

Dear Editor,

You will find our point-by-point reply to both reviewers' comments below. Most of the requested amendments have been incorporated into the manuscript. In cases where we disagree with the reviewers' suggestions, we have provided a justification in the text below. Additionally, we corrected some typos and minor errors in the main text.

We look forward to your decision on the manuscript.

On behalf of the authors' team.

Łukasz Kozub

Reviewer 1:

The manuscript by Dembicz et al. addresses a timely and important topic, focusing on how accelerated anthropogenic CO₂ emissions and eutrophication have been recorded in plant tissues over more than a century using an isotopic and stoichiometric approach. In the Introduction, the discussion is largely limited to artificial fertilizers, with little attention given to organic manure, including both solid and liquid forms. Considering their well-known contribution to nutrient enrichment, I recommend incorporating this aspect to provide a more comprehensive background.

We are grateful for this positive assessment of our manuscript. Indeed our results suggest that organic nitrogen enrichment may be the main source of eutrophication in wetland habitats. However we did not focus on this issue in the introduction (we added one additional sentence to signalize the issue of other than fertilisers nitrogen sources) as the existing data (including the data on livestock numbers) suggests that the amount of organic nitrogen reaching the ecosystems has remained quite stable throughout the last century in our study area and what changed was the introduction and increase in use of artificial fertilisers. The reasons behind more pronounced organic nitrogen eutrophication in wetlands may be driven by other factors than just its amount, such as destruction of buffer zones around wetlands impeding denitrification or replacement of dispersed sources by point sources such as malfunctioning of communal, industrial and agricultural sewage treatment plants. As suggested by the reviewer we elaborated the abovementioned issues in the discussion section.

The study is thorough, based on a large number of sites and well-executed methods. However, in the Methods section, I have raised a few questions and suggestions that should be addressed. The results are clearly presented, but in Figures 3, 4, and B1, I recommend indicating statistically significant differences more explicitly. While values clearly different from zero may be considered significant, adding markers (e.g., asterisks) would improve the clarity and reliability of these figures. The discussion is sound and well-written.

We are pleased to hear those positive views on our work again. To make the abovementioned figures easier to interpret, we added an indication of the significant differences by using different colours for significant and insignificant ones.

The specific comments line-by-line with some editorial suggestions are listed below:

Line 11 and the whole manuscript: I strongly recommend using italics for small delta, as it is a common use in the stable isotopic-oriented literature.

Corrected accordingly.

Line 25: I strongly recommend briefly explaining the Suess effect in the abstract for clarity.

We followed this recommendation and provided brief explanation of the Suess effect in the abstract and expanded our rationale for accounting for it in the introduction, as also requested by the second reviewer.

Line 66-72: What about organic fertilizers, particularly liquid forms? I suggest also providing information on their relevance at both the EU and Poland scales.

We added information on the current use of organic fertilisers in Poland. However, an analysis of the existing literature (including the cited publication Tian et al. 2022) suggests that the use of natural fertilisers in the form of manure has remained fairly consistent in the study area for over 100 years. The increase in nitrogen input was primarily due to the introduction and increasing use of artificial fertilisers, while the use of manure remained stable.

Line 85 and the whole manuscript: A different font type was used than in the previous description of the nitrogen isotopic signature; please unify the style throughout the entire manuscript.

Corrected accordingly.

Line 103 – Please change "organic" to "inorganic".

Corrected accordingly.

Line 106 - In the reference list, the 2024 publication has only one author (Pronin), so “et al.” should not be used.

Corrected accordingly.

Line 119: Since the authors described this above, I suggest replacing "below" with "above".

We rephrased the whole sentence to make it more understandable and removed the term “below”.

Line 146: The species name is used here instead of the Latin family name, unlike in other cases. Please clarify this inconsistency.

This was done on purpose. That is why we mentioned “taxa” not “families”. There are known members of the Nymphaeaceae family, which have mycorrhizal associations, but the Nymphaea alba is known to be not-mycorrhizal.

Line 190: Did the authors test whether the samples contained CaCO₃ that could influence the carbon isotope results? Carbonates may be present, particularly in lake samples. Please clarify how this potential issue was addressed.

This issue was raised also by the second reviewer so we tried to address it more thoroughly. In our analysis we did not perform pre-treatment of samples with acid to remove carbonates as it can affect both total nitrogen and nitrogen isotopic composition, as well as $\delta^{13}\text{C}$ values of organic carbon (<https://besjournals.onlinelibrary.wiley.com/doi/10.1111/2041-210X.12183> <https://www.sciencedirect.com/science/article/abs/pii/S0009254111000222?via%3Dihub>). Most of our samples were collected from the parts of the plants growing above the water and we wanted to follow a standardized protocol for all of the samples. In our dataset we had only two submerged species which could contain CaCO₃ on their surface: Stuckenia pectinata and Elodea canadensis.

We performed additional analyses requested by Reviewers 1 and 2 regarding the impact of the lack of acid treatment in our methodology. We used 12 contemporary samples: six from Elodea canadensis and six from Stuckenia pectinata. We employed a modified approach suggested by Kennedy et al. (2005) (<https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/10.1002/rcm.1889>). Due to the

*lack of visible CO₂ release, we could not use this as an indicator of complete carbonate removal. This enabled us to assume a very low carbonate content, so we used a weaker acid (0.3 M) to minimise the loss of organic matter. We weighed four aliquots from each homogenised sample into silver capsules. We then added 100 ml of 0.3 M HCl directly to two of the aliquots in the capsules. After one hour, we dried the samples at 40°C overnight. This process was repeated once more. We then closed all the capsules, and analysed one pair of acid-treated and control samples for total carbon and nitrogen, and the second pair for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. The results show no effect of acid treatment on $\delta^{15}\text{N}$ and total nitrogen (TN) content, nor on $\delta^{13}\text{C}$ for *Stuckenia pectinata*. The only statistically significant difference was observed in *Elodea canadensis*; however, the mean difference in $\delta^{13}\text{C}$ between treated and untreated samples was 0.44‰ (90% confidence interval: 0.141 to 0.739), whereas the mean difference observed in our study between historical and contemporary samples was -8.38‰ (90% confidence interval: -15.22 to -1.55). Thus, the possible error (including its uncertainty and the uncertainty of the results) is no greater than the difference observed between contemporary and historical samples. Moreover, as we treat contemporary and historical samples of plants inhabiting similar habitats in the same way, systematic methodological error would not lead to the detection of false patterns.*

We described the whole procedure and its results and embedded it in the manuscript as the Appendix A.

Line 194-195: The names and isotopic values of the standards used for calibration should be reported.

We used four standards for the calibration curve ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values respectively in brackets: USGS65 (-23.29‰, 20.68‰), USGS40 (-4.52‰, -26.39‰), USGS41a (47.55‰, 36.55‰) and Urea#3a (5.89‰, 42.05‰) from Schimmelmann Research and IAEA-600 (-27.77‰, 1.02‰) for recalculation and precision assessment. We included these information in the reviewed manuscript as it was also requested by the second reviewer.

Line 225-226: Please provide the name of the software package used, along with the appropriate citation.

As the models were just simple linear models we did not use any packages but just functions available in 'stats' R-package embedded in base R, which was cited in the manuscript.

Line 240: Figure 2 Caption: What do the whiskers in these boxplots represent? Please clarify.

The boxplots were created using the default settings of 'ggplot2' package where whiskers extend from the hinges to the largest/smallest values within 1.5 times the Interquartile Range (IQR) and the points represent the samples with values outside of this range. We added this information to the figure caption.

Line 318: The upper index in the nitrogen signature is missing.

Corrected accordingly.

Line 320-321: The upper index in the nitrogen signature is missing.

Corrected accordingly.

Line 323: The upper index in the carbon signature is missing.

Corrected accordingly.

Line 325: It would be beneficial to include, in the introduction, relevant information on this issue in Poland in recent years, particularly after 2022, when the prices of artificial fertilizers increased dramatically due to the escalation of the war in Ukraine.

Indeed, the use of artificial fertilisers decreased by 11% in 2022 compared to 2020, and then by a further 6% in 2023. However, there is no data to indicate whether this was due to lower fertilisation levels or the replacement of artificial fertilisers with natural ones. In the regions studied, the ratio of nitrogen provided by artificial and natural fertilisers varies from nearly equal proportions (with slightly more organic fertiliser) in the Podlaskie region, to less than a third provided as natural fertiliser in the Lubelskie region. This pattern is probably even more complicated at smaller spatial scales, with sub-regions specialising in animal production, where manure is available and widely used, and locations where crop production dominates and artificial fertilisers are used more frequently. We discussed this issue in more detail in this section, using the information provided here. In the introduction we provided most up-to-date data on use of organic fertilisers in Poland.

Line 374: The lower index of 3 in nitrite is missing

Corrected accordingly.

Line 440 Figure B2: The equation of the regression lines, as well as the R² and P values, should be presented.

We added requested information to the abovementioned figure.

Reviewer 2:

Overall, the manuscript by Dembicz et al., entitled “Contrasting isotopic responses of dryland and wetland plants to a century of global anthropogenic changes in nutrient cycling,” represents a valuable contribution and is worthy of publication. The study is supported by a robust dataset encompassing diverse vegetation types and ecosystems, and it provides important insights into nutrient cycling and its response to anthropogenic influences.

However, I recommend that the manuscript undergo revisions prior to publication, particularly to clarify several aspects and improve overall clarity. Detailed comments and suggestions are provided below:

We would like to thank you for the overall positive assessment of our work and we hope that we managed to improve the issues that have been raised in the provided revision.

Introduction:

The background of the study and the research gap are clearly articulated and well-supported by several relevant previous studies. However, I suggest that several improvements should be considered, including the following:

I understand that the authors aim to outline the limitations and research gaps in the use of carbon and nitrogen isotopes. However, this section is overly detailed and may cause readers to lose focus on the main highlights of the study.

We tried to shorten this section without losing any important content.

I suggest including an explanation of the Suess effect in the background section, as this factor constitutes a substantial part of the discussion.

We provided more elaborate explanation of the Suess effect in the background section and our rationale behind removing this effect as it was also required by the first reviewer.

Lines 125–130: I recommend restructuring this paragraph. It would be clearer to present the study objectives explicitly rather than framing them as hypotheses.

To be honest, we would prefer to present the objectives or research questions rather than the hypotheses. However, our general impression is that most journals currently require hypotheses to be stated clearly. Therefore, we have retained the hypotheses and will leave this issue to the handling editor's decision.

Laboratory Analysis:

Did the authors perform acid treatment prior to the isotope analysis? If not, how do the authors ensure that the samples are free from contaminants or external influences?

*We did not perform any treatment, as it can affect both total nitrogen and nitrogen isotopic composition, as well as $\delta^{13}\text{C}$ values of organic carbon (<https://besjournals.onlinelibrary.wiley.com/doi/10.1111/2041-210X.12183> <https://www.sciencedirect.com/science/article/abs/pii/S0009254111000222?via%3Dihub>). We cleaned the samples (leaf fragments) manually by gently scratching their surfaces with a tool to remove possible contamination. However, as the first reviewer also raised the issue of possible contamination of submerged species (*Stuckenia pectinata* and *Elodea canadensis*) samples with inorganic carbon from CaCO_3 precipitation, we decided to check the impact of sample pre-treatment*

with acid on the results of the analysis, especially those related to carbon isotopes, for these two species. The detailed results of this check were provided in the answer to the first reviewer as well as in the Appendix A, which we added to the manuscript body.

Please specify the internal standards used, along with the analytical precision and accuracy based on these standards. Furthermore, I would like clarification on how frequently the standards were analyzed to ensure data reliability (e.g., after every 10 unknown samples). Were blank samples also analyzed?

We measured a blank (empty) run every day to check for background contamination, and one empty tin capsule from each batch to ensure capsule cleanliness.

We used four standards for the calibration curve: USGS65, USGS40, USGS41a and Urea#3a from Schimmelmann Research and IAEA-600 for recalculation and precision assessment. A calibration curve was prepared at the beginning of each day of measurements, along with 3 IAEA-600 standards. Measurements only started if the results of all three samples do not differ to the certified values by 0.2‰ for $\delta^{13}\text{C}$ and 0.1‰ for $\delta^{15}\text{N}$. Daily, about 40-60 samples were analyzed. Each 10 samples a IAEA-600 standard was measured, and sample results were only included, if the standard result fell within the abovementioned threshold. We included the above information in the manuscript.

Did the authors separate specific plant components prior to isotope analysis, or were the samples processed as bulk material (e.g., via whole-sample grinding)?

We only analysed leaf samples (not stems, roots or rhizomes), but the leaf fragments were ground using whole-sample grinding. We rephrased some sentences to make it more clear.

Discussion:

1. The current discussion section is overly long, which may reduce readability and obscure the main points. I recommend dividing it into several subsections aligned with the study objectives outlined in the Introduction.

We divided the discussion into subsections aligned with the study objectives according to the reviewer's suggestion.

2. The authors consistently refer to the data as being derived from leaves; however, this is not clearly specified in the methodology. Please address the comment in the Laboratory Analysis section. For example, in Line 295 (“...we found a significant decrease in $\delta^{15}\text{N}$ values by 3.14‰ on average in contemporary plant leaf tissues compared to those from the pre-Green Revolution period”), did the analysis indeed focus specifically on leaf tissues?

We would like to point out that in lines 180-181 we explicitly state that “Each contemporary sample, similarly to the historical ones, consisted of two to five leaf fragments collected from several individuals per locality (Fig. 1).” However, we added this information also to the laboratory analysis section to make it clearer.

3. Depletion of Nitrogen Isotopes in Grasslands:

I agree with the authors’ proposed interpretations; however, prior to presenting the two hypotheses, it would be beneficial to acknowledge that species differences between grassland and wetland ecosystems can inherently lead to variations in isotopic signatures. For instance, C_4 plants or shrub-dominated vegetation may rely more on atmospheric nitrogen fixation, resulting in $\delta^{15}\text{N}$ values closer to atmospheric N ($\sim 0\text{‰}$) or lower than those typically observed in C_3 plants.

*We thank the reviewer for this valuable comment. However, all of the species studied are herbaceous C3 plants, which is typical of grassland and wetland habitats throughout the Palearctic. None of them are nitrogen-fixing either. The nitrogen-fixing strategy may be present in both wetland (woody genera such as *Alnus*) and grassland (*Fabaceae* family) habitats, but the contribution of nitrogen derived from nitrogen fixers may vary between sites. We must also consider the multiple microbial processes through which this nitrogen must pass before it can be incorporated into our focal plants, which alter its isotopic composition. There is also no indication that the proportion of nitrogen-fixing organisms in the focal communities has changed over time, which could lead to a change in the isotopic composition of the modern community compared to the historical one.*

I also support the hypothesis of grasslands as vulnerable ecosystems. This interpretation could be further strengthened by incorporating the concept of canopy gaps, which may enhance atmospheric nitrogen fixation dominance. Furthermore, I recommend expanding the discussion by integrating relevant literature to support these arguments, such as the study available at: <https://www.nature.com/articles/s41598-025-01123-x> and references therein.

We thank the reviewer for this suggestion and recommendation of the valuable literature. However, it may be that the reviewer's experience comes from humid tropical ecosystems, where forests are the dominant vegetation type and other types only occur as short-term stages following natural or human-caused disturbances. Due to fast biomass turnover and intensive leaching, living plants are the main nutrient stores, so secondary succession may be strongly limited by nutrient availability, promoting nitrogen-fixing strategies. Our study systems seem different. Although their primary productivity may be limited by nutrient availability (predominantly nitrogen), these shortages may be due to slow mineralisation rates (in wetlands, due to anoxic soil conditions; in dry grasslands, due to insufficient water supply) or denitrification processes (in wetlands). Even though, under the current climate and wild herbivore densities, the studied grasslands are semi-natural and secondary habitats, with appropriate management they can remain stable for long periods and develop mature, organic-matter-rich soil profiles. Thus, nitrogen-fixing strategies are present in both wetlands (which may seem like 'climax' habitats) and dry grasslands, but they are seldom dominant. The aforementioned C4 plants could also be present in both habitats, but their presence is negligible due to the cool climate of the region under study. Moreover, as mentioned before, we do not assume any major shifts in species composition or dominant nutrient acquisition strategies in the studied habitats over the last century thus we attribute the observed patterns to the alteration of nutrient cycling in a broader landscape.

4. Enrichment of Nitrogen Isotopes in Wetlands:

I agree with the authors' interpretation that nitrogen isotope enrichment may be associated with fertilizer use during the pre-Green Revolution period. However, it is important to acknowledge that natural processes can also generate nitrogen enrichment in wetland ecosystems. This aspect appears underexplored and warrants further discussion beyond the anthropogenic factors already well presented.

We thank the reviewer for this valuable comment. However, our study focuses on changes in the nitrogen isotopic signature over time for specialist species with quite narrow niches that occur in the same or nearly the same locations. We have no evidence or reason to assume that natural processes of nitrogen enrichment (runoff and mineralisation) and cycling (denitrification) in these ecosystems have substantially changed over the last century, resulting in changes to their isotopic composition other than those caused by humans.

The degree of organic matter decomposition is commonly reflected by TN and $\delta^{15}\text{N}$ values. Under aerobic conditions, peat decomposition promotes preferential utilization of ^{14}N by microorganisms, resulting in residual peat enriched in ^{15}N and total nitrogen. Moreover, vegetation growing in aerobic peat conditions may preferentially utilize atmospheric nitrogen sources, as plants tend to assimilate lighter ^{14}N , leading to relatively low $\delta^{15}\text{N}$ values (e.g., $<3.0\%$) in plant tissues and litter. Therefore, these patterns may also represent signals of natural organic matter decomposition processes.

To strengthen this discussion, I recommend incorporating relevant literature, such as:

<http://dx.doi.org/10.1002/jqs.2541>

<http://dx.doi.org/10.5194/bg-12-2861-2015>

We thank the reviewer for this valuable insight and for providing the relevant literature. It is true that the degree of peatland drainage has increased tremendously in the study area over the last century (most large-scale drainage campaigns were carried out after the Second World War). However, as our study species have quite narrow niches with regard to wetness, we can assume that the moisture conditions in which they grow today are similar to those in the past. Those species that could be associated with peatlands (in our case, Cardamine amara and Carex paniculata) are likely to grow in permanently wet sites with anaerobic conditions today as well. Nevertheless, we acknowledge that landscape-scale peatland drainage and agricultural management could impact nitrogen and carbon in wetland habitats (including fully aquatic ones) outside peatlands, through the input of inorganic nitrogen resulting from peat aerobic mineralisation, as well as the input of particulate and dissolved organic matter, which can decompose in water. We have discussed these issues in lines 329–330 and have already cited the publication by Krüger et al. (2015).

5. Line 330: “the observed pattern in wetland species could be attributed to increased runoff of artificial nitrogen fertilisers into water systems, leading to elevated nitrogen concentrations and resulting in higher rates of organic nitrogen mineralisation in water, followed by increased rates of nitrification, leading to elevated $\delta^{15}\text{N}$ values (Diebel and Zanden, 2009

Such a model, in which increased runoff enhances nitrogen concentration and subsequently promotes higher rates of organic nitrogen mineralization and nitrification under natural conditions, has been proposed by Patria et al. (2025; <https://www.nature.com/articles/s41598-025-01123-x>). This framework could further support the interpretation of the present results.

We would like to thank the reviewer for supporting our model of nitrogen transformations (including denitrification), which leads to elevated $\delta^{15}\text{N}$ values in nutrient-enriched waters. However, we could not find relevant support for this model in the proposed paper, as it deals with the isotope composition of a peat core. It therefore studies residual nitrogen and carbon that is unavailable to plants, whereas in our case, the signature of mobilised nitrogen and carbon available to plants would be more relevant.

6. Line 355: “especially important in dry habitats where primary productivity could be limited by drought”

Nitrogen productivity limitation under drought conditions has been widely documented across various ecosystems and is often associated with $\delta^{15}\text{N}$ depletion signals. This pattern is generally attributed to shifts in nitrogen cycling and isotopic fractionation under water-limited conditions, including changes in microbial activity and nitrogen availability.

We thank the reviewer for this valuable comment. Generally, we do not anticipate significant changes to habitat conditions, except for nutrient supply and potentially management patterns (cessation of grazing), when comparing the habitats studied today with those in the past. Dry grasslands are habitats that develop in locations with a water deficit, both now and in the past. However, due to climate change leading to increased temperatures and evapotranspiration, we could assume that drought stress in these habitats has increased over the last 100 years, which may also contribute to the lowering of $\delta^{15}\text{N}$ in dry grasslands. We added additional sentence with this interpretation to our discussion.

7. Line 365:

“In the case of wetland plants, there was an overall visible increase in total nitrogen content in plant tissues when contemporary plants were compared with herbaria vouchers from the pre-Green Revolution period.”

Did the authors conduct tissue-level analysis in this study? If so, this should be clearly described in the methodology. Alternatively, please clarify what is meant by “tissue” in this context. As a reader, the term tissue analysis suggests a more detailed examination of specific plant structures (e.g., epidermis, parenchyma), which is not currently evident from the methods described.

To strengthen this argument, I recommend incorporating relevant studies, such as:

<https://doi.org/10.1016/j.revpalbo.2021.104482>

[https://doi.org/10.1016/S0009-2541\(98\)00105-3](https://doi.org/10.1016/S0009-2541(98)00105-3)

By 'tissues', we only meant 'leaf tissues'. We did not conduct a tissue-level analysis. We rephrased some sentences in the methodology section to emphasise that we used bulk-processed samples (whole-sample grinding) of plant leaves, as this issue was also raised in one of the initial comments.

8. Line 385: “significant decrease in $\delta^{13}\text{C}$ since historical times in dry grassland plant tissues. This is contrary to the expected increase in water-use efficiency (Seibt et al. 2008) caused by water shortage, and is hard to explain.”

Methanogenesis may lead to ^{13}C enrichment in residual organic matter, as methanogenic microorganisms preferentially utilize ^{12}C under anaerobic conditions. Moreover, rainfall and groundwater levels are typically negatively correlated with $\delta^{13}\text{C}$ values in C_3 plants and peat, reflecting shifts in photosynthetic processes under water-stress conditions.

[http://dx.doi.org/10.1016/S0012-821X\(00\)00367-8](http://dx.doi.org/10.1016/S0012-821X(00)00367-8)

<https://scispace.com/pdf/stable-isotopes-and-organic-geochemistry-in-peat-tools-to-4dvgzqrkh7.pdf>

<https://doi.org/10.1002/jqs.254>

We would like to thank the reviewer for their valuable comments on the impact of methanogenesis on $\delta^{13}\text{C}$ values in residual peat. However, the aforementioned sentence on line 385 refers to the results for dry grassland plants, which obtain their carbon solely from the atmosphere and thrive in fully aerated soil habitats where methanogenesis is hardly possible. With regard to the impact of drought on $\delta^{13}\text{C}$ values, the publications presented also interpret increased $\delta^{13}\text{C}$ values in plant remains as signs of water deficits and decreased values as signs of water surpluses. Perhaps our results should be interpreted as signals of increased water use efficiency in plants due to increased ambient CO_2 concentrations, leading to more efficient CO_2 uptake and thus lower water loss, which reduces drought stress and lowers $\delta^{13}\text{C}$ values in plants, even though climate change is leading to

more severe droughts in terms of absolute water availability, as mentioned previously. We included this interpretation in the discussion.