Publisher's note: this comment was edited on 31 March 2025. The following text is not identical to the original comment, but the adjustments were minor without effect on the scientific meaning.

Major Comments

One of the highlights of the manuscript "Enhanced isotopic approach combined with microbiological analyses for more precise distinction of various N-transformation processes in contaminated aquifer – a groundwater incubation study" is the simultaneous use of isotopic labeling and natural abundance stable isotope methods to investigate the nitrogen cycling processes in groundwater and the sources of nitrous oxide (N_2O) production. The study found that nitrite (NO2-) may originate from the mineralization of dissolved organic nitrogen (DON) under conditions of high dissolved organic nitrogen. Furthermore, the nitrogen transformation processes are highly sensitive to the availability of organic carbon. The increase in the C/N ratio due to the additional organic carbon promotes the transition of nitrification processes to denitrification processes in groundwater. This manuscript is significant for a deeper understanding of the sources of nitrogen pollution in groundwater and the migration and transformation processes of nitrogen. The conclusions of this study provide theoretical support for explaining the sources and mechanisms of high nitrate pollution in groundwater.

Thank you for your positive evaluation of our study and all the comments which helped us to further improve this work. We have addressed all the critical comments as explained below.

Specific comments

1. Line 45-48: It is suggested to explain the specific anthropogenic sources and natural processes involved in groundwater.

   Thank you for your comment. We have revised the manuscript and added the lines as below:

   Controlling NO_3^- levels in aquatic systems presents substantial environmental challenges, particularly in groundwater, due to the complexity of differentiating between its anthropogenic sources- such as fertilizer runoff, waste from livestock manure, industrial waste water discharges- and natural processes including soil organic matter mineralization, precipitation and biological nitrogen fixation by microorganisms.

2. Line 138-139: Supplement the salinity and dissolved oxygen concentration values of the groundwater samples collected from the monitoring wells.

Thank you for your comment. The dissolved oxygen (DO) concentration range of 2.2–4.3 mg L^{-1} provided in the manuscript reflects typical suboxic groundwater conditions reported for this aquifer system and is based on previous field campaigns in the Wołczyn region (Olichwer et al., 2012). In the present study, individual DO values were not systematically measured for each piezometer during sampling, and therefore, this range is intended to provide a general reference for groundwater conditions. We are aware that this is a major shortcoming of our dataset, but unfortunately this was due to unexpected problems. We did the sampling campaign in collaboration with routine monitoring action performed by private company, who should have measured all the physicochemical parameters. Only afterwards we learned that O2 was not included and due to major logistics problems this could not be repeated. But from the archival data we see quite a narrow and stable range of these values, so we strongly believe that this range is well representative for our current samples.

3. Line 139-142: Why were these four monitoring well water samples selected for laboratory cultivation? Please clarify the selection criteria.

The major criterium was the nitrate concentration, but the second one was also the possible water gain from a piezometer. Some of the wells were quickly pumped out and no more water could be collected. This was eg. the case for P-0 and P-3 which exhibited comparably high nitrate concentration levels, but must have been excluded from the incubation experiments due to limited water availability at the time of sampling.

4. Line 181-188: Indicate the detection limits for the inorganic nitrogen analysis methods.

For our analysis, wavelengths of 520 nm, 560 nm, and 610 nm were selected for NO_3^-, NO_2^-, and NH_4^+ concentration, respectively, with detection limits of 0.1–50.0 mg L-1 for NO_3^-, 0.02–1.0 mg L-1 for NO_2^-, and 0.01–5.00 mg L-1 for NH_4^+.

This information was added in the manuscript.

5. Line 204-207: What is the spatial relationship between these monitoring wells and the discharge point (the retention area for yeast production wastewater)? Is there a possibility that the high DON in the yeast wastewater mineralizes to ammonium and subsequently nitrifies to NO3-? Table 1 shows that the monitoring wells with high

NO3- concentrations also have higher DON concentrations (except for the discharge point P-L1). If this is the case, it is necessary to also focus on monitoring wells with high ammonium and low NO3- to compare with high NO3- water samples, thus revealing the process of high DON converting to NO3- in groundwater and the nitrogen transformation conditions.

Thank you for this important comment. Yes, the process undergoes as you described. We have revised the manuscript and added the following paragraph in the discussion section to address this point:

'The increased concentrations of both DON and NO_3^- concentrations in several piezometers—particularly those located near the yeast wastewater lagoon, such as P-7 and P-23—suggests that organic nitrogen input may contribute to higher NO_3^- concentration. Although direct groundwater flow paths were not explicitly studied, the spatial positioning of these wells to the lagoon, along with their elevated DON levels, supports the possibility of influence from wastewater discharge. One possible explanation could involve microbial mineralization of DON to NH_4^+, with rapid nitrification to NO_3^- under suboxic conditions. This is also further supported by low NH_4^+ concentrations across most wells indicating either rapid nitrification or low NH_4^+ content. Only one piezometer P-4 showed higher NH_4^+ along with high DOC and DON. But due to very low NO_3^- levels and significantly higher conductivity (~4794 µS/cm) compared to other wells, it was unsuitable for comparison with the selected incubation samples and was therefore excluded from the incubation experiments. While other piezometers such as P-16 and P-20 are more distant from the lagoon, their chemical concentrations may indicate higher nitrogen inputs from varied agricultural sources. Future studies should integrate groundwater level measurements or tracer-based studies to confirm source connectivity between lagoons and piezometer, along with sampling of ammonium-rich, nitrate-poor locations for better analysis of nitrogen transformation pathways.'

6. Line 213-215: Does this mean that the DO concentration in the water of the monitoring wells falls within this range? Since this data is not shown in Table 2, it is suggested to include the DO concentration values of the field monitoring wells in Table 1.

Thank you for your comment. The dissolved oxygen (DO) concentration range of 2.2–4.3 mg L^{-1} provided in the manuscript reflects typical suboxic groundwater conditions reported for this aquifer system based on previous field campaigns in the Wołczyn region (Olichwer et al., 2012). In the present study, individual DO values were unfortunately not systematically measured for each piezometer during sampling, and

therefore, this range is intended to provide a general reference for groundwater conditions.

We have clarified this point in the revised manuscript to avoid confusion with the DO values reported in Table 2, which reflect dissolved measurements dynamics during laboratory incubation, at different time points.

7. Line 220-221: Why is the volume of the added labeled liquid different here?

The added volume of ^{15}N-labelled tracer solution was adjusted according to the nitrate concentration in each sample to achieve a comparable level of isotopic enrichment across all samples while minimizing alteration to the natural isotopic composition.

8. Line 263-266: Please specify the detection limit for the denitrifying bacteria method.

The denitrifier method using Pseudomonas aureofaciens enabled isotope analysis of NO3- at concentrations as low as 40 nmol NO3− L-1 (Stock et al., 2021) (Deb and Lewicka-Szczebak 2024), while using Stenotrophomonas nitritireducens allowed for NO2- analysis at concentrations as low as 150 nmol NO2− L-1 (Deb and Lewicka-Szczebak, 2025).

This information was added in the manuscript.

9. Line 308-309: The NO3- concentration in the P-0 monitoring well is higher than that in P-7, yet it was not selected for further cultivation studies. Why?

This has been already explained under section 2.2. Although P-0 and P-3 also exhibited comparably high nitrate concentration levels, they were could not be further studied in the incubation experiments due to limited water availability at the time of sampling.

This clarification was added in the manuscript:

'Due to restricted water availability in some piezometers, where the aquifer was quickly pumped out and the water amount required for the further incubations (min. 750 mL) could not be collected, some potentially interesting piezometers must have been omitted. This was the case for eg. P-0 and P-3 where despite high NO3- concentrations the incubations were not possible due to too less water gain.'

10. Line 314-316: Please add the detection method for NH4+ isotopes and its detection limit in the methods section.

Thank you for the comment. We would like to clarify that although NH_4^+ concentrations were measured in our study, stable isotope analysis $\delta^{15}N\text{-}NH_4^+$ was not performed due to concentrations being too low.

This clarification was added in the manuscript.

11. Line 600: Should it be "labelled NO3- reduction"?

Corrected

12. Line 632-633: According to Figure 7, before the addition of glucose, some points (despite their low NO2- concentrations) tended towards heterotrophic nitrification, while after the addition, they clustered more in the autotrophic nitrification range. Please verify the data and correct the result description.

Thank you for your comment, you are right. We have revised the discussion in the manuscript as follows:

'Before the addition of glucose, the isotopic signatures (empty circles) were more scattered with several points within or near the heterotrophic nitrification zone, suggesting a mix of both autotrophic and heterotrophic pathways to nitrite production under low-carbon conditions. . Some clustered points were also observed near autotrophic nitrification area indicating that autotrophic bacteria, such as Nitrosomonas europaea were likely involved in conversion of ammonia (NH_3) to nitrite (NO_2^-) as an energy-generating process (Deb et al., 2024). During this process, CO_2 serves as the sole carbon source for these bacteria, assimilated into their biomass to support cellular growth, independent of the chemical reaction used for energy generation (Hommes et al., 2003). In the groundwater samples, CO_2 likely originated from the decomposition of organic matter in the yeast sewage (Section 2.1) or from the carbonate system naturally present in groundwater (Section 3.1.1).

The application of yeast-based sewage as fertilizer in the agricultural site likely introduced organic nitrogen into the groundwater, which undergoes microbial decomposition to release ammonium (NH4+) through the mineralization of proteins and amino acids (Watanabe et al., 2023). However, NH4+ was not detected in the groundwater samples (Table 1), indicating its rapid transformation within the nitrogen cycle, such as nitrification and assimilation.. Microbial assimilation likely contributed to NH4+ exhaustion, as microbes utilized it for biomass synthesis. However, this is not the case for sterile samples, where we observe slight accumulation of NH_4^+, indicative of biological uptake in NH_4^+ turnover.

Following glucose addition, the isotopic signatures (filled circles) were more concentrated within the autotrophic nitrification zone, which indicates that autotrophic nitrification continued to dominate nitrite production despite under elevated carbon conditions. While a shift towards heterotrophic nitrification might be expected under increased carbon availability, the isotope data suggest that autotrophic ammonia-oxidizing bacteria remained more metabolically active than the heterotrophs under the given incubation conditions. Together, these findings demonstrate the rapid transformation of NH4+ from yeast-based fertilizers into intermediate nitrogen compounds, driving nitrification and subsequent nitrogen cycling processes in groundwater'.

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13. Line 804-806: This conclusion is very interesting. Was there simultaneous monitoring of DON during the cultivation period? What was the magnitude of the DON changes?

Thank you for this comment.

Dissolved organic nitrogen (DON) concentrations were determined in the initial groundwater samples prior to incubation and are presented in Table 1, but variation in DON during the incubation period were not assessed. We admit it should have been done, but at the time of performing the incubations we did not really expect possibly important role of DON mineralization. We constructed this incubation experiment rather to check denitrification rates and the associated isotope effects. Only when evaluating the results, which were partially very surprising, we discovered the importance of DON unusually high concentration, as for groundwaters. Therefore, we based our conclusion about DON's role in nitrite production on the high initial DON concentrations and also the isotopic data, which suggest that nitrogen from natural (unlabelled) sources — likely including DON — was actively involved in the nitrification process.

14. Line 845-846: The samples collected in this study are all high in NO3- or DON, and samples with high NH4+ concentrations were not analyzed. Therefore, the sources of NO2- under high ammonium conditions and the relationship between NO2- and DON at that time cannot be shown. This is a significant limitation of the study. It is suggested to address this in the discussion.

Thank you for this comment. We have revised the manuscript.
'While this study focused on samples with elevated NO_3^-/DON concentrations to investigate nitrogen transformation processes, samples with higher NH4+

concentrations were not included in the incubation experiments as they typically showed low NO_3^- levels and below detection isotope signatures, further limiting their utility for isotopic-based analysis. As such, the role of NH4+ in NO_2^- formation under such conditions could not be evaluated and requires further research'.

Citation: https://doi.org/10.5194/egusphere-2025-754-RC1

RC2: 'Comment on egusphere-2025-754', Anonymous Referee #2, 27 Apr 2025

The work by Deb et al. presents an isotope study of inorganic N and N2O turnover from a contaminated groundwater system. With both natural abundance and light 15N-tracing methods, they determined the source/process contributions based on empirical isotope fractionation effects. In addition, by adding glucose to several groundwater samples, they examined the response in N turnover processes. Overall, this is a detailed mechanistic work exploring nitrate, nitrite and N-gas production dynamics from groundwater system. However, much remains to be improved and clarified.

Thank you for your positive evaluation of our study and all the comments which helped us to further improve this work. We have addressed all the critical comments as explained below.

- This work is written in a loose manner, and organized in a sophisticated way. For example, lots of "raw" data were presented in a simple table without better organization and comparison. A lot of paragraphs include only one or two sentences. For sections 4.1 and 4.2, results/discussions were simply piled up without better sorting for highlighting important outcomes. Further, almost half of the figures and tables, together with descriptive contents for methods, should be moved to SI.

  Thank you for this comment. We have revised the manuscript – we reorganized the sections and improved the clarity.

- The closed-system assumption is very important for such source partitioning work based empirical isotope fractionation effects. While the key assumption is not too different from previous work, the major uncertainties still exist. It is important to limit the discussion of source contributions, and provide some uncertainty discussions.

Thank you for this comment. We have revised the manuscript. Discussion on this issue was added:

'Isotope-based source partitioning in this study assumes a closed system approach. However natural groundwater environments may often exhibit open-system behavior due to water movement, nutrient inflow, and microbial activity and therefore, the estimated source contributions- especially based on isotope mass balances and FRAME modeling may represent results under controlled laboratory conditions. Although tracer application and microbial data helped minimize uncertainties from concurrent processes such as DON oxidation or oxygen exchange, certain limitations still apply and require future field applications to validate closed-system approaches under varying conditions.'

- The water-incubation experiments were somewhat disconnected with the real-field scenarios, at least based on the current manuscript version. The authors should further rationalize why glucose addition is important. Also, how would the knowledge of the incubation study be transferred to real-field understanding?

Thank you for this comment. We have revised the manuscript by adding the links between field and laboratory studies and additional explanation of glucose application was added as follows:

'To simulate microbial denitrification under suboxic conditions, glucose was added as a carbon source. This approach is also supported by previous research (Liu et al., 2022) where external carbon addition, particularly glucose, can significantly enhance biological denitrification and nitrate removal efficiency in groundwater. This helps understand how elevated organic carbon (e.g., from wastewater or agricultural leachate) could influence N-transformations in the field. In our study, the observed shift from archaeal-driven nitrification to bacterial denitrification highlights the role of carbon availability in nitrogen cycling pathways. While lab incubations cannot fully mimic complex field-scale conditions, they provide insights into microbially mediated processes. Future in situ studies incorporating natural carbon amendments would help validate these findings under real, open-system aquifer conditions'

Title: the current tile is too long. In addition, "enhanced" may not fully represent the research approach here and it is difficult to define which is more "enhanced". Considering

that microbial analysis and incubation have been applied, why not call this approach a "combined" or something similar?

The title has been changed to shorter and clearer version: 'N-transformations in nitrate-rich groundwaters tracked with combined isotope and microbial approach'

Line 50-56: While NO3- and NO2- are two major components in the groundwater environment, some more literature review regarding inorganic N (particularly NO2-) pollution and related turnover process should be mentioned.

Thank you for this comment. We have revised the manuscript

'Also, Nitrite (NO_2^-), often a transient intermediate in the nitrogen cycle can accumulate under certain environmental conditions and pose significant environmental and health risks such as methemoglobinemia (blue baby syndrome) in infants and carcinogenic nitrosamines (Ward et al., 2018). Further, elevated NO_2^- levels in water bodies and agricultural soils, can be toxic to aquatic life and humans, and therefore underscore the importance of monitoring NO_2^- alongside NO_3^- in groundwater systems.'

Line 240-250: What were the calibrate standards used in both this study and in the comparison at Thünen Institute? How many calibration points were available?

We applied the recently produced and distributed standards in frame of DASIM project (DASIM 16, DASIM 17, 1ppm N2O) (Well et al., 2024). In both laboratories two standards have been used with high and low N2O isotope values, so that isotope normalization of each isotope value is performed based on 2 point calibration line. Additionally, as a quality check standard the commercially available technical N2O gas mixture was applied – to check for stability, drifts and calibration control. Additionally, the set of different N2O concentration standards (produced from technical N2O gas bottle) was applied to perform the amount correction, especially needed forCDRS analysis.

A clarification on the standards applied has been added into the manuscript.

Line 316-318: To indicate denitrification process, the NO3- concentration also matters. According to Kendall et al. (2007), the reduction of NO3- concentration should coincide with the increase of 15N-NO3-, which allows the estimation of isotope enrichment factor.

Thank you for this comment. We have added this information with the respective citation in the manuscript

'. According to (Kendall et al., 2007; Kendall and Aravena, 2000) a parallel decrease in NO_3^- concentration and increase in $\delta^{15}N–NO_3^-$ is characteristic of denitrification and allows

estimation of nitrogen isotope enrichment factor, and helps quantify microbial NO_3^- reduction.'

Section 4.1: As for quite affirmative deductions of NO3- or NO2- sources/processes (e.g.,Line 461-463; Line 481-483; Line 502-504), it is important to note that such source partitioning may only apply to close-system assumption. In groundwater studies, one-time sampling may be rather uncertain and cannot fully avoid the uncertainties from water exchange and nutrient diffusion.

Thank you for this comment. We definitely agree that a close system assumption is not valid for groundwater filed studies and we added the critical discussion about this issue.

However, we believe that the deductions of NO2- sources and the relation NO3-NO2 gives a true insight into the ongoing processes, because NO2- is a very short-living compound and actually provides a snapshot of actually occurring processes, it can be really transported on the longer distance, so even in open system the formation of this compound (NO2-) most probably only depends on the in-situ available substrates.

We have revised the manuscript as follows:

'Although the isotope signatures provide strong evidence for active denitrification and mixed nitrification pathways in the aquifer, it is important to acknowledge that these isotope-based interpretation of NO_3^- and NO_2^- transformations are based on single-timepoint groundwater sampling in open aquifers. Therefore processes such as water exchange, nutrient diffusion, and variations in nitrogen transformation may also influence the observed isotopic signatures. To better understand which potential N transformations are active in the aquifer we performed the laboratory incubations under controlled conditions of the selected groundwater samples.'

Section 4.2: The part for 4.2.1 is too long and written in a complexed way. It is better to move lots of descriptive contents into the result section.

Thank you for this comment. We have revised the manuscript.

Line 700-709: How to rationalize the difference among P-7, - 16, -20 and -23, regarding the source contributions after glucose addition?

Thank you for this valuable comment. We have revised the manuscript.

'The observed variations across the piezometers after glucose addition were not uniform and can be attributed to site-specific conditions such as initial nitrate concentrations, DON levels, and also variation in microbial communities. Notably, nosZI gene abundance

increased across all piezometers, with higher enrichment in P-16 and P-20, and suggests enhanced denitrification potential. Conversely, archaeal amoA gene abundance declined—particularly in P-7 and P-23—indicating a microbial shift from archaeal-driven nitrification to bacterial denitrification. These patterns highlight how suboxic, carbon-rich conditions can selectively enhance denitrification, depending on environmental conditions.'

As seen in the revision, most concerns have been addressed. Regarding the assumption of closed-system assumption and possible uncertainties, it would be good to address this in the discussion, comparing with other studies in groundwater systems, instead of only mentioning in the final conclusion.

Thank you for this comment. We have revised the manuscript.

'4.2.5. Comparability of laboratory incubation and field groundwater studies

Laboratory incubation studies are typically conducted under controlled, closed-system conditions for detailed investigation of specific nitrogen transformation pathways and quantify isotopic and microbial changes without external mass exchange. However, applying the results to natural groundwater systems —which may often have variable flow, recharge, and complex structures—requires careful evaluation. However, the closed-system assumptions are often applied for the simplification of the case study.

Several field studies have explored how flow and mixing processes play an important role in nitrogen cycling in the aquifers. For example, (Well et al., 2010) examined N_2O isotopologue signatures ($\delta^{15}N$, $\delta^{18}O$, and δ 15NSP) in a sandy aquifer and observed near-complete N_2O reduction to N_2, attributing this to slow diffusion and limited N_2O escape in saturated groundwater- resulting in isotopic signatures comparable to those seen in closed-system incubations. Similarly, (Weymann et al., 2008) observed N_2 and N_2O accumulation in nitrate-contaminated groundwater and studied changes in gas concentrations to estimate nitrate loss. Their mass balance calculations involved accumulation of gaseous denitrification products, assuming limited external exchange with surrounding water — effectively reflecting closed-system structure in specific aquifers.

Further (Mander et al., 2014) applied isotopologue ratio analysis ($\delta^{15}N$, $\delta^{18}O$, and $\delta^{15}NSP$) to evaluate denitrification in riparian alder forest aquifers. The study reported denitrification as the dominant nitrogen removal pathway and demonstrated substantial accumulation of N_2 and N_2O in water-saturated, low-oxygen zones. Although not explicitly studied as a closed system, the mass balance methodology assumed limited mixing and dilution during transformation processes. In another study, (Well et al., 2012) applied a closed-system nitrogen balance to estimate N_2O emissions. While the approach was

based on the stoichiometric balance of nitrogen species to evaluate denitrification extent, the findings also indicated that recharge and groundwater mixing contributed to isotopic and concentration variability- indicating natural groundwater systems are only partly closed.

Collectively, these studies suggest that while certain aquifer zones— with low permeability or strong redox gradients—may exhibit reduction-dominated closed-system behavior, most groundwaters are not completely closed and are affected by water flow, mixing, and recharge. In our study, the use of low-level ^{15}N-labelling in a closed, suboxic incubation system enabled detailed analysis of nitrate reduction pathways in groundwater systems. However, real aquifer conditions—particularly affected by wastewater inputs or organic carbon—are more complex and may involve complex mixing and transport processes which can affect nitrogen transformation products. Therefore, even though closed-system lab incubations are useful for elucidating microbial nitrogen processes, their results should be supported by field measurements or transport models that better reflect the open and variable nature of groundwater systems.'

References"

Well, R., Buchen-Tschiskale, C., Burbank, J.,Dannenmann, M., Lewicka-Szczebak, D., Mohn, J., Rohe, L., Scheer, C., Tuzzeo, S. and Wolf, B., 2024. Production of standard gases for routine calibration of stable isotope ratios of N2 and N2O. EGU General Assembly 2024, EGU24-11996.

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