isotope offsets—Across all plant samples, $\delta^{15}N_{pheo}$ of live foliage was significantly, linearly correlated with bulk $\delta^{15}N_{leaf}$ (slope = 0.9; y-intercept = −1.1; adjusted $R^2 = 0.8$; p = 0.000002507) (Fig. 4). $^{15}\varepsilon_{pheo\text{-}bulk_{leaf}}$ was equal to 1.4‰ (± 2.3‰) across all plant samples (Eq 1).

Of the six sampled species, all but the two trees exhibited mean $^{15}\varepsilon_{pheo\text{-}bulk_{leaf}}$ values ≤ 1.5‰ (Table 3): $^{15}\varepsilon_{pheo\text{-}bulk_{leaf}}$ for *P. pallida* was 6.5‰ and $^{15}\varepsilon_{pheo\text{-}bulk_{leaf}}$ for *M. polymorpha* was 2.5‰. If the *P. pallida* sample is omitted, $^{15}\varepsilon_{pheo\text{-}bulk_{leaf}}$ drops to 0.71‰ (±1.3‰).

$^{15}\varepsilon_{pheo\text{-}bulk_{leaf}}$ was largest at site C (4.0‰) and smallest at site F (0.12‰). For a given species, average $^{15}\varepsilon_{pheo\text{-}bulk_{leaf}}$ tended either to remain flat or slightly decrease into the wettest sites (Fig. 5).

$\delta^{15}N_{pheo}$ *offsets across leaf-litter-soil*—$\delta^{15}N$ of Pheo *a* in litter was on average 0.3‰ higher than the $\delta^{15}N$ of pheo *a* of live foliage at a common site. Average difference between the $\delta^{15}N$ of bulk litter and foliage were slightly higher, at ~ 2.6‰ at a common site (Table 4). Pheo *a*-specific soil $\delta^{15}N$ values were on average 1.3‰ higher than pheo *a* litter values at a common site; bulk $\delta^{15}N$ soil values are 2.6‰ higher than bulk litter. The average offset between $\delta^{15}N_{pheo}$ $\delta^{15}N_{pheo}$ of soil and live foliage at a site was1.1‰; the average offset between bulk soil and bulk vegetation $\delta^{15}N$ was 4.9‰.

3.4 Soil depth profiles

Soil $\delta^{15}N_{bulk}$ was, on average, higher than the $\delta^{15}N_{bulk}$ of overlying litter, and there was a slight trend of increasing $\delta^{15}N_{bulk}$ with increasing depth in the upper ~25 cm of soil pits (Fig. 6). $\delta^{15}N_{pheo}$ of soil also displayed higher values relative to overlying litter in sites F and I and in part of the profile at site M (Fig. 6). Soil $\delta^{15}N_{bulk}$ values returned to slightly more negative values deep in the profiles; particularly notable at site F. At site C, $\delta^{15}N_{pheo}$ along the soil profile do not deviate significantly from that of the overlying litter. At site M, $\delta^{15}N_{pheo}$ values increased slightly with depth in the upper profile, but then decreased while $\delta^{15}N_{bulk}$ steadily increased with greater depth. In sum, $\delta^{15}N_{pheo}$ of soil did not follow $\delta^{15}N_{bulk}$, nor did it constantly track $\delta^{15}N_{pheo}$ of modern plants at a common site.

4. Discussion

4.1 Evaluation of $\delta^{15}N$ fractionation within leaves

Nitrogen isotopic offsets between pheophytin *a* and live foliage ($^{15}\varepsilon_{pheo\text{-}bulk_{leaf}}$) are generally small (mean of 1.4‰) and well constrained (s.d. ± 2.3‰, Fig. 3) across our sites, marking $\delta^{15}N_{pheo}$ as a useful proxy for bulk $\delta^{15}N_{leaf}$. The robustness of $^{15}\varepsilon_{pheo\text{-}bulk_{leaf}}$ across the wide range in environmental variables and values of $\delta^{15}N_{leaf}$ observed across these sites (Fig. 4) suggests that isotope effects from species or physiological changes will be relatively small sources of variation in $\delta^{15}N_{pheo}$ compared with changes to primary $\delta^{15}N_{leaf}$. For example, average community foliar $\delta^{15}N_{leaf}$ varies by about ~10‰ across terrestrial biomes in the modern environment (Craine et al., 2009), and paleo records have inferred comparable shifts in $\delta^{15}N_{leaf}$ of 10‰ between glacial-interglacial cycles (Stevens et al., 2008). The possible effect of species shifts, and particularly growth of forests, should be considered when evaluating proxy $\delta^{15}N_{pheo}$ records, however. One species, *P. pallida*, has $^{15}\varepsilon_{pheo\text{-}bulk_{leaf}}$ of 6.5‰. The other tree studied here, M. *Polymorpha*, had the next largest $^{15}\varepsilon_{pheo\text{-}bulk_{leaf}}$ (2.5‰), while herbaceous plant values were all considerably smaller (-0.29 to 1.4‰), raising the ~~question of whether there may be~~possibility that this is a ~~positive~~systematic isotope effect ~~of cellulose accumulation on $\delta^{15}N_{pheo}$~~in trees, perhaps due to their greater size, longevity, and resulting N storage and redistribution requirements relative to herbaceous plants.

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4.2 Evaluation of $\delta^{15}N_{pheo}$ from soil as a proxy for $\delta^{15}N_{leaf}$

Pheo *a* was present in quantities sufficient for isotopic analysis in litter and the uppermost mineral soil sample at all sites, and down to 30 cm at the wettest site (Fig. 4), inviting exploration of soil $\delta^{15}N_{pheo}$ as a proxy for $\delta^{15}N_{leaf}$. $\delta^{15}N_{pheo}$ of soil and litter match $\delta^{15}N_{pheo}$ of overlying leaves at only one studied site (site C). Two alternatives could explain the lack of coherence in leaf, litter, and soil pools across most sites: 1) there is isotopic alteration during senescence and mineralization of chlorophyll, and 2) litter and soil pheo *a* pools have other sources than the plants we sampled, either on the current landscape, or from previous vegetation covers.

To investigate the expression of fractionation on demetallation of chlorophyll in these samples, we can compare the $\delta^{15}N_{pheo}$ with chlorophyll *a* ~~(chl *a*)~~ from the same sample. ~~Chl~~Chlorophyll *a* was only in sufficient abundance for isotopic measurement in live plant leaves. ~~Chl~~Chlorophyll *a* is about 0.05‰ depleted in ^{15}N relative to bulk leaves in this study. This is consistent with previous studies, which found chlorophyll in plants to be 1.2‰ depleted in ^{15}N relative to bulk leaf tissue (Kennicutt et al., 1992; Chikaraishi et al., 2005). Pheo *a* is accordingly about 2‰ enriched in ^{15}N relative to ~~chl~~chlorophyll *a* in our leaf samples, which could point to fractionation either within the leaf or in the laboratory (Sachs, 1997). Because chlorophyll abundance is greatly reduced between leaves and litter, any fractionation is likely to be expressed as a difference between leaves and litter, not as a difference between litter and soil or within soil. Fractionation on demetallation from chlorophyll should therefore not account for changes in $\delta^{15}N_{pheo}$ in the soil.

Soil is enriched in ^{15}N relative to litter at our sites, consistent with observations from a wide range of environments, including temperate rainforest (Menge et al., 2011), temperate deciduous forests (Templer and Dawson, 2004), boreal forests (Hyodo and Wardle, 2009), tropical forests (Martinelli et al., 1999), temperate grasslands (Baisden et al., 2002; Brenner et al., 2001), and elsewhere (Hobbie and Ouimette, 2009). Because post-depositional processing of N involves the preferential loss of ^{14}N from the soil N pool without replacement, through removal of the products of mineralization and denitrification, bulk soil $\delta^{15}N$ is highly dynamic and will tend to increase along a decomposition gradient. In contrast, if there is no fractionation on breakdown of pheo *a,* $\delta^{15}N_{pheo}$ in soil should not depart from the $\delta^{15}N_{pheo}$ of litter inputs.

In soil profiles across the climosequence, $\delta^{15}N_{bulk}$ and $\delta^{15}N_{pheo}$ do not exhibit similar depth patterns (Fig. 6). This suggests that different processes govern these two records in soil. While $\delta^{15}N_{bulk}$ values drift increasingly positive across the leaf-litter-soil continuum, pheo *a* values remain centered around site average leaf values, and sometimes show slight decreases in value from leaf to litter and soil (Table 4). This suggests that mixing of inputs may account for litter and soil pheo *a* $\delta^{15}N$ values, while fractionating losses from the bulk soil N pool are required to explain $\delta^{15}N$ bulk values.

$\delta^{15}N_{pheo}$ of litter is a window into possible isotopic effects of decomposition on $\delta^{15}N_{pheo}$ of plant leaves. The difference between $\delta^{15}N_{pheo}$ of leaves and litter averaged close to zero and was substantially smaller than the difference in $\delta^{15}N_{bulk}$ (0.3‰ *versus* 2.3‰), supporting greater isotopic fidelity along the decay continuum in pheo *a* $\delta^{15}N$ than bulk $\delta^{15}N$. Some of the site variability in isotopic offsets between litter and leaves/soil is likely due to differences in how well decomposed the collected litter was from site to site: at sites C, F, and M, C/N of litter is closer to that of vegetation than to soil, while at site I C/N of litter is closer to that of soil than to vegetation.

It is likely that litter samples do not reflect equal contributions from the plants sampled at the same site. Litter was collected from the surface of the soil pits, while vegetation was sampled from a radius of many meters, and plant taxa were not identified in litter samples to allow for source attribution. Soil algae and bacteria are considered insignificant contributors to soil pheo *a*, as the absorbance spectra of soil extracts showed that chlorophyll degradation products are dominated by higher plant contributions.

Pheophytin has two pathways of generation: Like chlorophyll *a*, it is biosynthesized in plant leaves from glutamate where it serves as an electron acceptor in photosystem II. (Klimov et al., 1977). Additionally, it is a product of chlorophyll degradation, whether via senescence, grazing, or decomposition across the leaf-soil continuum~~, in which the central Mg is replaced by two H atoms. Transformation from chlorophyll to pheophytin~~ (Kräutler and Hörtensteiner, 2014; Sanger, 1971b), in which the central Mg is replaced by two H atoms. Transformation from chlorophyll to pheo *a* involves breaking N-Mg bonds, and so has potential for fractionation of N isotopes. However, the N-Mg bond is not a normal covalent bond, but a bond loosely connecting

a ligand and metal in complex. Because the energy needed to break this bond is substantially smaller than the covalent bond, Mg loss from ~~Chl~~chlorophyll would theoretically be expected to have little or no N isotope fractionation. Senescence is unlikely to impart significant fractionation from bulk leaves given the observation that bulk leaf $\delta^{15}N$ does not change on abscission (Kolb and Evans, 2002) and the understanding that N contained in the tetrapyrrole structure of chlorophyll is not recycled by the plant (Eckhardt et al., 2004). However, fractionation on demetallation of chlorophyll to ~~pheophytin~~pheo *a* has been observed in a laboratory setting with an effect of up to 2‰ (Sachs, 1997). Mineralization of pheo *a* is unlikely to alter $\delta^{15}N$ of the remaining pheo *a* pool because known products of chlorin defunctionalization retain the four atoms of tetrapyrrole N and their associated bonds (Keely, 2006).

It is unlikely that environmental changes that took place between production of litter and growth of current leaves explain the observed differences between $\delta^{15}N$ of plants and litter, given that litter at these sites is estimated to be no more than a few years old. Soil pools, however, could contain pheo *a* that is considerably older. There is reason to expect that deeper pheo *a* will be older than overlying pheo *a* in soil profiles, and increasing recognition that compounds can be preserved in the soil matrix much longer than their inherent lability would predict (Mikutta et al., 2006; Torn et al., 1997; Marin-Spiotta et al., 2011; Kramer et al., 2012).

While we do not know the age of the sampled soil pheo *a,* radiocarbon dates of 4130 and 8030 yBP ~~taken~~were measured on soil organic carbon (SOC) deep within the soil profiles at ~~other~~ sites A and D, respectively, along the climosequence ~~suggest that the pheo *a* compounds may exhibit a range of several thousand years over the depths of these profiles~~ (Chadwick et al., 2007)~~.~~, suggesting that our studied soils may reflect organic contributions from several thousands of years of soil development. Bulk soil carbon isotope values suggest that C4 pasture grasses, introduced over the last two hundred years, have been incorporated in the SOC in the top 40 cm of soil on these sites (Kelly et al., 1998a).

Patterns of radiocarbon ($\Delta^{14}C$) depletion of SOC in profile (Baisden et al., 2002; Townsend et al., 1995; Tonneijck et al., 2006; Trumbore, 2009) indicate that SOC age increases with soil depth. Departures from this trend have been explained by the introduction of modern material by plant roots (Baisden et al., 2002), but as ~~chl~~chlorophyll *a* and pheo *a* are photosynthetic pigments, we expect that inputs to the soil originate exclusively as litter accumulated at the soil surface, and for age of the chlorophyll derivatives ~~to~~ therefore to increase with depth. Bioturbation and episodic leaching could be disruptive of space-for-time trends, but based on the pattern observed in bulk SOC $\Delta^{14}C$, are unlikely to interfere with millennial scale patterns given appropriate vertical sampling distances. In fact, in aggrading profiles, bioturbation has been shown to contribute to the increase in SOC age with depth, by transporting SOC downward over short distances and migrating upwards as soil accumulates overhead (Tonneijck and Jongmans, 2008).

If down-profile $\delta^{15}N_{pheo}$ values did represent prior landscape $\delta^{15}N_{leaf}$ values, these data suggest greater changes to the N cycle at sites F and I than C and M, which could reflect heavier grazing at sites F and I. Climate has been more constant throughout the Holocene at lower elevations than higher elevations on Kohala (Hotchkiss, 1998), which could further account for the relatively constant $\delta^{15}N_{pheo}$ values at site C.

If the total soil $\delta^{15}N$ pool reflects fractionation on loss of N, but the soil $\delta^{15}N_{pheo}$ pool does not, we expect that the more important gaseous losses are relative to leaching losses at a site, the higher $\delta^{15}N_{bulk}$ and the greater the offset between $\delta^{15}N_{pheo}$ and $\delta^{15}N_{bulk}$ will be. Environments where denitrification is more important relative to leaching tend to be dry environments, due to much smaller isotope effects of non-gaseous losses (Houlton and Bai, 2009), and drier sites do indeed have higher $\delta^{15}N$ in this study. Differences between $\delta^{15}N_{pheo}$ and $\delta^{15}N_{bulk}$ do not show clear trends along the climosequence, however. This could be due to the complexity of soil moisture at these sites, reflected in the variability in down-profile soil bulk $\delta^{15}N$.

Direct testing of the hypothesis of increasing age with depth of pheo *a* would be enabled by improving the purification method of ~~pheophytin~~pheo *a* from soil. Removing contaminating C would make stable or radiocarbon of pheo *a* available tools to shed light on sources of pheo *a* in soil profiles, age of the compounds, and with what resolution change in N cycling over the temporal window provided by the chlorins depth profile is observable (Ishikawa et al., 2015). These questions could also be averted by measuring $\delta^{15}N_{pheo}$ in soil profiles with constrained ages, such as buried horizons.

4.3 Implications of the $\delta^{15}N_{pheo}$ proxy

We suggest two key opportunities for application of a soil-based $\delta^{15}N_{pheo}$ proxy data to advance understanding of past terrestrial N dynamics. First, the ability to track changes in foliar $\delta^{15}N$ over time implies comparatively direct insight into factors affecting $\delta^{15}N$ of plants, notably the availability of nitrogen. Central questions concerning the timescale of N cycle responses to elevated CO_2 concentration in the atmosphere, and whether availability of this limiting nutrient increases or decreases with climate change, could be explored by selecting a time series that covers changes in atmospheric pCO_2 (Goulden, 2016).

Second, comparison of compound-specific $\delta^{15}N_{pheo}$ with other $\delta^{15}N$ proxies over the same time domain could provide insights into processes that cause them to deviate from one another. Alternative sources of $\delta^{15}N$ proxies include subaqueous sediment deposits in lakes, ungulate tooth enamel, and bulk wood, black carbon, or soil. Deviations in records of $\delta^{15}N$ of pheo and tooth enamel at a common site would highlight changes in factors affecting dietary isotope fractionations, such as animal growth rates. $\delta^{15}N_{pheo}$ records could provide information on terrestrial sources of $\delta^{15}N$ relevant to aquatic sediment $\delta^{15}N$ records, and allow aquatic and terrestrial signals to be distinguished. $\delta^{15}N_{pheo}$ could validate bulk proxy records, or highlight diagenetic limitations of the record. Within soil horizons, comparing $\delta^{15}N_{pheo}$ with $\delta^{15}N_{bulk}$ could provide information both on N availability to plants and dominant pathways of N loss, hydrologic or gaseous, at a site, allowing for comparison of multiple N cycle dynamics over time.

Conclusions

$\delta^{15}N_{pheo}$ in leaves provides a molecular recorder of foliar $\delta^{15}N$, and provides a means to trace leaf nitrogen signatures in litter and soils. The compound is found in litter and upper soil horizons in abundance sufficient for isotopic analysis, where it shows greater fidelity to leaf $\delta^{15}N$ than does bulk material. $\delta^{15}N_{pheo}$ of soil may reveal temporal changes in nitrogen cycle ~~behavior~~behaviour—e.g. the availability of nitrogen to plants, and whether nitrogen losses from the ecosystem had a dominant atmospheric or hydrologic fate. Due to the well-constrained photoautotrophic sources of chlorins, their lack of confounding heterotrophic enrichment, and their minimum N isotope fractionation during burial processes, CSIA of chlorins offers several advantages over bulk isotope analysis on the study of soil C and N cycles. $\delta^{15}N_{pheo}$ of soil is a most promising proxy for $\delta^{15}N_{leaf}$ where organic matter inputs are high and profiles aggrade. The potential to investigate decadal-to-millennial scale N cycle dynamics will depend largely on conditions for preservation of soil organic matter.

Data availability

The data can be accessed by email request to the corresponding authors~~.~~ and have been submitted to the open access database PANGAEA (Data submission 2019-08-01T06:12:00Z https://issues.pangaea.de/browse/PDI-21268).

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Author Contributions

SG and BH acquired funding for the project. SG, BH, and OC collected samples. NO, KF, and BH contributed laboratory space and materials. SG, NO, KF, YC, NO, and HS collaborated on laboratory methodology; SG, NO, HS, and YC conducted analyses of samples. SG performed data analysis, generated figures, and wrote the initial draft; BH, NO, KF, YC, HS, and OC contributed to the manuscript.

Competing interests

The authors declare that they have no conflict of interest.

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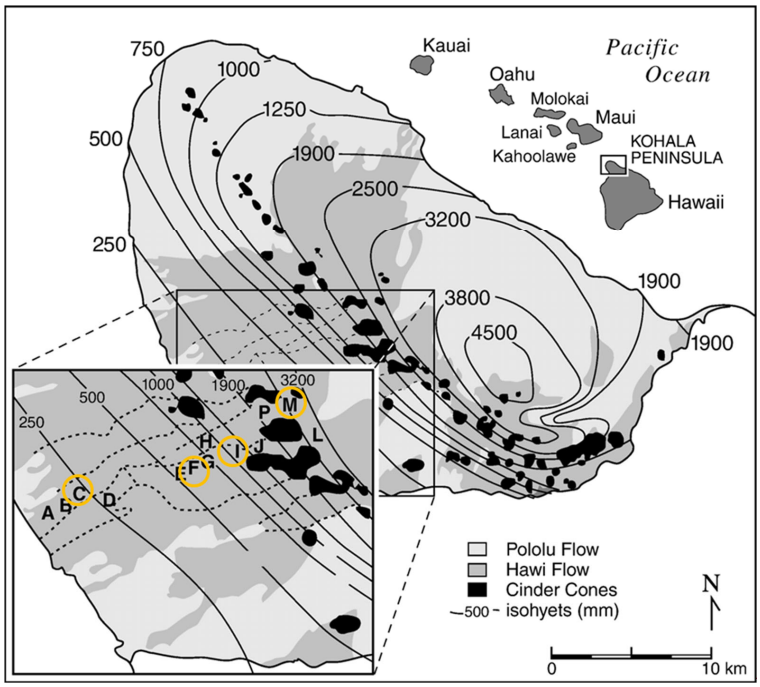
Figures


750
1000
1250
500
1900
2500
3200
250
1900
3800
4500
1900
Kauai
Oahu
Molokai
Maui
Lanai
Kahoolawe
KOHALA
PENINSULA
Hawaii
Pacific
Ocean
500
1000
1900
3200
250
A
B
C
D
F
H
I
L
M
P
Pololu Flow
Hawi Flow
Cinder Cones
500 isohyets (mm)
N
0
10 km

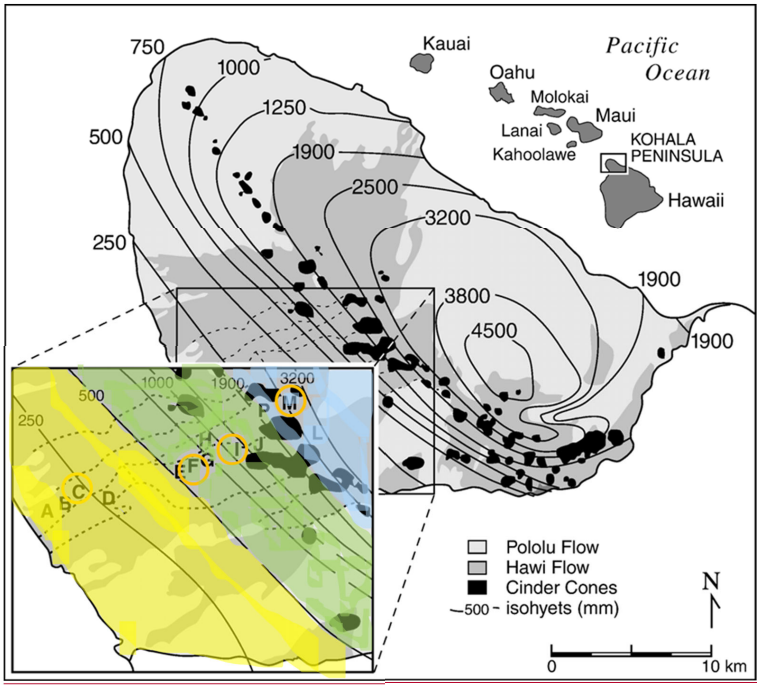
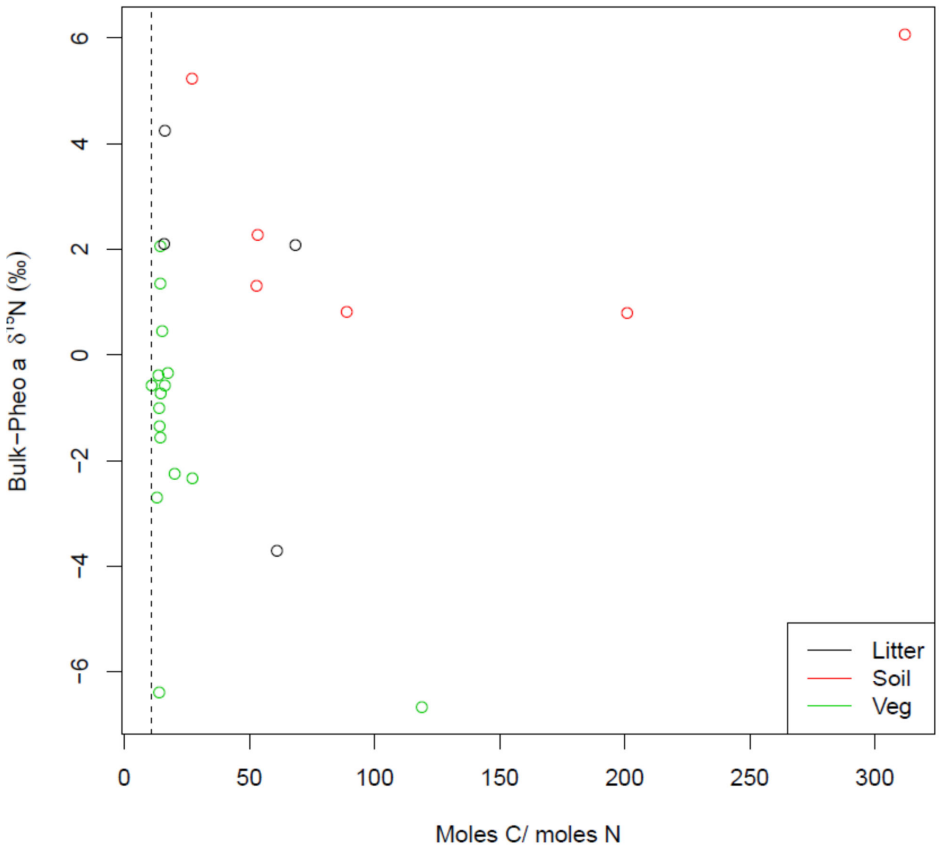


Figure 1: Map of Kohala climosequence sites with respect to rainfall isoclines, reproduced from Chadwick et al. (2007). Sites sampled for this study (C, F, I, and M) are circled in orange. Vegetation zones are highlighted in the inset map: lowland dry scrubland and grassland between 150 and 500 mm of rainfall (yellow); lowland dry and mesic forest, woodland and shrubland from 500 mm to 2000 mm (green); and wet forest and woodland above 2000 mm (blue).


Bulk−Pheo a δ15N (‰)
6
4
2
0
−2
−4
−6
0
50
100
150
200
250
300
Moles C/ moles N
Litter
Soil
Veg

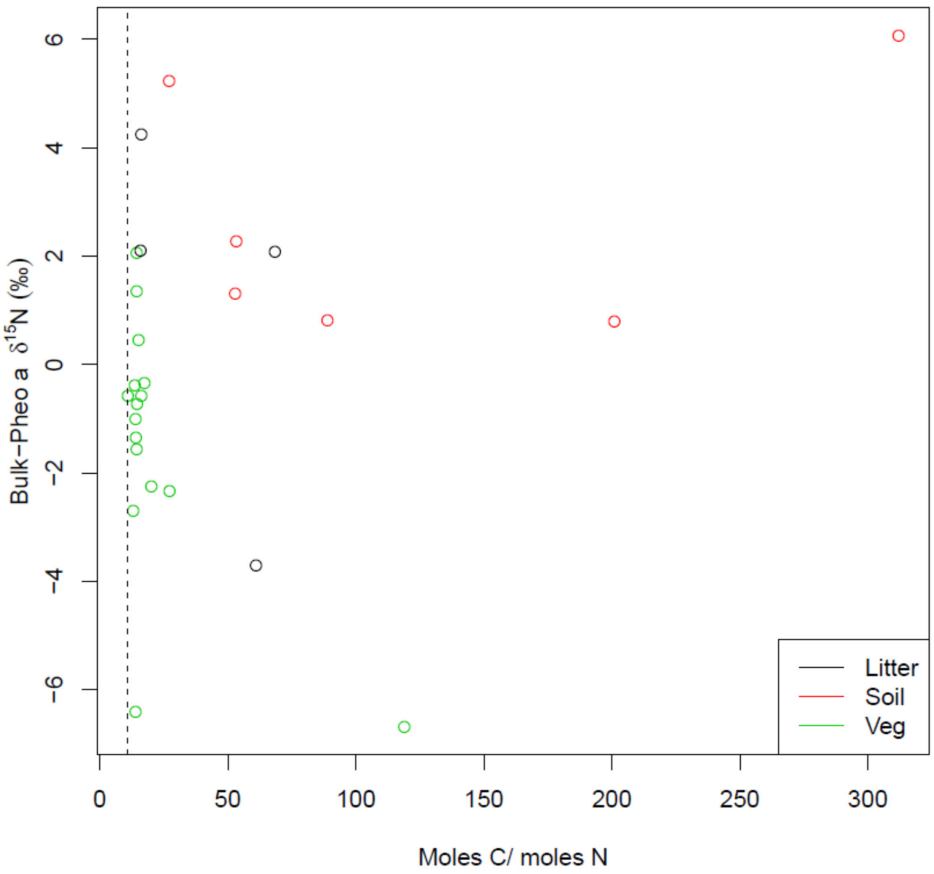
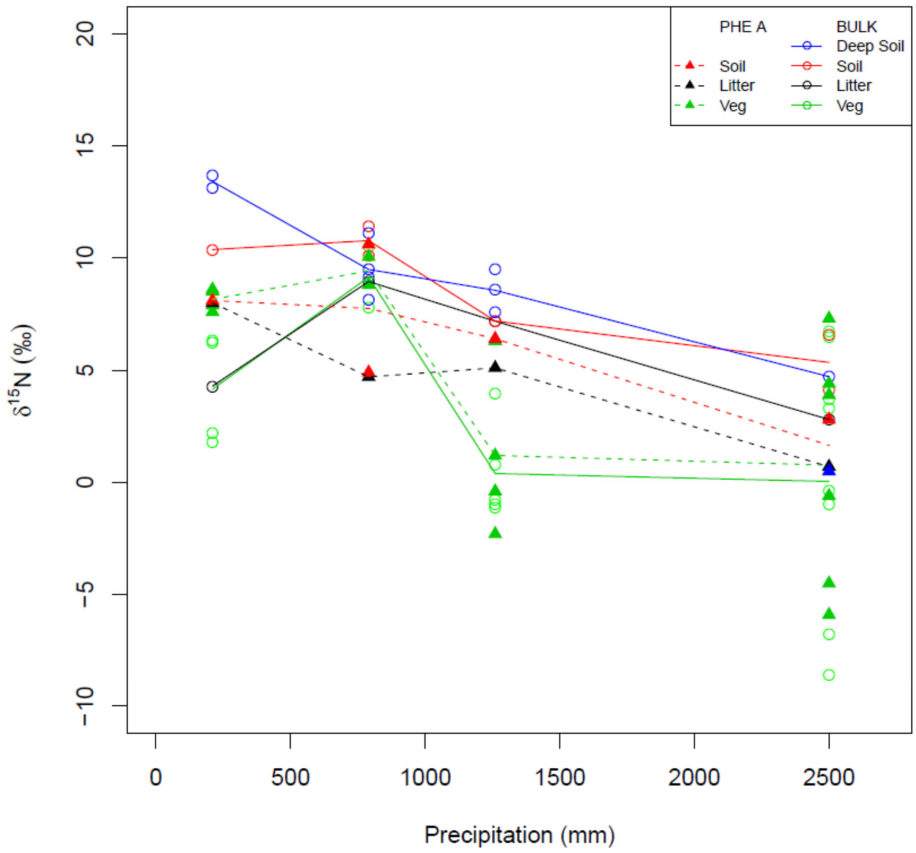


Figure 2: C/N ratios for pheo *a* isolates higher than 11 (dashed line) reveal contamination by carbon compounds. Effect of this contamination on pheo a $\delta^{15}N$ is investigated by comparing "pheo *a*" molar C/N with the offset between pheo *a* and bulk $\delta^{15}N$ for litter (black), soil (red), and vegetation (green) samples. Lack of a systematic relationship between high C/N ratios and greater similarity of pheo *a* $\delta^{15}N$ to bulk values is evidence against contamination of the pheo *a* samples by N from the greater bulk sample.


PHE A
Soil
Litter
Veg
BULK
Deep Soil
Soil
Litter
Veg
δ15N (‰)
20
15
10
5
0
−5
−10
0
500
1000
1500
2000
2500
Precipitation (mm)

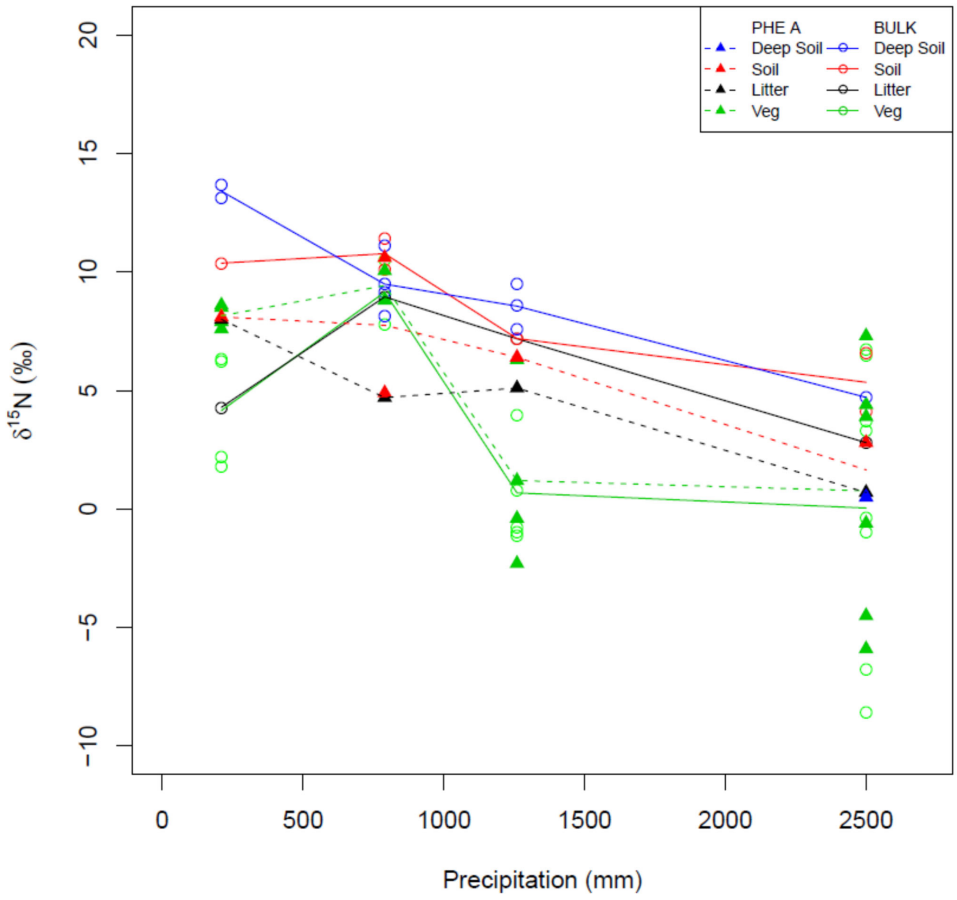


Figure 3: Patterns in isotopes of bulk material (open circles) and pheo *a* extracts (closed triangles) from plant leaves (Veg, green), litter (black), and soil (red). Deep soils (blue) have a depth of greater than 30 cm and only one pheo *a* ~~bulk~~ isotope measurement was made for these samples. Lines connect site averages between bulk (solid) and pheo *a* (dashed) measurements.

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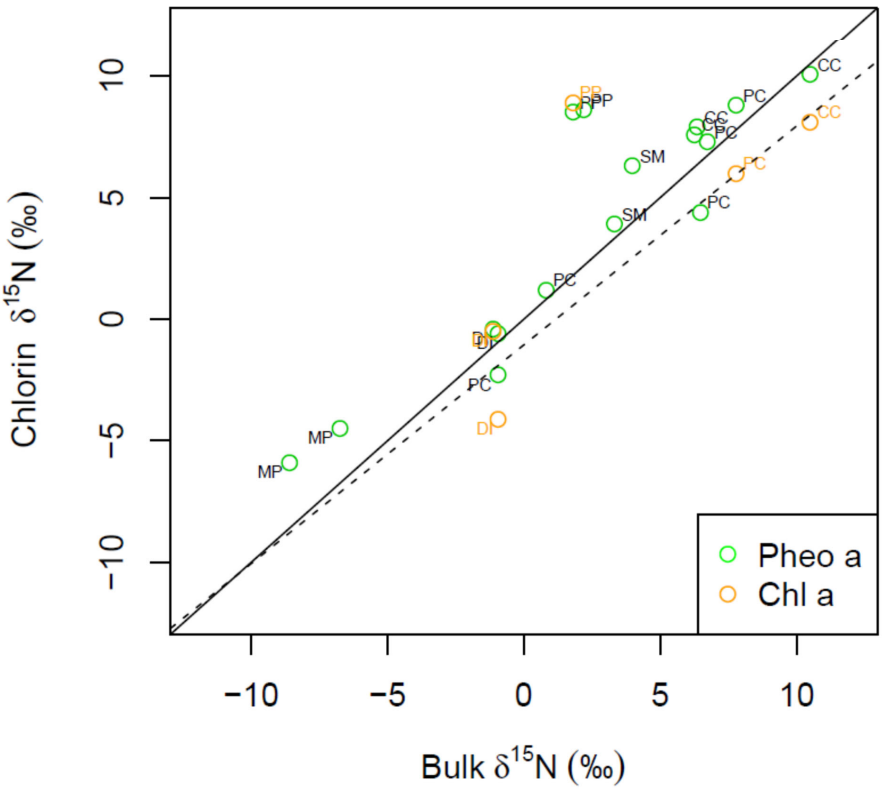
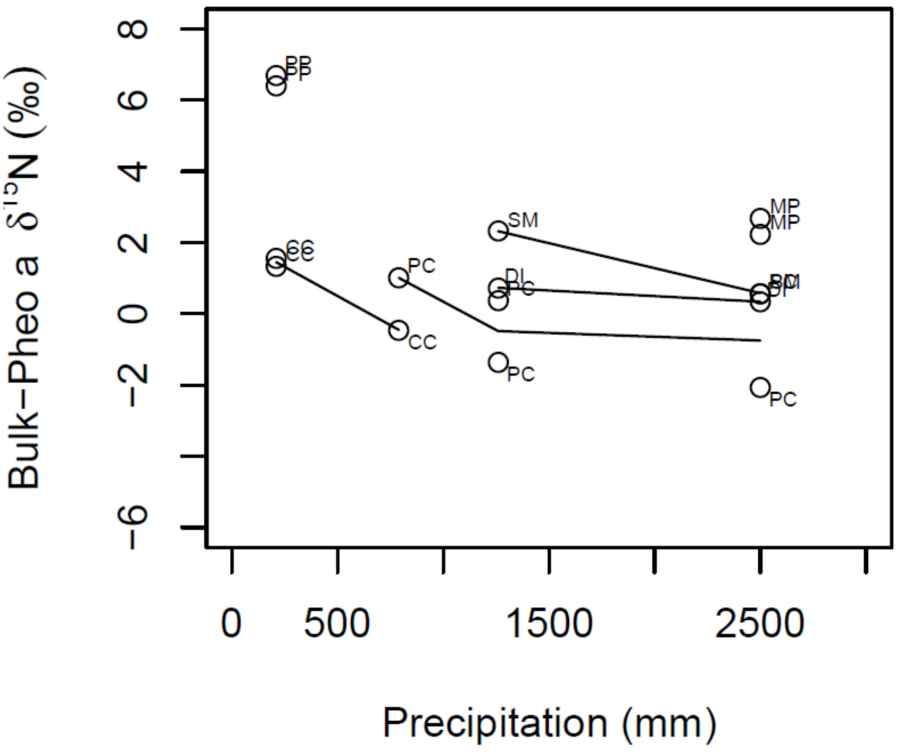


Figure 4: $\delta^{15}N$ of pheo *a* (green) and chlorophyll a (orange) correlates linearly with bulk leaf $\delta^{15}N$. The linear model for $\delta^{15}N$ of pheo *a* as a function of bulk leaf $\delta^{15}N$ is printed as a dashed line. A line with a slope of 1 (solid line) is plotted for reference. Each sample's species is identified with a label: CC = C. ciliaria*, DI = D. incanum†, MP= M. polymorpha‡, PC= P. clandestinum*, PP = P. pallida†‡, SM = S. madagascariensis.*=C4 pathway, †=N fixer, ‡=tree


Bulk−Pheo a δ15N (‰)
8
6
4
2
0
−2
−6
0
500
1500
2500
Precipitation (mm)
SM
PC
CC
MP
MP
PC
PC

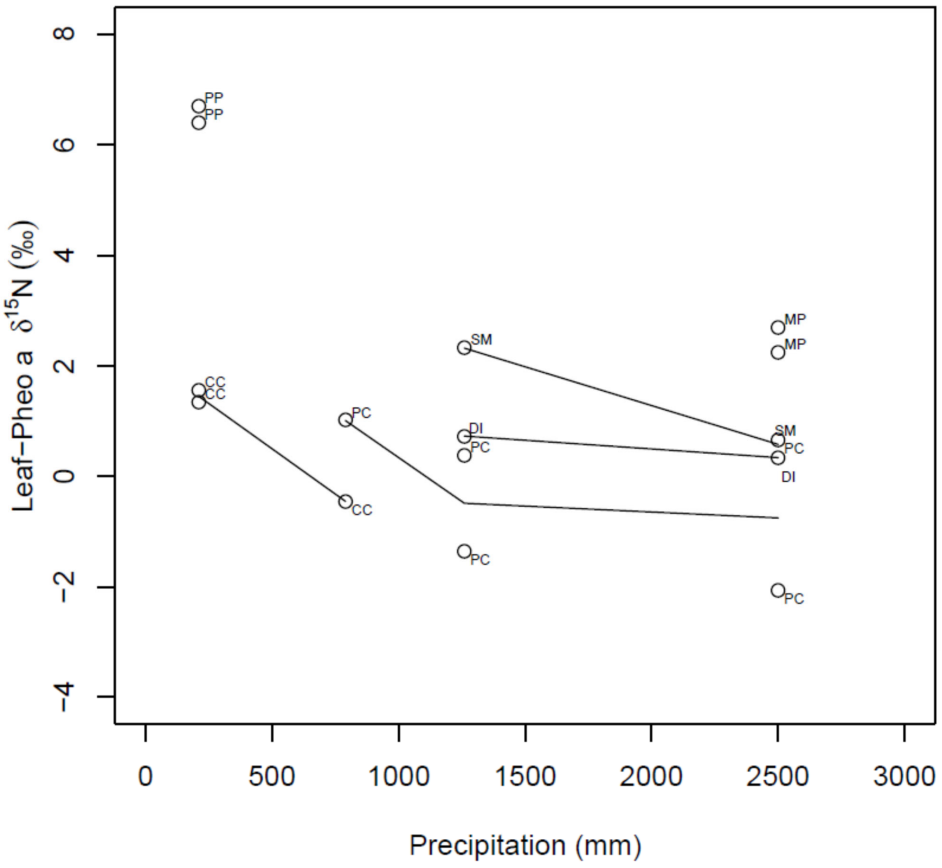


Figure 5: Comparison of species average intra-leaf $\delta^{15}N$ offset between pheo *a* and bulk ($^{15}\varepsilon_{pheo\text{-}bulk\text{-}leaf}$) across sites. CC = *C. ciliaria**, DI = D. *incanum*†, MP= *M. polymorpha*‡, PC= *P. clandestinum**, PP = *P. pallida*†‡, SM = *S. madagascariensis*.*=C4 pathway, †=N fixer, ‡=tree

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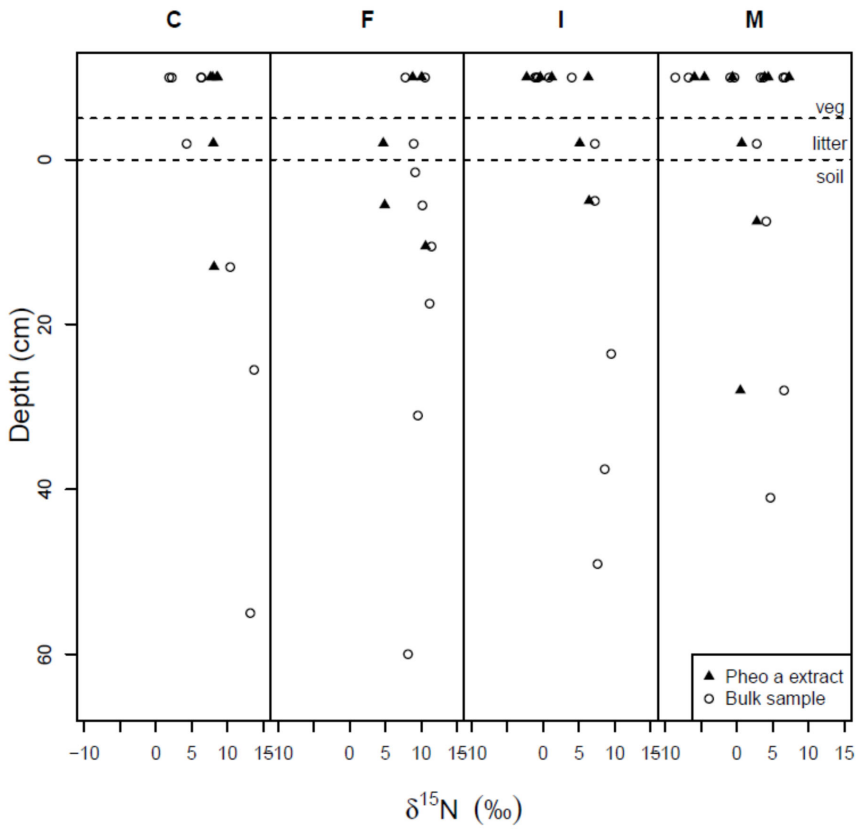

Figure 6: Bulk samples (open circles) and pheo *-a*-specific (closed triangles) $\delta^{15}N$ in depth profiles across the rainfall gradient from dry (C) to wet (M) sites. Dashed lines set apart the vegetation samples (top) from litter samples (middle) and soil samples (bottom). The surface of the mineral soil is at a depth of 0.

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Tables

	C	F	I	M	Site average
Precipitation (mm)	210	790	1260	2500	1190
$\delta^{15}N_{bulk}$ leaf	4.15	9.14	0.39	0.46	3.54
$\delta^{15}N_{bulk}$ litter	4.29	8.95	7.18	2.8	5.8
$\delta^{15}N_{bulk}$ soil	12.39	9.91	8.21	5.13	8.91
soil %C	1.93	9.67	10.13	17.24	9.74
soil %N	0.17	0.83	0.95	1.19	0.78
soil C/N	11.51	11.31	10.62	16.25	12.42
litter %C	21.54	29.52	18.67	36.28	26.5
litter %N	1.38	1.94	1.48	1.44	1.56
litter C/N	15.66	15.24	12.61	25.17	17.17
veg %C	41.48	37.06	39.92	43.11	40.39
veg %N	2.69	2.65	2.32	2.28	2.49
veg C/N	15.5	14.99	20.92	28.18	19.9

Table 1: Site averages, for all bulk samples. Percent C and N and C/N are weight-based.

Site	Name	Chl *b*	Chl *a**	Pheo *b*	Pheo *a*	Sum All	%Pheo *a*	%Chl *a**
C	*C. ciliaria*	22119	81074	39	52545	260225	44	62
	Litter/O				5173	9622	54	0
	00-16				3225	5178	62	0
F	*P. clandestinum*	3980	36469		21452	75487	28	48
	Litter/O	525	5496		4625	14995	31	37
	03-08		559		2483	4391	57	13
	08-13		1104		1590	3687	43	30
I	*S. madagascariensis**	15264	92967		4106	144176	3	64
	Litter/O		855		2820	10036	28	9
	00-10			225	1058	1711	62	0
M	*D. incanum*	1146	9385		2432	16299	15	58
	Litter/O	276	1260		963	2952	33	43
	00-15			2357	7437	13698	54	0
	24-32			88	332	656	51	0

Table 2: Relative compound abundance in a plant sample and the litter and soil horizons on which $\delta^{15}N_{pheo}$ was measured, grouped by site. Values reported are summed peak areas of absorbance at 660 nm in units of mAU, summed for all collected injections of the sample. Values are first reported for individual compounds and then compared with the summed area of all detected chlorin peaks, first as absolute values and then as a percentage of chlorin peak area. *Chlorophyll is abbreviated Chl. *Chlorophyll *a* and ~~Py Chl~~Pyrochlorophyll *a* are combined.

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	mean ε	min ε	max ε	range	n
*C. ciliaria**	0.81	-0.45	1.54	-1.99	3
*P. clandestinum**	-0.29	-2.06	1	-3.06	5
P. pallida†‡	6.53	6.39	6.68	-0.29	2
D. incanum†	0.54	0.34	0.73	-0.39	2
S. madagascariensis	1.44	0.58	2.31	-1.73	2
M. polymorpha‡	2.48	2.26	2.71	-0.46	2

Table 3: Average, minimum, maximum, and range of intra-leaf $\delta^{15}N$ offsets between pheo *a* and bulk ($^{15}\varepsilon_{pheo\text{-}bulk_{leaf}}$) for n measurements of each sampled species across all sites. *=C4 pathway, †=N fixer, ‡=tree

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	C	F	I	M	Site average
$\delta^{15}N_{bulk}$ leaf	4.15	9.14	0.68	0.04	3.5
~~$\delta^{15}N_{phe}$~~$\delta^{15}N_{pheo}$ leaf	8.15	9.43	1.2	0.77	4.89
$\delta^{15}N_{bulk}$ litter	4.29	8.95	7.18	2.8	5.8
~~$\delta^{15}N_{phe}$~~$\delta^{15}N_{pheo}$ litter	8	4.7	5.1	0.7	4.62
$\delta^{15}N_{bulk}$ soil	10.37	10.77	7.19	5.35	8.42
~~$\delta^{15}N_{phe}$~~$\delta^{15}N_{pheo}$ soil	8.1	7.75	6.4	1.65	5.97
ε ~~phe~~pheo-leaf	3.99	0.28	0.52	0.73	1.44
Δ_{phe} veg-litter	0.15	4.73	-3.9	0.07	0.26
Δ_{bulk} veg-litter	-0.14	0.2	-6.5	-2.76	-2.3
Δ_{phe} litter-soil	-0.1	-3.05	-1.3	-0.95	-1.35
Δ_{bulk} litter-soil	-6.08	-1.82	-0.01	-2.55	-2.61
Δ_{phe} leaf-soil	0.05	1.68	-5.2	-0.88	-1.09
Δ_{bulk} leaf-soil	-6.22	-1.62	-6.51	-5.3	-4.91

Table 4: Isotope values, offsets between bulk and pheo *a* $\delta^{15}N$ values, and differences in pheo *a* and bulk $\delta^{15}N$ values across the leaf-soil continuum, across sites. Bulk values are included only for samples for which a corresponding pheo *a* measurement was made.